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Preface

The International Congresses of Immunology have become for the science of the immune system what Olympic Games are for sports: the performance of the best that the different disciplines can offer, in a great friendly reunion of immunologists from all over the world. The Gesellschaft für Immunologie of the Federal Republic of Germany is grateful and proud that it was given the opportunity to be host to the international world of immunology.

The five themes of this Congress – structure and function of recognition and effector molecules; cell developments; effector phases; defense; and preventive and therapeutic manipulations – deal with the traditional areas of immunological research: biochemistry, cell biology, microbiology, and pathology. Despite the veritable explosion of knowledge in all disciplines of immunology, a strong sense of unity in scientific effort can be felt. Modern molecular and cellular biology and the exciting technical advances in our capacity to clone genes and cells, to express and engineer proteins, and to transfer, modulate, and eliminate single specific cells of the immune system have drawn basic, clinical, and technical immunology more closely together. Medicine is encouraged that the basic discoveries of the molecular and cellular functioning of the immune system may be applicable to the diagnosis, treatment, and management of many diseases, such as bacterial, parasitic, and viral infections, autoimmune diseases, immunodeficiencies, and cancer. Basic research is beginning to understand nature's unfortunate and often terrifying experiments. The hope is strong that our ever-increasing knowledge will improve the quality of our lives.

The proceedings, in particular the introductions to the 27 symposia, convey the impression of the maturity of this field which was felt so strongly at the congress in Berlin. They relive the excitement of new discoveries, not least in the late additions to the symposia. Perusing the reports in their written form helps us to absorb the enormous wealth of experimental data, leading to concepts of the structure and function of the immune system which provide unexpected views of the future of immunology. The aim of any international congress of this size and scope must be to disseminate the scientific information as rapidly and accurately as possible. Published in the same year in which the congress took place, these proceedings will allow thousands of scientists interested in the immune system – those who attended the Congress, and those who could not – to "take part" in all the symposia.

We expressed our gratitude at the beginning of the Congress (see p. XXX) to all the national and international advisors who helped assemble the program. We now wish to thank the 188 symposium speakers and 265 workshop chairpersons, who laid the basis for the scientific success of the Congress. We appreciate the cooperative spirit and disci-

pline of more than 90% of the symposia speakers who had their manuscripts ready at the congress or shortly thereafter. It is remarkable that 2 weeks after the congress these contributions are in our hands, although 36 of the 188 symposium speakers were only chosen shortly before or even at the congress itself.

Our heartfelt thanks go to Springer-Verlag, especially to Dr. Dietrich Götze and Ms. Barbara Montenbruck, and to the secretary of the program committee, Ms. Leslie Nicklin, for their extraordinary efforts to make Progress in Immunology, Volume 7, available in 1989. They, and all of us, are rewarded with a truly historic document representing a milestone in immunological research.

August 1989

For the Editors
Fritz Melchers

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Frau Bürgermeisterin,

President Nossal,

Guests of honor,

Ladies and gentlemen,

It is certainly one of the most significant moments in my life to welcome you, the international immunological community, to the city of Berlin. Today the 7th International Congresss of Immunology is being opened. On this occasion, I extend a very warm welcome, from the depths of my heart, to all immunologist colleagues who have found their way to Berlin.

Although I would like to welcome all of you individually, I cannot do so for obvious reasons. I must therefore restrict my individual welcomes to a number of guests whose presence here is either an outstanding honor for the Organizing Committee of the Congress or a particular personal pleasure, or both. We are very much honored by your presence, Frau Bürgermeisterin Stahmer, as representative of the Governing Mayor of the City of West Berlin under whose patronage this congress takes place. I welcome you, Sir Gus Nossal as president of the International Union of Immunological Societies, which has given us the honorable task of organizing this congress. In addition, a very warm welcome to Alain de Weck, past president of IUIS and Jacob Natvig, vice president and president elect of IUIS. I should like to include in this welcome all other council members and official delegates, not only of IUIS but also of the European Federation of Immunological Societies and of each national immunological society.

We have, among the members of this congress, 5 Nobel laureates as guests of honor. These are Prof. David Baltimore, Prof. Baruj Benacerraf, Prof. Niels Jerne, Prof. Georges Köhler, and Prof. Suzumu Tonegawa. I welcome you and I am very happy that you accepted our invitation bo be guests of honor to this congress. In recent years, Nobel prizes for physiology and medicine have been awarded with increasing frequency to immunologists. After the award to Edelman and Porter in 1972 it took 8 years until Benacerraf, Dausset and Snell earned it again for immunology in 1980. Only 4 years later the prize was awarded to Jerne, Köhler and Milstein and just 3 years later to Tonegawa. Nothing demonstrates better the impact that immunology presently has in medicine and biomedical research.

Guests of honor include further Prof. Michael Sela, former president of IUIS, Prof. Paul Klein, honorary member of the Gesellschaft für Immunologie, and Prof. Otto Westphal, and Prof. Niels Jerne honorary chairmen of this congress.

Prof. Michael Heidelberger, 101 years of age and honorary member of the Gesellschaft für Immunologie, was also invited as a guest of honor, but did not feel quite up to the strain of travelling. It is my special pleasure at this moment to convey the greetings and good wishes of this entire assembly to Dr. Heidelberger in New York, witness to almost the entire history of immunological research.

In the preparation of the congress, which started approximately 10 years ago but which reached a climax of activity in the last 3 years, several generations of immunologists have taken part. When IUIS decided to give the 1989 Congress to the Gesellschaft für Immunologie, Prof. Klaus Rother had just succeeded Prof. Otto Westphal as Chairman of our Society. I should like to use this opportunity to firstly thank our senior colleagues, not only for bringing this Congress to Berlin but also, in a much broader sense, for the scientific and organizational foundations they laid in the reestablishment of immunology in postwar Germany. Secondly, I would like to thank my contemporary colleagues Jochen Kalden and Fritz Melchers, with whom I shared most of the responsibility in my task. Thirdly, although I cannot mention all the members of the various organizing and program committees by name, these colleagues took most of the workload upon their shoulders and I thank them sincerely for their efforts. All of these people have worked tirelessly on the preparation of this Congress. Now the period of preparation is over, I think that I speak in all our names when I say that the overwhelming worldwide interest in this Congress more than compensates the burden of its preparation.

I must take this opportunity to mention gratefully the contributions of organizations, foundations and companies to the financial support of this congress, without which many of the scientific activities as well as some of the hopefully enjoyable social activities would not have been possible. Outstanding contributors were the Senate of the City of West Berlin, the Deutsche Forschungsgemeinschaft, the Federal Ministry for Science and Technology, and the companies Behringwerke, Hoffmann-La Roche, and Sandoz AG. I cannot mention all the other contributors, but their names are compiled in the Program booklet and I would like to express my gratitude equally to them. Each contribution has made our life a little easier.

It has become customary on this occasion to commemorate the immunologist colleagues who have died since the time of the previous congress. I should like to pay my respects to all colleagues deceased since the congress in Toronto in mentioning the names of four eminent immunologists whose contributions to science and whose reputation as teachers were outstanding. These are Ruggiero Ceppelini, John Humphrey, Peter Medawar, and Jacques Oudin. Their names will be remembered, as will those of all other deceased friends and colleagues. The members of the Gesellschaft für Immunologie also remember with affection our friend and Honorary Member Paul Kallos, who died in 1988.

In the phase of the preparation of the congress in which we collected the abstracts, we were amazed to see that about 14000 immunologists have contributed well over 5000 abstracts. Hence, judged from the number of abstracts, and perhaps also from the number of actual participants, this may be among the biggest of the international congresses thus far. Somehow I feel that this must have something to do with the attraction of a place like the City of Berlin, which lies between East and West Europe and which is thus an ideal place of contact between them. The Organizing Committee has taken every effort to facilitate the participation of as many colleagues from East European countries as possible, and I extend a special

welcome to these colleagues, hoping that their participation exceeds that of previous congresses. We are living in a time in which there seems to be increasing hope for a greater freedom of exchange between the West and the East, not only politically and economically but perhaps also in science. The 7th International Congress of Immunology, and the forthcoming 8th Congress in Budapest, could become landmarks in this development.

As you will see, Berlin is an enjoyable city and has much to offer for your relaxation after the scientific work. Nevertheless, August is the time of the general summer vacation in Germany, so that you may find the cultural program of the city not overwhelmingly crowded. Thus, the Organizing Committee felt obliged to offer an extensive social program highlighted by the boat party on Tuesday, the summer night party on Thursday, and the classical music concert on Friday. I would very much like to encourage everyone's participation, and I very much hope to see many of you there. I promise that it will be difficult to find equivalent entertainment for the same price in Berlin.

I now have the privilege of declaring the 7th International Congress of Immunology open. I wish you all very fruitful and informative days as well as relaxation and "Gemütlichkeit" in the evenings. I thank you for your attention.

K. EICHMANN

Professor Nossal, President of the IUIS,

Professor Kalden, President of the Gesellschaft für Immunologie,

Professor Eichmann, Chairman of the Congress,

Nobel laureates,

Ladies and gentlemen,

I am happy that you have come to Berlin and happy to open your Congress. Even for a city which has experienced as many conventions as Berlin, it is a special event to be accepted into the impressive range of venues of the International Congress of Immunology.

As mayor of this city and also on behalf of Governing Mayor Walter Momper, I bid you a cordial welcome. With your Congress opening you met exactly the day where Mr. Momper is already on vacation and I was supposed to be still on vacation. So I came back a little earlier to let this be my first official act.

Despite strong competition from other countries I understand it is no coincidence that Berlin was chosen as the venue for this. The location of the city, at the seam between East and West, lends itself to cross-border scientific dialogue. Against the background of a Europe that is on the move, Berlin is an excellent meeting place for scientists and artists, economic experts and politicians. The great number of representatives from the countries of Eastern Europe — I welcome you with special warmth — is proof that the choice of Berlin was right.

In this city lie the roots of immunology, which originated more than a century ago from the discoveries of Robert Koch and his disciples Ehrlich, Behring and Kitasato. The practical applications of their discoveries have since not only saved the lives of thousands of millions of people and eradicated epidemic diseases, but have been the basis of our present understanding of immunology. This is something of which Berlin — and especially the Robert-Koch-Institut — can rightly be proud.

But it cannot be passed over in this context, that immunologists at the Robert-Koch-Institut in particular, as historical records document, participated after 1933, following the expulsion of many of the most able members of the institute, in crimes against humanity, such as lethal human experiments with inoculation sera in concentration camps.

Following the exodus of distinguished scientists from Germany during the National Socialist years, the center of immunological research moved mainly to the USA and Great Britain.

In the Federal Republic of Germany, above all in Berlin, there have been great efforts since the 1960s to regain our traditional position in immunological re-

search. The 7th International Congress for Immunology will certainly strengthen Berlin's reputation as one of the leading research centers in the heart of Europe. The great resonance which this event has found in Berlin shows that Berlin is on the right path toward regaining its former prominence in science. It is in accordance with this goal that the chair of immunology, now occupied by Professor Diamantstein, was created at the Steglitz Clinic of the Free University Berlin in 1987.

I should like to express my thanks to the organizers of this international congress, mentioning particularly Professor Kalden, Professor Eichmann and Professor Melchers in the name of all the others.

Ladies and gentlemen, allow me to briefly outline the principles of the Senate of Berlin concerning the subject that brings you together here: The Senate puts at the forefront of a social and ecological renewal of public health policy for Berlin the striving to prevent disease, and not merely alleviate and heal it. We want to extend preventive care against disease-causing environmental conditions such as pollutants in air, water and soil. These are projects in which the achievements of immunology can help us.

There is one particular topic from the wide range of your schedules I want to refer to: You are going to discuss among other things, clinical immunology. To this field belong inherited and acquired immune deficiency diseases, such as AIDS. It is a further objective of this Senate to close as soon as possible the gaps in AIDS policy, particularly in the field of drugs, by new measures in the sense of protecting the most affected groups. This means group-specific measures such as a Stop AIDS project and low-threshold offers to drug addicts not willing to subject themselves to a therapy of total abstinence.

We hope that basic immunological research will show up ways of understanding disease-causing principles, and also ways of treating and preventing these and other chronic diseases.

I wish you successful and stimulating discussions and a richly interesting stay in our city. I also hope that you will not be immune to the variety existing in multicultural Berlin, that, on the contrary, you allow yourselves to be infected and succumb to the vitality of the city.

I thank you for your attention.

I. STAHMER

Herr Vorsitzender,

Frau Bürgermeisterin Stahmer,

meine Herren Ehrenpräsidenten Jerne und Westphal,

Dr. Kalden,

Dr. Melchers,

meine Damen und Herren,

Es ist für mich eine ganz besondere Ehre und auch eine große Freude, als symbolischer Kapitän der 25000 Personen starken Schar der Weltimmunologen, sie hier in Berlin herzlich willkommen zu heißen. Die Immunologie bringt uns zusammen; aber hier in Berlin gibt es doch soviel mehr zu genießen: Kunst, Kultur, die Schönheit der eleganten Großstadt, Geschichte und auch einen gewissen, hochinteressanten Blick in die Zukunft, auf den ich später noch eingehen werde.

So in welcoming you to Berlin as your President and as we begin this serious and adventurous week, the first and also the most important thing that I have to do, in the name of the IUIS, is to thank our German colleagues for the three years of hard and painstaking work that have prepared the way for our deliberations. No one could have done more to ensure that the presentations have a true international perspective, a fine balance between the many elements of our great discipline, and a modernity which gives due emphasis to recent discoveries and to younger workers. As an Australian — our country had the privilege of hosting the 3rd Congress — and as an avid student of all 7 Congresses since Washington in 1971, I know very well the immense amount of patient and self-sacrificing effort involved in ensuring that all the pieces of the jigsaw puzzle fit together.
To the democratic co-leadership of the Congress, Professors Eichman, Kalden and Melchers, and to all the many who worked on committees, subcommittees and as advisors, our heartfelt thanks from IUIS.
Ladies and gentlemen, a tragic pandemic has given immunology a new prominence in society, even a kind of fame. However, for those of us who have been in world immunology for some time, it really did not require the tragedy of AIDS for us to realize that communicable diseases, including vaccine-preventable communicable diseases, continue to take a devastating toll, particularly in the developing countries. I want to say to this large gathering here that one of the most satisfying things of my three years as president has been the excellence, warmth, depth and breadth of our relations with WHO. Yes, we must make every effort to control AIDS, of course, sincerely; but also YES we must link arms with WHO in the struggle against other communicable diseases, the struggle to devise new and im-

proved vaccines. Dr. Lambert of WHO has informed us of new research plans in this field, which we must strongly support.

All of us share, I think, a huge sense of excitement about the progress of immunology. The revolutions in DNA technology, protein chemistry including structural analysis, and cell biology have given us depths of understanding not dreamt of even a decade ago. So it is with a huge sense of confidence and adventure that we begin our week at the 7th Congress.

We are meeting here in Berlin, the city, as others have said, of heroic figures in the history of our discipline. (I warmly recommend to you the historical display on ground level.) Is it not also the case that we approach political life with a new sense of hope and adventure when we are here in Berlin? Some of the developments in superpower relationships, in the arms race, in the rhetoric governing discourse between nations, have recently taken a truly revolutionary turn. And, somehow, the new Berlin, a crucible of social change, symbolizes so much of what is in the air. There is history in the making at this meeting point of east and west; a sense of exhilaration about the possibility of a peaceful future that I have not experienced in my adult life, lived totally in the nuclear age.

As immunologists, we can do so much to promote and accelerate goodwill among nations. As IUIS, we have a long tradition of breaking down national barriers, finding our collaborations where the scientific impulse demands; finding our friendships where shared intellectual concerns bring us together. We have our own particular brand of glasnost that we can practice with vigor and optimism, here in Berlin and beyond.

Meine Damen und Herren, nach diesem Kongress verlasse ich meinen Posten als Präsident der Internationalen Vereinigung der Immunologischen Gesellschaften. Ich kann Ihnen tatsächlich versichern, daß sich die Immunologie in einem brillant guten Zustand befindet. Meine "State of the Union" message ist vollkommen positiv.

In closing, I want to thank the many colleagues who have made my three years as President so happy and, I believe, productive: my fellow officers of the Council; all the Council members; the Chairpersons of the many hard-working Committees; the Assembly; the whole wide family of the IUIS. This afternoon, we elected Dr. Jacob Natvig of Norway to succeed me as President of the Union immediately at the conclusion of the Congress. His dynamic personality, dedication, energy and true internationalism will ensure that the Union is led with distinction, and my congratulations and very good wishes go with him.

Thank you.

G. J. V. Nossal

Frau Bürgermeisterin,

Sir Gustav,

Nobel laureates,

Ladies and gentlemen,

Dear friends,

As President of the Gesellschaft für Immunologie, I would like to extend a warm welcome to all of you who have come to the 7th International Congress of Immunology in the City of Berlin.

Over the last century, this City has experienced very different times, good ones as well as bad ones, and its eventful history reflects — at least in certain aspects — the situation of immunology in Germany over the last 8 — 9 decades.

Among the different activities during this congress, an exhibition has been organized which is called Berlin — Roots of Immunology. It highlights major scientific contributions which originated from this city at the turn of the century. They are connected with names such as von Behring and Kitasato, Koch, Ehrlich and von Virchow. However, when we display in an exhibition major achievements of immunology in Germany from earlier in this century, it does not mean that we, the Society for Immunology, do not remember that those early flourishing times of immunology came to an abrupt end in 1933 when about 500 medical professors had to leave this country. They represented almost half of all the professors teaching at German universities at that time, and included quite a number of famous immunologists. You might forgive me if I just mention, as pars pro totum, Ernest Witebsky.

After the Second World War, the emerging science of immunology at the universities of the Federal Republic was substantially and overwhelmingly supported by all the major countries doing research in immunology, offering and providing training facilities for young German scientists. I hope you will forgive me for not naming all the famous institutions in the different countries which provided assistance and with which — in many cases — scientific exchange still exists.

I myself, as one of many who were trained in immunology abroad, and the Society for Immunology are deeply grateful for this help in reestablishing immunology research in the Federal Republic of Germany, and without any doubt this support helped to found the Gesellschaft für Immunologie in 1967, which — I believe — is quite a young Society for Immunology, although two years older than IUIS.

The present activities and contributions to Immunology from our Society are summarized in the August issue of *Immunology Today*, including a special section on immunology in the Federal Republic of Germany, published on the occasion of

this Congress. The scientific contents of that journal correctly reflect — in my opinion — the immunological activities which are being pursued in the Federal Republic of Germany. However, although German immunologists too know that eagles have an immune system, I would have preferred to see the cover and logo for this special issue similar to the poster designed by Jean Tinguely for this congress, which in my opinion, expresses in a fantastic way the ever-moving immune system, and which much better reflects the feelings, "die Stimmung", of the members of the Society.

By now, the "Gesellschaft für Immunologie" has roughly 1000 members, and more than half of them are in their early thirties and enthusiastically involved in immunology research. However, with regard to the institutionalization of departments in basic as well as clinical immunology at German universities there is still a deficit, so a lot of work still has to be done. Perhaps this 7th International Congress of Immunology will help to further improve the situation.

The "Gesellschaft für Immunologie" is proud that it has been entrusted by the IUIS to organize the 7th International Congress of Immunology. Since organization means people, I would like to thank Klaus Eichmann and Fritz Melchers who took over the task of organizing this Congress as Chairman of the Organizing Committee and Chairman of the Scientific Program respectively. Furthermore, I would like to thank all the members of our Society who have been actively involved in the organization helping us to make this 7th International Congress of Immunology a success.

The Society for Immunology and I hope that all of you are not only going to experience a week full of exciting scientific information and exchange, but that you will also find time and opportunity to see and explore the uniqueness of Berlin's life by day and maybe also by night, if time permits.

Let me finish with a personal wish: I do hope that the young members of our Society as well as doctoral and postdoctoral fellows attending this Congress will take the opportunity of establishing fruitful relations with scientists from all the different countries who are here in Berlin, in order to exchange ideas and to discuss possible cooperation projects. This, hopefully, would result in a continuing exchange of scientists, helping us to further develop immunology in this country in the same international way as has been the case in the past.

J. R. KALDEN

Frau Bürgermeisterin Stahmer,

Sir Gustav Nossal,

Honorary Chairmen Niels Kaj Jerne and Otto Westphal,

Guests of honor,

Friends and colleagues,

More than three years ago, when the Gesellschaft für Immunologie set up a Program Committee for the 7th International Congress of Immunology, one of the first decisions taken was to organize a scientific program which would combine topics of basic, clinical and industrial immunology — all under one roof. This was decided because it was felt that the often divergent interests of these fields of immunology are clearly in need of each other. None of them can prosper without knowing the progress of the others.

In your program book you will find the three years of our efforts summarized in 27 symposia and 130 workshops. We hope that they will cover the most important issues of immunology today, and we hope that you will find this program interesting, stimulating, informative, exciting and rewarding.

At 8.30 in the morning and at 2.00 in the afternoon no other scientific activities are scheduled than the 30-minute long introductions given by the chairpersons of 27 fields of immunology covered by the symposia. We hope that these half-hour introductions will serve as overviews of recent exciting discoveries and of current problems, and will give the nonspecialist a chance to learn where a given field of immunology stands — and in which direction it is moving.

It has often been regretted that symposia of large international congresses must be planned so far in advance that the latest news is most often omitted, or is not publicized widely enough. We have therefore reserved at least one half hour of each symposium for such latest news, chosen at the last minute by the chairpersons. You can find these late additions on display at the entrance of the congress center, and in the symposia halls. We hope that this gives recent exciting discoveries a chance to be presented to a wider audience, and will heighten the curiosity for what is really new. I would like to thank all the symposia chairpersons for these additional efforts to make the congress scientifically even more lively.

The programs of the workshops, and the styles in which they are to be held, have been planned entirely and only by the chairpersons. In fact, the chairpersons were encouraged to solicit contributions. This, we hope, will give life and excitement to the workshops — and I thank all the chairpersons for their efforts.

All participants who so wished were given the opportunity to present a poster. As a result, over 4300 posters will be presented in the one week of the congress, and

this requires skilful and devoted planning. I would like to thank Angelika Reske-Kunz and Reinhard Burger for their enormous organizational efforts.
It takes the cooperation and attention of all the poster presenters to make this part of the congress a success. Imagine that every morning and every noon, 400 to 600 posters will have to be mounted, and at the same time an equal number will have to be removed! I urge all poster presenters to follow the instructions in the program very carefully indeed.
At lunchtime, four respected immunologists will talk about their scientific and personal recollections of important milestones in the discovery of the immune system, and the four national societies of the USSR, China, Hungary and Brazil will introduce you to their interests and efforts to conduct immunological research in their countries.
Now that the congress is ready to begin, I wish to thank those who helped to assemble the scientific program of 27 symposia, 130 workshops, 4300 posters and 5140 abstracts, submitted by over 14000 authors.
First, I would like to thank the 17 subcommittee chairpersons, the over 100 national, and the over 250 international advisors for their suggestions and criticisms.
Then I wish to thank Boehringer-Mannheim and the Deutsche Forschungsgemeinschaft for their financial support of the planning meeting at Schloss Elmau, and Hoffmann-La Roche for their support of the Basel Institute for Immunology which housed the scientific secretariat.
I want to thank the staff of the secretariat for their devoted efforts. More than anyone else I would like to thank Leslie Nicklin, secretary of the program committee, for the three years of her life which she devoted to this congress — and I do so from the bottom of my heart. She kept order in the correspondence with over 350 advisors and over 500 chairpersons and speakers. She ordered and edited the program, received and ordered the abstracts, and assembled the authors' index. She was always in command of the situation, and stayed calm, helpful and friendly. It is very simple: she deserves a big hand of applause from all of us.
Let me also thank two people who are not scientists. Anne-Sophie Mutter and Jean Tinguely, two outstanding artists of our times, are giving their precious time and their unique talents to our congress. Jean Tinguely has created our congress poster, and Anne-Sophie Mutter will play Dvořák's Violin Concerto on Friday night. Finally, I would like to thank the Gesellschaft für Immunologie and all its members, particularly my two friends and colleagues Klaus Eichmann and Jochen Kalden, for entrusting me with the organization of the scientific program of this congress, held in the city where I was born. I have felt the fun and excitement of discovering new areas of immunology and the frustration that all of the areas of immunological research had more talented scientists than could be considered to participate as chairpersons and speakers.
And I would like to thank the many immunologists who chose to attend this congress, and thereby to reward us as organizers by making this one of the most popular international congresses of our field held so far.
The immune system protects us from the hostile environment which is full of infectious diseases. A hundred years ago Koch, Behring and Ehrlich, in this town, were already fascinated by the idea that we could understand the structure and function of our immune system so well that we could protect ourselves against this hostile environment even better than we do naturally. One hundred years have

brought tremendous advancement of our knowledge of the immune system, and with it the realization that it sometimes does not function properly, and that it sometimes attacks what it should not attack: our own body. We still need much more basic knowledge of the system, and we need to apply this knowledge: to get better protection, to fight autoimmune disease, and to strengthen against immunodeficiencies. I hope that this congress will publish new knowledge, air new views, discuss new strategies, and start new cooperations between scientists all over the world with the aim of achieving these goals. Welcome to Berlin.

This is the moment for which we have been waiting for three years. We now light the immunological flame with talks by the two Honorary Chairmen of the Congress.

F. MELCHERS

The first Honorary Chairman who is going to speak to you is Niels Kaj Jerne. He was born in 1911 in London. He became a research worker at the Danish State Serum Institute in 1943 and stayed there until 1954. After a year as a research fellow at the California Institute for Technology, he became the head of the Section of Biological Standards and Immunology at the World Health Organisation from 1956–1962, and was a Professor of Biophysics at the University of Geneva at the same time. He then became Professor of Microbiology at the Medical School of the University of Pittsburgh, and from there went to Frankfurt as a Professor of Experimental Therapy at the Johann Wolfgang Goethe University and Director of the Paul Ehrlich Institute. In 1969 he moved to Basel to become the first director of the Basel Institute for Immunology. He now is Emeritus Member and Honorary Chairman of the International Board of Scientific Advisors of that Institute. It gives me the greatest of pleasure, as his successor in the directorship of the Institute, to introduce him and now ask him to deliver his lecture.

The discovery of antibodies here in Berlin 99 years ago created a wave of enthusiasm in the medical profession, with the hope that antibodies in the serum of immunized animals would provide powerful therapeutic agents against infectious diseases. Serum institutes were established in many countries, and many students of medicine, biology, and chemistry decided to devote their talents to research in immunology.

When I joined the Danish State Serum Institute half a century later, in the 1940s, a large spectrum of phenomena related to antibody formation and to antibody-antigen reactions had been described, and antibodies were still the main, if not the only, element on which all immunological research was focussed. Phenomena which did not involve antibodies were thought not to belong to immunology. Thus, what now may seem curious, one of the best textbooks on fundamental immunology, by William Boyd, in its second edition of 1947, does not even mention transplantation, because the rejection of transplants did not seem to involve antibodies, and therefore was not thought to be an immunological phenomenon.

One may ask why, in the first part of this century, so little effort was made to find the type of cells that produce antibodies. I think this was a side effect of Paul Ehrlich's side-chain theory of antibody formation. Since all cells of the body, according to Paul Ehrlich, would display side-chains for catching food molecules for the cell, this implied that all cells were capable of antibody formation, and that it would be useless to look for a particular cell type. The side-chains were molecules that were physiologically engaged in the nourishment of all cells, and that functioned only secondarily as antibodies.

This double function avoided the wastefulness of the later selective theories of antibody formation, which imply that the immune system makes millions of different antibodies, most of which will never be called upon to function. I think it was this apparent wastefulness that made it hard for the later selective theories to gain acceptance.

In the 1940s, protein chemistry was still in its infancy. It had been shown that antibodies belong to the serum globulins, and mainly to a fraction named γ-globulin. It was not proposed, however, that all γ-globulins are antibodies.

On the contrary, even in the 1950s, globulin, like albumin, was looked upon as a class of identical molecules which were normal constituents of the blood. Antibodies, as well as myeloma proteins, were regarded to be "modified" γ-globulins. The later selective theories of antibody formation suggested that in fact all γ-globulins are antibodies. This then led to the change of their name to "immunoglobulins".

As an old-timer, I myself still have a particular weakness for antibodies. In spite of the tremendous advances that have been made during the last two decades, I still consider that one of the most striking facts of the immune system is the presence in every ml of our blood of several milligrams of immunoglobulin, which is more than 10^{16} antibody molecules. Even with a repertoire of, say, ten million different specificities, there will be, on the average, 10^9 antibodies of every specificity present in every ml of blood, ready to attach to an arriving antigen particle.

For the system as a whole to remain stable and functional, all the millions of different antibody specificities must continuously be produced, the vast majority in the absence of corresponding foreign antigens. This is not possible unless the antibodies themselves are the antigens that, by their idiotypic epitopes, stimulate the lymphocytes and endow the system with a diversity that strives to retain its identity. This also removes the stigma of wastefulness, because every antibody molecule, even if it never encounters a fitting foreign antigen, will be engaged in this selective stimulatory process.

Belonging to an earlier generation than most of you, and speaking to this congress of several thousand immunologists here in 1989, I am struck not only by the enormous increase in the number of scientists now studying this field, but also, looking through the scientific program, by the great diversity of subjects that will be discussed in the coming week.

In spite of this great variety of subjects, we have come together here in Berlin because of the overriding concept that all these subjects belong to one science: immunology. All discussions will reflect our common effort of describing the immune system, the way it develops and functions, and the ways it might be made to yield further medical advances. I hope that everyone of you will endeavor not to become a specialist in only one branch of the immune system, perhaps because of your particular competence in certain methods or techniques, but that you will all remain aware of the entire field of immunology that unites us all.

N. K. JERNE

I now would like to introduce the second Honorary Chairman of our congress, Otto Westphal. He was born in 1913 here in Berlin. He later moved to the south of Germany and obtained a D. Sc. from the University of Heidelberg in 1938 with a thesis on blood group substances, done in the laboratory of Professor Freudenberg. In 1942 he became a lecturer at the University of Göttingen. In 1946 he became the director of the Wander Research Institute in Säckingen near Basle, which moved to Freiburg i. Br. in 1958. In 1962 the Wander Institute was transformed into the Max Planck Institut für Immunbiologie, and Otto Westphal its first director. Since 1984 he has been an Emeritus Member of the Max Planck Gesellschaft. Otto Westphal was instrumental in founding the Gesellschaft für Immunologie and became its first president. He thereby influenced the development of immunology in postwar Germany in a very decisive way. I am sure that I speak in the name of all members of the Gesellschaft when I express happiness and satisfaction that Otto Westphal has agreed to be one of the two Honorary Chairmen of the Congress, and to deliver an opening lecture.

Immunology and the "Belle Epoche" in Berlin

Adding Some Personal Memories

Ladies and gentlemen,

May I invite you to go back in history and pay a visit to Berlin about 100 years ago? You arrive by train at the central station, Friedrichstraße (Fig. 1), and you may have made a reservation at one of the elegant hotels, such as the "Grand Hotel" (Fig. 2). You spend the night and enjoy a good breakfast, costing together not much more than 2 Reichsmark. Then you call for a horse carriage (Fig. 3) and ask the coachman to take you on a round trip with stops at the famous institutes and, maybe by chance, meet one or other of the great scientists now working in Berlin. Almost every coachman — be it in Berlin or in Paris — knew the places and names of the great professors, and they knew the jokes that made them popular. At the end of the nineteenth century the general attitude towards science and research had a very optimistic undertone. Everybody was convinced that science would lead to better health and more comfortable life. People were all for science.

Louis Pasteur (1822—1895; Fig. 4) had recently expressed that general feeling in a presentation before the Academie Française: ..."Consider the consecrated places which are named by that meaningful word, Laboratory. They are the temples of the future, of prosperity and growth. It is there that humanity will become greater, stronger and better. There man learns to read the works of nature, works of genuine progress and universal harmony."

Your coachman drives you down the main street, Unter den Linden (Fig. 5), passing the University (Fig. 6), and further to the complex of university hospitals, the Charité (Fig. 7), with many attached institutes. Here you might meet Professor Rudolf Virchow (1821—1902; Fig. 8), the famous pathologist (Fig. 9), now already aged, the founder of cellular pathology, scientifically and socially a great authority and very active also in political life.

You are driven further to the Institute of Physiology and there you might bump into Professor Emil du Bois-Reymond (1818—1896; Fig. 10), the founder of electrophysiology and neurophysiology, since thirty years a brillant teacher who mastered also physics and even history and philosophy. It was du Bois-Reymond who presided over the memorable session of the Berlin Physiological Society on

Fig. 1 Arrival at the central station, Friedrichstrasse

Fig. 2 Grand Hotel Announcement

Fig. 3 Horse carriage

Fig. 4 Louis Pasteur

Fig. 5 Unter den Linden

Fig. 6 University main building

Fig. 7 Charité, general view

Fig. 8 Rudolf Virchow

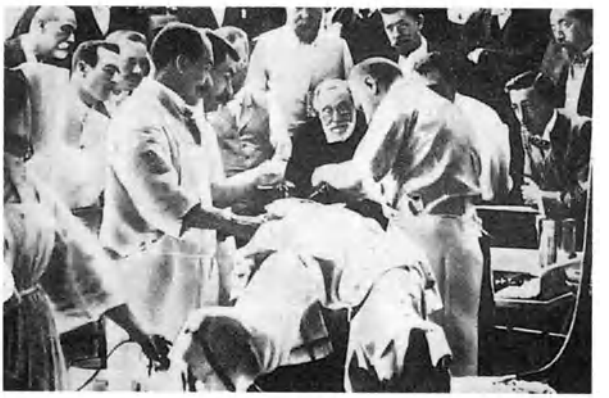


Fig. 9 Virchow following operation

Fig. 10 Emil du Bois-Reymond

Fig. 11 Library of the Institute of Hygiene

Fig. 12 Robert Koch (around 1882)

Fig. 13 Paul Ehrlich (around 1882)

the 24th March 1882 in the library of the Institute of Hygiene (Fig. 11) in which Robert Koch (1843–1910; Fig. 12) presented the discovery ot the tubercle bacillus. Koch's talk and demonstrations stimulated great excitement and also critical discussions with Virchow, the pathologist. Amongst those attending was the 28-year-old Dr. Paul Ehrlich (1854–1916; Fig. 13), at that time assistant at the Medical Clinic of the Charité. From Ehrlich we know that after the session he went back into his small laboratory to work out a staining of tubercle bacilli in sputum, based on their acid-fastness which Ehrlich discovered. His technique proved to be superior to the one presented by Koch – very much to Koch's satisfaction.

We go on to the Surgical Clinic of the University headed, since 1882, by Professor Ernst Bergmann (1836–1907; Fig. 14). He was not only an excellent operator (Fig. 15) who developed brain surgery, but also very interested in putrid wound infections. He extensively studied septic processes and he described pharmacologically highly active (and rather toxic) material, which he called Sepsin, isolated from inflammatory exudates. (He might have already dealt with impure cytokine mixtures.) Bergmann was the first in Germany to recognize the significance of Joseph Lister's (1827–1912; Fig. 16) antiseptic operations, published in 1866/67. Bergmann went further, and until 1892 he formulated the principles of aseptic surgery. He introduced the steam sterilization of surgical and other equipment, and Virchow supporting him – with his enormous energy and political engagement – strongly furthered the modernization of general hygiene, also in the township of Berlin.

Our coachman now drives us to the Institute for Infectious Diseases (Fig. 17), near the Charité, the so-called Triangel. It was here where Robert Koch and his pupils worked. The building had been reconstructed according to his plans – with research laboratories and rooms for microbiological courses for many students. Many excellent young men would you meet in Koch's Triangel! It was just at that time, around 1890, when Dr. Emil Behring (1854–1917; Fig. 18) and Dr. Shibasaburo Kitasato (1852–1931; Fig. 19) from Tokyo discovered tetanus and diphtheria antitoxins in the sera of toxin-treated animals.

By coincidence, bacterial toxins – later called exotoxins – had just been described by Emil Roux (1853–1933) and Alexandre E. Yersin (1863–1943; Fig. 20) at the Pasteur Institute. Both Behring and Kitasato cooperated with Paul Ehrlich who, after having left the Charité, joined Koch's Institute. Here he worked on quantitative aspects of antitoxin production during immunization, and it was Ehrlich who told his colleagues how to produce high-titered antisera. They all had their share in the introduction of serum therapy after 1890, especially Behring who started the treatment of diphtheria in children – as a first beautiful demonstration of a humoral immune reaction in human therapy.

Behring also recognized early the significance of the role of phagocytes and phagocytosis which had been described by Elie Metchnikoff (1845–1916; Fig. 21), working since 1888 at the Pasteur Institute in Paris. Between Behring and Metchnikoff there developed a close friendship, documented by many handwritten letters. Behring made Metchnikoff a godfather of one of his sons.

Everybody knew that Geheimrat Koch was intensely occupied by his work on tuberculosis (Fig. 22) – long one of the greatest hazards to human health. Everybody looked to Koch, who had just described tuberculin, which he proposed as a possible agent against tuberculosis. On January 14, 1891, the Berlin Medical So-

Fig. 14 Ernst Bergmann

Fig. 15 Bergmann operating (1906)

Fig. 16 Joseph Lister

Fig. 17 Institute for Infectious Diseases (Triangel)

Fig. 19 Shibasaburo Kitasato

Fig. 18 Emil Behring in the laboratory

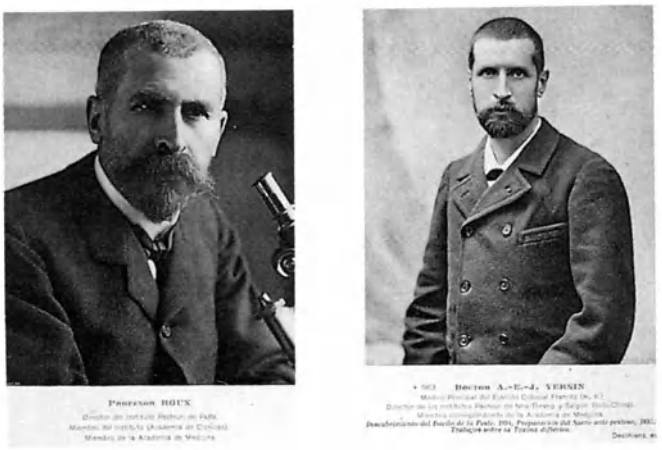


Fig. 20 Emil Roux and Alexandre Yersin

Fig. 21 Elie Metchnikoff

Fig. 22 Robert Koch in his laboratory

Fig. 23 Virchow with magnifying glass

Fig. 24 Virchow in big lecture hall

Fig. 25 August Wilhelm Hofmann (28 years old)

Fig. 27 A. W. von Hofmann (ca. 50 years old)

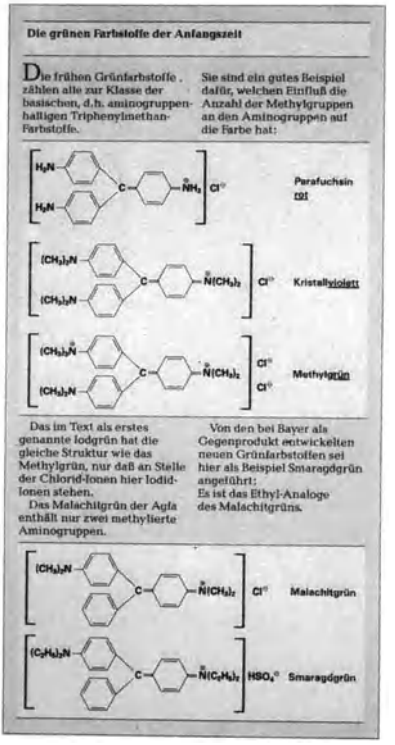


Fig. 26 Formulas of some aniline dyes

ciety under Bergmann's chairmanship had a meeting where almost all those attending shared the optimism about the potential role of tuberculin. As is known, this optimism faded within the next years. At that session, only Virchow (Fig. 23) showed a critical attitude. In the discussion (Fig. 24) he carefully described the local tuberculin reaction. For the first time, he spoke of "focal reactions"; and he said... "Already by superficial inspection, the action of Koch's tuberculin on the affected tissue is mainly of irritative nature, insofar as acute local irritations are being initiated with very strong swelling" ... It was a classical example of a local cellular immune reaction!

But our coachman insists on paying a visit to the Chemical Institute. Director of the institute was August Wilhelm von Hofmann (1818—1892). Originally a pupil of Justus von Liebig (1803—1873), he had been invited at the age of 27 (Fig. 25), by the British Royal family, to move to England, where he became director of the Royal College of Chemistry in London. He remained there for an extremely fruitful 20 years before he finally moved back to Berlin. One of his pupils, William Henry Perkin (1839—1907), at the age of 18 years, discovered by chance the first aniline dye (Fig. 26)!

Hofmann was a great expert in the chemistry of tar products, and thus of aniline and its derivatives. He and Perkin, the two became pacemakers of synthetic dyestuff chemistry and technology. After his return to Berlin, Hofmann (Fig. 27) gave a popular lecture before a distinguished audience under the title "Organic Chemistry and the Teaching of Medicinals". He said that for the logic development of medicinals we may witness changes similar to the so impressive ones in the field of dyestuffs. And Hofmann gave an illuminating example: ... "Take carmoisin which can be derived from rosaniline; add one methyl group and the splendid red changes into the richest violet. With the introduction of a second and a third methyl group the red-violet changes into blue-violet and finally into blue. Another two more methyl groups, and we arrive at a beautiful green! When used for the dying of clothes, these colours have gained the greatest enjoyment of our handsome ladies"... Hofmann anticipated the development of pharmaceutical industries, and it is no wonder that the dyestuffs and pharmaceutical industries have since often developed hand in hand. In Germany, think of Farbenfabriken Bayer, Farbwerke Hoechst, or the Badische Anilin- und Sodafabrik (BASF).

Special dyes had specific dyeing properties with different materials, like wool, silk or linen. It was Carl Weigert (1845—1904), an elder cousin of Paul Ehrlich, assistant pathologist at Breslau University, who first systematically tried many of the new aniline dyes for the staining of histological material. Already as a schoolboy, Ehrlich spent much time during the holidays with Weigert in his laboratory (Fig. 28). Weigert's influence on Ehrlich became crucial. Based on these early experiences, Ehrlich discovered the remarkably selective affinity of certain dyes for certain cell types. He, thus, became the founder of modern blood cell teaching. As you know, he early discovered the mast cells by staining their granules, and soon afterwards the eosinophils; with neutral red he selectively stained the neutrophil granulocytes, and he differentiated between granulocytes and lymphocytes. Think of his introduction, in his thesis already, of vital staining, for example the methylene-blue staining of nerve fibers, and the related studies on redox systems using redox dyes!

Now, here in Berlin, the aged von Hofmann (Fig. 29) and the young Paul Ehrlich

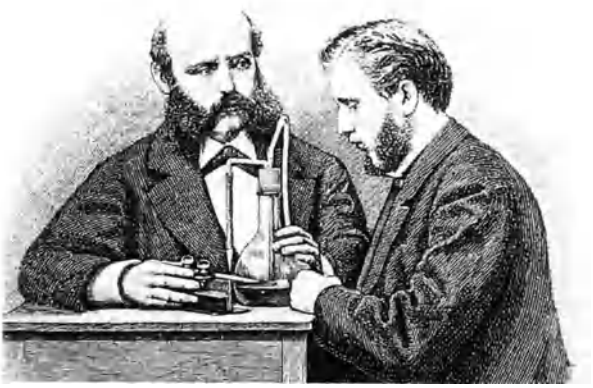


Fig. 28 Carl Weigert and Paul Ehrlich (around 1870)

Fig. 29 A. W. von Hofmann (72 years old)

Fig. 30 P. Ehrlich (around 1892)

Fig. 31 Emil Fischer

Fig. 32 Chemical Institute Hessische Strasse

Fig. 33 J. H. Van't Hoff

Fig. 34 Max Planck (around 1892)

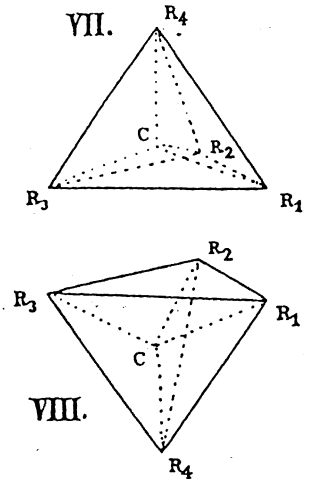


Fig. 35 Tetraeder model of carbon-atom

Fig. 36 Svante Arrhenius and Paul Ehrlich (around 1905)

Fig. 37 Nobel laureates 1901

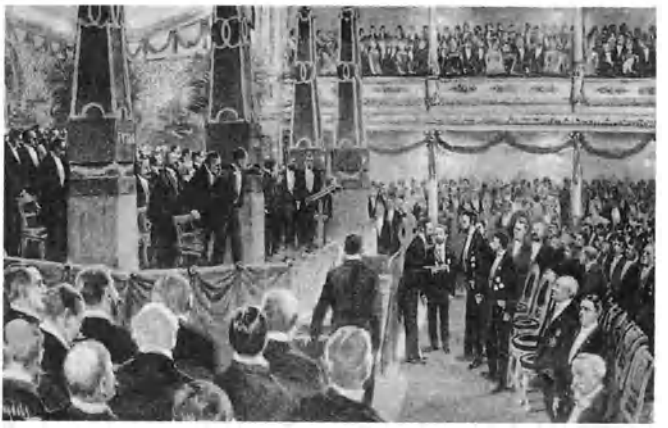


Fig. 38 Painting of Nobel ceremony 1901

Fig. 39 Oswald Avery
(around 1945)

Fig. 40 Michael Heidelberger (1917 — 1954 — 1988)

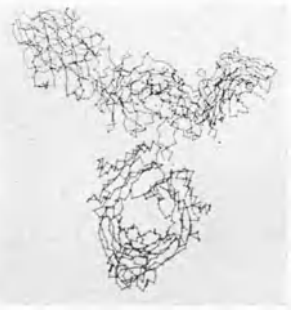


Fig. 41 Stereo Structure of IgG (Huber, Nobel prize 1988)

(Fig. 30) were geographically and spiritually very near to each other. We do not know when and where they might have met personally.
Hofmann died in 1892, and another genius, Emil Fischer (1852–1919; Fig. 31), almost of the same age as Ehrlich and Behring, entered the newly created big institute of chemistry (Fig. 32), near the Charité. Greatly influenced by Pasteur, Fischer was just engaged in a systematic study of the chemical specificity of enzyme reactions in the field of isomeric sugars, glycosides and oligosaccharides as substrates – many of which he had synthesized. It was Fischer who, in a lecture given before the Berlin Chemical Society, coined the since oft-quoted metaphor "that enzyme and substrate must structurally fit like the keyhole and its key... in order to be optimally reactive"... "and this leads us", he continued, "into stereochemical research after the phenomenon of enzyme action is now shifting from the biological to the purely chemical field. Stereochemistry allows an extension of the theory of asymmetry".
Under Fischer's influence the same ideas grew in Paul Ehrlich's mind for the structural relation between toxin and antitoxin, or generally between antigen and antibody.
Emil Fischer soon urged his colleagues to invite the greatest scientist in theoretical stereochemistry to join the Berlin Faculty: the Dutchman Jacobus Henricus Van't Hoff (1852–1911; Fig. 33), exactly of Fischer's age, then professor in Amsterdam. The faculty in Berlin sent its dean, the young physicist Max Planck (1858–1947; Fig. 34). Van't Hoff, already at the age of 22, had published a short pamphlet about the extension of the then used structural formulas in organic chemistry into tridimensional space. In 1874, he wrote his famous article entitled "The Position of Atoms in Space". Van't Hoff – and independently Joseph Achille Le Bel (1847–1930) in France – for the first time clearly proposed the Tetraeder model (Fig. 35) of the carbon atom. It was the birth of stereochemistry, the basis of all future organic chemistry in theory and practice.
Under Max Planck's and Fischer's influence, the Berlin Academy of Sciences appointed Van't Hoff as a honorary professor, an unique procedure at that time. Finally accepting the call to Berlin in 1896, Van't Hoff quoted Faraday, who once had said to his young friend Tyndall: "Regular personal meetings of scientists, of which I think very highly, advance science chiefly by bringing scientific men together and making them known and to be friends with each other". These were, indeed, the atmospheric conditions offered by the scientific Berlin around the late 19th century. Here many great scientists were now united. It was, indeed, a "Belle Epoche", not only in the arts (from which the expression is borrowed), but also in science!
Ehrlich developed great enthusiasm for Van't Hoff's stereochemical visions. Once he told a friend that benzene rings, or dyes, and all kinds of organic molecules, floated to and fro before his spiritual eye as sterical formulas. He called it his sense for the "plastic" in chemistry. – With the new trends in organic chemistry, immunology slowly changed into a branch of science, and all further developments can be looked at as logic and historically compelling. In Sweden, Svante Arrhenius (1859–1927; Fig. 36), the founder of modern physico-chemistry, well known for the theory of solutions, introduced the term "immunochemistry" in 1904. He formulated immune reactions as chemical equilibria and started systematic investigations into the conditions of the static adaptation of antigen and antibody

Fig. 42 Horse carriage waiting for us

Fig. 43 Unter den Linden

Fig. 44 Opera hall

Fig. 45 A ladies box in the Opera

Fig. 46 Sunday in Berlin at the Halensee bridge

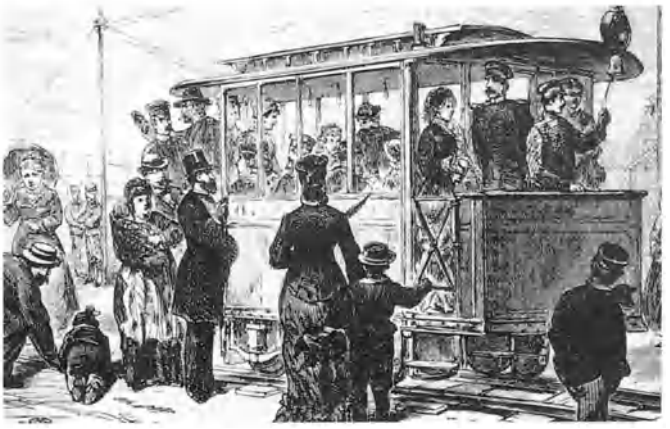


Fig. 47 Early suburb Tramway in Berlin

Als ich 1911 jung verheiratet nach Berlin kam, fand ich dort einen mir nahestehenden schlesischen Kreis vor, dessen Haupt Max Planck war. Da er neben seiner Phys. K auch der Musik huldigte, (er hatte einen schönen Chor, er spielte wunderbar Klavier) so haben wir viel Musik gemacht. Zitternd saß ich neben ihm, wenn wir vierhändig spielten. Die g moll Symphonie v. Mozart ist mir unvergesslich. In seinem Chor (Liederkranz von Brahms) habe ich so selig mitgesungen, dass mein Sohn Otto 10 Tage zu früh geboren wurde. Auch mit Einstein habe ich musiziert. Er spielte sehr schön Geige. Und wiederum saß ich zitternd am Klavier, um keine Fehler zu machen. Es war eine bewegte, wenn auch schöne Zeit!

Fig. 48 From my mother's notebook

Fig. 49 Max Planck at piano (in the 1940s)

Fig. 50 Einstein around 1913

and the involved chemical forces. He had vigorous discussions with Ehrlich, and both agreed in rejecting the application of "colloid chemistry" to specific immune phenomena.

When the first Nobel prizes were awarded in 1901 (Fig. 37), two of our heroes from Berlin made their trip to Stockholm: Emil Behring for medicine, Van't Hoff for chemistry, together with Wilhelm Roentgen for physics, for his discovery of X-rays. The ceremony at the Royal Academy of Music in Stockholm was immortalized by a picture showing Roentgen receiving the prize, with Behring and Van't Hoff in the background (Fig. 38). One year later, in 1902, Emil Fischer followed in chemistry; in 1903 it was Arrhenius. Robert Koch was honored for medicine in 1905, Paul Ehrlich together with Metchnikoff in 1908.

Emil Fischer received the Nobel prize for his fundamental work on carbohydrates and purines. Later he also went into the field of aminoacid and proteins; he was the first to synthesize polypeptides. Although the concept of macromolecules came up only much later, Fischer found one principle realized again and again in nature, namely the formation of polymers from monomers by characteristic types of linkages: from sugars to oligo- and polysaccharides, from amino acids to polypeptides and proteins, and from purines and pyrimidines to polynucleotides and nucleic acids. With regard to the latter, he gave a lecture in Berlin (1914) from which I can't resist quoting the following: ..."With the synthetic approaches to this group (of purines and pyrimidines) we are now able to obtain numerous compounds that resemble, more or less, natural nucleic acids. How will they affect various living organisms? Will they be rejected or metabolised or will they participate in the construction of the cell nucleus? Only the experiment will give us the answer. I am bold enough to hope that, given the right conditions, the latter may happen and that artificial nucleic acids may be assimilated without degradation of the molecule. Such incorporation should lead to profound changes of the organism, resembling perhaps permanent changes or mutations as they have been observed before in nature" ...

Thirty years later, Oswald Avery (1877—1955; Fig. 39) in New York found DNA as the type-transforming principle of pneumococcal types, and another ten years later Watson and Crick, and also Lederberg, substantiated Emil Fischer's vision!

Van't Hoff in 1906 visited his fellow Nobel laureate Behring in Marburg, where he had built up his serum factory (today the Behringwerke, Germany's biggest and most prominent serum and vaccine manufacturer). Both scientists agreed about the necessity of getting away from antibody titers and serum dilutions to chemically defined entities and their quantitative chemical estimation. As is known, it took some 30 years more — and the life's work of other heroes, around Oswald Avery at the Rockefeller Institute in New York — until Michael Heidelberger (born 1888; Fig. 40) characterized antibodies as gamma-globulins. Heidelberger liked to stress: "We must overcome the tyranny of titers", and he did so. Think how the stereochemistry of IgG has evolved during the last 50 years (Fig. 41)! — In 1988 Michael Heidelberger celebrated his 100th birthday in the best spiritual and physical health. The whole big family of immunologists is thinking of Michael with admiration and great affection.

Our coachman with the horse carriage is still waiting for us (Fig. 42) near the chemical institute. After our long day in old Berlin, we end up our round trip

Fig. 51 August Wassermann

DEUTSCHE MEDIZINISCHE WOCHENSCHRIFT

REDAKTEUR: PROF. DR. J. SCHWALBE — VERLAG: GEORG THIEME

BERLIN W. AM KARLSBAD 8 — LEIPZIG, RABENSTEINPLATZ 2

No. 19. Donnerstag, den 10. Mai 1906. 32. Jahrgang.

Aus dem Institut für Infektionskrankheiten in Berlin (Direktor: Geheimrat Prof. Dr. Gaffky) und der Dermatologischen Klinik der Universität in Breslau (Direktor: Geheimrat Prof. Dr. A. Neisser).

Eine serodiagnostische Reaktion bei Syphilis.

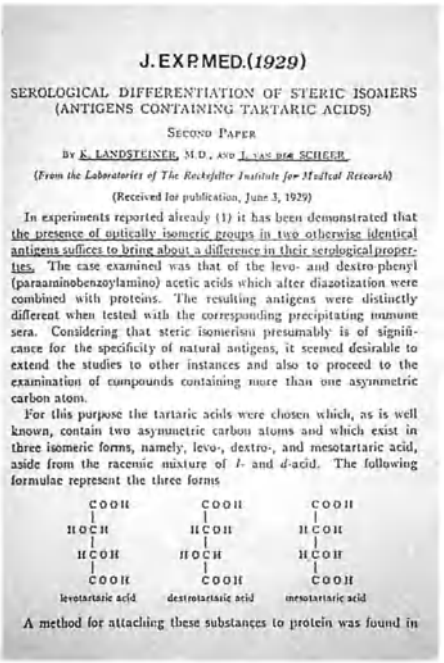
Von A. Wassermann, A. Neisser und C. Bruck.

Fig. 52 Publication of the Wassermann reaction

Fig. 53 Inauguration of the Kaiser-Wilhelm-Institut für Experimentelle Therapie in 1913

Fig. 54 Rockefeller Institute, New York, in the 1920s

Fig. 55 Karl Landsteiner



J. EXP. MED. (1929)

SEROLOGICAL DIFFERENTIATION OF STERIC ISOMERS
(ANTIGENS CONTAINING TARTARIC ACIDS)

SECOND PAPER

BY K. LANDSTEINER, M.D., AND J. VAN DER SCHEER

(From the Laboratories of The Rockefeller Institute for Medical Research)

(Received for publication, June 3, 1929)

In experiments reported already (1) it has been demonstrated that the presence of optically isomeric groups in two otherwise identical antigens suffices to bring about a difference in their serological properties. The case examined was that of the levo- and dextro-phenyl (paraaminobenzoylamino) acetic acids which after diazotization were combined with proteins. The resulting antigens were distinctly different when tested with the corresponding precipitating immune sera. Considering that steric isomerism presumably is of significance for the specificity of natural antigens, it seemed desirable to extend the studies to other instances and also to proceed to the examination of compounds containing more than one asymmetric carbon atom.

For this purpose the tartaric acids were chosen which, as is well known, contain two asymmetric carbon atoms and which exist in three isomeric forms, namely, levo-, dextro-, and mesotartaric acid, aside from the racemic mixture of *l*- and *d*-acid. The following formulae represent the three forms

levotartaric acid | dextrotartaric acid | mesotartaric acid

A method for attaching these substances to protein was found in

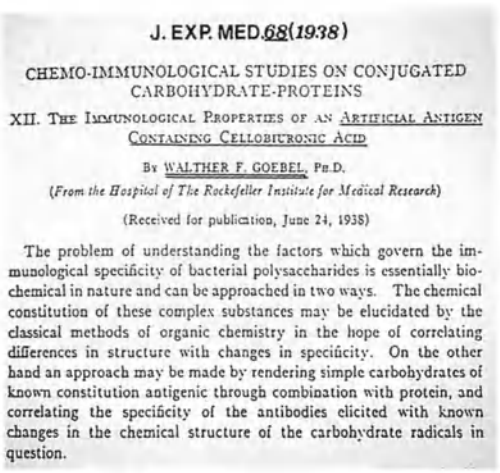
Fig. 56 Title in the Journal of Experimental Medicine (1929)

Fig. 57 Karl Freudenberg

Fig. 58 Hans Sachs

13 · *Members of Avery's department about 1932. Left to right: seated, Thomas Francis, Jr., Avery, Walther F. Goebel; standing, Edward E. Terrell, Kenneth Goodner, René J. Dubos, Frank H. Babers.*

Fig. 59 Oswald Avery with Walther Goebel and other coworkers, photographed in the early 1930s



J. EXP. MED. 68 (1938)

CHEMO-IMMUNOLOGICAL STUDIES ON CONJUGATED CARBOHYDRATE-PROTEINS

XII. THE IMMUNOLOGICAL PROPERTIES OF AN ARTIFICIAL ANTIGEN CONTAINING CELLOBIURONIC ACID

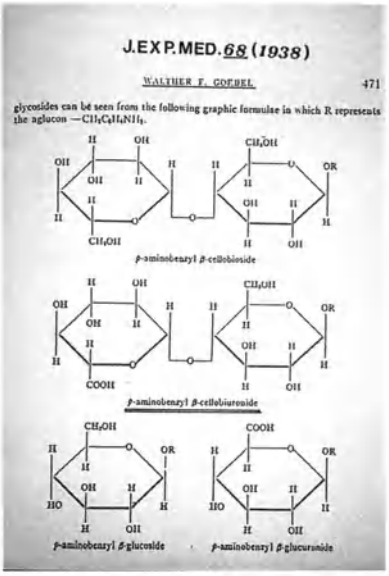
BY WALTHER F. GOEBEL, PH.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

(Received for publication, June 24, 1938)

The problem of understanding the factors which govern the immunological specificity of bacterial polysaccharides is essentially biochemical in nature and can be approached in two ways. The chemical constitution of these complex substances may be elucidated by the classical methods of organic chemistry in the hope of correlating differences in structure with changes in specificity. On the other hand an approach may be made by rendering simple carbohydrates of known constitution antigenic through combination with protein, and correlating the specificity of the antibodies elicited with known changes in the chemical structure of the carbohydrate radicals in question.

Fig. 60 Title in the Journal of Experimental Medicine (1938)



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glycosides can be seen from the following graphic formulae in which R represents the aglucon —$C_{11}H_{14}NH_2$.

p-aminobenzyl β-cellobioside

p-aminobenzyl β-cellobiuronoside

p-aminobenzyl β-glucoside

p-aminobenzyl β-glucuronide

Fig. 61 Formula of the cellobiuronic acid determinant (middle)

Fig. 62 Walther Goebel in the 1950s

Fig. 63 Walter Morgan photographed 1968

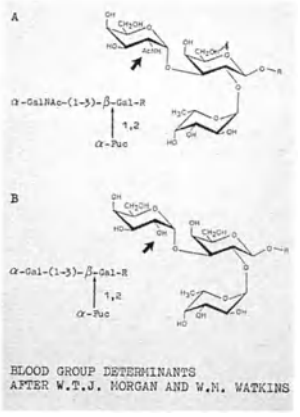

Fig. 64 Blood types A, B, O(H) structures

knowing that there would be more places and more excellent scientists worth visiting. We pay less than 2 Reichsmark for the whole day and say good-bye to the friendly coachman who has brought us back along the main street, Unter den Linden (Fig. 43), to our hotel. There they had been able to reserve tickets for that evening in the opera (Figs. 44, 45). On the next day, the first of a weekend, we may walk through Berlin doing some sight-seeing, or take one of the new tramways to the suburbs (Figs. 46, 47).

Ladies and gentlemen, the First World War, 1914–18, brought a great interruption and change of German and European life. The Belle Epoche was certainly over. However, already before the war, physics in Berlin developed to a high level, with men like Max Planck, Albert Einstein, Max von Laue, Walter Nernst, James Franck and Gustav Hertz, and, in physico-chemistry, Fritz Haber – all sooner or later honored with Nobel prizes. My father, Wilhelm Westphal (1882–1978), was a member of the faculty to which these famous physicists belonged. Many of them visited our home – I myself, at that time, having no idea how privileged I was to share their company as a young boy. In the memoirs of my mother, who was a gifted pianist, I found a passage (Fig. 48) which you may allow me to quote:

"When young married, in 1911, I came to Berlin. I found there a scientific community, the head of which was Max Planck. Besides his physics he was also devoted to music (he had a small choir, and he played the piano marvellously; Fig. 49). So we played a lot of music. I sat trembling beside him when we played pieces for four hands. The G minor symphony of Mozart I will never forget! In his choir (we sang the Liebeslieder waltzes of Brahms) I joined in the singing so intensely that my son Otto was born 10 days too early. ... Also with Einstein (Fig. 50), who played the violin, I played a lot of chamber music, and – again – I sat at the piano trembling in fear of making mistakes. It was a wonderful time, full of emotions."

An attraction for Berlin became the institutes of the newly founded Kaiser-Wilhelm-Gesellschaft, created in 1911 to allow eminent scientists to be free of any other duties, devoting themselves only to research chosen by themselves and with no interference by the state.

In the 1920s, despite great economic problems (remember the inflation in Germany), these institutes began to flourish. Immunology was represented by the Institute for Experimental Therapy with Professor August Wassermann (1873–1934; Fig. 51) as director, widely known for the Wassermann reaction (Fig. 52) for the diagnosis of syphilis. As a pupil of Paul Ehrlich, Wassermann was an expert in complement and immune cell lysis, and in general serologic techniques.

At the inauguration of his institute, in the presence of Kaiser Wilhelm II (Fig. 53), on October 28, 1913, Wassermann gave an address in which he stated: "..Using the defense substances of the organism – which Ehrlich had called antibodies – we can now build up sophisticated serodiagnostics by aid of which it is possible to diagnose many diseases without bothering the patient, namely in the laboratory with the certainty of a chemical reaction". Antibodies as reagents for specific chemical structures, the basis of immunochemistry.

But Wassermann's idea, propagated in 1913, only became reality in the late 1920s

Fig. 65 Kaiser-Wilhelm-Gedächtniskirche, before 1945

Fig. 66 Kaiser-Wilhelm-Gedächtniskirche, 1945

Fig. 67 Kaiser-Wilhelm-Gedächniskirche, after 1945

and far away from Berlin, in New York, at the Rockefeller Institute (Fig. 54), when Karl Landsteiner (1868–1943; Fig. 55) introduced the preparation of artificial antigens by azo-coupling determinants of known structure to immunogenic carrier proteins. He wrote his famous book entitled "The Specificity of Serological Reactions". In 1929, at the Rockefeller Institute, together with J. van der Scheer (Fig. 56), he showed that the three stereo-isomeric tartaric acids – originally discovered by Pasteur and which had so much occupied Van't Hoff – when coupled to protein via the respective p-amino-tartranilic acids, could be serologically clearly distinguished by aid of their specific antibodies!

Landsteiner's publication came to be of crucial influence on my own scientific life – and, here, you may allow me a few more personal remarks. Several years later I visited the well-known stereochemist, Professor Karl Freudenberg (1886–1983; Fig. 57), a pupil of Emil Fischer, in Heidelberg to ask him whether he could accept me for a thesis in organic chemistry. Invited for breakfast at his home, he started to speak enthusiastically about Landsteiner's work on the serology of tartaric acids. He said, that recently he had a kind of dream in which vaguely an idea arose in his mind that the three main human blood types, A, B and O(H) (discovered 30 years ago by the same Landsteiner), might be something like a d-, l- and meso-configuration. "Would you like to work on that question?", he asked me very kindly. Spontaneously I said "yes". Why, I cannot reconstruct. But this set me off on a long way of no return right into immunochemistry.

At Heidelberg University, Professor Hans Sachs (1877–1945; Fig. 58), a highly esteemed pupil of Paul Ehrlich, and his coworkers gave lectures on the basic principles of general immunology and serological techniques as they were understood at that time, in the 1930s. Well-known immunologists like Ernst Witebsky and Erwin Neter, later working in Buffalo, came from Sachs' institute. Thanks to him and his excellent library, I soon felt rather competent with regard to facts and theories about the immune response and the respective literature. But, after they all had left, there were, at that time, only few with whom one could discuss relevant questions.

Realize that in the 1930s and 1940s immunology did not create much interest. Vaccination and serum therapy were more and more replaced by chemo- and antibiotic therapy: first by the sulfonamides around 1934/35 (by the way, the first sulfonamide was a dye, Prontosil rubrum), then penicillin and so on. But in contrast, immunochemistry as a science began to develop beautifully, and this especially at the Rockefeller Institute in New York. Landsteiner's concept of the general make up of antigens was brilliantly exemplified by Walther Goebel (born 1899; Figs. 59, 62) who, in 1938, on the basis of Oswald Avery's work on pneumococcal polysaccharides, synthesized an artificial antigen with the type III-specific disaccharide, cellobiuronic acid, as the determinant (Figs. 60, 61). Goebel's anti-cellobiuronic antibodies protected against manifold lethal doses of highly pathogenic type III pneumococci. Pneumonia, before the times of sulfonamides and penicillin, was killer no. 1 in the United States. How excited we were when reading Goebel's publication in the Journal of Experimental Medicine, now half a century ago! And, may I say, what a "pity" for immunobiology that chemo- and antibiotic therapies (the latter were also developed around the same time and at the same Rockefeller Institute, by René Dubos (born 1901), who discovered tyrocidine and gramicidine) had just efficiently been created... to make the practical

consequences of Goebel's synthetic antigen irrelevant! Only recently, in the hands of Michael Sela in Rehovot – and some others – have such approaches experienced a revival, especially in the field of polypeptide determinants.

Landsteiner kindly took notice of our blood group endeavor in Heidelberg. However, the time was not really ripe. We were only able to pinpoint the carbohydrate nature and show the presence of aminohexoses, galactose and fucose in crude preparations of blood group substance A. After 1945 Walter Morgan (born 1900; Fig. 63) in London took over, and it was mainly him who finally clarified the essential oligosaccharide structures for A, B and O(H) specificity (Fig. 64) – not quite fulfilling Professor Freudenberg's dream. Morgan's and my group, during World War II, independently cross-changed our subjects: Morgan at the Lister Institute changed from enterobacterial O-antigens to blood group carbohydrates – and my group changed from blood group research to bacterial polysaccharide antigens, thus getting into the field of enterobacterial O- and R-antigens and endotoxin (the latter originally discovered by Richard Pfeiffer (1858–1945) while working with cholera bacilli around 1892 in Robert Koch's institute in Berlin).

By sheer chance or luck, for quite some time I was, thus, one of the very few people in Germany scientifically interested in immunology and especially in immunochemistry. When in the 1950s and 1960s the new immunology arose, mainly in the Anglo-American countries, and began also to attract young scientists in Germany, quite a proportion of them went through our institute, since 1962 the Max-Planck-Institut für Immunbiologie in Freiburg, with the late Herbert Fischer (1921–1981) and Otto Lüderitz and myself as directors. In 1967 a handful of immunologists founded the Gesellschaft für Immunologie in Frankfurt. Its history has recently been described by Professor Gerhard Schwick, our first treasurer and secretary, and director of the Behringwerke in Marburg. For the first 10 years I had the honor to act as president, and meanwhile the number of members has reached almost 900. – From my story, you may understand and appreciate why the most prestigious prize of our Gesellschaft für Immunologie was named the Avery-Landsteiner prize. It was first awarded in 1970, at our annual meeting in Strasbourg, to Walther Goebel from New York and to Jacques Oudin (1908–1985) from Paris.

Ladies and gentlemen, the old Berlin around the end of the 19th century exists no more. The First World War drastically changed its social structure, and the second great war led finally to its almost complete destruction (Figs. 65, 66, 67). But all these events did not wipe out the vital spirit of the Berliner and of Berlin as a cultural center – science being understood as part of human culture. The intuition and insight of the great scientists of the Belle Epoche, 100 years ago, are still alive. The great scientists themselves would be astonished to realize the great progress that has been achieved in immunology during our century, and how many scientists in the meantime are participating, a progress that could hardly have materialized without the life's work of our scientific forefathers. They would probably have a lot of difficulty understanding what active immunologists today are speaking about.

I hope you can appreciate the message that without understanding our history we cannot really understand our own times. We are all links in a long chain connecting past and future scientific achievements. This chain must never break.

O. WESTPHAL

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I Structure and Function of Recognition and Effector Molecules

The T Cell Receptor Complex

T Cell Receptors

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INTRODUCTION AND REVIEW

Mammmals have evolved a number of different ways of recognising the arrival in their bodies of a foreign invader. At least 3 of these appear to be antigen specific, by virtue of the fact that the foreign invaders are recognised by receptors which vary in sequence from one cell to another.

These clonally variable receptors are, of course, B cell immunoglobulins (Ig) and two different types of receptors found on T cells (TCR), the so-called $\alpha\beta$ and $\gamma\delta$ receptors. All of these proteins are constructed from functional genes which are assembled in precursor B or T cells by rearrangement of genes coding for their component parts. Although each type of receptor gene is made up of different component parts, the constructions of the different receptors do have some common features. These are illustrated in Table 1, which lists the approximate numbers of the genes which are used to make up mouse Ig and TCRs.

For example all of the receptors contain constant (C) region sequences, regions of little variability from one clone to another. Crystallographic solution of the structure of Igs, and a fit to this solution of TCR sequences, suggests that the C region portions of these receptors do not contact antigen but rather are concerned with some other function of the receptors, such as membrane binding, or, in the case of Ig, with the known functions of the different Ig isotypes. In spite of the fact that TCRs are not (usually) secreted molecules it is worth noting that both $\alpha\beta$ and $\gamma\delta$ receptors are made up of one chain with a single constant region, and another which has 2 or more options for its C region. The 2 β chain C regions are very similar in sequence in both mouse and man. At the moment we are at a loss to explain why 2 such genes exist. Other species may have more, but no example, besides the NZW mouse, of fewer β chain C region genes/haploid set has yet been described. The γ chain C region sequences are less like each other (in man there are only 2). Nevertheless, there is still no clear understanding of why the γ chain locus would require more

than 1. Since particular Vγ genes can only rearrange to particular JγCγ combinations, perhaps the multiple Cγ sequences are required to allow the animal to rearrange and construct different γ chain sequences at different times ontologically (Havran and Allison 1988).

Table 1. Components of Clonally Variable Receptors on Mouse Lymphocytes

Receptor	Chain	Numbers* of Available Genes V	D	J	C
Ig	Heavy	~200	10-20	4	8
	Light	~200	none	4+2	3 or more
TCR $\alpha\beta$	β	21 or less	6	12	2
	α	~50	none	~50	1
TCR $\gamma\delta$	δ	? >7	2	2	1
	γ	6	none	3	3

* Numbers are approximate and do not always include considerations such as the fact that δ chain rearrangements can include one, both or neither of the available D regions. Data are from Honjo 1983; Tonegawa 1983; Kronenberg et al. 1986; Elliott et al. 1988.

As illustrated in Table 1, it is a fact that each type of receptor is made up of 2 clonally variable chains, one of which is made up of 2 types of varying genes, V and J, and the other of which is made up of 3 such genes, V,D and J. Additional variability is created for each chain by insertion of nongerm line encoded bases or removal of germ line encoded bases (N region bases) at the junctional points between D and J, V and J and V and D. Genes that can contain D regions, heavy chain, β or δ, rearrange in precursor cells before those which do not, light, α or γ. In most cases D to J rearrangements precede V to DJ (Alt et al. 1981; Born et al.) , although there is some evidence that this is not always true in δ gene construction. There is also evidence, not only from the DNA signal sequences involved, but also functionally, that the same enzymes are involved in rearrangements of all these genes.

T cell receptor $\alpha\beta$ and $\gamma\delta$ genes are rearranged in the thymus. Rearrangement and expression of these genes can also occur extrathymically, but T cells bearing these receptors appear much more slowly in the absence of a thymus, particularly in the case of those bearing $\alpha\beta$ receptors. A number of studies are in progress to decipher the relationship between $\alpha\beta$ and $\gamma\delta$ precursors. Delta chain genes rearrange first, and successful α chain rearrangements delete δ genes, because the δ locus lies within that of α (Chein et al. 1987), so expression of these 2 gene complexes may be mutually exclusive.

Apart from the fact that $\alpha\beta$ and $\gamma\delta$ cells arise in the same location, probably from the same precursors, these cells do share 2 other features. First, both types of receptors require for surface expression association with a complex of polypeptides known as CD3. CD3 is made up of at least 5 different kinds of polypeptide and seems to be required not only for good surface expression of the clonally variable receptors, but also for transduction to the inside of the T cell of signals consequent upon the binding of $\alpha\beta$ or $\gamma\delta$ to their ligands outside the cell. CD3 therefore serves essential functions for all T cells.

It is worth considering why T cells require this complex collection of proteins for signal transduction. Other receptors bear ligand binding domains and signal transducing domains on a single polypeptide chain. Some receptors may require more than one polypeptide for both functions, but certainly do not involve as many as 7 or more components. We would like to suggest that the complexity of the TCR/CD3 receptor stems from the fact that binding to this receptor is required to have different consequences for the T cell bearing it at different stages in the cell's life history. This is particularly true in the case of $\alpha\beta$ T cells, which are known to be subject, via their $\alpha\beta$/CD3 receptors, to positive selection, for maturation, and negatively selection, for clonal deletion, in the thymus. Also, binding to this same receptor complex must signal proliferation and activation or perhaps inactivation, by anergy, on peripheral T cells. It is possible that the TCR manages to deliver all these different signals to the cell which bears it because of differences in the component parts of the TCR/CD3 complex at different stages in the history of the T cell. It has been shown, for example, have shown that cells lacking ζ respond differently to stimulation of their TCRs than do cells which express ζ (Mercep et al., 1988). We have recently found that at a particular stage of thymocyte development the TCR is not coupled to CD3 in the same way as it is on mature cells (Finkel et al., 1989). Perhaps this reflects some differences in the components of CD3.

A second similarity between $\alpha\beta$ and $\gamma\delta$-bearing T cells is that both can bear the so-called accessory molecules, CD4 or CD8. In both cases this appears to be optional, although mature $\alpha\beta$ cells bearing neither are much less frequent than their $\gamma\delta$-bearing counterparts. CD4 and CD8 are thought to contribute to T cell reactivity by binding to Class II or Class I molecules respectively on target cells. Such an idea is entirely in line with the known distribution of CD4 or CD8 on Class II or Class I restricted $\alpha\beta$ T cells. However, the rationale for the presence of these accessory molecules on $\gamma\delta$ cells is less clear, given our current cloudy understanding of the ligands for such cells (see below).

It seems that 3 different types of clonally variable receptors exist on lymphocytes because each has evolved to recognise antigen in a distinct fashion. Immunoglobulin binds native antigen and $\alpha\beta$ TCRs bind antigenic peptide fragments bound to major histocompatibility complex proteins (MHC). The usual ligands of $\gamma\delta$ TCRs have yet to be identified, but for a subclass of these receptors they appear to be heat shock proteins derived either from mycobacteria or self, or perhaps some class of MHC related products (O'Brien et al. 1989; Janis et al. 1989; Matis et al. 1988).

SPECIFICITY FOR ANTIGEN AND MHC OF $\alpha\beta$ T CELL RECEPTORS

Studies on the specificities of $\alpha\beta$ TCRs have led to some surprising conclusions. First, there are no obvious differences between the components of the TCRs on Class I- or Class II-restricted cells. Immunologists, therefore, are currently ignorant of the criteria (apart from expression of CD4 or CD8) which determine the restriction specificities of T cells.

Some efforts have been made to screen T cells with specificity for a particular allogeneic MHC protein to find out whether or not such cells bear receptors with common features. With a single type of exception discussed below, receptors for a particular allogeneic MHC molecule have turned out to be very heterogeneous, with no obvious common features. It has been suggested in the past, and in light of current knowledge this suggestion is probably correct, that allogeneic MHC is probably a vast array of ligands made up of a foreign MHC molecule bound to many different peptides, not all of which may themselves be allelic (Matzinger and Bevan 1977). If this is true, then it is not at all surprising that the repertoire of $\alpha\beta$ T cells for allogeneic MHC is very large.

In fact the same is usually true for T cells specific for a given antigenic protein, and even, in some cases, for a given peptide. It is only when the peptide is whittled down to a bare minimum or is for some other reason relatively non immunogenic that a limited T cell repertoire can be found (Fink et al. 1986). Under these circumstances it is possible to determine which residues on the $\alpha\beta$ TCR contribute to its specificity for antigen and MHC.

In general the conclusions from this type of experiment have been that all the variable components of the TCR contribute to its ability to bind its antigen/MHC ligand. Although several models have been suggested for the structure of the TCR/antigen/MHC complex, our own feeling is that the complex will have structure very similar to that described for the binding of Ig to a protein target, with contact residues over a large area of both Ig or TCR and their ligands, derived by folding from many different points on the linear sequence of both receptor and ligand.

SPECIFICITY FOR SELF SUPERANTIGENS AND MHC OF $\alpha\beta$ T CELL RECEPTORS

Recently we and others have come across some interesting exceptions to the rules discussed above. Some antigens are able to bind to MHC Class II molecules and thus form a ligand which is able to stimulate almost all T cells bearing a particular Vβ as part of their receptor, almost regardless of the rest of the variable components of the receptor. Such antigens are therefore able to stimulate a significant proportion of all T cells, especially in the mouse, which possessing 21 or less Vβs, and expresses each Vβ on average on about 5% of all T cells. Because antigens of this type stimulate so many T cells we have suggested the name superantigen to describe them.

The first superantigens to be discovered are made by mice themselves and are either themselves allelic, or depend upon an allelically expressed MHC molecule for recognition by T cells. An apparently nonallelic B cell-derived antigen, for example, which reacts with almost all T cells bearing Vβ17a, is dependent upon binding to IE for this property (Kappler et al. 1987). For various reasons IE is not expressed by all MHC haplotypes, and therefore some mice express this Vβ17a-stimulating ligand, and others do not. Mls-1^a, on the other hand, is an allelic product which can bind to most MHC Class II molecules, and thereby stimulate almost all T cells bearing Vβ6 and 8.1 (MacDonald et al. 1988; Kappler et al. 1988).

Animals which express a particular Vβ ligand delete all T cells bearing that Vβ during development in the thymus, and

the resulting restriction in T cell repertoire is surely the most significant consequence of expression of a superantigen for the mouse concerned (Kappler et al. 1987, 1988).

Of the mouse Vβ regions which can be studied accurately at the moment, ie those against which monoclonal antibodies are available, nearly all demonstrate evidence that a superantigen/MHC combination expressed in some mice is able to delete T cells bearing that Vβ. The Vβs deleted include Vβ3, 5.1, 5.2, 6, 7, 8.1, 9, 11 and 17a. The only exceptions to this rule at the moment are Vβ2 and 14.

So far superantigens have not been found which affect Vα expression in a similar way. This could be because Vαs are much more difficult to study in mice than are Vβs. What little evidence there is, however, suggests that self superantigens might not have such a profound affect on Vα expression as they do on Vβ.

Because the effect of self superantigens is the deletion of a group of T cells, and consequent diminution in the peripheral T cell repertoire available to a mouse, one might imagine that self superantigens would not be advantageous to wild mice, which have to contend with a universe of pathogens. To test this idea we have recently examined a collection of wild mice for expression of such antigens. Thirty nine wild mice, trapped in Florida, were tested for expression of Vβs on their peripheral T cells and thymocytes. Several conclusions could be drawn from the data we obtained:

1. About 1/3 of the animals were homozygous for a large deletion at the Vβ locus, involving 12 mouse Vβs. This deletion had different boundaries from that previously described for SJL, SWR, C57L and C57BR mice, and wild mice trapped on islands north of Scotland (Behlke et al. (1986). The deletion also differed in its boundaries from that recently described by Haqqi et al. (1989) for the RIII strain of mice. Therefore Mus musculus expresses at least 4 different Vβ genotypes, at least 3 of which contain large, independent deletions of Vβs. Interestingly, all 3 deletions involve many of the same Vβs, whether this is due to a characteristic of the DNA sequence around the region of deletion, or to natural selection of animals which have deleted these Vβs remains to be determined.

2. We found evidence for expression in individual animals for all except one of the mouse self superantigens known from studies of laboratory mice. Thus self superantigen/MHC combinations caused deletion of Vβs 3, 5.1, 5.2, 6, and 8.1. Surprisingly Vβ11, which is deleted in laboratory mice by a superantigen plus IE, was not often absent in the wild animals even though all but one of the

mice expressed IE. Whether this was due to a variant Vβ11 gene, or self super antigen in the wild mice could not be tested.

3. The wild mice contained 2 variant forms of the Vβ8.2 gene. One of these led to almost complete interaction of TCRs which used it with the mouse superantigen, Mls-1^a. Consequently mice homozygous for this Vβ8.2 and expressing Mls-1^a, contained very few peripheral T cells bearing Vβs 6, 8.1 and 8.2. Another variant of the 8.2 gene contributed to receptors with markedly increased reactivity with Mls-1^a by comparison with the normal Vβ8.2 (50% reactivity). The Vβ8.2 genes in question were sequenced and found to contain 5 and 1 amino acid changes respectively by comparison with Vβ8.2 in laboratory mice. These changes may be the first indications of the sites of interaction of the self superantigen/MHC ligand with Vβ.

4. All the wild mice expressed at least one self superantigen. The fact that these antigens all map independently of each other, and of TCR and MHC genes led in the wild mice to tremendous diversity between individuals in the expression of their Vβ repertoires. Thus the wild mouse populations we studied had within it functional genes for all but one of the known mouse Vβs, but no individual animal expressed them all, and each animal differed in the collection of Vβs it could use as part of its T cell repertoire.

5. We have begun to examine man for similar Vβ-deleting self superantigens. In limited sampling of middle class Americans no such antigens have become apparent.

So far no satisfactory model has been built to account for the structural association of self superantigens, such as Mls-1^a, with Class II MHC proteins, and TCR Vβs. This is partly because the structure of the self superantigens themselves is unknown. There is some evidence that these entities can be transferred, but not very efficiently, from one cell to another. Apart from this we do not even know whether or not the molecules are proteins, they may be peptide derivatives of proteins, and it has even been suggested that they are in fact lipid components of the cell membrane.

Whatever the nature of the Vβ-deleting self superantigens one property is apparent. They represent a very sophisticated and flexible method of distributing Vβ expression throughout the mouse population. Since tolerance mediated by clonal deletion is dominant, absence of T cells bearing a particular Vβ because of superantigen-mediated deletion will be apparent even in heterozygous animals. This is not the case for Vβ absence mediated by gene

deletion. Moreover 2 heterozygous, $V\beta^-$ animals can give rise, in a single mating, to offspring in which the $V\beta$ will be expressed. Again, this is not possible for gene deleted $V\beta$s.

MICROORGANISMS CAN PRODUCE SUPERANTIGENS

In the absence of a cloned self superantigen gene, immunologists have had to turn for evidence about how superantigens might operate to what seems to be a related system. It has been known for some years that some bacteria produce toxins which are powerful T cell stimulants, in mouse and man. Recently, several groups have shown that these toxins bind to Class II proteins, and that this is required in order for T cell stimulation to occur. We and others have studied these toxins, and shown that each acts as a superantigen, stimulating T cells in a $V\beta$ specific way (White et al. 1989; Janeway et al. 1989). Staphylococcal enterotoxin B (SEB), for example, stimulates all mouse T cells bearing $V\beta$s 3, 7, 8.1, 8.2 and 8.3. The staphylococcal toxin responsible for induction of toxic shock, on the other hand, stimulates mouse T cells bearing $V\beta$3 and 15. In man, likewise, each toxin is $V\beta$ specific in its action (Kappler et al. 1989).

Many of the sequences of these toxins are known. We have therefore studied the structural features of the toxins which contribute to the ability of the proteins to act as superantigens. Our finding, at that of others, is that on the whole the intact protein is required for activity. Denaturation and proteolytic digestion destroys the ability of these toxins to stimulate T cells. Moreover the toxins themselves do not appear to bind to Class II molecules at the same site as conventional antigenic peptides, nor do the toxins compete for presentation with such peptides. The toxins, therefore, do not seem to bind to Class II MHC molecules in the groove described by Bjorkman et al. (1988). They may well wrap themselves around Class II proteins in some unconventional manner and thereby interact with TCRs, primarily through $V\beta$.

Functionally the Staphylococcal toxins appear to be very good models for the action of mouse self superantigens. Structurally, we are not so sure that they mimic such entities. If self superantigens, such as Mls-1^a, were to be 240 amino acid proteins with tight binding to Class II as the toxins are, we might have expected such entities to be identified by now, either by straightforward protein chemistry and coprecipitation, or by antibodies.

Whether or not the Staphylococcal toxins, and other microbial products like them, are good structural models for

self superantigens, they do provide a rationale for the existence of the self superantigens. Our studies have shown that mice which have deleted all T cells bearing a Vβ with which a particular toxin can interact are resistant to the pathological effects of that toxin. Therefore self superantigens may exist in wild mice to contribute to resistance to these types of toxin. We must admit that we find it surprising that the products of a few bacteria, such as the species of Staphylococcus or Streptococcus found in mice, could have such a profound effect on the repertoire of peripheral T cells but in the absence of any other explanation for the phenomena we observed in wild mice, this seems to be the case.

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Positive and Negative Selection of T Cells: A Transgenic Model

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INTRODUCTION:

Functional T cells have the ability to respond to a myriad of antigens. This recognition is accomplished via a heterodimeric cell surface receptor called the antigen-specific T cell receptor (TCR). However, unlike the immunoglobulin molecules that can recognize the nominal antigen directly, the TCR can recognize antigens only in the context of self-MHC molecules, a phenomenon called MHC restriction. To generate the sequence diversity necessary to recognize the many antigens, the TCR genes undergo somatic DNA rearrangement similar to those of the immunoglobulin genes. Because this rearrangement event appears to be random, we surmised that certain combinations of the TCR rearrangement may be self-recognizing and thus autoreactive. To determine the fate of such autoreactive cells, we introduced a TCR from an alloreactive T cell clone, 2C, into a transgenic mouse and followed its fate.

CONSTRUCTION OF THE TCR TRANSGENIC MOUSE:

2C is a CD8 dependent cytotoxic T cell clone that was obtained by immunizing Balb/b mouse with Balb/c cells. Its specificity has been mapped to the Ld MHC Class I molecule. Two monoclonal antibodies are available to track the 2C TCR. F23.1 antibody recognizes members of the $V_\beta 8$ family and thus the 2C β chain ($V_\beta 8.2$). 1B2 Antibody is a clonotypic antibody that recognizes the combination of 2C α and β chains. After initial failure in attempts to create a transgenic mouse whose transgene was expressed in vivo, we constructed TCR α and β constructs which contained up to 40 KB of DNA using cosmid vectors.

After confirming the authenticity of the transgenic animals by breeding experiments, RNase protection experiments were performed to analyze for the expression of the transgenes. These studies showed that expression was indeed tissue specific--high in thymus and lower in spleen while brain, heart, kidney and liver were negative. To ascertain the expression at the protein level, we performed fluorescent-activated cell sorting experiments using F23.1, 1B2, anti-CD8, and anti-CD4 antibodies. (Sha, 1988a)

SELF-TOLERANCE TO MHC ANTIGEN PRESENT IN THE THYMUS IS ACCOMPLISHED BY CLONAL DELETION:

Initially, the transgenic mice analyzed were of the H-2b haplotype. In these mice, the clonotype positive cells were found almost exclusively as $CD4^-CD8^+$ cells in the spleen. To follow the fate of these cells in an autoreactive environment, these mice were mated to H-2d mice. The resulting H-2(bXd) progenies were analyzed for the type and distribution of the clonotype positive cells. In the spleen, there were clonotype positive cells but these were all $CD4^-CD8^-$ although $F23.1^+CD8^+$ were present. In the thymus, clonotype positive cells were only found among the $CD4^-CD8^-$ populations. No functional clonotype positive $CD4^-CD8^+$ were found. These findings support the clonal deletion hypothesis of tolerance development against antigens that are present in the thymus (otherwise called negative selection). (Sha, 1988b)

MHC RESTRICTION--A POSITIVE SELECTION MODEL:

To our initial surprise, when the 2C transgenic mice were crossed into the H-2s background, clonotype positive $CD4^-CD8^+$ failed to emerge in the spleen. To rule out tolerance development by negative selection in the thymus, mice of the H-2(bXs) were analyzed and found to contain functional clonotype positive $CD4^-CD8^+$ cells in the spleen. In addition, the distribution of the clonotype positive cells in the thymus of H-2s mice differed markedly from those in the H-2(bXd) mice--namely, the clonotype positive cells were almost exlusively $CD4^+CD8^+$. To explain these data, we hypothesized that the absence of functional cells in the H-2s mice was not due to negative selection but rather due to lack of positive selection. We postulated that an element in the H-2b was necessary for the normal development of the 2C receptor bearing T cells allowing interaction in the thymus and giving them a "positive" signal. This would explain why the cells develop normally in the H-2(bXs) animal.

IMPLICATIONS OF THE POSITIVE SELECTION MODEL:

Positive selection model of T cell development implies that the thymocytes interact with self-MHC molecules as part of normal differentiation. It suggests that this interaction is H-2 allele specific and is a mandatory step. Little is known of the molecular interactions and subsequent signalling that occurs. Based on earlier data, we postulate that it is the radioresistant epithelial cells in the thymus that provide the MHC molecules involved in this positive selection step. Whether specific forms of MHC molecules (possibly due to peptides bound) unique to these cells accomplish this task cannot be ascertained at the present moment. Alternatively, it is possible that the thymocytes are at a distinct stage of development as it encounters the thymic epithelial cells and thus are uniquely susceptible to signals resulting in positive selection. These and other hypotheses dealing with the exact nature of the molecular interactions between the TCR and the MHC molecules must await further experimentation.

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Selection of an $\alpha\beta$ T Cell Antigen Receptor In Vivo and Engineering a Solulizable Form

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INTRODUCTION

Much progress has now been made in terms of identifying and characterizing the molecules important in T cell recognition, both receptors and ligands. Many issues remain unresolved, however, among these are: how and on what basis T cell receptors are selected in the thymus? and the precise nature of the T cell receptor-antigen-MHC recognition event. Towards addressing these issues we have developed two types of experimental systems. The first involves mice transgenic for specific T cell receptor genes crossed onto different MHC backgrounds. With suitable serological probes we can follow the expression of both α and β chains and the progress (or lack thereof) of T cells bearing these receptors in the animal. Together with the work of von Boehmer and colleagues and Loh and colleagues (this volume) the results of these studies give us some very clear ideas about the reality and efficiency of both positive and negative selection in the thymus. In addition, these transgenic systems should make possible the biochemical characterization of these phenomenon in the near future.

A second area we have been interested in for some time concerns how best to make soluble forms of T cell receptor heterodimers in order to move the bases for discussion from a cellular readout (e.g. T cell activation assays) to a molecular one (such as binding and structural studies). Towards this end we have recently been successful in expressing both a mouse and a human $\alpha\beta$ heterodimer in a lipid-linked fashion that can then be cleaved off the cell-surface to produce soluble protein. Prelimimary indications are that this type of expression of $\alpha\beta$ TCR (in the absence of CD3) is faithful to the original and can take us a long way toward the goal of better understanding the troika of T cell recognition (TCR-Ag-MHC) at a molecular level.

POSITIVE SELECTION

While the ligand for $\alpha\beta$ T cell receptor in the periphery (antigen-MHC) seems clear, what T cell receptors see in the thymus has been a very controversial issue. Kisielow et al.

(1988b) have provided data indicating that positive selection for a class I + HY specific T cell receptor can be provided by the original restricting element (D^b). Similarly, Loh et al. (this volume) have obtained evidence that the D molecule may also be the positively selecting element in their transgenic system. In our own work, we have recently obtained convincing evidence that the original restricting element of the 2B4 T cell receptor, namely, I-E, is necessary and sufficient for the efficient export of transgene expressing T cells from the thymus. We have demonstrated this in two ways (Berg et al., 1989b):

1) Peripheral T cells expressing both α and β chain TCR's of 2B4 are much more abundant in $H\text{-}2^k$ bearing mice than in $H\text{-}2^b$ homozygotes (which lack functional I-E expression) as judged by surface staining. This effect can also be seen in $H\text{-}2^b$ mice that carries a functional E_α transgene.

2) The frequency of moth cytochrome c reactive T cells in the periphery of $H\text{-}2^k$ mice is at least 10 times higher than in $H\text{-}2^b$ homozygotes. Again, this can also be seen on b haplotype animals bearing E_α transgenes.

In addition E_α deletion mutants which express I-E predominantly in either the medullary or cortical regions of the thymus (Van Ewjik et al., 1988) show clearly that positive selection can occur even with MHC expression only on cortical epithelial cells. In contrast, medullary MHC expression has no apparent effect (Berg et al., 1989b; Benoist and Mathis et al., 1989). The general thymic phenotype of $\alpha\beta$ transgenic mice in the absence of an appropriate restricting element is that of an arrest at the double positive ($CD4^+8^+$) stage of differentiation. That is, very few single positive thymocytes are detectable.

In addition, we see a strong disposition towards CD4 expression, especially in peripheral cells in the presence of the restricting element (I-E). This is consistent with the observations of Kisielow et al. (1988a) and Sha et al. (1988a) in which class I MHC specific T cell receptor transgene expression promotes a pronounced skewing towards CD8 expression. Thus it seems that the MHC class specificity of a given T cell receptor somehow determines its accessory molecule (CD4 or CD8) phenotype. It would be interesting to determine how this is accomplished.

NEGATIVE SELECTION

In the 2B4 transgenic system we have also encountered negative selection based on Mls reactivity, which helps to resolve some of the current issues in the literature (Berg et al., 1989a). In particular, there is a quantitative removal of cells expressing a

high level of the 2B4β chain because of the combination of its $V_\beta 3$ component and the Mls $2^A/3^A$ genotype (C3H/HeJ) of one of the parental strains (as defined by Pullen et al., 1988). We found deletion of mature 2B4β positive cells to be evident in the thymuses of both 2B4 $\alpha\beta$ TCR mice, as well as 2B4β mice. What was interesting, however was the very different phenotype exhibited by these two types of mice, both of which were undergoing massive and efficient negative selection. The thymic CD4/CD8 profile indicates that the β mice had a very similar arrest in the double-positive stage as seen in the H-2^b $\alpha\beta$ 2B4 transgenics or in normal mice undergoing Mls mediated deletion (Kappler et al., 1988). In contrast, the $\alpha\beta$ mice which deleted $V_\beta 3$ had a greatly reduced percentage of double positive cells (2% of the total) and overall less than 1/10 of the normal number of thymocytes, exactly as reported by Kisielow et al. (1988a) for HY + D^b, and as seen in the high-expressing mice of Sha et al. (1988b). We therefore conclude that the presence of $\alpha\beta$ TCR transgenes greatly augments the affects of negative selection, perhaps by speeding up the kinetics of T cell differentiation and selection in the thymus (Berg et al., 1989a). This is in fact predictable from the observations that the TCR α chain is the last TCR to be rearranged and expressed during thymic development and thus the presence of a rearranged α chain is bound to have an effect on maturation of at least some T cells. In fact, current data (Fazekas de St. Groth, et al., in preparation) indicates that TCR α transgenics express $\alpha\beta$ TCR fully 2 days before normal (d15 vs. d17) in fetal mice and at inappropriately high levels. This effect may be due to the absence of normal controlling elements, especially the silencer regions 3' of C_α, recently described by Winoto and Baltimore (personal communication).

Another important point is that this study of negative selection shows the essential equivalence of Mls-mediated deletion versus antigen + MHC (Kisielow et al., 1988a) and alloreactive (Sha et al., 1988b) deletion in $\alpha\beta$ transgenic mice. This indicates that Mls mediated deletion is, in fact, a valid model for the developmental aspects of self-tolerance.

SOLUBLE T CELL RECEPTOR HETERODIMERS

Because TCR heterodimers are assembled with the CD3 polypeptides to form complexes of at least seven polypeptides before appearing in the surface (Minami et al., 1987) they may be one of the more difficult types of molecules to produce in a soluble form. On the other hand, the very Ig-like character of its V, D, J and C region elements, makes it very likely that it: 1) can bind to Ag-MHC by itself and 2) that structurally it could exist in solution much like an antibody. Thus the challenge has been to find conditions in which TCR chains can be expressed and form heterodimers free of CD3 molecules, either secreted from cells in culture or in a membrane-associated form that could be easily cleaved from the surface. Initial attempts in our lab took the

form of TCR(V)-Ig(C) hybrids expressed in myeloma cell lines (Gascoigne et al., 1987). Interestingly, only V_α (TCR) C_H (Ig) chimeras could be assembled or secreted as apparently normal Ig molecules (being assembled and expressed with light chains). Neither V_β (Gascoigne et al., 1987), V_γ nor V_δ (R. Wallich et al., unpublished, MacNeil et al., unpublished) gene segments could be expressed in that same context, suggesting that there is some structural barrier to proper folding. Recently, we became aware of the increasingly large list of surface proteins known to be lipid-linked (as reviewed in Ferguson and Williams, 1988). All such proteins can be cleaved from the surface with a specific enzyme, PI-PLC, to produce soluble forms. Caras et al., (1987) had shown that the C-terminal 37 amino acids of decay accelerating factor (DAF), could serve as the signal sequence for the lipid linked expression of a Herpes Simplex virus membrane protein. We decided to apply this finding to T cell receptors in the hopes that expressing TCR polypeptides as PI-linked molecules might allow them to associate in the plane of a membrane to maximize heterodimer formation and to be cleaved off the surface of expressing cells to produce a soluble form.

Thus far, we find that, in most cases, TCR-PI-signal sequences chimeras are expressed well in both COS cells (for transient transfections) and CHO cells (for long-term transfectants). Single chains of the TCR could be expressed with high efficiency and cleaved off the surface of expressing cells with PI-PLC (in early experiments, the kind gift of Dr. Martin Low). Expression could be demonstrated by either the immunoprecipitation of surface iodinated cells or fluorescence tagging and analysis by microscopy or FACS. Immunoprecipitation indicates both dimers and monomers, in most cases (A. Lin et al., in preparation; B. Devaux et al., in preparation). To demonstrate that heterodimers between α and β were being formed that juxtapose V_α and V_β determinants, we made use of the fact that preincubation of 2B4 TCR bearing cells with saturating amounts of anti-$V_{\alpha 2B4}$ (A2B4-2 from Samelson et al., 1983) antibody abolished staining with the anti-$V_{\beta 3}$ antibody (KJ25 from Pullen et al., 1988). This same cross-blocking phenomenon can also be shown to occur in CHO cells expressing the 2B4 α and β chains in a lipid-linked form (Lin et al., in preparation). Currently we are able to purify hundreds of micrograms of soluble receptor heterodimers. Perhaps the most crucial test is whether TCR expressed in this way can bind to antigen-MHC complexes. Preliminary indications are that it can, with iodinated soluble 2B4 TCR able to bind to $I\text{-}E^k$ bearing cells in the presence of the appropriate moth cytochrome c polypeptide. Thus, we are very hopeful at being able to bring T cell recognition into the biochemical and biophysical realm.

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Mice Transgenic for the T Cell Receptor Cγ4 Gene are Altered in T Cell Development

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INTRODUCTION

Lymphocyte interaction, induced and maintained by antigenic stimuli, is mediated in B cells by immunoglobulin and in T cells by the αβ T cell receptor (TcR). It is well established that in B cells, free antigen is sufficient for binding to immunoglobulin (Davies and Metzger 1983). However in T cells, foreign antigens peptides must be associated with the appropriate major histocompatibility antigens (MHC of "self" molecules) in order for the αβ TcR to be activated biologically (Ohashi et al. 1985; Dembic et al. 1986; Saito et al. 1987).

In the process of searching for the α-β T cell receptor , a second TcR, γδ, has also been discussed (Brenner et al. 1986; Saito et al. 1984; Chien et al. 1987a, 1987b; Iwamoto et al. 1986) and shown to be on the surface of T cells in various anatomical locations (Asarnow et al. 1988; Elliott et al. 1988; Goodman and Lefrancois 1988; Havran and Allison 1988; Steiner et al. 1988). As this receptor was identified mainly based on its structural similarly to the α-β receptors, the physiologic role of cells that bear these receptors is still a mystery. The failure to arrive at a role for these receptors is also partly due to the lack of biological models available to measure their function. Rapid progress, however, has been made in the identification of γ and δ genes, their temporal and spatial expression, the cloning of T cells that express γδ receptors, the development of monoclonal antibodies against γδ receptors and the generation of in vivo models that alter the expression of these receptors. These efforts have generated an enormous amount of data bout the γδ receptor that is being used to design experimental models to characterize their elusive biological function(s).

TcR γδ Diversity and Chromosomal Structure. As shown in Figure 1, there are many fewer variable (V) and joining (J) gene segments available to the γ and δ loci suggesting a more limited number of receptor binding conformations for the γδ TcR. However, if one takes into account that both Dδ segments can exist in tandem or separately and can be read in all three reading frames; plus the extensive amount of N-region diversity observed within rearranged δ chain genes, then a comparable number of possible unique binding conformations can exist for γδ as compared to αβ cells.

T CELL RECEPTOR REPERTOIRE IN MAN

	Alpha - Beta		Gamma - Delta	
V	100	100	10	6
D	?	≥2	?	2 (3)
J	100	13	4	3
C	1	2	2	1
	10^4	10^4	40	40
Recombinations	10^8		10^3	
* N-sequences	10^4	10^8	10^4	10^{12}
Total Diversity	10^{20}		10^{19}	

* assume 3 a.a. at each junction

Fig. 1. The potential diversity of human γδ and αβ T cell receptors generated from the aproximate total number of gene segments available and taking into account N-region diversity.

A rather unique and intriguing aspect of both receptors is that the δ chain locus resides in the middle of the α locus, such that, whenever α chain rearrangement occurs the intervening δ gene segments are deleted . This chromosomal organization and recent experimental evidence showing the existance of a δ deleting element (de Villartay et al. 1988a, 1988b; Ohashi et al. submitted), have in fact led to the suggestion that lineage determination between αβ and γδ may occur before rearrangement. This implies that whatever regulatory mechanism exists for the determination of entry into either the αβ or γδ TcR lineages, the site of control most likely exists in the delta locus. In addition, this would suggest that there is not a direct precursor relationship between the γδ and αβ lineages. A schematic diagram for the development of α-β and γ-δ cells is illustrated in Figure 2.

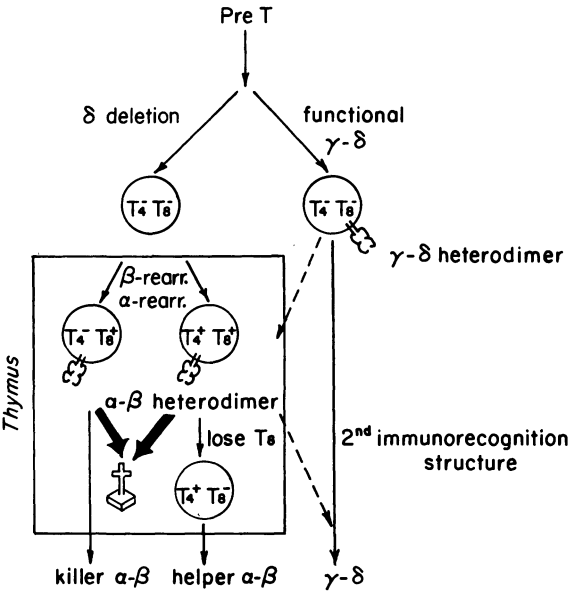

Fig. 2. Possible scheme for lineage commitment of α-β and γ-δ cells.

Fetal and Spatial Expression of $\gamma\delta$ TcR. Cells that express $\gamma\delta$ receptors are the first immigrants to colonize the thymic rudiment (Havran and Allison 1988) and exist in mice as a major population starting at day 14 of fetal development and declining substancially by day 18 of gestation. Thymocytes that express $\gamma\delta$ are for the most part double negative ($CD4^-$ $CD8^-$) (Pardoll et al. 1987; Cron et al. 1988) and in the periphery exist as a minor T cell population of about 1-5% (Brenner et al. 1987; Cron et al. 1988). Three areas where $\gamma\delta$-bearing cells exist essentially in the basence of $\alpha\beta$ is in the skin ($Thy\text{-}1^+$ dendritic epidermal cells)(Asarnow et al. 1988; Steiner et al. 1988), the gut (intestinal epithelial $CD8^+$ T cells) (Goodman and Lefrancois 1988) and in athymic mice ($Thy\text{-}1^+$ cells) (Yoshikai et al. 1986). In fact, the finding that athymic mice contain relatively high level of γ-δ cells with reduced $\alpha\beta$ cells suggest that their $\gamma\delta$ cells can develope outside the thymus. It is intriguing to speculate that the deletion of the δ locus may occur within the thymus, thus committing a large majority of the cells to the α-β lineage. A summary of the development of their α-β and γ-δ cells is illustrated in Figure 2.

Generation of $C\gamma4$ Transgenic Mice. One approach to measure gene effect and function, in an in vivo environment, is to generate transgenic mice. The over- or inappropriate expression of a TcR γ or δ chain gene may produce a phenotype that is related to the normal function of that receptor chain. We recently reported the effect of a $C\gamma4$ ($V\gamma1.1J\gamma4C\gamma4$) transgene on T cell reactivity in mice (Ferrick et al. 1989). The $C\gamma4$ gene was isolated by A. Iwamoto et al. (1986) and is located within the $C\gamma$ locus. This gene shares little similarity with either $C\gamma1$ or $C\gamma2$ (50%). There is low expression of this gene in the adult thymus (Elliott et al. 1988). However, high levels of $C\gamma4$ TcR exists in the spleen beginning at 2-4 weeks and is maintained throughout the life of the

mouse, representing approximately half of the spleenic γδ cells (J. Bluestone, personal communication).

Previously we demonstrated that Cγ4 transgenic mice displayed a significant T cell-mediated reactivity at a younger age in the periphery as compared to controls (Ferrick et al. 1989). In fact, newborn transgenic splenocytes were capble of generating allo-reactive CTL's in primary culture (D. Ferrick, unpubl.). This difference in T cell reactivity disappeared with time and was greatest between 2 and 4 weeks after birth (Ferrick et al. 1989).

Phenotype of Cγ4 Transgenic Mice During Development. In Table 1, the ratios of the absolute number of transgenic over control thymocytes are shown for various cell surface phenotypes. It is interesting to note that at the time when the greatest difference exists in peripheral T cell function between transgenic and control (2-4 weeks of age), there is a 3-4 fold increase in the absolute number of thymocytes in the transgenic thymus compared to control (ex. $2.1x10^8$ thymocytes for transgenic mice 4 weeks old versus $7.5x10^7$ thymocytes for control mice at the same age). However, the absolute number of T cells in the spleen and lymph nodes was not significantly different between control and transgenic for all time points tested (data not shown). From Table 1 it is apparent that there was a greater percentage as well as absolute umber of γδ T cells at all time points suggesting that this excess is due to expression of the Cγ4 transgene on the surface. In addition, there is an increase in the percentage of CD4 single positive thymoctyes at 2 and 4 weeks of age that correlates with a decrease in the percentage of $CD4^+$ $CD8^+$ double positive cells at these time points (Table 1). However, what may be most important is that the presence of the Cγ4 transgene is not only affecting the number of γδ-bearing cells as well, especially between 2 and 4 weeks of age (Table 1). In fact, for transgenic mice at 2 and 4 weeks of age, the greatest increase occurs within the subset of thymocytes expressing αβ.

Table 1. Thymic Development of T cells in Cγ4 Transgenic Mice

Trans/Cont	Birth	2 wk	4 wk	6 wk	16 wk
Cells	1.0	1.8	2.8	1.2	0.9
CD3	1.0	1.9	2.7	1.4	0.9
TCR αβ	0.9	1.8	2.7		
TCR γδ	1.7	2.7	3.5		
CD 4^-8^-	1.0	3.8	3.7	2.5	1.5
4^+8^+	0.9	1.6	2.6	1.3	0.8
4^+8^-	1.4	3.1	3.8	1.7	1.1
4^-8^+	1.0	1.8	2.7	0.9	0.9

Thymocytes were isolated and stained with monoclonal antibodies against various surface molecules. The absolute number of postive cells/mouse thymus was calculated and the ratio of transgenic (Trans) over control (Cont) is shown for the ages indicated.

In addition to the phenotypic changes in the T cell compartment of the transgenic mice, there is also a growth effect as well. In Table 2, preliminary data suggest that the size of the transgenic mice and their negative littermates is quite different. From early on the transgenic mice increase in body weight much sooner than their negative littermates as well as becoming much more alert and active between 1 and 2 weeks after birth. For example, at 6 months of age, a normal male $H\text{-}2^q$ x $H\text{-}2^b$ mouse will weigh approximately 32.8 ± 1.3 grams (average of 4 mice) while a transgenic mouse at the same age will weigh approximately 49.2 ± 2.6 grams (average of 5 mice).

Table 2. Weight of Cγ4 Transgenic Mice During Development

Trans/Cont	2 wk	6 wk	12 wk
Whole Body[a]	1.52	1.31	1.26
Thymus[b]	1.86	2.33	1.01

[a]Six male littermates (3 control and 3 transgenic) were weighed at various time points and the ratio of transgenic (Trans) over control (Cont) is shown. [b]The thymus weights were the average of 4-6 mice for each time point and is represented as the ratio of transgenic over control.

Discussion

Recently, the second described TcR, γδ, has been the subject of intense investigation. The physiologic role of cells that bear this receptor remains an enigma as there are still no good experimental models available to test for their function. In an attempt to alter the expression of one TcR γ gene we have generated mice transgenic for the Vγ1.1Jγ4Cγ4 TcR chain gene. Our previous results suggested that the T cell compartment is accelerated during development in these mice (Ferrick et al. 1989).

In this report the phenotype of the T cells present and their abundance as well as some preliminary data on the overall size of these mice as they age, suggest that the transgene is having a drastic effect on the overall development of these mice. The transient increase in the absolute number of thymocytes, peaking between 2 and 4 weeks, along with the increases in both the γδ and αβ T cell subsets (Table 1) correlates with the acute differences in T cell reactivity measured previously in the periphery at those early time points (Ferrick et al. 1989). Being that there is no significant increase in the number of T cells in the periphery of transgenic mice between 2 and 4 weeks of age makes it difficult to hypothesize what effect this increase in absolute number has on the observed early appearance of T cell reactivity. Even more difficult to envision are the reasons why the transgenic mice are larger and appear to develop sooner than their negative littermates. One possibility is that the effect of the transgenic γδ receptor, when engaged, is to signal the cell to produce growth factors such as interleukins that can have a positive effect on cells other than lymphocytes. This is not so improbable since it is known that γδ-bearing cells can produce biologically active factors such as interleukins and γ-interferon (Nixon-Fulton et al. 1988). We are currently measuring the levels of growth factors in the transgenic mice as well as looking at the relative abundance of hemopoietic precursors to shed some light on these differences.

Given the difficulty of designing biological models for a set of cells that bear a receptor whose physiologic ligand has not been identified has not hindered scientists from developing theories based on the current data. Some of the most widely accepted models based on the data available at present are as follows: 1) The γδ receptor is a primitive first line of defense against highly conserved molecules (ex. endotoxins, heat-shock proteins). 2) The γδ receptor recognizes class I or II molecules complexed with antigen (Ia, K, D, Qa, TL). 3) The γδ receptor is an adhesion molecule (ie. homing receptor). 4) And finally, it is an anti-self receptor involved in the augmentation of αβ TcR development and responses. Due to the probable heterogeneity of cells that bear γδ receptors some or all of the above may be correct.

In terms of our Cγ4 transgenic mice, although they do cast doubt on the possibility that they are horming receptors, they are not inconsistent with any of the above potential roles of γδ-bearing cells, although they do favor the role of these cells as an anti-self receptor capable of augmenting T cell development and reactivity. One might suggest, based on ours and others data, that γδ cells (most likely a subset) could recognize MHC molecules (such as Qa or TL) complexed with foreign or self peptides on activated T cells in both the thymus and periphery. As a result of this binding, the γδ-bearing cell would produce and secret biological factors to aid in the differentiationa and/or activation of the corresponding lymphocyte. Alternatively, these γδ cells could be stimulated by endotoxin and/or heat-shock protein like substances which can in turn influence the development and proliferation of α-β T cells. The hypothesis is now testable with transgenic mice carrying different chains of γ and δ TCR genes.

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Lymphocytes Bearing Either γδ-TCR or αβ-TCR Can Recognize Non-MHC Encoded CD1 Molecules

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Two T cell populations exist which express distinct T cell receptors (TCR), TCR αß (Marrack and Kappler, 1986) and TCR γδ (Brenner et al. 1986). T cells bearing the TCR αß complex are numerically predominant in the blood and organized lymphoid organs and recognize foreign peptides presented in the context of self-major histocompatibility complex (MHC) molecules. As mature lymphocytes, they are "single positive" and express either CD4 or CD8 which participate in recognition of MHC class II or class I molecules, respectively. In contrast, the recognition specificity of lymphocytes bearing the γδ-TCR are unknown, and these cells are frequently both CD4 and CD8 negative ("double negative") (Groh et al. 1988). Besides γδ-TCR lymphocytes, a population of $CD4^-8^-$ lymphocytes bearing the αß-TCR also exists in man (Shivakumar et al. 1989). In cytofluorographic analyses, staining with anti-CD4 together with anti-CD8 in one color compared to staining with anti-CD3 in a second color, we observed the expected presence of a subpopulation of $CD3^+CD4^-8^-$ cells in human peripheral blood. When also staining with mAb anti-TCRδ1 (pan TCR γδ reactive), a majority of these double negative T cells were shown to bear the γδ-TCR. However, in all normal subjects examined a population of 0.5-2.0% of these $CD4^-8^-$ T cells was found to lack reactivity with anti-TCRδ1. These cells were isolated and were shown to express the αß-TCR. $CD4^-8^-$ TCR-αß bearing cells have been reported to be increased in number in certain genetically autoimmune strains of mice that have generalized lymphadenopathy (gld, lpr) (Miescher et al. 1987). These mice share some clinical features in common with the human autoimmune disease systemic lupus erythematosus (SLE) where an increase in the number of $CD4^-8^-$ T cells in peripheral blood has also been found (Shivakumar et al. 1989). Our analyses suggest that the number of double negative αß-TCR bearing lymphocytes were increased and represented more than 10% of the $CD3^+$ cells in several individuals with SLE. Thus the $CD3^+4^-8^-$ phenotype occurs on a majority of γδ-TCR lymphocytes and on a small fraction of αß-TCR bearing lymphocytes, particularly in SLE.

The antigen recognition of both these subsets of double negative T cells is not well characterized. Human γδ T cells cultured in the presence of interleukin-2 (IL-2) have the ability to lyse a variety of tumor target cells in ^{51}Cr release assays (Borst et al. 1987, Brenner et al. 1987, Brenner et al. 1988). This lysis is thought to be antigen-nonspecific and may be analogous to the LAK activity exhibited by CD3$^-$ lymphocytes after exposure to high levels of IL2. In addition, several examples of γδ-TCR bearing lymphocytes with MHC-linked recognition have been reported suggesting recognition of classical MHC antigens or MHC-linked TL encoded antigens (Bluestone and Matis, 1989). Yet, only a few such examples have appeared, and it has been difficult to obtain classical MHC-restricted antigen-specific γδ-TCR lymphocytes in either mouse or man. Moreover, earlier we identified a γδ-TCR cytotoxic T cell (CTL) line designated "immunodeficiency deficiency patient 2" (IDP2) which lacks expression of both CD4 and CD8. In contrast to the more generalized killing of many ^{51}Cr-labeled tumor targets, cytolysis assays against a panel of tumor and allogeneic target cell lines revealed specific lysis only of one or two cell lines, notably the MOLT4 T leukemia cell line. This killing was not inhibited by mAb against monomorphic determinants present on MHC class I or class II molecules (indeed MOLT4 cells do not appear to express class II antigens). Based on such a specific cytolysis profile and the failure to inhibit the recognition with mAb, we proposed that IDP2 cells displayed specific MHC-unrestricted recognition (Brenner et al 1987).

Recently we investigated further the ligand recognized on MOLT4 cells by the IDP2 CTL. In addition to MOLT4 cells, we found that IDP2 cells lysed JURKAT and HPB-ALL cells, two other T leukemia cell lines. All three of these thymic-derived cell lines express the first cluster of differentiation, CD1 (T6) antigens. CD1 molecules were first described as markers present on immature, cortical human thymocytes that are lost by medullary (and mature peripheral) T cells (McMichael et al. 1979, Reinherz and Schlossman 1980, Bahn et al. 1980). Structurally, the CD1 protein is expressed noncovalently associated with ß2 microglobulin (Terhorst et al. 1981, van Agthoven and Terhorst 1981). Five cross-hybridizing CD1 genes have been identified (Martin et al. 1986, Martin et al. 1987, Balk et al. 1989). Three of which correspond to serologically and biochemically distinct CD1a, CD1b, and CD1c molecules. Protein products for the other two genes have not yet been described. CD1a, b, and c genes predict polypeptides whose sequence corresponds to a domain structure (α1, α2, and α3) that is roughly similar to that of MHC class I molecules. Yet the homology to MHC molecules is very limited with less than 32% identity at consensus residues of MHC class I molecules in the α2 and α3 domains, and even lower homology in the α1 domain. No homology could be detected between CD1 genes and MHC class I genes at the nucleotide level. Moreover, CD1 molecules have shorter cytoplasmic tails than MHC class I molecules. Although both murine TL antigens and CD1 antigens are expressed on thymic leukemias, sequence homology analysis did not reveal CD1 proteins to be more related to TLa than to Qa, H2-K, D, L or HLA-A, B, or C. Finally, CD1 genes are not encoded in or linked to the MHC, but are encoded on chromosome 1 in man (Calabi and Milstein 1986).

CD1 molecules are expressed on dendritic cells in the epidermis (Langerhans cells) and the dermis, on activated B cells in mantle zones, and possibly at low levels on resting B cells (Fithian et al. 1981, Small et al. 1987). Given the expression of CD1 molecules on cells that are of immunological interest and which may function as antigen-presenting cells, investigators have wondered if CD1 antigens could function as antigen-presenting molecules. To date no evidence for recognition of CD1 molecules by T cells has come to light and no examples of antigen presentation by CD1 molecules have been found.

Since CD1 antigens are expressed on the thymic leukemia lines recognized by IDP2 cells, we attempted to block lysis of the target MOLT4 cell line with anti-CD1 mAbs. Anti-CD1c mAb such as M241 block lysis of MOLT4 cells by IDP2 by more than 80%. Furthermore, transfectants of mouse L cells and By155.16 T-T hybridoma cells expressing CD1c, but not CD1a molecules were specifically lysed by IDP2 cells. This killing was completely inhibited by anti-CD1c mAb. These results indicate that IDP2 cells recognize CD1c molecules on the surface of target cells (Porcelli et al. 1989).

In another series of experiments, we obtained eight double negative T cells expressing the αß-TCR. One such cell line, BK6, derived from the peripheral blood of a patient with SLE was studied in detail because it was observed to lyse MOLT4, JURKAT and HPB-ALL all of which express CD1 antigens. Lysis of MOLT4 cells by CTL BK6 was inhibited by more than 80% by mAb directed against CD1a specific determinants, such as OKT6. Moreover, CD1a, but not CD1c transfected mouse L cells or human rhabdomyosarcoma (RD) cells were lysed by CTL BK6. Lysis of the CD1a bearing transfectants was specifically and completely blocked by CD1a-specific mAb (Porcelli et al. 1989). Since the appropriate CD1 molecules were recognized in target cells derived form various donors as well as in xenogeneic transfectant cell lines, and since mAb against monomorphic MHC antigens failed to block lysis, the recognition of CD1 by IDP2 and BK6 CTL appeared to be MHC unrestricted (Porcelli et al. 1989).

Together, these experiments show that a γδ-TCR CTL and an αß-TCR CTL clone lyse targets cells through recognition of CD1c or CD1a molecules respectively. Further experiments showed that the αß-TCR CTL line was inhibited from killing its target cells by mAb directed against the αß-TCR complex (BMA031) while the γδ-TCR CTL line was inhibited from lysing its targets by mAb directed against the γδ-TCR complex (anti-TCRδ1 or anti-Tiγ A). These results, together with the specificity of different T cell clones for distinct CD1 antigens suggests the recognition may be mediated by the T cell receptors present on each CTL. The nature of the CD1 recognition described here remains unclear. It seems unlikely to represent alloreactivity since CD1 antigens appear to display little polymorphism. It may represent an autoreactive phenomenon especially since the BK6 CTL was derived from a patient with autoimmune disease. In addition, we have recently isolated and partially characterized $CD4^-8^-$ T cell lines derived from synovial tissue of rheumatoid arthritis patients. These T cell lines are 80-90% TCR $\gamma\delta^+$ and show specific cytotoxicity of $CD1c^+$ transfected target cells. The isolation of such cells from inflamed synovium and of the CD1a reactive CTL BK6 from the blood of an SLE patient raises the possibility of a role for CD1 reactive $CD4^-$

8^- T cells in autoimmune diseases. Alternatively, CD1 molecules presenting foreign antigens may be recognized by TCRs in vivo, and the assays performed here in vitro may detect this general ability to recognize CD1 molecules. The frequency with which peripheral T cells show CD1 reactivity is unknown. However, the finding of two examples in our panel of 22 $CD4^-8^-$ T cell lines derived without intentional selection for CD1 recognition indicates that this may be a fairly common reactivity. This reactivity may be limited to the double negative T cell subset examined here, or alternatively may also be found in the $CD8^+$ T cell subset since CD8 molecules have been shown to associate with some CD1 molecules. The studies summarized here present the first evidence that T cells can recognize CD1 molecules. However, further work in needed to define both the physiologic role for CD1 in normal immune responses as well as its potential role in autoimmune disease.

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Assembly of the T Cell Receptor/CD_3 Complex

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INTRODUCTION

The human T cell receptor for antigens is a multichain cell surface receptor consisting of variable (TCR -α/ß or TCR -γ/δ and constant elements (CD3 - $\gamma, \delta, \varepsilon, \zeta$). The exquisite specificity for antigen plus MHC (or CD1) resides in the variable regions of the TCR α/ß (or TCR -γ/δ) glycoproteins (1, 2, 3). Since T cell receptors and MHC products (4) (or CD1 glycoproteins (5)) are anchored in the plasma membrane of T lymphocytes and antigen presenting cells, respectively, antigen recognition takes place on the interface between the two cells. A localized and TCR-independent set of adhesion events provides a stablizing environment for the subtle ternary interaction which is dependent upon the fine specificity of its participants. Thus, antigen recognition by T cells is a carefully orchestrated and tightly regulated event.

A T cell canot recognize antigen unless the multichain receptor complex /TCR/CD3) is correctly assembled and transported to the plasma membrane. Assembly of the TCR/CD3 complex during intrathymic differentiation as well as in the functionally mature T lymphocyte appears to be regulated in a precise manner. Taken together, the existing evidence favours the notion that a hierarchy of assembly exists which permits a stringent control of cell surface expression of the antigen receptor on the surface of T lymphocytes.

THE CD3 GENES

The cells of the immune system arise from a common precursor stem cell. The earliest definitive signal of commitment to the T cell lineage is expression of the CD3 genes. As members of the TCR/CD3 complex these proteins appear on the cell surface relatively late in development after all of the component chains are synthesized. Transcription of the CD3 genes however begins much earlier, it precedes TCR rearrangement and may even occur prior to migration to the thymus. Early thymocytes and mutant thymoma lines with an immature phenotype express low levels of CD3 mRNA while more mature T cells express higher levels. This suggests that transcription of CD-3γ, -δ and -ε is controlled by the activities of nuclear regulatory proteins which are modulated during T cell development.

We have previously reported that a 400 bp region 3'of the CD3δ gene functions as a transcriptional enhancer with strong specificity for T cells. We have now identified two cis acting elements δA and δ B in this region responsible for mediating the enhancer function. Element δA is a T cell specific enhancer which in the context of the CD3δ enhancer requires element δ B for high levels of activity. We have studied the nuclear protein factors that bind to these elements and found that their concentration levels correlates with enhancer activity and expression of the endogenous gene (7). Element δ A was also found in the TCR-α enhancer (8) and the δA binding proteins may therefore play an important role in T cell development.

The transcription start site of CD3-γ is 1.4kb from the CD3-start site. A strong non-tissue specific enhancer has recently been found 200 bp upstream from the CD3-δ start site (J.Versteegen, unpublished). It is plausible that δA and δ B also govern the transcription of CD3-γ in thymus derived lymphocytes.

The human CD3-ε gene contains nine exons and spans 12 kb (9). The gene encoding the CD3-ε chain of the human T cell receptor (TCR) complex, when introduced into the mouse germ line, was expressed in T lymphocytes only. The gene was screened for T lymphocyted-specific cis-acting elements in transient CAT assays. The promoter (-228 to +100) functioned irrespective of cell type. A 125 bp enhancer with strict T cell specificity was found in a DNase I-hypersensitive site downstream of the last exon, 12 kb from the promoter. Two CD3-ε⁻ T cell lines were identified in which the enhancer was not active (10).

INTRINSIC STABILITY OF THE SUBUNITS OF THE TCR/CD3 COMPLEX

To study intercellular behaviour of the TCR/CD3 complex individual chains coding for the human α, ß, δ, γ and ε chains were transfered into COS and CHO cells. Analysis of pulse - chase labeled cells used immunoprecipitation by monoclonal antibodies specific for the transferred subunit. TCRα was visualized as three precipitable forms of approximately 40kD, that migrated between an upper actin band seen at 45kD and a lower non-specific band seen at 30 kD. TCR ß resolved as two precipitating proteins that were smaller at 35 kD. These bands represented the different glycosylated forms of the proteins and they migrate with the same molecular weights as TCR α and ß chains precipitated from human T cells (11, 12). CD3 δ and ε were precipitated as proteins of 20 kD while CD3 γ was slightly larger at 23-25kD. When the pulse-labelled cells were chased the relative stabilities of the subunits became evident. The quantity of immunoprecipitable TCR α and ß remained constant during the first hour following the pulse label. After this lag period they were degraded rapidly. CD3δ on the other hand was degraded without a noticeable lag period.

TCRα and ß and CD3δ had similar intracellular half lives during the degradation phase estimated by laser scanning sensitometry to be at approximately 1 to 1.5 hours (13). In contrast, CD3 γ and ε were stable.

To see how far along the secretory pathway the chains were transported prior to their degradation, pulse-chase immunoprecipitations were repeated for the glycoprotein subunits. At no time during the chase did Endo-H resistant forms of the glycoproteins appear. This implied that the TCRα and ß and CD3 δ were degraded without passing through the medial Golgi. The stable γ chain remained sensitive Endo-H indicating its retention in an early compartment of the secretory pathway. CD3εdoes not have N-linked oligosaccarides and was not tested.

Immunofluorescence analysis was used to further define the intracellular structures involved in the transport of the subunits of the receptor. By virtue of the antibodies available we were able to study the steady state distribution of the stable CD3 γ chain and the labile CD3 δ subunit. In parallel experiments oligosaccarides processed in the Golgi forms containing N-acetyl glucosamine and sialic acid were visualised by incubation of cells with wheat germ agglutinin conjugated to Texas Red (13). The distribution of immunofluorescence was similar for both proteins and was in agreement with the Endoglycosidase-H analysis recorded above. Staining was restricted primarlily to a reticular membrane network that extended from the nucleus to the periphery of the COS cell. This pattern strongly resembled the known structure of the endoplasmic reticulum of COS cells. These data confirm the notion derived from studies in T-cells that the majority of the α, ß, γ, δ and ε chains reside within the Endoplasmic Reticulum.

Degradation of TCR-α, ß, and CD3-δ takes place in an unknown compartment. Based on studies involving drugs that affect lysosomal degradation pathways in T cells (14) and Cos cells (15) it appears as though the lysosomes are not involved. that observation is supported by the notion that these proteins do not pass through the medial Golgi for the large majority of the oligosaccarides attached to TCR-α, ß, and CD3-δ are not processed and remain Endo-H sensitive (13). Preliminary data show that the propensity for direction towards pre-Golgi degradation is not limited to distinct parts of the molecules. For instance, fusion proteins prepared with a soluble form of the IL-2 receptor and the ectodomains of TCR-α and -ß are degraded (15). Similarly, the transmembrane and intra-cellular domains of the TCR-ß direct the soluble IL-2 R to the pre-Golgi degradation. It will be of great interest to establish whether distinct signals exist in the TCRα, ß, and CD3-δ proteins which allow them to be destined for pre-Golgi-degradation.

Indirect immunofluorescence studies showed that none of the single polypeptide chains reaches the cell surface by itself. Since the presence of charged amino acid residues in the transmembrane region of integral membrane proteins have been shown to affect their transport to the cells surface, site directed mutagenensis experiments were conducted. When the negatively charged residues of CD3-γ,δ and -ε were replaced by Ala or Gly the majority of each polypeptide chains were still found in the endoplasmic reticulum. Similarly, when the two positively charged residues in the transmembrane region of the TCR-α chains (Arg and Lys) were replaced by glycine and isoleucine the polypeptide chain was retained intracellularly. As in the case of the wild type polypeptide chains the TCR-α, ß and CD3-δ were degraded rapidly. Thus, like commitment to pre-Golgi-degradation, retention in the endoplasmic reticulum and pre-Golgi degradation is not likely to be caused by a simple "retention signal". Retention in the endoplasmic reticulum most likely serves a purpose in that it allows for an efficient assembly of the complete TCR/CD3 complex.

THE CD3-γ AND CD3-ε PROTEINS STABILIZE THE OTHER POLYPEPTIDE CHAINS

In these experiments we used vectors containing different resistance markers to co-transfer CHO cells and Cos cells with precise combinations of subunits. The cells were labelled metabolically and then lysed using mild detergent conditions to maintain associations between chains. Immunoprecipitation with subunit-specific antibodies allowed us to assess specific interactions between subunits and see if interactions between subunits would affect their intracellular transport. Thus, we showed that interations between CD3γ, δ, and ε can take place, and that CD3-ε confers a stability on CD3-δ after association. Similarly, association between the CD3-ε and TCR-ß results in a stable subcomplex.

HIERARCHY OF ASSEMBLY OF THE TCR/CD3 COMPLEX

Many T cell variants that lack one or more polypeptide chains of the TCR/CD3 Complex have been described (18,19). For the most part partial complexes do not arrive at the cell surface, although in two distinct cases an escape of the retention mechanisms has been reported. In one murine T cell line MA 5.8 (CD3-ζ) mutant expresses low level of the pentameric complex on the surface. And in some TCR-α/ß or TCR-α/CD3-δ AKR thymomas small amounts of CD3-γ and-ε were detectable at the cell surface (20). In all these cases the N-linked oligosaccarides attached to the cell surface chains were of the complex (Endo-H resistant) type. But in most TCR/CD3 variants which lack the expression of one of the polypeptide chains the partial complexes do not arrive at the cell surface.

The CD3-γ, δ, -ε core complex can be found in human and murine T cells which lack the TCR-α and -ß chains (21,22). In TCR-α and TCR-ß cells the $\beta\gamma\delta\varepsilon$ and $\alpha\gamma\delta\varepsilon$ subcomplexes are found, respectively.

Whilst in human T cells CD3-ζ does not associate with either of the tetrameric partial complexes one report describes that CD3-ζ associates with some partial complexes in a murine cell line (23). After short pulses with radioactive amino acids a pentameric complex with disulfide bridged TCRα/ß heterodimers can be found in both CD3-ζ^- and CD3-ζ^+ cells. Moreover, in the presence of the drug monensin the TCRα-ß chains associated with CD3 are disulfide bridged, although their oligosaccarides remain Endo-H sensitive. Thus, it appears that the partial complex α, ß, γ, δ, and ϵ is assembled within the endoplasmic reticulum. Similar studies have shown that CD3-ζ associates within the endoplasmic reticulum and that only the hexameric complex migrates to the cis-Golgi.

In order to study the relationship between assembly, surface expression and signal transduction of the α/ß T cell Antigen/CD3 complex (TCR/CD3), a series of T-cell mutants with a partial block in assembly of the complex was generated. Using chemical mutagenesis we produced somatic cell variants of the human T-leukemia cell line, HBP-ALL, which expressed low amounts of TCR/CD3 complexes on their surface. RNA and protein analyses demonstrated that some variants synthesised normal amounts of the individual members of the complex, i.e. TCR-α, TCR-ß, CD3-γ, δ, ϵ and ζ. However, less than 10% of the TCR/CD3 complexes inside the cell contained the CD3-ζ_2 homodimer due to an intrinsic deficiency in the formation of the TCR-α/ß heterodimer. These latter two defects plus a slow transport of the TCR/CD3 complex out of the Endoplasmic Reticulum explained the low surface expression of α/ß receptors in these mutants. only cells which expressed the complete set of subunits of the TCR/CD3 complex on their surface, were capable of transducing CD3-mediated signals as determined by calcium mobilization.

These results provide the first evidence for differential cell-surface expression of CD3-ζ_2 in cells which synthesize the complete set of subunits of the TCR/CD3 complex and are of importance to the prevailing model attributing a signal function to CD3 (24).

I Structure and Function of Recognition and Effector Molecules

MHC and Antigen Presentation

Structural and Functional Analysis of Human Class I and Class II Major Histocompatibility Complex Proteins, with Special Emphasis on Alloreactivity

J.L. Strominger

Class I MHC proteins

The class I histocompatibility antigen from human cell membranes (Ploegh et al., 1981) has two structural motifs: the membrane-proximal end of the glycoprotein contains two domains with immunoglobulin-folds that are paired in a novel manner, and the region distal from the membrane is a platform of eight antiparallel ß-strands topped by α-helices. A large groove between the α-helices provides a binding site for processed foreign antigens. An unknown "antigen" is found in this site in crystals of purified HLA-A2 (Bjorkman et al., 1987a). Most of the polymorphic amino acids of the class I histocompatibility antigen, HLA-A2, are clustered on top of the molecule in a large groove identified as the recognition site for processed foreign antigens. Many residues critical for T-cell recognition of HLA are located in this site, in positions allowing them to serve as ligands to processed antigens (Bjorkman et al., 1987b).

A relatively large number of mutants of HLA-A2 have been constructed either (i) by site-directed mutagenesis of residues in the peptide binding cleft or (ii) by deletions of mini-exons 6 and/or 7 encoding portions of the intracytoplasmic region and have been used to analyze the structure and function of this molecule in the light of the structure revealed by x-ray crystallography.

A. Mutations in the foreign antigen binding cleft.

Four immunological phenomenon in which these molecules participate have been examined.

1. Recognition by monoclonal antibodies (Santos-Aguado et al. 1988).

The localization of the amino acid residues involved in the serologic specificity of the HLA-A2 molecule has been investigated. At least three non-overlapping serologic epitopes

were identified. Mutations in the highly polymorphic region at amino acids 62 to 66 completely eliminated binding of mAb MA2.1 (A2/B17 cross-reactive). Mutation at position 107 resulted in complete loss of mAb BB7.2 binding (A2 allospecific). The recognition of other allotypic mAbs was not affected by these mutations and they therefore represent at least a third serologic epitope. Mutations at positions 152 and 156, known to be important for T cell recognition, did not affect serologic recognition. Introduction of residues of HLA-B7 origin in the polymorphic segment spanning amino acids 70 to 80 created a molecule carrying the -Bw6 supertypic determinant as demonstrated by mAb SFR8-B6 binding but no determinant recognized by B7 allospecific mAbs was detected.

2. Recognition by alloantisera (Santos-Aguado et al., 1989a).

A small number of pregnancy alloantisera are allospecific and recognize the same epitopes detected by mAbs at residues 62-66 and at residue 107 (which earlier data had indicated was a non-linear epitope also involving residue 161).

3. Recognition by influenza virus-directed HLA-A2-restricted CTL (in collaboration with Andrew McMichael and members of his laboratory).

a. Epitope in matrix protein of a Type A strain (McMichael et al., 1988).

Cytotoxic T lymphocytes (CTL) specific for influenza A virus were prepared from 15 donors. Those with HLA-A2 recognized autologous or HLA-A2-matched B-lymphoblastoid cells in the presence of synthetic peptide representing residues 55-73 or 56-68 of the virus matrix protein sequence. Influenza A virus-specific CTL from donors without HLA-A2 or with an HLA-A2 variant type failed to respond to this peptide. CTL lines specific for HLA-A2 plus peptide did not lyse peptide-treated target cells from HLA-A2 variant donors. They also failed to lyse peptide-treated cells with point mutations that had been inserted into HLA-A2 at positions 62-63, 66, 152, and 156 and, in some instances, mutations at positions 9 and 70. CTL lysed peptide-treated target cells with mutations in HLA-A2 at positions 43, 74, and 107. The results imply that this defined peptide epitope therefore interacts with HLA-A2 in the binding groove so that the long α-helices of HLA-A2 make important contacts with the peptide at positions 66, 152, and 156. Different amino acids at position 9, which is in the floor of the peptide binding groove of HLA-A2 and the closely related position 70, modulate the peptide interaction so that some T-cell clones react and some do not. These data raise the possibility that the same peptide may bind in the groove in two different ways.

b. Epitope in nucleoprotein of a Type B strain (Robbins, et al., 1989).

An influenza B virus nucleoprotein (BNP) peptide, residues 82-94, identified by very limited sequence homology with the influenza A matrix protein epitope, was recognized by HLA-A2-restricted cytotoxic T lymphocytes. Reciprocal inhibition of T cell recognition by the two peptides suggest that the BNP peptide may have lower avidity for HLA-A2 molecules than the matrix peptide. The weak competitor activity of the BNP peptide for the matrix peptide may be explained by its location within the HLA-A2 binding cleft, which has been mapped using T cell recognition of target cells expressing natural variants and site-specific mutants of HLA-A2. Mutations at residues 9, 99, 70, 74, 152, and 156 were found to abolish T cell recognition of the BNP peptide. These results taken together with results obtained with the influenza A matrix peptide suggest that the two peptides bind differently in the peptide binding site and in particular that binding of the BNP peptide may involve residues 9, 99, 70, and 74 in a way that they are not involved in the binding of the matrix peptide.

4. Recognition by a panel of allogeneic CTL (Santos-Aguado et al., 1989b).

The complexity and fine specificity of the allospecific T cell response generated against the human HLA-A2.1 molecule and the characterization of the antigenic determinants that anti-HLA-A2 alloreactive CTLs recognize in the molecule has been analyzed using as targets cell lines expressing HLA-A2 CTL-variants and the human rabdomyosarcoma cell line (RD) transfected and expressing HLA-A2 mutants obtained by site-directed mutagenesis. The data were correlated with the three-dimensional structure of the HLA-A2.1 molecule and led to the following conclusions: 1) Every clone displayed a different recognition pattern, illustrating the enormous epitope-diversity of this allogeneic T cell response (Figure 1). The heterogeneity of the CTL clones was also demonstrated when mAbs against monomorphic or polymorphic determinants in HLA molecules were used in cytotoxicity inhibition assays; and 2) Residues shown to be important for allogeneic CTL recognition were located either in the $\alpha 1$ or $\alpha 2$ helix pointing into the site or in the bottom of the putative antigen binding recognition site, just as they are for virus peptide-directed HLA-A2.1-restricted CTL recognition. Thus, allogeneic recognition must also involve recognition of some material in the foreign antigen binding site of HLA-A2.1, most likely a self peptide. The multiplicity of recognition patterns of the mutants seen with different CTL clones is compatible with a model in which a large number of different self peptides are bound in different ways in the peptide binding cleft of HLA-A2 (just as virus-derived peptides may be bound in distinct ways) and serve as the ligands for allogeneic recognition.

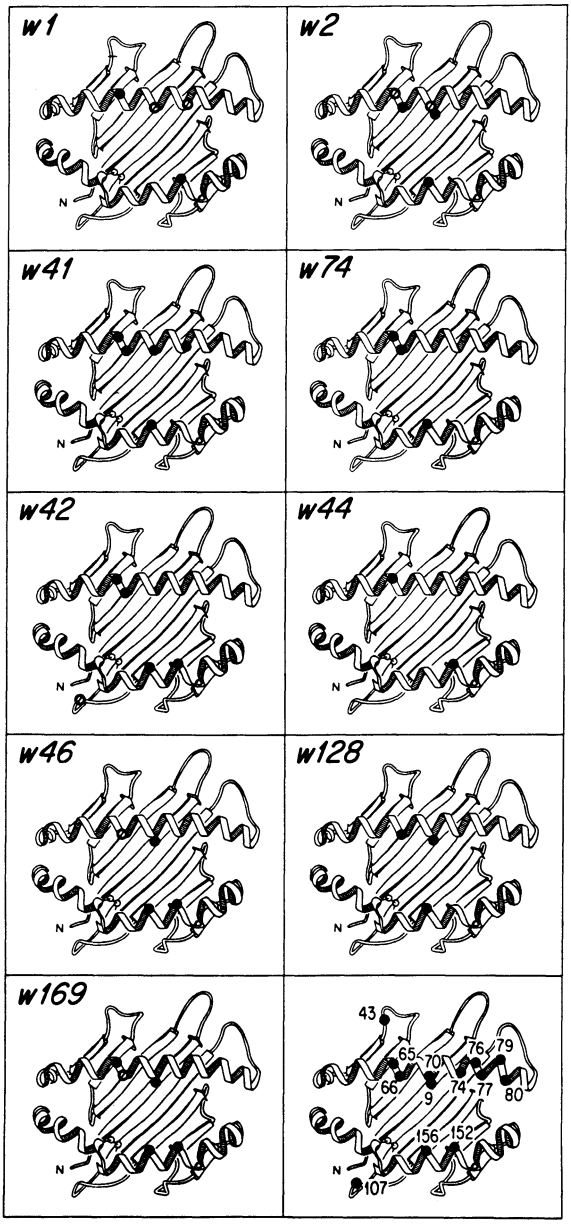

Figure 1. The pattern of mutations that affected several clones. •, 0-30% lysis relative to the RD-A2.1 transfectant recognition, i.e. same as background on RD-mock cells; O, 30-60% lysis; Not shown, mutations resulting in 60-100% lysis (no effect). Only the single point mutations were considered in making this figure.

The effects observed, including the very large repertoire of allogeneic CTL, can be explained most readily by a model in which a number of different self-peptides derived from the allogeneic presenting cell are presented by the allogeneic (or mutant) MHC molecule and in which different self-peptides are bound at different specific subsites within the antigen bindng cleft. Such a model is compatible with the size of the cleft and with the biological observation that a very large universe of foreign peptides can be presented to the immune system by a large, but in this sense relatively limited, number of MHC molecules. Each molecule must then be able to present a variety of different peptides. Studies of the recognition of two different peptides from a Type A and a Type B influenza virus strain led to the conclusion that a single peptide, as well as two different peptides, can be bound to HLA-A2 in different ways and possibly in different subsites.

Are the data also compatible with a model in which the allogeneic CTL recognizes HLA-A2 molecules which contain no peptide in the antigen binding cleft? In such a model, the variety of CTL would be explained by specific recognition of different portions of the cleft acting as T-cell epitopes. This model is less attractive, however, because many of the residues analyzed are pointing into the cleft, where they should be less accessible to CTL, and at least one, residue 9, is on the floor of the cleft (although alterations in this residue could affect the conformation of more accessible residues in the $\alpha 1$ helix). In addition, this model implies no specific recognition of an MHC molecule, and it might be anticipated that altered residues outside the area of the antigen-binding site, such as, for example residues 43 and 107, could also be targets for allospecific CTL.

The problem, however, of how a <u>non-self</u> MHC molecule can be recognized remains. Positive selection in the thymus is believed to ensure that only those T cells bearing receptors for self MHC molecules will reach the periphery. However, the number of polymorphic amino acid residues that point upward from the α helices and are potential ligands for T cell receptors is relatively limited, i.e. five; in the $\alpha 1$ helix four of the seven positions that point upward can have three or more different amino acid residues, whereas in the $\alpha 2$ helix it is only one position out of eleven. Possibly all T-cell receptors do not contact all five of these residues. Moreover, some changes may be tolerated by some T-cell receptors (as was evident in data obtained for changes at residues 65 and 76); this would be an example of cross-recognition. The self peptide in the peptide-binding cleft of an MHC molecule is derived from the allogeneic tissue. For an allogeneic response to occur this peptide must be different from that to which the T cells of the recipient have become tolerant because the individual must have become tolerant to most, if not all, self-peptides bound by self-MHC

during negative thymic selection, presumably by clonal deletion. A different set of peptides derived from processed self proteins would have been selected by a different (allogeneic) MHC molecule. In addition, polymorphism for the self-peptides derived from allogeneic tissues including those derived from MHC molecules may also play a role; polymorphic self proteins may be minor histocompatibility antigens. Finally, the same self peptide may be bound in distinct ways by self and allogeneic MHC molecules, presenting different conformations or side chains to T-cell receptors, and, therefore, resulting in different thymic selection. Thus, alloreactivity is the recognition by the host T-cell receptors of a foreign MHC molecule (possibly imperfect recognition) containing either a new set of self peptides or possibly a polymorphic, and therefore foreign, self peptide in its antigen-binding cleft. In this view T-cell epitopes for allorecognition are unusual self peptides bound in the foreign peptide-binding site of an allogeneic MHC molecule; they do not exist on the MHC molecule itself.

Similar studies of mutant MHC molecules have been carried out recently by a number of investigators although their interpretations are not identical to that in the model presented here.

All of the data obtained are compatible with the proposed structure of HLA-A2. Antibodies recognize only residues which are exposed in the structure. Recognition of two different flu virus peptide epitopes (one from a type A strain and one from a type B strain) indicate that these two quite different peptides which are presented by HLA-A2 interact with different residues in the peptide binding cleft and may be located in slightly different positions. The repertoire of allogeneic CTL is extremely large. The data are compatible with a model in which allogeneic CTL "cross-recognize" the allogeneic MHC molecule containing an unusual set of self peptides bound in different ways in its cleft.

B. Deletions of portions of the intracytoplasmic region.

Finally, the deletion of mini-exons encoding portions of the 32 intracytoplasmic amino acids of HLA-A2 have been employed in a study of the constitutive endocytosis of HLA-A2 in a human T cell line. First, HLA class I antigens present in the human leukemia T-cell line HPB-ALL were shown to be endocytosed in the absence of specific antibodies (constitutive endocytosis). In 1 hr, ≈10% of class I molecules initially present at the cell surface were found intracellularly. Genetically engineered mutants of the HLA-A2 gene lacking exon 6 or 7 or both were used to analyze whether the cytoplasmic region contributes to the internalization. The results indicate that amino acids encoded by exon 7 (spanning amino acid residues 323-340) are required for internalization, while deletion of exon 6 had no effect. In addition, a comparison

of the cytoplasmic sequences of receptors that are known to be internalized via coated pits and the present data revealed that they share a structural feature that could constitute a specific signal required for endocytosis (Vega et al., 1989). The biological significance of endocytosis and its possible relevance to antigen presentation remain to be explored.

Structure of class II MHC antigens

Data obtained for the structure of class II antigens from protein sequencing and cDNA analyses had suggested an overall similarity of structure of class I and class II antigens (Kaufman et al., 1984, Korman et al., 1985). A structure of class II MHC antigens has been modeled (Brown et al., 1989) on the structure determined for the class I antigen, HLA-A2. A very similar structure has been proposed. The class II antigens, DR1 and DR4, have been isolated in large amount, using a recently described procedure (Gorga et al., 1987) and solubilized. Crystals of both have been obtained (J. Gorga and J. Brown, unpublished) and efforts to elucidate the structure are in progress. In the meantime the secondary structures of human class I and class II histocompatibility antigens in solution have been examined by Fourier transform infrared spectroscopy and circular dichroism (Gorga et al., 1989) in order to compare the relative amounts of α-helix, ß-sheet, and other structures, which are crucial elements in the comparison of the protein structures. Quantitation of infrared spectra of papain-solubilized HLA-A2, HLA-B7, and DR1 in phosphate buffer gave α-helix contents of 17%, 8%, and 10% and ß-sheet contents of 41%, 48%, and 53%, respectively. By circular dichroism, papain-solubilized HLA-A2, HLA-B7, and DR1 were also found to have comparable α-helix contents (e.g., 8%, 20%, and 17%, respectively). Circular dichroism analysis for ß-sheet gave 29% for papain-solubilized HLA-B7 and 42% for papain-solubilized DR1. The value for papain-solubilized HLA-A2 (74%) was anomalous. It is proposed that Trp-107 of HLA-A2, missing in both HLA-B7 and DR1, may be responsible for much of the anomaly. Due to the uncertainties inherent in quantitation of the amounts of secondary structures by both spectral methods, the differences in the contents of α-helix and ß-sheet in the three proteins are not considered significant. However, differences in the nature of the ß-sheet structures are suggested by infrared spectroscopy. These results provide physical evidence for an overall structure of class II antigens modeled on that of class I antigens.

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The material presented is, in part, abstracted from the references cited. Full documentation to the work of others can be found in the papers cited.

Further Analysis of the Role of MHC Molecules in Antigen Presentation

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This is a summary of some of the research taking place in our laboratory. The paper represents a condensed version of a recent review paper (Unanue et al., 1989).

We regard the histocompatibility molecules as a special transport system in antigen presenting cells (APC) that functions to rescue denatured or processed peptides from extensive intracellular digestion (Unanue and Allen, 1987). This transport system operates by virtue of the property of the molecules of the major histocompatibility complex (MHC) to interact with peptides and unfolded proteins. The peptide-MHC bimolecular complex also constitutes the antigenic determinant that engages the αß T cell receptor.

The cellular immune system evolved to recognize antigenic determinants formed by linear sequences of amino acids. This recognition system required not only a vast diversity but at the same time the capacity to recognize self vs. non-self proteins. Protein antigens require a processing event in APC before they can be recognized by T cells. This processing takes place in acid intracellular vesicles and consists of either the unfolding of the molecule or its fragmentation to small peptides. We concentrated on the examination of hen-egg white lysozyme (HEL), an immunogenic protein in H-2^k mouse strains (Allen et al., 1987a). HEL recognition by T cell hybridomas required its internalization by APC and a chloroquine-senstive processing event. However, peptides derived from HEL were presented by live macrophages in the presence of chloroquine. Macrophages lightly fixed in paraformaldehyde presented peptides but not the native HEL molecule. In contrast, macrophages that internalized and processed HEL presented it to T cells if fixed after 30-60 minutes, indicating that the peptide-MHC-II complex became available and was resistant to the fixatives.

The molecular basis of processing became apparent when it was found that processed fragments interact with purified MHC-II molecules (Babbitt et al. 1985, 1986). Using equilibrium dialysis, immunogenic peptides of HEL bound in detergent solution to affinity-purified I-A^k molecules. The binding was saturable with K_D of 2-4 μM. While HEL(46-61) could bind to I-A^k it did not bind to I-A^d. I-A^k but not I-A^d is the restriction element for presentation of HEL(46-61). Thus there is agreement between the binding pattern of a peptide and its immune gene response. This observation first implied that peptide binding was one essential function of MHC molecules. It was later confirmed and extended primarily by the laboratory of H. Grey (Buus et al., 1986). MHC-II molecules show a single binding site as evidenced from competition experiments with a range of unlabeled peptides (Babbitt et al., 1986; Buus et al., 1987; Guillet et al., 1987). The site, although unique, can interact with a variety of peptides, and can contain peptides of at least 8-12 aminoacids. The tertiary structure of the HLA-A2 molecule (Bjorkman et al., 1987 a,b) has revealed the combining site of the molecule. A similar structure has been proposed for class II-molecules (Brown et al., 1988). The existence of a single combining site that binds to one peptide at a given time explains the phenomenon of competition

among protein antigens noted both in vivo and in vitro (Babbitt et al., 1986). In "antigenic competition", simultaneous immunization with two proteins results in a decrease in the response to one of them. This competition may take place at the level of uptake of the protein by the APC as well as at the level of binding of processed peptides to MHC-II molecules. In the latter case antigenic competition probably takes place in the milieu of the endosome. A single binding site implies that many peptides must share the property of interacting with the same binding site of a given class II molecule. Several peptide sequence motifs have now been proposed to explain the pattern of peptide binding to particular MHC alleles.

Our experience with lysozyme peptides is summarized in Table I: 1) the immunodominant sequence for H-2^k mice was found in the tryptic peptide HEL(46-61). 2) the minimal size functional peptide in the tryptic fragment HEL(46-61) was from residues 52-61. We made an "Ala map" by making peptides that had Ala substitutions at each position, which then enabled us to identify residues that contacted I-A^k and those that contacted the T cell receptor (Allen et al. 1987b). Three MHC-II contact residues were identified, Asp^{52}, Ile^{58} and Arg^{61}, and three T cell receptor residues, Tyr^{53}, Leu^{56} and Gln^{57}. These residues were intermingled in the molecule. We have proposed that peptides mold and adopt particular conformations as they bind into the MHC-II combining site. For example, folding the peptide in an α helix results in a segregation of each set of residues in the two planes of the helix.

Finally, 3) it is of key importance to note that MHC molecules did not discriminate between self and non-self peptides (Babbitt et al., 1986). Thus, the murine lysozyme sequence 52-61 that differs from HEL (52-61) by a Phe to Leu change at residue 56 was not immunogenic yet was able to bind to I-A^k. Not all self-peptides bind to I-A^k (Lorenz and Allen, 1988). For example the murine lysozyme peptide (34-45) will not bind to I-A^k because of Arg → Gln change at residue 41 (Lambert and Unanue, 1989). Thus foreign and self-peptides behave similarly in terms of their interaction with MHC molecules. Some bind, but others do not, to a given MHC allelic form, depending on their structure and the MHC haplotype.

TABLE I. BINDING PROPERTIES OF HEL

PROTEIN	BINDING TO MHC-II	MHC-HAPLOTYPE	COMMENT
Native HEL	No binding	--	Needs processing
Peptide HEL(46-61)	2-4 μM	k	Immunodominant peptide
Peptide HEL(52-61)	2-4 μM	k	Minimal size of immunodominant peptide
Murine(52-61)	Competes with HEL peptide	ND	Self-peptides bind to MHC-II
HEL(34-45)	2 μM	k	A second epitope in HEL
Murine(34-45)	No binding	--	An Arg in murine peptide instead of Gln at residue 41 affects the binding

A critical issue in antigen presentation is the balance between presentation of self and non-self peptides. Since the MHC-II molecules do not make such differentiation at the level of their binding site, some other system must operate to insure favorable presentation of foreign determinants during antigenic challenge. The intracellular processing events may be critical in order for the APC to effectively present foreign proteins and override the antigenic competition brought about by self proteins. The plasma membrane contains MHC-II molecules that are free and unoccupied, but the exact number is uncertain. With binding affinities of HEL peptides of ~10^{-6}M and using B lymphoma cells and our T cell hybridomas, we estimated that about 600-1000 peptide-MHC complexes per APC were required to stimulate T cells. This number represents about 1% of the available MHC-II sites on an APC surface which will need to be occupied by an antigenic epitope to stimulate an immune response.

Self proteins that compete with foreign peptides derive from two sources: i) exogenous - either proteins that are internalized and processed in endosomes (in a process akin to HEL) or peptides that are in the circulation and bind directly to surface MHC-II molecules or ii) endogenous - i.e., proteins that are synthesized and assembled in the ER and are then handled in the Golgi. Now we have little idea of the extent to which any of these sources provides peptides for binding to MHC molecules. The degree of internalization of plasma proteins and generation of self-peptides is also unclear; we believe that this is a significant but limited process. There is also little evidence for significant amounts of circulating peptides in blood that bind to MHC.

The internally made proteins could be a major component in the competition with foreign peptides. The experiments mainly from Dr. T. Braciale's laboratory (Morrison et al., 1986) indicate that the pathway for presentation via MHC-I molecules is different from that of MHC-II. Endogenous proteins appear to be presented by MHC-I. If both MHC-I and MHC-II molecules are in ER-Golgi, then why doesn't the MHC-II get saturated with peptides that result from partial proteolysis of some of the endogenous protein? Our speculations are that, i) the MHC-II proteins may get loaded with self peptides but they unload as they reach the endosome in a manner similar to the turnover of foreign peptide-MHC-II complexes (see below; Harding et al., 1988); ii) in ER-Golgi the MHC-II proteins do not bind peptides because their binding sites are not open, perhaps because of their interaction with other components. One possibility is that the invariant chains may bind and protect the MHC-II and prevent the binding of peptides until the endosome is reached; there the invariant chain dissociates from the MHC-II molecules which are now free to bind peptide.

Therefore, in endosomes, MHC-II proteins become available for peptide binding; these derive from a nascent pool or by way of the plasma membrane [either free or occupied by peptides that are subsequently released]. Their binding to foreign antigens will depend on the relative amounts of foreign and self proteins. The manipulations that result in effective immunization typically involve altering the antigen molecules to increase their uptake by the APC system. Further experimentation on the in vivo processing events should give valuable information on how the presentation of self/non-self is balanced at the level of the APC.

Cellular Studies on Antigen Presentation - It is important to place the interaction of peptide with MHC-II molecules in the context of

the live APC. Recent experiments compared the behavior of MHC-II molecules in macrophages and the B cell lymphoma TA3 ($H\text{-}2^{kxd}$) (Harding and Unanue, 1989; Harding et al., 1988, 1989). The turnover rate of the peptide-MHC-II complex was compared in these cells. The complex was very long lived on the membranes of fixed TA3 cells but had a short life in live cells. The experiment was done by pulsing fixed cells with HEL (46-61) peptide, washing and incubating for various periods at 37°C and then assaying for the amounts of $I\text{-}A^k$-HEL (46-61) remaining on the surface using a T cell hybridoma as our read-out system. Alternatively, live cells were pulsed, washed, and then fixed at later times. After 72 hrs there was no major reduction in the amount of immunogenic complex displayed by the fixed B lymphoma cells; in contrast, the half-life in live cells varied from 15 to 50 min in different experiments. In similar experiments with macrophages there was no significant loss in the amount of peptide-MHC-II complex in fixed macrophages after 60 hours while the half-life in viable macrophages was ~5 hours. The turnover rate of radiolabeled MHC-II molecules indicated a half-life of 11 hours in the macrophage and 14 hours in TA3 cells.

The dissociation of isolated complexes of peptides and MHC-II molecules was also studied. Purified $I\text{-}A^k$ molecules were incubated with HEL(52-61); the bimolecular complex was isolated by Sephadex G50 chromatography and concentrated; a 10,000 fold excess of unlabeled HEL(52-61) was added; at different times the solution was fractionated in order to determine the amounts of free and complexed peptide. There was minimal dissociation of the peptide, in accordance with the results published with an ovalbumin peptide by the Grey laboratory (Buus et al., 1986b).

The turnover of Ia-peptide complexes in viable cells was much more rapid than that observed with fixed cells or with isolated peptide-MHC-II complexes. Thus, an active mechanism must mediate the turnover of these complexes. In B lymphoma cells the disappearance of $I\text{-}A^k$ peptide complexes was very rapid relative to the turnover of the $I\text{-}A^k$ molecule. It suggests that the active turnover of the complexes was mediated by their dissociation to free peptide and $I\text{-}A^k$, which could then be recycled to present new peptides. In macrophages, the half life of the complexes was somewhat longer (1/3-1/2 the half life of the $I\text{-}A^k$ molecule). Recycling of $I\text{-}A^k$ may be less prevalent in macrophages and largely reflects the turnover of the Ia molecule. These conclusions fit with other studies using cycloheximide and Brefeldin A that imply a greater degree of "recycling" of MHC-II molecules in B lymphoma cells than in macrophages (Table II).

TABLE II. COMPARISON BETWEEN MACROPHAGES AND B LYMPHOMA CELLS

	MACROPHAGES	B LYMPHOMA CELLS
CYCLOHEXIMIDE	Inhibits	Does not inhibit
BREFELDIN-A	Inhibits	Does not inhibit
DEXTRAN	Inhibits	Does not inhibit
PEPTIDE MHC-II HALF LIFE	4-5 hours	15-55 minutes
MHC-II RECYCLING	Slight	Predominant

Treatment of B lymphoma cells and macrophages with cycloheximide produced strikingly different results. Doses of cycloheximide that entirely inhibited protein synthesis did not affect the presentation of HEL by B cells but markedly inhibited that of the macrophage. The dramatic reduction in presentation in macrophages following inhibition of protein synthesis was first reported by Jensen (1988). In recent experiments we tested the effect on antigen processing of the fungal product Brefeldin-A, which inhibits the egress of molecules from endoplasmic reticulum - Golgi to plasma membranes (Lippincott-Schwartz et al., 1989). Brefeldin-A inhibited the presentation of HEL by macrophages but not by TA3 cells. Other differences between macrophages and B lymphoma cells are found with regard to the inhibitory effects of some polysaccharides. Incubation with several polysaccharides such as dextran inhibited presentation of HEL by macrophages and not by B lymphoma cells (Harding and Unanue, unpublished experiments, 1989). Taken together, these results indicate that B lymphoma cells may use a recycling pathway of MHC-II much more than macrophages; the latter would depend more on nascent MHC-II molecules (Table II).

We have also provided experimental evidence in support of internalization of MHC-II molecules. Both B lymphoma cells and macrophages contained MHC-II in an intracellular compartment that remained in the cell for long periods after cessation of protein synthesis (Harding and Unanue, 1989). This compartment was revealed by the binding of anti-MHC-II monoclonal antibodies to cells in the presence of saponin; saponin permeabilized the cells to allow the antibody to enter and bind intracellular MHC-II, causing an increment in antibody binding. The intracellular compartment was stable in both the B lymphoma cells and macrophages kept in suspension but was markedly reduced when the macrophages adhered to plastic dishes. In other experiments we directly observed endocytosis of MHC-II molecules in both B cells and macrophages: 25-30% of I-A^k molecules were internalized as measured by the uptake of monoclonal antibodies or their monovalent fragments. The internalized I-A^k molecule was identified by Percoll density gradient fractionation in a light endosomal vesicle that sedimented in identical position as vesicles containing transferrin (c.f. Creswell, 1985). These experiments indicate that while the majority of I-A^k molecules are on the plasma membrane, there is a significant number (20-30%) found in a light endosomal organelle. Endosomes are those intermediate organelles which harbor internalized proteins in their sojourn through the cell and prior to their fusion with lysosomes. In our view it is likely that endosomes have key roles in protein processing and in the coupling of peptides to I-A^k as well as in the peptide interchange suggested from the turnover studies described above.

Recent Biochemical Aspects of Peptide-MHC Interaction - The endosomes, where peptide-MHC-II interaction may take place, may contain components that can modulate primary interaction between peptide and MHC-II molecules. Studying the interaction of MHC-II with photoaffinity conjugates of lysozyme peptides, we have shown that some phospholipids play an important role. The data suggests that the combining site of I-A^k, which holds HEL (52-61), is conformationally not fixed, but is pliable, resulting in variable interactions with peptides.

In these experiments, cell membranes from TA3 cells were exposed to HEL(46-61) conjugated to a photoaffinity moiety either at its amino or carboxyterminus. After UV irradiation, to generate a covalent bond of the peptide to I-A^k, the I-A^k was detergent-solubilized

and isolated using monoclonal antibodies; its component chains were then separated by SDS-PAGE. All the label was found associated with the α chain (Luescher et al., 1988). The same results were obtained when the cell membranes were first solubilized, followed by incubation of the crude material with the probe and subsequent analysis. In contrast, handling of the I-A^k molecule during its purification led to decreased labeling, as well as labeling of the ß chain. Simple dialysis of the crude membrane preparation resulted in a decreased labeling, suggesting that a small soluble component was critical for the binding properties of the MHC-II molecule. Addition of certain lyso-phospholipids resulted in the re-acquisition of strong labeling or labeling predominantly of either of the two chains (Luescher and Unanue, unpublished studies, 1989). The binding kinetics of radiolabeled peptide and I-A^k indicated that the affinity constant for HEL(52-61) binding to I-A^k was 1-3 x 10^{-7} M in the presence of lysophosphatidylserine, as compared to 2-4 x 10^{-6} in their absence. The increase was the result of a higher rate of association. The rate of dissociation was similarly slow with or without phospholipids (experiments to be published by Luescher, Roof and Unanue, 1989).

Thus, class II molecules, particularly in detergent, may have a certain conformational flexibility that affects their interaction with antigens. Conformational changes in the antigen binding site conceivably originate in different mutual orientations in which the subunit chains assemble. These orientations may be affected by particular lipid/detergent interactions of the spanning domains of the subunits.

The analysis of the interaction between HEL(34-45) and I-A^k suggests that a single peptide bound to MHC-II may give rise to more than one antigenic site. HEL (34-45) is a second immunogenic peptide to which T cell hybridomas have been developed. We synthesized peptides having Ala substitutions at each position and then tested each peptide for its binding to I-A^k as well as for stimulation of two T cell hybridomas. Peptides with Ala substitutions at residues 43, 44 or 45 bound to I-A^k and so did peptides with substitution at residues 34, 35 or 36. Indeed, binding assays showed that the Ala substituted peptides competed for the binding of radiolabeled HEL (52-61) to I-A^k. However, the Ala substitutions at the C terminus stimulated only one of the two hybridomas while the peptides with Ala at the N terminus stimulated the other. Thus, although the substituted peptides were able to bind to I-A^k, each had different stimulatory capacity for each T cell hybridoma.

HEL(34-45) could be binding to I-A^k in different conformations or at different places in the combining site of MHC-II. The binding of different T cell receptors, specific for each particular situation, would be affected differently by the Ala substitutions. Another alternative explanation is that the differences are explained by the T cell receptor itself - the peptide could be located in the combining site in a single configuration, but T cells may show higher specificity for either the N or C terminal portion of the peptide. The conclusion, regardless of the final explanation, is that small peptides do generate more than one antigenic specificity when bound to I-A^k. This point may be critical in the generation of diversity for T cell systems. If indeed the T cells recognize two or three amino acids within a peptide (Allen et al, 1987b), it implies that there should be other factors, aside from the random sequence of amino acid, that serve to generate the antigenic diversity of peptides.

Finally, a variety of polysaccharides did not bind to the combining site of I-A^k, I-E^k or I-A^d. The binding was tested either by competition on direct binding of radiolabeled HEL (52-61) to detergent-solubilized, affinity purified I-A^k or by competition for binding of immunogenic peptides to fixed APC (for antigen presentation to T cell hybridomas). Tested compounds included dextrans of various sizes, dextran sulphate, ficoll, lipopolysaccharides, the capsular polysaccharides of H-influenzae, Neisseria meningitidis, and Diplococcus pneumoniae, heparin and a variety of di-and tri-saccharides (Roof and Unanue, experiments to be published, 1989). The hydrophilic nature of the carbohydrate moieties was not conducive to their interactions with the MHC-II combining site. The lack of association of carbohydrate antigens with MHC-II molecules explains their incapacity to stimulate CD4 T cells.

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Studies on the Nature of Physiologically Processed Antigen

S. Demotz, H.M. Grey, and A. Sette

INTRODUCTION

Activation of class II-restricted T cells requires the formation of a trimolecular complex between antigenic peptide fragments, class II MHC molecules, and the T cell receptor (Buus et al. 1987). In contrast to the substantial body of evidence characterizing the interaction between synthetic peptides and class II molecules, essentially no data are available on the nature of the antigen resulting from in vivo processing of protein molecules. It has been postulated that fragments arise from limited proteolytic degradation of native antigens inside acidic compartments of the antigen-presenting cells; however, up to now no direct chemical characterization of physiologically processed peptides has been presented. In this paper we review experiments that we have performed that provide the first partial characterization of a physiologically processed antigen.

RESULTS

Taking advantage of the high stability of the complexes between MHC molecules and antigenic peptides, we attempted the isolation and characterization of a naturally processed antigenic determinant from hen egg lysozyme (HEL) pulsed A20-1.11 B lymphoma cells (A20 cells). First, IE^d molecules were purified by affinity chromatography from a lysate of HEL-pulsed A20 cells (HEL/IE^d), and an aliquot of this IE^d preparation was inserted into lipid planar membranes (PM). These complexes strongly stimulated IL-2 production by the IE^d-restricted T cell hybridoma 1 H-11.3, for which the sequence HEL 107-116 had previously been identified as the minimal antigenic determinant (Adorini et al. 1988). Thus, this IE^d preparation contained naturally processed material that included the HEL 107-116 determinant (Table 1, first row).

The naturally processed antigen contained in such IE^d preparations was isolated by acid treatment of acetonitrile-precipitated HEL/IE^d material, followed by Sephadex G-10 chromatography (Buus et al. 1988). As assessed by sodium dodecylsulfate-polyacrylamide gel electrophoresis, this step completely depleted the acid-eluted HEL/IE^d peptide preparation of IE^d.

Table 1. Isolation of naturally processed antigenic material from antigen-pulsed B lymphoma cells

Source of IE^d	Antigen added to purified IE	U/ml IL-2 produced by 1 H-11.3 T cells
HEL-pulsed A20[a]	nothing	1280
Unpulsed A20	20 ng synthetic HEL 107-116	20
Unpulsed A20	200 ng synthetic HEL 107-116	640
Unpulsed A20	acid-eluted peptides[b] from HEL/IE^d	80

[a] A20 cells grown in RPMI medium supplemented with 5% fetal calf serum, 100 µg/ml streptomycín, 100 U/ml penicillin G, and 5 x 10^{-5} 2-mercaptoethanol were pulsed for 24 hours with 1 mg/ml hen egg lysozyme (HEL, Grade I, Sigma, St. Louis, MO). IE^d molecules were purified by affinity chromatography on 14.4.4 anti-IE^d monoclonal antibody column.

[b] To prepare acid-eluted IE^d peptides, 350 µg IE^d purified from 1.5 x 10^{10} HEL-pulsed A20 cells (HEL/IE^d) were acetonitrile precipitated, treated for 30 minutes at 37°C with 2.5 M acetic acid, and then loaded on a Sephadex G-10 column equilibrated with acetate buffer. The peptide material eluted in the void volume was collected and lyophilized twice.

In vitro prepared complexes between IE^d and peptides were obtained by dissolving the lyophilized peptides in 10 µl phosphate buffer saline/100 mM tris, pH 7.2, supplemented with 1 mg/ml sodium azide and a cocktail of protease inhibitors (16 mM EDTA, 2.6 mM 1,10-phenanthroline, 0.15 mM pepstatin A, and 2 mM PMSF), and 10 µl IE^d preparation 1-2 mg/ml. After one day of incubation at room temperature, the samples were diluted to 250 µl with PBS containing 1 mg/ml sodium azide, 1% n-octyl-β-D-glucopyranoside (Sigma, St. Louis, MO), 175 µg/ml L-α-phosphatidylcholine (Sigma, St. Louis, MO), and 25 µg/ml cholesterol (Sigma, St. Louis, MO).

The acid-eluted HEL/IE^d peptides were then incubated with IE^d molecules purified from A20 cells grown in the absence of HEL. The putative complexes were then inserted into PM. These antigen-presenting structures stimulated IL-2 release from 1 H-11.3 T cells, indicating that peptidic material containing the HEL 107-116 sequence was eluted from the IE^d isolated from cells incubated with HEL, and these peptides could be rebound to IE^d molecules and detected by their capacity to stimulate T cells (Table 1, last row). This, then, provided an assay with which naturally processed antigen could be characterized. To this end, an acid-eluted peptide preparation was subjected to reverse-phase HPLC, and each fraction was tested for its capacity, following incubation with IE^d molecules, to form T cell stimulatory complexes. Material antigenic for 1 H-11.3 T cells was eluted within a restricted portion of the solvent gradient, between 34-40% acetonitrile. The activity profile suggested that at least three peptide species were produced by A20 cells during processing of HEL molecules (Fig. 1). The synthetic peptides HEL 107-116 and HEL 105-120, respectively, eluted at 34% and 40% acetonitrile, suggesting that the HEL fragments generated by A20 cells were structurally related to these peptides.

Next, the molecular weights of acid-eluted HEL/IE^d peptides were estimated by Sephadex G50 chromatography. Each fraction was tested for its capacity to produce, when combined to IE^d molecules, complexes antigenically active for 1 H-11.3 T cells. Stimulatory material was found in a single homogeneous peak, with an apparent molecular weight of about 2 kDa (Fig. 2).

As a control, a radioiodinated synthetic peptide corresponding to ^{125}I-Y-HEL 105-120 was subjected to the purification procedures described above, to evaluate the possibility that limited degradation of the antigenic material occurred during the isolation procedure. As assessed by reverse-phase HPLC, no significant degradation could be detected, indicating that the antigenic HEL peptides characterized above were representative of that which were produced by processing within A20 cells.

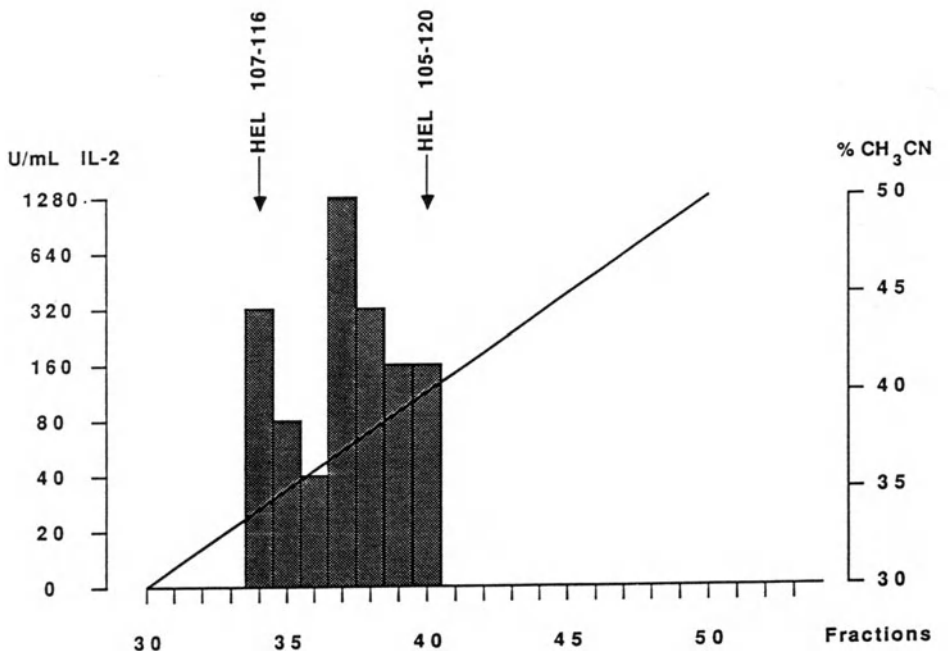

Fig. 1. Reverse-phase HPLC analysis of a naturally processed MHC class II-restricted determinant of lysozyme. Acid-eluted material obtained from 350 µg of HEL/IE^d complexes was separated by reverse-phase HPLC. One-ml fractions were collected, lyophilized, and tested. The elution positions of synthetic HEL 107-116 and HEL 105-120 peptides are indicated.

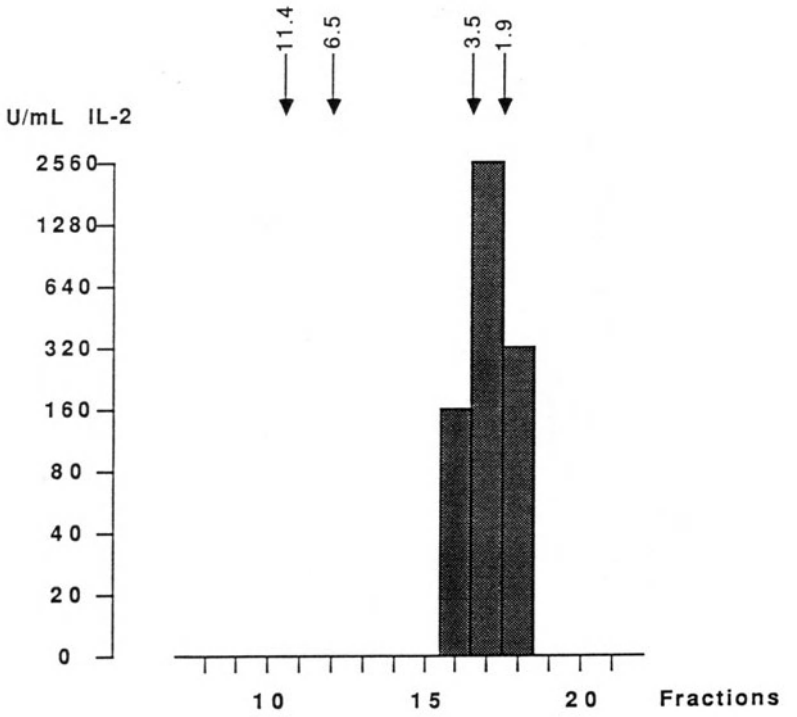

Fig. 2. Gel filtration analysis of a naturally processed MHC class II-restricted determinant of lysozyme. Acid-eluted material obtained from 350 µg of HEL/IE^d complexes was chromatographed on a Sephadex G50 column. Eight-ml fractions were collected, which were lyophilized twice. One-fifth of each fraction was tested. The positions of molecular weight markers are indicated (11.4 kDa, cytochrome c; 6.5 kDa, aprotinin; 3.5 kDa, protamine A; 1.9 kDa, Y-HEL 105-120). In this experiment, IE^d complexes prepared with 20 ng and 200 ng synthetic HEL 107-116 peptide stimulated the production of 1280 U/ml IL-2 and >2560 U/ml IL-2 by 1 H-11.3 T cells, respectively.

Proportion of IE^d occupied by HEL 107-116 containing peptides

To estimate the proportion of IE^d molecules on HEL-pulsed A20 cells that were occupied by peptides containing the HEL 107-116 determinant detected by 1 H-11.3, we compared the antigenicity of an IE^d preparation from HEL-pulsed A20 (HEL/IE^d) with synthetic HEL 107-116/IE^d complexes prepared *in vitro*. The amount of synthetic HEL 107-116 peptide bound to IE^d molecules was first determined by Scatchard analysis, as previously described (Buus et al. 1986). Thus, it was calculated that under saturating concentrations of HEL 107-116 peptide (>10 µg/ml), 7.5% of IE^d molecules were occupied by this peptide. Then, various amounts of HEL 107-116/IE^d complexes were inserted into PM, and for each of them the corresponding 1 H-11.3 T cell stimulation was measured. The total amount of IE^d inserted into each PM sample was kept constant by adding IE^d purified from unpulsed A20 cells, to obtain a direct correlation between the T cell response and the density of antigenic complexes present in the PM (Fig. 3).

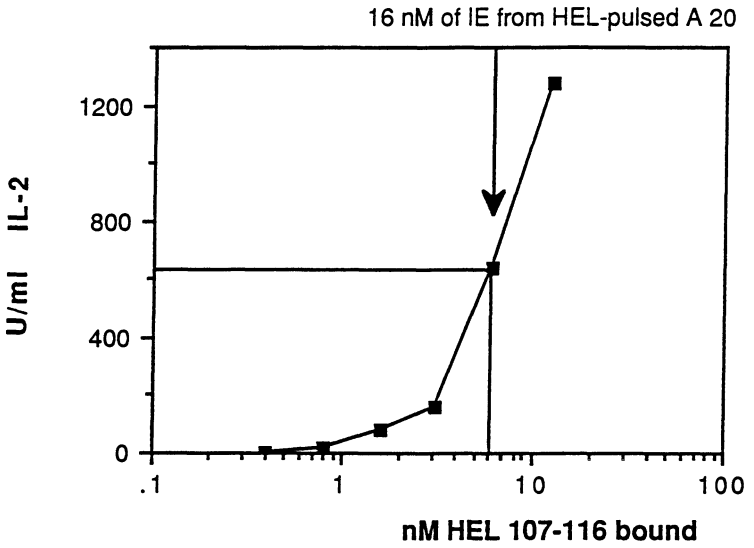

Fig. 3. Occupancy of MHC class II molecules purified from lysozyme-pulsed B lymphoma cells by lysozyme 107-116 determinants. Various concentrations of synthetic HEL 107-116 peptide bound to IE^d molecules and inserted into PM were tested for their ability to trigger 1 H-11.3 T cell stimulation. A known concentration of IE^d molecules purified from HEL-pulsed A20 cells (16 nM) was inserted into PM and used to stimulate 1 H-11.3 T cells.

It was found that naturally processed HEL/IE^d complexes inserted at 16 nM into PM stimulated 1 H-11.3 T cells to produce 640 U/ml IL-2. A similar T cell activation was reached with 6 nM HEL 107-116 peptide bound to IE^d molecules. Assuming that HEL 107-116/IE^d complexes were as stimulatory as HEL/IE^d complexes, these data would indicate that 38% of the IE^d molecules expressed by HEL-pulsed A20 cells were occupied by HEL 107-116 determinants. An independent method of estimating the degree of occupancy was based on the amount of antigenic peptides eluted from an HEL/IE^d preparation after its separation on G50 gel filtration. In this experiment, antigenic material obtained from 2 nmoles of IE^d yielded T cell stimulatory activity similar to that obtained by using approximately 0.2 nmoles of synthetic HEL 107-116 peptide. Thus, by this calculation it would appear that approximately 10% of the IE^d molecules purified from HEL-pulsed A20 cells carried HEL 107-116 determinants. This figure is a minimal estimate, since the G50 fractions probably also contained non-HEL-derived IE^d binding peptides that would tend to compete with, and lower the efficiency of, binding of the HEL peptides to IE^d.

DISCUSSION

In this report, we have described techniques for the preparation of complexes between MHC molecules and naturally processed antigenic peptides that allowed us to initiate characterization of a naturally processed determinant of HEL. The micro-heterogeneity obtained upon HPLC analysis of the antigenic peptides produced by A20 cells indicates that processing does not lead to single unique peptide fragments, but rather to a series of related peptides of approximately 15-20 residues in length. The observed heterogeneity may be due to cleavages at different positions within the HEL sequence, reflecting the usage of either different proteases or proteases displaying a broad substrate specificity.

Attempts to quantitate the proportion of HEL/IE^d complexes bearing HEL 107-116 determinants yielded surprisingly high estimates (10-40%). These data can be at least in part rationalized, considering that HEL represented under the experimental conditions used about one-third of the total extracellular proteins (1 mg/ml HEL in 5% total calf serum). Furthermore, the HEL 107-116 peptide region is the major IE^d binding site of HEL (Adorini et al. 1988). Nevertheless, the efficiency of *in vivo* binding of HEL-processed fragments by IE^d molecules appears to be quite high. If, however, naturally processed antigen had, for some unknown reason, an increased antigenic potency compared to the synthetic peptide, this could also contribute to the high biologic activity observed (Falo et al. 1986). Direct sequencing of naturally processed antigenic peptides should allow us to solve these issues.

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Role of MHC Polymorphism in Autoimmune Disease

H.O. McDevitt, D.C. Wraith, D.E. Smilek, and L. Steinman

INTRODUCTION

The immune response to foreign protein antigens, and to self-protein antigens in autoimmunity, begins with an interaction between a peptide fragment of the protein antigen and an MHC class II molecule. Binding of the peptide in the binding site of the class II molecule is followed by recognition of this complex by T cells with receptors complementary for the peptide-class II molecular complex. This induces the development of memory helper T cells, and leads to both T cell and β cell immune responses to the peptide fragments of the antigen and to the intact three dimensional protein (Schwartz, R.H., 1986).

Immunogenicity of a protein, and selection of the immunodominant peptides of the protein, are functions of the amino-acid sequences of the MHC class II α and β chains (Schwartz, R.H., 1986). Therefore, ability to respond to peptide, and susceptibility to many autoimmune diseases in both mouse and man is strongly influenced by the allelic polymorphism of MHC class II molecules (Schwartz, R.H., 1986; Todd, et al., 1988).

Thus, in patients with type 1 insulin dependent diabetes mellitus (IDDM), rheumatoid arthritis (RA), and pemphigus vulgaris (PV), allelic polymorphism plays a major role in susceptibility to each of these autoimmune diseases (Todd, J.A., et al., 1987; Todd, J.A., et al., 1988; Sinha, A.A., et al., 1988; Scharf, S.J. et al., 1988). Disease susceptibility, (and in some cases resistance to an autoimmune disease) is associated with a particular first domain allelic hypervariable region sequence that is often shared by several HLA-DR or DQ alleles. Specific allelic hypervariable region sequences, presumably by conferring ability to bind a self peptide with high affinity, confer susceptibility to specific autoimmune diseases.

The alleles and allelic hypervariable region sequences predisposing to autoimmune disease can be used in strategies designed to prevent treat these diseases.

In principle, the interaction between MHC molecules, peptides, and T cell receptors can be attacked at several points. When T cell receptor (TcR) Vα or Vβ gene usage is restricted, antibodies to the Vα or Vβ gene product can be used to prevent the autoimmune disease (Acha-Orbea, H., et al., 1988; Urban, J.L., et al., 1988; Owhashi, M., and E. Heber-Katz, 1988). In some situations, T cells capable of inducing autoimmune disease may use multiple Vα and Vβ genes (Sakai, K., et al., 1988). Here, an alternative approach to preventing the autoimmune disease might employ 'blocking' peptides, with a high affinity for the binding site of the MHC susceptibility allele, preferably with no cross-reactivity with peptides inducing the autoimmune disease. Such competitive inhibition has been demonstrated at two levels. Gefter, et al., have reported that it is possible to inhibit T cell activation <u>in vitro</u> through the use of peptides which compete with the specific T cell antigen for MHC binding (Guillet, J.G., et al., 1987). More recently, Adorini, et al., have 'blocked' the <u>in vitro</u> priming of murine I-A^k binding mouse (self) lysozyme peptide 52-61 (Adorini, L., et al., 1988).

One animal model in which the peptide epitopes, T cell receptor (TCR) repertoire, and MHC susceptibility alleles have been fully characterized is experimental autoimmune encephalomyelitis (EAE). This disease is induced by immunization with myelin basic protein (MBP), or with peptides of MBP. The disease is characterized by lymphocyte infiltration into the CNS resulting in demyelination and paralysis.

The acetylated N-terminal peptide 1-11 of rat MBP (Ac1-11) shares the first nine amino acids with mouse MBP, and is able to induce encephalitogenic T cells in mice expressing I-A^u antigens. T cell recognition of Ac1-11 is an important model for immune intervention since this epitope is immunodominant for disease induction in both Pl/J (H-2^u) and (PL/J x SJL) Fl (H-2^{uxs}) mice, even though the latter strain is able to mount a T cell response to both Ac1-11 and 89-101 (Figure 1). The experimental goal is thus, to define MHC and TCR interaction residues in order to design peptides which might inhibit the autoimmune response leading to EAE.

It has been shown (Acha-Orbea et al., 1988), that encephalitogenic T cell clones from PL/J and (PL/J x SJL) Fl mice use only four related TCR types for recognition of Ac1-11, all utilizing Vβ8.2. These clones all displayed a similar pattern of recognition when tested on a panel of peptides constructed by single amino acid substitution of the first nine amino acids of Ac1-11 with serine (residue 1)

or alanine (residue 2-9) peptide. Most of these peptide analogs stimulated encephalitogenic T cell clones at the same concentration as the Ac-11 peptide. Thus, these peptides are capable of binding to I-A^u. Substituting alanine for lysine at position 4 generated a heteroclitic peptide Ac1-11[4A], which stimulated the T cell clones much better than Ac1-11. Substituting alanine for glutamine at position 3 (Ac1-11[3A]) or proline at postition 6 (Ac1-11[6A]) abolished the response of the clones to the peptide analogs.

To distinguish whether these differences in T cell clone responsiveness to the analogs were based on MHC-peptide or TCR-peptide interactions, an MHC binding assay was developed (Cell, in press). A radiolabelled, photoaffinity probe was designed which would both bind and crosslink to the I-A^u. The ability of unlabelled peptide to inhibit binding of the probe then provided a measure of the peptide's ability to bind to I-A^u. Ac1-11[4A] was found to bind to I-A^u with greater than ten fold higher affinity relative to Ac1-11, thus explaining this peptide's heteroclitic stimulation of the T cell clones. Ac1-11[3A] and Ac1-11[6A] also bound to I-A^u, even though unable to activate encephalitogenic T cell clones. Lymph node proliferation studies showed that the two latter peptides activated T cells which were specific for each immunizing peptide, and were not cross-reactive with the Ac1-11 peptide. These data define the N-acetyl group and residue 4 as determinants important in I-A^u binding, and residues 3 and 6 as determinants important in TCR interactions (Figure 2 and Table 1).

Ac1-11[4A] is heteroclitic both for I-Au binding and T cell activation. Unexpectedly, this peptide is a poor immunogen when tested in lymph node T cell activation experiments. It appears that the heteroclitic analog induces a state of specific unresponsiveness in potentially encephalitogenic, Ac1-11 specific T cells <u>in vivo</u>. On co-immunization with an encephalitogenic dose of Ac1-11, a three fold molar excess of Ac1-11[4A] significantly inhibits the disease induced by injection of Ac1-11 alone (Cell, in press). Further experiments are required to determine whether heteroclitic 'blocking' analogs will provide a feasible means of immune intervention. Precise characterization of disease associated T cell epitopes may well provide a means of predictably designing MHC 'blocking' peptides, as shown in this study for the N-terminal peptide of MBP.

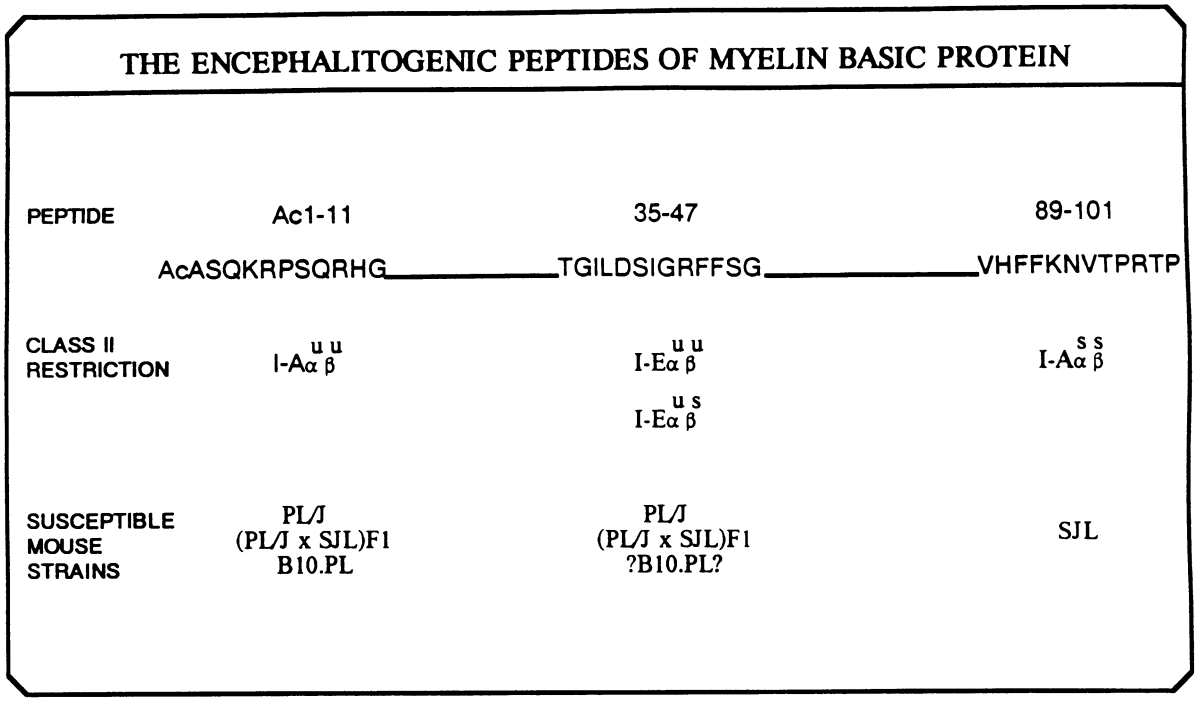

Figure 1. Amino acid sequences of myelin basic protein encephalitogenic peptides are listed with their corresponding restriction elements and susceptible mouse strains.

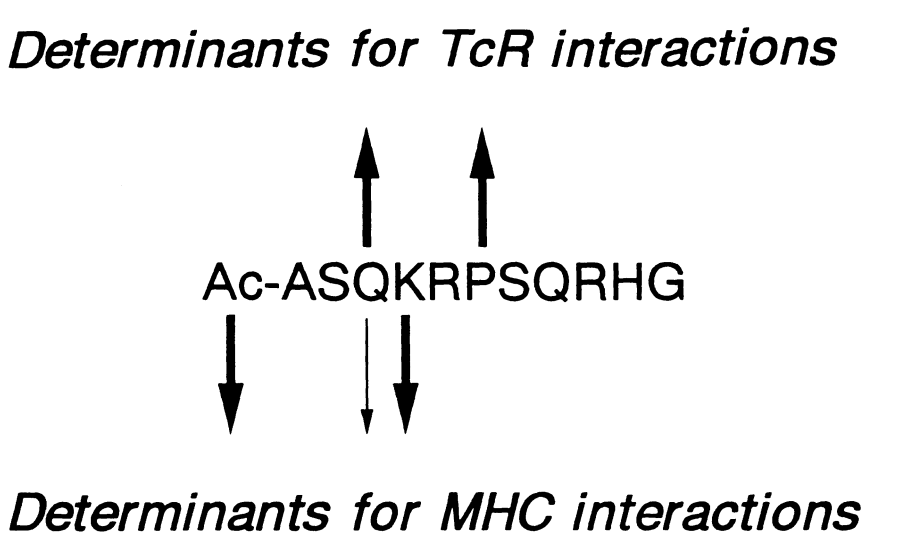

Figure 2. Arrows point from each amino acid residue of Ac1-11 to its role as either a T cell or an MHC interaction determinant.

In vitro I-A binding and T cell activation properties of Ac1-11 substituted peptides			
		T Cell * Activation	$I\text{-}A^{u}$ Binding
Ac1-11	ASQKRPSQRHG	++	++
Ac1-11[1S]	SSQKRPSQRHG	+	nd
Ac1-11[2A]	SAQKRPSQRHG	++	nd
Ac1-11[3A]	ASAKRPSQRHG	-	+
Ac1-11[4A]	ASQARPSQRHG	++++	++++
Ac1-11[5A]	ASQKAPSQRHG	+	nd
Ac1-11[6A]	ASQKRASQRHG	-	++
Ac1-11[7A]	ASQKRPAQRHG	++	nd
Ac1-11[8A]	ASQKRPSARHG	++	nd
Ac1-11[9A]	ASQKRPSQAHG	++	nd
1-11[4A]		++	++
1-11[3A,4A]		-	++
Ac1-11[3A,4A]		-	++++

*Data for T cell activation by substituted peptides [1A] through[9A] are summarized from Acha-Orbea, H. et al., 1988.

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Complex Regulation of MHC Class I Expression: Constitutive and Modulated Patterns of Expression

B. David-Watine, M. Kieran, F. Logeat, A. Israël, and P. Kourilsky

The major transplantation antigens encoded by class I genes of the major histocompatibility complex (MHC) play a key role in a number of immunological processes, particularly in the recognition of foreign antigens by cytotoxic T cells of the host (Zinkernagel and Doherty 1980). The MHC class I heavy chain (37-45 kd) are non covalently associated on the cell surface with the non-polymorphic beta-2-microglobulin (beta-2m) light chain (12 kd).

The expression of the MHC class I genes of the mouse is developmentally regulated : class I mRNA and proteins are not detected until the midsomite stage of mouse embryogenesis (Ozato et al. 1985; Morello et al. 1985). Then, they are expressed on most somatic cells of the adult organism except on brain cells and certain cell population of the placenta (David-Watine 1989a). They are not expressed in undifferentiated embryonal carcinoma cells which exhibit a variety of molecular and cellular properties characteristic of the early embryo (Silver et al. 1983; Morello et al. 1982). Similarly the beta-2m is expressed by virtually all adult cells, but not by embryonal carcinoma cells. However, its synthesis follows a different time course during embryogenic development, so that it is not clear whether common regulatory mechanisms control the expression of H-2 (Histocompatibility-2 : mouse MHC) and beta-2m genes (Morello et al. 1985; Sawicki et al. 1981).

Molecular cloning studies have revealed that most of the class I genes (30 to 40 depending on the mouse strain) are located in the Qa-Tla region (Winoto et al. 1983). To this group of genes belong those coding for the serologically defined Qa and Tla differentiation antigens (Flaherty 1981). They are much less polymorphic than the transplantation antigens like H-2K or H-2D and some of them display restricted tissue distribution : Qa-2 antigens are only expressed in certain subsets of lymphoid cells (Rabinowitz et al. 1986) and Q10 expression is restricted to liver and fetal yolk sac (Cosman et al. 1982; David- Watine et al. 1987). Furthermore, the expression of Qa-Tla antigens has been documented at stages of embryonic development as early as the oocyte and 2-cells stage (Warner et al. 1987). The function of the proteins encoded by these genes remains unknown.

The diversity in tissue distribution and developmental regulation among class I molecules may reflect transcriptional control due to various trans-acting nuclear factors interacting with different cis-acting elements. For several years, our interest has been focussed on the regulation of expression of the ubiquitously expressed H-2K^b gene. More recently, we have begun to analyse the regulatory elements of the Q10 gene, in order to get some insight of the mechanisms by which class I genes may be so diversely expressed.

DELETION AND EXPRESSION ANALYSIS OF THE H-2K^b PROMOTER

Deletion analysis of the H-2K^b promoter has defined several regions that are important for the expression and regulation of this gene. These include an enhancer-like sequence (enhancer B : -120, -61) and another enhancer sequence (enhancer A : -193, -158), which overlaps an interferon response sequence, IRS (-165, -137), homologous to the sequence found in the promoter region of several human genes responsive to IFN-alpha (Friedman and Stark 1985). Both of these enhancer sequences are conserved inside the promoter of several genes coding for classical transplantation antigens (H-2K^k, H-2L^d) but not in the promoter of class I genes located in the Qa-Tla region, as for example in the promoter of the Q10 gene. Two mismatches in the sequence corresponding to enhancer A in the Q10 promoter strongly decrease its stimulating activity when compared to that of H-2K^b in mouse fibroblasts (Kimura et al. 1986). These sequences display enhancer activity in cells that normally express H-2 class I genes but not in undifferentiated embryonal carcinoma cells like F9 cells.

The same positive trans-acting factor binds to enhancer sequences in the promoters of the H-2K^b and beta-2m genes

By various methods (gel retardation, DNAse footprint, methylation interference) we have characterised a protein which binds to a palindromic sequence located in enhancer A (-171 to -158). This protein, called KBF1, can also bind to a second imperfect palindrome located in the same region (-187 to -176). Interestingly, this factor also binds to an analogous sequence located in the beta-2m promoter and exhibiting enhancer activity (-127 to -116) (Israël et al. 1987). In vivo competition experiments using cotransfection with an excess of the palindromic sequence indicate that KBF1 is a positive transacting factor involved in the expression of both H-2 and beta-2m genes (Israël et al. 1987).

PURIFICATION OF KBF1

By using a combination of conventional chromatography and affinity columns, we have purified the KBF1 factor to homogeneity : it is a 48 kd protein which displays binding characteristics similar to those observed in crude extracts (Yano et al. 1987).

Enhancer binding activities present in F9 cells, before and after differentiation

KBF1 activity has been detected in all cell types tested so far, except in undifferentiated carcinoma cell lines like F9 cells. These cells can be induced to differentiate after treatment by retinoic acid and dibutiryl cyclic AMP. This differentiation is accompanied by the expression of class I antigens at the cell surface. It was then interesting to check the status of KBF1 activity before and after differentiation.

By band shift assays we demonstrated that KBF1 binding activity is absent in undifferentiated F9 cells, but is induced after treatment with retinoic acid and dibutyryl cAMP. These results were correlated with the lack of activity in undifferentiated cells of expression vectors harbouring binding sites for KBF1, while these constructs are active in various differentiated cells (Israël et al. 1989).

PURIFICATION OF KBF2

Another protein, designated KBF2, binds to the same sequence as KBF1 and is present in undifferentiated F9 cells, where it was first selected, as well as in all differentiated cells tested so far. In gel shift assay, KBF2 displays a higher affinity for the enhancer sequence of beta-2m than for the homologous sequence of K^b. This property was used to purify this factor to near homogeneity. It is a 58 kd protein which can bind to its target sequence either as a monomer or as a dimer, depending on its concentration, while KBF1 only binds as a dimer (Israël et al. 1989).

Different strategies have been developed to clone the genes coding for these two factors. Putative KBF1 cDNA clones have been isolated and are analysed at the moment in our laboratory.

REGULATION OF CLASS I GENE EXPRESSION BY DIFFERENT FACTORS

A complex set of nuclear factors interact with the H-2K^b enhancer and is modified after treatment of the cells by TNF

By methylation experiments carried out on the entire enhancer A, we confirmed the binding of KBF1 to the proximal palindrome. However, a second factor interacts with the interpalindromic region and part of the distal palindrome, then blocking the interaction of a second dimer of KBF1. Using nuclear extracts derived from HeLa cells induced by TNF, we observed that these two factors no more bind to their related sequences and that a different set of proteins interact with the palindromic sequences.

The IRS sequence potentiates the activity of the enhancer sequence

The H-2K^b promoter can be induced by all three types of interferon. The IRS sequence is necessary for induction to occur but it is active only when associated with a functional enhancer A sequence (for example, that of K^b or L^d), and is no more active when associated with the homologous Q10 sequence nor with the 72 bp repeat of SV40. However, the IRS sequence of Q10 is potentially functional, that is it can also increase the activity of an enhancer A from L^d or K^b upon IFN stimulation (Israël et al. 1986).

Three cAMP responsive elements are present in the H-2K^b promoter

One element has been mapped within the enhancer A region corresponding to an AP2-like binding site and two others in the (-210, -181) and (-109, -84) regions.

REGULATORY ELEMENTS INVOLVED IN LIVER SPECIFIC EXPRESSION OF THE Q10 GENE

We have compared the sequence of the proximal promoter region of Q10 to that of the ubiquitously expressed H-2K^b; these sequences are well conserved and can be aligned. We have found that nearly all the sequences which are known to be involved in the ubiquitous expression of H-2K^b are pontually altered or in some instances deleted

in the case of Q10. Conversely, most of the sequences of Q10 that bind liver-specific factors are partially deleted or mutated in the H-2K^b promoter. No liver factor binds to the sequence homologous to enhancer A in the Q10 promoter (David-Watine et al. 1989b). Our results are summarised in figure 1.

As already discussed, the IRS sequence is functional. We are currently investigating the role of the different sequences which bind liver-specific factors in the regulation fo Q10 expression by in vitro transcription experiments and transfection experiments in hepatoma cells and primary hepatocytes.

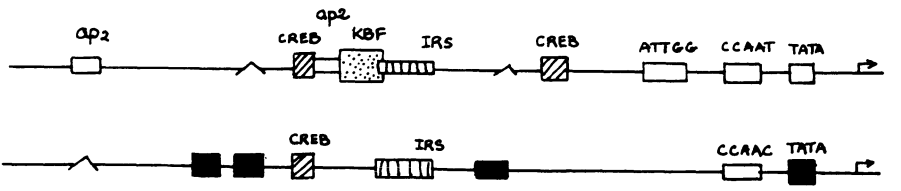

Fig. 1. The sequence upstream of the transcription initiation site of the H-2K^b and the Q10 genes have been aligned and compared. Sequences of the H-2K^b promoter known to be involved in the ubiquitous expression of this gene have been boxed. Identical sequences (i.e. having the same function or binding the same factor) have been boxed in the same fashion for Q10; sequences binding liver specific factors are also indicated with black boxes.

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I Structure and Function of Recognition and Effector Molecules

Somatic Changes of Ig and T Cell Receptor Genes

Organization and Reorganization of Antigen Receptor Genes

R.M. Perlmutter

INTRODUCTION

The successful elaboration of immune responses in vertebrates is critically dependent on the generation of a satisfactory repertoire of antigen receptor structures capable of discriminating the universe of pathogens from a similarly complex set of autologous determinants. Diversification of antigen receptors is achieved through somatically propagated gene rearrangement events that juxtapose discontinuous germline gene segments, forming lymphocyte-specific transcriptional units. The combinatorial assembly of antigen receptor gene segments permits a limited amount of germline genetic material to encode an enormous repertoire of distinct recognition structures.

Four fundamental mechanisms underlie the diversification of antigen receptors. These are: (1) the maintenance of several hundred germline gene segments that contribute to the structure of receptor variable regions, (2) combinatorial association of these germline gene segments (3) flexibility in the DNA rearrangement process that juxtaposes germline elements specifically in lymphocytes, and (4) combinatorial association of polypeptide chains that together determine the structure of the mature receptor. These basic features of receptor diversification were initially defined a decade ago for antibody genes (reviewed in Tonegawa, 1983) and have since been shown to apply equally well to T cell receptor genes (Davis and Bjorkman, 1988). In addition, the antibody repertoire is further diversified through a process of somatic hypermutation which introduces substitutions, insertions and deletions into the rearranged antibody gene sequence (Kim et al., 1981). During the three years since the Sixth Congress of Immunology, considerable progress has been made in characterizing the long-range structures of the antibody and T cell receptor loci. In addition, a series of experiments have illuminated the mechanism of DNA rearrangement in these gene families, and have focused attention on the physical properties of the recombinase that catalyzes this process. The process of repertoire assembly has been dissected in considerable detail, and has been shown to be developmentally regulated for both T cell and B cell antigen receptors. Finally, an increasingly persuasive data set favors the view that allelic differences in germline receptor elements may contribute to disease susceptibilities in man. These advances are summarized below.

ORGANIZATION OF ANTIGEN RECEPTOR GENES

There are seven known sets of rearranging genes that encode

antigen receptors. These are the antibody heavy (H), κ and λ genes, and the α, β, γ, and δ genes of the T cell antigen receptor. In each of these gene systems, gene rearrangement events, which occur only in developing lymphocytes, are required to create a functional transcriptional unit. Figure 1 diagrams the gene rearrangement events that occur in the antibody heavy chain locus during B cell development. Rearrangement begins with juxtaposition of D_H and J_H gene segments. A V_H gene segment is then joined to the D-J unit. This brings transcriptional regulatory sequences positioned 5′ to each V_H segment into proximity with an enhancer sequence ("E" in the Figure) located in the intron between J_H and C_μ. There is some evidence that transcriptional activity may be correlated with the rearrangement process (Blackwell et al., 1986; Alt et al., 1987).

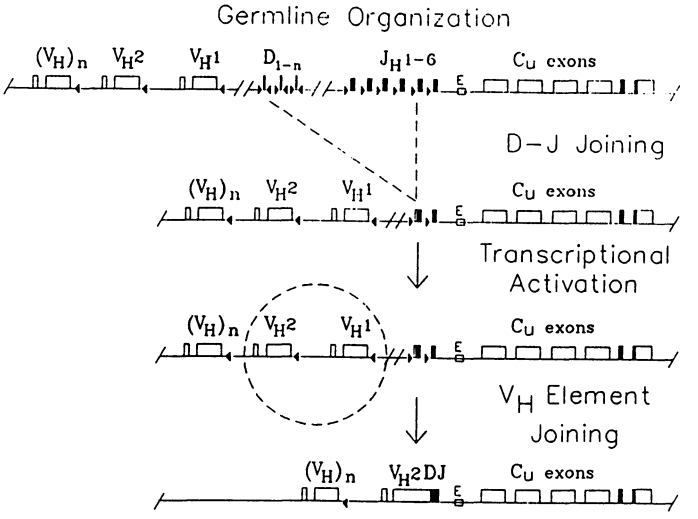

Fig. 1. DNA rearrangements form a functional antibody heavy chain gene. The positioning of variable (V_H), diversity (D_H), joining (J_H) and constant region (C_μ) elements is schematized, and the order of rearrangement outlined.

Details of the physical organization of T cell receptor and antibody genes have become increasingly available. In man, V_H, D_H, and J_H gene segments have been physically linked on a 120 kb DNA segment, where all are positioned in the same transcriptional orientation (Schroeder et al., 1988). The total human V_H repertoire includes at least 100 elements which span more than 10^6 bp on chromosome 14 (Berman et al., 1988; Lee et al., 1987). Interestingly, the most 3′ V_H element in the human genome is expressed early in fetal development (Schroeder et al., 1987), emphasizing the relationship between physical proximity to J_H gene segments and the timing of rearrangement of V_H elements during fetal ontogeny (Perlmutter et al., 1985; Yancopoulos et al.,

1988). Alignment of the known human germline V_H sequences reveals that there exist at least seven small families of V_H elements, members of which share greater than 80% overall nucleotide similarity (Fig. 2). This familiy organization has been conserved throughout much of the mammalian radiation, as has the overall organization of the heavy chain locus (Becker et al., 1989; Schroeder et al., 1989; Wood and Tonegawa, 1983).

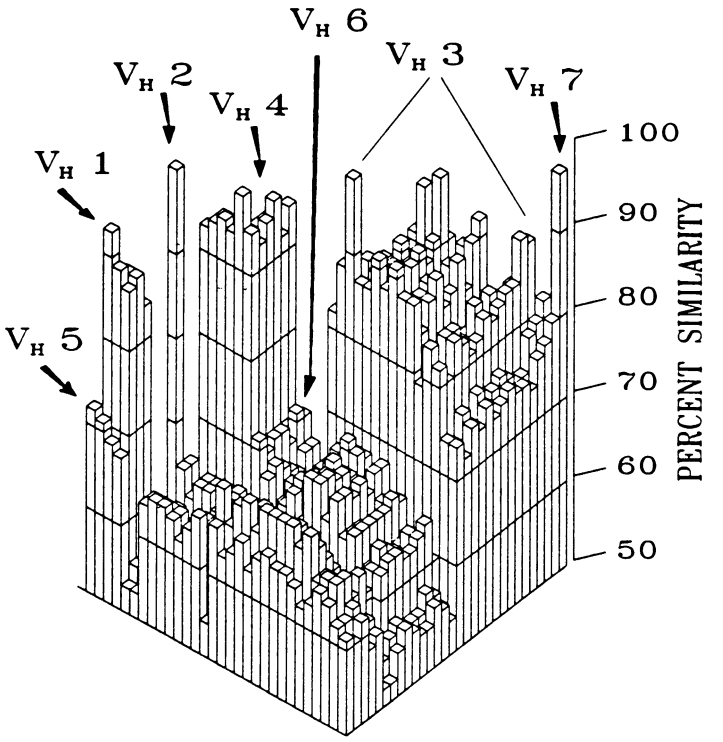

Fig. 2. Family organization of human V_H sequences. Shown is a pairwise alignment of 30 presumed or confirmed germline human V_H sequences with the percent similarity indicated for each comparison on the Z axis. Redrawn from Schroeder et al., 1989.

A similarly detailed analysis of the human κ light chain locus has been conducted by Zachau, permitting the definition of more than 50 germline V_κ gene segments (Straubinger et al., 1988; Lorenz et al., 1987; reviewed elsewhere in this volume). Rearrangement of V_κ gene segments proceeds in some cases through an inversional mechanism, as does rearrangement of the V_β14 gene segment of the T cell antigen receptor (Malissen et al., 1986).

Organization of T Cell Receptor Genes

Two forms of T cell receptor have been defined. These are heterodimers of either α and β chains, or γ and δ chains. Examination of the genes encoding these chains reveals the

multiple different configurations that rearranging gene families may adopt to promote diversification. The human β locus is positioned on chromosome 7 and is known to span at least 600 kb of germline DNA (Lai et al., 1988). The murine β locus is similarly organized (Chou et al., 1987; Lai et al., 1987). Diversification of the β genes is achieved by use of J_β, D_β and V_β gene segments, the latter of which rearrange in some instances by inversion. Like antibody heavy chains, T cell receptor β chain junctions frequently contain additional nucleotides ("N regions") that are added by a template independent process (Davis and Bjorkman, 1988). Diversification of the β chain repertoire also results from the fact that D_β gene segments are functional when read in any reading frame, a phenomenon rarely encountered in D_H gene segments.

The α chain locus is unusual in two respects: it contains a very large number of J_α gene segments spanning more than 80 kb of germline DNA (Hayday et al., 1985; Winoto et al., 1985; Yoshikai et al., 1985), and it contains the δ locus imbedded within it (Davis and Bjorkman, 1988). By simply enumerating the number of gene segments in the α and β loci, and estimating the extent of N region diversification, the combinatorial variability for these receptors can be calculated to provide a repertoire of more than 10^{15} different structures (Davis and Bjorkman, 1988).

A similar type of analysis can be performed for the γ genes, believed to span about 200 kb of germline DNA in man (Lefranc and Rabbitts, 1989), and for the δ locus. Here the combinatorial joining of multiple D_δ regions combined with N region diversity permits assembly of a repertoire that may exceed 10^{18} distinct structures (Davis and Bjorkman, 1988).

MECHANISTIC FEATURES OF RECEPTOR GENE REARRANGEMENT

Fujimoto et al. (1985) first noted that thymocyte nuclei contain extrachromosomal circular DNA elements. Cloning of these circular DNA segments revealed that most derived from T cell receptor gene rearrangement events (Okazaki et al., 1987). These studies directly demonstrated that antigen receptor gene recombination occurs through intramolecular deletion of intervening DNA. Similar studies have since been reported for the λ light chain gene rearrangements in immature chicken B cells (McCormack et al., 1989), and for the α chain rearangements of the T cell receptor in the mouse (Winoto and Baltimore, 1989). The latter analysis also revealed that α chain rearrangement was not preceded by δ chain rearrangement, implying that α/β and γ/δ T cells represent distinct lymphocyte lineages.

A higher resolution view of the process of antigen receptor gene rearrangement has resulted from studies of Abelson virus-transformed pre-B cell lines which will satisfactorily catalyze the rearrangement of heterologous recombination substrates introduced by transfection or infection (reviewed in Alt et al., 1987). These analyses have been particularly informative when performed in a quantitative fashion using a shuttle vector system that permits identification of a satisfactory rearrangement through the acquisition of drug resistance in a bacterial host (Hesse et al., 1987). From these studies it is clear that the conserved heptamer and nonamer sequences which flank rearranging gene segments are absolutely required for recombinase recognition. Moreover, rearrangement in the majority of cases yields products

in which the recombinase recognition sequences are seamlessly joined in a head-to-head fashion [Lieber et al. (1988) and Lewis et al. (1988) describe some significant exceptions]. Such studies are placing important constraints on models of recombinase function.

Three different experimental strategies promise to yield considerable information regarding the structure of the recombinase itself. First, a mouse strain bearing a recessive mutation that results in a form of severe combined immunodeficiency (SCID) has been shown to be defective in joining the coding regions of antibody and T cell receptor genes (Lieber et al., 1988). Thus detailed examination of the SCID mutation promises to illuminate the genetic basis for recombinase function. A more direct approach has been taken by Sakano and collaborators who have preliminarily defined a nuclear protein in pre-B cell lines that specifically interacts with immunoglobulin recognition sequences (Aguilera et al., 1987). This binding activity appears to be distinct from cleavage activities previously described by the same group (Hope et al., 1986) and by others (Desiderio and Baltimore, 1984). More dramatically, Schatz and Baltimore (1988) have recently reported that fibroblasts can be induced to express recombinase activity after transfection with exogenous human DNA. Structural characterization of the "recombinase activator gene" defined by this experiment is presented elsewhere in this volume. By definition, this gene must either encode the recombinase itself (a surprising result since the multiple enzymatic activities of the recombinase might be presumed to be mediated by a complex of many polypeptides), or a trans-acting inducer of recombinase function. Molecular definition of the antigen receptor recombinase and its mechanism of action will continue to be an exciting area of research in the coming years.

REGULATION OF ANTIGEN RECEPTOR GENE REARRANGEMENT

All antigen receptor gene segments are flanked by essentially identical recombinase recognition signals, and yet rearrangements of these gene segments are constrained by cell lineage and by ontogeny. For example, T cell receptor gene rearrangements do not, in general, occur in B lymphocytes. This phenomenon can even be demonstrated following transfection of V_β, D_β, and J_β recombination constructs into pre-B cell lines expressing an active recombinase machinery: D_β-to-J_β rearrangement events occur, but V_β-to-D_β rearrangement does not (Ferrier et al., 1989). Such observations have led to the view that "accessibility" of substrate to recombinase is somehow regulated, perhaps by controlling transcription (Yancopoulos and Alt, 1986; Perlmutter, 1987). A dramatic example of preferential rearrangement of particular gene segments during ontogeny occurs in the case of dendritic epithelial cells, which derive from an early set of thymic emigrants and which express γ/δ receptors of extraordinarily limited diversity (Asarnow et al., 1989; Ito et al., 1989).

Regulation of recombinase substrate choice may explain the developmentally programmed rearrangement of antibody heavy and light chain genes, and of T cell receptor β and α chain genes. Experiments conducted in transgenic animals or using Abelson virus-transformed cell lines indicate that expression of a functionally rearranged heavy chain gene suppresses further heavy chain rearrangements, a phenomenon referred to as feedback regulation (reviewed in Storb et al., 1988; Taussig et al., 1989).

Similarly, lymphocytes from mice made transgenic for a functional T cell receptor β chain do not exhibit endogenous β chain gene rearrangements (Uematsu et al., 1988). Thus it is proposed that the presence of a productively rearranged allele somehow alters the accessibility of gene segments to recombinase. At the same time, it is widely recognized that variable region gene replacement can occur in the context of a previously functional antibody gene sequence (Levy et al., 1989), and suppression of endogenous rearrangements in transgenic animals bearing functional antigen receptor transgenes is often "leaky" (Bluthmann et al., 1989; reviewed in Taussig et al., 1989). Hence the mechanisms responsible for the regulation of recombinase activity and for recombinase substrate selection remain obscure.

Even more mysterious is the enzymic system that permits juxtaposition of antibody heavy chain switch sequences and hence the expression of a previously rearranged V_H-D_H-J_H combination in association with a new constant region sequence. Previous studies have demonstrated that switch regions are positioned immediately 5′ to each set of constant region exons, with the possible exception of $C\delta$. These switch regions are composed of multiple small repetitive sequences, and rearrangements involving these sequences can occur at any of a large number of sites spanning more than 1,000 bp in each case (reviewed in Radbruch et al., 1986). Jack and Wabl (1988) have demonstrated that switch recombination probably occurs via intramolecular deletion, in a fashion analogous to that which occurs during variable region assembly. However the molecular basis for this phenomenon and the means whereby appropriate regulation is achieved have yet to be defined. Recently, it has been possible to direct isotype switching through treatment of B cells with mitogen or IL-4 (Lutzker et al., 1988). Since this treatment also induces transcription from the target gene sequence, it is possible that switch recombinase accessibility is also regulated by transcription. There is, however, no formal proof that transcriptional activation is in any way required for subsequent switch recombination.

SOMATIC HYPERMUTATION

The extraordinary diversification of receptor repertoire that results from gene rearrangements is further amplified in the case of antibody molecules by a process of somatic hypermutation. An intriguing view of this phenomenon derives from the work of Weill and colleagues who have demonstrated that the chicken λ light chain locus contains only a single functional V_λ segment. Diversification of the chicken light chain repertoire results from segmental gene conversion events using a series of pseudogenes located 5′ to the functional V_λ gene segment as templates (Reynaud et al., 1987). Measurements performed in an Abelson virus-transformed pre-B cell line suggest that rearranged antibody heavy chain genes can accumulate substitutions at a rate of 10^{-3} events per base pair per generation (Wabl et al., 1987). The majority of these substitutions are almost certainly not the result of gene conversion events. In the murine antibody response to phosphorylcholine, substitutions do not appear to be donated by closely-related germline sequences and in fact occur in regions of the genome that are known to exist in only a single copy per haploid genome (Perlmutter et al., 1984). Intriguingly, a functionally rearranged transgene is an acceptable target for the somatic mutation process (O'Brien et al., 1987). There is at this

writing no satisfactory model to explain hypermutation in rearranged antibody genes, however it is interesting to note that the process may be regulated during the lifetime of responding B cells, presumably to permit emergence of antibody secreting cells producing molecules with high affinity for antigen (Siekevitz et al., 1988).

FUTURE DIRECTIONS

Research conducted during the last three years has provided considerable insight into the structure of antigen receptor gene families and the mechanisms responsible for the somatic diversification of these sequences. With the nearly complete dissection of the human T cell receptor and κ light chain loci, it will be interesting to learn whether allelic differences in antigen receptor repertoires contribute in any way to variations in disease susceptibility. Preliminary data supporting this view have been adduced through the analysis of individuals suffering from multiple sclerosis (Beall et al., 1989). It will be interesting to learn whether differences in primary antibody repertoires, easily demonstrated as allelic variation in human V_H gene segments (Willems van Dijk et al., 1989), affect the incidence of rheumatologic illness or of recurrent bacterial infection.

A more immediately revealing line of inquiry will result from direct studies of the antibody recombinase. Structural characterization of the protein(s) responsible for this activity can be confidently anticipated in the next few years. The regulation of substrate choice and of recombinase activity, currently observed at the level of cell populations, may soon be understood in molecular terms. These studies will also improve our understanding of some forms of B and T cell malignancy in which inappropriate selection of rearrangement targets results in proto-oncogene activation (Cory, 1986). Finally, it will be interesting to learn whether other gene systems utilize the efficient strategies for diversification that are employed in the antibody and T cell receptor gene families.

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The Human Immunoglobulin Kappa Genes

H.G. Zachau

INTRODUCTION

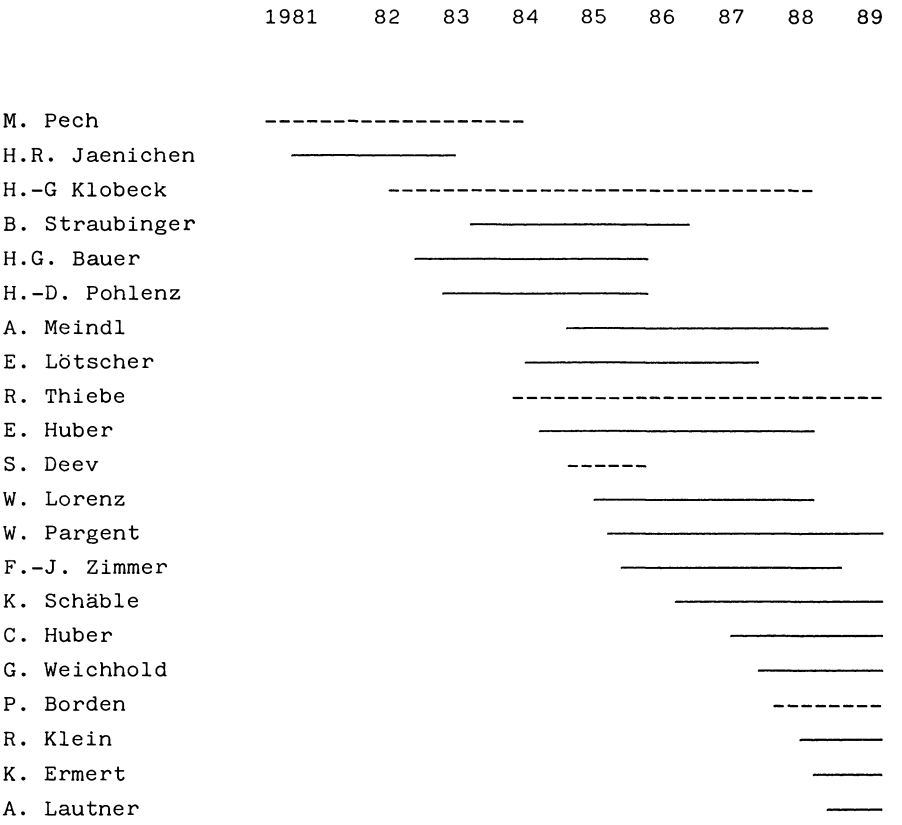
Most aspects of the work of our group on the human kappa locus have been reviewed recently. Many results obtained up to last year were included in an extensive review of the kappa genes of man and mouse (Zachau 1989a). Evolutionary aspects of the kappa genes and our work on the regulation of transcription of immunoglobulin genes have been the topics of two other reviews (Zachau 1989b; Mocikat et al., 1989). Finally, a brief survey of recent structural work, including a simplified map of the kappa locus, appeared in the special issue of Immunology Today, prepared for this Congress (Zachau 1989c). I shall therefore not write another general review but rather concentrate on two aspects: the development of the work on the human kappa locus and its current state as presented in the form of tables on gene numbers and related facts.

THE DEVELOPMENT OF THE WORK ON THE HUMAN KAPPA LOCUS

The human J_K and C_K gene segments were cloned first by Hieter et al. (1980) and the early work on the human V_K gene segments was by Bentley and Rabbitts (e.g. 1980, 1983). At this time our group was mostly involved in chromatin work. The object had been the chromatin structure of repetitive DNA and of the mouse immunoglobulin genes of the kappa type. The study of the inactive and active forms of immunoglobulin gene chromatin in mouse liver and myelomas, respectively, required the availability of various gene fragments for hybridization experiments. The cloning and sequencing work soon acquired itsown momentum. Since the results obtained with the immunoglobulin genes themselves were fascinating to us, the work on immunoglobulin chromatin was slowly phased out. In 1981 we began to look at human kappa genes in addition to the mouse genes. In fact, the human gene segments were cloned with the help of the respective mouse gene segments available in our lab. Our work of this time was summarized a few years ago (Zachau et al., 1984).

In Table 1 the senior colleagues and Ph.D. students are listed (dashed and full lines, respectively) who have worked on the structure, the rearrangements and the translocations of the human kappa genes. Not included are diploma and medical students, but they have of course contributed valuable results in the limited time available for preparing their theses, i.e. mostly sequencing data in this case. Also not included are those members of our group who worked on mouse and human immunoglobulin gene chromatin and on the regulation of transcription of the immunoglobulin genes. The contributions of

Table 1. Contributors to the current picture of the human kappa locus



the various members of our group to the understanding of the kappa locus are documented in the publications. Just three of the senior members should be mentioned here specifically. Michael Pech screened the first human DNA libraries for V_K genes, established the first contigs and found the duplication of large parts of the V_K locus. Gustav Klobeck isolated the V_KII, III, IV, J_K and C_K gene segments and contributed significantly to the understanding of the V_K-J_K and Kde rearrangements as well as the chromosomal translocations involving the kappa locus. Rainer Thiebe was and is responsible for much of the V_K gene sequencing in our lab.

It can be taken from Table 1 that up to now 18 post-doctoral and 43 Ph.D. student years were spent on the human kappa gene project. The figures, as interesting as they are, can be used only with great care in cost-efficiency considerations and probably not at all for

Table 2. Cloned V_K gene-containing regions, as of summer 1989

Kappa Locus					
La, λ1, B-J-C-Kde14		V_K genes	in	260 kb	70 V_K genes in 930 kb
Ob - Ab	23	"	"	280 kb	
Oa - Aa	24	"	"	250 kb	
Lb	9	"	"	100 kb	
Orphons					
W (Chr2)	11	"	"	250 kb	25 V_K genes in 800 kb
cos108 (Chr2)	1	"	"	40 kb	
Chr1	1	"	"	45 kb	
Chr22	5	"	"	115 kb	
Z	7	"	"	350 kb	
ZIII in YAC	1	"	"	200 kb	
ZI/II "	1	"	"	780 kb	
Not yet assigned					
cos138	1	"	"	40 kb	

Homology Regions without V_K Genes				
Wx	-	-	-	70 kb
Homox	-	-	-	80 kb

extrapolation to other medium or large scale mapping and sequencing projects. The reason is simply that at all stages of our work new methods and concepts had to be developed; similar projects starting now would require much less time and effort. It also should be kept in mind that the work was done not in a pure research institution but in an institute which is in charge of basic teaching of medical students with lecturing and course work duties for the senior workers and the Ph.D. students, respectively.

Table 3. Cloned human V_K gene loci, as of summer 1989

	Subgroups						
	I	II	III	IV	others	Total	
Within the K locus							
- sequenced bf	15	8	6	1	3	33	70
- " y(ψ)	8	12	4	-	1	25	
- isolated only	3	6	2	-	1	12	
Orphons, sequenced							
- W	4	6	1	-	-	11	20
- V108 Chr2	1	-	-	-	-	1	
- Chr1	1	-	-	-	-	1	
- Chr22	2	2	1	-	-	5	
- Z	2	-	-	-	-	2	
Orphons, isolated only	5	-	-	-	-	5	5
Not yet assigned							
V138	-	-	1	-	-	1	1

THE CURRENT STATE OF STRUCTURAL WORK ON THE HUMAN KAPPA GENES

The 70 V_K genes which were isolated up to now and assigned to the kappa locus on the short arm of chromosome 2 are located on seven contigs (Table 2 and Fig 1 in Zachau 1989c). The contigs were mapped by pulsed field gel electrophoresis and work on linking them by chromosomal walking is in progress. 25 V_K genes were found to be located outside of the kappa locus; they are called orphons. The 11 genes of the W regions were known to be located on chromosome 2; recently they were assigned to the long arm of this chromosome (F.-J. Zimmer, H. Hameister and H.G. Zachau, manuscript in preparation) and have therefore to be considered orphons. Two V_KI gene containing yeast artificial chromosmes (YACs) were donated by D. Schlessinger, St. Louis, MO (Little et al., 1989); unfortunately they turned out not to contain parts of the kappa locus but rather orphon V_K genes of the so-called Z family (W. Pargent, unpublished; Straubinger et al., 1988). The two contigs without V_K genes were isolated in the course of chromosomal walking experiments (Pohlenz et al., 1987, Klobeck et al., 1989). They cross-hybridize strongly with the walking probes and the region which we call homox even contains a 533 bp segment that is 96 % identical in sequence to a

Table 4. Sequenced gene and intergenic regions (bp) as of summer 1989

	in Munich	elsewhere
V_K gene regions within the locus	76.749	2.771
" " " ,allelic sequences	9.208	8.971
" " " ,W and V108	14.919	
" " " ,W alleles	3.358	
" " " ,orphons	17.681	
Rearranged V_K alleles	11.308	6.573
Kappa locus, other sequences	7.213	3.166

segment in the V_K-J_K intergenic region, but they are clearly not part of the kappa locus.

In Table 3 the sequenced and not yet sequenced V_K gene loci are classified according to subgroups. Only germline genes isolated from genomic DNA are listed here. It should be noted that the number of pseudogenes (ψ) is high and that not all bona fide (bf) genes may be used in the repertoire. The number of functional V_K genes may turn out eventually to be lower than was previously expected.

With Table 4 a piece of statistics is presented which is interesting mostly to the people directly involved in the work. In our lab all sequences were determined by the dideoxinucleotide chain termination method. For the genes, the regulatory elements and also for most of the intergenic regions both strands of the DNA were sequenced. The term allelic sequences is used for such germline sequences which were determined in more than one individual and which show allelic differences. Also the rearranged V_K genes have to be considered allelic to the germline genes since they are derived from other individuals, in the past usually from cell lines, at present also from cDNA libraries. The sequences determined elsewhere include for instance the early data from the laboratories of Leder and Rabbitts and the more recent ones of a group in San Diego (Liu et al., 1989 and earlier papers).

All sequencing in our lab up to now was directed towards gene segments, regulatory elements, UHO's (unidentified hybridizing objects), translocation breakpoints or other features of interest. No random sequencing was undertaken. Sequencing has become easier since the seventies and early eighties when it took us several years by the chemical method to accumulate 15 kb each of repetitive DNA and of mouse immunoglobulin gene sequences (and, of course, since the sixties when it took us from 1960-66 to determine the sequence of 85 nucleotides of a tRNA). So it may become feasible soon to sequence large intergenic regions or even the whole kappa locus. It remains to be seen whether such data turn out to be interesting.

CONCLUDING REMARKS

In the current work of our group we try to close the gaps between the contigs and, with that, to establish the definite number of V_K genes in the germline. In parallel the characterization of cloned and still uncloned V_K genes by blot hybridization has been refined as reported on a poster at this Congress (Meindl 1989); it is comforting to gather from the data that the number of still elusive genes within the kappa locus seems to be small. A step towards understanding the mechanism of transposition of V_K genes to other chromosomes is reported on another poster at this Congress (Borden 1989); certain sequence pecularities at the borders or insertion breakpoints between orphon regions and the receiving chromosomes were found. A topic of major interest is still the mechanism of V_K-J_K rearrangement which can be described in sufficient detail only when the structure of the kappa locus in the germline and in some lymphoid cell lines is clarified. We would also like to find out which V_K genes are used in which developmental or perhaps pathological states of the immune system; in this context various cDNA libraries are being screened.

Since we have worked in the past with DNA from a number of individuals, we can state with some confidence that not only the basic features of the kappa locus but also many of its structural details are conserved throughout the human species. However, it becomes increasingly important now for several aspects of our work to define more closely the differences between the genomes of individuals, cell lines and possibly patients.

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Somatic Changes in the Immune Response to the Hapten 2-Phenyl Oxazolone

C. Berek and M. Apel

INTRODUCTION

One of the longstanding central problems of immunology has been whether the multitude of antibodies which can be produced within an individual are all encoded in the germline or whether some part of this diversity is generated by somatic mechanisms. By now it is established that the V-regions of mouse antibody molecules are assembled from different gene segments, V_κ and J_κ for the L-chain and V_H, D and J_H for the H-chain. These gene segments exist in multiple copies in the germ line. During differentiation from the pre B-cell to the mature antigen specific B-cell, V- (D-) and J-segments are somatically rearranged to give rise to the functional V_H- or V_L-region of an antibody molecule (Tonegawa, 1983). Combinatorial diversity results from the use of alternative combinations of these gene segments. Junctional diversity occurs from variations in the joining. These somatic recombination mechanisms make it in principle possible to generate an antibody repertoire of up to 10^{10} different molecules using a germline repertoire of only a few hundred gene segments.

A further mechanism which might generate antibody diversity, somatic mutation, does not seem to play an important role in the generation of the primary B-cell antibody repretoire. One pre B-cell line, 18-81, has been described in which V-region mutations occur at a rate of 10^{-5}/base pair/ generation (Wabl 1985). The phenomenen of hypermutation with rates up to 10^{-3}/basepair/ generation has been seen only in B-cells after antigenic stimulation (Mc Kean et al 1984; Allen et al 1987; Berek and Milstein 1987). It has been shown that during the course of an immune response somatic mutations accumulate and that this is accompanied by an increase in the affinity for antigen. Hypermutation therefore plays a crucial role in the maturation of the immune response.

The maturation of the immune reponse to the antigen 2-phenyl-oxazolone

Over the last few years the development of antibody diversity in the immune reponse to the antigen 2-phenyl-oxazolone (phOx) has been intensively studied. BALB/c mice were immunized with the antigen phOx and at different timepoints after immunisation spleen cells were fused. Hybridoma lines, which secreted antibodies with specificity for the antigen phOx, were selected and mRNA of these cell lines was prepared. Using specific oligonucleotides as primers for cDNA synthesis the mRNA encoding the H- and L-chains of the antibody molecules was directly sequenced (Griffiths and Milstein, 1985). The analysis of primary, secondary and tertiary antibody molecules demonstrated that in the immune response to phOx diversity within the antigen specific repertoire increases with time. This diversification is characterised by:

a) a shift to alternative H- /L-chain combinations;
b) the accumulation of somatic mutations

Antibody diversity in the early primary response to phOx

The early primary response to the hapten phOx is rather restricted. Kaartinen et al (1983) showed that the majority of the antibody molecules had one particular H- and L-chain combination, V_H-Ox1 with V_κ-Ox1. In the L-chains the V_κ-Ox1 segment was nearly always joined to J_κ-5 presumably to conserve Leucine at position 96, the V_κ- to J_κ-joining point. Indeed in the one exception where V_κ-Ox1 was combined with a J_κ-4 segment, a Leucine codon was again created by the joining process.
For the Ox1 H-chains similiar restrictions were found. Although the sequences differed in their D/J joinings by several nucleotides certain features were strictly conserved (see Table 1).

Table 1 canonical sequence of H-chains found with V_κ-Ox1 L-chains

D-segment	Asp X Gly
D + FRW4	length always 16 amino acids
J_H-segment	only J_H-2, 3 or 4

The first amino acid of the D-segment was always Aspartic acid and the last residue Glycine. Only the middle residue showed variability. In addition in all of these antibodies the J_H-segment was combined to the D-segment in such a way, that CDR3's of identical length were formed.

7 days after immunisation there was practically no evidence for somatic mutation. Out of the 11 IgG antibodies sequenced only one single nucleotide exchange which could be the result of somatic mutation was found in a V_{κ}-Ox1 L-chain.

Antibody diversity at day 14 of the primary response

A different picture was found during the late primary response. By day 14 the majority of the antibodies still had the canonical sequence shown in table 1. However these antibodies differed in their V-regions by 1 to 4 nucleotides from each other (Griffiths et al 1984). In the H-chains the mutations seem to be more or less randomly distributed over the V-region. Nucleotide exchanges were found both in the CDR and the FRW regions. A rather different picture was obtained for the L-chains. Table 2 shows that in the fusion NQ7 all Ox1 antibodies had nucleotide exchanges only in the border region of CDR1 to FRW2. Actually they all occured in only 4 different triplets (Table 2, hybridoma lines from fusion NQ7). This is even more surprising because these antibodies are not clonally related as evidenced by their different joining regions.

Table 2 Mutations in V_{κ}-Ox1 L-chains of day 14 antibodies

	V_{κ}-Ox1							V_H			
	31		34	35	36	37		40		D			J_H
hybridoma	S		H	W	Y	Q		S					
	AGT	....	CAC	TGG	TAC	CAG	....	TCA		GAT	CGG	GGG	
	T		**N**		H								
NQ7/5.3	-C-		A--	---	C--	---		---		---	-AT	---	4
	T		**N**										
34.3	-C-		A--	---	---	---		---		---	A-C	--A	3
			N		**F**			T					
1.3	---		A--	---	-T-	---		A--		---	---	--T	2
			N		**F**								
41.3	---		A--	---	-T-	---		---		--C	-AC	---	2
			Q		**F**								
3.3	---		--G	---	-T-	---		---		---	-CT	---	3
			Q		**F**								
24.6	---		--G	---	-T-	---		---		--A	G--	---	4
			N		**F**								
H17/2.14	---		A--	---	-T-	---		---		---	TT-	---	4
			N		**F**								
L17/10.13	--C		A--	---	-T-	---		---		---	???	---	3

data for hybridoma lines from fusion NQ7 taken from Griffiths et al 1984), for fusions H17 and L17 this manuscript

Diversity in the secondary and tertiary response antibodies

Antibodies of the secondary response showed a further increase in the number of somatic mutations (Berek and Milstein, 1987). Ox1 antibodies had the same mutations as described for the day 14 response in their L-chains. However at this stage of the immune response additional mutations were observed at various positions of the V_{κ}-gene segment. The number of somatic mutations in Ox1 H-chains had likewise increased with time (Griffiths et al 1984).

A further increase of somatic mutations was found in the tertiary response antibodies (Berek et al 1987). A striking result was that without exception the V_{κ}-Ox1 L-chains had the characteristic mutations at residues 34 and 36 already seen in the late primary response plus up to 8 further nucleotide exchanges.

However somatic mutation was only one factor in the diversification of the immune response to phOx. Whereas in the primary response more than 70% of the antibodies had the V_H-/V_{κ}-Ox1 combination this number decreased to less than 20% in the secondary and tertiary response antibodies. This shift from a relatively homogenous primary response to the much more heterogenous secondary and tertiary responses is an integral part of the maturation of the immune response (Berek et al, 1985). The shift is presumably not caused by somatic changes of the immunoglobulin genes. A switch from one V_H- gene to a different one has so far only been described for an early B-cell line (Reth et al 1986) or for a Ly1 B-cell lymphoma (KLeinfield et al 1986). What is happening seems to be the result of complex cellular interactions about which we currently know very little. Antigen selection, idiotypic regulation, compartmentalisation of B-cells at various differentiation stages, senescence of clones, all may contribute to the change in the antibody repertoire from the primary to the secondary and tertiary response.

Hypermutation in primary and memory B-cells

In the early primary response, 7 days after immunisation, practically no somatic mutations were seen (Kaartinen et al 1983; Cumano and Rajewsky, 1985; Wysocki et al 1986). However a high frequency of mutations were found at day 14 (Griffiths et al 1984). These results suggested that the hypermutation mechanism is activated in B-cells only after antigenic stimulation. This viewpoint was further supported by the work of Manser and Gefter (1986), whose idiotypic suppression experiments indicated, that B-cells express germline encoded V-genes prior to antigenic stimulation. In the experiments of Siekevitz et al (1987) memory B-cells, which harboured somatic mutations were transferred into recipients and reactivated by anti idiotypic antibodies. The interesting result was that no further mutations were introduced into the V-regions of these memory B-cells. In the adoptive secondary response memory B-cells proliferated in the absence of somatic hypermutation which indicates that the hypermutation mechanism has been turned off with

the differentiation of the primary B-cell to the memory cell. It is an open question whether antigen under physiological conditions could reactivate the hypermutation mechanism in memory B-cells. In favour of this view are the results obtained in the immune response to phOx where it was found that the number of somatic mutations accumulates from the primary, to the secondary and tertiary response (Table 3).

Table 3 accumulation of somatic mutations in Ox1 antibodies

timepoint	No of antibodies sequenced	No of somatic mutations	no of somatic mutation/ 1000 base pairs
1° day 7	11	1	0,2
day 10	3	6	3
day 14	6	29	8
2°	3	25	14
3°	3	47	26

The data imply that after each antigenic boost memory cells may re-enter the hypermutation and selection process (Berek and Milstein, 1988). The stepwise accumulation of somatic mutations with increasing exposure to antigen, is parallel by increasing affinity. Therefore it becomes unlikely that affinity maturation is achieved solely by the outgrowth of a few hypermutated and at the same time high affinity B-cell clones.

The onset of somatic mutations

Antigen activated B-cells are believed to settle in the follicles where strong B-cell proliferation takes place. This leads to the formation of germinal centers (Nieuwenhuis and Opstelten 1984). It has been suggested, that it is at this stage of B-cell differentiation that the somatic hypermutation mechanism is initially activated (MacLennan and Gray 1987). Germinal centers desintegrate as B-cells leave the follicles to develop into plasma- and/or memory B-cells. This may be the period when antigen selection takes place. It has been suggested that only those germinal centre B-cells further differentiate whose antigen receptors are have been modified by advantagous mutations (Berek and Milstein 1988).

In order to look at the onset of somatic hypermutation, germinal center B-cells, defined by their strong binding to Peanut agglutinin (PNA^{hi}), were isolated from the spleens of phOx immune mice. PNA^{hi} and PNA^{lo} B-cells were sorted 10 days after primary

immunisation of mice when germinal centers are strongly developed. Cells were fused, hybridoma lines with specificity for the antigen isolated and mRNA sequenced to determine the primary structure of H- and L-chain molecules.

The results show that at this timepoint antigen specific hybridoma lines can be obtained mainly from the PNA^{hi} subset of B-cells (manuscript in preparation). The variable regions in these antibodies are mutated, but the pattern of somatic mutations is different from that of the day 14 antibodies. For example in the V-regions of the V_{κ}-Ox1 L-chains the characteristic mutations at positions 34 and 36, shown in table 2 for splenic hybridoma lines from day 14, were missing. The other striking result was, that 65% of the mutations were silent, which is far more than one would expect by a random distribution (25%).

From a fusion of PNA^{hi} (fusion H17) and PNA^{lo} (fusion L17) binding B-cells 14 days after immunisation, three phOx specific hybridoma lines were isolated, which all expressed the V_{κ}-Ox1 L-chain. One of the lines derived from PNA^{hi} B-cells had as many silent mutations as found for the day 10 antibodies. The other two lines H17/2.14 and L17/10.13 had in the their L-chains the mutations only at the positions 31, 34 and 36, confirming the data of the day 14 splenic fusion (Table 2).

These data suggest that 10 days after immunisation somatic hypermutation has diversified the V-regions of the PNA^{hi} binding B-cells, however selection by antigen has not yet led to the preferential expansion of B-cell clones expressing avantageous mutations. This was found only when PNA^{hi} and PNA^{lo} expressing B-cells were isolated 14 days after immunisation.

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The Lymphoid V(D)J Recombination Activity: Studies of Exogenous Plasmid Substrates in Whole Cells

M.R. Lieber, J.E. Hesse, S.M. Lewis, D.R. Brown, M. Sadofsky, K. Mizuuchi, and M. Gellert

The V(D)J recombination activity recombines V, D and J segments at the Ig and TCR loci to generate the variable domain exons of the genes encoding the antigen receptors for humoral and cell-mediated immunity. We have been interested in understanding the mechanism of this DNA recombination reaction and how the activity which mediates it is targeted to the loci and regulated during B and T-cell differentiation. For our investigations, we developed an assay that allows the recombination activity in cells to be measured and the products of the reaction to be readily recovered. The assay uses DNA substrates that remain extrachromosomal after transfection into eukaryotic cells and are recombined by the V(D)J recombination activity (Hesse et al. 1987). These substrates contain V(D)J "joining signals", the characteristic DNA sequence motifs that are found adjacent to each V, D and J segment at the Ig and TCR loci and that serve as recombination signals. Each signal sequence consists of a heptamer and an A/T-rich nonamer separated by a nonconserved spacer sequence. A recombination event is directed by two signals, one with a 12-base spacer (a 12-signal) and the other with a 23-base spacer (a 23-signal). Standard V(D)J recombination generates two recombinant junctions; coding elements are fused in a "coding joint", generating a region of continuous coding sequence, and joining signals are fused at their heptamers in a "signal joint". Typically a few nucleotides are lost from one or both coding ends in a coding joint. Non-template directed nucleotide addition is also usual. This base loss and addition adds to the diversity generated by coding segment joining.

Features of the Reaction Mechanism

Recombination of extrachromosomal substrates in this assay has suggested several mechanistic features of the V(D)J recombination reaction. In early studies, we found that V, D or J coding element sequences are not necessary for the reaction and can be replaced by other sequences at will; the joining signals themselves are sufficient to direct recombination (Hesse et al. 1987). Similar results have been obtained by Akira et al. (1987). (Note that the DNA sequences in our plasmid substrates that are positionally analogous to coding element DNA are still referred to here as "coding ends", and their junction as a "coding joint").

Joining signals in either direct or opposed alignment can be used. Directly aligned signals result in inversion of the intervening DNA and formation of both a coding and a signal joint. Opposed signals lead to deletion, with either a signal joint or a coding joint retained on the recombinant plasmid, depending upon whether the

signals had heptamers or nonamers proximal. On plasmid substrates, we find inversion and deletion at comparable frequencies (Hesse et al. 1987), suggesting that signal and coding joint formation may be coupled in the recombination reaction.

More recently, we have observed that signal ends are also subject to nucleotide addition, although unlike coding ends, base loss at signal ends remains quite rare (Lieber et al. 1988a). The observation that signal ends can be modified by base addition, even if somewhat less frequently than coding ends, has suggested that coding and signal ends may not be treated so dissimilarly in a reaction intermediate as was originally thought. The inserts at signal joints, like those at coding joints (called N-regions) are GC-rich, and their incidence is directly related to the cellular level of terminal deoxynucleotidyl transferase (Lieber et al. 1988a).

Terminal transferase has previously been postulated to be responsible for the inserts at coding joints (Alt and Baltimore 1982; Desiderio et al. 1984). However, we find the frequency of coding joints with inserts to be high over a wide range of terminal transferase activity levels. Terminal transferase in vitro has a known preference for adding G residues. The fact that the inserts remain GC-rich even in cell lines with very low terminal transferase activity suggests either that extremely low levels of terminal transferase may be sufficient to produce a high incidence of nucleotide insertion at coding joints, or that other activities may contribute to coding joint inserts and may become apparent in cells with no terminal transferase. However, the inserts produced by such activities would have to display the length distribution and base composition typical of immune inserts. One activity reported by Roth et al. (1985) makes inserts that are A/T -rich and typically one base long, and would not be a plausible candidate.

There is a noteworthy sequence asymmetry of the signal joint insertions, a great excess of G over C when reading 5' to 3' from the 12-signal toward the 23-signal. If terminal transferase is adding the nucleotides, its preference for adding G residues would indicate that it is starting at the 12-signal end (Lieber et al. 1988a).

Other recent experiments with plasmid substrates have revealed unusual products of V(D)J recombination which demonstrate that the partners joined in this reaction are not stringently specified, as is sketched in Fig. 1. (Lewis et al. 1988).

In addition to the standard products in which coding ends are joined to each other and signals ends are joined to each other, a coding end can join either to the partner signal forming a "hybrid joint" or to its own signal forming an "open and shut" joint. (Open and shut junctions are non-recombinant but can be detected through the nucleotide loss and addition at the joint.)

Hybrid joints occur at 20% of the frequency of the standard reaction, but the frequency of open and shut joints is much lower (about 2%). As at coding and signal junctions, base insertion is also common at both these types of novel junctions, and it is again the coding ends that show nucleotide loss whereas signal ends usually do not. This consistent treatment of coding and signal ends in all the types of junctions suggests that the V(D)J-mediated reaction forming hybrid junctions may be closely related to standard joint formation.

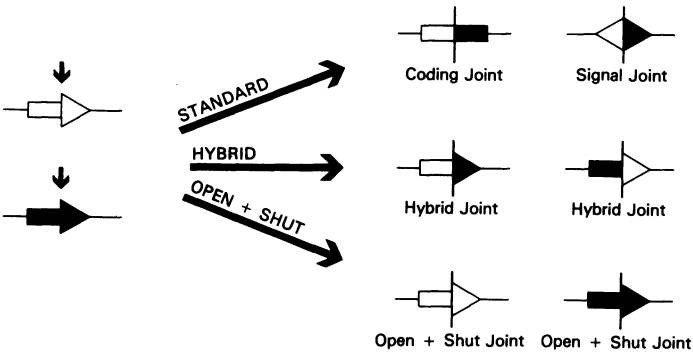

Fig. 1. Cartoon comparing standard recombination, hybrid joint formation, and open and shut events. A portion of the plasmid substrate containing a 12-signal (open triangle) and a 23-signal (filled triangle) is shown at the left. The boxes designate the DNA sequences positionally analogous in the reaction to coding element DNA flanking the 12-signal (open box) and flanking the 23-signal (filled box). V(D)J-mediated products are shown at the right and include all conceivable combinations of coding and signal ends, underscoring a remarkable flexibility in the V(D)J recombination reaction.

Severe Combined Immune Deficiency (SCID) as a Tool for Studying V(D)J Recombination

Murine severe combined immune deficiency is an autosomal recessive defect in which homozygous mutant mice fail to develop mature B and T cells (Bosma et al. 1983). Stimulated by the findings of abnormal rearrangements at Ig and TCR loci (Schuler et al. 1986), we studied the V(D)J recombination reaction in scid lymphoid cells with extrachromosomal substrates (in collaboration with M. J. Bosma's laboratory) and found abnormalities in both coding and signal joint formation (Lieber et al. 1988b). Our results are summarized in Fig. 2.

Coding joints were not detected at all, either in inversional or deletional recombination; their frequency must be reduced at least 100 to 1000 fold. Signal joints were formed and, although 50% of these were structurally like the signal joints formed in normal cells, the remainder had lost nucleotides from one or both signal ends. These deletions were mostly confined to the heptamers, but some larger deletions were also recovered. In those signal joints that showed base loss within the heptamers, nucleotide addition occurred nevertheless and at an increased frequency relative to signal joints from normal cells and non-deleted signal joints from scid cells. These deleted signal joints demonstrate that retention of the heptamer nucleotides in the final product is not necessary for base addition. When signal deletions extend more than one base pair beyond either heptamer, however, nucleotide addition drops markedly.

Though coding joints were not detectable in scid cells, coding ends were joined if they shared DNA sequence homology (Lieber et al. 1988b). Instead of being joined end-to-end as in V(D)J recombination, the two DNA ends underwent homologous recombination and retained only one of the two DNA sequence homology blocks. The fact that the signal joint could be generated by V(D)J recombination,

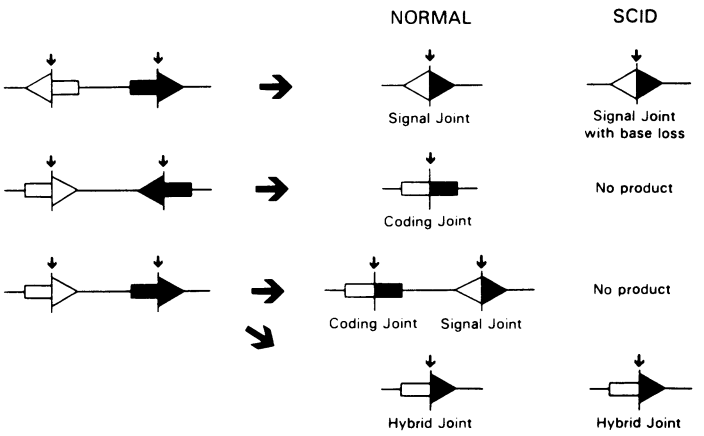

Fig. 2. Summary of recombination reactions in normal and scid lymphoid cells. Plasmid substrates, shown at the left, differ only in the arrangement of the joining signals. In normal cells, depending upon the signal arrangement, V(D)J recombination results in deletion, forming a signal or a coding joint, or inversion, forming both standard joints or a hybrid joint. In scid cells, signal and hybrid joints are recovered, but no V(D)J-mediated coding joints from either deletion or inversion. Symbols are as in Fig. 1.

with a junction of coding ends resolved by homologous recombination, indicates that the two halves of the V(D)J recombination reaction can be uncoupled from one another.

Although we found no V(D)J-mediated coding joints in scid cells, we did detect hybrid joints, indicating that coding ends can participate in alternative V(D)J recombination reactions even though they fail to join to one another. This and the fact that the ends can undergo homologous recombination suggests that the coding ends are not being destroyed.

We find that the single genetic defect in scid mice affects signal and coding joint formation identically in pre-B and pre-T cell lines from these mice. This is the firmest evidence for a common V(D)J recombination activity for B and T cells.

Mutational Analysis of the Signal Sequences

By testing extrachromosomal substrates with systematically altered joining signals in our recombination assay, we have studied the signal sequence requirements of the V(D)J recombination reaction (Hesse et al. 1989). Although we find no signal variant to recombine more efficiently than the consensus joining signal sequences, many recombine nearly as well. This flexibility in the signal sequence emphasizes the existence of mechanisms other than signal sequence recognition for targeting genomic loci for recombination, because of the large number of potentially acceptable signal sequences expected to occur within the genome.

Many other signal variants are moderately tolerated, displaying a wide range of recombination efficiencies. These differences may be

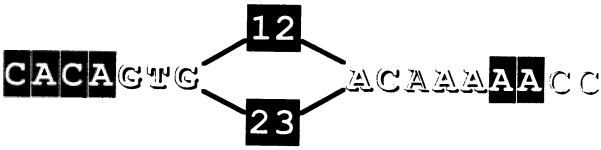

Fig. 3. Contribution of individual nucleotides to V(D)J signal use. The heptamer and nonamer consensus sequences are shown. Signals with altered nucleotides were tested for recombination. Dark, medium, light, and no stippling indicate, respectively, the most essential nucleotides, the less essential nucleotides, the nucleotides whose alteration had only slight effect, and nucleotides that were not tested. Dark stippling of the boxes enclosing spacer length indicates that changes in these lengths were accommodated poorly.

relevant to temporal and developmental biases observed in coding element usage during variable domain exon assembly. The deviation of some signals flanking endogenous coding elements from the consensus sequence, and its possible effect on recombination efficiency, might contribute to the nonrandom use of coding elements observed during endogenous gene assembly at some loci (see Hesse et al. 1989 for references). Figure 3 gives a graphical summary of our results.

The nucleotides most critical in the reaction include the 3 coding-proximal bases of the heptamer and positions 6 and 7 of the nonamer, counting from the heptamer-proximal side (Figure 3). It is not important to maintain the 5 consecutive A's that characterize the consensus nonamer sequence, suggesting that neither melting nor bending in the nonamer plays a role in the reaction. In general, the heptamer is the most important part of the signal; although inefficient, a "nonamer-less" signal still works, while a "heptamer-less" signal does not. The length of the spacer sequence is a critical feature for efficient recombination; when spacer length is changed by more than one base pair, the recombination frequency drops to the level of a "nonamer-less" signal.

Directionality of the signal is determined by the position of the nonamer relative to the heptamer. In the absence of an effective nonamer, the middle A/T of the otherwise palindromic heptamer influences which side of the heptamer is used for the recombination crossover. Except for allowing either end of the heptamer to be used, heptamer symmetry is unimportant in the reaction; a signal in which the rotational symmetry of the heptamer has been completely disrupted still works quite well.

We find no evidence of a role in recombination for homology between the signals; in no example was the activity of a defective signal restored when it was tested with a partner signal altered so as to restore the homology between them. Thus, models that invoke direct DNA-DNA interaction via base pairing between the signals, such as the "stem-and-loop" model, are inconsistent with our mutational analysis. Our results favor instead a model in which the joining signals serve as recognition and binding sites for recombination proteins. In addition, our results suggest the possibility that the two signals are recognized by the same protein species; not only are the heptamer and nonamer motifs common to both signals, but in our experiments both signal types display very similar profiles of base identity requirements, perhaps reflecting essential protein contact points in common.

Regulation of the V(D)J Recombination Activity

By testing extrachromosomal substrates for recombination in a wide variety of cell lines, we determined that the V(D)J recombination activity is restricted to hematopoietic lineages, and present in early B, early T and early myeloid cell lines (Lieber et al. 1987). Our results are summarized in Fig. 4. Within the B-cell lineage, the activity level is highest at the earliest committed stage, lower in the later stages, and shut off at or around the mature B cell stage. However, it appears that surface Ig alone is not sufficient to shut the activity off, weighing against simple models of feedback inhibition.

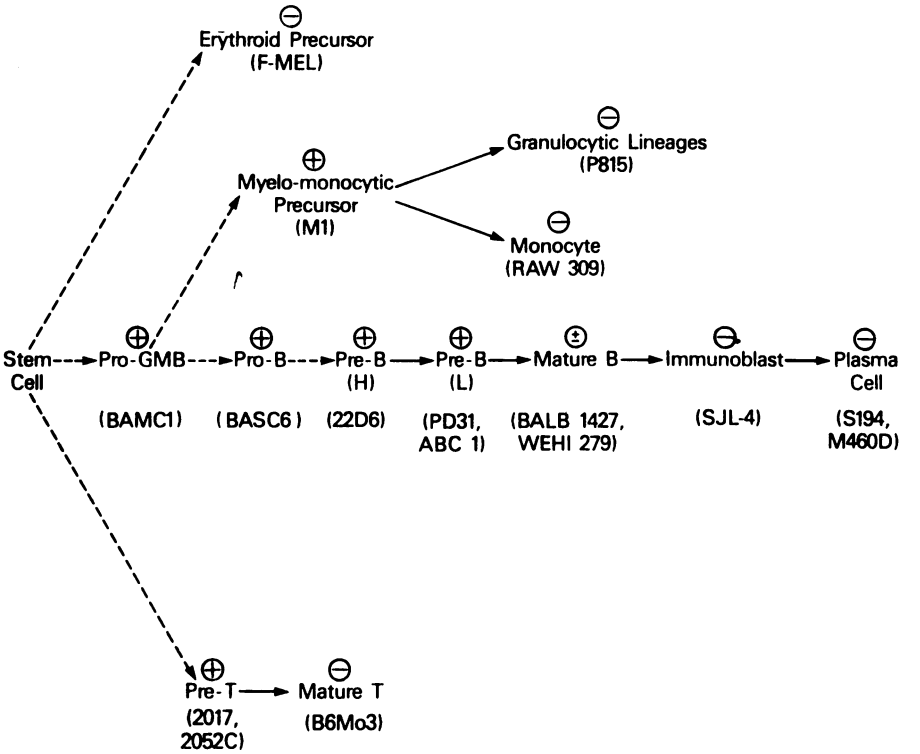

Fig. 4. Distribution of V(D)J recombination activity among hematopoietic cell types. The pathway of hematopoietic differentiation is summarized. Known lineage relationships are indicated by solid lines, more tenuous relationships by dashed lines. A circled (+) or (-) above a cell stage indicates the presence or absence of detectable V(D)J recombination activity in that cell stage. Specific cell lines representing each stage (only a subset of those tested) are named below it.

Plasmid Substrates: A System for Exploring V(D)J Recombination

The plasmid assay system has revealed many interesting features of V(D)J recombination. At the same time, our results are consistent with events detected, though with much greater difficulty, at the endogenous loci and in exogenous substrates that integrate into the cellular genome. The parallels confirm that recombination of plasmids accurately mimics normal V(D)J recombination.

Among these parallels are the use of heptamer-spacer-nonamer signals to direct V(D)J-mediated inversion or deletion, with the coding joints displaying base loss and GC-rich insertions, and the signals being precisely retained in the signal joints. While signal junctions isolated from endogenous gene rearrangement often show precisely fused heptamers, many others have bases between the heptamers (Korman et al. 1989; see Lieber et al. 1988a for other references). These latter examples fit well with the results from plasmid substrates which show that signal joint inserts are not unusual.

The other types of V(D)J junctions generated on plasmid substrates have also been detected elsewhere (see Lewis et al. 1988 for references). Hybrid junctions have been isolated at endogenous loci, and in exogenous recombination substrates that are integrated into the host cell genome. An example of an open and shut junction has been reported at the TCR delta locus.

Consistent with our findings with plasmid substrates in lymphocytes derived from scid mice, V(D)J-mediated coding junctions are not recovered from cultured bone marrow cells (Okazaki et al. 1988) or from cell lines derived from these mice (Kim et al. 1988; Malynn et al. 1988; Blackwell et al. 1989). However, signal joints can be recovered (Blackwell et al. 1989). Hybrid joints, detected with plasmid substrates in scid lymphocytes, may also have been detected at the IgH locus of scid cells (Okazaki et al. 1988; Kim et al. 1988).

Our results on mutations in the joining signals fit well with the conservation of joining signal sequences observed among the endogenous genes. All those nucleotides identified in our study as functionally significant are known to be highly conserved (Hesse et al. 1989). The requirement for two different signal types with defined spacer lengths, a critical feature according to our plasmid assay results, is also well-conserved. We find "nonamerless" signals to be inefficient but functional; consistent with this are the many examples of recombination involving a lone heptamer that have been reported at the endogenous loci.

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Early Expression of Ig-Related Genes in the Human B-Cell Lineage

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INTRODUCTION

B cell differentiation may be studied by analysis of Ig gene expression that follows sequential gene rearrangements (VH-D-JH; VL-JL) of the classical H,κ,λ loci. This has been extensively studied in the mouse (Alt et al. 1986) for which a preferential utilization of the most proximal 3' VH genes early in ontogeny has been reported (Yancopoulos et al. 1984). Kinetics of human Ig gene expression is less substantiated, although it was reported by Gathings et al. (1977) that Ig-expressing B cells occurred as early as the 7th week of gestation in human fetal liver. Recent characterization of human Ig V (Kodaira et al. 1986; Pohlenz et al. 1987) and Ig C (Daviavach et al. 1987) genes has been described, making specific human Ig probes available. We have identified early Ig transcripts from human fetal tissues, revealing a discrete expression pattern of V genes as compared with the adult repertoire. A germline gene and cDNAs of the λ-like family have been isolated and shown to be the mouse λ5 equivalents. Heavy chain transcripts have been obtained, providing evidence for the occurrence of an early switch mechanism in ontogeny.

SEQUENTIAL EXPRESSION OF VH AND Vκ FAMILIES IN HUMAN ONTOGENY

Ig-specific RNA expression has been analyzed by dot blot hybridization. At 6 weeks of gestation, there was no detectable signal with either probe (Cμ, Cκ, Cλ). Cμ transcripts only were found in 7 week old human fetal liver (Fig. 1) suggesting that a majority of pre-B cells was contributing most of the Ig gene mRNAs. When tested with V probes covering the 6 VH (Berman et al. 1988) and the 4 Vκ (Pohlenz et al. 1987) families, only VH5 and VH6 transcripts were identified, pointing to a preferential expression of these 3' terminal genes (Cuisinier et al. 1989) at the onset of Ig gene transcription. This distribution is in sharp contrast with that observed in the adult (Fig. 1) for which the major subgroups were VH3 ≫ VH2 > VH1. Major Vκ families were Vκ1 and Vκ4, the latter result being somewhat surprising since this family contains only 1 member (Klobeck et al.1985). EBV clones have been derived from 11 week old bone marrow, and were individually tested for expression. The average values of 20 clones are represented in figure 1. Values for both the VH and the Vκ families indicated that the expression pattern was close to that of the adult, suggesting a rapid development of the V repertoire between the 7th and the 11th week of gestation. EBV clones were tested for natural antibody specificity on a panel including 8 antigens (actin myosin, tubulin, HSA, transferrin, thyroglobulin, myoglobin and DNA) by B. Guilbert and S. Avrameas (manuscript in preparation).Whatever

the origin of clones (fetal or adult), the same percentage, i.e. 10 to 15% was found to be polyspecific, as defined by fixation of at least 3 antigens. The usage pattern of VH and Vκ families of these clones did not differ from that of "non specific" clones, suggesting that polyspecificity was not linked to a restricted repertoire.

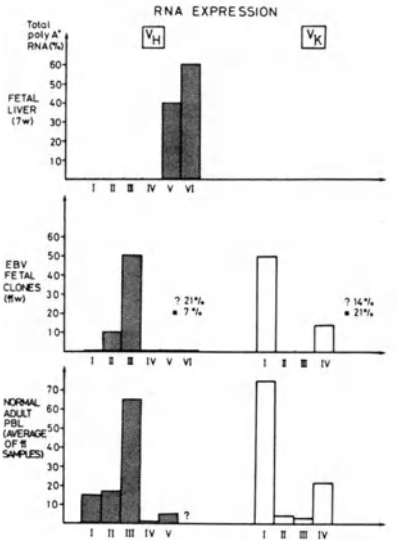

Fig. 1. Relative expression of VH and Vκ families in 7 week old human fetal liver, in EBV clones derived from 11 week old bone marrow (average of 20 clones), and in adult peripheral blood cells.

ISOTYPE SWITCHING OCCURS EARLY IN ONTOGENY

Screening of a human fetal liver cDNA library ($\leq$ 90 days) with C-Ig (μ,γ,κ, λ)probes led to the isolation of 10 heavy chain clones that were characterized by restriction mapping and partial sequencing. As no regular light chain clone was found, it seems likely that the B-lineage library was mostly derived from pre-B cells. Three transcripts were potentially functional, with a complete V-D-J-C coding region, one μ, one γ2 and one γ4, using VH4, VH3 and VH1 family genes respectively. The presence of γ2 and γ4 members was unexpected since these isotypes have been reported to occur late in development (Burrows et al. 1981). This observation strongly suggests that a "switch like" mechanism may operate early in ontogeny, possibly in the absence of light chains. Seven sterile heavy chain transcripts have also been isolated, one Cμ and 6 Cγ1. The sterile Cμ contained the 5' UT germline region. Theγclones possessed, 5' to Cγ1, a stretch that was spliced from the Sγ1 switch region. They may show the opening of the γ1 locus and may indicate that a classical switch to γ1 is about to operate in a number of cells (Stavnezer et al. 1988). Altogether, these observations suggest that a switch mechanism may operate early in ontogeny, i.e. before the 12th week of gestation.

THE λ-LIKE CLUSTER (14.1; 16.1; Fλ1) IS THE HUMAN COUNTERPART OF THE MOUSE λ-5 GENE

Screening of the human fetal liver cDNA library with an Ig-Cλ probe also allowed us to isolate 2 clones, Fλ1 and Fλ8 that contained an identical Cλ-like region 85% homologous to the classical Cλ genes. Fλ1 and Fλ8 differed from one another by a splicing event that joins a Jλ-like to a Cλ-like exon, in the absence of any DNA rearrangement (Fig. 2). These 2 clones represented transcripts of a λ-like gene that was shown to be the third non allelic member of the 14.1/16.1 Cλ-like family (Chang et al. 1986). The 3 genes were clustered on a 200 kb Sst II fragment, as shown by pulsed field gel electrophoresis. They were located by in situ hybridization on the q-11 band of chromosome 22. Homologies between Fλ1 and the mouse λ5 gene strongly suggest that the Jλ-like and the Cλ-like regions correspond to exons 2 and 3 of the mouse λ5 described by Kudo et al. (1987). Since Fλ1/Fλ8 appeared to be a pseudogene, because of a single nucleotide deletion in the Cλ-like exon, the actual candidate for a human equivalent to λ5 appears to be either the 14.1 or 16.1 (or, alternatively both) gene. Elucidation of the complete human λ-like gene was obtained at the germline level, in a cosmid clone given by M-P Lefranc et al. (unpublished results), using a DNA fragment that contained an homologous exon of similar length located 5' of Cλ1 onto which it occasionally spliced (P. Guglielmi, in preparation).

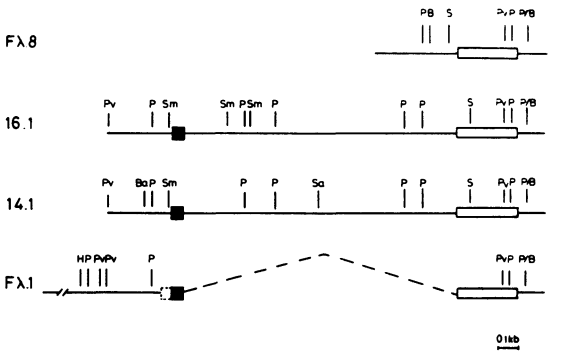

Fig. 2. Comparison of the restriction maps of Fλ1/Fλ8 cDNAs to 14.1 and 16.1 λ-like germline genes. Jλ and Cλ-like exons are boxed. Restriction sites: B, Bam HI; Ba, Bal I; H, Hinc II; P, Pst I; Pv, Pvu II; S, Sst I; Sa, Sal I; Sm, Sma I.

This clone, which had the 14.1 reported Cλ-like sequence, contained 3 putative exons with an open reading frame and the canonic splicing signals. The 3 exons were 61, 66 and 75% homologous to exons 1, 2 and 3 of mouse λ5, with lengths of 69, 38 and 106 codons, respectively. A specific probe, containing exon 1 was shown to selectively hybridize with pre-B cell line mRNAs, at 1 kb (Fig. 3). The 14.1 gene would thus encode a "fetal" light chain that may associate with the μ chain in pre-B cells, such as the 18,000 ω chain (Pillai and Baltimore 1987). Since the 14.1 gene contains 213 codons, correspon-

ding to a polypeptide of ca 22,000, the encoded chain might necessitate a splicing event or the removal of a NH_2-terminal signal peptide.

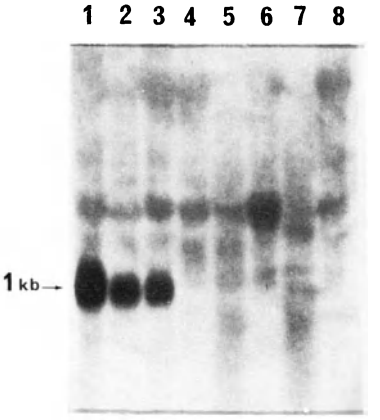

Fig. 3. The 14.1 gene is expressed in pre-B (Nalm 6, Nall 1, and REH-KM3, lanes 1, 2 and 3, respectively), but not in B nor T cell lines (lanes 4-8). Exon 1 of the 14.1 gene was used as a probe in this Northern blot.

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I Structure and Function of Recognition and Effector Molecules

Cell Interaction Molecules

Adhesion Receptors Regulate Antigen-Specific Interactions, Localization, and Differentiation in the Immune System

T. A. Springer

Antigen-specific killer T cell interactions require cooperation between the T cell receptor for antigen (TcR) and the adhesion molecules on the T cell known as LFA-1, CD2, and CD8 (Springer et al.1987; Martz, 1987; Kishimoto et al.1989; Bierer et al.1989). The high degree of cooperation required between these molecules for successful T cell interactions is illustrated by the ability of monoclonal antibodies (mAb) to each of these structures individually to inhibit T cell mediated killing. All of these molecules are involved in adhesion, and many are also involved in the signalling events attendant upon antigen recognition. Thus, they are most properly referred to as adhesion receptors. The properties of these receptors, and the counter-receptors to which they bind, all of which are glycoproteins, are summarized in Table 1.

TABLE 1: CHARACTERISTICS OF T CELL ADHESION RECEPTORS & COUNTER-RECEPTORS

Receptor	Mass (KDa)	Distribution	Counter-Receptor	Mass (KDa)	Dist.
LFA-1 (CD11a/CD18)	α180,β95	Thymocytes, T&B lymphocytes, LGL monocytes, activated macrophages, neutrophils	ICAM-1 (CD54)	90-110	Restricted, widely inducible by IL-1, TNF, IFN-γ & LPS
			ICAM-2	45	Wide? Constitutive on endothelial cells.
CD2 (LFA-2/T11)	50-58	Thymocytes, T lymphocytes, LGL	LFA-3 (CD58)	55-70	Wide
CD8	30-38,α-α or α-β dimer	Subset of thymocytes & lymphocytes, LGL	MHC Class I	α44,β12	Wide, increased by IFN-α, β, γ
CD4	55	Subset of thymocytes & T lymphocytes, monocytes, macrophages	MHC Class II	α34,β29	Restricted, widely inducible by IFN-γ

References: (Springer et al.1987; Bierer et al.1989; Kishimoto et al.1989)

Although much remains to be learned about how activation regulates lymphocyte interactions in vivo, two distinct molecular adhesion mechanisms recently defined in vitro are likely to be important (Springer et al.1987; Spits et al.1986; Shaw, and Luce, 1987). If CTL lines are maintained in culture in an activated state by weekly stimulation with foreign antigen and addition of T-cell growth factors, they will conjugate with B-lymphoblastoid "target cells" even when the target cells do not express the antigen to which T cells are immune. Such adhesion is not shown by resting T lymphocytes (Dustin, and Springer, 1989). Activation-regulated adhesion is due to binding of the CD2 and LFA-1 molecules on the T cell to LFA-3 and ICAM molecules on the target cell, respectively. As shown by mAb blocking, together these two mechanisms account for all the adhesion; there is little (Spits et al.1986) or no (Springer et al.1987; Shaw, and Luce, 1987) contribution by TcR and the CD4 and CD8 molecules. Although the CD2/LFA-3 and LFA-1/ICAM adhesion mechanisms are required for or greatly contribute to antigen-specific killing, CTL only lyse the target cells if the TcR recognizes specific antigen on the target cell. Thus the TcR is required for triggering. Although TcR (and CD4 or CD8) interaction with peptide-MHC must make a contribution to adhesion, the lack of measurable antigen-specific adhesion by CTL clones suggests that the adhesive contribution by binding to peptide-MHC is at least 10-fold lower than the contribution by CD2 and LFA-1 binding to their counter-receptors.

LFA-1 was first identified based on ability of mAb directed to it to inhibit T cell-mediated killing (Springer et al.1987; Kishimoto et al.1989). It was subsequently found that LFA-1 is required for the adhesion step in CTL-mediated killing, and that it is required for a broad range of leukocyte functions involving adhesion, including T helper and B lymphocyte responses, natural killing, monocyte and granulocyte antibody-dependent cytotoxicity, and adherence to endothelial cells, fibroblasts, and epithelial cells.

A counter-receptor for LFA-1, ICAM-1, was identified using a simple assay called homotypic adhesion, in which homogeneous cell populations such as B or T cell lines adhere to one another to form multicellular clusters (Dustin et al.1988; Springer et al.1987). Although resting lymphocytes do not form homotypic aggregates, they do so when stimulated with phorbol esters; transformed lymphoid cell lines aggregate weakly, or if stimulated, strongly. Homotypic adhesion is completely inhibited by LFA-1 mAb, and is not observed with cell lines established from genetically LFA-1-deficient patients (see below). The ability of $LFA\text{-}1^+$ cells to coaggregate with $LFA\text{-}1^-$ cells in the homotypic adhesion assay showed LFA-1 is not a homophilic receptor which binds to itself, but rather a heterophilic receptor binding to a distinct counter-receptor. A counter-receptor was defined by immunizing mice with $LFA\text{-}1^-$ cells, and selecting mAb which would inhibit LFA-1-dependent homotypic adhesion. This counter-receptor was designated an intercellular adhesion molecule (ICAM-1) (Table 1). Confirming the receptor/counter-receptor relationship, lymphocyte binding to purified ICAM-1 is inhibited with LFA-1 mAb (Kishimoto et al.1989), and purified LFA-1 protein micelles bind to purified ICAM-1 on artificial substrates (Dustin, and Springer, 1989). In contrast to LFA-1 which is an integrin (see below), ICAM-1 is a member of the Ig superfamily with 5 Ig domains (Dustin et al.1988). ICAM-1 is 20% identical to the nervous system adhesion molecules NCAM and myelin associated glycoprotein, which also have 5 Ig-like domains. The binding site for LFA-1 lies within the two, most membrane-distal, Ig

domains of ICAM-1 (D.E. Staunton, M.L. Dustin, T.A. Springer, in preparation).

Induction of ICAM-1 in inflammation is one important means of regulating the LFA-1/ICAM interaction (Dustin et al.1988; Kishimoto et al.1989). In contrast to LFA-1 which is restricted to leukocytes, ICAM-1 has the potential to be expressed on a wide variety of cells. In absence of stimulation, however, ICAM-1 is expressed only on a few cells in a pattern correlating with MHC class II expression (Dustin et al.1986) and which therefore may facilitate antigen-presenting cell interactions. Consistent with its importance in in vitro immune responses (Makgoba et al.1988; Altmann et al.1989), in vivo ICAM-1 is well expressed in lymph node germinal centers both on follicular dendritic cells and on the activated B lymphocytes which congregate in these centers (Dustin et al.1986). Germinal centers are formed in lymph nodes during immune responses to specific antigen and homotypic adhesion involving LFA-1 and ICAM-1 may contribute to their formation. Inflammatory mediators, including lipopolysaccharide, interferon-γ, IL-1, and TNF-α and β cause strong induction of ICAM-1 in a wide variety of tissues (Springer et al.1987; Dustin et al.1988; Kishimoto et al.1989). Expression can reach $>10^6$ sites/cell. Induction of ICAM-1 greatly increases lymphocyte and monocyte binding via their cell surface LFA-1. Plots of lymphocyte binding to purified ICAM-1 reconstituted in planar lipid bilayers are sigmoidal, with no adhesion below a threshold value of 100 ICAM-1 molecules/μm^2. Between 150 and 1,000 ICAM-1 molecules/μm^2, lymphocyte binding rises sharply to a plateau; ICAM-1 expression on unstimulated and maximally stimulated endothelial cells falls below (40 sites/μm^2) and above (1,600/μm^2) these levels, respectively (Dustin, and Springer, 1988). Endothelial, fibroblastic and epithelial cells vary in which cytokines are capable of inducing ICAM-1 expression, and the types of mediators released may therefore help regulate differing patterns of cell localization induced by inflammatory stimuli. Binding of leukocytes to endothelium is the first step in localization of circulating cells at an inflammatory site. In vivo, ICAM-1 induction accompanies T cell-mediated hypersensitivity (allergic) reactions (Wantzin et al.1988), and after administration of γ-IFN and IL-1, its appearance on endothelial cells correlates with sites of mononuclear cell infiltration (Munro et al.1989).

A second LFA-1 ligand differing in tissue distribution from ICAM-1 was originally defined functionally by ability of LFA-1 mAb but not ICAM-1 mAb to inhibit certain cell adhesion assays. Lacking a mAb or any information about the structure of ICAM-2, it was nonetheless cloned based on its functional properties. A cDNA expression library was screened for ability to confer on COS cells the ability to bind to purified LFA-1 coated on Petri dishes. Screening was done in presence of ICAM-1 mAb. An ICAM-2 cDNA clone was isolated which encodes a transmembrane protein which binds to LFA-1 and shares no antigenic determinants with ICAM-1 (Staunton et al.1989). ICAM-2 has 2 Ig-like domains, in contrast to ICAM-1 which has 5, and these are 35% identical to the first 2 domains of ICAM-1. ICAM-1 and ICAM-2 are much more similar to one another than to other members of the Ig superfamily, and thus represent an Ig subfamily specialized to interact with LFA-1. A family of LFA-1 counter-receptors emphasizes the importance of this adhesion mechanism and may be a means of imparting fine specificity and functional diversity. Unlike ICAM-1, ICAM-2 is well expressed basally on endothelial cells and mRNA level is not increased by inflammatory mediators. Whether further ICAM's exist is an open question. The

functional cDNA isolation approach should have wide application for other as yet unidentified adhesion counter-structures.

LFA-1 has noncovalently associated α and β subunits; two other leukocyte adhesion receptors, Mac-1 and p150,95 have the same β subunit and different α subunits. They function in adhesion of neutrophils and monocytes to other cells and Mac-1 is also a complement receptor specific for iC3b (Kishimoto et al.1989) (Table 2). The important role of these glycoproteins is illustrated in congenital "leukocyte adhesion deficiency" (LAD), in which all 3 $\alpha\beta$ complexes are deficient due to mutation of the common β subunit (Anderson, and Springer, 1987; Kishimoto et al.1989). Patients have recurring infections which are often fatal in childhood unless corrected by bone marrow transplantation. Patient monocytes and neutrophils are unable to bind to and cross the endothelium at sites of infection, leading to a lack of pus formation. Chemoattractants both increase surface expression of Mac-1, and make it qualitatively more active (Anderson, and Springer, 1987; Kishimoto et al.1989; Wright, and Meyer, 1986; Buyon et al.1988; Lo et al.1989). β subunit mAb administration in vivo mimics defects in LAD, and appears clinically useful in inhibiting leukocyte extravasation and neutrophil-mediated tissue injury in myocardial infarction and ischemic shock (Kishimoto et al.1989).

Sequencing of the β subunit common to LFA-1, Mac-1, and p150,95 revealed 45% identity to a subunit of a chicken receptor for fibronectin, and conservation of all 56 cysteine residues (Kishimoto et al.1989). This provided evidence for the existence of a family of receptors which mediate both cell-cell and cell-matrix interactions, and which are now called the integrins (Hynes, 1987). Sharing of a common β subunit by LFA-1, Mac-1, and p150,95, which may also be called the leukocyte integrins, or β2 (CD18) subfamily, also provided a model for understanding the subunit relationships of 2 other integrin subfamilies which share the distinctive β1 (CD29) and β3 (CD61) integrin subunits (Table 2).

Each integrin contains a single noncovalently associated α subunit of ~130-180 kD and β subunit of ~90-110 kD. The α subunits are 25-65% identical in amino acid sequence and the β subunits are 37-45% identical; the structural and functional similarities are so strong that integrins should be considered a protein family rather than a superfamily (Kishimoto et al.1989). The association of multiple α subunits with the same β subunit generates distinctive ligand specificities. Although there is no promiscuity among β1, β2, and β3 in interacting with one another's α subunits, recently 2 β subunits designated β4 and β5 have been found to associate with α6 and αv subunits alternatively to β1 and β3, respectively (Kajiji et al.1989; Cheresh et al.1989). This also alters ligand specificity, suggesting both α and β subunits interact with ligand, which has been confirmed by crosslinking to small ligand peptides (D'Souza et al.1988).

A number of β1 subfamily members are expressed on leukocytes of different stages of differentiation; the designation VLA (very late activation) denotes the appearance of VLA-1 and VLA-2 on lymphocytes 2-4 weeks after antigen stimulation in vitro (Hemler, 1988). However, VLA is not an apt acronym because some VLA molecules are basally expressed on leukocytes, and expression on nonhematopoietic cells does not require activation (Table 2). The ligands recognized by β1 integrins (Table 2) show interesting patterns of expression in the extracellular matrices,

TABLE 2. THE INTEGRIN FAMILY OF CELL-CELL AND CELL-MATRIX RECEPTORS

SUBUNITS, NAMES		LIGANDS[a]	RGD ROLE	DISTRIBUTION NON-LEUK[b]	LEUK[c]
$\alpha 1\beta 1$	CD-/CD29,VLA-1	LM,CO	-	F,BM	B*,T*
$\alpha 2\beta 1$	CD49b/CD29,VLA-2	CO	-	P,F,EN,EP	T*
$\alpha 3\beta 1$	CD-/CD29,VLA-3	FN,LM,CO	-	EP,F	
$\alpha 4\beta 1$	CD49d/CD29,VLA-4	FN	-	NC,F	B,T,M,LGL
$\alpha 5\beta 1$	CD-/CD29,VLA-5,FNR	FN	+	F,EP,EN,P	Th,T*
$\alpha 6\beta 1$	CD49f/CD29,VLA-6	LM	-	P	
$\alpha 6\beta 4$	CD49f/CD-,αEb4	LM	-	E	
$\alpha L\beta 2$	CD11a/CD18,LFA-1	ICAM-1,2	-		B,T,M,G
$\alpha M\beta 2$	CD11b/CD18,Mac-1,CR3	C3bi,FX?,FB?	?		M,G
$\alpha X\beta 2$	CD11c/CD18,p150,95	?	?		M,G
$\alpha IIb\beta 3$	CD41/CD61,gpIIb,IIIa	FB,FN,vWF,FB	+	P	
$\alpha V\beta 3$	CD51/CD61,VNR	VN,FB,vWF,TSP	+	EN	
$\alpha V\beta 5$	CD51/CD-	VN,FN	+	C	

a. LM, laminin; CO, collagen; FN, fibronectin, FB, fibrinogen; FX, Factor X; VN, vitronectin; vWF, von Willebrand factor; TS, thrombospondin.
b. EN, endothelial cells; EP, epithelial cells; F, fibroblasts; NC, crest, melanocytes; P, platelets; C, carcinomas; BM, basement membrane associated.
c. B, B lymphocytes; T, T lymphocytes; *, activated lymphocytes only; Th, thymocytes; M, monocytes; G, granulocytes; LGL, large granular lymphocytes.

References: (Kishimoto et al.1989; Hynes, 1987; Ruoslahti, and Pierschbacher, 1987; Hemler, 1988; Kunicki et al.1988)(Wayner et al.1988; Takada, and Hemler, 1989; Holzmann et al.1989; Takada et al.1989; Sonnenberg et al.1988; Kajiji et al.1989; Plow, and Ginsberg, 1989; Cheresh et al.1989)

basement membranes, and lamina of different tissues (Ruoslahti, and Pierschbacher, 1987). Induction of VLA-1, 2, 3, and 5 expression during leukocyte activation may be of great importance in controlling localization of lymphocytes and monocytes in inflammation. In contrast, VLA-4 (CD49d/CD29) is present on resting as well as activated lymphocytes (Hemler, 1988), is a fibronectin receptor (E. Wayner, unpublished), a Peyer's patch homing receptor (Holzmann et al.1989), participates in T cell-mediated killing (Takada et al.1989), and can mediate lymphocyte homotypic adhesion stimulated by certain VLA-4 mAb (B.W. McIntyre, unpublished). There may be some functional redundancy among VLA-4 and LFA-1. A role for VLA-5 in T lymphocyte activation suggests the importance of the extracellular matrix in regulating immune responses (Matsuyama et al.1989).

All integrin α subunits have 3-4 tandem repeats of a putative divalent cation binding site motif, and require Ca^{2+}, Mg^{2+}, or Mn^{2+} for function (Kishimoto et al.1989). LFA-1 α has 3 such repeats and has been shown to bind Mg^{2+}, correlating with the requirement for Mg^{2+} in T cell adhesion and in binding of purified LFA-1 to purified ICAM-1 (Dustin, and Springer, 1989). All 3 leukocyte integrin α subunits, and one of the VLA α subunits, have a domain of 200 amino acids not present in other integrin α subunits, and hence termed the "inserted" or I domain.

The I domains are homologous to ligand binding repeats in von Willebrand factor and other proteins, and may confer modes of ligand recognition in addition to those shared by all integrins (Kishimoto et al.1989).

The amino acid sequence arginine-glycine-aspartic acid (RGD in the one letter code) is a key motif recognized by 4 different integrins within at least 6 different ligands (Ruoslahti, and Pierschbacher, 1986; Ruoslahti, and Pierschbacher, 1987) (Table 2). Short peptides containing this sequence inhibit binding to this sequence within ligands as well as to related sequences within fibrinogen, but do not affect LFA-1 binding to ICAM's (Kishimoto et al.1989); Mac-1 binding to C3bi and fibrinogen however involves RGD-like sequences (Wright et al.1988).

As discussed above, one mechanism for regulating the LFA-1/ICAM-1 interaction is by changing the surface density of ICAM-1 after cytokine stimulation. In vitro or in vivo, increased expression of ICAM-1 is first seen after 4-6 h and is maximal by 9-24 h (Dustin et al.1988; Munro et al.1989; Kishimoto et al.1989). This is typical of regulation at the mRNA level of surface adhesion receptor density, and appears true for CD2 and LFA-3 as well. Alteration of cell surface charge, another mechanism for regulating cell interactions, involves a change in glycoprotein sialylation; replacement of cell surface sialic acid requires de novo glycoprotein biosynthesis (Reichner et al.1988) and glycoprotein turnover, which is on the order of 12-24 h. However, T cells can regulate adhesion over a much shorter time scale, adhering to target cells, delivering a lethal hit, deadhering, and engaging in repeated target cell interactions, with a cycle time as short as 15-30 min (Martz, 1977). What mechanism is involved? Data on CTL primed in vivo, show that in contrast to in vitro stimulated cells, they can adhere in an antigen-specific manner (Martz, 1987). This would only be compatible with the importance of adhesion receptors in cell interactions if their activity was stimulated by the TcR (Martz, 1987; Springer et al.1987; Rothlein, and Springer, 1986). A second mechanism for regulating the LFA-1/ICAM-1 interaction, which is stimulated by the TcR and changes the avidity of LFA-1 over a timescale of minutes has now been defined (Dustin, and Springer, 1989).

Using cells coexpressing LFA-1 and ICAM's, and testing binding to plastic substrates coated with either purified ICAM-1 or purified LFA-1, regulation of the avidity of cellular LFA-1 and of cellular ICAM-1 can be separately tested (Dustin, and Springer, 1989). Stimulation of resting T lymphocytes with TcR crosslinking with mAb converts cellular LFA-1 from a low to high avidity state, whereas cellular ICAM-1 is constitutively avid. There is no change in LFA-1 surface density. In contrast to T cell clones, resting peripheral blood T lymphocytes do not conjugate with B lymphocyte target cells. However, TcR stimulation induces conjugate formation due to an increase in LFA-1 avidity; there is little or no increase in CD2 avidity. The high avidity state peaks 5-10 min after TcR stimulation and returns to the low avidity state by 30 min; kinetics are influenced by the amount of TcR crosslinking. Subsequent addition of phorbol ester returns LFA-1 to the high avidity state, demonstrating the adhesion machinery is still intact. In contrast to TcR stimulation, after phorbol ester stimulation LFA-1 does not return to the low avidity state. Inhibition by dibutryl cAMP of stimulation via the TcR, and stimulation by phorbol esters which act on protein kinase C, strongly suggest that the TcR and LFA-1 are linked by intracellular signalling pathways.

Based on these findings the following model can be proposed for cooperation between the TcR and adhesion molecules to mediate antigen specific recognition (Dustin, and Springer, 1989). On contact with cells bearing specific antigen, TcR ligation generates intracellular signals which lead to energy-dependent conversion of LFA-1 to a high avidity state, favoring LFA-1/ICAM dependent adhesion. Antigen specificity is maintained because the input of energy to convert LFA-1 to the high avidity state, whether this energy is used to fuel protein phosphorylation, LFA-1 redistribution, or some other mechanism, is controlled by the TCR. Cellular energy expended in converting LFA-1 to a high avidity state helps drives the adherence/nonadherence equilibrium toward stable adherence, and is analogous to the use of ATP to favor an otherwise energetically unfavorable reaction in intermediary metabolism. Since TcR binding to peptide MHC does not have to stabilize cell-cell adhesion but instead triggers adhesion amplification, this provides a mechanism for greatly increasing the sensitivity of T cells by lowering the number of TcR-ligand interactions required for antigen recognition. This view of adhesion strengthening is consistent with the recent observation in murine T cell clones that LFA-1 and talin, a cytoskeletal protein which co-localizes with a number of integrins at sites of adhesion, redistribute to sites of interaction with antigen bearing B cells, but not antigen-negative B cells (Kupfer, and Singer, 1989). It is intriguing that redistribution of LFA-1 and talin has been shown to be highly sensitive to low antigen concentrations and may correlate with the high avidity state of LFA-1.

The transience of TcR-stimulated increase in LFA-1 avidity provides a mechanism for regulating the adhesion/deadhesion cycle (Dustin, and Springer, 1989). It is proposed that the TcR triggers a cascade of phosphorylation events or second messengers such that early events lead to an increase in LFA-1 avidity, while later events are responsible for lowering LFA-1 avidity. The kinetics of LFA-1 avidity changes measured after TcR activation by mAb crosslinking are in good agreement with those previously measured for CTL contacting antigen-bearing target cells. Highly active CTL bind to targets rapidly (0.2-2 min.) and can deliver the lethal hit and disengage from the target within an additional 6 min (Martz, 1977; Poenie et al.1987). Antigen density and hence the number of TcR engaged may influence the kinetics of the signalling cascade and thus the kinetics of avidity regulation. Duration of adhesion may also be influenced by the level of ICAM expression and whether ICAM-1 or ICAM-2 is the ligand. It is important to remember that since ICAM-1 is inducible by cytokines (Springer et al.1987; Dustin et al.1988), T cell stimulation could lead to induction of ICAM-1 on antigen presenting cells, and secondarily alter the kinetics of T cell interactions.

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From Marker Antigens for T Lymphocyte Subsets to Molecules that Regulate Cell Activation

A.F. Williams and A.D. Beyers

Introduction

Determinants of the CD4, CD8 and CD45 antigens provide markers that distinguish subsets of T lymphocytes. In each case these antigens were identified at early stages in the development of different technical approaches to the analysis of the lymphocyte cell surface. Then the definition of T cell subsets became the focus of interest to be followed by structural analysis of the antigens. Most recently the structural studies have led to new functional insights and these antigens can now be grouped together as molecules that appear to regulate the state of phosphorylation of tyrosine residues in cytoplasmic domains of receptors involved in signal transduction. Given that tyrosine phosphorylation plays a key role in the activation cascade, the subset markers are likely to be important regulatory molecules in cell activation.

Mouse Alloantigens

Studies on lymphoid cell-surface molecules began when tumor-specific antigens were sought in the early 1960s. Such antigens were not found but polymorphic determinants of normal lymphoid cell surface molecules were identified. The genetic approach to specificity was used where-by animals were back-crossed to produce congenic strains in which the polymorphic difference was isolated against the genetic background of one of the parental strains. The initial set of antigens that were discovered included Thy-1, which was to be the first T lymphocyte marker to be described, and the Ly-A (later to be called Ly-1, and now CD5) and Ly-B (Ly-2 and now CD8 α) antigens (Boyse et al,1968; reviewed Boyse & Old,1969). At the outset the antigens were considered to be of key interest because they might play crucial roles in the specialised functions of lymphocytes rather than because they might mark different lymphocyte types (Boyse & Old,1969). Later the Ly-C (Ly-3 and now CD8 β) and Ly-5 (now CD45) antigens were identified (Boyse et al,1971; Komuro et al,1975). A rat alloantigen, ART-1 (Lubaroff,1973) or Ly-1 (Fabre & Morris,1974) now known to be CD45 was also described.

The key finding that there were subsets of T lymphocytes with differing functions was made by use of the Ly-2,3 and Ly-1 antigens (Kisielow et al,1975). Cytotoxic cells were largely Ly-2,3+ and helper cells were Ly-2,3-. However a detailed pattern of T cell differentiation that is not in accord with modern data was proposed (Cantor & Boyse,1977). The confusion arose because cytotoxicity assays were used and with these partial killing of T cells occurred with Ly-1 antisera even though this molecule was expressed on all T cells (Mathieson et al,1979; Ledbetter & Herzenberg,1979). The mouse data came into line with human and rat data when a mAb to mouse CD4 was prepared by Dialynas et al (1983). Although in

retrospect it is clear that CD45 had been identified in mouse and rat with alloantisera these sera were not useful in identifying CD45 as an antigen of interest in marking T subsets.

Use of Xenogeneic Sera

Immunisation across the species barrier where all molecules were likely to be antigenic was potentially of great value with regard to the goal of routinely using antibodies to identify cell surface molecules (reviewed Williams,1977). The problem was to produce specific antisera. Despite the difficulties key early studies on the identification of CD45 were done with the use of rabbit antisera in mouse (Trowbridge et al,1975) and rat (Fabre & Williams,1977).

The Modern Era: Monoclonal Antibodies and The FACS

The discovery of the monoclonal antibody (mAb) technique allowed the immortalisation of single antibody-forming cells (Köhler & Milstein,1975) and potentially this method could solve the specificity problem of xenoimmunisation. This was tested in a mouse anti-rat thymocyte fusion and in the first productive experiment the W3/25 antibody (anti-rat CD4) was identified (Williams et al,1977; White et al,1978; Bernstein et al,1980). The use of quantitative binding assays and the fluorescence-activated cell sorter (FACS) resolved the serological problems that were inherent in cytotoxicity assays.

The mAb approach allowed the discovery of human CD4 (OKT4) and CD8 (OKT8) antigens (Reinherz et al,1979;Reinherz & Schlossman,1980) and rat CD8 (MRC OX8) (Brideau et al,1980) and the pattern of two non-overlapping lineages in peripheral T cells was established for both these species. Cytotoxic cells were mostly of CD4-CD8+ phenotype and T helpers were CD4+CD8-. In the thymus most cells were CD4+CD8+. These patterns have now been seen in all vertebrate species where mAbs are available.

It was established that CD45 antigens included a family of molecules that differed in apparent mol.wt.(Strandring et al,1978; Trowbridge,1978), antigenicity (Coffman & Weissman, 1981; Dalchau & Fabre,1981,1983) and carbohydrate structures (Brown & Williams,1982; Childs et al,1983) and the finding that restricted determinants of CD45 (called CD45R) could distinguish subsets with different functions within CD4+ T cells was made in rat (Spickett et al,1983), human (Rudd et al,1987; Terry et al,1988) and mouse (Bottomly et al,1989). Current data indicate that the CD45R+ cells are primary T cells whilst the CD45R- cells have previously been stimulated by antigen (Tedder et al;1985; Powrie & Mason,1989).

Functional Effects of Antibodies

Mouse alloantisera against CD8 were shown to inhibit killing by cytotoxic T cells (Shinohara & Sachs,1979; Nakayama et al,1979) and this was the first data indicating that antibodies against differentiation antigens could affect T cell functions. The first result with a mAb was the finding that W3/25 (anti-rat CD4) in the form of IgG or $F(ab')_2$, blocked responder T cells in the rat MLR (Webb et al,1979) and blocking of cytotoxicity with anti-mouse CD8 mAb was also seen (Hollander et al,1980; Sarmiento et al,1980). Anti-human CD4 mAbs blocked CD4+ cytotoxic T cells (Biddison et al,1982) and the general result is that both anti-CD4 and anti-CD8 mAbs inhibit functions of appropriate T cells. Different mAbs against

CD45 do not have a consistent effect on T cell functions.

Ligands for the Subset Markers

Initially the mature T subsets were correlated with cytotoxicity and helper functions but later it became clear that the correct correlation was between expression of CD4 or CD8 on the mature T cell and MHC class 2 or class 1 respectively on the target or accessory cell (Engleman et al,1981; Swain,1981; Krensky et al,1982; Meuer et al,1982). This further led to the possibility that CD4 interacted directly with MHC class 2 antigen and CD8 with MHC Class 1 antigen. This interaction has been difficult to establish but in recent studies specific adhesion with appropriate target cells has been observed with cell lines expressing CD4 (Doyle & Strominger,1987) or CD8 (Norment et al,1988) after transfection of cDNA. The affinity of interactions would appear to be low since very high levels of antigen expression are required for these adhesion reactions to be observed. The goal of demonstrating interactions directly between the appropriate molecules in solution is yet to be achieved.

For CD45 antigen there is as yet no information concerning ligands interacting with the extracellular part of the molecule.

Molecular Aspects

The primary cDNA cloning of the subset markers was achieved for human CD4 and CD8 α chain via DNA transfection (Kavathas et al,1984; Littman et al,1985; Maddon et al,1985); for rat CD8 β chain and CD45 antigen via determination of protein sequence (Johnson & Williams,1986; Thomas et al,1985); and for mouse CD45 via mRNA size selection and screening of clones by RFLP analysis (Shen et al,1985). Subsequently the antigens have all been fully characterized in human, mouse, and rat (reviewed:CD4 and CD8, Parnes,1989; CD45, Thomas,1989).

In Fig.1 models drawn roughly to scale are shown for the antigens at a cell surface. The CD4 antigen can be argued to consist of four Ig-superfamily (IgSF) domains (Clark et al,1987; Maddon et al,1987) but only the first domain, which is V-like, shows typical sequence patterns. The structure of CD4 is more like the Poly Ig receptor than any antigen recognition structure and there is no convincing sequence match to J pieces of IgSF V-domains as has been suggested by Maddon et al (1985,1987).

The CD8 antigen is a heterodimer structure with a single V-like domain in each chain. In the CD8 chains there are hinge regions between the V-like domain and the transmembrane sequence that consist of about 40 (α-chain) and 28 (β-chain) residues. If these sequences were in an extended conformation as shown in Fig 1 then the distance between the membrane and the V-related domains could be as much as 9.5 nm (see legend to Fig 1). Extended sequences have been directly observed in mucin-like molecules (Bramwell et al,1986). The CD8 β chain is particularly notable for the presence of a strong match to IgSF V-domain J piece sequences (Johnson & Williams,1986). However the genomic sequence of the J-like region is in the same exon as the rest of the V-related sequence. The CD8 structure and antigen receptors may have had an immediate common ancestor in evolution.

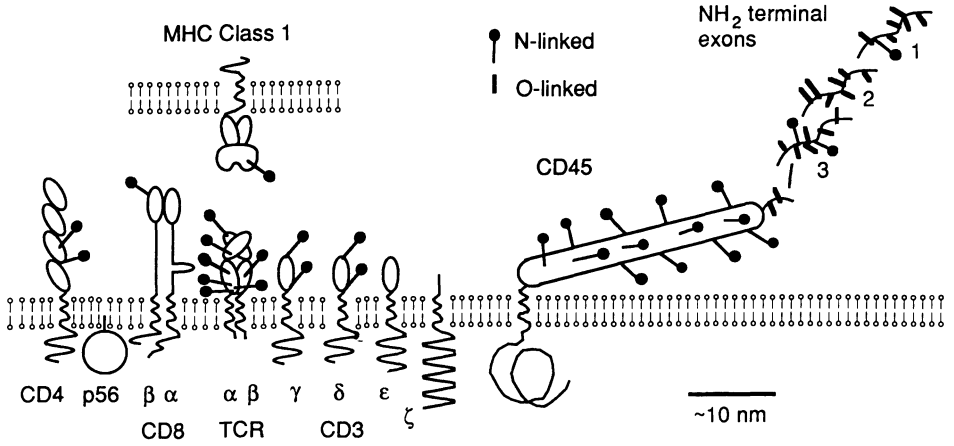

Fig 1 Models for CD4, CD8, the TCR complex and CD45 at a cell surface along with MHC Class 1 antigen on an opposing cell surface. The dimensions shown are roughly to scale on the basis of X-ray structures for Ig domains as used in Williams & Barclay (1986), and on the length of an extended peptide backbone for the hinge regions of CD8 and the cytoplasmic regions of all structures, except CD45, for which dimensions are based on low-angle shadowing electron microscopy data (Woollett et al, 1985) and p56lck where a globular conformation has been assumed.

It is perhaps surprising that the CD4 and CD8 structures are not particularly closely related within the IgSF given the fact that their MHC ligands are "first-cousin" IgSF molecules. It seems unlikely that a precursor molecule that interacted with an MHC-like structure gave rise to both the CD4 and CD8 antigens. Details concerning the sites of interaction between CD4 and CD8 and the MHC antigens are beginning to emerge (Potter et al,1989; Salter et al,1989; Clayton et al,1989) and the data are most clear for the CD8 α chain site on the MHC class 1 molecule. This appears to involve a region in the α3 domain of the class 1 H chain. It is believed that this interaction occurs at the same time that the TCR interacts with the MHC class 1 molecule presumably via the top faces of each of these molecules. An interaction between the CD8α V-like domain and α 3 of MHC class 1 would appear to be topologically feasible if there is an extended hinge structure as shown in Fig 1. Similarly the first domain of CD4 could presumably interact with parts of MHC class 2 that are not involved in interactions with the TCR.

The CD45 antigen is not an IgSF structure but is notable for its large cytoplasmic domain (Thomas et al,1985). In the extracellular part there is a common region that is likely to be tightly folded with disulphide bonds and a part that varies in different cell types via alternative splicing of three exons that code for sequence near the NH_2-terminal end (Barclay

et al,1987; Ralph et al,1987; Saga et al,1987; Streuli et al,1987a). N-linked carbohydrate structures are found throughout the sequence but O-linked carbohydrate is restricted to the region of varying sequence which is likely to show an extended conformation. The CD45R antigenic determinants are encoded in the exons that show alternative splicing (Barclay et al,1987; Johnson et al,1989; Streuli et al,1987b).

Phosphatases and Tyrosine Kinases

Cell biologists have focused on phosphorylation of tyrosine residues in structures at the inner face of the membrane as being a key factor in the regulation of cell activation signals (Alexander & Cantrell,1989). The steady-state level of phosphorylation is a result of the opposing activities of kinases and phosphatases.

Recently a lymphocyte specific protein-tyrosine kinase called $p56^{lck}$ has been identified (Marth et al,1985) and this molecule can be found associated with CD4 or CD8 in detergent lysates (Rudd et al,1988; Veillette et al,1988; Barber et al,1989). Thus it can be postulated that the activity of $p56^{lck}$ is modulated by interaction with CD4 or CD8 particularly in view of the tyrosine phosphorylation of the CD3 ζ chain during T cell activation.

Phosphatases oppose the kinase activities and recently a soluble tyrosine phosphate phosphatase from human placenta was sequenced and the sequence was found to be similar to both the homology units of the cytoplasmic domain of CD45 (Charbonneau et al,1988). Phosphatase activity has now been established for CD45 (Tonks et al,1988). Thus via the structural studies a function for CD45 has been precisely identified.

Immunological Functions

Antibody blocking data for CD4 and CD8 mAbs could have been interpreted as follows. (1) The molecules solely mediate down-regulation signals that are mimicked by the binding of mAbs. (2) The molecules function mainly as adhesion structures and mAbs inhibit by blocking the interactions. (3) The molecules associate with other molecules on the cell suface (e.g. TCR) and this is blocked by the mAbs. (4) The molecules give activation signals and mAbs interfere with this process.

Point (1) was ruled out by the finding that activation via TCR transfected into a CD8minus cell was greatly potentiated by the co-transfection of the CD8 α chain (Dembic' et al,1987; Gabert et al,1987). These data support point (4) and similar results have also been found for the CD4 molecule(Gay et al,1987; Sleckman et al,1988). Point (2) seems unlikely given the difficulty in showing interactions between CD4 or CD8 and their MHC ligands. Point (3) has been strongly supported by the finding that TCR and CD4 co-localise into the interface between a T cell clone and its target cell (Kupfer et al,1987). Thus the current concept is that CD4 and CD8 interact with the TCR to deliver signals to the cell (Emmrich et al,1986) and this view is compatible with the data on tyrosine kinase associations. In addition to this the CD45 molecule is a phosphatase that presumably modulates the signalling reactions.

Support for concerted positive and negative effects comes via studies in which signalling is assessed by activation of an increase in cytoplasmic free calcium, $[Ca^{2+}]i$. Triggering by

combinations of mAbs can be studied and in some cases a co-operative effect is seen while in others inhibition occurs (Ledbetter et al, 1988a, 1988b). One set of data is shown in Fig 2 for triggering of a CD4+ rat anti-myelin basic protein cell line (Beyers et al,1989; Beyers et al,unpublished). Anti-CD4 mAbs give a weak signal but a greatly potentiated response is seen with anti-CD4 plus anti-TCR added at a level that is subthreshold for signalling via TCR alone. In contrast to this, anti-CD45 mAb crosslinked with anti-CD4 leads to inhibition of the anti-CD4 response. It is interesting that in other experiments anti-CD4 plus anti-CD45 gave a co-operative response (Ledbetter et al,1988b). Although these data are difficult to interpret they suggest that complex interactions that rely on the precise geometry and kinetics of association between molecules will be occuring in the course of physiologically relevant cell interactions.

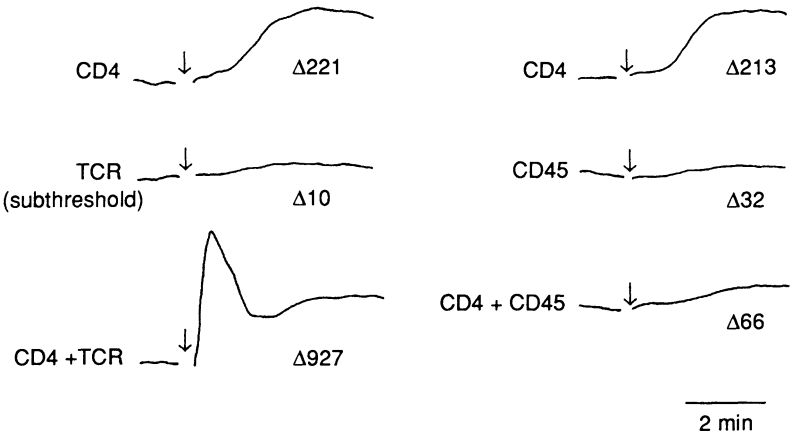

Fig 2 Changes in [Ca^{2+}]i induced by crosslinking various cell surface antigens. A CD4+ rat cell line, loaded with fura 2, was preincubated with mAb directed against the indicated antigens. Arrows indicate the addition of rabbit anti-mouse Ig and the horizontal bar indicates time. Δ values denote [Ca^{2+}]i increases in nM, calculated as in Grynkiewicz et al (1985). Details of mAbs and methods are in Beyers et al (1989).

Future possibilities

Key areas for future studies include:
(1) Determination of the structures of the molecules by X-ray crystallography and proof of proposed interactions by determination of affinities in solution.
(2) The search for ligands for CD45 with the possibility that molecules will be found that interact with the various CD45R forms.
(3) Analysis of the role of the cytoplasmic domains in the signal transduction pathways.
(4) Discovery of the precise points in the ontogeny of T cells where key events are triggered by the CD4, CD8 and CD45 molecules.

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The T-Cell Antigen Receptor and CD2 in Rat T-Cell Activation and Ontogeny

T. Hünig, G. Tiefenthaler, E. Schlipköter, and A. Lawetzky

INTRODUCTION

MAbs to the TCR complex have provided a useful model system in which activation of human and mouse T-cells can be studied irrespective of their specificity. In addition to the TCR, several other cell surface molecules have been identified as triggering structures by stimulatory mAbs. The best characterized of these "alternative pathways" of T-cell activation is that mediated through the "E-receptor", now called CD2 (first described by Meuer et al. 1984). Thus, both a natural ligand, LFA-3 (Hünig 1985, Dustin et al. 1987), that can participate in CD2-mediated T-cell activation (Tiefenthaler et al. 1987), and an intracellular domain necessary for signal transduction (He et al., 1988; Bierer et al. 1988) have been identified for this molecule.
Until recently, it was impossible to test the role of CD2 in T-cell ontogeny in an animal model for lack of the appropriate reagents. In the meantime, Williams and coworkers have generated several anti-rat CD2 mAbs two of which (OX-54 and OX-55) are stimulatory when used in combination (Clark et al. 1988), closely resembling the situation in humans. In addition, we have produced a mAb to a constant determinant of the rat α/β-TCR that efficiently activates T-cells (Hünig et al. 1989a). We here describe our initial experiments in which the expression of the TCR and of CD2 on immature and mature T-cells and the effects of Mabs to these receptors are studied in the rat model system.

EXPRESSION OF THE α/β-TCR AND OF CD2 ON PERIPHERAL RAT LYMPHOCYTES.

Correlation of the cell surface markers OX-52 (pan T and NK), CD5 (pan T), CD4, and CD8 with the α/β–TCR on peripheral rat lymphocytes allows the identification of the subpopulations shown in Fig. 1. In addition to the two major T-cell populations of α/β–TCR+ T-cells (CD4+8- and the CD4-8+) which account for roughly 90% of OX52+ cells in the spleen, two minor populations were detected: 1. α/β-TCR-negative CD5+ cells, at least some of which express the rat γ/δ-TCR (Hünig et al., 1989b). Over 90% of cells in this minor subset express the CD8 antigen in spleen, lymph node and blood. 2. CD5-CD8+ cells which do not express CD3-associated TCR molecules (Hünig

et al. 1989b). In agreement with others (Vujanovic et al. 1988), we have identified NK activity of freshly isolated lymphocytes exclusively in this population (Hünig et al. 1989b).
All four populations thus defined are are CD2+. CD2-negative T-cells, found in great numbers in sheep (MacKay et al., 1988, Giegerich et al. 1989) were not detected. In contrast to what has been recently reported for mice (Yagita et al. 1989), rat B-cells are CD2-negative (Williams et al. 1987). With the exception of CD2-expression on macrophages in the spleen (Williams et al. 1987), the cellular distribution of rat CD2 thus closely resembles that found in humans. In addition, despite the discovery of CD2 homologues in rats, mice, and sheep, the rat represents the only animal model where activation through CD2 has been demonstrated to date.

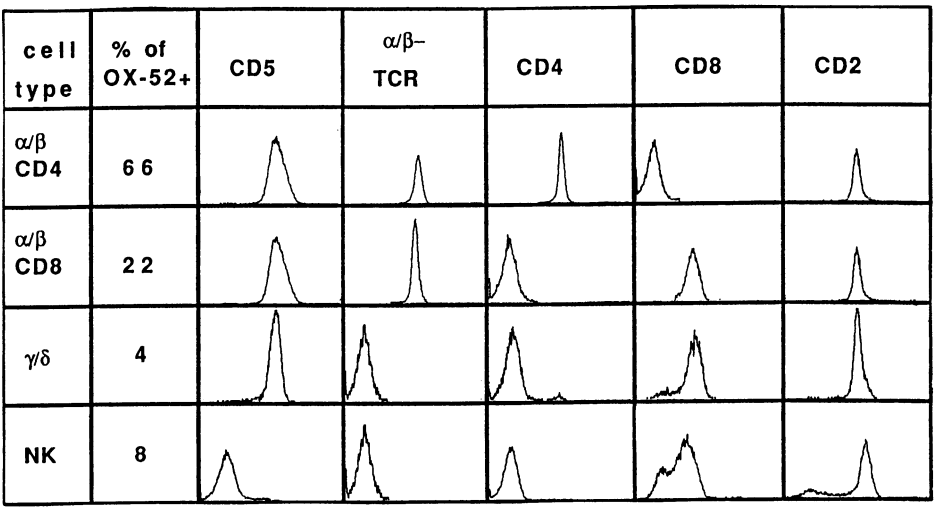

Fig. 1. Subpopulations of T-cells and NK cells in rat spleen.

EXPRESSION OF THE α/β-TCR AND OF CD2 ON THYMOCYTE SUBPOPULATIONS.

As previously shown for humans and mice, three populations of thymocytes expressing distinct levels of the α/β-TCR are resolved by immunofluorescence and flow cytometry in the adult rat thymus (Fig. 2, and Hünig et al., 1989a). About 10% of cells express as much TCR as peripheral T-cells; these are the mature medullary thymocytes. About 70% express roughly ten-fold less TCR; all of these cells are immature, CD4+8+ cortical thymocytes. The remaining 20% of α/β-TCR-negative thymocytes also contain mostly CD4+8+ cells, but in addition most of the CD4-8- immature thymocytes (CD4-8+ immature blasts from which CD4+8+ cells are generated make up only 2% of thymocytes; they express the TCR at a very low level. Hünig, 1988).

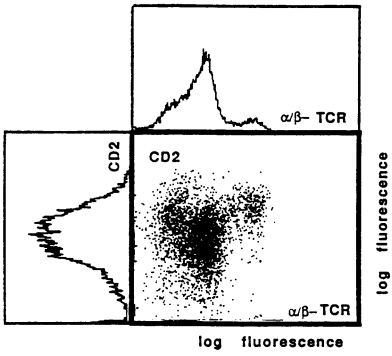

Fig. 2. Correlation of TCR and CD2 expression on adult rat thymocytes.

Correlation of CD2 with TCR-expression on adult rat thymocytes reveals an interesting distribution: Both TCR-negative and TCR-high cells are uniformly CD2 high. The intermediary, TCR-low population, however, expresses distinctly less CD2 on average and some of these cells express very little if any CD2. Interestingly, CD4+8+ cells generated from their CD4-8+ precursors in cell culture are uniformly CD2-high (not shown), raising the possibility that downregulation of CD2 on a fraction of this population in vivo may be the result of intrathymic repertoire selection (or lack of of selection).

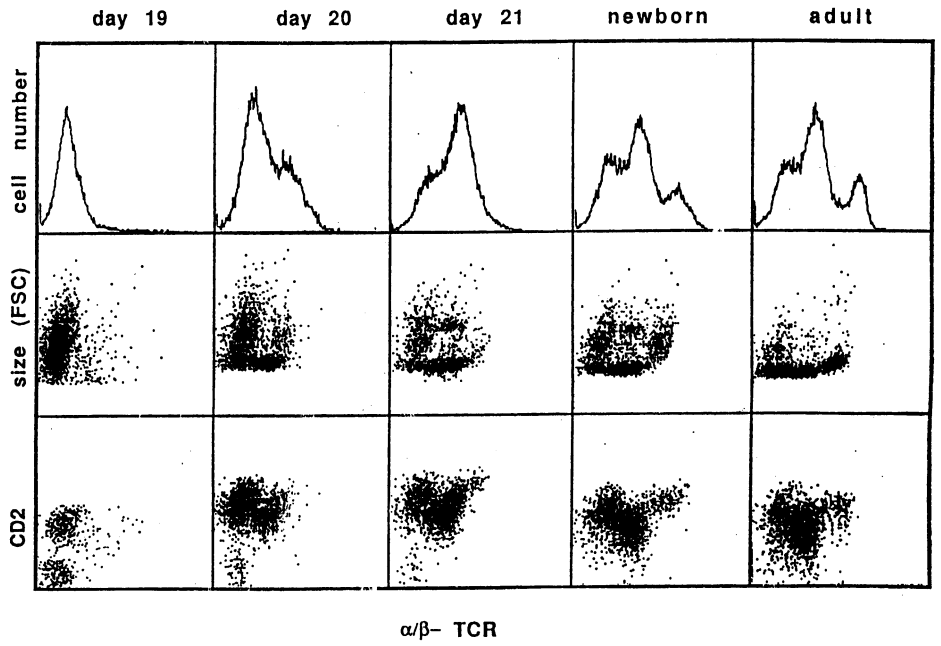

Fig. 3. Expression of the α/β-TCR and of CD2 in rat thymopoiesis. Thymocyte suspensions were prepared from embryonic, newborn, and adult thymuses and were stained with mAbs to the α/β-TCR (R73) and to CD2 (OX-34).

EXPRESSION OF THE α/β-TCR AND OF CD2 IN RAT THYMOPOIESIS.

The stepwise generation of TCR-low and TCR-high cells from TCR-negative precursors is readily observed in rat prenatal life (Fig. 3, top and center). With the exception of a population of CD2- and TCR-negative cells at early stages of thymopoiesis, CD2 expression on the successively appearing subpopulations follows that expected from the expression of CD2 on adult thymocytes. I.e., as TCR-low cells are "growing out" from the TCR-negative blast population, they partially downregulate CD2, whereas all of the first wave TCR-high cells express as much CD2 as the TCR-negative population.

ACTIVATED PHENOTYPE OF FIRST WAVE α/β-TCR-HIGH THYMOCYTES.

As can be seen in Fig. 3, thymocytes with a mature level of TCR expression first appear around birth in the rat. Interestingly, these "first wave" TCR-high cells are much larger than resting lymphocytes, suggesting an activated state. Accordingly, their cell surface phenotype and cell cycle status were investigated. In Fig. 4, these results are shown in comparison to those obtained with resting lymph node T-cells. In addition to their blastoid phenotype, newborn TCR-high thymocytes differed from resting adult T-cells in that they contained about 20% CD4+8+, and a small but significant fraction (about 5%) of IL-2R+ cells. In addition, 21% of TCR-high blasts were in the S and G2/M phases of the cell cycle as compared to only 0.6% of adult lymph node T-cells.

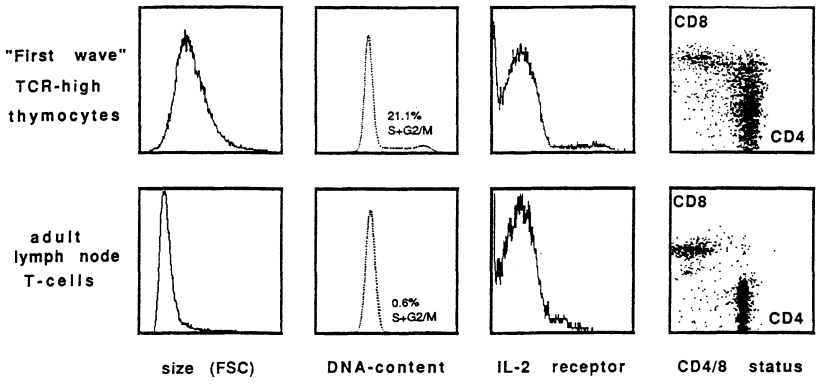

Fig. 4. Phenotype and cell cycle status of first-wave TCR-high rat thymocytes. TCR-high cells were enriched from new born thymocytes by collecting the low densitity fractions of a percoll gradient and selecting for cells expressing the OX-44 antigen restricted to medullary and CD4-8- thymocytes (Paterson et al. 1987). Nylon wool passed lymph node cells are included for comparison. Gates were set on TCR-high cells that were either stained for additional surface markers or for DNA-content with Hoechst stain.

IN VITRO REACTIVITY OF FIRST WAVE TCR-HIGH THYMOCYTES

The proliferative response of highly enriched TCR-high new born thymic blasts to TCR- and CD2-induced triggering was compared to that of resting peripheral T-cells. The results in Fig. 5 show: 1. As expected from cell cycle analysis, some "spontaneous" thymidine incorporation was observed during the first 16 hrs in the thymocyte cultures but not in those of peripheral T-cells. 2. The thymic blasts, but not the peripheral T-cells, responded to IL-2 with proliferation, confirming the presence of IL-R+ cells in the former population. 3. A much stronger response was observed to crosslinked anti-TCR mAb plus IL-2. The maximum response of new born TCR-high thymocytes was already observed on day 1, in contrast to the peak response of peripheral T-cells that occurred on day 2 of culture. 4. Stimulation with anti-CD2 plus IL-2 also followed accelerated kinetics in "first wave" TCR-high thymocytes as compared to lymph node T-cells.

Additional studies (not shown) have indicated that cultivation of newborn TCR-high blast cells with anti-TcR mAb in the absence of IL-2 rapidly induces cell death. These functional and phenotypic properties of "first wave" TCR-high thymocytes, and the presence of such blast cells as a very minor population among adult thymocytes (not shown) suggest that after or during upregulation of the TCR to a mature level, thymocytes pass through an activated state in which they may still be sensitive to signals involved in repertoire selection.

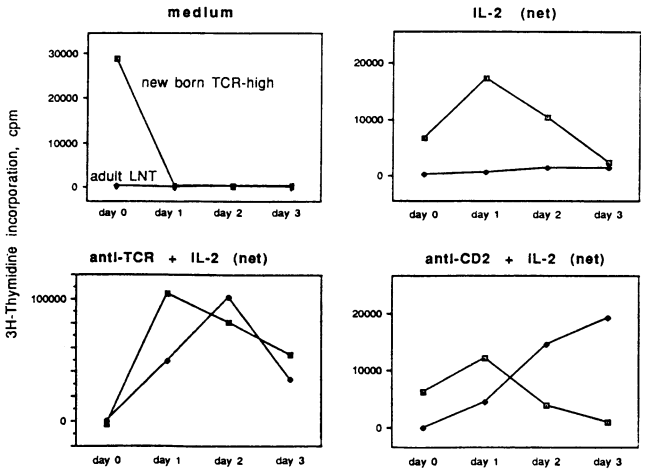

Fig. 5. Proliferative response of first-wave TCR-high thymocytes and of adult lymph node T-cells to IL-2 and to TCR- and CD2-mediated stimulation. The responses of newborn thymic blasts (85% TCR-high) are compared to those of nylon wool-passed adult lymph node cells (85% TCR+). For "net" cpm, values obtained in medium only were subtracted from the IL-2 response, and values obtained with IL-2 were subtracted where responses to crosslinked anti-TCR mAb plus IL-2 or to anti-CD2 mAbs plus IL-2 are shown.

EFFECTS OF NEONATAL INJECTION OF ANTI-TCR AND OF ANTI-CD2 MABS ON THE GENERATION OF MATURE RAT T-CELLS

Rats were treated postnatally with mAbs to the α/β-TCR or to CD2. Biweekly intraperitoneal injections of mAb doses were administered that had been found to saturate all peripheral binding sites. In the thymus, however, only partial saturation was achieved with these doses of mAb.
Fig. 6 compares the T-cell composition in the lymph nodes of 6 wk old rats that were either injected with PBS, anti-α/β-TCR mAb R73, or the non-stimulatory anti-CD2 mAb OX-34.
Injection of R73 mAb virtually eliminated α/β-TCR+ cells. Most of the residual CD5+ (i.e. T-cells) remained α/β-TCR-negative when cultured for 2 d in vitro. They were mostly CD4-8+ (not shown) and thus were of the same phenotype as the γ/δ T-cells identified in normal animals (see above). A small fraction of freshly isolated CD5+ cells in R73-suppressed animals did, however, express a low level of the α/β-TCR. These cells had modulated their antigen receptor, since upon cultivation, they upregulated the R73 antigen (not shown). In the thymus, α/β-TCR-high cells were largely depleted (not shown) in R73-treated animals, whereas the other subpopulations were not visibly affected. Since thymic α/β-TCR epitopes had not been saturated, no firm conclusions can be drawn from these results.
Injection of anti-CD2 mAb from birth led to a completely different picture: The periphery of treated animals was severely (at least four-fold) depleted of α/β-TCR+CD4+ cells but contained normal frequencies of α/β-TCR+CD8+ and of α/β-TCR-negative, CD5+ cells. It appears unlikely that this selective reduction in the "helper" T-cell compartment reflects a difference between T-lymphocyte subpopulations in their sensititvity to peripheral mechanisms of mAb-mediated elimination of cells (complement, ADCC, opsonisation). Accordingly, at least three other possibilities have to be considered: 1. Occupancy of CD2 by mAb OX-34 interferes more severely with maturation of CD4 than of CD8 T-cells, 2. Migration of newly formed CD4 cells to their peripheral sites is more dependend on CD2-LFA-3 interaction than that of CD8 T-cells, and 3. CD4, but not CD8 cells have to expand peripherally in order to reach normal numbers and this proliferation is inhibited by anti-CD2 mAb. Since the fraction of CD4+8- thymocytes was not dramatically altered even when very high mAb doses were injected that did saturate all 0X-34 epitopes in the thymus (not shown), specific inteference with maturation of CD4 cells appears as the least likely of these hypotheses.

IMMUNE REACTIVITY OF ANTI-α/β-TCR AND ANTI-CD2 TREATED RATS

The results obtained in a large number of experiments with neonatally α/β-TCR-suppressed rats and in a limited number of anti-CD2-treated rats can be summarized as follows (data not shown):
1. Alloreactivity. Even after enrichment of CD5+ cells by nylon wool

filtration, lymphocytes from α/β-TCR-suppressed did not mount a proliferative response to allogeneic stimulator cells even in the presence of IL-2. Thus, α/β-TCR-negative T-cells (including, or identical with, γ/δ–T-cells) are unlikely to contain a high frequency of alloreactive cells. In contrast, T-cells from anti-CD2 treated animals did respond in MLR. This response was, however, dependend on the addition of exogenous IL-2, suggesting that under our experimental conditions, provision of IL-2 from CD4+ cells (which had been largely depleted) was limiting.

2. T-cell help for B-cells. Injection of neonatally anti-α/β-TCR-suppressed rats with the strong T-dependent antigen KLH in alum plus pertussis resulted in no measurable specific antibody response. Thus, γ/δ-T-cells cannot replace the α/β-TCR+ helper T-cells when the latter compartment is suppressed from birth.

3. NK-cell activity. Neonatal suppression of α/β-TCR+ T-cells had no adverse effect on NK activity. Rather, a 2-8 fold enhancement was observed in individual animals, suggesting that NK cells may be upregulated to some degree in compensation for the lack of the major T-cell compartment.

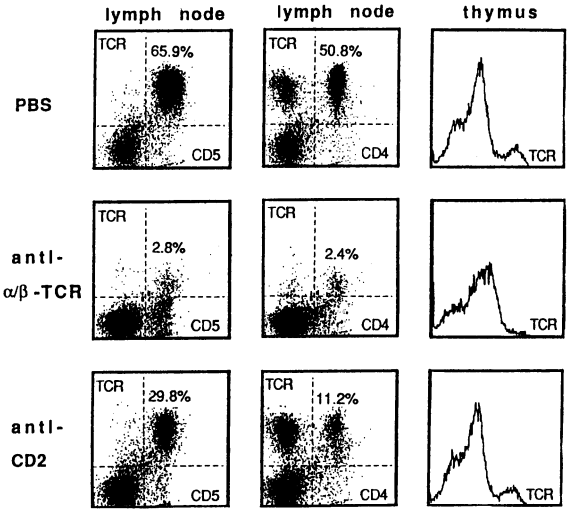

Fig. 6. Lymphocyte subsets in rats treated from birth with mAbs to the α/β-TCR or to CD2. Flow cytometric analyses of cells from 8 wk old animals. Indirect staining was used for the mAb used for in vivo treatment.

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Tolerance Induction Using CD4 and CD8 Monoclonal Antibodies

H. Waldmann, S.P. Cobbold, and S. Qin

INTRODUCTION

The immune system maintains a constant capability to respond to the outside world while remaining unresponsive to self. There may be two broad explanations for self-tolerance. First there must be some self-molecules which are simply invisible or cryptic to the immune system (see Waldmann et al. 1989a). In this case ignorance maintains unresponsiveness. Second, some self-molecules become visible to lymphocytes with complementary receptors and are able to inactivate or "tolerise" those cells. For T-cells the visible antigens would be processed peptides presented on cell surfaces together with the individual's own MHC Class I and Class II gene products. It is not known for any individual cell or even tissue what number of different peptides are used to define "self". Some peptides are likely to be ubiquitous (present in all cells), while others may be restricted to defined cell types. Endogenous peptides may be invisible (cryptic) for reasons of quantity, capacity to bind to MHC, and failure to compete in processing, transport and access to MHC molecules.

In the thymus (**central**) self-tolerance appears to arise by deletion of T-cells with receptors for any visible-self peptides that can be expressed in there. The T-cells which eventually leave the thymus will then encounter further self antigens in the periphery. Tolerance must be established to these. The mechanisms which are used to achieve peripheral tolerance remain to be defined.

In clinical practise it would be highly desirable to exploit knowledge of the self-tolerance pathways to permit long-term acceptance of foreign grafts and correction of autoimmunity and allergy. Practical realities dictate that, for humans, tolerance-therapy should largely operate through exploitation of peripheral tolerance mechanisms, as central (thymic) tolerance may be unachievable except in the case of bone-marrow grafting where donor cells/ antigens may actually access recipient thymus.

Most peripheral T-cell responses to foreign antigens require participation of a number of so-called "adhesion molecules" in concert with the T-cell receptor. These include CD4, CD8, CD11a/CD18 (LFA-1) and CD2 (LFA-2). Monoclonal antibodies to these molecules can block T-cell function both in-vitro and in-vivo. Certain monoclonal antibodies (Mabs) to CD4 and CD8 can also deplete each of the T-cell subsets in-vivo (Cobbold et al. 1984). In these circumstances of T-cell depletion, and/or blockade; it has been possible to introduce a foreign antigen to the immunosuppressed animal and to determine what record the immune system had kept of the antigen encounter when antibody therapy was stopped and full immune function restored.

We have found, and will review here the evidence, that CD4, CD8 and CD11a Mabs can create within mice a tolerogenic milieu which permits tolerance to be acquired to a range of foreign proteins, and to marrow

and skin allografts. In some of these models tolerance must have been induced in peripheral T-cells. These provide novel experimental systems for studying mechanisms of peripheral tolerance as well as pointing to the use of comparable strategies for tolerance induction as a therapeutic procedure in humans.

ANTIBODIES

We will be referring to antibodies as either **depleting** or **blocking/non-depleting**. Where we have wished to definitely prove that cell-depletion was not essential to tolerance induction we have used $F(ab')_2$ fragments. The Mabs capable of **depleting** CD4+ T-cells (YTS 191 and YTS 3), and those able of eliminating CD8+ cells (YTS 169, YTS 156 and YTS 105) have been previously described (Cobbold et al. 1986; Qin et al. 1989). To provide the **non-depleting** Mab to CD11a we used the hybridoma FD441.8 (Sarmiento et al. 1982) and to CD4, the rat IgG2a Mab YTS 177 (Qin et al. 1989). To follow the behaviour of T-cells with serologically defined receptors we used the Vß6 Mabs 44-22-1 and 46-6B5 (Paynes et al. 1989; MacDonald et al. 1989).

TOLERANCE TO XENOGENEIC RAT AND HUMAN IMMUNOGLOBULINS.

All forms of CD4 mab (depleting, non-depleting, fragments) permit tolerance to rat and human xenogeneic immunoglobulins if these antigens are given under the umbrella of a short course of Mab therapy (Benjamin and Waldmann, 1986; Benjamin et al. 1986; Gutstein and Wofsy, 1986; Qin et al. 1987; Carteron et al., 1988; Benjamin et al. 1988. The **non-depleting** CD11a Mab was also permissive for HGG tolerance (Benjamin et al. 1988). Tolerance could be induced in the peripheral CD4 T-cell pool itself without involvement of any newly generated T-cells, as neither the induction of tolerance nor its expression required CD8 cells; nor the presence of a thymus (Wise and Waldmann in preparation). Adoptive transfer studies revealed tolerance at the level of T-helper cells with no demonstrable suppression. The duration of tolerance was unlimited if the antigen (the xenogeneic immunoglobulin) was reinjected into tolerant mice at regular intervals. No further CD4 Mab therapy was required beyond the first tolerance-inducing regime.

The basic observations outlined above suggested that Mabs to T-cell adhesion molecules might be similarly tolerance-permissive for more complex antigenic systems such as cellular allografts. As CD8 cells can themselves reject allografts (Cobbold et al, 1986), we combined CD8 Mabs together with CD4 antibodies in this series of studies.

TRANSPLANTATION TOLERANCE

Classical-type Transplantation tolerance

Billingham et al. (1953) demonstrated that the injection of allogeneic haemopoietic cells into newborn mice could produce both donor chimerism and donor-specific transplantation tolerance. If allogeneic bone marrow transplants could do the same in the adult, then simulataneous marrow + organ grafting might provide an alternative to the long-term immunosuppression presently required for sustaining allogeneic tissue grafts. Numerous attempts to achieve classical transplantation tolerance in adult rodents have failed except where ablative regimes of irradiation, chemotherapy and broad specificity anti-lymphocyte sera have been used to destroy host immune and marrow function (Rappaport et al. 1987; Slavin et al. 1987; Thomas et al. 1983; Ilstad and Sachs 1984; Mayumi et al. 1986). Recently, however we

have demonstrated Classical-type Transplantation Tolerance in a number of mouse strain combinations using very short courses of **depleting** (CD4 and CD8) antibodies to smuggle in the foreign marrow. This was achieved without any other lympho-or myelo-ablative agents. Although we have succeeded with strain combinations differing at multiple-minor or Class I + minor histocompatibility differences, we have been less successful across complete MHC+ minor incompatibilities. Here, long-term chimerism and donor-specific unresponsiveness required the addition of a CD11a Mab and 300 rads (non-lethal) whole body irradiation (Benjamin et al. in preparation). Similar results have been obtained by Sharabi and Sachs (1989) who have used depleting CD4 and CD8 mabs together with thymic irradiation to produce mixed chimerism and tolerance across complete H-2 differences.

In the H-2 matched multiple-minor mismatch (B10.BR to CBA/Ca) combination both the CD4 and CD8 mabs were required to prevent rejection of donor marrow, thus permitting low-level donor chimerism and donor specific tolerance. Although our initial studies had involved **depleting** antibodies it became clear that **non-depleting** antibodies were sufficient (Qin et al 1989), just as for the xenogeneic proteins. Again the likeliest explanation was that antigen could tolerise peripheral T-cells if the CD4 and CD8 adhesion molecules were pre-occupied with antibody.

Tolerance Of Recipient-type T-cells to Donor Alloantigens: Where we had used **depleting** protocols it was theoretically possible that any new T-cells that developed might have arisen from donor stem cells. If so we would not have been able to claim true transplantation-tolerance. In a combination (AKR into CBA) where we could monitor the origin of the reconstituting T-cells, we observed that the majority of T-cells were Thy 1.2+ although there were clearly donor Thy1.1+ cells detectable. Recipient T-cells must, therefore, have been tolerant to donor antigens.

Mechanisms of Tolerance: CBA/Ca (Mls^b) mice tolerant of AKR were unable to mount a proliferative response to AKR stimulators (Mls^a) in-vitro even though we could show that they had normal levels of Vβ6+ T-cells. In contrast previous work (Kappler et al.1988; MacDonald et al. 1989) had suggested that **central** tolerance induced in the thymus to Mls alloantigens was associated with loss of T-cells expressing particular Vβ specificities. In our antibody-facilitated chimeras we have been able to show that all the Vβ6+ cells are of recipient type (Qin et al. in preparation), while in radiation chimeras where all T-cell reconstitution proceeds through the thymus, Vβ6+ cells are in fact deleted. This suggests that in tolerant animals all the Vβ6+ cells were derived from the peripheral T-cell pool of the CBA/Ca recipients. In other words the peripheral T-cells had become tolerant to Mls^a but had not been deleted. By analogy with B-cell tolerance this might be coined "anergy". Surprisingly, Vβ6+ cells of half of the tolerant animals could be stimulated to proliferate with a mitogenic Vβ6 antibody. T-cells from other mice all remained anergic even with such a strong stimulus. This either means that anergic cells can be triggered if given a sufficient stimulus, or that the responsive cells are not Mls specific.

We have found that neither chimerism nor tolerance generated in this way is easily broken by boosting with donor cells, or even infusion of normal non-tolerant cells. It can however be broken by infusion of primed cells. This suggests that anergy may be only one of the mechanisms by which **peripheral** tolerance is maintained , and that other forms of regulation may be operating.

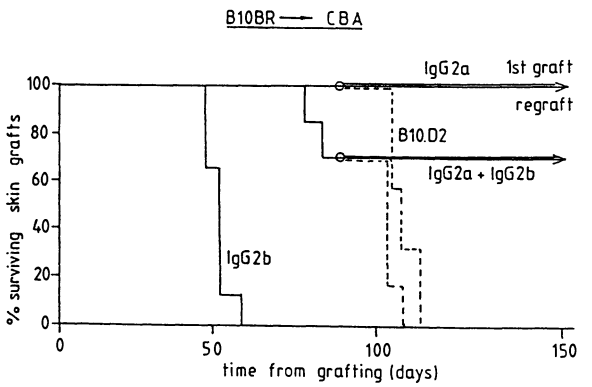

Fig. 1. Tolerance of CBA/Ca mice to B10.BR skin transplanted under cover of CD4 and CD8 monoclonal antibodies. Mice were transplanted on day 0 with tail-skin. One group received 3 injections of synergistic rIgG2b **depleting** CD4 and CD8 mabs (1.2mg/ mouse total) from the time of grafting. Two other groups received 3 weeks therapy with the **non-depleting** rIgG2a CD4 and the rIgG2a CD8 Mabs only (9mg/ mouse total) or 1 week of rIgG2b **depleting** Mabs followed by 2 weeks of the rIgG2a Mabs. Fresh grafts were transplanted onto these same recipients after 3 months from B10. BR strain (solid lines) or B10. D2 strain (dotted lines).

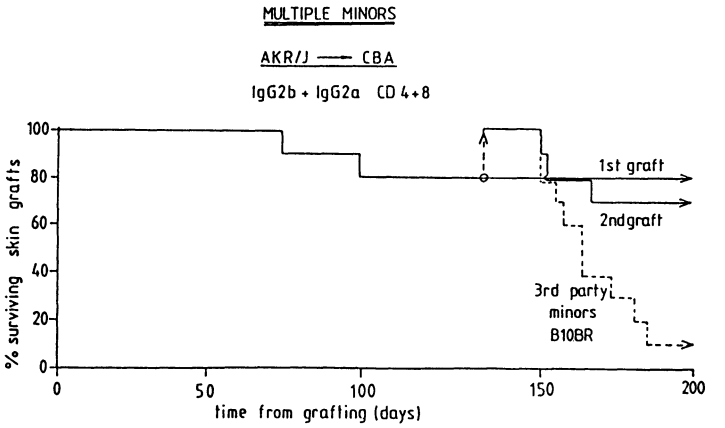

Fig. 2 Tolerance of CBA/Ca mice to AKR/J skin transplanted under cover of CD4 and CD8 mabs. Groups of mice were transplanted as above under cover of the combined rIgG2b (2 doses) and rIgG2a CD4 and CD8 Mab regimen. Fresh grafts were transplanted after 4.5 months. 8/10 first grafts have remained normal (MST > 200 days). A proportion of tolerant animals rejected their second AKR graft (1/8), and all eventually rejected a third party B10. BR graft (MST=28 days).

Tolerance to Skin-Grafts

In the models we described above the "tolerogen" was disseminated through the body in the form of a donor haemopoietic system. It would not be difficult, therefore to imagine that all host T-cells could have easy access to donor antigens for tolerance induction and maintenance. This would not always be expected with a small patch of donor skin as the only available source of donor antigen. In the situation where donor and recipient show MHC identity but differ in minors then reprocessing of donor-skin minors and their continued presentation on host haemopoietic cells would allow a geographically constrained source of donor antigen to exert a diverse influence. In contrast, when donor and host are mismatched at MHC then only the graft should be able to provide the contact-antigen. (This assumes that reprocessing of majors {+donor minors} is not operating for tolerance).

We sought to establish whether a limited course of CD4 and CD8 Mab therapy would create a tolerance-permissive environment for skin grafts. To our surprise we have been able to demonstrate skin-graft tolerance not only in MHC-matched minor-mismatched combinations, but also across complete MHC + minor differences. (Cobbold, Qin, Martin and Waldmann, in preparation; Qin, Cobbold, Kong, Parnes, and Waldmann, in preparation)

Tolerance Across Multiple-Minor-Mismatch Combinations: In Fig. 1 we show skin graft survival of B10.BR skin onto CBA/Ca using three different Mab treatment protocols (Cobbold et al.; Qin et al. in preparation). The short course of three injections of depleting synergistic pairs of rIgG2b CD4 and CD8 mabs (3 injections over 4 days), was insufficient to produce tolerance to skin alone, even though this protocol was adequate for tolerance induction to a combination of marrow and skin (Qin et al. 1989). In constrast the combination of a **non-depleting** rIgG2a CD4 mab with CD8 Mab given over 3 weeks was able to induce long-term skin graft tolerance. The substitution of a short course of **depleting** antibodies for the first week did not alter the outcome. The same antibody combination allowed CBA/Ca mice to become tolerant of AKR/J skin (Fig. 2). As CD4 cells are the major effectors of rejection in these combinations we must conclude that blockade of CD4 cells with monoclonal antibodies over a period of three weeks will allow tolerance to occur. Short periods of blockade are insufficient (Qin et al. in preparation). Presumably prolonged blockade provides time for antigen to be disseminate and tolerise all relevant T-cells. We know that CD8 Mabs are required but have not yet examined whether **depletion** or **blockade** was necessary.

Tolerance to Skin Grafts Mismatched for both Major and Minor Transplantation Antigens: In Fig. 3 we show skin-graft survival data where B10 ($H\text{-}2^b$) donor skin was grafted onto CBA/Ca recipients ($H\text{-}2^k$). We have analysed a number of different antibody combination protocols. The successful one required three weeks of antibody therapy, where a short course of depleting rIgG2b antibodies (CD4 + CD8) was followed by the rIgG2a CD4 and CD8 (**non-depleting for CD4**). Tolerance to the first skin graft lasted indefinitely (MST $>$ 200 days). Remarkably, second grafts from fresh B10 donors were virtually all rejected at a very slow rate (MST 44 days). In other words tolerance was fully maintained to the first "tolerance-inducing" graft, whilst it could only have been partial to the challenge graft. The only plausible explanation was that the process of grafting itself exposes new visible peptides to which the recipient is not tolerant. These are able to induce a rejection response that can only exert itself on the challenge graft. The slow

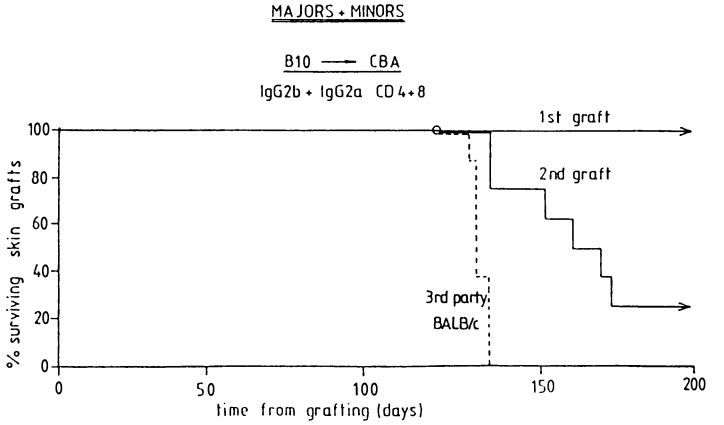

Fig. 3. Tolerance of CBA/Ca mice to skin grafts from H-2 + minor mismatched donors (C57/BL.10 to CBA/Ca). CBA/Ca mice received tail skin from B10 mice under cover of CD4 and CD8 Mab therapy. Test groups received 2 **depleting** doses of the rIgG2b CD4 and CD8 synergistic sets (800ug total Mab per mouse), followed by the rIgG2a Mabs up to 3 weeks. Controls received no antibody. All animals received fresh grafts after 4 months. All the first B10 grafts have remained intact (> 200 days). Second B10 grafts have rejected at a relatively slow rate (MST = 44 days). Third party BALB/c grafts were rejected promptly (MST = 13 days).

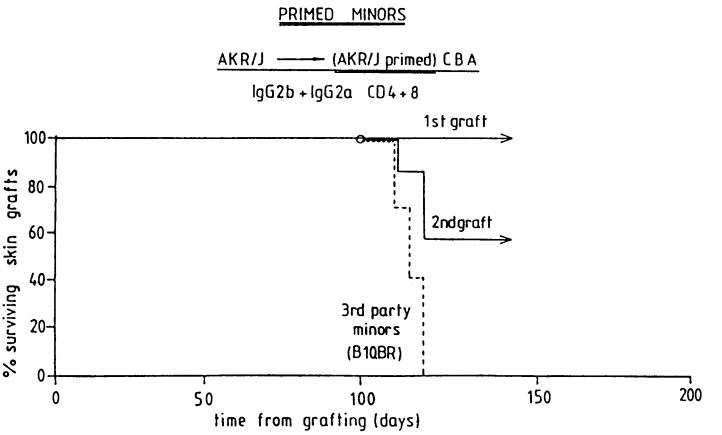

Fig. 4 Tolerance induction in mice previously primed to the alloantigens of the donor. CBA/Ca mice were previously primed to irradiated (2500 rads) AKR spleen cells and two weeks later grafted with tail skin from the same donors under cover of the rIgG2b/ rIgG2a CD4 and CD8 combination as above. Fresh skin grafts were again transplanted after 100 days. All the first grafts remain intact (MST > 140 days); a proportion of the second grafts (3/7) have rejected while third party B10.BR grafts rejected promptly (MST = 14 days).

rejection rate would then reflect the frequencey of host alloreactive cells able to respond to these few peptides/minors.

We have shown that tolerance to MHC-mismatched skin allografts is possible with a short course of monoclonal antibody therapy. In a different strain combination BALB/c ($H\text{-}2^d$) to CBA/Ca ($H\text{-}2^k$) the same Mab protocol produced prolonged graft survival but not absolute tolerance. Given the empiricism of the approach it may be that more aggressive antibody protocols would be required to achieve a method robust enough to transcend all possible strain combinations.

Tolerance to Second-Set Grafts: In previous published work (Cobbold and Waldmann, 1986) we observed prolonged survival of second-set grafts in the minor-mismatch combination B10.BR onto CBA/Ca where recipients had been given a short course of **depleting** CD4 and CD8 antibodies. Graft survival extended well beyond the immunosuppressive window of the antibodies, implying that partial tolerance had been induced. We have now found that the same combination of antibodies (**depleting** and **non-depleting**) that permitted skin-graft tolerance across MHC differences (see Fig 3) was also capable of establishing tolerance in a second-set multiple-minor-mismatch combination (AKR into primed CBA/Ca-Fig. 4 and B10.BR in to primed CBA/Ca- Cobbold et al. unpublished). As before the primary grafts have survived for more than 150 days, while some (3/7) of the second test grafts (grafted at 100 days) were rejected. The possibility of transiently visible neoantigens applies as before.

DISCUSSION

We have here shown that CD4 and CD8 Mabs can be used as both **depleting** and **non-depleting** agents for tolerance induction. Tolerance could be induced to xenogeneic immunoglobulins, and to allografts of bone marrow and skin, even in second set systems. Since the initial description of CD4 Mabs facilitating tolerance to xenogeneic immunoglobulins (Benjamin and Waldmann, 1986; Gutstein et al. 1986), and prolonging graft survival (Cobbold and Waldmann, 1986; Shizuru et al. 1987; Mottram et al. 1987) we and others have extended the findings to allograft-tolerance (Madsen et al. 1988; Herbert and Roser, 1988; Qin et al. 1989). In both mouse and rat, tolerance to vascularised or foetal heart grafts has been straightforward (Masden et al. 1988; Herbert and Roser, 1988). A major challenge to the power of antibody-facilitated tolerance has been to create tolerance to skin allografts as these are the most immunogenic of all rodent grafts. We have shown this to be possible without the need for any other immunosuppressive agent. The finding that tolerance could even be induced in primed cells is exciting. It predicts that similar Mab protocols could be applied to ongoing autoimmunity where T-cells are already immunised to particular self-peptides. As long as those peptides remain "visible" then restoration of tolerance should be possible.

The finding that non-depleting CD4 antibodies can so powerfully mediate these effects for CD4 cells has obvious clinical advantages. In principal one should be able to achieve a therapeutic effect without elimination of cells - the ideal form of "low-impact" therapy.

As for mechanisms - we have a glimpse of one form of **peripheral** tolerance which seems to arise by T-cell anergy. Our goals for the future will be to dissect these novel models of peripheral tolerance and see what number of failsafe systems **peripheral** T-cells can use to safeguard and assert self-tolerance. An hypothesis for how CD4 and CD8 Mabs may be operating to establish tolerance has been fully discussed elswhere (Waldmann et al.1989; Waldmann 1989).

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A Lymphocyte Homing Receptor: A Member of an Emerging Class of Cell Adhesion Molecules

S.D. Rosen

WHAT IS LYMPHOCYTE HOMING?

The body contains a collection of scattered lymphoid organs, which drain all potential portals of entry for foreign substances. Antigens that enter through the skin are carried by the lymph to cutaneous lymph nodes; antigens that enter through the gut are collected by gut-associated lymphoid organs such as Peyer's patches, and antigens in the blood are filtered out by the spleen. Any particular antigen can activate only a small percentage of the immunocompetent lymphocytes. To maximize the probability that this small number of cells comes into contact with a cognate antigen that becomes localized in a particular lymphoid organ, there is a continuous and massive recirculation of lymphocytes between the blood and all the lymphoid organs (see review by Yednock and Rosen 1989). In this way the body's repertoire of immunocompetent B- and T-cells are eventually brought into contact with the antigen, in whichever of the widely scattered lymphoid organs the antigen has become sequestered. Lymphocytes move from one lymphoid organ to the next by alternatively traveling via the blood and the lymph. In murine, human and other mammalian species, the portal of entry of blood-borne lymphocytes into secondary lymphoid organs (except the spleen) is through high endothelial venules or HEV. These are postcapillary venules characterized by tall or cuboidal endothelial lining cells. Lymphocytes adhere to these specialized endothelial cells and subsequently migrate into the parenchyma of the lymphoid organ. Since the HEV was first recognized as the primary site of lymphocyte emigration from the blood into secondary lymphoid organs (Gowans and Knight 1964), there has been great interest in the nature of the adhesive interaction between the lymphocytes and the endothelial cells of the HEV.

It became possible to study this interaction in detail through the development of an in vitro adherence assay by Stamper and Woodruff (1976). When lymphocytes are overlaid on a cryostat section prepared from a lymphoid organ (e.g., lymph node), the cells attach highly selectively to exposed profiles of sectioned HEV. Extensive studies by Woodruff and colleagues (reviewed by Woodruff, Clarke and Chin 1987) and by Butcher, Weissman and colleagues (reviewed by Butcher 1986) have thoroughly validated this assay as a reliable and accurate measure of the in vivo interaction. Based largely on studies with this assay, it has been established that lymphocyte-HEV binding is both a highly regulated and highly specific cell-to-cell interaction. The important parameters (reviewed by Yednock and Rosen 1989) include the nature and state of the lymphocyte (i.e, class, subclass, maturational state, activation state, history of antigenic stimulation) and the anatomical site of the HEV. Various lymphocytes

and lymphomas demonstrate selective migration or "homing" into particular lymphoid organs. The selective homing is believed to be dictated to an important extent by the selective binding of the lymphocytes to HEV in different lymphoid organs. The adhesive receptors on lymphocytes involved in this interaction are termed homing receptors. The complementary adhesive sites of the endothelium are named HEV-ligands or vascular addressins (Streeter et al. 1988). Three distinct adhesive specificities are now known to exist involved in lymphocyte binding to HEV in peripheral lymph nodes, gut-associated lymphoid organs, and lung-associated lymphoid organs (reviewed in Yednock and Rosen 1989). LFA-1 on lymphocytes appears to serve as an accessory adhesion molecule, augmenting lymphocyte attachment to HEV without imparting organ selectivity (Hamann et al. 1988; Pals et al. 1988).

IDENTIFICATION OF gp90^{MEL} AS THE PERIPHERAL LYMPH NODE HOMING RECEPTOR IN MOUSE

The MEL-14 mAb blocks lymphocyte attachment to PN HEV but not to PP HEV in the in vitro adhesion assay (Gallatin, Weissman and Butcher 1983). The antibody immunoprecipitates a lymphocyte surface protein of ≈90 kDa, referred to as gp90^{MEL}. The occurrence of this epitope on various lymphocytes and lymphoma cell lines correlates, without exception to date, with the ability of the cells to bind to PN HEV. gp90^{MEL} is reported to be modified covalently with ubiquitin (Siegelman et al. 1986), but this claim has not as yet been rigorously confirmed. Strong additional evidence for a direct homing receptor function for gp90^{MEL} derives from the recent demonstration that the solubilized molecule can react with PN HEV and prevent lymphocyte attachment (Geoffroy and Rosen 1989). The molecule has no effect on the lymphocyte attachment activity of PP HEV, consistent with the expectation of organ-selectivity for a homing receptor. These experiments provide the first direct evidence that gp90^{MEL} mediates lymphocyte attachment to HEV by forming a direct bridge from the lymphocyte to the endothelium.

gp90^{MEL} FUNCTIONS AS A LECTIN

Our early experiments showed that the attachment of lymphocytes to PN HEV (in mouse, rat and human) is selectively inhibited by millimolar levels of mannose-6-phosphate (M6P) and its structural homologue fructose-1-phosphate (F1P) but not by comparable concentrations of other phosphorylated monosaccharides (Stoolman and Rosen 1983; Stoolman, Tenforde and Rosen 1984; Stoolman, Yednock and Rosen 1987). A M6P-rich polysaccharide called PPME blocks attachment at 10 nM levels. PPME-derivatized fluorescent spheres decorate lymphocytes in a M6P- and F1P-inhibitable manner (Yednock, Stoolman and Rosen 1987). Moreover, PPME-bead binding to lymphocytes shows an absolute requirement for extracellular calcium, just as does the attachment of lymphocytes to HEV. The quantitative dependency on calcium concentration is identical for both interactions. A relationship between the receptor detected by PPME-beads and gp90^{MEL} was initially shown by the finding that the MEL-14 mAb blocks bead decoration of lymphocytes, whereas a series of antibodies directed against other cell surface components do not (Yednock, Butcher, Stoolman and Rosen 1987). Furthermore selection of lymphoma cell variants for increased

binding to PPME-beads resulted in a highly correlated increase in the expression of the MEL-14 epitope on the cell surface.
Recently, molecular cloning of gp90^{MEL} (Lasky et al. 1989; Siegelman et al. 1989) has provided considerable additional support for a lectin-like function of this molecule. The cDNAs predict a transmembrane protein of 372 amino acids. The predicted extracellular domain of 295 amino acid contains 3 tandem protein motifs consisting of a calcium-type (C-type) lectin domain (117 residues) in the most membrane-distal location, an EGF-like motif (34 residues), and two identical "complementary-regulatory" motifs (62 amino acids each) . Complement-regulatory domains are found in a number of proteins that exhibit binding to complement factors C3b or C4b. The putative carbohydrate-recognition motif is highly homologous to domains found in an extensive series of calcium-dependent animal lectins. Formal proof that isolated gp90^{MEL} possesses lectin activity has been achieved with an ELISA assay that measures the interaction of PPME with purified gp90^{MEL} immobilized on a plastic surface (unpublished). As predicted, this interaction is inhibited by calcium chelation, by MEL-14 mAb and by M6P and F1P. Although the lectin domain is obviously integral to the function of this receptor, the EGF and complement-regulatory domains presumably also contribute to function. This contribution may simply be structural or it may involve recognition of cognate "ligands" on the endothelium or conceivably on the lymphocyte.

THE INVOLVEMENT OF CARBOHYDRATES ON HEV

The discovery that gp90^{MEL} is a lectin-like receptor has focused attention on the possibility that carbohydrate residues function as the actual recognition determinants of HEV-ligands. The periodate sensitivity of the lymphocyte attachment sites is consistent with such a role (Rosen et al. 1985). More compelling evidence comes from the demonstration that sialidases from 5 different sources selectively inactivate attachment sites on PN HEV without affecting those on PP HEV (Rosen et al. 1985). Inactivation of the PN HEV sites is observed upon exposure of the HEV to sialidase either in vitro or in vivo (Rosen et al. 1989). The importance of sialic acid is further indicated by the finding that Limax agglutinin, a sialic acid-specific lectin, functionally inactivates HEV sites when it is incubated with tissue sections prior to the in vitro adhesion assay (True and Rosen 1986). In contrast, a variety of lectins with other sugar-binding specificities are inactive. These results suggest a role for sialic acid either as a recognition determinant of the PN HEV ligand or as a modulator of ligand activity. The former possiblity is especially attractive in view of the multiple forms and linkages of sialic acid and thus its great potential as an informational molecule. A prominent candidate for the HEV-ligand is the molecule recognized by the MECA 79, a monoclonal antibody that reacts selectively with PN HEV and prevents lymphocyte attachment (Streeter et al. 1988). It remains to be determined whether this molecule or a physically associated structure bears a carbohydrate determinant (a unique sialyloligosaccharide?) that is recognized by gp90^{MEL}.

CLOSE RELATIVES OF gp90^{MEL}

An attractive possibility is that lectin-like receptors may underlie other homing specificities. This speculation gains impetus from the

recent finding that two other proteins, ELAM-1 (Bevilacqua et al. 1989) and GMP-140 (Johnston et al. 1989) are strikingly similar to $gp90^{MEL}$ in their molecular organization. Each consists of an amino terminal C-type lectin domain- highly homologous to the one in $gp90^{MEL}$ - an EGF-like domain, several tandem complement-regulatory domains, a putative transmembrane domain and a short COOH-tail projected to be within the cytosol. ELAM-1 is biosynthetically induced on cytokine-treated endothelial cells and mediates neutrophil attachment to the "activated" endothelium. GMP-140 is present in platelets and endothelial cells in storage granules from which it is rapidly elicited to the cell surface by thrombin stimulation. The regulation of GMP-140 expression as well as its structural homology to two known adhesion proteins suggests an adhesive function for this protein. By analogy to $gp90^{MEL}$, it is strongly suspected that the amino-terminal lectin domains of ELAM-1 and GMP-140 will be central to their functions. A provocative question is whether this emerging family of proteins can be expanded to include other homing receptors.

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I Structure and Function of Recognition and Effector Molecules

Complement

Introduction to the Symposium on Complement

M.P. Dierich

It is the intention of this symposium to present the C system and to review some of its actual questions and not primarily to present the speakers' own work. Along this line and due to the short space reserved for this introduction I will make reference primarily to reviews for further information of the reader and as a source of the original references.

Some times there has been a debate whether C is really a part of immunology in its strictest sense. It does not have antibody or T- or B-cell dependent specific immune reactions. But, it is in so many ways interlinked with these specific reactions that it is difficult to separate it. During the last years it has become obvious that aside from the lytic function complement is the source of a broad spectrum of biological activities (Ross 1986; Rother and Till 1988). It mediates adherence phenomena (Dierich 1988), triggers a lot of cell activities, among these - for this congress most relevant - the activation of B and T lymphocytes (Melchers et al. 1985; Melchers et al. 1986; Dierich et al. 1987). It even can distinguish between "self" and "foreign" with respect to different species (Atkinson and Farries 1987). Furthermore, the biological significance is proven by the fact that C defects in many ways result in diseases, such as multiple infections, chronic disease such as SLE, glomerulonephritis and others (Rother K. and Rother U. 1986; Rother and Till 1988). Also, if pathogens can overcome complement effects then they typically are more virulent. More and more publications document that many pathogens make use of mechanisms to circumvent complement effects (Joiner 1985; Dierich et al. 1989; Cooper and Nemerow 1989).

Let me start this session by introducing you to the activation pathways and its control mechanisms.

Originally complement became known as the factor completing the lytic action of antibodies (Recently, about 90 years later, perforine, a factor similar to C9, was shown to be essential for the lytic action of T-cells (Podack and Dennert 1983; Müller-Eberhard 1988)).
Thus it seemed that complement is a resting system, waiting to be activated by antibodies. In contrast to this, I like to stress that the complement system is never in a resting state, but permanently activated. The continuous activation originally observed by Peter Lachmann and termed "tick over" by him is brought about by H_2O and other nucleophiles (Müller-Eberhard 1988).
Under healthy conditions this continuous activation is controlled by a multifactorial control system operating in the fluid phase as well as on cell surfaces.

The key component of these processes is C3, the third component of the complement system (Lambris 1988).
C3 consists of an alpha- and beta-chain, linked by a disulphide bridge. In the alpha-chain C3 contains an internal thioester. H_2O, methylamine and other nucleophiles can brake this esterbond (Müller-Eberhard 1988).
As a consequence C3 gains the capacity to behave like C3b, i.e. it can interact with factor B (Müller-Eberhard 1988).

$$C3 + H_2O \longrightarrow C3(H_2O)$$

$$C3(H_2O) + B \longrightarrow C3(H_2O),B \xrightarrow[Mg^{++}]{D} \overline{C3(H_2O),Bb} \longrightarrow \overset{C3}{\underset{C3b}{\downarrow}}$$

$$C3b + B \longrightarrow C3b,B \xrightarrow[Mg^{++}]{D} \overline{C3b,Bb} \longrightarrow \overset{C3}{\underset{C3b}{\downarrow}}$$

Fig. 1. Initiation of the alternative pathway of complement activation by H_2O and its amplification

The interaction of $C3(H_2O)$ with factor B sets in motion the alternative pathway of the complement system. This action generates C3b, which interacts with B, generating finally $\overline{C3b,Bb}$, the alternative pathway convertase. This enzyme starts an amplification process, which would continue until all C3 is consumed. To avoid unlimited C3 consumption, a number of control proteins is active.

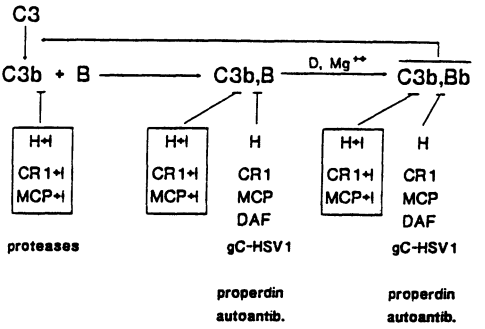

Fig. 2. Factors controlling the alternative pathway of complement activation

Factor H serves as a cofactor for factor I, which cleaves C3b and turns it into C3bi. Membrane bound CR1 and MCP, the membrane cofactor protein, serve similar purposes. Aside from this, H, CR1, MCP and DAF, the decay accelerating factor, are capable to disrupt the alternative pathway convertase by detaching factor B (Atkinson and Farries 1987; Müller-Eberhard 1988; Rother and Till 1988).
On some cells, such as certain tumor cells, C.W. Vogel demonstrated that proteases destruct the deposited C3b such that it loses its activation potential. Herpes simplex virus type 1 carries glyco-

protein C, which functions as a destabilizer of this convertase by binding to C3b (Dierich et al. 1989; Friedman et al. 1984).

On the other hand I should mention that properdin acts as a stabilizer under normal conditions (Rother and Till 1988); in certain diseases autoantibodies may stabilize this alternative pathway convertase, partly overruling the before mentioned control proteins (Rother and Till 1988).

Inactivation of C3b to C3bi is achieved by two cleavages between aminoacids 1281-1282 (Arg-Ser) and 1298-1299 (Arg-Ser) releasing C3f; one further cleavage at position 932-933 (Arg-Glu) frees C3c and leaves behind C3d,g (Lambris 1988).
C3d,g may be even further cleaved into C3g and C3d, the latter staying covalently attached to cell surfaces, a water molecule or others.
Complement-resistant bacteria or our own cells for instance, use these control mechanisms. A C3b that becomes covalently attached to a surface, quickly binds H and C3b inactivation by factor I follows. To be effective in this way on surfaces H must obviously also bind to surface elements.
On streptococci, as shown by Horstmann, the M protein may serve as the H binding element.
On other surfaces neuraminic acid or heparin sulfate act similarly (Kazatchkine et al. 1979).
But not only binding of H is helpful for the pathogens to control C activation, a fascinating spectrum of restriction mechanisms and further strategies to avoid complement effects are used by pathogens. M.M. Frank reports about these ways.
But there are bacteria, LPS and various proteins, which bind C3b and very inefficiently inactivate it. Thus, on their surfaces C3b escapes the control. They are called activators of the alternative pathway; many of them are T independent antigens (Bitter-Suermann et al. 1975). Either C3b is offered in such a configuration that H hardly can bind; maybe the H binding site in C3b is hidden. Or H does not bind well to such surfaces. Both mechanisms may be operating at the same time.
Also, the lipid composition of membranes may be critical. Many cells, once infected by different viruses, are activators (Cooper and Nemerow 1983); what they have in common is unclear.

If the availability of C3b is not effectively controlled, the activation process continues on to activation of C5, C6, C7, C8 and C9 to form the membrane attack complex, MAC (Müller-Eberhard 1988). But this terminal sequence is also very carefully controlled by a number of factors:

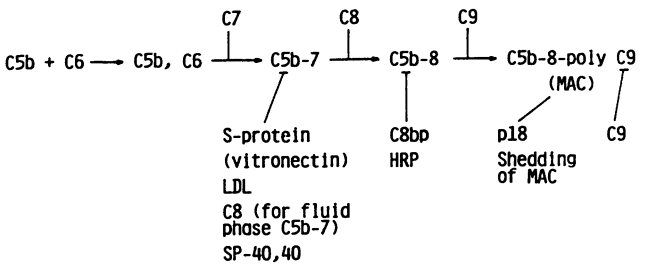

Fig. 3. Terminal sequence and its control

Among these are the human restriction factor, HRF (also termed C8-binding protein), the S-protein (vitronectin), and the proteins SP 40/40 and p18 (for further information see consecutive contributions, Müller-Eberhard 1988; Rother and Till 1988). You will learn later from Z. Fishelson about the impact of phosphorylation on inactivation of the terminal sequence.

Several of the factors before mentioned are effective in a homologous system, for instance on human cells against human serum, but do not act across species barriers. J. Atkinson will show the potency of complement to differentiate between "self" and "foreign" (Atkinson and Farries 1987 and following contribution).
T. Kinoshita discusses the special way in which some of these factors are anchored in the membrane (following contribution).

Parallel to the alternative pathway the classical pathway of complement activation (CPCA) exists, the biochemistry of which is known for quite some time (Müller-Eberhard 1988).
Typically, C1 binds via C1q to Fc portions of antibodies, becomes activated and generates the active fragments of C4 and C2, leading to the formation of the classical pathway C3 convertase, C4b,2a (Loos 1982). The enzymatic activity, residing in C2a, may effect cleavage of C3b and activation of the terminal sequence.
Activation of complement by immune complexes is critical for the size of these immune complexes. Complement deposition renders immune complexes smaller and more soluble (Nydegger and Kazatchkine 1986). Cells get lysed. But, small amounts of C5b-9 may induce cells to secrete e.g. prostaglandins (Hänsch et al. 1984).
Also on the CPCA several control proteins act inhibitory.

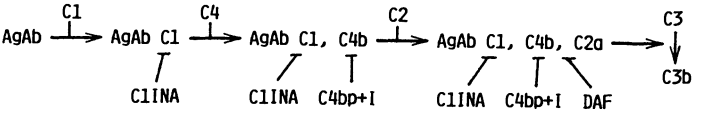

Fig. 4. Classical pathway of complement activation and its control

The classical pathway is sometimes used without antibody. As one example I like to mention the activation by retroviruses.
Bartholomew, Esser and Müller-Eberhard demonstrated binding of C1q and C1s and consecutive activation of C4 and C2 by the envelope protein of Moloney Mouse Leukemia Virus (Bartholomew et al. 1978).
We were able to show that also the AIDS-virus triggers complement activation and consumes complement (Soelder et al. 1989b).
We could specify that the envelope protein, p160, is responsible for this effect and that this effect is mediated via the classical pathway (Soelder et al. 1989b).

If complement activation is not controlled fast enough a significant amount of biologically active fragments may be generated.
These may cause smooth muscle contraction, chemotaxis, secretion of histamin, effects on B lymphocytes and others.
These fragments are subject of D. Bitter-Suermann's contribution.

As a consequence of complement activation also material opsonized and thus modified by complement arises. In very general terms this may be material X forming X-C1q, X-C4b, X-C3b, X-iC3b, X-C3d,

X-C3b.H, X-C5b-9.
This is very relevant because binding structures or even proper receptors exist for such complement-modified material (Cooper et al. 1988; Kazatchkine and Dierich 1988; Dierich 1988). In several of the consecutive contributions these binding structures and receptors will be delt with.
I like to give one example, which is possibly very important in the AIDS-infection.
Human retroviruses - although activating complement - are not lysed by human serum (Soelder et al. 1989b). On the contrary they become coated with C3, bind to phagocytes.
Soelder and Reisinger in my lab could show that for small amounts of HIV this is an effective mechanism to enter and to infect macrophages and other complement receptor positive cells (Soelder et al. 1989a).

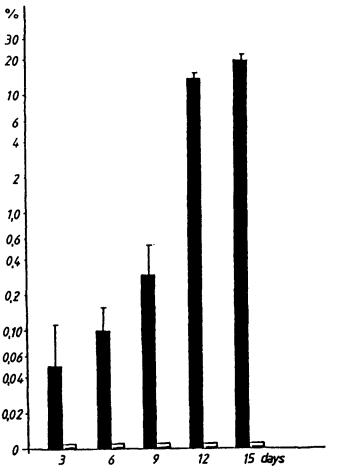

Fig. 5. Complement-dependent infection of macrophages (U937) by HIV-1. ■ represents infection rates (in %) induced by complement-treated HIV-1; □ = infection rates by HIV-1, treated with buffer

This complement-dependent entry into cells may be an important pathway also for other pathogens, such as listeria, legionella, chlamydia, rickettsia and other intracellularly growing pathogens.

Another aspect of complement is - aside from the activation of whole sequences - the use of individual components or even peptides thereof for certain biological effects.
Four years ago we showed that polymerized human C3d acts as a growth promoting factor by stimulating the S-phase during mouse B-lymphocyte proliferation (Melchers et al. 1985; Melchers et al. 1986; Dierich et al. 1987).
Today W. Lernhardt will describe that lymphoid cells may produce their own C3 or C3 peptides, possibly as autocrine growth factors. The ensuing signal transduction from the engaged CR2 to the nucleus is reported by R. Frade.

Another example that awaits further clarification is our earlier observation that factor H can trigger B-cells (Hammann et al. 1981). Clq may yet be an additional stimulator for various cells.

I anticipate that in the coming years complement, a system which is basically clarified as far as the activation sequences are concerned, will remain a highly exciting field.
It participates in the preparation of the immune response and certainly also in its effector phases.
Analysis of complement genes and their regulation by hormones, interleukins, interferons and others draws a lot of interest (Colten 1986).
Disease-related problems of complement are every 3 years subject of a continuously growing international meeting. We have learnt a lot from C defects. C4 may serve as a prominent example: an association between certain allotypes of C4 and slow virus and autoimmune disease has been described (Rother K. and Rother U. 1986).

Another argument, which renders complement so exciting, is the fact that complement components and complement receptors are in many instances composed of certain domains which are found in molecules on first glance very unrelated to complement components (Reid and Day 1989). D. Campbell will report that various complement components are composed just like mosaics of different motives, suggesting interesting interrelationships between the complement system, the clotting system, intercellular adhesion molecules and possibly others.

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The Regulators of Complement Activation (RCA) Gene Cluster

D. Hourcade and J.P. Atkinson

INTRODUCTION

The complement system is a potent recognition and effector pathway that plays a major role in the immune response. It mediates the destruction and disposal of immune complexes and foreign substances through interactions between complement proteolytic fragments deposited on targets and complement receptors on cell surfaces. To faciliate this process, and to prevent damage to self-tissue, a remarkable family of control proteins (the regulators of complement activation or RCA proteins) has evolved (see recent reviews of Hourcade et al., 1989; Campbell et al., 1988 and Reid and Day, 1989). Each member interacts with a derivative of C4 and/or C3, shares a repeating polypeptide motif, and is encoded at a single genetic region, the RCA cluster.

BIOCHEMICAL INTERACTIONS OF THE RCA PROTEINS WITH C3b/C4b AND THE C3 CONVERTASES

There are two major complement pathways, the classical pathway and the alternative pathway. The classical pathway is activated by immune complexes and results in the covalent attachment of C4b and C3b to foreign surfaces. The alternative pathway is activated by microbes, resulting in attachment of C3b. C4b and C3b serve as the foci for the classical and alternative pathway convertases, respectively, which in turn generate more C3b. Since the attachment of C4b and C3b is relatively non-specific, and can direct damage to normal tissue as well as pathological material, two distinct biochemical mechanisms, decay accelerating activity and factor I-dependent cofactor activity, have evolved to discriminate self from non-self (Farries and Atkinson, 1987) by deactivating C3b and C4b and/or their convertases (Fig. 1).

At least six proteins mediate complement activation through interactions with C3b and/or C4b (Table 1). Membrane cofactor protein (MCP) and decay accelerating factor (DAF) are widely distributed membrane proteins available to down regulate C3b and C4b on cell surfaces. Factor H (H) and C4 binding protein (C4bp) inhibit fluid phase activation and assist in control on tissues. C3b/C4b receptor or complement receptor 1 (CR1) on leukocytes binds C3b/C4b-bearing immune complexes and on erythrocytes mediates transport of complexes to the liver and spleen for clearance, and complement C3b and C3d receptor or complement receptor 2 (CR2) binds C3d-bearing immune complexes and thereby activates B cells.

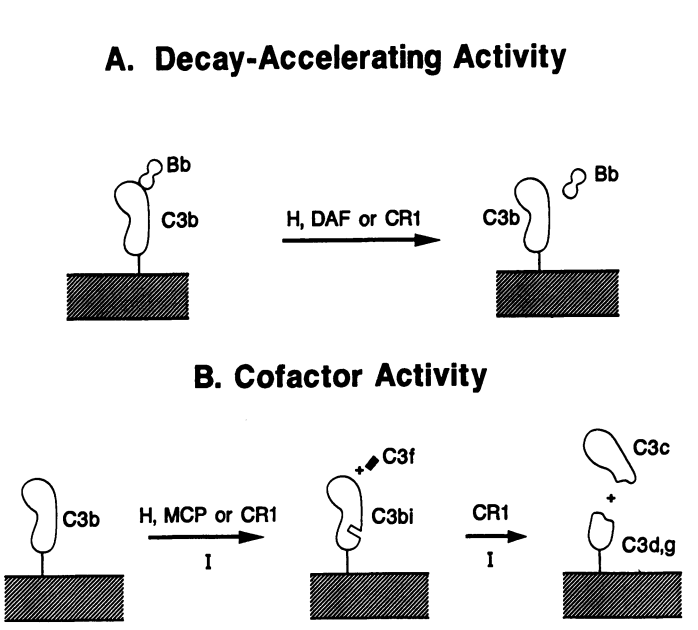

Fig. 1. Biochemical interactions of the RCA proteins with C3b. Figure indicates membrane-bound C3b, but similar reactions occur with fluid phase C3b. Parallel reactions occur with C4b and its binding to C2. In that case, C4b interacts with C4bp in the same way that C3b interacts with H (above). Binding of H, DAF, MCP or CR1 to C3b is not specifically shown

Table 1. RCA PROTEINS: BIOCHEMICAL ACTIVITIES

	Primary Ligand(s)	Receptor Activity	Decay Acceleration	Cofactor Activity
H	C3b	-	+	+
C4bp	C4b	-	+	+
CR1	C3b, C4b	+	+	+
CR2	C3dg	+	-	?
DAF	C3b, C4b	-	+	-
MCP	C3b, C4b	-	-	+

DISCOVERY OF THE REGULATORS OF COMPLEMENT ACTIVATION (RCA) GENE CLUSTER

It was not at all clear, when the RCA proteins were first characterized, how closely related they actually were. Their M_rs were very different, some were plasma proteins and others membrane proteins, and although binding specificities overlapped to a degree, a primary specificity was apparent for each (Table 2). The first strong indication of their common evolutionary origins was shown in a collaboration between our group in St. Louis and that of Santiago Rodriguez de Cordoba and Pablo Rubinstein in New York. We had discovered an unusual structural polymorphism by SDS-PAGE of the C3b/C4b receptor of CR1 and they had defined polymorphisms of C4bp and factor H by isoelectric focusing. Classical genetic methods established that the structure of genes for CR1 and C4bp were linked (de Cordoba et al., 1984) and then that CR1 and C4bp in turn were

Table 2. RCA PROTEINS: STRUCTURAL FEATURES AND DISTRIBUTION

	$M_r \times 10^3$		
	non- reduced	reduced	Distribution
H	160	170	Plasma; Synthesized in Liver, Fibroblasts, and U937 cells
C4bp	590	70	Plasma; Synthesized in Liver
CR1[a]	190	220	Membranes of Erythrocytes, Monocytes, Granulocytes, some T cells, B cells (pre-B, mature and plasma), Follicular-Dendritic cells, Glomerular Podocytes
CR2	130	140	Membranes of B cells (mature), Follicular-Dendritic cells, Epithelial cells (stage specific), T cell lines (absent from peripheral T cells), platelets (?)
DAF	70	75	Membranes of Erythrocytes, T cells, B cells, Monocytes, Granulocytes, Platelets, Endothelial cells, Epithelial cells, Fibroblasts,
MCP	58 63	63 68	Membranes of T cells, B cells, Monocytes, Granulocytes, Platelets, Endothelial cells, Epithelial cells, Fibroblasts,

a) Most common phenotypic variant.

linked to factor H (de Cordoba et al., 1985). These observations prompted our group to propose in a review (Holers et al., 1985) that CR1, C4bp and factor H were members of a multigene family of receptor and regulatory glycoproteins that were derived from a single ancestral C3b-binding protein. In this review the functional similarities among these proteins were highlighted and we predicted, based largely on their C3b and C4b binding properties, that DAF and MCP would also be members of this family.

THE SHORT CONSENSUS REPEATS (SCRs) OR COMPLEMENT CONTROL PROTEIN REPEATS (CCPs) AND OTHER STRUCTURAL FEATURES OF THE RCA PROTEINS

Within a year of our review, the derived primary structures of several of RCA proteins were reported and the striking finding was a common structural motif (Fig. 2). The major building block of the

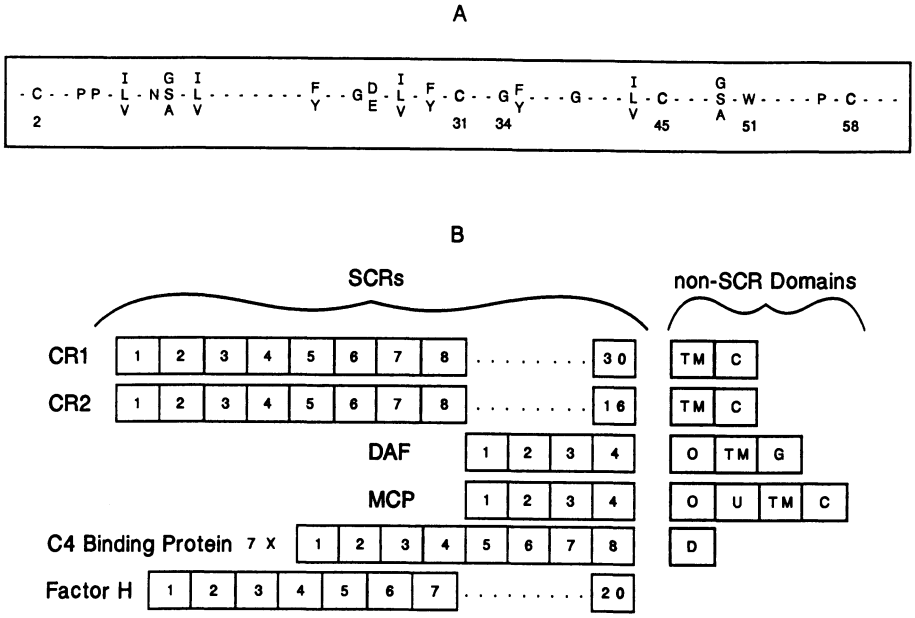

Fig. 2. A. Complement control protein repeat (Short consensus repeat). Those amino acids shown are conserved in at least 44% of 102 CCPs analyzed (Perkins et al., 1988). The cysteines at positions 2, 31, 45 and 58 and the tryptophan at position 51 are found in 95%-100% of the CCPs analyzed. Positively or negatively charged residues (not shown) occur in more than 40% of the CCPs at positions 9, 12, 26, 28, 30, 32, 59, 60. B. Organization of the RCA proteins into CCP-containing and non-CCP-containing regions. (NH_2-terminal ends at the left) TM = transmembrane domain. C = cytoplasmic domain. O = O-linked glycosylation domain. G = glycolipid anchor. U = domain with unknown functional significance. D = disulphide bridge-containing domain

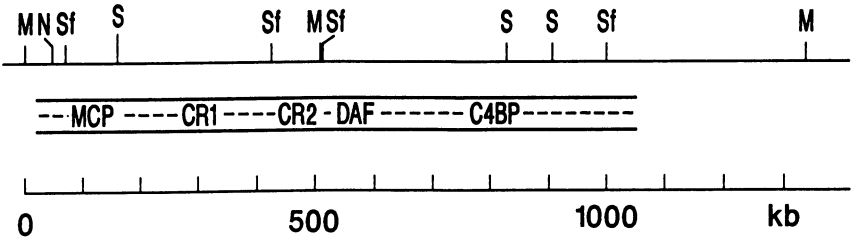

Fig. 3. Physical map of RCA cluster. Data from Rey-Campos, et al., 1988; Carroll et al., 1988; Bora et al., 1989

RCA proteins, termed a short consensus repeat (SCR) or, more recently, a complement control protein repeat (CCP), is an approximately 60 amino acid long, cysteine rich (four per repeat) element. It is found in all six proteins, beginning at the amino-terminus (Reid et al., 1986) in a single array of four to 30 tandem copies. Based on studies of β-2 glycoprotein 1 (Lozier et al., 1984) and C4bp (Janatova et al., 1989), cysteine one is linked to cysteine three and cysteine two to cysteine four, giving a multiple loop structure. Each CCP is expected to have a similar overall secondary structure, given the highly conserved primary structure.

A model has recently been proposed in which the RCA proteins are visualized as semi-rigid, elongated rods and each CCP is a globular bead that is composed predominantly of anti-parallel beta sheet structures (Perkins et al., 1988). The available evidence suggests that the active sites of the molecules are often at the amino-terminal ends (Dahlback et al., 1983; Dahlback and Muller-Eberhard, 1984; Alsenz et al., 1985; Klickstein et al., 1988; Krych et al., 1989).

Other features of these proteins which must also contribute to their activities include different anchoring systems (CR1, CR2 and MCP are type I membrane glycoproteins, while DAF is anchored by a glycolipid), differential glycosylation (all possess N-linked sugars but DAF and MCP also are rich in O-linked sugars), cell and tissue specific expression and alternative RNA processing.

ORGANIZATION AND EVOLUTION OF THE RCA GENES

Recently, *in situ* hybridization studies have revealed that CR1, factor H and C4bp as well as CR2, MCP and DAF, are all encoded at the q32 band of human chromosome 1 (the RCA cluster) (Weis et al., 1987; Lublin et al., 1987, 1988). In addition, pulsed-field gel electrophoresis has shown that 5 of these genes are located within an 800 kb region (Fig. 3; Rey-Campos et al., 1988; Carroll et al., 1988; Bora et al., 1989).

In general, each CCP is encoded by a separate exon (Kristensen et al., 1986) but split (two equal sized exons encoding for a single repeat) and fused exons (exon encoding for two repeats) have been described, (see Hourcade et al., 1989).

It is clear that DNA duplication has played a major role in the evolution of the RCA cluster. Intragenic duplications have been responsible for the construction of long CCP arrays, most likely from exons that each encoded a single CCP. Some of the steps of this process are indicated by the homologous internal repetitions in CR1 (Klickstein et al., 1987, 1988; Hourcade et al., 1988; Wong et al., 1989) and CR2 (Weis et al., 1988; Moore et al., 1987; Fujisaku et al., 1989). In those cases intragenic duplications of seven SCR's and four SCR's respectively, have resulted in much longer receptors. In addition, gene duplication has been responsible for the generation of new RCA genes. This is most clearly seen in the homologies between CR1 and CR2 (See Weis et al., 1988) and, in a more recent case, CR1 and the CR1-like partial replica (Wong et al., 1989; Hourcade et al., 1988).

Perhaps not surprisingly, examples of viruses encoding structural and functional units with similarities to the CCPs have been noted in vaccinia (Kotwol and Moss, 1988); and in herpes (McNearney et al., 1987). Presumably, the resulting proteins provide a virulence factor for infection by down-regulating the complement system. The vaccinia sequence is highly homologous to the amino-terminal end of C4bp and, thus, may have evolved through the transposition of the C4bp gene to the vaccinia genome.

CONCLUSIONS AND FUTURE DIRECTIONS

The complement cascade is controlled at the critical C3b and C4b steps by a family of at least six regulatory genes. Recent molecular analysis has shown that all six genes are located at a single genomic region, the RCA cluster. In addition, the protein encoded by these genes are composed chiefly of a tandemly repeated polypeptide domain. Future work will elucidate the fine structure of the RCA cluster as well as the structure and function of the repeated polypeptide domains. This information will be critical in our understanding of the function of complement system, and the evolution of its regulatory genes.

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Host Cell Protection from Complement by Glycosyl-phosphatidylinositol-Anchored Complement Inhibitors and Their Deficiencies in Paroxysmal Nocturnal Hemoglobinuria

T. Kinoshita

INTRODUCTION

Complement plays an essential role in eliminating microorganisms and immune-complexes from tissues and blood. Its three major functions are targeting microorganisms to cells bearing complement receptors, recruiting phagocytic cells to the area where complement activation is taking place and destruction of target membranes. Since these effector functions mediated by C3 and C5 convertases and membrane attack complexes (MAC) are very effective, activation of complement should be focused on target surfaces. However, an inherent characteristics of complement is that its active fragments or their complexes, such as C4b, C3b and C5b-7, can bind to host cell surfaces and lead to host cell damage. This potentially harmful characteristics is prevented by widely distributed membrane-bound complement inhibitors that protect host cells from the action of autologous complement by inhibiting C3 and C5 convertases and MAC.

Recently at least three types of complement inhibitors have been found to have a common membrane anchoring mechanism, glycosyl-phosphatidylinositol-anchor (GPI-anchor). The importance of the GPI-anchored complement inhibitors in host cell protection is highlighted by a hemolytic disorder, paroxysmal nocturnal hemoglobinurea (PNH). Blood cells from patients with PNH are deficient in these inhibitors and this is causally related to increased susceptibility of their blood cells to complement. This chapter deals with the GPI-anchored complement inhibitors and their deficiencies in PNH.

COMPLEMENT INHIBITORS RESPONSIBLE FOR HOST CELL PROTECTION

Decay-accelerating factor (DAF), an inhibitor of C3 and C5 convertases

DAF is a 65,000 Da membrane protein, termed CD55. It has been shown to be expressed on a wide variety of cells in contact with complement; that is, erythrocytes, platelets, monocytes, granulocytes, T and B lymphocytes (Nicholson-Weller et al., 1982, 1985b; Kinoshita et al., 1985), natural killer cells (Ueda et al., 1989), umbilical vein endothelial cells (Asch et al., 1986), various epithelial cells (Medof et al., 1987c; Quigg et al., 1989) and blood cell progenitors, such as CFU-Mix, CFU-GM, BFU-E and CFU-E (Moore et

al., 1985; Kanamaru et al., 1988).

DAF accelerates decay dissociation of preformed C3 convertases of both the classical (C4b2a) and alternative (C3bBb) pathway and also prevents assembly of these enzymes (Nicholson-Weller et al., 1982; Medof et al., 1984; Fujita et al., 1987). DAF also inhibits C5 convertases (Medof et al., 1984). Its mechanism of inhibition is not fully understood, but one idea is that it interacts with C4b or C3b, and thereby either induces dissociation of prebound C2a or Bb, or prevents stable binding of C2 or B, or of nascent C2a or Bb. This idea is supported by reports that complexes of DAF and C4b or C3b are found on the erythrocyte surface after treatment with a cross-linking reagent (Kinoshita et al., 1986) and that DAF contains four units of short consensus repeats (SCR) or complement control protein repeats (CCP), which is a common character of most proteins with ability to bind C3, C4 or their fragments (Caras et al., 1987; Medof et al., 1987b; Reid and Day, 1989).

DAF functions only on cell surfaces on which C3 convertase is being formed; i. e., it does not inhibit C3 convertase assembly on proper targets of complement (Medof et al., 1984). Therefore, DAF appears to function only for host cell protection.

65,000 Da-inhibitors of MAC

Hänsch and colleagues purified an erythrocyte membrane protein of 65,000 Da and called it C8-binding protein or C8bp (Schönermark et al., 1986). This protein has binding affinity for C8 and its alpha-gamma subunit and inhibits MAC formation (Schönermark et al., 1986, 1988). The primary structure of C8bp is not known, but anti-C8bp reacts with C9 and the C8 alpha-chain, suggesting that C8bp shares a similar structure with its ligand C8 and C9 (Schönermark et al., 1988).

Müller-Eberhard and colleagues identified and purified an erythrocyte membrane protein, homologous restriction factor (HRF), of 65,000 Da (Zalman et al., 1986). HRF has binding affinity for C8 and C9 and inhibits MAC formation (Zalman et al., 1986; Müller-Eberhard et al., 1986). Its inhibitory activity is restricted to human C8 and C9, and hence its name. The primary structure of HRF is not known, but it reacts with anti-C8 antibodies and anti-C9 antibodies (Zalman et al., 1986; Müller-Eberhard, 1988). The relationship between HRF and C8bp has not been established directly, but the above findings suggest that the two proteins are identical.

C8bp is present on platelets, monocytes, granulocytes and lymphocytes as well as erythrocytes (Blaas et al., 1988). HRF has been found on erythrocytes, granulocytes, stimulated T lymphocytes and a small proportion of natural killer cells (Zalman et al., 1986; Martin et al., 1988).

18,000 to 20,000 Da-inhibitors of MAC

Three groups have independently found another type of MAC inhibitor. Tomita and colleagues purified an erythrocyte membrane protein of 18,000 Da that inhibits MAC formation. Originally they called this P-18 (Sugita et al., 1988), but recently they renamed it the membrane attack complex inhibitory factor (MACIF) (Sugita et al.,

1989). In western blotting experiments polyclonal antibodies to MACIF identified a single protein of 18,000 Da in a detergent extract of erythrocyte membranes, indicating that MACIF is not a fragment of C8bp or HRF.

Okada and colleagues established a mouse monoclonal antibody, 1F5, that binds to human erythrocytes and neuraminidase-treated erythrocytes and induces hemolysis of the latter in human serum containing Mg-EGTA (Okada et al., 1989a,b). 1F5 antibody recognizes a 20,000 Da-protein named 1F5 antigen (1F5Ag). Purified 1F5Ag inhibits reactive lysis of guinea pig erythrocytes when human C8 and C9, but not rabbit C8 and C9, are used, indicating its species restriction, like that of HRF. Therefore, they renamed the protein HRF20 (homologous restriction factor of 20,000 Da) (Okada et al., 1989b). Amino acid sequence analyses of purified MACIF and HRF20 demonstrated the identity of the two (Okada N et al., 1989a; Sugita et al., 1989; Okada H et al., in press).

Parker and colleagues purified an erythrocyte protein of 18,000 Da that inhibits reactive lysis, and they named this protein membrane inhibitor of reactive lysis (MIRL) (Holguin et al., 1989). The primary structure of MIRL is not known, but its other structural and functional properties strongly suggest its identity with MACIF and 1F5Ag.

The NH_2-terminal amino acid sequences of MACIF and 1F5Ag indicate that these proteins are both identical with a protein recognized by a monoclonal antibody MEM-43 (Stefanova et al., 1989), which was recently termed CD59 (Shaw, 1989).

MACIF/HRF20/MIRL is expressed on all blood cells (Okada et al., 1989a,b; Stefanova et al., 1989). Therefore, most blood cells bear both the 65,000 Da-MAC-inhibitor and the 18,000-20,000 Da-MAC-inhibitor. The reason for this redundancy is not known.

DEFICIENCIES OF HOST-CELL-PROTECTING COMPLEMENT INHIBITORS IN PNH

PNH is an acquired hematopoietic stem cell disorder characterized by intravascular hemolysis and hemoglobinuria. Somatic mutation in a clone or clones of hematopoietic stem cells is thought to give rise to the abnormal blood cells. The abnormal red cells are sensitive to the action of complement, because they are deficient in activity to destabilize C3 convertases and so take much C3b when activation takes place on their surfaces, and in most cases they are highly sensitive to reactive lysis, which involves C5 through C9 (Rosse and Parker, 1985). Platelets and neutrophils that have no activity to destabilize C3 convertases formed on their surface have also been found.

Various proportions from a few % to nearly 100 % of the erythrocytes in most patients with PNH are very sensitive, so called type III PNH cells. After being sensitized with antibodies, type III cells are lysed with 1/20 to 1/30 as much serum as that required to lyse the same number of sensitized normal erythrocytes (Rosse and Parker, 1985). Consistent with previous reports that type III cells are deficient in activities to destabilize C3 convertases assembled on their surface and to prevent reactive lysis (Rosse and Parker,

1985), DAF, HRF/C8bp and MACIF/HRF20/MIRL are undetectable on type III cells (Pangburn et al., 1983; Nicholson-Weller et al., 1983; Zalman et al., 1987; Hänsch et al., 1987; Holguin et al., 1989; Okada et al., 1989a; Sugita and Tomita, personal communication).

Moderately sensitive cells are present in a few patients. These cells, so called type II PNH cells, are lysed with 2- to 3-fold less serum than that necessary for lysis of normal cells. Studies have shown that the surface of type II cells has no activity to destabilize C3 convertases, whereas the activity to prevent reactive lysis seems normal (Rosse and Parker, 1985). Consistent with this, DAF is undetectable or present at only very low level in type II cells (Nicholson-Weller et al., 1983; Kinoshita et al., 1985; Medof et al., 1987a). The expression of HRF/C8bp has not been studied, but recently the level of MACIF/HRF20/MIRL was also found to be significantly reduced in these cells (Holguin et al., submitted for publication). This apparent discrepancy between the results of functional and immunochemical studies is explained by the observation that the level of MACIF/HRF20/MIRL on normal erythrocytes is four to five times that necessary to protect the cells completely from reactive lysis (Holguin et al., submitted for publication).

The cells of patients with apparently normal sensitivity to complement are called type I PNH cells. Quantitative analysis of DAF and MACIF/HRF20/MIRL demonstrated that some type I cells are not really normal, but have significantly reduced levels of DAF (Medof et al., 1987a; Kinoshita et al., 1987; Ninomiya et al., 1988) and MACIF/HRF20/MIRL (Holguin et al., submitted for publication).

A unique common feature of purified DAF, C8bp/HRF and MACIF/HRF20/MIRL is that they all bind spontaneously to the erythrocyte membrane resulting in reconstitution of type II and III cells. Type II cells reconstituted with DAF to approximately 20 % of the normal level showed nearly normal sensitivity to lysis by whole complement, indicating that deficiency of DAF is causally related to the increased sensitivity of type II cells, which have sufficient activity to prevent reactive lysis. Reconstitution of type III cells with DAF restored the activity to inhibit C3 convertases, but had little effect on sensitivity to lysis (Medof et al., 1987a). Reconstitution of type III cells with C8bp/HRF rendered them resistant to reactive lysis (Hänsch et al., 1987; Zalman et al., 1987). There is no report on whether sensitivity to lysis by whole complement is decreased. Reconstitution with MACIF/HRF20/MIRL rendered type III cells resistant to reactive lysis (Holguin et al., 1989) and also significantly decreased sensitivity to lysis by whole complement (Holguin et al., submitted for publication). These results indicate that deficiencies of MAC inhibitors are causally related to the increased sensitivity of type III cells to reactive lysis and that MAC inhibitors are more important than DAF in maintaining resistance to lysis by whole complement. Results also show that the absence of DAF affects lysis sensitivity when the level of MAC inhibitors is greatly reduced, but not when it is normal. This is consistent with reports that individuals with hereditary DAF-deficiency, who probably have normal level of MAC inhibitors, do not have a hemolytic disease (Spring et al., 1987; Telen et al., 1988; Merry et al., 1988).

GPI-ANCHOR, A COMMON MEMBRANE ANCHORING MECHANISM OF DAF, C8BP/HRF AND MACIF/HRF20/MIRL.

There is increasing evidence that many proteins are anchored to membranes by a GPI structure rather than a hydrophobic peptide (Ferguson and Williams, 1988; Low, 1989). The polypeptide portion of this group of membrane proteins is not inserted into the lipid bilayer, but is anchored to the membrane by covalent attachment of a GPI structure to a COOH-terminus. Interestingly, DAF (Davitz et al., 1986; Medof et al., 1986), C8bp (Hänsch et al., 1988) and MACIF/HRF20/MIRL (Okada et al., 1989a,b; Stefanova et al., 1989; Sugita and Tomita, personal communication; Holguin et al., submitted for publication) all belong to this group of proteins.

The functions of the GPI-anchor are not well understood, but one function of this type of membrane anchor that might be related to complement inhibitors is that it gives proteins high lateral mobility (Ferguson and Williams, 1988). This could facilitate the functional efficiencies of these inhibitors by increasing the frequency of their association with ligands on the cell surface. Indeed, the mobility of DAF on HeLa cells, measured by analysis of fluorescence recovery after photobleaching, is nearly as high as those of lipids, and ten times higher than those of peptide-anchored membrane proteins (Thomas et al., 1987).

Biosynthesis of GPI-anchored proteins is now being studied intensively. The polypeptide portions of the proteins are first synthesized with hydrophobic COOH-terminal signal peptides of 20 to 30 residues that are cleaved by a putative membrane-bound enzyme or enzyme complex. The newly formed COOH-terminal residue is then ligated to the preassembled GPI structure, perhaps by the same enzyme or enzyme complex. Assembly of the GPI structure must include multiple enzymes that are still to be characterized (Ferguson and Williams, 1988; Low, 1989).

NATURE OF PNH DEFECTS

In PNH, deficiencies of erythrocyte acetylcholinesterase (Auditore et al., 1960; Kunstling and Rosse, 1969) and neutrophil alkaline phosphatase (Beck and Valentine, 1951; Lewis and Dacie, 1965) have long been known. The demonstrations that these proteins and DAF are GPI-anchored proteins suggested that PNH defects must reside in a common structure, the GPI-anchor (Davitz et al., 1986; Medof et al., 1986). Consistent with this idea, several other membrane proteins with a GPI-anchor were found to be deficient, namely lymphocyte function-associated antigen 3 (LFA-3) on erythrocytes and leukocytes (Selvaraj et al., 1987), type III Fcgamma receptor (CD16) on neutrophils (Selvaraj et al., 1988; Huizinga et al., 1988) and CD14 on monocytes (Haziot et al., 1988; Simmons et al., 1989). As described above, C8bp and MACIF/HRF20/MIRL have also been shown to be GPI-anchored. Conceivably the biosynthetic pathway of the GPI structure or a mechanism that transfers the GPI structure to peptides is deficient in blood cells of patients with PNH.

Deficiency of DAF has been found in platelets, granulocytes and monocytes from all patients tested (Nicholson-Weller et al., 1985a; Kinoshita et al.,1985). The lymphocytes of most patients are also DAF-deficient (Kinoshita et al., 1985). Detailed analysis showed that fractions of the T and B lymphocytes and natural killer cells of these patients were DAF-deficient (Ueda et al., 1989; Ueda, Kinoshita, Inoue and Kitani, unpublished observation). Therefore, hematopoietic cells of all lineages show deficiency of GPI-anchored proteins. These findings, together with a report that complement sensitive erythrocytes are of clonal origin (Oni et al., 1970), suggest that the abnormalities of this acquired disease arise from a clone or clones of totipotent hematopoietic stem cells in most patients and of pluripotent stem cells in a few patients. The nature and cause of the somatic mutation is not known.

Phenotypic expression of the somatic mutation is heterogeneous in severity; that is, deficiencies of GPI-anchored proteins may be either complete or partial. As described above, all three inhibitors are missing in type III cells but in type II cells a subnormal level of MACIF/HRF20/MIRL is expressed and there is little or no DAF. Even in type III cells of some patients, subnormal levels of acetylcholinesterase and/or LFA-3 are expressed (Ueda et al., submitted for publication; Selvaraj et al., 1988). Low levels of DAF have been found in monocytes and granulocytes of some patients (Kinoshita et al., 1985). Therefore, the severity of deficiency varies in different patients, in different cells in a single patient and even in different GPI-anchored proteins in a single cell.

CONCLUSIONS

Active fragments and complexes of complement have the inherent characteristics of binding to host cell surfaces physiologically or accidentally, and this could lead to host cell damage. However, host cells, particularly those in contact with plasma, have cell surface inhibitors of complement that inhibit C3 and C5 convertases and MAC, thereby protecting the cells from complement. DAF (CD55) destabilizes C3 and C5 convertases on the cell surface and C8bp/HRF and MACIF/HRF20/MIRL (CD59) inhibit MAC formation. Three inhibitors are members of a group of proteins that are attached to the membrane through a phosphatidyl-inositol containing glycolipid (GPI). GPI-anchored proteins have high lateral mobility, which may be important for complement inhibitors for their efficient survey of cell surface.

The importance of GPI-anchored complement inhibitors in host cell protection is clearly demonstrated in PNH. In patients with PNH, red cells are deficient in GPI-anchored complement inhibitors and are lysed intravascularly, often resulting in hemoglobinuria. The restoration of normal resistance to complement of type III erythrocytes, which do not have any of these three inhibitors, by MACIF/HRF20/MIRL but not by DAF indicates the relative importance of MAC inhibitors in protection. The presence of DAF is essential to maintain resistance when the levels of MAC inhibitors are much below normal, as in type II erythrocytes. The nature of the somatic mutation in a clone or clones of multipotential hematopoietic stem cells that gives rise to deficiency of GPI-anchored proteins in fractions of blood cells is not known.

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Inflammation-Promoting Complement Fragments

D. Bitter-Suermann and J. Köhl

INTRODUCTION

The bulk of inflammatory potential of the complement system is characterized by the following items:

1. Along the reaction sequence of the complement system split products (activation peptides) of well characterized complement proteins are generated and released which by themselves don't influence the further activation cascade. But, as soluble factors they represent one of the three major effector mechanisms mediated by complement, i.e. inflammation.
2. The other mechanisms are adherence, antigen- and IC-processing mediated via complement receptors for the covalently fixed C3b, iC3b and C3d, and cytolysis mediated by the membrane inserted terminal complex C5b-9 (membrane attack complex). Adherence to and membrane perturbation by C3b fragments and C5b-9 respectively contribute to pathologic sequelae exerted by actively attracted and passively affected cells in the inflammatory situation.
3. The inflammatory complement peptides strictly speaking are:
C2b-derived peptide, enhancing vascular permeability and smooth muscle contraction, possibly associated with the pathogenesis of hereditary angioneurotic edema.
C3e, a leukocytosis inducing or leukocyte mobilizing fragment generated from the α-chain of C3b by further enzymatic degradation.

C4a and C3a on the one side and C5a on the other side, the three anaphylatoxic peptides (AT-peptides) cleaved from the α-chain of C4, C3 and C5 by proteolysis at nearly identical sites. Homologous in structure and some functions to a large extent but quite different with regard to receptors and some other biological effects, e.g. chemotaxis C5a desarg is the only AT-peptide with a residual activity despite loss of the common carboxyterminal arginin, cleaved by the serum carboxypeptidase N.
4. All these peptides, especially the AT-peptides are well characterized with regard to their primary structure. Some of the respective genes are cloned and recombinant or synthetic peptides are available. A few of the receptors are identified and considerable efforts are made to further analyse their structure and genes. Ligand-receptor-interactions and signal transduction pathways are in the centre of interest. A lot of in vitro data on the biological effects have been accumulated but in vivo data are rare. There is a boom of highly sensitive and specific detection systems on the basis of monoclonal antibodies.

5. The continued interest in these peptides does exist irrespective of the fact that they are not the only or even central ingredients of the inflammatory menu. Instead they really appear and act at the very beginning of a localized or systemic inflammatory reaction, have a true initiating or trigger-function. Therefore great efforts are made to analyze the ligand-receptor interactions in order to modulate this early event by probing the pharmacological principle of agonistic and antagonistic effects at the receptor side for therapeutic purposes. In the following a state of the art summary for the above mentioned peptides is given.

C2-Derived Peptide

The interest in C2-derived peptides (C2 kinin) stems from the analysis of the pathogenetic principles in hereditary angioneurotic edema. The decreased synthesis of C1 inhibitor or its dysfunctional variant both result in an unregulated activity of the C1s enzyme with secondarily uncontrolled cleavage of C2 and C4. Previous work by Donaldson et al. (1983) and Strang et al. (1988) has suggested that C2b, the smaller activation peptide of C2, is further degraded by plasmin resulting in peptides which increase vascular permeability, produce an acute non-inflammatory, non-painful edema of mucosa and skin and as a further bioassay induce contractile responses of smooth muscle (estrous rat uterus or guinea pig lung parenchymal strips). Recently these studies were substantiated (Cholin et al. 1989) by use of short synthetic peptides (17-residue peptides) delineated from the carboxy-terminal region of human C2b (released from C2b by plasmin cleavage as a 38-residue peptide) with the following sequence:

M L G A T N P T Q K T K E S L G R .

Although the C-terminal amino acids L G R are identical to the human C5a sequence the C2 peptide differs with regard to functional activities and nature of the respective receptors.
This C2 peptide (C2 207-223) at a relatively high concentration for contractile responses ($5x10^{-4}$ M) is active only with an intact C-terminal arginine, is neither tachyphylactic to itself nor to C3a and C5a (preincubation with C2 peptide does not desensitize to itself nor to C5a or C3a and vice versa), is not blocked by anti-histamines (pyrilamine maleate) in skin vasopermeability and contractile assays and therefore possibly not acting on mast cells, and is finally not blocked by cyclooxygenase inhibitors (indomethacin). Specific target cells are not identified yet. In so far the C2 derived peptides with quite dissimilar pharmacological characteristics compared to the AT-peptides represent a new and relevant pathogenetic principle.

C3e

A leukocyte mobilizing factor (LMF) derived from C3 was first described by Rother (1972) and further characterized by Ghebrehiwet and Müller-Eberhard (1979) as a leukocytosis inducing acidic 10-12 kd fragment from the α-chain of C3b and clearly distinct from C3a. This fragment is physiologically generated in serum after activation of C3 and by further proteolytic processing of C3b. Cleavage of iC3b with kallikrein results in the fragment C3d-K, slightly different from the C3dg after cleavage by Faktor I.
As bioassay the perfusion of the isolated rat or guinea pig femur with C3e results in mobilization of leukocytes from the bone marrow. Infusion of C3e into experimental animals causes a massive and rapid leukocytosis without preceeding leukopenia as with the AT-peptides.

Intradermal injection evokes increased vascular permeability (for rev. see Rother and Buhl 1987).
From the aminoterminal sequence of C3d-K Hoeprich et al. (1985) delineated a synthetic nonapeptide with the same biological acitivities as the above mentioned fragments. The sequence:

T L D P E R L G R

is postulated to represent the active centre of all C3-derived leukocytosis factors. The three carboxyterminal residues again are identical with that of the C5a peptide. But, at a final concentration of $4x10^{-6}$ M both the nonapeptide and its desarg octapeptide were active to induce leukocytosis in rabbits and to increase skin vasopermeability. Therefore these peptides are distinct from C5a. It is surprising that since 1985 in this research area no further data with regard to characterization of receptor bearing target cells and ligand receptor interactions were published.

Anaphylatoxic Peptides (C4a, C3a, C5a, C5a desarg)

With regard to the long known and well characterized AT-peptides emphasis is put on recent developments only. For comprehensive reviews on AT-peptides see Hugli (1984 and 1986) and Bitter-Suermann (1987). In this context we will focus

- on efforts to approach the active centre of these peptides by methods like peptide-synthesis and recombinant gene technology combined with site directed mutagenesis and
- on the actual and available information concerning the different AT-receptors on neutrophils, eosinophils, macrophages, mast cells, U937 cells and guinea pig platelets

The in vivo plasma concentration AT-peptide levels at maximal (theoretical) activation are as follows:

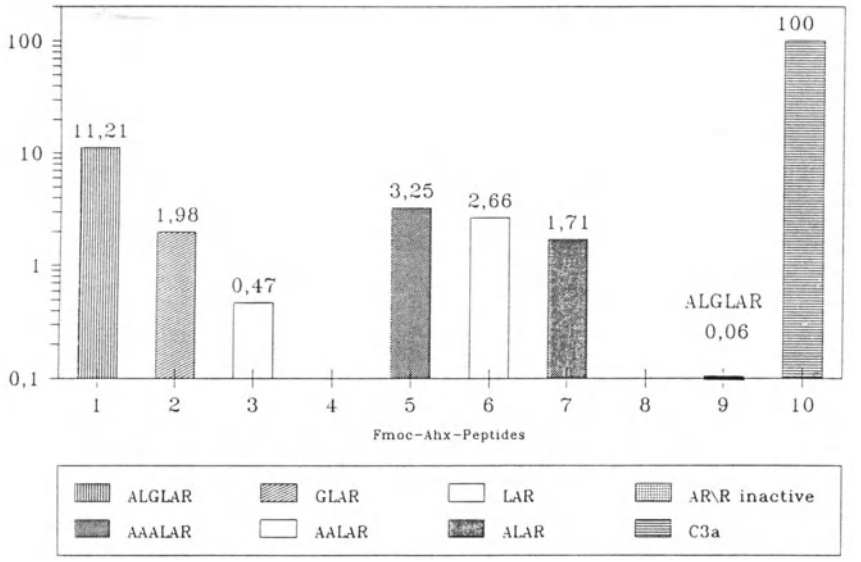
Table 1. In vivo concentrations of human anaphylatoxinogens and AT peptides

	mol. wt.	Approximate plasma concentrations
C4	206 000	500 µg/ml
C3	180 000	1400 µg/ml
C5	180 000	80 µg/ml
		at maximal activation
C4a	9 000	22 µg/ml = 2.4×10^{-6} M
C3a	9 000	70 µg/ml = 7.7×10^{-6} M
C5a	11 000	4.9 µg/ml = 4.4×10^{-7} M

The concentration of C3a and C5a in plasma of normal individuals is about 10 ng and 1 ng respectively. Having in mind that the range of significant functional in vivo and in vitro activity for C3a is 10^{-9}-10^{-10} M, for C5a 10^{-10}-10^{-13} M and that in clinical situations such as extracorporal circulation C3a levels of 10 µg/ml (about 10^{-6} M) and C5a levels of 100 ng/ml (about 10^{-8} M) are often found, this equals to 0,1-10% of the maximally possible yield (see Table 1).

Synthetic peptides and mutant recombinant AT-peptides: When the primary sequence of C3a, C4a and C5a between 1975 and 1980 was analyzed by TE Hugli and coworkers (for rev. see Hugli 1984 and 1986) and the crystal structure of C3a was elucidated (Huber et al. 1980) efforts were made to characterize the active centre of all AT-peptides. Starting with the work of Caporale et al. (1980) and continued by Unson et al. (1984) synthetic C4a and C3a analogues were designed. The C-terminal pentapeptide with the sequence LGLAR was shown as the minimal structure with a C3a specific biological activity and both leucines and arginine were postulated to be essential. A 21 residue C-terminal peptide of the natural sequence had 100% of the C3a activity (Hugli 1986). The introduction of helix promoting residues like aminobutyric acid further increased activity (twice that of C3a).

Our own efforts (Gerardy-Schahn et al. 1988) culminated in a 13 residue peptide with sixfold the C3a activity. This was possible by introduction of nonpeptidic elements with a strong hydrophobic domain like the photolabel Nap or Fmoc linked with a spacer of aminohexanoic acid to an optimized C-terminal 13 residue peptide. In addition we have recently shown that the minimal physiological structure of C3a is the C-terminal tripeptide LAR only when linked to the above mentioned nonpeptidic elements (Fig. 1).



In this figure peptide activities are given relative to native C3a. It can be seen that the Fmoc-Ahx-peptides in lanes 1 to 4 starting with a hexapeptide have decreasing biological activities. The tripeptide LAR is the last active one whereas the AR- and R-derivatives are inactive. Filling the amino acids at positions 72-74 with alanine (lanes 5-7) again increase the activity. The unsubstituted hexapeptide without Fmoc-Ahx (lane 9) has only marginal activity (peptides were synthesized by Dr. M. Casaretto, DWI, Aachen). In lane 10 native C3a is set as 100%.

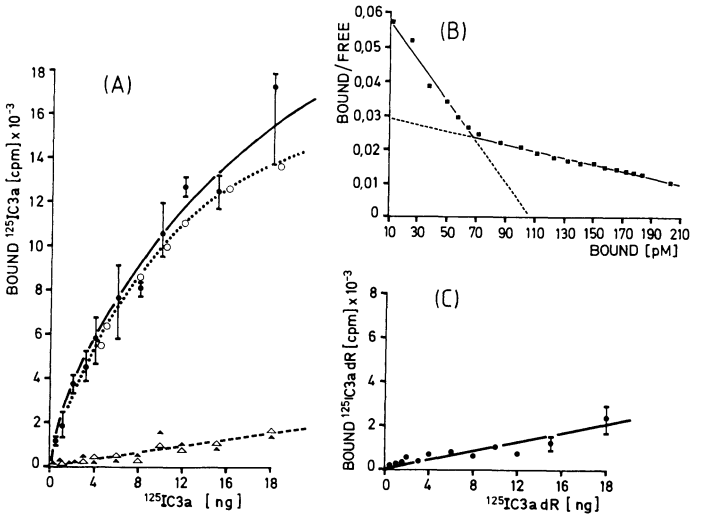
Table 2 summarizes the data and conclusions that can be drawn from synthetic peptide studies of C3a (from Hugli, 1986, Gerardy-Schahn, 1988, filled up with unpublished results).

Table 2

Taken from Hugli (1986),	from Gerardy-Schahn (1988)
1 Residues that are essential for C3a activity (i.e. receptor binding) are contained in a C-terminal linear sequence including LGLAR	LAR is the minimal active structure
2 The C-terminal arginyl residue can be substituted by no other amino acid	=
3 Substitution of residues N-terminal to the LGLAR region in C3a has little influence on activity	Cationic residues at positions 64-69 increase activity
4 Fragments longer than the pentapeptide LGLAR exhibit progressively greater activity suggesting that conformation may be important	A hydrophobic membrane anchor and a spacer of either peptidic or non-peptidic residues with sufficient length and flexibility (without need for an ordered α-helical conformation) to manoeuvre the active centre LAR into the C3a receptor pocket optimizes functional activity.
5 Peptides of sufficient length to assume a metastable helical conformation exhibit considerably higher levels of activity than shorter analogues	
6 Introduction of helix-promoting residues resulted in enhanced activity of synthetic C3a analogues	
7 Folding of the peptide backbone adjacent to the C-terminal pentapeptide LGLAR into a helical conformation appears to optimize functional expression	

With regard to C5a short synthetic C-terminal peptides equivalent to C3a proved to be inactive with the exception of a 21-residue peptide (Erickson et al. 1986) with a residual C5a activity below 0,1%. Therefore the construction of a C5a gene by synthesis of short oligonucleotides (32 residues) and ligation enabled the expression in E.coli and purification of recombinant human C5a with full C5a activity (Mandecki et al. 1985; Franke et al. 1988). With this C5a gene and by site-directed mutagenesis Mollison et al. (1989) produced and purified rec-hu-C5a and its mutants and tested the peptides by chemokinesis and competition for specific C5a binding with purified membranes of human neutrophils. By this approach evidence is given by these authors that C5a achieves high affinity receptor binding through interaction of its carboxy-terminus (Lys-68, Leu-72, Arg-74) in concert with widely spaced residues in the disulfide linked core (Arg-40) and possibly a turn region connecting the core to the amino-terminal helix (His-15).

AT-receptors: Because C4a and C3a have the same functional activities, compete for the same receptor binding site and show cross tachyphylaxis (in contrast to C5a) the C3a receptor is the reference for both. Until recently all attempts to characterize C3a receptors were unsuccessful (Huey et al. 1986). By use of the guinea pig platelet system (for rev. Bitter-Suermann 1987) two groups (Fukuoka and Hugli, 1988; Gerardy-Schahn et al. 1989) contributed to the first characterization. From our data (see Fig. 2) highly specific and saturable high affinity C3a receptors on guinea pig platelets with about 200 binding sites/cell (K_d $1,7 \times 10^{-9}$ M) and low affinity receptors (500 sites/cell, K_d 3×10^{-8} M) were characterized. In addition a homogenous receptor class on human PMNL with 40.000 sites/cell and a K_d of 3×10^{-8} M were found.



Legend to Fig. 2: Binding of ^{125}I-gp C3a and ^{125}I-gp C3a-des-Arg to gp platelets. (A) (closed circles) total binding to gp platelets; (open triangles) nonspecific binding to gp-platelets, (open circles) specific binding to gp platelets; (closed triangles) total binding to gp C3a-receptor deficient platelets. (B) Scatchard plot of the specific binding curve of ^{125}I-gp C3a to gp platelets shown in (A). The Scatchard plot indicates two receptor classes, a high-affinity class with K_d = 1.7 x 10^{-9} M and 160 copies/cell, and a relatively low affinity class with K_d = 1 x 10^{-8} M and 500 copies/cells. (C) Binding behaviour of ^{125}I-gp C3a-des-Arg to gp R^+ platelets.
Taken from Gerardy-Schahn et al. (1989)

Molecular characterization of the C3a receptor on guinea pig C3a receptor positive platelets (in contrast to platelets from a recently described guinea pig C3a receptor deficient strain, Bitter-Suermann and Burger, 1986) was achieved by cross-linking experiments with photoaffinity labelled C3a or synthetic C3a analogues, and with chemical cross-linkers like DSS. Three distinct bands with molecular masses of about 86, 99 and 114 kDa (ligands subtracted) reproducibly could be shown and have to await further characterization.

Molecular characterization of the C5a receptor, of its kinetics and saturability started in 1985 when three independent groups by use of cross-linking experiments identified a C5a receptor on human PMNL with a molecular weight of about 42 kDa (after subtraction of the ligand). For rev. see Huey et al. (1986). With more refined tools and techniques the C5a receptor was further characterized and extracted in active form from membranes of human PMNL by Rollins et al. (1988). The receptor seems to exist in two forms of 150 kDa (probably an oligomeric form) when C5a is bound and in addition a smaller form of 40 kDa in the non-ligated state which is thought to be the binding subunit of the receptor. This subunit is complexed after binding to C5a with the second molecule, possibly the α-subunit of a GTP-binding protein. The number of binding sites/cell is on average $8x10^4$-$1x10^5$ with a K_d of $8x10^{-11}$ M - $5x10^{-10}$ M (Rollins et al. 1988; Gerard et al., 1989).

Until now the discussion on C5a receptors leaved open whether two types of C5a receptors, one for C5a and one for C5a desarg or whether a common receptor type with different affinities for both ligands do exist. All experiments performed to characterize and isolate the C5a receptor(s) were done with serum-derived C5a, always containing to a variable degree C5a desarg. In addition neutrophils or macrophages were used, both interacting with C5a and C5a desarg. The data by Rollins et al. (1988) might alternatively argue for two receptors instead of subunits of the same receptor or a linkage with G-proteins. The fact that mast cells and guinea pig platelets in contrast to phagocytes exclusively interact with C5a but not with C5a desarg is another hint for two different C5a receptors.

CONCLUSION

The above mentioned inflammatory peptides, especially the AT-peptides, dependent on both pathways of complement activation, represent an essential part of the biological capacity of the complement system. The spectrum of important activities (beneficial as well as pathological) is far from being resolved. However, when considering physicochemical and genetic properties, these peptides belong to the most advanced fields in complement research regarding primary, secondary and tertiary structural elements, structure function relationships, synthetic peptides, ligand receptor interactions, and genetic engineering.

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Evasion Strategies of Microorganisms

M.M. Frank

There are three principal mechanisms by which complement controls invasion by microorganisms(Frank 1988). Binding of complement to the surface of an organism leads to the sequential activation of components C5 to C9 with formation of a complex of these proteins (the membrane attack complex). The complex has a hydrophobic surface and inserts into cell membrane lipid of the microorganism. This cylinder like structure has a hydrophilic central axis and destabilizes the osmotic regulation of the organism causing cell death. In the case of gram negative organisms, insertion of the C5b-9 complex appears to occur principally in the outer membrane. The precise mechanisms for dissolution of the inner or cytoplasmic membrane is unknown and the ultimate cause of death of these organisms is unknown(Taylor 1988). Enveloped viruses may have typical complement lesions formed in their membranes upon attack leading to loss of viral integrity(Cooper 1986). Parasites such as, promastigotes of Leishmania and Schistosoma mansoni cercaria undergo direct membrane attack and dissolution(Joiner 1988).

A second mechanism of complement mediated host defense is via opsonization, coating the organism with complement peptides recognized by specific receptors on phagocytic cells(Frank 1988; Fries 1987). Interaction of the coated organism with the phagocyte leads to binding to the phagocyte surface, followed by phagocytosis and death. In many cases death of the ingested pathogen is due to products of the oxidative burst released into the phagolysosome, but there are additional microbicidal mechanisms operative.

A third mechanism by which complement activation aids host defense relates to the generation of complement peptides with phlogistic activity. Phlogistic peptides increase blood flow to an area of infection, induce adherence of phagocytes to endothelial cells in vessels near inflammatory focus, and cause the directed in migration of neutrophils and monocytes into the area of infection. This in turn promotes microbial ingestion.

It appears that the alternative complement pathway is the most primitive pathway of complement mediated, microbial attack. This pathway is operative in the absence of antibody, although high concentrations of complement proteins are required and the activation process is a slow one(Frank 1988, Fries 1987). Alternative pathway mediated destruction of an organism is not thought to involve direct activation of the pathway by the organism; for this reason the attack is not well focused. Slow physiologic hydrolysis of circulating C3 leads to generation of hydrolyzed C3, which binds factor B and futher activates the pathway. This nonspecific activation of the alternative pathway deposits small amounts of C3b on the organism surface as a random event. This in turn is followed by alternative pathway amplification, leading to opsonization and microbial lysis.

Organisms have developed a complex set of mechanisms for dealing with complement attack. For example, the outer membrane of gram positive organisms often activates complement leading to complement component binding and opsonization. The evolution of the bacterial capsule covering the outer membrane represents the formation of a barrier preventing complement peptides on the bacterial outer membrane from interacting with complement and immunoglobulin receptors on phagocytes, thereby inhibiting phagocytosis(Brown 1988; Frank 1988). Gram positive organisms have evolved a thick peptidoglycan layer that provides structureal rigidity but also prevents insertion of the C5b-9 complex into the bacterial outer membrane and lysis.

In evolutionary development, the primate host has evolved the ability to form bactericidal antibody, which provides a focus for alternative pathway activation, increasing the efficiency of this process. The presence of antibody allows alternative pathway activation to induce lysis of some gram negative organisms like Salmonella and Hemophilus influenzae, and parasites including, Leishmania promastigotes. Evolution of classical complement pathway proteins provides further protection to the host. Under most circumstances the classical pathway is activated by IgG and IgM, C1 binding antibodies. Unlike the alternative pathway the classical pathway is operative when complement component concentrations are low. Moreover, the specific recognition steps provided by antibody and C1 allows for rapid and focused anti microbial attack. The recognition proteins of the classical pathway appear to have evolved by sequential mutation of the genes that provide alternative pathway activation.

Antibody serves many functions in this microbicidal system. It may allow a poor activating surface to be coated with complement. For example, the capsule of Escherichia coli K1 is rich in sialic acid and is a poor complement activator(Vermuelen 1988) (discussed further below). Similarly the capsules of group B streptococcus, Neisseria meningitides group B and Hemophilus influenzae type b and many types of pneumococci are poor complement activators(Jarvis 1987; Frank 1988). In the presence of antibody, complement activation may be focused at the surface of the capsule and complement binding may occur. Antibody augments both the rate and extent of alternative pathway activation and activates the classical pathway(Fries 1987). As mentioned, it recruits the early components of the classical pathway to cause more efficient and more rapid complement activation. In addition, antibody provides a site for complement component binding. In studies of type 7 pneumococci, as much as 30% of bound C3 associated with the capsule was not bound directly to the capsule but to anti-capsular antibody(Joiner 1984; Brown 1988). Bound to IgG, C3 is a far more efficient opsonin and lysin(Malbran 1987). The formation of a heterodimer composed of one molecule of C3b bound to the Fd fragment of one molecule of IgG acts as a more efficient opsonin because it engages two receptors simultaneously. It is interesting to note that, unlike C3 bound to red cells, C3 on some microorganism surfaces such as on cryptococci and some group B streptococci mediates ingestion in the absence of detectable IgG antibody(Baker 1982; Bobak 1988). Presumably this represents engagement of complement receptors by organism bound C3 and engagement of another set of receptors, perhaps lectin receptors by the organism surface. Because activated C3 binds efficiently to IgG, we have questioned whether one mechanism of action of intravenous immunoglobulin used in high dose to treat autoimmune diseases may be to divert complement attack. In our studies immunoglobulin appears to act as an acceptor for activated complement components, preventing their attachment to the target. Finally, antibody directs complement attack on targets. In a series of studies with Salmonella, E. coli, Neisseria gonorrhoeae and Borrelia Burgdorferi it has been shown that conditions may be established such that equivalent numbers of late acting complement component molecules bind in the presence of or absence of

bactericidal antibody. In the presence of antibody the complement may cause organism lysis but not in its absence(Joiner 1984; Frank 1988; Kochi 1988). The most reasonable explanation for this effect is that antibody directs the placement of the earlier components, allowing the later components to bind to the microorganism surface in the correct location or orientation to successfully cause death.

Just as some organisms activate the alternative pathway in the absence of antibody leading to lysis, a number of organisms activate the classical pathway by the direct binding of C1(Loos 1987). In general the organisms that bind C1 directly grow with rough morphology and are relatively weak pathogens. Some experiments suggest that the C1 is bound to the organism surface by multiple sites of contact. Interestingly, it has been shown that C1 inhibitor interacts with and inhibits this C1 bound directly to the microorganism surface poorly and thereby allows continued classical pathway activation. C1 inhibitor fails to regulate C1 on rough forms of E. coli (Tenner 1984) and Salmonella (Ryan 1989) as well as on type 1A group B streptococci(Levy 1986).

Similar C1 binding and activation may occur with smooth forms of the organism however the C1 may not be bound via multiple sites of contact. C1 inhibitor efficiently inactivates this C1. The remaining components of the classical pathway are not activated and lysis does not ensue. Thus, these more virulent smooth forms of microorganisms such as E. coli and Salmonella initiate activation of the classical pathway in the absence of antibody but no destruction occurs. One function of antibody in this setting is to provide a site for C1 activation where it is not easily inhibited by the C1 inhibitor. If C1 remains active, C4 and later components can cause lysis. We have shown that antibody partially protects activated C1 from activity of the C1 inhibitor(Tenner 1986). Interestingly the further binding of C4 protects that C1 bound to antibody even more efficiently from inactivation by C1 inhibitor.

Studies of parainfluenza virus show similar direct C1 binding(Vasantha 1988). The organism activates the classical pathway directly leading to complement binding to the virion but the organism is not damaged. The presence of antibody augments classical pathway activation and killing via antibody directed complement attack. Many viruses including retroviruses, Sindbis virus, New Castle virus and parainfluenza virus activate the classical pathway directly(Cooper 1986). The alternative pathway may be activated by Epstein Barr virus as well as Sindbis virus, RS virus and Herpes viruses. The consequences are virus specific. In some cases such as in feline leukemia virus, activation is followed by viral death. In other cases, such as the parainfluenza example given, viricidal antibody is required for killing. The situation with virally infected cells appears to be similar. It has been shown that measles virus infected culture cells interact with components of the alternative pathway with efficient component binding to the virally infected cell membrane. The cell is not damaged in the absence of antibody and antibody is required to successfully direct complement attack(Perrin 1976).

For the alternative pathway to be effective, C3b, the form of C3 active in the C5 convertase, must remain stable on the microorganism surface. During complement activation C3b binds to the surface of host cells as well. To avoid host cell destruction the C3b is rapidly cleaved by serum factors H and I to form iC3b, a protein that is incapable of continuing complement cascade activation. The factors that determine whether C3b on a host cell will be degraded by factors H and I are not completely known. It is known that on sheep erythrocytes sialic acid plays a major role in augmenting factor H binding(Fries 1987). This in turn facilitates factor I mediated cleavage of the host cell bound C3b

to prevent late component recruitment. A number of organisms have sialic acid rich surface components either on the capsule, outer membrane or cell wall. As in the host cell, these sialic acid rich surface molecules facilitate the binding of factor H and I and the destruction of microorganism bound C3b. Organisms such as Neisseria meningitides group B, E. coli with K1 capsule, Group B Streptococci type 3 and Treponema pallidum (Fitzgerald 1987) have all have surface structures rich in sialic acid and rapidly cleave surface bound C3b. Often removal of the sialic acid will lead to more successful alternative pathway attack. Thus, the microorganism has developed mechanisms for facilitating surface C3b destruction that appear to mimic physiologic control mechanisms. In the case of pneumococci, the consequences of C3b binding to the capsule has been examined in terms not only of ability to bind factor H to promote C3b cleavage but in terms of ability to bind factor B, an essential component for alternative pathway activation(Joiner 1984; Brown 1988). It has been shown that unlike C3b bound to the usual cellular membrane, such C3b is a poor acceptor for B. The biochemical reasons for this are unknown, but such C3b does not promote alternative pathway activation and C5-9 binding.

C3 is central to both the alternative classical pathway and is an important opsonic molecule. A wide variety of mechanisms have been developed to avoid C3 attack. These mechanisms include failure to activate C3. As mentioned, capsules in general are poor C3 activators (examples listed above). Modification or substitution of the sugars in the O antigenic side chains of lipopolysaccharide (LPS) has been shown to have a marked affect on C3 activation and binding by Salmonella spp.(Jimenez-Lucho 1987; Makela 1988). Minor changes in the O antigen side chain sugars of otherwise isogeneic strains of Salmonella lead to marked differences in C3 activation and binding; the more pathogenic organisms activate complement less efficiently and bind C3 less well. In the case of Pseudomonas aeruginosa pathogenic organisms activate complement as well as less pathogenic organisms, however, the capsules of the more virulent organisms tend to bind C3 poorly(Schiller 1989). A similar situation is present with Campylobacter fetus but here surface proteins appear responsible for the poor C3 binding(Blaser 1988).

If C3 is bound it may be degraded. In some Leishmania degradation appears to occur particularly rapidly. The increased binding of factor H by sialic acid rich capsules has been mentioned. Once C3 is bound it may be shed. Schistosoma mansoni cercaria activate complement and bind C3 but are able to eliminate the C3 by shedding the glycocalyx which acts as an acceptor for C3. Failure of amplification of the alternative pathway by pneumococcal capsules has been mentioned.

A fascinating example of bacterial evolution of complement defense mechanisms is M protein of streptococci. Recent studies suggest that M protein acts as an acceptor of factor H(Horstmann 1988). Bound factor H located near C3 bound on the organism surface rapidly induces its cleavage preventing further complement activation. M protein also binds fibrinogen and its fragments(Whitnack 1988). The binding of host proteins appears to cover sites that may act as complement activators or acceptors. Thus, M protein can assist in evasion of opsonization by coating the microorganism with protein that are not seen as antigenic and cleaves complement components that penetrate this defense. Schistosoma mansoni have been reported to bind host proteins including proteins of the major histocompatibility locus, thus in theory covering antigens which may induce complement attack(Joiner 1988).

Perhaps the most carefully studied system is the LPS on gram negative organisms. In general gram negative organisms with long chain LPS resist serum lysis and those that grow with rough morphology and that

have truncated LPS are serum sensitive. One of the functions of the LPS is to prevent complement attack at susceptible sites on the organism surface. The role of variation in the O antigenic side chains of the LPS has been mentioned. A further function of the LPS is to provide a harmless site for C3b binding. Activated C3 tends to bind to the end units of the longest LPS side chains, presumably far from the bacterial outer membrane(Joiner 1986). Here such bound C3b activates late components that bind far from sites where they can cause lysis.

It has been shown that smooth gram negative organisms with long LPS side chains including E. coli, Salmonella spp., Pseudomonas, induce complement activation but resist complement attack. The late acting components bind to the surface of the organism but are easily eluted by buffers with high ionic strength. On these organisms bound complement proteins are easily attacked by proteolytic enzymes. Interestingly, serum sensitive organisms often activate and bind fewer complement molecules. However, when late components bind to the surface of serum sensitive organisms they tend to bind in a stable fashion (Joiner 1984), are not susceptible to proteolytic digestion, and are not eluted by high salt containing buffers. With serum sensitive organisms, the late components are inserted into the lipid of the bacterial outer membrane where they can assemble an effective membrane attack complex. Here they are not susceptible to proteolytic enzymes or elution by high ionic strength buffers. With serum resistant organisms the C5b-9 complex is shed once it is completed. The organism can accept and shed additional complement components without undergoing damage. In this way serum sensitive organisms lysed by complement may utilize fewer complement molecules than serum resistant organisms and the resistant organism can deplete virtually all of the complement activity at a site of infection.

Destruction of complement proteins in the fluid phase at inflammatory sites may also be induced by bacterial and parasitic proteases. Sarratia marcescens is reported to release such a protease(Molla 1989). Some bacteria such as the heavily encapsulated, relatively avirulent pseudomonas that are associated with chronic bronchial inflammation in patients with cystic fibrosis, are protected at inflammatory sites by destruction of inflammatory cell complement receptors. In these patients the bronchi are filled with neutrophils. Neutrophil proteases such as elastase and perhaps also bacterial proteases cleave receptors for complement proteins from the surface of invading neutrophils thereby crippling the cell's defensive machinery. A specific streptococcal C5a protease located in the bacterial genome close to the gene coding for M protein has been identified. This protein can rapidly cleave any generated C5a destroying its chemotactic activity (Chen 1989).

Some microorganisms have developed fascinating mechanisms for molecular mimicry. They mimic complement and control proteins and thereby protect themselves from complement attack. In some cases the ability of the microorganism to produce such proteins mimics not only function but structure. Thus, Epstein Barr virus codes for a 350 kDa surface glycoprotein that shares sequence homology with C3dg(Nemerow 1987). This protein allows the virus to bind to cellular CR2, thereby providing a site for its insertion into the B cell to continue its life cycle. The protein acting as a cofactor may control other aspects of activation(Mold 1988). Vaccinia virus has been found to code for a C4 binding protein like molecule that is secreted by virally infected cells(Kotwal 1988). This C4 binding protein has marked sequence homology with true C4 binding protein and functions like that molecule in partially regulating complement attack. Perhaps this represents a mammalian protein that the virus has incorporated into its genome. Candida albicans has on its surface a protein which binds iC3b and that is recognized by some monoclonal antibodies to CR3;(Heidenreich 1985;

Edwards 1986) thus it structurally and functionally resembles CR3. This protein in general is only present on pathogenic Candida species. Although not formally proven, it is believed to act to prevent complement attack on the infectious candida organisms. Histoplasma capsulatum binds and enters macrophages via CR3(Bullock 1987).

Herpes Simplex type I codes for a C3 binding molecule that augments alternative pathway convertase decay(Fries 1986). This protein has no co-factor activity for factor I, but clearly decreases the effectiveness of C3 in promoting complement attack. In the case of Herpes simplex virus type II it has been shown that the protein partially protects against viral neutralization(McNearney 1987). Trypanosoma cruzi codes for a developmentally specific decay accelerating factor like molecule that causes decay of the alternative pathway convertase, thereby protecting the organism from attack(Joiner 1988).

Neisseria gonorrhoea has proteins that specifically bind complement proteins and prevent their insertion into the outer membrane(Joiner 1984; Frank 1988). In the case of this organism it is important to note that blocking antibody, which blocks successful complement mediated lytic activity, actually increases complement binding to the organism surface. Once again antibody functions to direct complement attack, here to areas on the membrane where little damage occurs.

Some organisms do not attempt to neutralize or divert complement proteins. Rather they use complement proteins to enter cells via attachment to complement receptors. Leishmania donovani and Leishmania major both enter cells after activation of complement and coating of the organism with C3(Joiner 1988). In the case of Leishmania donovani one means of uptake is via CR3. Following activation of serum complement C3b is rapidly cleaved to iC3b. The iC3b coated parasite binds to the phagocyte and enters via the CR3 receptor. In the case of Leishmania major C3b is a prominent form of deposited C3 on the organism and the organism appears to enter cell via CR1. Mycobactium tuberculous, Mycobacterium leprae (Horwitz 1988) and Babesia (Ward 1981) appear to use complement proteins to enter cells. Legionella pneumophila also coats itself with complement and uses both CR1 and CR3 for cell entry(Horowitz 1988). In this case CR3 appears to be the predominant receptor for entry. As mentioned EBV uses CR2 to enter cells and Flavivirus is reported to use complement (iC3b) coating and CR3 for cell entry(Porterfield 1986). It has been suggested that HIV coated with antibody and complement is more virulent than unopsonized HIV(Robinson 1988). Intracellular parasitism is an important part of the life cycle of this organism and replication within phagocytes may be facilitated by complement coating. The fact that interaction with complement receptors itself is a poor activator of the oxidative burst may aid the pathogen in avoiding intracellular destruction. Complement proteins and associated factors may aid organisms not only in binding to phagocytes but in binding to other cells as well although this has not been explored in detail. Streptococci are reported to adhere to endothelial cell monolayers via binding of S protein (vitronectin) from plasma with subsequent binding to vitronectin receptors on the endothelial cell surface(Valentin-Weigaud 1988).

Phagocytosis often does not lead to destruction of intracellular pathogens. The function of complement proteins in determining the fate of an organism following ingestion are still conjectural but three general consequences of ingestion have been observed(Horwitz 1988). For some organisms like Trypanosoma cruzi, ingestion of a complement coated organism appears to be followed by penetration of the organism from the phagocytic vacuole into the cytoplasm of the cell where it divides. With Legionella pneumophila, the organism enters via a process called coiling phagocytosis. Leishmania donovani may enter a

cell via a similar complex process in which the organism appears to assemble a coil of membrane about the phagocytic vesicle with mitochondria associated with this coiled vesicle. In the case of Legionella no fusion of phagosome with the lysosome is observed and no acidification occurs. The organism grows in the phagosome and is not exposed to lysosomal enzymes or the acidification process that leads to organism death. It has been suggested that Toxoplasma gondii and Chlamydia psittaci also prevent lysosomal-phagosomal fusion and acidification.

In the case of Leishmania donovani the phagosome once formed appears to fuse with lysosomal containing vesicles and acidification follows. Nevertheless the organism grows in this vacuole without difficulty and ultimately destroys the cell.

The classical and alternative complement pathways are relatively efficient mediator systems that have evolved to opsonize microorganisms or mediate lysis directly. Antibody is a further elaboration of a complex system of host defense. It should be clear from this discussion that various microorganisms have evolved complex and highly specific mechanisms for avoiding complement attack at each step in the sequence. These range from the evolution of proteins that mimic normal cellular control proteins to direct shedding of complement from the cell surface. The interaction between microorganisms and man appears to have undergone a continuous evolution with a development of evermore complex methods of mediating and avoiding the host defense process. Clearly, evolved defense mechanisms are quite effective, but in the case of highly virulent organisms, the ability to avoid complement attack has lead to the development of a niche in which the organism successfully competes. An understanding of these mechanisms will presumably lead to a more intelligent approach to an understanding of pathogenesis and ultimately to more well directed therapy.

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Production of Complement Component C3 by Lymphoid Cell Lines: Possible Function of C3 Fragments as Autocrine Growth Regulators

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ABSTRACT

Complement component C3 synthesis by two human lymphoid cell lines was investigated at the mRNA and protein levels. We report that the C3 gene is constitutively transcribed and that C3 protein is synthesized, secreted and proteolytically processed in cell lines of the lymphoid lineage. Because C3 fragments are implicated in lymphocyte growth control, we will also discuss the significance of C3 fragments as potential autocrine growth regulators.

INTRODUCTION

The third component of complement (C3) is a central element in the classical and alternative pathways of activation of the complement system. C3 is cleaved into active peptide fragments by the proteolytic action of other complement components. The activated C3 fragments mediate several important biological functions including stimulative and suppressive effects in the immune response (Weigle et al., 1985).

C3 is translated as a single-chain precursor protein from a 5.1 kb mRNA (Domday et al., 1982; DeBruijn and Fey, 1985) and is post-translationally cleaved into mature two-chain molecules with disulfide-linked α and β subunits of 115,000d and 75,000d, respectively. C3 is synthesized by several cell types. About 90% is synthesized by hepatocytes (Fey et al., 1985). Monocytes/macrophages are considered to be the major source of C3 present in tissues other than the liver (Fey et al., 1985; Zimmer et al., 1982; Minta and Isenman, 1987; Goodrum, 1987). Fibroblasts also produce low amounts of C3 (Senger and Hynes, 1978). In this report we summarize our results which have demonstrated the constitutive C3 gene transcription and C3 protein synthesis by two human lymphoblastoid cell lines.

RESULTS AND DISCUSSION

Complement C3 production in the human B lymphoblastoid cell line Raji and the T cell lymphoma HSB2 was analyzed at nucleic acid and protein synthesis levels. Northern blot analysis revealed that both Raji and HSB2 cells contain the 5.1 kb C3 mRNA species found in our control monocytic cell line U937 (Nichols, 1984). We corroborated our finding by isolating two cDNA clones from a Raji λgt10 cDNA library using the human C3 probe HC3-11 (DeBruijn and Fey, 1985). Comparative restriction endonuclease analysis and nucleotide sequence

determination revealed that these clones were derived from C3 mRNA. The Raji C3 gene is a polymorphic variant as compared to the published sequence derived from a human liver. These results confirm the transcription of the C3 gene in the macrophage cell line but also indicate that the C3 gene can be active in cells of the lymphoid lineage.

In addition to the 5.1 kb C3 mRNA shown in Raji and HSB2 cells, a 6.1 and a 1.7 kb mRNA species hybridizing to the C3 probe were observed. Previously, we reported the occurrence of truncated C3 mRNA species in murine lymphoid cell lines (Lernhardt et al., 1986; Lernhardt et al., 1987). Although some murine cell lines contain a 4.2 kb species, the predominant C3 mRNA species is 1.9 kb in length. The molecular mechanisms leading to truncated C3 mRNA species are unclear. RNA mapping studies indicated that the 1.9 kb truncated message encodes part of the C3 α chain (Lernhardt et al., 1987). The translation product of the 1.9 kb mRNA would contain the complement receptor type 2 (CR2, CD21; Barel et al., 1981; Iida et al., 1983) binding site on C3 (Lambris et al., 1985) but not the thiolester region (Thomas and Tack, 1983). It remains to be shown whether the truncated human and murine mRNAs give rise to proteins with the structural features just described. Nevertheless, our findings suggest that alternative modes of C3 gene transcription and/or alternative RNA processing pathways may result in polypeptides with structures and functions different from the known C3 fragments.

C3 protein synthesis by human cell lines was analyzed by biosynthetic labelling and immunoprecipitation. Using an anti C3 antiserum we were able to determine whether C3 mRNA is translated into C3 protein and secreted. We found that Raji and HSB2 synthesize and secrete C3. Secretion of the C3 molecules appears more pronounced from Raji cells. Protein analysis showed that the C3 polypeptide is cleaved into the mature two-chain C3 molecule. Lymphoid cells must therefore possess the appropriate protease to carry out the cleavage at the boundary of α and β chain consisting of four arginine residues (DeBruijn and Fey, 1985). Our results are in apparent contradiction with those of Whitehead and coworkers (1985). They were unable to detect C3 in Raji and HSB2 cells using a monoclonal anti-C3 antibody. A possible explanation for the apparent contradiction is the inability of the monoclonal antibody to detect the polymorphic variant(s) expressed by Raji and HSB2 cells.

C3 fragments C3b and C3d have been shown to exert growth-regulating properties by controlling entry into S phase of the cell cycle in PMA-stimulated human B lymphocytes (Bohnsack and Cooper, 1988) and lipopolysaccharide-activated murine B lymphocytes (Melchers et al., 1985). The complement receptor on B lymphocytes mediating this effect is CR2 (CD21) (Cooper et al., 1988). CR2 is capable of binding the C3 activation products C3b and C3d. Earlier studies showed that a synthetic decapeptide with the amino acid sequence responsible for binding of C3 to CR2 (Lambris et al., 1986) inhibits the S phase entry of murine B lymphoblasts mediated by aggregated C3d (Lernhardt et al., 1987). Therefore, the ligand receptor combination C3/CR2 appears to be directly involved in the clonal expansion of activated B lymphocytes. Hatzfeld et al. (1987) have recently shown that Raji requires C3 as a growth factor when cultured in serum-free medium at cell concentrations below 1000 cells per ml. At higher cell concentrations a dependency on exogenous C3 was not observed. Taking the above observations in conjunction with the Raji cell

line's ability to synthesize C3, it appears plausible that C3 may function in an autocrine fashion.

We will now investigate whether the transcriptional activation of the C3 gene plays a role in the malignant transformation of lymphocytes.

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Protein Phosphorylation as a Mechanism of Resistance Against Complement Damage

Z. Fishelson, E. Kopf, Y. Paas, L. Ross, and Y. Reiter

INTRODUCTION

Complement inflicts cell damage by insertion of a membrane attack complex (MAC) comprised of the C5b, C6, C7, C8 and C9 proteins into the plasma membrane of target cells (Muller-Eberhard 1986). Nucleated cells vary in their sensitivity to complement-mediated lysis, however, generally, they are more resistant to immune damage than erythrocytes (Koski et al. 1983). It has been suggested that this resistance of nucleated cells is due to a postulated capacity of the cells to actively resist and repair complement damage (Ohanian and Schlager 1981). Recently, a Ca^{2+}-dependent process of rapid MAC removal from the surface of nucleated cells has been suggested to be involved (Carney et al. 1986; Morgan et al. 1987). Yet, the cellular regulatory mechanisms which support these and other postulated resistance/repair processes are poorly characterized.

Sub-lytic concentrations of MAC may elicit triggering events in nucleated cells, many of which are mediated by Ca^{2+} (Hänsch et al. 1988; Imagawa et al. 1983; Morgan 1989). MAC binding to platelets stimulates secretory responses and phosphorylation of platelet proteins (Wiedmer et al. 1987). Results presented herein demonstrate that sub-lytic concentrations of complement induce phosphorylation of membrane and cytosolic proteins in human leukemic cells. Furthermore, reagents which inhibit protein kinase activity are also shown to increase sensitivity of these leukemic cells to complement damage. Based on these results and others, we are proposing that protein kinases and protein phosphorylation play a major role in the resistance of nucleated cells to complement-mediated damage.

RESULTS AND DISCUSSION

Interference with protein, DNA and RNA synthesis can increase sensitivity of tumor cells to lysis by antibody and complement (Ohanian and Schlager 1981). That 3'5' cAMP can protect cells already damaged by complement from lysis (Boyle et al. 1976), further supported the notion that nucleated cells are equipped with a damage-repair mechanism. As shown in Fig. 1, in agreement with the latter finding, pretreatment (for 30 min at 37°C) of human leukemic cells with dibutyryl cAMP or reagents known to induce elevated intracellular cAMP level (3-isobutyl 1-methyl xanthine (MIX) and forskolin) (Montague and Cook 1970; Daly 1984) reduced their sensitivity to lysis by antibody and human complement. Similar protection was induced in both K562 and U937 cells by phorbol 12-myristate 13-acetate (PMA), a known activator of protein kinase C (Nishizuka 1984). In contrast, four inhibitors of protein kinases, i.e. polymyxin B (PMB), N-(6-aminohexyl)-5-chloro-2-naphthalene sulfonamide (W-7), 1-(5-isoquinolinyl sulfonyl)2-methylpiperazine

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(H-7) and tamoxifen (Nel et al. 1985; Hidaka et al. 1984; O'Brian et al. 1985) markedly enhanced complement-mediated cell lysis (Fig. 1). These results led us to propose (Fishelson et al. 1987) that processes of protein phosphorylation effected by cAMP-dependent protein kinase, protein kinase C and possibly other serine/threonine kinases (Edelman et al. 1987; Cohen 1988) promote cell resistance to complement damage.

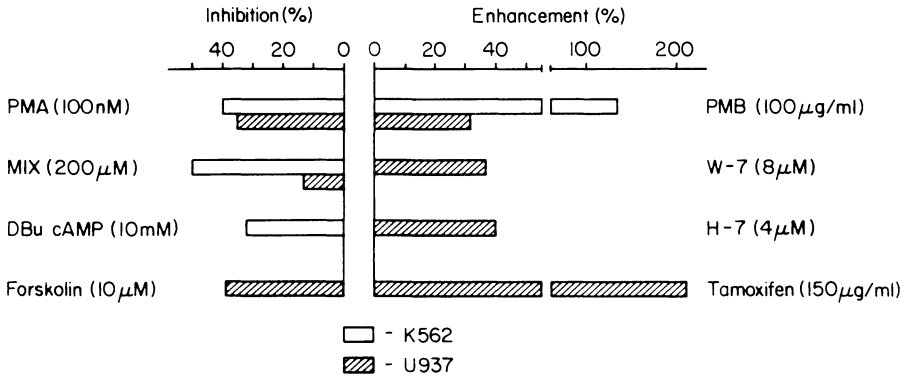

Fig. 1. Effect of chemical pretreatment on the sensitivity of K562 and U937 cells to lysis by antibody and complement. Human erythroleukemic K562 and histiocytic leukemic U937 cells (1 x 10^6) were treated for 30 min at 37°C with various drugs (concentrations indicated in parenthesis) in 100 μl PBS. Rabbit antibodies were then added followed by NHS at concentrations which yielded 30-70% lysis (trypan blue inclusion after 60 min incubation at 37°C) of control cells preincubated without drugs. Results are expressed as the percent deviation from controls. Only the drug concentrations which produced the maximal effect are presented, i.e. higher and lower concentrations were less or as effective. None of the drugs was toxic to cells at these concentrations.

To determine whether complement activation can indeed trigger protein phosphorylation in cells under attack, K562 cells were pre-equilibrated for 60 min at 37°C with [^{32}P]-orthophosphoric acid (0.5 mCi, NEN, Boston) in phosphate-free medium, washed and exposed to normal human serum (NHS) or heat-inactivated (HI, 30 min at 56°C) NHS for 60 min at 37°C. The cells were pretreated or not with rabbit anti-K562 cells antibodies (Ab) during the last 30 min of the pre-equilibrtion with [^{32}P]-orthophosphate. In all cultures, cell viability at the end of the experiment was above 85%. The cells were then washed, disrupted by sonication and sedimented at 100,000 x g for 30 min. The supernatant (cytoplasmic fraction) was removed and the membrane pellet was solubilized in 1% NP-40 and sedimented (as above) to remove insoluble material. All procedures were performed at 4°C in presence of protease and phosphatase inhibitors. Samples of the cytoplasmic and membrane preparations were subjected to TCA (10%) precipitation on ice. The radioactivity and protein concentration of the resulting protein pellets were determined; and the quantity of ^{32}P incorporated per mg protein in each sample was then calculated. In absence of antibody, NHS caused a 46% increase in phosphorylation of membrane proteins when compared with HI-NHS ($p < 0.01$ in a t-test) (Fig. 2). A similar increase in membrane proteins phosphorylation ($p < 0.05$) was induced by NHS in antibody-pretreated cells. An increase in phosphorylation of cytoplasmic proteins also occurred upon NHS and Ab + NHS treatment as compared with HI-NHS AND Ab + HI-NHS, respectively, but the differences were not statisti-

cally significant ($p < 0.1$). However, analysis of the cytoplasmic preparation of these NHS-treated K562 cells by SDS-polyacrylamide gel electrophoresis followed by

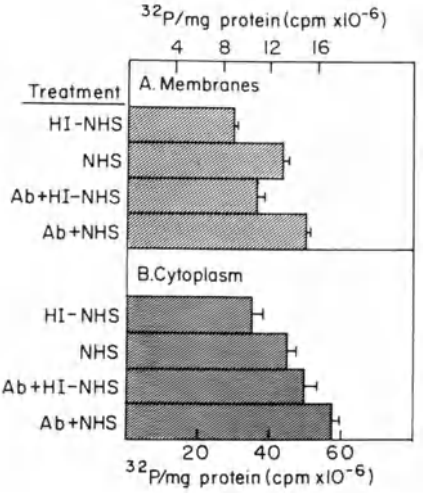

Fig. 2. Complement-induced phosphorylation of membrane (A) and cytoplasmic (B) proteins in K562 cells. See text for further details.

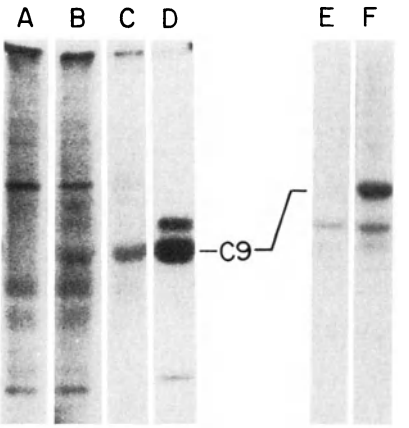

Fig. 3. In vitro phosphorylation of C9. Purified human C9 (10 μg) was incubated for 15-30 min at 30°C with (a) crude cytoplasmic fraction of K562 cells, undiluted or at 10 x dilution (lanes B or C, respectively), (b) catalytic subunit of cAMP-dependent protein kinase from bovine heart (Sigma) (lane D) or (c) ecto-kinase-containing supernatant of K562 cells (lane F). The phosphorylation reaction mixture contained 50 μM ATP, 1-5 μCi [γ-^{32}P]ATP, 10 mM Mg^{2+}, 25 mM NaF, 100 μM $NaVO_3$ and 1 mM theophylline in Tris-buffered saline, pH 7.4. The proteins were analyzed by SDS-PAGE (10% gels) and autoradiography. Lanes A and E represent endogenous phosphorylation of the crude undiluted cytosol and the ecto-kinase preparation, respectively, in absence of C9.

autoradiography (results not shown) revealed several proteins which were either phosphorylated or dephosphorylated. These results indicate that sub-lytic complement action on K562 cells may activate protein kinases (and phosphatases) leading to phosphorylation/dephosphorylation cascades in the cytoplasmic and membrane compartments.

Cellular proteins are likely endogenous substrates for the postulated complement-activated protein kinases. However, complement proteins which bind to cells following complement activation may themselves serve as substrates for these kinases. Indeed, Dan-Gaspar and Esser (1987) reported that C9 can be phosphorylated in vitro by the catalytic subunit of cAMP-dependent protein kinase. We confirmed their finding (Fig. 3, lane D) and further demonstrated that purified C9 can be also phosphorylated by protein kinase(s) present in a crude preparation of K562 cytoplasm (lanes B and C). Since complement proteins are usually in contact with the extracellular milieu, it was of interest to examine whether C9 could also be phosphorylated by protein kinases present on the outer surface of cells. Such an ecto-kinase has been described in HeLa cells by Kübler et al (1982). Upon incubation with intact and fully viable K562 cells in presence of [γ-^{32}P] ATP (NEN), C9 became phosphorylated (Fishelson et al. manuscript in preparation). Furthermore, K562 cells constitutively released protein kinases which efficiently phosphorylated C9 (Fig. 3, lane F). Interestingly, much more ecto-kinase activity was measured in the supernatant of complement-resistant K562 cells than of a complement-sensitive K562 variant (Fishelson et al. manuscript in preparation). The effect of phosphorylation on C9 functions is not known and is being examined.

In summary, nucleated cells appear to respond to complement damage by activation of phosphorylation/dephosphorylation cascades. The results also suggest that protein phosphorylation processes protect the cells from lysis, possibly by interfering with the lytic machinery and/or by facilitating damage repair. The protective mechanism(s) and the molecule(s) involved still remain to be elucidated.

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The Mosaic Nature of the Complement Proteins

A.J. Day, R.D. Campbell, and K.B.M. Reid

INTRODUCTION

The primary sequences of nearly all the proteins associated with the complement system have now been established (Reid, 1986; Campbell et al., 1988). The majority of these proteins are mosaic in structure being built up from a number of different types of consensus sequence motif: generally 40-110 amino acids in length (Reid and Day, 1989; Day, 1989). The mosaic nature of the complement proteins is illustrated in Fig. 1. At present only two of the motifs are specific to the complement system (i.e., the repeat found only in C1r and C1s; and the repeat present in factor I and at the C-termini of C6 and C7. See Fig. 1), whereas all the other motifs are also found in non-complement proteins. For example the epidermal growth factor (EGF) domain, which is present in a single copy in C1r and C1s, is found in a large superfamily of diverse proteins including the blood clotting cascade and fibrinolysis system, membrane-bound receptors and growth factors (Rees et al., 1988). The EGF domain has recently been described in the endothelial leukocyte adhesion molecule 1 (ELAM-1), the 140 KDa granule membrane protein (GMP-140), the lymphocyte-associated cell surface molecule (LAM-1) and the mouse lymph node homing receptor (LHR), as shown in Fig. 1, which belong to a new family of cell adhesion proteins (Marx, 1989; Tedder et al., 1989; Johnston et al., 1989; Lasky et al., 1989; Bevilacqua et al., 1989).

The two most common motifs found in the complement system are the Complement Control Protein repeat (CCP) [also called a Short Consensus Repeat (SCR)] and the Thrombospondin Repeat (TSR). These two types of motif are discussed below.

THE COMPLEMENT CONTROL PROTEIN REPEAT

The CCP motif is present in 12 of the complement proteins, with a total of 96 individual repeats (Fig. 1), and is therefore the most common structural feature of proteins associated with the complement system. The number of repeats that are found in a single polypeptide chain range from 2, for C1r and C1s, to 30 for the major polymorphic variant of complement receptor type 1 (CR1 A). The repeats, which are arranged in a contiguous fashion, are about 60 amino acids in length and have a characteristic consensus sequence with 4 invariant cysteines and other highly conserved residues (Campbell et al., 1988; Day, 1989). The CCP motif is thought to be an independently folding domain with the framework cysteines forming intra-domain disulphide bridges in the pattern Cys1-3 and Cys2-4 (Day et al., 1987; Day, 1989; Janatova et al., 1989). CCPs

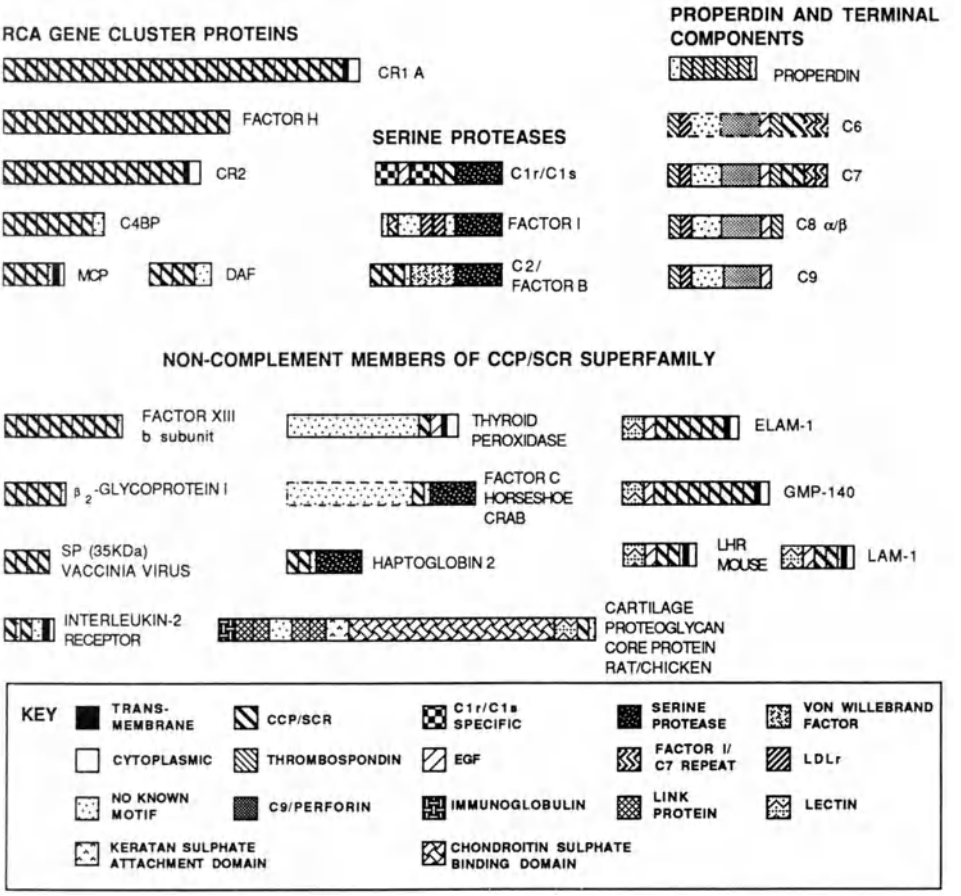

Fig. 1. A schematic representation of the primary structures of those complement proteins which contain consensus sequence motifs and the non-complement members of the CCP/SCR superfamily. All proteins shown are from humans unless otherwise specified. Dashed boxes indicate a sequence that is either not determined or not yet published. Abbreviations are defined in the text.

are generally encoded by discrete exons at the gene level (Campbell et al., 1984; Leonard et al., 1985; Vik et al., 1988), however some CCPs are encoded by 2 exons (Hourcade et al., 1988; Vik et al., 1988). The complement control proteins CR1, factor H, C4b-binding protein (C4BP), membrane cofactor protein (MCP) and decay accelerating factor (DAF) and the complement receptor type 2 (CR2) are composed almost entirely of CCPs (Fig.1). These proteins, which are encoded in the 'regulation of complement activation' (RCA) gene cluster, on human chromosome 1q (see Campbell et al., 1988), are all involved in the control of C3b and/or C4b. Three CCP motifs are also found at the N-termini of the serine proteases, C2 and factor B, which are involved in complement activation.

In C2 and factor B the CCP motif has been shown to be involved in C4b and C3b interaction, respectively. The CCPs found in C1r and C1s may also have a functional role which involves C4-binding. Therefore the CCP motif is found in a number of C3b/C4b-binding proteins. However this structure is evidently not only associated with a C3b/C4b-binding function as these proteins have many repeats but are thought to be univalent for C3b/C4b (Campbell et al., 1988). The CCP motif is therefore likely to be a structural building block onto which function-related sequence variations are superimposed. This is further emphasised by the fact that this motif is found in a growing number (12 to date) of non-complement proteins (Fig. 1) which are not believed to interact with C3b or C4b. The non-complement proteins that belong to the CCP superfamily include the blood clotting factor XIIIb subunit, β2-glycoprotein I, interleukin-2 receptor, haptoglobin 2 precursor, the core protein from cartilage proteoglycan and thyroid peroxidase (Day, 1989). A single CCP has been described in factor C from the horseshoe crab which indicates that this motif is relatively old in evolutionary terms. A 35 KDa protein (SP) encoded by vaccinia virus, and secreted from cells infected by the virus consists of 4 contiguous CCPs (Kotwal et al., 1988). These CCPs have a very high degree of sequence similarity with the first 4 repeats of C4BP, which suggests that the virus may be utilising these CCPs to avoid complement activation (i.e., due to the inhibitory effect that a C4BP-like protein could have on the activation of the classical complement pathway).

Recently a new family of cell adhesion proteins has been described which are important in targeting white blood cells to their appropriate sites of action. These homing receptors, ELAM-1 (Bevilacqua et al., 1989), LAM-1 (Tedder et al., 1989), mouse LHR (Lasky et al., 1989) and GMP-140 (Johnston et al., 1989), have a similar structural organisation. All consist of a N-terminal lectin domain followed by an EGF domain, a varying number of CCPs, a transmembrane region and a short cytoplasmic domain (Fig. 1). The CCPs in these proteins are somewhat unusual in that they contain 2 extra consensus cysteines, which are presumably disulphide bonded. These proteins probably mediate the binding of cells on which they are expressed to target tissues via their lectin domain which may recognise anionic polysaccharides (Lasky et al., 1989; Bevilacqua et al., 1989).

A clear function has not been ascribed to the CCP motif in most of the non-complement members of the CCP-superfamily. The CCP may be important in a wide range of binding specificities or may purely have a structural role. In this respect it is known from physical studies that proteins containing many repeats (e.g., factor H and C4BP) are very elongated (see Sim et al., 1986) and their structures can be likened to a string of beads (i.e., each CCP being an independently folded domain with dimensions 30 x 42.5 Å (Perkins et al., 1986)). Therefore in cell-surface molecules (e.g., the adhesion family) CCPs may act as spacer units, projecting the binding domain (e.g., lectin motif) away from the cell membrane.

At present no 3-dimensional structure for the CCP is known. However work is in progress to determine the CCP structure by nuclear magnetic resonance spectroscopy of single CCPs expressed in yeast (Baron et al., 1989).

THE THROMBOSPONDIN REPEAT

The second most common motif found in complement system proteins is the TSR, which was first described in thrombospondin (Lawler and Hynes, 1986), a 420 KDa adhesion protein secreted from activated platelets. This motif is about 60 amino acids long and is largely based on a consensus sequence of 6 cysteine and 3 tryptophan residues (see Day, 1989). Five complement proteins contain TSRs as shown in Fig. 1. These include the terminal components C6, C7, C8 and C9 and the control protein properdin. Properdin is almost entirely composed of 6 contiguous TSRs, whereas the terminal components each have a N-terminal TSR and C6, C7 and C8 also have a TSR in their C-terminal half. At present less is known about the TSR than the CCP domain (e.g., the disulphide bridge organisation has not been determined). However, electron microscopy studies (Smith et al., 1984) on properdin, composed of 6 TSRs, indicate that it has an elongated structure (25 x 260 Å) and the TSR therefore may have similar dimensions to the CCP domain. The roles of the TSRs in properdin and the terminal components are not known and a comparison of the properties of these proteins does not reveal an obvious common function. The tertiary structure of the TSR (on which studies are in progress) may provide a clearer picture of the role of the TSR in the complement system.

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II Cell Developments

Development of T Cells

1. Phylogeny, Stem Cells, Lineages

The Role of the Thymus in the Development of T-Cells

J.J.T. Owen, E.J. Jenkinson, R. Kingston, G.T. Williams, and C.A. Smith

INTRODUCTION

The crucial role of the thymus in the generation of T lymphocytes has been suspected for some time. Experiments carried out over 25 years ago, showed that neonatal thymectomy in rodents results in severe immune deficiency (Miller and Osoba, 1967). These results were confirmed in an analysis of mice homozygous for the nude gene where an association between immune deficiency and defective thymic development was shown (Pantelouris, 1971). In patients, the rare condition of thymic aplasia (di George syndrome) results in severe immune deficiency. When separate populations of T and B lymphocytes were identified, it became clear that the thymus is important for the generation of T lymphocytes. Absence of the latter leads to a functional deficiency in immune responses.

Recent studies have amply confirmed these original findings. They support the notion that the thymus is a highly efficient site for the generation of T lymphocytes. However, this does not rule out the possibility that some T cells are generated in extra thymic sites. Indeed, some T cell maturation including T cell receptor expression is found in nude mice (MacDonald et al., 1987). However, the process is inefficient and generation of T cells takes time so that T cell receptor expressing cells are found mainly in old animals. Furthermore few T cell receptor gene rearrangements seem to occur and so the diversity of T cells for recognition of foreign antigens is very limited. It seems likely that the thymus has evolved as a site for the provision of efficient signalling for T cell maturation. Signals for proliferation, for T cell receptor gene rearrangement and for phenotypic maturation e.g., expression of molecules such as CD2, CD4, CD8, etc., are all required.

However, there is a further major role of the thymic environment in T cell immunity. A number of recent studies have demonstrated the importance of antigen expression (especially expression of MHC antigens) by the thymic environment in shaping the T cell receptor repertoire (von Boehmer et al., 1989) by positive selection (i.e., selection of useful receptors) and negative selection (i.e., removal of potentially autoreactive cells).

Thus production of T-cells in the thymus can be divided into three main phases. First, there is the establishment of a stem cell pool within the thymus. Second, there follows a period of intense cell proliferation accompanied by T cell receptor (TCR) gene rearrangements and phenotypic maturation of cells. Finally, there is a phase of selection, as a result of which, only some cells migrate to the peripheral lymphocyte pool (Fig. 1).

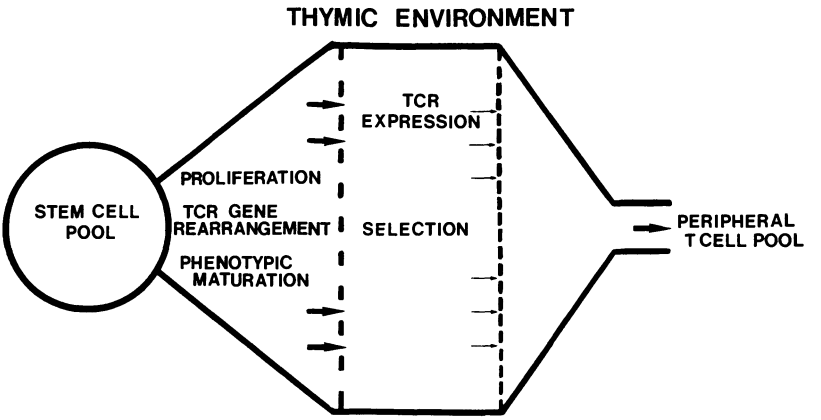

Fig. 1. Three main cell compartments exist in the thymic environment. The stem cell pool occupies the outer cortex of the thymic lobe. The proliferative compartment is also in the outer cortex. The compartment of TCR expression occupies most of the cortex and the medulla. Some cells may die before they enter the compartment of TCR expression - if, for example, they fail to make productive gene rearrangements. Many cells may die in the phase of TCR expression - if they express auto reactive receptors (negative selection), or fail to interact with thymic MHC antigens (positive selection).

CELLS OF THE THYMIC ENVIRONMENT.

Many of the cells of the thymic environment are derived from epithelium of the pharyngeal pouches (van Vliet et al., 1985). These epithelial cells make close contact with developing lymphocytes and they are suspected to provide important signals for T cell maturation. The stem cells which give rise to thymic lymphocytes migrate into the epithelial stroma from the blood stream and have their origin in haemopoietic tissues such as the foetal liver (Moore and Owen, 1962). There is little doubt that these stem cells derive from multipotential haemopoietic stem cells but whether the cells that enter the thymus are still multipotential or whether they have already become restricted to lymphoid development is still a matter of debate.

There are several types of epithelial cell in the thymic environment. Thus monoclonal antibodies to surface antigens on thymic epithelial cells have demonstrated differences between those epithelial cells in the outer part of thymic lobes - the cortical epithelium, and those epithelial cells in the inner part of lobes - the medullary epithelium (van Vliet et al., 1984). The significance of this epithelial cell diversity remains uncertain.

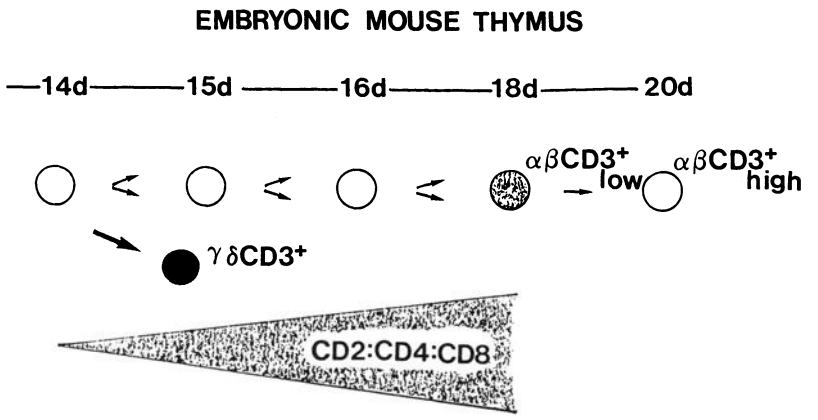

Fig. 2. Early γ and δ gene rearrangements in the 12-14d mouse embryo thymus result in the production of $\gamma\delta$CD3 expressing cells at the 15d stage (Pardoll et al., 1987). β and later α gene rearrangements lead to the production of cells expressing low levels of $\alpha\beta$CD3 in the cortex of the embryonic thymus. These cells also express CD2, CD4 and CD8. Signalling of the TCR at this stage induces cell suicide - the process of apoptosis involving the breakdown of DNA. This provides a mechanism by which autoreactive cells can be negatively selected. Positive selection by interaction with thymic MHC antigens is required for the generation of "single positive" $CD4^+$ or $CD8^+$ cells expressing high levels of $\alpha\beta$CD3.

In addition to epithelial cells, there are two other stromal cell types that are thought to be important in lymphocyte maturation. Macrophages are distributed throughout cortex and medulla of thymic lobes and dendritic (interdigitating) cells are distributed throughout the medulla (Barclay and Mayrhofer, 1981). Interest in these cells centres on their efficient role in antigen presentation.

CELL GROWTH AND T CELL RECEPTOR GENE REARRANGEMENT SIGNALS WITHIN THE THYMUS

Once they are within the thymic environment, stem cells proliferate and their progeny begin an ordered programme of T cell receptor gene rearrangements. Initially, a small percentage of $\gamma\delta$CD3 expressing cells are produced in the embryonic thymus (at about day 15 in the mouse embryo). These cells are a separate lineage to the $\alpha\beta$CD3 expressing cells which appear later (Winoto and Baltimore, 1989). The significance of the early appearance of $\gamma\delta$ cells in the thymus is unknown - one possibility is that they are generated prior to migration to peripheral tissues. At the time of appearance of

$\gamma\delta$ cells in the thymus, many developing lymphocytes already contain cytoplasmic CD3 molecules and it is interesting to note that the addition of anti CD3 antibodies to organ cultures of embryonic thymus blocks full rearrangement of β chain genes which normally occurs at this time (Owen et al., 1988). The cellular and molecular basis for this effect is currently unknown.

Within the $\alpha\beta$ cell lineage, α chain gene rearrangements follow those of the β chain. Precursor cells with cytoplasmic β chains and CD3 molecules are found within the embryonic thymus and presumably reflect a stage of development where α chain expression is awaited prior to expression of $\alpha\beta$CD3 on cell surfaces (Fig. 2).

These events are accompanied by intensive cell proliferation and considerable growth of the embryonic thymus. The stem cells that first enter the thymus have a considerable, but finite, proliferative capacity and new waves of migrant stem cells enter the thymus at subsequent phases of development in order to maintain the generation of new lymphocytes. There are a number of claims with regard to the identification of growth signals within the thymus. IL-1, perhaps delivered by macrophages, is thought to be important in the earliest events (Papiernik et al., 1987). IL-2 and IL-4 can both cause the proliferation of lymphocytes derived from the embryonic thymus and in situ hybridization studies have shown the presence of mRNA for these growth factors within developing lymphocytes themselves (Carding et al., 1989). Thus, in part, growth control might be autocrine involving production of growth signal from lymphocytes. Recent studies have implicated the growth factors IL-6 and IL-7 in lymphocyte proliferation (Takeda et al., 1989), thus suggesting a complex pattern of overall growth regulation. Clearly, further work will be required to unravel the complexities of this system.

T CELL RECEPTOR EXPRESSION ON DEVELOPING THYMUS LYMPHOCYTES

T cell receptor expression can be detected on the surfaces of a majority of lymphocytes within the thymus. Within the cortex, where the least mature lymphocytes reside, T cell receptor expression is low. Within the medulla, where the maturer cells are present, T cell receptor expression reaches levels found on peripheral T cells (Fig. 2). The newly generated cortical thymus lymphocytes with low receptor expression are not in cell cycle (Parkin et al., 1988). Furthermore, unlike mature T cells they do not enter cell cycle on activation with mitogens or antigens. They express a number of other membrane molecules including CD4, CD8, CD2, CD5, and in humans CD1 and CD7 molecules. There is evidence that some of these "double positive" $CD4^+$ $CD8^+$ cells are precursors of mature "single positive" $CD4^+$ or $CD8^+$ cells with higher levels of T cell receptor expression. Recent studies suggest that these developmental phases of T cell receptor expression may be important in selection of the T cell receptor repertoire.

DELETION OF POTENTIALLY AUTO REACTIVE CELLS BY APOPTOSIS - NEGATIVE SELECTION.

Several studies have shown that self antigens that react with the β chain of the T cell receptor induce deletion of developing thymus lymphocytes expressing the particular Vβ elements that are bound during maturation in the thymus (Kappler et al., 1987; Hengartner et al., 1988). Furthermore, these observations have been extended by showing that exogenous antigens which react with Vβ elements also delete developing T cells expressing these elements when the antigens are administered to neonatal animals (White et al., 1989). Both self and exogenous antigens of this type react with selected Vβ elements of the T cell receptor and are presented by MHC class II expressing cells. Because of their ability to react with a considerable proportion of the repertoire of T cells, they have been given the name "superantigens". Although it is not yet clear whether other self antigens might act in the same way, these superantigens provide a useful model for the analysis of the deletion process.

Several inter related questions arise. Firstly, is there a defined developmental stage in T lymphocyte ontogeny when cells are susceptible to deletion? What type or types of cell present self antigens to susceptible cells during the deletion process? Is the deletion process itself restricted to the thymus or can it occur in peripheral lymphoid tissues? In addition, there is the more general question as to whether a deletional process operating during T cell maturation can explain tolerance to all self antigens. Recently there has been considerable interest in the notion that cells might be rendered unresponsive (anergic) to antigen in peripheral lymphoid tissues in circumstances where antigens are not presented in a immunogenic form (Rammensee et al., 1989).

However to return to the matter of clonal deletion within the thymus, we have shown that antibodies which react with the CD3 component of the T cell receptor initiate the process of apoptosis in developing thymic T cells (Smith et al., 1989). Apoptosis is a common form of physiological cell death seen for example during embryonic development where tissue remodelling is required. It is characterised by the activation of endogenous endonucleases which procede to fragment DNA into oligonucleosomal bands. The affected cells collapse and are phagocytosed by macrophages. We have found that the predominant cell type that is affected is the immature "double positive" low T cell receptor expressing cell type (Fig. 2.). This cell can also be influenced to undergo the same process by agents which increase calcium mobilization within cells (e.g., calcium ionophores) Indeed, we have preliminary evidence that the activation of immature cells with anti CD3 antibodies results in phosphoinositol hydrolysis, as in mature T cells, but the result in immature cells is death rather than activation. In other preliminary studies, we have found that the exogenous superantigen staphylococcal enterotoxin B also induces apoptosis within thymus organ cultures but here the effect is limited to Vβ8 cells with which this agent reacts. These results provide the first direct evidence for the induction of apoptosis within immature thymus cells by an exogenous antigen.

Thus our results suggest that immature T cells are susceptible to deletion if their receptor is engaged by antigen during thymic development. Most of the cells of the thymic cortex are in this early developmental phase but the question as to precisely where the

deletion process occurs also relates to the site of antigen presentation. For example, in the studies on deletion of T cells by Mls^a antigens, many cortical thymocytes expressing the $V\beta6$ receptor with which Mls^a interacts, are present until a late stage of maturation (Hengartner et al., 1989). However this late deletion might reflect the limited tissue distribution of Mls antigens i.e., on B lymphocytes but not on macrophages and dendritic cells. Since B lymphocytes are localized to the thymic medulla, deletion may predominantly occur at the corticomedullary junction. In other situations, for example in transgenic mice carrying a T cell receptor for the male H-Y antigen, deletion of autoreactive cells in male mice occurs at an early stage of cortical thymus lyphocyte development (von Boehmer et al, 1989). This might reflect the presence of the H-Y antigen on cortical thymus epithelium.

Negative selection alone does not account for all of the cell death within the thymus. Positive selection for lymphocytes with receptors which can interact with self MHC antigens is known to be important in the generation of T cells which will be useful in immune responses i.e.,in the recognition of foreign antigens in the context of self MHC (von Boehmer et al, 1989). Positive selection will be discussed in other sections of these proceedings. At the moment, it is difficult to map the precise point where positive selection occurs onto the developing lineage of T-cells within the thymus except to say that positive selection is involved in determining whether cells become single positive $CD8^+$ or $CD4^+$ cell and that MHC antigens expressed by thymic epithelial cells are crucial in this process. If positive selection operates on immature cells, it is difficult to understand how interaction of the T cell receptor with antigen plus MHC induces cell death as described above, whilst at the same time being a necessary requirement following interaction with MHC antigens for positive selection. Perhaps positive selection might occur after immature cells have passed through the development window when they are susceptible to negative selection (apoptosis). Clearly this is likely to be a topic of intense interest in the future.

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Ontogeny of T Cells and a Third Lymphocyte Lineage in the Chicken

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INTRODUCTION

Comparative analysis of immune system development in birds and mammals revealed that development of lymphoid stem cells proceeds along T and B cell lines (Cooper et al. 1965; Moore and Owen 1967). In order to begin T cell development, blood-borne stem cells in birds periodically enter the epithelial thymus in response to locally-produced chemoattractants (Le Douarin 1978; Jotereau and Le Douarin 1982). Stem cell colonization of the amphibian and mammalian thymuses also appears to occur in waves (Turpen and Smith 1989; Jotereau et al. 1987).

The development of T cells and their function has been extensively analyzed in mice and humans (Davis and Bjorkman 1988; Marrack and Kappler 1987; Roger and Reinherz 1987). We have relied heavily on this information to begin a comparative analysis of avian T cell development. In these studies we have used a panel of mouse monoclonal antibodies against chicken T cell antigens to exploit some of the experimental advantages of the avian model. These include the ready accessibility of the embryo and the ability to create chick-quail chimeras by exchange of various embryonic tissues. The species specificity of the antibodies to chicken T cells has allowed us to trace the developmental potential of chick thymocyte precursors in recipient quail embryos.

This comparative analysis has revealed a remarkable conservation of T cell development and function in birds and mammals. Several novel features of T cell development were also observed in the chick, many of which are likely to be mirrored in mammals.

DEVELOPMENT OF TCR1 AND TCR2 CELLS IN THE CHICKEN

The development of chick T cells can be followed with a panel of mouse monoclonal antibodies specific for the avian homologues of CD3 (Chen et al. 1986), CD4, CD8 (Chan et al. 1988) and the different types of T cell receptors (TCR) (Sowder et al. 1988; Cihak et al. 1988; Chen et al. 1988). All of these T cell surface antigens are very similar to their mammalian homologues in secondary structure, tissue specificity of expression, and function (Reviewed by Lahti et

al. 1988). Information on the primary structure of the avian homologues and their encoding genes is still needed, however.

The antibodies directed against the chicken $\gamma\delta$ (TCR1) and $\alpha\beta$ (TCR2) epitopes on viable T cells, together with the anti-chick CD3 homologue, provided the first such set of antibodies, and allowed us to elucidate the developmental pathways followed by the TCR1 and TCR2 populations. As shown in figure 1, the TCR1 subpopulation is the first to be generated in the thymus. The TCR2 subpopulation appears later in development, following a pattern noted previously for the $\gamma\delta$ and $\alpha\beta$ T cells in mammals (Pardoll et al. 1987; Havran and Allison 1988; Haynes et al. 1988).

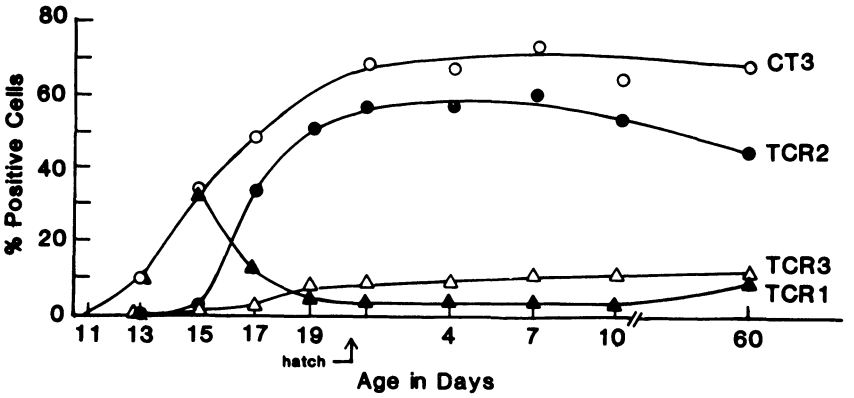

Fig. 1. Ontogeny of TCR1, TCR2 and TCR3 cells in the thymus

Also in parallel with T cell development in mammals (Brenner et al. 1986; Lew et al. 1986), avian TCR1 thymocytes rarely express the CD4 or CD8 accessory molecules, whereas the vast majority of TCR2 thymocytes express both (Sowder et al. 1988; Chen et al. 1988). Following their generation in the thymus, the TCR1 and TCR2 subpopulations of T cells are sequentially seeded to the peripheral lymphoid tissues. Arrival in the spleen begins on embryonic day 15 for TCR1 cells and on day 19 for TCR2 cells (Bucy et al., manuscript in preparation). The TCR1 subpopulation of T cells is relatively large in the chicken, where it may constitute from 20 to 50% of the circulating T cell pool in young adults (Sowder et al. 1988; Cihak et al. in press).

Our studies of the TCR1 subpopulation have revealed several interesting features that previously had not been appreciated in mammals. First, the level of receptor complex expression (CD3/TCR1) on this subpopulation is relatively high from the onset of their appearance in the thymic cortex (Chen et al. 1988; George and Cooper, manuscript in preparation). For example, the mean level of receptor expression on TCR1 cells in 14 day embryos is approximately 15-fold higher than that observed for TCR2 cells in 17 day embryos (George and Cooper, manuscript in preparation). Second, in comparison with the TCR2 subpopulation, the TCR1 subpopulation of cells appears to divide

relatively infrequently and rapidly traverse the cortex to enter the thymic medulla or migrate to the periphery (Bucy et al., manuscript in preparation). Third, TCR1 cells preferentially home to the sinusoidal areas of the spleen and to the intestinal epithelium whereas TCR2 cells accumulate around the splenic penicillary arteries and in the intestinal lamina propria (Bucy et al. 1988). Fourth, a majority of the TCR1 cells present in peripheral lymphoid tissues express the CD8 antigen (Chen et al 1988; Bucy et al. 1988). Fifth, development of the TCR1 subpopulation of peripheral T cells requires a relatively prolonged period of thymic seeding (Chen et al. 1989; Cihak et al. 1989). The reason for this requirement may be the relatively limited expansion of the TCR1 subpopulation in the periphery. Whereas TCR2 cells are often found in follicular distribution, the TCR1 cells are always relatively dispersed (Bucy et al., manuscript in preparation).

Parallels for several of these features of development have been observed in mammals (Goodman and Lefrancois 1988; Bucy et al. 1989). On the basis of this set of observations, we have postulated that the TCR1 thymocytes are not subjected to the rigorous selective pressures that shape the TCR2 repertoire. In both birds and mammals, the acquisition of CD8 accessory molecules by TCR1 cells when they home to particular microenvironments in peripheral lymphoid-tissues suggests that TCR1 cells play a special role in body defense and are triggered by antigen presenting cells expressing the MHC class I family of molecules (Strominger 1989).

TCR3: A THIRD T CELL SUBLINEAGE

In birds, a third T cell sublineage can be delineated on the basis of the expression of a CD3/TCR complex that is not reactive with either the TCR1 or TCR2 antibodies. T cells of this phenotype can be isolated by negative immunofluorescence cell sorting (Ia^-, $TCR1^-$, $TCR2^-$ lymphocytes) and their TCR analyzed by immunoprecipitation (Chen et al. 1989). The CD3-associated TCR on this subpopulation of T cells were slightly smaller than the TCR1 and TCR2 molecules: M_r 88,000 versus 90,000. The TCR3 heterodimer consists of disulfide-linked M_r 48,000 and 40,000 polypeptide chains, at least one of which appears to differ in amino acid composition from the M_r 50,000 and 40,000 disulfide-linked chains of the TCR1 and TCR2 heterodimers. The TCR3 cells isolated by negative immunofluorescence sorting were also used to generate monoclonal antibodies specific for a non-polymorphic determinant of the TCR3 heterodimer (Chen et al. 1989). The TCR3-specific antibodies have been used to trace the development of this subpopulation of cells.

The TCR3 cells appear first in the thymus of 17 day chick embryos. They are also the last to be seeded to the periphery where they gradually increase in numbers to constitute approximately 15% of the circulating pool of T cells in adult chickens. The TCR3 subpopulation can also be identified in other Galliforme species on the basis of antigenic cross-reactivity of the TCR3 molecule. Interestingly, it may constitute the major T cell type in some gallinaceous birds (Char et al., unpublished observations).

The TCR3 cells closely resemble the TCR2 subpopulation in many ways. In the thymus, they are predominantly $CD4^+/CD8^+$ cells, whereas most of the mature TCR3 cells in the periphery express CD4 only. The CD4/CD8 ratio for peripheral TCR3 cells is approximately 4:1. Immature TCR3 thymocytes express their CD3 receptor complex at relatively low

levels and gradually increase their surface receptor density as a function of maturation. TCR3 cells also home to the same areas of the spleen as do the TCR2 cells, and relatively few migrate into the intestinal epithelium.

The TCR3 cells apparently represent a distinct sublineage of T cells despite their resemblance to the TCR2 subpopulation. Their development is not impaired by suppression of either the TCR1 or TCR2 sublineages by embryonic treatment with the corresponding monoclonal antibodies. In fact, when development of the TCR2 subpopulation is severely impaired by repeated injections of TCR2 antibodies before thymectomy 8 days after hatching, the TCR3 cells become the major T cell type, constituting as much as 80% of the total T cell pool (Cihak et al. in press).

All three sublines of T cells are generated sequentially in the first wave of thymocyte development (Fig. 2; Coltey et al. 1989). This was demonstrated by transplantation of the thymus from 9-day chick embryos into quail embryos and subsequent charting of chick T cell development in these chick-quail chimeras.

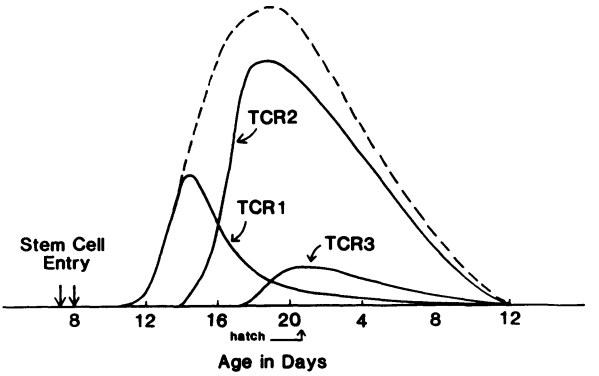

Fig. 2. Generation of the first wave of chick T cells in chick-quail chimeras

A THIRD LYMPHOCYTE LINEAGE

Lymphocytes of an unusual phenotype can be observed in the lymphoid tissues of chick embryos (Bucy et al. in press). These cells, which appear first in the spleen of the 8-day embryo, were noticed because of their cytoplasmic staining with the CT3 antibody (which identifies a determinant of the chick CD3 homologue) and their lack of surface TCR/CD3 receptor complexes. These cells, which we call TCRO cells for want of a better name, often express CD8 but apparently do not express the CD4 accessory molecule.

We have examined the migration and differentiation capabilities of the TCRO lymphocyte population in chick-quail chimeras (Bucy et al., in press). The most informative experimental design involved transplantation of the embryonic chick spleen into quail embryos. Spleens were removed as early as the 6th embryonic day (which is just before

the time when precursor cells begin to enter the thymus) and transplanted into 3-day quail embryos. In this model, the development of the chick TCRO population was unimpaired, and the TCRO lymphocytes migrated throughout the lymphoid system of the quail embryo recipients. They could be found in the spleen, thymus (medullary region only), bursa, and intestine; i.e., sites in which the TCRO cells are normally found in the chick embryo. None of the chick TCRO cells of splenic origin acquired cell surface TCR/CD3 or Ig receptors in the quail recipients, regardless of tissue location. These cytoplasmic $CD3^+$/surface $CD8^+$ cells thus appear to represent a separate lymphocyte lineage.

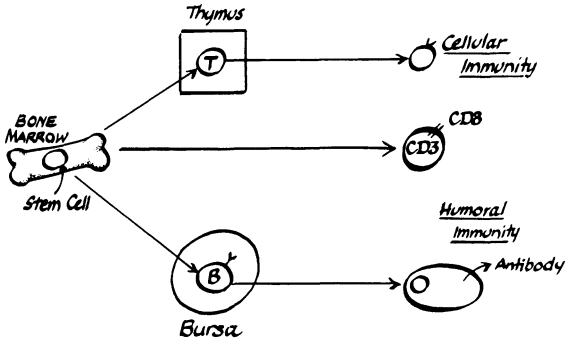

Fig. 3. Model of lymphoid development

The TCRO cells are also found in the adult. In the spleen, these cells represent a small minority of lymphoid cells (≈1%), but they constitute ≥30% of the intestinal epithelial lymphocytes.

CONCLUSIONS

The TCR1 subpopulation of T cells, which is highly conserved in birds and mammals, is a relatively large subpopulation in the chicken, comprising 20 to 50% of the peripheral T cell pool. The avian TCR1 subpopulation of cells is characterized by a 1) rapid maturation and transit through the thymus, 2) relatively high density of TCR1/CD3 expression, 3) relatively limited population expansion in both the thymus and periphery, 4) striking dependence on prolonged thymic seeding, 5) CD8 expression in peripheral lymphoid tissues,and 6) distinctive homing pattern. The TCR1 thymocyte subpopulation thus may not undergo the rigorous selection pressure that is so essential in shaping the TCR2 repertoire (Marrack and Kappler 1987). Their acquisition of the CD8 accessory molecule on homing to the peripheral lymphoid tissues is consistent with the hypothesis of a special role for TCR1 cells in body defense that is triggered by antigen presenting cells expressing the MHC class I or class I-like molecules.

The third type of TCR, generated relatively late in avian ontogeny, is especially intriguing. The avian TCR3 cells are similar to the TCR2 cells in their developmental pattern of TCR/CD3 acquisition and

their utilization of CD4 and CD8 accessory molecules. The TCR2 and TCR3 heterodimers could therefore be related isotypes, or they may reflect a different mode of TCR repertoire development, as is true for the generation of antibody diversity in birds (Weill and Reynaud et al. 1987). While information on the avian TCR genes is needed to resolve this issue, we favor the former possibility. We also postulate the TCR3 subpopulation of cells may exhibit specialized functional capabilities.

Our studies reveal the existence of a thymus-independent and bursa-independent lineage of lymphocytes that express a CD3-related antigen in their cytoplasm and CD8 on the cell surface. We propose that these cells represent a primitive lymphocyte population which persist in higher vertebrates because they serve an important role in body defense. The CD8 molecule on these cells, having both immunoglobulin variable and constant family domains, could represent the original antigen recognition molecule (Davis and Bjorkman 1988). Thymus-independent lymphocytes capable of mediating skin allograft rejection and a relatively imprecise memory response in Xenopus (Nagata and Cohen 1983) could represent cells of this lineage. It is also interesting to note that a population of lymphocytes in man with similar properties has recently been found to express CD3ϵ RNA but no TCR/CD3 receptors (Ciccone et al. 1988). The exploration of the obvious implications of a primitive lymphocyte lineage which uses a primordial antigen receptor molecule for target cell recognition is likely to merge ultimately with the trail of the amorphous natural killer cell.

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Analysis of Hematopoietic Stem Cell Development In Vivo

G. Keller

A. Structure of the Hematopoietic System

Hematopoiesis, the production of blood cells, begins early during embryogenesis and continues throughout adult life. Continuous hematopoietic activity is necessary since most blood cells have a short life span and need to be replaced as they die. The cells responsible for producing new blood cells are referred to as precursor cells and are found in the primary hematopoietic organs; the embryonic yolk sac, the fetal liver and the adult bone marrow. The structure of the hematopoietic system has been best defined in the mouse and most evidence now suggests that the precursors are organized in a hierarchy as shown in Fig. 1.

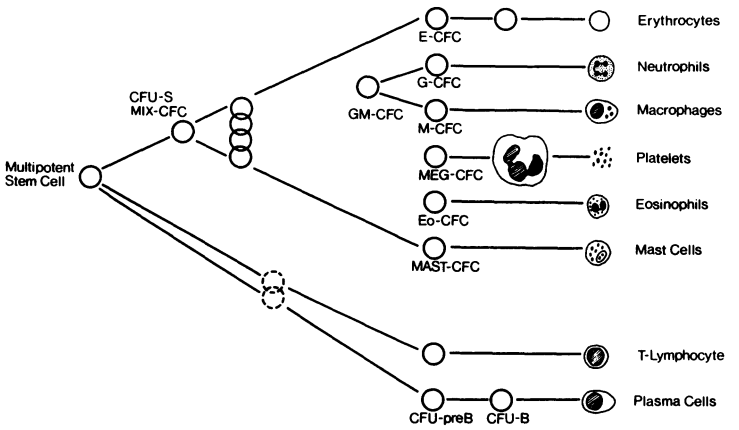

Fig. 1 Schematic representation of the hematopoietic system of the mouse

The most primitive precursors are multipotential and are unique within the system in that they have the capacity to generate progeny of all the myeloid and lymphoid lineages as well as the capacity to generate new multipotential cells i.e. self renew. These primitive multipotential cells can be considered as the true stem cells of the hematopoietic system. Although the term "stem cell" can and has been used for other classes of hematopoietic precursors, for the purpose of this paper it will be used to refer to those cells which display the capacity to maintain hematopoiesis over long periods of time *in vivo*. As these multipotential stem cells differentiate, they give rise to progeny that are more restricted in their developmental potential and in general have little or no self-renewal capacity. Among these progeny one can identify pluripotential precursors which retain the capacity to differentiate along all the myeloid lineages but lack

lymphoid potential, oligopotential precursors that are restricted to two or three myeloid lineages and unipotential precursors that are committed to a single blood cell lineage.

Most of these intermediate and late stage precursors have been identified by one of two colony assays. The first, the CFU-S assay, detects precursors which are able to generate a colony of hematopoietic cells in the spleen of an irradiated recipient mouse (Till and McCulloch 1961). Initially it was thought that these spleen colony-forming cells (CFU-S) were the most primitive stem cells within the hematopoietic system. However, most evidence now suggests that they are precursors already committed to the myeloid (non-lymphoid) pathway of differentiation and range in developmental potential from pluripotential cells to those committed to a single blood cell lineage (Paige et al. 1979; Harrison 1980; Kitamura et al. 1981; Magli et al. 1982). The second assay that has been used extensively to deliniate the various stages of development within the myeloid compartment of the hematopoietic system is the in vitro colony-forming cell (CFC) assay (reviewed in Metcalf 1977). This assay detects precursors that are able to generate colonies of mature blood cells in semi-solid cultures in response to specific growth factors. The spectrum of precursor cells detected by this assay is somewhat broader than that detected by the CFU-S assay and ranges from pluripotential cells which are able to generate large multilineage colonies to relatively mature restricted cells that form small colonies (<100 cells) of cells from a single lineage (Johnson and Metcalf 1977; Iscove 1978; Suda et al. 1983). As with the CFU-S assay, the most primitive stem cells are not detected by this in vitro colony assay.

These primitive stem cells can only be identified by their capacity to reconstitute the hematopoietic system of an irradiated or a genetically hematopoietic deficient W/W^v mouse. The fact that a population of cells can reconstitute the hematopoietic system of another animal does not in itself demonstrate the multipotential nature of the reconstituting cells, as cells already committed to the various lineages could be responsible for the reconstitution. The existence of multipotential cells has been demonstrated by experiments in which the cells used for reconstitution were "uniqely" marked prior to implantation into the recipient animals. The first series of this type of experiments was carried out using radiation induced chromosomal translocations as unique markers (Wu et al. 1968; Abramson et al. 1977). These studies demonstrated the existance of multipotential cells capable of generating both myeloid and lymphoid progeny. The existence of multipotential cells does not exclude the possibilty that cells restricted in their developmental potential can also repopulate limited numbers of lineages in these recipient mice. The concept of "restricted" stem cells has been proposed earlier from experiments which demonstrated the presence of a unique marker in a limited number of lineages of reconstituted mice (Abramson et al. 1977; Dick et al. 1985). However, as will be discussed in the following section, the fact that some clones derived from multipotential cells can be found in a limited number of lineages for significant periods of time following reconstitution indicates that further experimentation is required to conclusively demonstrate the existence of such restricted cells.

More recently a number of groups have used the retroviral integration site of recombinant retroviruses as a unique marker and have been able to confirm the existence of multipotential stem cells (Dick et al. 1985; Keller et al. 1985; Keller and Wagner 1986; Lemischka 1986; Snodgrass and Keller 1987). In addition to confirming the existence of these mutipotential cells, this method of uniquely marking these cells has provided an approach with which to address issues concerning lineage relationships, longevity of stem cells and clonal stability of the hematopoietic system that were previously difficult or impossible to study.

B. Analysis of Hematopoietic Stem Cell Development by Retrovirus-Mediated Gene Transfer

The method of marking hematopoietic stem cells with retroviruses is relatively simple and has been previously published (Keller et al. 1985; Lemischka et al. 1986; Keller and Wagner 1989). Briefly, bone marrow cells are infected by mixing them with a

supernatant containing the recombinant retrovirus or coculturing them directly with the virus producing cells. These infected cells can be either introduced directly into recipient mice (irradiated or W/W^v recipients) or, in those situations where the virus expresses the selectable *neo* gene, first preselected in G418 and then used for reconstitution. At appropriate times following reconstitution the animals are sacrificed and DNA is prepared from the various tissues and cell populations derived from them. This DNA is digested with a restriction enzyme that does not cut within the proviral sequences and analysed by standard Southern blotting techniques. Provided that only one virus has infected each stem cell, each of the resulting hybridizing bands represents a unique integration site and thus each can be regarded as representing a single clone.

One of the first intriguing observations made from the analysis of the hematopoietic system of mice reconstituted with retrovirus transduced stem cells was that relatively few clones could be detected in the myeloid and lymphoid lineages of many of these animals, suggesting that a small number of stem cells was sufficient for maintaining hematopoiesis (Dick et al. 1985; Keller et al. 1985; Keller and Wagner 1986; Lemischka et al 1986). One of the limitations of this type of analysis is that only the largest clones are detected and therefore it is possible that many small clones in addition to a few large ones could be present in these animals. However, when the intensity of the bands representing the various clones was compared to control DNA made from a cell which contained a single copy of the transduced gene it became apparent that most of the cells (50-90%) belonged to these limited numbers of clones indicating that indeed a small number of stem cells was sufficient for maintaining hematopoiesis in these recipient mice. One of the puzzling aspects of this observation was that in many instances the number of stem cell derived clones was significantly lower than one would have predicted from the number of cells used for reconstitution. There are at least two explanations that could account for this low number of stem cells in these reconstituted mice.

First, it is possible that the manipulations involved in infection of the bone marrow, i.e viral infection and/or preselection in G418 are not optimal for the stem cells and thus only a small proportion can survive to the reconstitution stage. This is most likely a contributing factor as the conditions required for the survival of reconstituting cells in culture are unknown.

A second explanation for the limited number of clones found in reconstituted mice is that at any given time, only a small portion of the total stem cell pool is active, the remainder being essentially dormant. If this is true, then one would predict that over a period of time or in situations of severe hematopoietic stress some of these inactive stem cells should become active and new clones should emerge. The time required for the appearance of the new stem cells would depend, to some extent, upon the life-span of normal stem cells in these transplanted animals. A number of groups have proposed that stem cells have a limited life span and as a consequence, the hematopoietic system is maintained by a succession of relatively short-lived clones (Kay 1965; Mintz et al. 1984; Lemischka et al. 1986). If this model is correct, it should be possible to detect clonal changes within the hematopoietic system of mice over an extended period of time. This issue can be addressed by analysing the clonal make-up of the hematopoietic tissues of mice reconstituted with retrovirus transduced stem cells at several points in time following the transplantation.

Lemishka et. al. (1986) analysed the clonal make-up of primary and secondary recipient animals to determine whether or not retransplantation of bone marrow could activate any "silent" stem cells. In one secondary animal they found clones that were not present in the primary mouse while in another secondary recipient only those clones present in the primary animal could be identified. In a previous study we passaged bone marrow cells from a primary, to a secondary and finally to a tertiary recipient and did not detect the appearance of any new clones (Keller et al. 1985). Thus at present it is difficult to determine to what extent new clones do appear upon transplantation of bone marrow

cells. In another series of reconstituted animals, this same group analyzed DNA from successive samplings of peripheral blood and compared the clonal make-up of the different time points. Although some changes were noted (mostly an increase in size of existing clones) the majority of the clones were present at all time points (2-4 months). These findings would suggest that there are not any dramatic changes in the clonal make-up of the hematopoietic system of reconstituted mice at least within this period of time.

To analyse, in more detail, possible changes in the clonal make-up of the hematopoietic system, we developed a protocol in which we removed part of the spleen from mice relatively soon after reconstitution and compared the clonal make-up of cell populations (myeloid and lymphoid) derived from it to the clonal make-up of the same lineages taken from the remaining portion of spleen or bone marrow at a later time point (Snodgrass and Keller 1987). In the first experiment, we analysed the clonal make-up of the hematopoietic system of mice at 7 weeks and 5 months following reconstitution. From this analysis we were able to demonstrate that within this period of time the hematopoietic system consists of clones which were present in all tissues and cell populations at both time points (stable clones) as well as of clones that underwent temporal changes. Within this latter category, one of the most interesting and frequent changes that we observed was the presence of a clone in a limited number of lineages (eg. myeloid) at one point in time and the emergence of this same clone in other lineages (lymphoid) at a later time point. This pattern of change could represent the expansion of a stem cell-derived clone into these lineages at different times following reconstitution. The time at which a clone can be detected in a particular lineage could be influenced, to some extent, by the need for differentiated cells within that lineage. Thus it is possible that a clone derived from a multipotential stem cell could contribute progeny to a limited number of lineages at one time and then to other lineages at another time. If these animals had been assayed at only one time point, the presence of a clone in a limited number of lineages could have been interpreted as evidence supporting the existence of stem cells with a "restricted" developmental potential. However this clearly would have been a wrong interpretation as the stem cells which gave rise to them were multipotential. This finding highlights the difficulty in addressing the issue of restricted stem cells and demonstrates the need for sampling tissues at multiple points in time to unequivocally demonstrate the existence of such cells. Those clones which showed no changes between the two points in time (stable clones) most likely represent the progeny of primitive stem cells which had already expanded into the various lineages at the time of the first analysis.

In an attempt to estimate the longevity of these primitive stem cells, in a second experiment we analysed hematopoietic tissues of mice at 2 and 10 months following reconstitution and then passaged the bone marrow to secondary recipients and analysed these animals following a further 7 month period (Keller and Snodgrass, manuscript in preparation). Thus the total time of clonal analysis spanned a period of 15 months. From this analysis, several important conclusions can be made concerning the long-term function of hematopoietic stem cells in reconstituted mice.

First, we were able to identify some clones at the 2 month time point that persisted for the duration of the experiment, demonstrating the existence of a class of primitive stem cells that can function for a significant portion of the lifetime (15 months) of a mouse. This observation would be consistant with data from a recent report of Harrison et al. (1988) which also supports the existence of long-lived stem cells. Our findings also indicate that by 2 months after reconstitution, one can identify the progeny of at least some of the primitive stem cells within the myeloid and lymphoid tissues of reconstituted mice. However, not all clones were readily detectible at the early 2 month time point. We found a number of clones which were very difficult to identify at 2 months but were present as major clones at 10 months following reconstitution and then persisted throughout the course of the experiment. This would indicate that not all stem cell-derived clones have expanded fully by 2 months following reconstitution and thus demonstrates the need to analyse recipient mice beyond this time when measuring stem cell function.

A second important observation came from the analysis of the secondary recipients in this experiment. Two secondary recipients were reconstituted with bone marrow from each of the primary animals. When the clonal make-up of the hematopoietic system of these mice was analyzed, it was found that many of the same clones could be found in both animals. These clones were also found in the primary recipients. This observation would indicate that as the stem cell in the primary recipient differentiated it gave rise to a minimum of 2 new stem cells which could reconstitute the hematopoietic system of the secondary recipients. This finding is the first indication that the primitive stem cell population can actually expand.

C. Conclusions

The ability to uniquely mark hematopoietic cells with recombinant retroviruses has provided a method for studying aspects of stem cell development *in vivo* that were previously difficult or impossible to study. The analysis of the hematopoietic system of mice reconstituted with retrovirus tranduced stem cells has demonstrated the existence of a class of primitive hematopoietic stem cells that can function for a significant portion of the lifetime of a mouse (15 months). The existence of these cells does not support the model which predicts that hematopoiesis is maintained by a succession of short-lived clones but rather suggests that hematopoietic activity is maintained by stable long-lived clones. Some changes can be detected in the clonal make up of the various hematopoietic lineages relatively soon after reconstitution. However, many of them appear to represent the expansion of mutipotential stem cell-derived clones into the various lineages at different times following reconstitution. The fact that large numbers of new clones do not appear with time would indicate that the small number of clones found within the hematopoietic tissues of some of the reconstituted mice reflects the actual number of stem cells that were transplanted and are maintaining hematopoiesis in these mice. Finally, the analysis of the secondary recipients has provided evidence suggesting that the primitive multipotential stem cell population can expand, at least during regeneration of the hematopoieitic system. The extent to which this population can expand and whether or not there exists a specific environment that favours this expansion will be the subject of future experimentation.

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Utilization of a Bone Marrow Suspension Culture System for the Analysis and Manipulation of T-Cell Development

S.D. Thompson, J. Pelkonen, J. Samaridis, and J.L. Hurwitz

INTRODUCTION

In an attempt to simplify the study and manipulation of T-cell development, many have examined the ability of cells to differentiate in a bone marrow suspension culture. Several years ago, it was reported that bone marrow cells generated T-cell markers and function following a short term incubation with thymosin or agents of non-thymic origin such as poly A:U (Scheid 1973). Subsequent reports from numerous other laboratories also indicated a generation of T-cell function from immature populations in various suspension culture systems. It was difficult, however, to prove that differentiating cells were indeed responsible for the T-cell activity. An alternative possibility was that a small number of mature T-cells contaminated the starting cultures and that these rare cells expanded in number to account for the T-cell function.

Debates regarding the possibility of T-cell development in suspension culture have persisted for many years. In order to resolve the issue, we have analyzed one bone marrow suspension culture system in much detail with regard to phenotypic, functional and molecular changes as well as precursor-product relationships between early and late populations (Hurwitz 1986, 1987, 1988 and unpublished results). By this means, the starting cells have been rigorously shown to differentiate along pathways similar in many ways to developing fetal thymocytes. It has become clear that a simple expansion of mature T-cells cannot account for the results. The culture components appear to provide an activation signal normally received in the thymus. The developmental program which follows may be innate to the bone marrow precursor as phenotypic changes occur in the absence of thymus cell contact.

Suspension culture systems may now be used to reveal the potentials of T-cell development and to direct follow-up in vivo experimentation. The cultures may further supply tools with which T-cells developing in vitro may be used to alter inadequate T-cell development in vivo. We discuss strategies for the potential replenishment of T-cell pools in vivo, promotion of tumor rejection and enhancement of success in bone marrow transplantation.

EXPERIMENTAL SYSTEM

The culture system discussed in this report was composed of T-cell depleted bone marrow cells (anti-Thy-1.2 antibody plus complement-treated cells, 1 x 10^6 cells/ml), irradiated splenic feeder cells (3600R, 2 x 10^6 cells/ml) and a Con A Supernatant (CAS) enriched medium (RPMI, 10% FCS, 4 mM glutamine, 50 μg/ml gentamicin, penicillin-streptomycin, 5 x 10^{-5} M 2-Mercaptoethanol, 10 mg/ml α-methyl mannoside, 10% Rat CAS). The cells were analyzed over a two week incubation period, during which feeders were replenished on day 2 and media was replenished on day 6.

The development of T-cell function was first monitored by use of a cytotoxic assay with the P815 mastocytoma cell as the target line. (Hurwitz 1986, 1987) There was no detectable killing activity at an 80:1 effector:target ratio during the first 5 days of culture, and yet by day 8, killing function was strong at an 0.3:1 effector:target ratio. This revealed a 10^2-10^3-fold increase of activity over a 3 day culture period. Function was also analyzed by an anti-T-cell receptor (TCR) antibody mediated killing assay. In this case, the F23 antibody (specific for Vβ8-encoded TCR) was used to link TCR-positive effector cells to the Fc-receptor positive-U937 human cell line (Leeuwenberg 1985). Again, killing in this assay was absent among early cultured cells, but was strong in late staged cultures. These results together revealed the acquisition of functional TCR cell surface molecules within the second week of the bone marrow culture.

Previous analyses of fetal thymocytes during ontogeny have provided a great deal of information regarding the distribution of T-cell markers on developing cells. For example Habu and Okumura (1984) have shown a progressive decrease in asialo-GM1-positive cells and a reciprocal increase in Thy-1 positive cells between days 13 and 20 of fetal thymus development.

We chose to test the Thy-1 and asialo-GM1 markers among developing populations in bone marrow cultures. To do so, late staged cultures were treated either with anti-Thy-1 antibody or anti-asialo-GM1 antibody plus complement immediately prior to a P815 directed cytotoxic assay. The killing activity was then compared to cells treated with complement only (Hurwitz 1987). Results are reviewed in Table 1. Anti-asialo-GM1 antibody plus complement treatment of day 10 cultured cells lead to a significant reduction in killing function compared to controls. The same treatment on day 12 had a lesser effect. An opposite result occurred following the use of anti-Thy-1 antibody plus complement. In this case, the reduction on day 8 was low compared to the reduction on days 10 and 12. Apparently, while the asialo-GM1 density was steadily decreasing in the effector population, the Thy-1 density was increasing similar to the situation among fetal thymocytes.

Table 1 also shows the effect of day 0 antibody treatments (prior to initiation of culture) on day 8 function. While anti-Thy-1 antibody had no effect (by definition) on the generation of killers, the additional asialo-GM1 treatment on day 0 completely abolished kill on day 8 (Hurwitz 1987). These results reinforced the trend of decreasing asialo-GM1 with increasing Thy-1 antigens on individual cells. Results could not simply be explained as a death of asialo-GM1 bearing cells and expansion of Thy-1 bearing cells. Should that have been the case, the removal of asialo-GM1-positive cells at the beginning of culture would have had a positive rather than negative effect on killer cell expansion.

Table 1. Antibody plus complement depletion of cytotoxic cells from bone marrow culture

	Day of Culture			
Antibody specificity	0	8	10	12
asialo GM1	↑↑↑↑[a]	NT	↑↑	↑
Thy-1	0	↑↑↑	↑↑↑↑	↑↑↑↑

[a] The effectiveness of antibody plus complement treatment on the depletion of P815-directed killing function is indicated by arrows. One, two, three and four arrows indicate 1-25%, 25-50%, 50-75% and 75-100% respective depletion of killing activity compared to complement-only treated controls. NT = Not tested.

A next antigen of considerable interest was the J11d antigen. Bruce et al. (1981) had previously shown that the J11d antigen was present on the majority of bone marrow cells, but was absent on stem cells. The antigen appeared on most thymocytes, but was absent on functional T-cells.

Studies were done (as above) to determine the presence or absence of the J11d antigen on cultured bone marrow cells. The treatment of cells with the J11d antibody in addition to the anti-Thy-1 antibody plus complement prior to culture caused no depletion of functional cells. In fact, the killing was greater than normal as tested on day 8. A treatment with the J11d antibody and complement on day 4 caused a severe depletion of killing function as tested on day 8. Finally, the treatment immediately prior to the killing assay on day 8 had no effect on function (Hurwitz 1987). The results suggested that J11d-negative cells gained J11d during the course of development, but lost the antigen as function was attained. Again, the situation matched that of T-cell development in vivo.

An alternative explanation for the previous experiment was that a J11d-positive accessory cell which was absent in the starting population developed in mid-culture and prompted expansion of a contaminating killer population. An experiment which disproved this possibility was done as follows: Bone marrow cells were isolated from two sets of mice which were congenic at the Thy-1 locus [C57Bl/6 mice (bearing the Thy-1.2 allele, referred to as B6) and

B6.PL Thy-1^a mice (bearing the Thy-1.1 allele, referred to as PL)]. Cells were treated with antibody and complement to remove T-cells and J11d-positive cells prior to culture. They were then placed in separate cultures in the presence of CAS and splenic feeders (day 0). On day 1, cells were removed from culture and treated either with J11d antibody plus complement or complement only. Cells were then mixed in three ways. 1) B6-'Complement only' treated and PL-'Complement only' treated. 2) B6-'Complement only' treated plus PL-'J11d antibody and Complement' treated, and 3) B6-'J11d antibody and Complement' treated plus PL-'Complement only' treated. By this method, each culture retained accessory cells from complement only-treated populations. Killer precursors from B6 and PL populations should therefore have survived equally well unless they were specifically depleted by the antibody treatment prior to mixing. On day 8, anti-Thy-1.2 and anti-Thy-1.1 antibody plus complement treatments were performed prior to a functional assay to determine from which bone marrow population killer cells derived.

Results are shown in Fig. 1. In the control culture shown in the

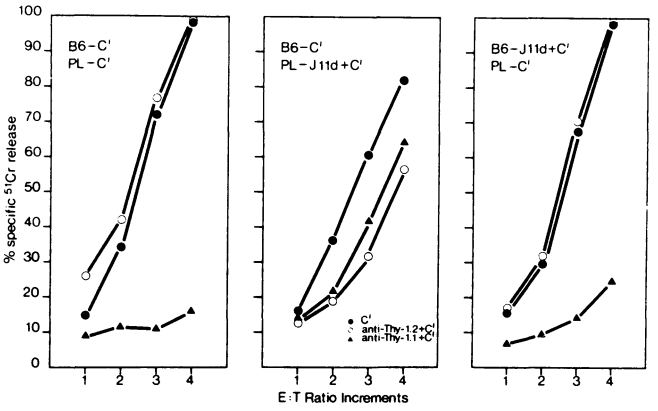

Fig. 1. J11d treatment in intermediate culture removes the precursor to the day 8 killer cell. Bone marrow cells were harvested from day 1 B6 and PL cultures. They were treated with: 1) complement only (first panel), 2) 'complement' and 'J11d antibody plus complement' respectively (2nd panel) or, 3) 'J11d antibody plus complement' and 'complement' respectively (3rd panel) prior to mixing for re-culture. On day 8, cultures were harvested and treated with either complement (closed circles), 'anti-Thy-1.2 antibody and complement' (open circles) or 'anti-Thy-1.1 antibody and complement' (triangles). Cells were then assayed for killing activity towards ^{51}Cr-labeled P815 target cells during an 8 hour incubation period. E:T ratio increments 1-4 represented successive 3-fold increases in effector cell numbers. The target cell number (5 x 10^3 cells/well) was unvarying. Increments #4 were E:T ratios of 6:1, 18:1, and 4:1 for complement treated effector cells (closed circles) in panels 1-3 respectively. Cells from each culture were aliquoted evenly prior to the three distinct treatments on day 8 and cell numbers were not readjusted prior to assay.

first panel (B6- 'complement treated',PL-'complement treated') the PL cells provided essentially all of the killing activity (as shown by depletion with the anti-Thy-1.1 and complement treatment directly prior to the killing assay). This result revealed a yet unexplained, but typical growth advantage of the PL cells in the mixed population. Treatment of the B6 cells with J11d antibody on day 1 (third panel) therefore had no effect on the functional profile. However, when J11d antibody plus complement treatment was performed on the PL population on Day 1 prior to mixing, the functional PL cells (as tested on Day 8) were depleted and the B6 cells contributed to the majority of the killing function (2nd panel). Had the J11d antibody and complement treatment depleted only an accessory cell, the degree of kill following treatment may have dropped, but the functional profiles of Thy-1.2 and Thy-1.1-positive killer cells should not have changed. The fact that the PL population lost an ability to generate function following treatment despite the presence of the B6 accessory cells showed that the J11d-positive cell was the true precursor.

T-cell Receptor Gene Rearrangements in Bone Marrow Cultures

Hybridomas were prepared by fusing cultured cells at various times with the BW5147 thymoma line. Southern blots of the hybridoma DNA showed that TCR β and γ rearrangements were present by day 6 of culture, but occurred at a greater frequency and exhibited a greater complexity on day 12 (Hurwitz 1988). β rearrangements for example were often Dβ1-Jβ1 joins on day 6, while on day 12, V-D-J joins were more common. The β rearrangements also encompassed the Jβ2 gene more often on day 12 than on day 6. Jγ1 rearrangements occurred more often on both chromosomes among day 12 versus day 6 hybridomas.

A next analysis involved Jα gene rearrangements. The Jα locus covers a region of approximately 65 kb on mouse chromosome 14. To determine whether rearrangements were randomly distributed among the Jα genes or skewed within isolated regions of the locus, a Southern blot analysis was done.

Two Jα probes (designated A and B) were used which hybridized with regions towards the 5' end and middle of the Jα locus, respectively. A Cα probe was used to determine the number of bone marrow-derived α-bearing chromosomes in each cell. Southern blot patterns revealed the position of Jα gene rearrangements on the homologous chromosomes derived from the bone marrow parent. Rearrangements were designated 5' if they occurred in the region near probe A or between probe A and B. Rearrangements were designated mid-3' if they occurred in the region near or 3' to probe B. Because the hybridomas were prepared from (C57BL/6 x DBA/2)F1 bone marrow cultures, the two bone marrow-derived chromosomes could be easily distinguished from each other and from the BW5147-derived chromosomes.

Hybridomas from four bone marrow cultures were tested. Within the first culture, eight hybridomas were shown to retain two α-bearing chromosomes with rearranged Jα genes. All but two bone marrow derived rearrangements in these cells appeared in the 5' region of the Jα locus. The opposite situation occurred within cultures 2-4. Among another eight hybridomas shown to bear two bone marrow derived α-bearing chromosomes, all but one rearrangement appeared in the mid-3' position. These results revealed an unusual skewing of rearrangements to different portions of the Jα locus correlating with the cultures from which they derived. There was only one

hybridoma in which rearrangements fell into two separate regions (5' and mid-3') on homologous chromosomes.

One hybridoma was further analyzed by detailed Southern blot analysis. Both bone marrow-derived rearrangements occurred either precisely at the Jα27 gene or within a 1 kb region bordering the gene. This further illustrated a tight association of Jα rearrangements during development.

DISCUSSION

The work described thus far has been done to determine the potentials of T-cell differentiation outside of the thymus. In the presence of CAS and splenic feeder cells, T-cell depleted bone marrow cells undergo a number of phenotypic changes typical of fetal thymus development. These include an increase in Thy-1 expression, a decrease in asialo-GM1 expression and a sudden acquisition of killing activity. Other events typical of in vivo development include a sequential gain and loss of the J11d antigen and presence of complex TCR gene rearrangements plus anti-TCR antibody-mediated function in late cultures.

Together the previous results unveil a number of differentiation events which take place during a two week culture of bone marrow cells. The changes reflect in vivo results, but add precursor-product information to the scheme. The fact that such schemes proceed in the absence of the thymus, but mimic thymus events, suggest that much of the differentiation pathway is a program inherent to the bone marrow cell. Triggering of the pathway either intra- or extra-thymically leads to a similar course of phenotypic changes.

An unexpected finding among hybridomas prepared from bone marrow cultures was skewing of Jα rearrangements within cells derived from single cultures. Multiple explanations exist for this result. One consideration is that the fine composition of factors which may vary between cultures may predispose certain regions of the J locus to rearrangement. There is precedent for this type of directed switch among B-cells stimulated in vitro, being that the factor composition during plasma cell development will skew the usage of C_H genes in joining events (Lutzker 1988).

It is not yet clear to what extent skewed Jα gene usage occurs during T-cell development in vivo. β, γ and δ TCR genes have already been shown to rearrange non-randomly during fetal ontogeny (Elliott 1988; Born 1985; Garman 1986). The restriction of Jα gene usage would further deplete the number of functional α-β pairs available to the T-cell pool in young animals.

Supplementation of T-cell Populations In Vivo with T-cells Generated from the In Vitro System

Experiments are underway to test the potential of bone marrow cultures for supplementation or manipulation of T-cell repertoires in vivo. As illustrated in Fig. 2, three main questions are presently being considered:

Question #1: Can T-cells which have differentiated in vitro be used to provide a T-cell pool to athymic individuals? As illustrated in Fig. 2A, We are presently approaching this question by isolating bone marrow from athymic mice, placing cells in culture for variable time intervals and re-injecting cultured cells into athymic mice. A good deal of evidence is now available showing that IL-2 enhances T-cell mediated responses in nude mice (for review see [Hünig 1983]). We will examine the possibility of stably repopulating athymic mice with a heterogeneous T-cell pool. If successful, we will evaluate the function and TCR repertoire provided by the procedure.

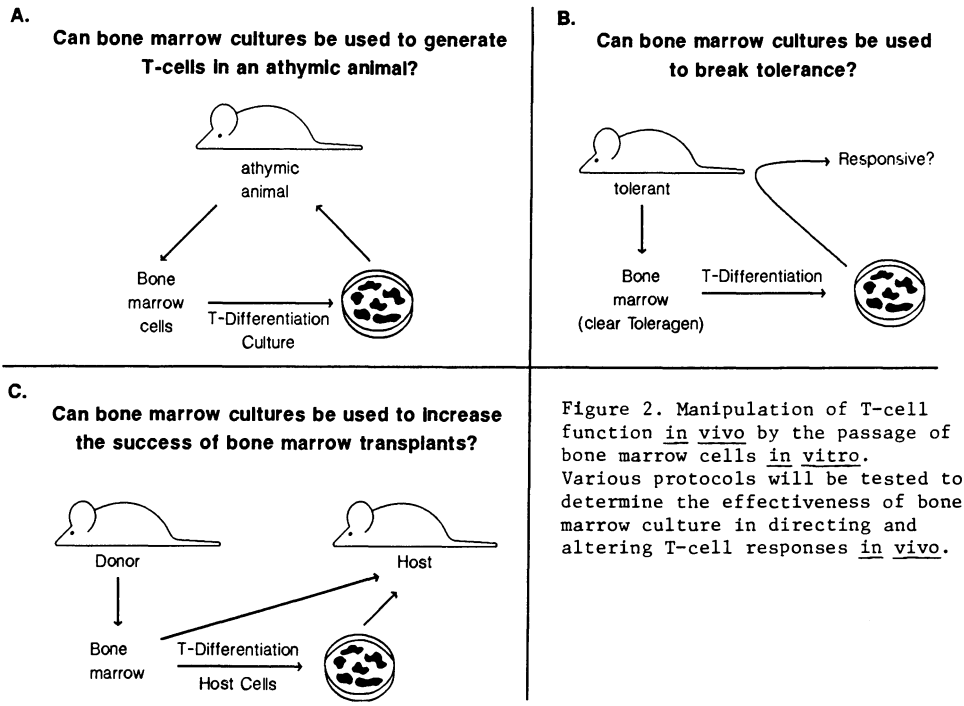

Figure 2. Manipulation of T-cell function in vivo by the passage of bone marrow cells in vitro. Various protocols will be tested to determine the effectiveness of bone marrow culture in directing and altering T-cell responses in vivo.

Question #2: Could the bone marrow culture be used to break tolerance in vivo -particularly to tumor antigens?(Fig. 2B) It is possible that the presentation of tumor cells in young or immunosuppressed individuals may render a persistent state of non-responsiveness. As new T-cells arise, tumor-specific T-cells may undergo clonal deletion (Kappler 1987) if they contact tumor antigens during an immature stage of development. This would yield an insufficient T-cell defense against tumor cells. Although the tumor may be easily depleted in vitro (perhaps by antibody and complement treatment), the same depletion in vivo may not be possible. If bone marrow cells were isolated, depleted of tumor cells and allowed to differentiate in vitro, functional tumor-specific cells might be generated which could attack the tumor target upon re-injection into the original donor. This method would

provide an alternative to present protocols aimed at enriching tumor-specific T-cell populations. Rather than to specifically expand populations thought to possess tumor-killing activity in tumor-bearing animals, the strategy would provide a newly differentiated and heterogeneous T-cell population (in the deliberate absence of tumor cells) within which tumor-specific cells may reside.

Question #3: Could bone marrow cultured cells be used to enhance allogeneic or semi-allogeneic bone marrow transplantation?(Fig. 2C) A common concern regarding bone marrow transplantation is that the depletion of mature T-cells from bone marrow grafts prevents graft versus host reactions in MHC mismatched individuals, but lessens the frequency of graft survival (Gale 1986). We are now testing donor marrow which is T-cell depleted and cultured with irradiated splenic feeders from the host. We hope to generate a T-cell population which will accept the host. The resultant population may be injected alone or in conjunction with fresh donor marrow into host animals to determine if graft survival is enhanced.

In conclusion, the bone marrow suspension culture provides a system which parallels by several criteria normal T-cell differentiation. Although the thymus may be the most sophisticated environment for T-cell development, alternative signals appear to initiate a differentiation program inherent to the bone marrow precursor. The system may be used to examine the potentials of T-cells throughout development and perhaps to alter T-cell responsiveness in vivo.

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Diversity, Development, Ligands and Probable Functions of γδ T Cells

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INTRODUCTION

T cell receptors (TCR) play a pivotal role in the immune system by recognizing and distinguishing diverse antigens. Until recently all TCR were thought to be made up of a heterodimer composed of α and β subunits. However, during the search for the genes encoding these TCR subunits, a third gene called γ which shares a number of characteristics with TCR α and β genes was discovered (Saito et al. 1984; Kranz et al. 1985; Hayday et al. 1985). Subsequent studies established that this gene encodes one polypeptide chain of the second TCR composed of a heterodimer γδ (Brenner et al. 1986; Bank et al. 1986; Weiss et al. 1986).

Despite the striking similarities in the overall structure of their genes and polypeptide chains, TCR γδ and the T cells which express it are significantly different from their αβ counterparts. For instance, γδ T cells are detected in both the thymus and peripheral lymphoid organs in relatively low numbers (< 5% of T cells), but predominate (50-100%) within epithelia, such as epidermis (Stingl et al. 1987; Kuziel et al. 1987) and small intestine (Bonneville et al. 1988; Goodman and Lefrancois 1988). In contrast to most αβ T cells, the majority of γδ T cells in the thymus and spleen do not express either CD4 or CD8 (Brenner et al. 1986; Bank et al. 1986; Lew et al. 1986; Nakanishi et al. 1987; Maeda et al. 1987). Neither the specificity of γδ TCR recognition nor γδ T cell function in immune defense is understood. In this paper we summarize results of our recent studies on the diversity, development, and specificity of γδ T cells, and discuss possible functions of these cells.

RESULTS

Anti Mouse γδ TCR mAbs

To study the nature of the TCR γδ and the role of γδ T cells, monoclonal antibodies (mAbs) directed against the native receptor are useful. To generate such antibodies we immunized Armenian hamsters with the anti CD3 immunoprecipitate of a lysate of γδ T hybridoma, KN6 (Ito et al. 1989) and prepared three anti γδ TCR mAbs (Itohara et al. 1989a): mAb 3A10 specific for a C_δ constant region determinant, mAb 8D6 specific for a $V_{\gamma 4}$- and $V_{\delta 5}$-encoded γδ TCR, and mAb 5C10 specific for a KN6 TCR idiotype.

Appearance of αβ and γδ T Cells in the Developing and Mature Thymus

Using appropriate mAbs, we determined the number of thymocytes bearing γδ TCR (abbreviated hereafter as γδ-thymocytes) or αβ TCR (αβ-thymocytes) as a function of age (Fig. 1). Only 0.4 to 0.6% of total thymocytes at E14.5 are γδ-thymocytes, increasing rapidly to its highest value (5%) at E16.5, then gradually decreasing through embryonic life and the first postnatal week until it reaches a stationary adult level of 0.3 to 0.5% at about ten days after birth. αβ-thymocytes are rare (<100 cells per thymus) at E14.5 but outnumber γδ-thymocytes after E16.5. By E18.5 they are the dominant thymocyte population. We analyzed the thymocytes by using γδ (3A10)

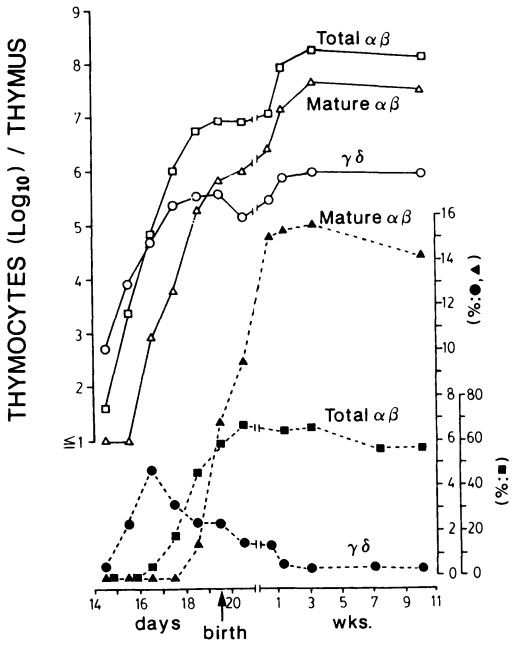

Figure 1. Ontogeny of TCR γδ, αβ thymocytes in C57BL/6 mice. Thymocytes of mice at different ages were stained with anti γδ (3A10) or anti αβ (H5-597) biotin conjugates followed by a streptavidin PE conjugate together with an anti-CD3 (2C11) FITC conjugate. The samples were analyzed with FACScan (Becton-Dickinson) using FACSCAN software.

and αβ (H57-597) mAbs in two color analysis. The result indicated that the αβ and γδ TCR double positive cells are extremely rare, if any, suggesting the existence of a mechanism restricting the surface expression of TCR to only one of the two types.

Intrathymic Distribution of γδ T Cells Alters Drastically During the First Postnatal Week

We analyzed sections of fetal and adult thymi using immunohistochemical technique. Adjacent sections were alternatively labeled with the mAb 3A10 or with the Ulex europeus agglutinin (UEA) which we have previously shown to be a reliable marker for medullary thymic epithelial cells in the adult murine thymus (Farr and Anderson 1985). We observed a striking co-localization of $\gamma\delta^+$ thymocytes and UEA^+ medullary epithelial cells during late fetal and neonatal periods of development (Fig. 2). In contrast, γδ thymocytes were scattered throughout cortical and medullary areas of the thymus (Fig. 2) and most concentrated in the subcapsular areas of the thymus in the thymuses of adult mice. Results of utrastructural immunohistochemistry showed that some γδ TCR molecules were patched to areas of contact with medullary epithelial cells. As shown below the pattern of γδ TCR expression in the thymus is limited in diversity and developmentally ordered. Thus these histochemical results indicate that changes in receptor repertoire correlates with the changes in the intrathymic distribution of γδ thymocytes.

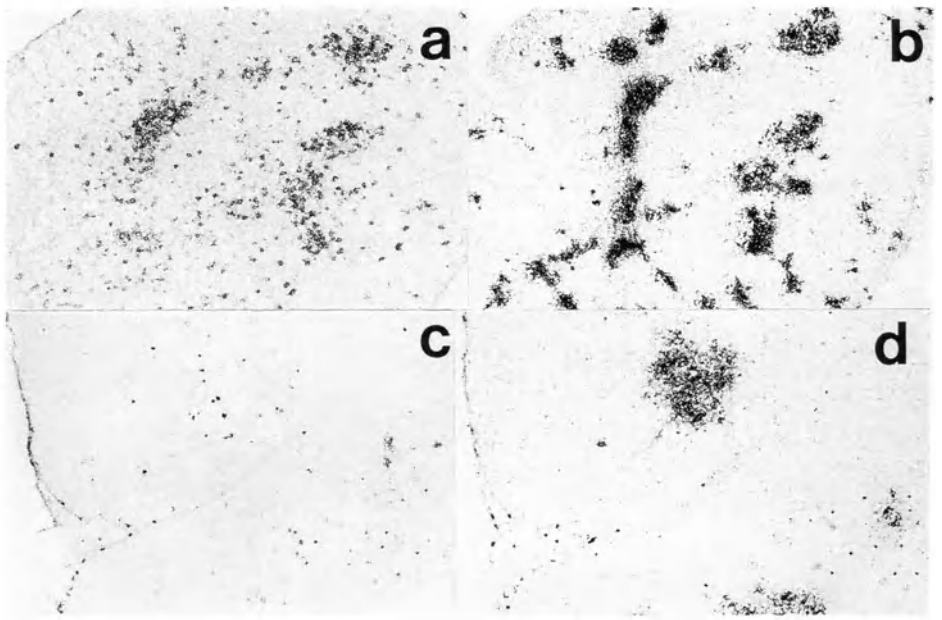

Figure 2. Intrathymic distribution of γδ cells in fetal and adult thymi. Adjacent serial sections of thymus tissue were labeled with anti-γ_w mAb 3A10 (a and c) or with Ulex europeus agglutinin (UEA) (b and d). Day 18, a, b; adult c, d.

γδ T Cells are Present in Many Organs Containing Epithelia

Since γδ T cells are relatively scant in peripheral lymphoid organs, we searched for these cells in sections of various organs and tissues. The results are summarized in Table 1. The γδ T cells were most abundant in small intestine, moderately abundant in tongue, stomach, and large intestine, and very scarce in esophagus. A substantial number of γδ T cells were also distributed in reproductive organs such as the uterus and vagina. In urinary organs, γδ T cells were observed in the bladder. Unlike the human and chicken epidermis (Bucy et al. 1988) the mouse epidermis is the site of a major γδ T cell subset. About $5x10^6$ γδ T cells are present within epidermis and hair follicles of each mouse. In many of the above organs γδ T cells were associated with the epithelial cells (Table 1).

For comparison, adjacent sections were stained with the mAb against αβ TCR. These cells were preferentially localized in the lamina propria of the intestine, in dermis of the skin, in the connective tissue of the vagina, and in the endometrium and myometrium of the uterus. Occasionally some αβ T cells were observed in association with the epithelia of the intestine, vagina, and uterus. However, the majority of $CD3^+$ intraepithelial lymphocytes are clearly γδ T cells. In light of these observations, we propose to refer to these T cells as intraepithelial lymphocytes (IEL) as a generic term and attach the initial of each organ to designate various IEL subpopulations, such as i-IEL, s-IEL, r-IEL, and t-IEL etc. for the IEL in intestine, skin, reproductive organs and tongue etc., respectively.

Table 1. Distribution of γδT cells in mouse.

Organs	Presence of γδT cells in mouse		Contact with epithelial cells	No. of cells per animal	Major Vγ gene segments used	Diversity of γδ TCR
	normal	nude				
Digestive system						
Tongue	+		+		6	-
Esophagus	+/-		+			
Stomach	+		+			
Small intestine	+	+	+	1×10^6	7	+
Large intestine	+	+	+	1×10^5	7, 4	
Liver	-					
Pancreas	-					
Reproductive system						
Ovary	+/-					
Uterus	+	-	+	1×10^5	6	-
Vagina	+	-	+	1×10^5	6	-
Testis	-					
Epididymis	+/-		-			
Seminal vesicle					7, 5	
Urinary system						
Kidney	-					
Bladder	+	-	+			
Others						
Skin	+	-	+	5×10^6	5	-
Lung					7, 4, 6	
Brain	-					
Heart	-					
Lymphoid organs						
Thymus	+			1×10^6	4, 7	+
Spleen	+	-		8×10^5	4, 7, 6	+
Lymph node	+	-		1×10^5	4, 7, 6	+
Blood	+			6×10^4	4, 7, 6	+

We extended our immunohistological analyses to 8 week old athymic nude mice. As expected, no αβ or γδ T cells were detectable in most organs analyzed, suggesting a thymus dependency of these T cells. However, γδ T cells were observed in the small intestines of these nude mice in about half of the number present in the strain- and age-matched normal mice (Table 1).

γδ T Cells of Different Peripheral Sites Utilize Different Vγ Gene Segments to Encode Their TCR

Previous studies have demonstrated that s-IEL (DEC) and i-IEL use distinct V_γ gene segments, V_5 (Asarnow et al. 1988) and V_7 (Takagaki et al. 1989b), respectively, to encode the γ subunits of their TCR. To determine whether there was a similar preferential usage of specific V_γ gene segments by γδ T cells present in other sites, DNA extracted from crude lymphocyte preparations from these sites was amplified by the PCR (polymerase chain reaction) technique (Saiki et al. 1988) using a V_γ ($V_{\gamma 4}$, $V_{\gamma 5}$, $V_{\gamma 6}$, or $V_{\gamma 7}$)-specific primer in combination with a $J_{\gamma 1}$-specific primer. The PCR products were then analyzed by the Southern blot method using a $J_{\gamma 1}$ oligonucleotide probe. The results are summarized in Table 1. As in adult thymi (Ito et al. 1989), the $V_{\gamma 4}$-$J_{\gamma 1}$ rearrangements were most abundant in peripheral lymphoid organs (blood, spleen and lymph nodes), but bands corresponding to $V_{\gamma 6}$-$J_{\gamma 1}$ and $V_{\gamma 7}$-$J_{\gamma 1}$ rearrangements were also detected in these organs. $V_{\gamma 6}$-$J_{\gamma 1}$ rearrangement was abundant in the vagina, uterus, and tongue. Cloning and sequencing of the PCR products indicated that most (12/14) $V_{\gamma 6}$-$J_{\gamma 1}$ clones isolated from vaginae and uteri contained in-frame junctions with an identical nucleotide sequence. These results strongly suggest that most γδ T cells in female reproductive organs use the single $V_6J_1C_1$ γ gene to encode the γ subunits of their TCR. Interestingly, γδ T cells associated with the tongue (t-IEL) also use the same γ gene.

γδ T cells associated with some epithelial organs are primarily derived from fetal thymocytes and carry an entirely homogeneous γδ TCR

It was previously shown that the utilization of γ and δ gene segments is developmentally ordered in thymocytes (Havran and Allison 1988; Ito et al. 1988; Itohara et al. 1989a). γδ thymocytes from early fetuses (i.e. at around day 15 of the embryonic life) preferentially express TCR encoded by $V_5J_1C_1$ γ and $V_1D_2J_2C$ δ genes (Ito et al. 1988; Havran and Allison 1988). These TCR are entirely homogeneous (Lafaille et al. 1989) and are identical to the TCR expressed on s-IEL (DEC) (Asarnow et al. 1988), suggesting that s-IEL probably originate from this first wave of fetal γδ thymocytes. At late fetal and newborn stages most γδ thymocytes bear TCR encoded by $V_6J_1C_1$ γ and $V_1D_2J_2C$ δ genes (Ito et al. 1989) which are also structurally homogeneous (Lafaille et al. 1989). Since the nucleotide sequence of this $V_6J_1C_1$ γ gene is identical to the sequence of the sole in-frame joined $V_6J_1C_1$ γ gene present in r- and t-IEL (Itohara et al. 1989b), we suspected that these cells are derived from the second wave of fetal γδ thymocytes. If this was the case, the δ chains of these T cell populations also should be identical. We therefore cloned and sequenced the PCR products of V_1DJ_2C δ genes from r-IEL preparations and found indeed that these genes are also homogeneous in their junctional sequences (Itohara et al. 1989b), and that the r-IEL δ gene sequence corresponds exactly to the sequence of the δ gene expressed on the surface of the late fetal thymocyte population (Lafaille et al. 1989). We conclude from these results that r-IEL are derived from the last fetal wave of thymocytes

γδ TCR Expressed in an Adult Thymus and Peripheral Lymphoid Organs are Diverse

Unlike the early and late fetal thymocytes or s-IEL, r-IEL, and t--IEL, adult γδ thymocytes and γδ T cells of peripheral lymphoid organs utilize various combinations of V_γ and V_δ gene segments to encode their TCR. Furthermore abundant V_δ-(D)-J_δ junctional diversity occurs among the TCR of these adult γδ T cells (Korman et al. 1988; Takagaki et al. 1989a; Lacy et al. 1989). Taken together these results indicate that in addition to the difference in the gene segments utilized for surface expression, there is a drastic fetal vs. adult shift in the extent of the junctional diversity in the γδ TCR.

Since i-IEL preferentially use $V_{\gamma 7}$ gene segment (Takagaki et al. 1989b) which is rarely utilized by fetal thymocytes but is used by some adult thymocytes, and since the TCR genes assembled in adult thymocytes and i-IEL show similar extensive deletions and insertions in the junctions (Takagaki et al. 1989a; Takagaki et al. 1989b), some adult thymocytes may home to intestinal epithelia.

The probable developmental relationship between the various γδ T cell subpopulations is summarized in Figure 3.

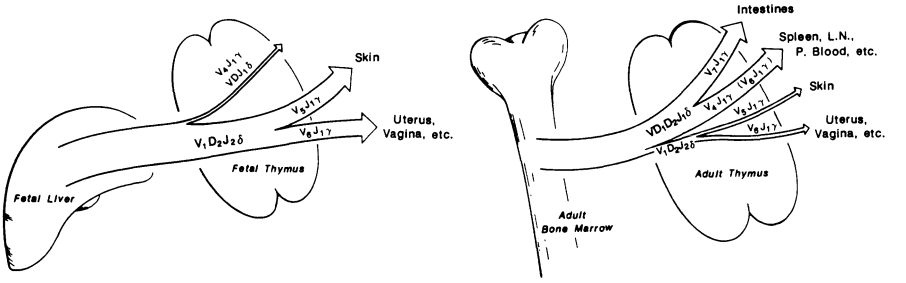

Figure 3. Development of various γδ T cell subsets. The arrows indicate the proposed pathways followed by γδ T-lymphocytes expressing the $C_{\gamma 1}$ region in fetal and adult mice.

Developmental Relationship Between αβ and γδ T Cell Lineages

One interesting issue raised by the discovery of γδ T cells is their developmental relationship with αβ T cells. Pardoll and his coworkers (1987) and Allison and Lanier (1987) proposed that if γ and δ genes rearrange productively (i.e. by in-frame joining), the cell proceeds to surface expression of γδ TCR, which, in analogy with the immunoglobulin system (Alt et al. 1987), inhibits further rearrangement of any other TCR gene. αβ T cells are generated only from those cells that failed to productively rearrange both γ and δ genes. We tested this model by constructing and analyzing transgenic mice (called KN6 transgenic mice) carrying in their germline productively rearranged TCR γ and δ genes. According to the Pardoll-Allison model, generation of αβ T cells should be disrupted in these mice, for all T cell precursors in these mice should have productively rearranged, transgenic γ and δ genes. However, contrary to this prediction neither the absolute number nor the proportion of αβ T cells is significantly altered in the thymus and spleen of the transgenic mice (Table 2). Furthermore, the thymocytes and splenocytes of these mice exhibit a normal expression pattern of CD4 and CD8 glycoproteins, suggesting that their maturation is normal.

Table 2. Surface expression of γδ TCR and αβ TCR on thymocytes and splenocytes of γδ-transgenic mice.

Mice	Age (weeks)	thymocytes ($x10^{-6}$) Total cells	thymocytes ($x10^{-6}$) stained with anti γδ	thymocytes ($x10^{-6}$) stained with anti KN6	thymocytes ($x10^{-6}$) stained with anti αβ	splenocytes ($x10^{-6}$) Total cells	splenocytes ($x10^{-6}$) stained with anti γδ	splenocytes ($x10^{-6}$) stained with anti KN6	splenocytes ($x10^{-6}$) stained with anti αβ
γδ-1313	9	79	1.26 (1.6)	1.02 (1.3)	56.8 (71.9)	153	6.32 (4.1)	6.27 (4.1)	43.0 (28.1)
LM	9	78	0.16 (0.2)	0.00 (0.0)	55.9 (71.7)	167	0.66 (0.4)	0.00 (0.0)	43.6 (26.1)
γδ-1355	8	100	2.66 (2.7)	2.66 (2.7)	72.7 (72.7)	161	4.96 (3.1)	4.83 (3.0)	36.1 (22.4)
LM	8	99	0.10 (0.1)	0.00 (0.0)	74.2 (74.9)	152	0.89 (0.6)	0.00 (0.0)	39.7 (26.1)

Thymocytes and splenocytes were stained with anti γδ, anti KN6, or anti αβ mAb. Proportion (%) of γδ TCR or αβ TCR bearing T cells among total thymocytes or splenocytes is also shown in parenthesis. LM designates nontransgenic littermates.

The apparently normal generation of αβ T cells is not due to a failure of the expression of the transgenes in these mice: These mice do contain an elevated level of γδ T cells and nearly all of their TCR are encoded by the transgenes (Table 2).

As in normal mice (see above), γδ-αβ double producers are not detectable in these transgenic mice (Ishida et al. 1989). We conclude that there must be a mechanism restricting the expression of TCR to one of the two types in a given T cell precursor which is active before either of the two types of TCR is expressed on the cell surface.

The Silencer Model of αβ and γδ T Cell Lineages

Both the γ and δ transgenes are retained by the αβ T cells of the KN6 transgenic mice, but no transcript derived from the transgenes accumulates in them. These results suggest that there exists a cis-acting DNA element(s) that mediates the repression of γ and δ RNA accumulation in αβ T cells. The element(s) seems to be contained within the 40 kb γ and/or 43 kb δ genomic DNA fragments used for the construction of the transgenic mice.

The validity of this hypothesis was strengthened by the finding that in the second set of transgenic mice (called "short γ" transgenic mice) constructed with a γ gene clone (pγ-L) with much limited flanking sequences, the γ transgene transcripts accumulate abundantly (Ishida et al. 1989).

In light of these findings we propose a model for the differentiation of αβ and γδ T cell lineages (Fig. 4). γ and δ gene rearrangements occur prior to the completion (i.e. V to DJ joining) of β gene rearrangement and the initiation of α gene rearrangement. In-frame rearrangements of both γ and δ genes are, of course, a prerequisite for the generation of γδ thymocytes. However, we propose on the basis of the normal appearance of αβ T cells in the γδ transgenic mice, that the failure to rearrange both γ and δ genes in-frame is not a requirement for the generation of αβ thymocytes. This implies that some αβ thymocytes could be generated from cells that harbor both in-frame rearranged γ and δ genes, and therefore precursors of some αβ T cells may express γδ TCR on their surface. While this possibility can not be ruled out, any γδ TCR expression in these cells

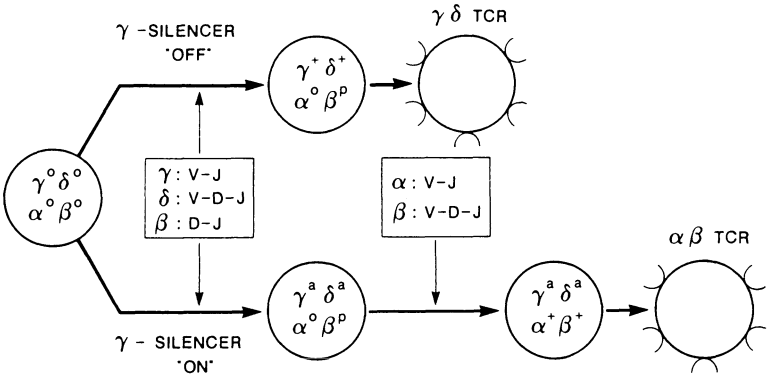

Figure 4. The silencer model of αβ and γδ T cell differentiation. The various states of rearrangement of four TCR genes are indicated by superscripts. O and + denote that the gene is in the germline configuration and productively rearranged, respectively, while a and p designate that the gene is in any state (i.e. in germline configuration, productively or nonproductively rearranged or deleted) and partially rearranged (i.e. D-J joined), respectively. The γ gene associated silencer is inactive in the γδ T cell lineage (top) and is activated in the αβ T cell lineage (bottom). The activation may occur either before any TCR gene is rearranged or after γ and δ genes are rearranged but before α and β gene rearrangements are completed. See text for more details.

is likely to be transient because we detect no thymocytes coexpressing both αβ and γδ TCR either in the γδ transgenic mice (this study) or normal mice (Itohara et al. 1989a).

We propose that the putative machinery acting upon the γ silencer is activated in a fraction of immature thymocytes and that it is from these cells that αβ thymocytes are generated. The activation of the silencer machinery could occur prior to γ and δ gene rearrangements. In this case αβ and γδ T cell lineages are completely separate and no αβ thymocyte precursors will ever express γδ TCR. The activation of the machinery, however, could occur between the completion of γ and δ gene rearrangements and that of α and β gene rearrangements. In this case precursors of some αβ T cells may express γδ TCR transiently.

A Third Set of Transgenic Mice Support the Silencer Model

One prediction that could be made by the silencer model of T cell development is that the generation of αβ T cells would be hampered in a transgenic mouse constructed with γ and δ genes carrying no silencer element. We tested this prediction by constructing the third set of γ/δ double transgenic mice (called DEC transgenic mice) using "short" γ and δ gene clones. As in the KN6 transgenic mice constructed with the "long" γ and δ gene clones, an overwhelming majority of the γδ TCR in these transgenic mice is encoded by the transgenes (Fig. 5a). While the number of γδ TCR^+ thymocytes is 10 to 100 fold higher in the DEC transgenic mice than in the nontransgenic littermates the total number of thymocytes is greatly reduced because the development of αβ T cells is severely hampered (Fig. 5b). In older DEC transgenic mice some αβ T cells become detec-

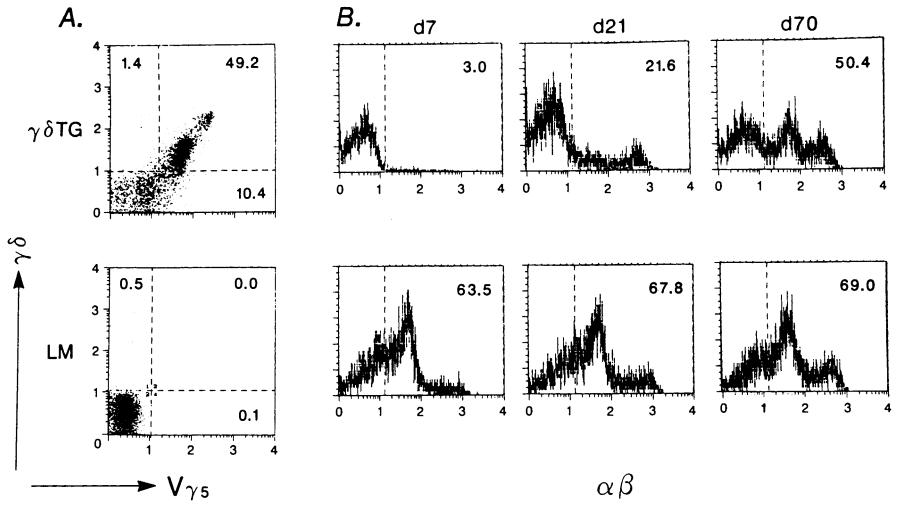

Figure 5. γδ and αβ TCR surface expression by thymocytes from DEC transgenic and nontransgenic mice. The number in each quadrant is the percentage of cells stained by one or both mAb(s). Fluorescence intensity is expressed in $\log_{10}$ units. (a) Thymocytes from 10 week-old DEC transgenic (left) and nontransgenic (right) littermate mice were analyzed by two color flow cytometry using anti-$V_{\gamma 5}$ mAb F536 and anti-γδ mAb 3A10. (b) Thymocytes from 7, 21, and 70 day-old DEC transgenic (left) and nontransgenic (right) mice were stained with anti-αβ mAb H57-597.

table, most of which coexpress γδ TCR. Southern blot analysis of DNA extracted from the total thymocytes indicated that β gene rearrangement is blocked in these transgenic mice.

Recognition of a Self Histocompatibility Complex TL Region Product by γδ T Cell-Receptors

In order to understand the function of γδ T cells it is essential to identify the ligand of the TCR. For this purpose we prepared a number of γδ T cell hybridomas from fetal and adult thymocytes and screened them for specificity using a growth inhibition array based on the previous finding that crosslinking the αβ TCR, which usually promotes growth of normal T cells, results in growth inhibition of T cell hybridomas (Ashwell et al.1987). As a result of this screening we identified one γδ T hybridoma, KN6, whose growth was inhibited by syngeneic (C57BL/6) but not allogeneic [BALB/c, CBA/J and AKR/J] spleen cells (Bonneville et al. 1989). The KN6 growth was also inhibited by thymocytes, peritoneal macrophages, cells from the Abelson transformed B6 T cell line 2052C, and by PCC3 embryonal carcinoma cells. That growth of TCR-negative variants of KN6 (KN619 and KN6-2) was not affected by syngeneic cells and growth inhibition was blocked in a dose-dependent fashion by the anti γδ TCR mAb (3A10) as well as by the anti KN6 γδ TCR clonotypic mAb (5C10) (Bonneville et al. 1989). These results provided evidence that the KN6 growth inhibition is mediated by the TCR.

Table 3. The ligand recognized by KN6 is controlled by MHC genes telomeric to the Q region.

Strain[a]	Region[b] K	D	Q	TL	Percent Phenotype[c]	Proliferation[d]
A.BY/SnJ	b	b	b	b	+	-75
C57BL/6J (B6)	b	b	b	b	+	-76±12
C57BL/10SnJ (B10)	b	b	b	b	+	-89±6
A.CA/Sn	f	f	f	f	±?	-37±19
B10.M/Sn	f	f	f	f	±?	-36±29
B10.D2/nSnJ	d	d	d	d	-	+30±17
B10.BR/SgSnJ	k	k	k	k	-	-5±12
P/J	p	p	p	p	-	+31±27
B10.G/Sg	q	q	q	q	-	+8±6
B10.RIII(7INS)/SnJ	r	r	r	r	-	+11±2
B10.S/Sg	s	s	s	s	-	+8±23
A/Boy	a	a	a	a	-	+4±8
B6/Boy	b	b	b	b	+	-85±4
A-Tla^{b}/Boy	a	a	a	b	+	-66±14
B6-Tla^{a}/Boy	b	b	b	a	-	-2±13

[a]Other + strains include: BALB.B/Li, B10.A(2R)SgSnJ, B10.A(R149)-Tla^{b}/Mrp, B10.A(R410)-Tla^{b}/Mrp, B10.P(13R)/Sg, B10.SM(70NS)/Sn, and TBR2. Other - strains include: AKR/J, A/WySnJ, BALB/c (what subline??), B6-H-2^{k}/Boy, B6.K1/Fla, B6.K2/Fla, B10.A/SgSnJ, B10.PL(73NS)/Sn, B10(R297)-Tla^{a}/Mrp, B10(R310)-Tla^{a}/Mrp, C3H/HeJ and MA.My/J. [b]Haplotype origin of region. [c]+ = strong inhibition. ±=possible intermediate inhibition. Results variable and not consistent. - = no inhibition. [d]Data shown are with a single clone, KN6-7. All experiments done at least twice, except with A.BY, which also tested + with the original KN6 line.

Since the KN6 hybridoma responds to syngeneic but not to allogeneic cells the gene encoding or controlling the KN6 ligand must be polymorphic. In order to confirm this and to map the gene, the KN6 hybridoma was screened for reactivity with a panel of spleen cells from various mouse strains (Table 3). Linkage to the MHC was demonstrated by results with congenic strains which differ only at H-2. Thus, KN6 cell proliferation was strongly inhibited by cells from H-2^{b} strains, partially inhibited by cells from H-2^{f} strains, and not affected by cells from H-2^{d}, H-2^{k}, H-2^{p}, or H-2^{s} mice. Most importantly, the analysis of recombinant strains showed that the gene controlling the KN6 ligand is located in or distal to the TL region. This conclusion is based on the observation that the hybridoma responds to B6/Boy ($K^{b}D^{b}Qa^{b}Tla^{b}$) and A.Tla^{b}/Boy cells ($K^{k}D^{d}Qa^{d}/TL^{b}$), but not to A/Boy ($K^{k}D^{d}Qa^{d}TL^{d}$) or B6-Tla^{a}/Boy ($K^{b}D^{b}Qa^{b}/TL^{d}$) cells (Table 3).

TL Gene That May Encode the Class I Molecule Recognized by KN6 γδ TCR

The embryonic carcinoma line PCC3 does not express class I H-2K, D, or L or class II I-A or I-E MHC molecules on their surface (Maher and Dove 1984; Stern et al. 1986), yet is specifically recognized by KN6 TCR. In order to determine the structure of the molecule recognized by KN6 γδ TCR, we made a cDNA library from this cell line and screened the library with a probe composed of the exon 4 region (Weiss et al. 1984) which is conserved among all MHC class I genes known to date. This led to the isolation of 12 cDNA clones which fell into seven kinds on the basis of the nucleotide sequences. The gene represented by one and only one of these seven kinds of clones was distinct from all known MHC class I genes and appears to belong to the TL gene family on the basis of the pattern of sequence homology in the various exons (Table 4). Thus, the product of this gene is a good candidate for the TL-mapped ligand recognized by KNG γδ TCR.

Table 4. Percent homology of the cDNAs encoding the putative KN6 ligand with known class I molecules.

Region	Gene	Exon					
		I	II	III	IV	V	VI
H-2	H2-K^d	53	73	53	92	38	39
Qa-2,3	Q10	50	72	50	95	35	42
Tla	T3	47	66	52	92	38	36
	T18	49	73	59	95	38	32

Fate of Autoreactive γδ Thymocytes in Transgenic Mice.

Recent studies suggest that T cell tolerance is accomplished, at least in part, by intrathymic deletion of self-reactive αβ T cells (Kappler et al. 1987; Kisielow et al. 1988; MacDonald et al.1988). We have begun studies to determine whether or not a similar deletion of self-reactive γδ T cells occurs. Since the KN6 γδ TCR recognize syngeneic (TL^b) thymocytes and splenocytes we analyzed thymocytes and splenic T cells from the KN6 transgenic mice expressing MHC^b or MHC$^{k/d}$ haplotype. As shown in Table 5, nearly all γδ T cells bear TCR encoded by the transgenes regardless of the MHC haplotype of the host (i.e., MHC^b or MHC$^{k/d}$). Furthermore, the number of γδ T cells present in either thymus or spleen is not lower in the MHC^b mice than in MHC$^{k/d}$ mice. Thus there was no obvious deletion of self--reactive γδ T cells. γδ thymocytes isolated from MHC^b hosts but not MHC$^{k/d}$ hosts propagate rapidly in vitro in the presence of "Con A supernatant" or recombinant IL-2. γδ-thymocytes derived from MHC$^{k/d}$ transgenic mice require, in addition to the lymphokine, stimulation by ligand-bearing cells. These results indicate that the γδ thymocytes are activated in TL-matched hosts presumably by their interaction with the intrathymic TL^b ligand.

Table 5. Surface expression of γδ TCR in KN6 γδ-transgenic mice.

Mice	H-2	Thymocytes (x10^{-6})			Splenocytes (x10^{-6})		
		Total cells	γδ$^+$	KN6$^+$	Total cells	γδ$^+$	KN6$^+$
LM	b/k	99	0.1	0	152	0.89	0
γδ	k/d	100	2.66	2.66	161	4.96	4.83
γδ	b/k	44	3.35	3.13	148	4.18	4.02
γδ	b/b	24	1.83	1.79	106	4.16	4.18

Thymus and spleen cell suspensions were stained with anti-δ (3A10) or anti clonotypic KN6 (5C10) mAbs. The H-2 haplotypes of nontransgenic and transgenic mice were determined by using anti H-2K mAbs.

DISCUSSION

Our study sheds considerable light on the characteristics of γδ TCR and T cells bearing this receptor. Despite this and other useful information accumulated during the past few years on γδ TCR or γδ T cells, the biological functions of these cells remain to be determined.

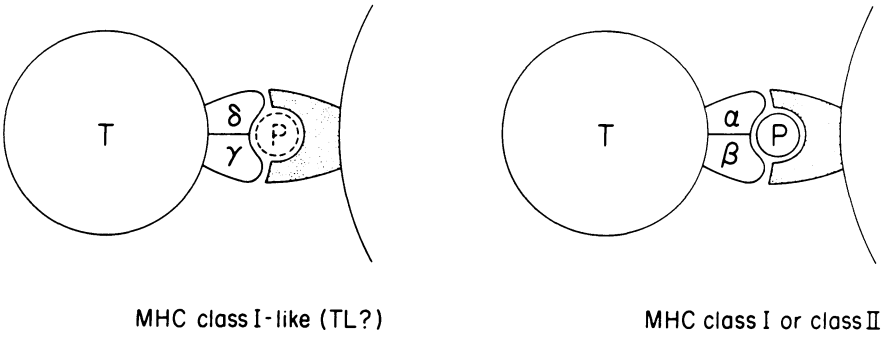

Figure 6. Comparison of the proposed recognition by a γδ T cell with the established recognition by an αβ T cell.

There is considerable evidence indicating that γδ T cells recognize molecules similar to but distinct from the MHC class I molecules that are utilized to present antigen-derived peptides to αβ T cells (this paper; Bonneville et al. 1989; Bluestone et al. 1989). While there also are reports that some γδ T cells recognize allogeneic K- or D-region class I molecules or the I-region class II molecules, these specificities may have resulted from crossreactivity, for strong selective pressure was applied to detect them (Matis et al. 1987; Bluestone et al. 1989). Indeed, the recent finding (Y. Utsunomiya, S.I., R.K., S.T., and O.K. unpublished results) that the subunit structure of CD8 molecules expressed on activated i-IEL and splenic γδ T cells is different from that expressed on αβ T cells supports the hypothesis that γδ and αβ T cells recognize distinct sets of class I (or class I-like) molecules (Fig. 6).

If γδ T cells generally recognize distinct class I (or class I-like) molecules, the next question is whether or not these class I molecules present peptides to γδ TCR, as do the MHC class I K, D, and L molecules to αβ TCR. While no direct evidence is available at the moment, the structural similarities both between the αβ and γδ TCR and between the class I (or class I-like) molecules recognized by the two types of TCR suggest that the ligands for γδ TCR generally include antigen-derived peptides (Fig. 6).

Are γδ T cells, then, specific to a variety of antigens as are αβ T cells, and the repertoire of the antigens recognized by γδ TCR more or less identical to that of the antigens recognized by αβ TCR? Here it would be useful to consider separately the two types of γδ T cell subsets - one with a high degree of TCR diversity such as i-IEL and splenic γδ T cells, and the other with invariant TCR such as s-IEL and r-IEL. In the former case it is likely that the antigens (hence the peptides derived from them) recognized by γδ T cells are structurally diverse. However, the antigenic repertoire for γδ T cells may be different from that for αβ T cells. Perhaps the class I molecules recognized by γδ TCR (hereafter abbreviated as REγδ, antigen-restriction elements for γδ TCR) have evolved to present a special set of antigens to the immune system. The selectivity of REγδ for a particular set of antigens might be explained by postulating a special intracellular pathway of peptide loading for REγδ and/or common structural features of the presented peptides. A hint to which set of proteins may be presented efficiently by REγδ has come from recent experiments that have shown the recognition of mycobacterial heat shock-like proteins by some γδ T cells (Holoshitz

et al. 1989; Janie et al. 1989; Modlin et al. 1989; O'Brien et al. 1989). It may be that γδ T cells with diverse TCR are primarily directed to a variety of mycobacteria and parasitical protozoa known to constitutively produce structurally related but distinct heat shock-like proteins. The effector function of the γδ T cells is unknown, but one might speculate that these cells play a role in the initiation or regulation of defense reactions by lymphokine secretion or elimination of undesirable cells such as persistent antigen-presenting cells.

In contrast to a γδ T cell subset capable of recognizing structurally diverse ligands, a γδ T cell subset with an invariable TCR must recognize an equally invariable ligand. Despite this important difference in the diversity of the ligands, it is likely that the basic composition (i.e., a peptide-RE complex) of the ligand recognized by the invariant γδ TCR is the same as that of the ligands recognized by diverse γδ TCR. Assuming that this is indeed the case, the key issue is again the origin of the peptide. Since it does not make sense for an organism to secure an entire subset of T cells localized in an organ for the protection against just a single foreign antigen, we should consider an alternative role and/or mechanism for the undiversified γδ T cells. Perhaps these γδ T cells recognize a tissue-specific host antigen whose synthesis may be induced in the epithelial cells by a variety of unfavorable stimuli such as viral infections, toxic chemicals, radiation, heat shock, and malignancy (Janeway 1988). One candidate of such a host antigen is a stress protein whose synthesis may be induced in a tissue-specific fashion. Since at least some of these proteins are known to be structurally related to mycobacterial heat shock-like proteins, it is conceivable that the peptides derived from them could also effectively bind to and be presented by REγδ. Since the postulated antigen is induced only under specific conditions, autoreactivity of these γδ T cells may not have any hazardous consequences but rather play an important regulatory and/or effector role. The peculiar specificity of γδ T cells envisaged in our model is somewhat reminiscent of the specificity of another class of lymphocytes, those called CD5 B cells. These B cells also appear to preferentially recognize common bacterial antigens as well as self antigens.

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Thymic Epithelial Cells Induce in Vitro Differentiation of PRO-T Lymphocyte Clones into TCR $\alpha, \beta/CD3^+$ and TCR $\gamma, \delta/CD3^+$ Cells

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INTRODUCTION

In the intact thymus, it is difficult to determine the molecular requirements for the induction of T-cell differentiation and to assess the roles of the various types of cells in the thymic environment (e.g. epithelial cells, macrophages, dendritic cells). Furthermore, precursor-product relationships can only be inferred indirectly.

In order to be able to study these issues we have attempted to develop an in vitro system that induce the differentiation of T-cell progenitor clones into T cell antigen-receptor bearing (TCR/CD3) cells. In the last few years, we have succeeded in developing T-cell progenitor clones from the bone marrow of young adult mice and the thymus of 14 day old mouse embryos. These clones represent the two earliest stages of T-cell development which have been indentified so far. We call them "marrow-type" and "thymus-type" PRO-T lymphocytes (1). Both types of PRO-T clones contain the TCR α,β,γ and δ genes in the germline configuration. Marrow-type clones do not transcribe any of the CD3 $\gamma,\delta,\varepsilon$ genes; thymus-type PRO-T clones express mRNA from the CD3γ but not from the CD3δ or ε genes (1). Both types of clones are $Thy1^+$, $L3T4^-$ $LyT2^-$, $PgP\text{-}1^+$, $LFA\text{-}1^+$.

IN VITRO DIFFERENTIATION

For four years we attempted without success to induce PRO-T clones to differentiate into $TCR/CD3^+$ cells with a variety of soluble factors including all known interleukins (from rIL1α to rIL7) or by coculturing them with irradiated macrophages/dendritic cells from spleen of nude mice or SCID mice regardless of the presence of exogenous interleukins. To test the possibility that thymic epithelial cells could be able to induce differentiation of the PRO-T clones, we attempted and succeeded in establishing four thymic epithelial clones from 16 day old mouse embryos. One had no detectable effect on either growth or differentiation and two epithelial clones were able to support proliferation but not differentiation of PRO-T clones. The fourth thymic epithelial clone, called ET, was found to be able to induce differentiation of both marrow-type and thymus-type PRO-T clones (2). Indeed, we have found that after 8-10 days of coculture, the three thymus-type clones (FTH5, FTD5, FTF1) and the three marrow-type clones (C4-77/3, C4-86/18, C4-95/16) tested, as well as freshly isolated bone marrow T-cell precursors from 5 weeks old nude mice, gave rise to significant numbers of $TCR/CD3^+$. Among which were $L3T4^+$ $F23.1^+$, $L3T4^+$ $F23.1^-$, $L3T4^-$ $LyT2^-$ $F23.1^+$ and $L3T4^-$ $LyT2^-$ $F23.1^-$ cells. Surprisingly, no $LyT2^+$ or $L3T4^+$ $LyT2^+$ cells were detected in these

cultures (2). Kinetic studies in which we followed the PRO-T clones from day 1 to day 21 of coculture with ET epithelial cells showed that TCR/CD3$^+$ cells were detected from day 4 when very few of them were L3T4$^+$. Subsequently there was a significant and progressive increase in the numbers of L3T4$^+$ TCR/CD3$^+$ and to a lesser but significant extent of L3T4$^-$ LyT2$^-$ TCR/CD3$^+$ cells as the cultures developed. However, no LyT2$^+$ cells were observed at any time in the same cultures. Thus, we concluded that the ET epithelial clone induces T-cell progenitors to differentiate in vitro into L3T4$^+$ LyT2$^-$ TCR/CD3$^+$ and L3T4$^-$ LyT2$^-$ TCR/CD3$^+$ cells but not into LyT2 bearing cells. The induced PRO-T clones generated polyclonal populations bearing TCR α,β/CD3 type of receptors encoded by the vß8, vß17a, vß11 gene families as F23.1$^+$, KJ16$^+$, KJ23a$^+$ and KT11$^+$ cells as well as CD3$^+$ cells which did not bind any of these vß-specific antibodies were found by FACS analysis (2 and unpublished results). The progeny of induced PRO-T clones also comprised CD3$^+$ cells that bound the Vγ3-specific antibody F536, indicating the presence of TCR γ,δ/CD3-bearing cells in the cultures. Confirming this result, normal sized mRNA from the TCRδ and the TCRγ genes were expressed by the induced PRO-T cells as assessed by Northern blot analysis (2). Also, the induced but not the uninduced PRO-T clones expressed RNA transcripts from the CD3δ gene, providing further molecular evidence of the differentiation of these cells (2). These results provided direct evidence that one PRO-T cell can give rise to both α,β^+ cells and γ,δ^+ cells.

The TCR/CD3$^+$ cells generated by the PRO-T clones in vitro proliferated in response to stimulation by a CD3-ε-chain-specific antibody in the presence of irradiated spleen cells from nude mice or Phorbol Myristate Acetate (2). These data further confirmed the presence of TCR/CD3$^+$ cells among the progeny of induced PRO-T clones and showed that the TCR/CD3 receptors expressed on them were functionally competent.

Next we performed experiments aiming to find out the requirements to induce the PRO-T clones to give rise to LyT2$^+$ cells. We found that lack of neither the several interleukins tested (From rIL1 to rIL7, IFN-γ) nor macrophages/dendritic cells in the cultures constituted by PRO-T cells and ET thymic epithelial cells could account for the absence of LyT2$^+$ cells in the cultures (2). Remarkably, we found that the PRO-T clones gave rise to all L3T4$^+$ LyT2$^-$, L3T4$^+$ LyT2$^+$ and LyT2$^+$ cells (48-61% of which were CD3$^+$) if cocultured with monolayers of heterogeneous populations of thymic epithelial cell preparations. The same PRO-T clones cocultured with the ET epithelial clone generated L3T4$^+$ TCR/CD3$^+$ and L3T4$^-$ LyT2$^-$ TCR/CD3$^+$ but not LyT2$^+$ nor L3T4$^+$ LyT2$^+$ cells. This finding indicates that thymic epithelial cells other than those represented by the ET thymic epithelial clone are required before a PRO-T cell can develop in vitro into L3T4$^+$ LyT2$^+$ and LyT2$^+$ cells. Our results obtained with the six PRO-T clones studied provide direct evidence that the same T-cell progenitor can give rise to all thymocyte subsets upon interaction with the appropriate thymic microenvironment. Kingston and colleagues (3) arrived to the same conclusion working with a different experimental system. While our results directly show that a L3T4$^+$ LyT2$^+$ intermediate step is not an obligatory stage in the development of functionally competent L3T4$^+$ LyT2$^-$ TCR/CD3$^+$ and L3T4$^-$ LyT2$^-$ TCR α,β/CD3$^+$ cells, they do not necessarily argue against the putative precursor potential of some L3T4$^+$ LyT2$^+$ thymocytes (4,5).

A MODEL FOR INTRATHYMIC T-CELL DEVELOPMENT

Based on the results summarized above and those of other groups we would like to propose a new model for T-cell development within the thymus. We suggest that there are two pathways by which a given PRO-T cell generates the different thymocyte subsets and peripheral T lymphocytes (Figure 1). In one pathway, if the PRO-T cell interacts with a thymic epithelial subset different from ET epithelial cells, it develops first into $L3T4^-$ $LyT2^+$ $TCR/CD3^-$ precursors, a proportion of which subsequently become $L3T4^-$ $LyT2^+$ $TCR/CD3^+$ (6). The $LyT2^+$ precursors which crosslink their TCR/CD3 receptors upon interaction with self MHC-class I survive and remain $LyT2^+$ and will then be subjected to selection against self antigen and class I MHC on haematopoietic cells (7,8). Those $LyT2^+$ precursors whose TCR/CD3 do not fit self MHC-class I, would as a last attempt to survive, express L3T4 (i.e. they become $L3T4^+$ $LyT2^+$) and now armed with L3T4, would seek to crosslink their TCR/CD3 by interacting with MHC class II. The successful $L3T4^+$ $LyT2^+$ cells switch off expression of LyT2 and are subsequently subjected to selection against self antigen plus MHC class II. The unsuccessful $L3T4^+$ $LyT2^+$ $TCR/CD3^+$ thymocytes die.

In the other pathway, the PRO-T cell by interacting with thymic epithelial cells like the ET cells described here, would be induced to give rise to $L3T4^+$ $LyT2^-$ $TCR/CD3^+$ (a precursor $L3T4^+$ $LyT2^-$ $TCR/CD3^-$ stage in this pathway can be also entertained), $L3T4^-$ $LyT2^-$ TCR $\alpha,\beta/CD3^+$ and $L3T4^-$ $LyT2^-$ TCR $\gamma,\delta/CD3^+$ cells which would then be selected. Those $L3T4^+$ $LyT2^-$ cells which crosslink their TCR/CD3 by interacting with self MHC class II survive and remain $L3T4^+$. If the TCR/CD3 does not fit self MHC-class II, the cell would as a last resort to survive, express LyT2 (i.e. becomes $L3T4^+$ $LyT2^+$) and try to achieve crosslinking of its TCR/CD3 again, but this time armed with LyT2, by attempting to interact with self class I MHC. The $L3T4^+$ $LyT2^+$ cells which succeed in this second attempt would switch off expression of L3T4 and thus end up as $L3T4^-$ $LyT2^+$ $TCR/CD3^+$ cells; those $L3T4^+$ $LyT2^+$ that failed die. The successfull $L3T4^+$ $LyT2^-$ initially generated and the $L3T4^-$ $LyT2^+$ cells developed following the second attempt which are reactive against self antigens and MHC class II or I, respectively, would then be eliminated (7,8). The TCR γ,δ^+ thymocytes and the $L3T4^-$ $LyT2^-$ TCR $\alpha\beta/CD3^+$ thymocytes would be also subjected to selection, perhaps by interacting with MHC and/or MHC-like gene products (Figure 1). According to this model, the $L3T4^+$ $LyT2^+$ $TCR/CD3^-$ thymocyte population would represent cells that have failed to make productive rearrangement of α and/or β TCR genes or have defects in the process of assembling their TCR/CD3 components. The presence of surface L3T4 and LyT2 on these $TCR/CD3^-$ cells would simply reflect the proposed mechanism of expressing L3T4 and/or LyT2 as a last resort to achieve crosslinking of TCR/CD3 upon interaction with self MHC and consequently, survive. As these cells do not "know" that they have no TCR/CD3 on their cell membrane, in functional terms, they behave as those $TCR/CD3^+$ thymocytes whose receptors do not fit self MHC class II and I.

The proposed model can account for most of the information available on both development and selection and it explains the generation of all thymocyte subsets by a single PRO-T cell. The essence of the model is the critical and essential role of different thymic epithelial cell subsets in instructing and inducing the T-cell progenitor to follow one or the other pathway of development. This has additionally the appealing implication that the interaction with one or the other type of epithelial cells may also

result in the expression of different genetic programs by L3T4$^+$ LyT2$^-$ and L3T4$^-$ LyT2$^+$ T cell lineages (e.g. helper function for B lymphocytes in L3T4$^+$ and cytolytic function in LyT2$^+$ T cells); thus, also accounting for the different functional properties of each T lymphocyte population.

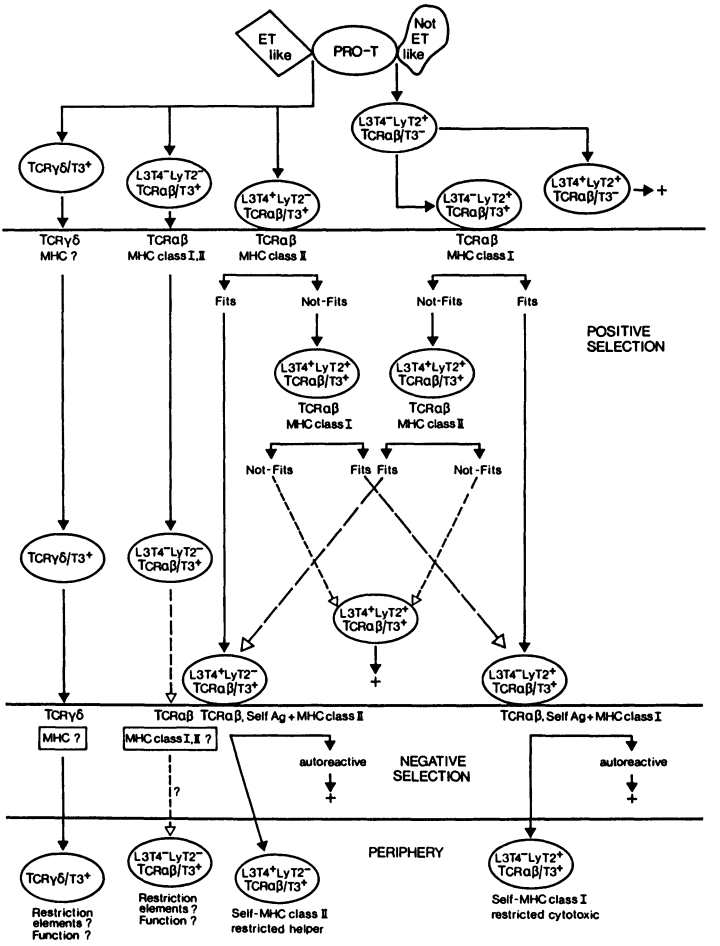

Figure 1. A suggested model of T-cell development within the thymus.

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The following contribution can be found on page 1276

Expression of T Cell Receptor Associated Proteins During Human T Cell Development (D. Campana, E. Coustan-Smith, L. Wong, and G. Janossy)

II Cell Developments

Development of T Cells

2. Repertoire Selection

Selection of the T Cell Repertoire in the Thymus

J.W. Kappler, T.H. Finkel, and P. Marrack

INTRODUCTION

The T cell αβ receptor (αβTCR) generally recognizes a ligand composed of a fragment of antigen complexed to a product of the major histocompatibility complex (MHC). Although the precise nature of the binding of the receptor to its ligand is unknown the available data, including the recently solved structure of a human class I MHC molecule (Bjorkman et al. 1988), suggest a complex interaction in which the binding depends on the contact of residues in both the MHC and antigen portion of the ligand with the variable portion of the αβTCR. The polymorphic residues of the MHC molecule appear to be particularly important in this interaction because a particular αβTCR which recognizes an antigen bound to one allelic form of an MHC molecule is unlikely to recognize the same antigen bound to a different allelic form of the MHC molecule. This phenomenon has been called "MHC restricted" antigen recognition.

The upper limit of the αβTCR repertoire is determined by the germline encoded elements which make up the variable portion of the receptor. Five elements contribute: Vα, Jα, Vβ, Dβ, and Jβ. Based only on the random combinations of these elements the maximum repertoire can be estimated at 5-10 million in mice and man. However, imprecision in the recombinational joining of these elements and non-germline encoded nucleotides often added at the junctions expands this number by at least several orders of magnitude. Although there is every reason to believe that the gene recombinations which lead to an expressed αβTcR are more or less random, the functional repertoire of mature T cells appears to be much smaller than the calculated germline repertoire. Two processes occur during T cell development in the thymus which select from the germline repertoire a limited set of receptors for expression on peripheral T cells.

The first of these has been called "positive selection" or "thymic restriction", to reflect the observation that the presence of a particular MHC molecule in the thymus during T cell development markedly enriches for the production of T cells capable of recognizing antigens bound to that MHC

molecule. The second process has been called "negative selection" or "tolerance" to describe the observation that T cells which recognize a particular antigen/MHC complex are neutralized if they encounter the complex during their development. This process is of course essential to eliminate self-reactivity from the αβTCR repertoire.

The attempt to understand MHC-restricted antigen recognition and the processes of positive selection and negative selection has been a major driving force in T cell research. Initially each of these phenomena was demonstrated at the population level (Bevan 1977; Zinkernagel et al. 1978). However, with the development of methodology for producing T cell clones and hybridomas and with the identification of the structural elements of the αβTCR, these processes have also been studied at the clonal level using serological and molecular probes to observe the behavior of T cells bearing particular receptors (Kappler et al. 1987; Kisielow et al. 1988b; Sha et al. 1988). Great strides have been made particularly in our understanding of αβTCR recognition of antigen/MHC ligands during T cell activation. However, despite such recent dramatic confirmations of the phenomenology of positive and negative selection, there have been many controversies and apparent contradictions concerning these two processes. Some of these issues have been resolved recently but many unanswered questions remain. Some of these issues are considered below.

AT WHAT STAGE IN T CELL DEVELOPMENT DO POSITIVE AND NEGATIVE SELECTION OCCUR?

The phenotypes of thymocytes in various compartments of the thymus and at various stages of the developing thymus have been determined in experiments over many years. The earliest cell in the thymus does not express an αβTCR and lacks the surface markers CD4 and CD8 (Fowlkes et al. 1985). Progeny from these cells rapidly express both CD4 and CD8. These cells, the major population of thymocytes, fill the cortex of the organ and during ontogeny are the first to express an αβTCR; however, the level of receptor on these cells is only about 10-20% of that on mature T cells and these cells are not immunocompetent. The medulla of the thymus fills last in ontogeny. These cells are similar in many ways to mature T cells in that they express either CD4 or CD8, have high levels of αβTCR similar to peripheral T cells and are immunocompetent. This population presumably contains the cells exported to the periphery (Scollay and Shortman 1984).

A major controversy over the years has been whether the $CD4^+CD8^+$ stage is an obligatory intermediate on the way to the mature medullary thymocyte. The kinetics of appearance

of the $CD4^+CD8^+$ cell and the fact that these are the first cells to express αβTCR would seem to indicate that they are an intermediate stage; however, the massive death of $CD4^+CD8^+$ cells in the cortex, their biochemical dissimilarity from mature medullary thymocytes and the difficulty in demonstrating mature T cell progeny arising from intrathymicly transferred $CD4^+CD8^+$ cells led to the suggestion that these were a differentiation backwater in the thymus with mature T cells springing full blown from a $CD4^-CD8^-$ precursor. This issue has been nearly resolved with evidence from several lines of research indicating that the $CD4^+CD8^+$ stage is intermediate between precursors and mature thymocytes (Smith 1987; Kisielow et al. 1988a; Carbone et al. 1988). Importantly for the discussion here, several types of experiments have now demonstrated that negative selection can operate on the $CD4^+CD8^+$ cells of the thymus and support the view that positive selection also occurs at this stage (White et al. 1989; Kisielow et al. 1988b; Sha et al. 1988; Fowlkes et al. 1988; see below).

WHAT IS THE MECHANISM OF POSITIVE SELECTION?

Positive selection was first demonstrated in chimeric mice in which the repertoire of peripheral T cells was shown to be heavily skewed toward recognition of antigens bound to MHC molecules that matched those expressed on the stromal cells of the thymus in which the T cells developed (Bevan 1977; Zinkernagel et al. 1978). These results argued strongly that ligation of the αβTCR by MHC molecules on the thymic stromal cells was essential for T cell development. This conclusion was supported by subsequent experiments which showed that blocking either the T cell receptor or MHC molecules in the thymus with monoclonal antibodies prevented the differentiation of mature T cells (McDuffie et al. 1988; Kruisbeek et al. 1985). Ultrastructural studies have shown thymocyte/epithelial conjugates with αβTCR capped in the junctional region (Farr et al. 1986). Most recently, positive selection was dramatically demonstrated in transgenic mice in which complete αβTCR's of known origin and specificity were introduced into the germline and became expressed on most T cells. Although the transgenic receptor was shown to be expressed on $CD4^+CD8^+$ cells regardless of the MHC type of the thymic stromal cells, high level expression on mature T cells required that the host thymic stromal cells be of the same MHC type as that of the mouse from which the original receptor was isolated (Kisielow et al. 1988; Sha et al. 1988).

These experiments leave little doubt that positive selection involves a specific interaction between the αβTCR and thymic MHC molecules. However, there is still no direct data on what biochemical events that receptor engagement initiates.

Most of the cells in the thymus cortex die there without maturing through a process known as apoptosis or "programmed cell death", so that minimally one can predict that positive selection induces an alteration in the cell which prevents the onset of this program. However, there is no evidence that any induction of proliferation is involved, since most of the proliferation seen among cortical thymocytes occurs in cells prior to surface αβTCR expression.

The most significant other event associated with positive selection may be the coordination of CD4 and CD8 expression with receptor specificity. Maturing T cells switch from $CD4^+CD8^+$ to either $CD4^+$ or $CD8^+$, such that T cells with αβTCR's selected to recognize antigen bound to class I MHC express primarily CD8 and those selected to recognize antigens bound to class II MHC express primarily CD4. This finding suggests that the CD4 and CD8 molecules play a role in positive selection and that the αβTCR/MHC interaction involved dictates which accessory molecule will continue to be expressed after maturation of the T cell. This conclusion has been supported in studies using anti-CD4, anti-CD8, anti-class I MHC or anti-class II MHC monoclonal antibodies to block differentiation of the appropriate mature T cell population and by findings in αβTCR transgenic mice that in the presence of the appropriate thymic MHC molecules mature T cells bearing the transgenic receptor were also heavily skewed toward expression of the appropriate accessory molecule (Smith 1987; Zuniga Pflucker et al. 1989; Kruisbeek et al. 1985; Kisielow et al. 1988a; Sha et al. 1988).

WHAT IS THE MECHANISM OF NEGATIVE SELECTION?

Despite the fact that tolerance to self antigens has been known for year to involve somehow the neutralization of self-reactive T cells, it has only been recently that direct evidence for the mechanism of tolerance has emerged. Numerous studies in the past several years have followed the fate of self-reactive T cells through thymic development. In one type of experiment self-antigens which interact with the large portion of T cells expressing a particular Vβ element were identified and T cells bearing these Vβ elements were tracked with monoclonal anti-Vβ antibodies (Kappler et al. 1987). In another self-reactive receptors were introduced into mice as transgenes (Kisielow et al. 1988; Sha et al. 1988). In both cases the results showed conclusively that T cells bearing the self-reactive cells first appeared in the thymic $CD4^+CD8^+$ cells, but were destroyed before they matured.

More recently, experiments have been designed in which tolerance to a self-antigen expressed only in extra-thymic

tissue has been shown sometimes to involve the apparent inactivation of the reactive T cell without its elimination (Lo et al. 1989). Although there has been considerable attention paid to the possibility that suppressor T cells may control the activity of self-reactive T cells their is little direct evidence to support this view.

HOW CAN A SINGLE RECEPTOR MEDIATE THREE FUNCTIONS?

If engagement of the αβTCR is involved in positive selection, negative selection and mature T cell activation, what determines which of these functions will be initiated? There are several possibilities. For example, the receptor may deliver a constant second message to the T cell in any of these processes, but this signal may be modified differently depending on the activation state of other genes in the T cell or on external signals provided by the ligand presenting cells. Alternatively, the second message transmitted by the αβTCR may change at different times in development. These are not mutually exclusive possibilities.

There is considerable evidence that different cell types primarily function in MHC presentation for positive and negative selection. Numerous experiments in chimeric mice have identified the thymic cortical epithelial cell to be important in positive selection and much less efficient in negative selection (Jenkinson et al. 1984; Marrack et al. 1988). Likewise bone-marrow derived cells (B cells, T cells, macrophages, dendritic cells, etc.) have been shown to be very efficient in inducing tolerance, but not in positive selection (Lo et al. 1986; Sprent and Webb 1987). The important features which distinguish these cell types have not been discovered. Although these cells may secrete factors which specifically enhance positive or negative selection, they may have special functions because of their particular location in the thymus and, therefore, the differentiative stage of the thymocytes they contact (See below).

Recent experiments on the cell biology of αβTCR signalling and tolerance induction suggest that the receptor changes the way it signals the T cell at different stages of differentiation. Engagement of the αβTCR on mature T cells results in a rapid rise in intracellular Ca^{++} coming from both intra- and extracellular sources with the concomitant turnover of phosphytidyl inositol and protein kinase C activation seen in many receptor systems (Weiss et al. 1884; Samelson et al. 1986; Mercep et al. 1988). Most data suggest that in mature T cells the transmission of the signal into T cells from the αβTCR occurs through the series of receptor associated proteins in the CD3 complex. In

support of this conclusion is the observation that direct engagement of the CD3 complex by crosslinking with an antibody to one components will activate T cells and lead to the same type of Ca^{++} mobilization as seen by engagement of the $\alpha\beta$TCR.

Similar studies on $\alpha\beta TCR^{+}CD4^{+}CD8^{+}$ thymocytes have revealed both quantitative and qualitative differences. About half of these cells mobilize Ca^{++} poorly if at all when their $\alpha\beta$TCR is engaged. The other half mount do mobilize Ca^{++}, but primarily from intra- rather than extracellular sources. Surprisingly, when the $\alpha\beta$TCR is bypassed on these cells and CD3 is ligated directly, all $\alpha\beta TCR^{+}$ cells mobilize Ca^{++} similarly to mature T cells (Finkel et al. 1989a). This result suggests a progressive alteration in the signal coupling between the $\alpha\beta$TCR and CD3. The possible significance of this maturation in coupling was indicated in negative selection studies. In vivo tolerizing antigens present during thymocyte development eliminated all mature T cell reactive to the antigen, but only about half of the $CD4^{+}CD8^{+}$ cell with reactive receptors (White et al. 1989). In similar experiments about half of cortical $\alpha\beta TCR^{+}$ cells were eliminated in vitro in fetal thymic organ culture whether an antigen or an anti-$\alpha\beta$TCR antibody was used to induce negative selection. Importantly, when an anti-CD3 antibody was used all $\alpha\beta TCR^{+}$ cells were eliminated (Finkel et al. 1989b). These results again indicated a progressive coupling of signalling between the $\alpha\beta$TCR and CD3 during differentiation, suggesting that the intracellular mechanism for negative selection is in place in all $\alpha\beta TCR^{+}$ cortical thymocytes, but is only set in motion by $\alpha\beta$TCR ligation when coupling between the receptor and the CD3 complex has progressed to a point where Ca^{++} mobilization is initiated.

These results indicate that the state of the $\alpha\beta$TCR may in part control whether engagement results in positive or negative selection and raises the possibility that positive selection may only be possible in the window between initial expression of the $\alpha\beta$TCR and the maturation of its coupling to CD3 to the point of obtaining the ability to mobilize Ca^{++}.

WHY DO POSITIVELY SELECTED CELLS ESCAPE NEGATIVE SELECTION?

The greatest conceptual dilemma in understanding positive and negative selection has been explaining how any of the T cells which are positively selected by engaging self-MHC then escape negative selection mediated by the same self-MHC to produce peripheral population with a pool of receptors enriched for those which will recognize self-MHC with a bound foreign antigen. Although this problem has been considered for many years and several hypothesis have been

proposed, there is little direct evidence shedding light on this quandary. Two types of models have been suggested.

The first has a number of variations which can be called collectively "affinity hypotheses". These proposals suggest that during positive selection only a very low affinity of the $\alpha\beta$TCR for MHC is required. Subsequently during negative selection and even later during antigen/MHC activation a higher affinity is required. Therefore, negative selection removes from the positively selected pool T cells bearing high to medium affinity receptors, but T cells bearing very low affinity anti-MHC receptors survive to make up the peripheral pool of T cells. Since these cells have already been selected for low affinity to self-MHC, they are enriched for those $\alpha\beta$TCR's which will have medium to high affinity when the additional contribution from foreign antigen bound to self-MHC comes into play.

A second group of proposals can be called "ligand hypotheses". These suggest that the MHC ligand recognized during positive selection is not identical to that recognized during tolerance. For example, the positively selecting MHC element may undergo some unique post-translational modification or become associated with a unique set of self-peptides, not present in the cells which tolerize or present foreign antigens later on. Since the ligand encountered during tolerance is different many positively selected T cells will survive. Since these were never-the-less selected for recognition of peptides bound to self-MHC, this surviving pool will be enriched for receptors capable of recognized foreign antigens bound to self-MHC

Neither of these hypotheses is totally satisfactory either in concept or in explaining the existing data. Despite its wide-spread acceptance, there is no direct evidence to support the affinity hypotheses. For example, no data support the notion that the $\alpha\beta$TCR's on mature T cells have any measurable low affinity for self-MHC in the absence of a foreign antigen. Furthermore, the demonstration that the level of $\alpha\beta$TCR on $CD4^+CD8^+$ T cells is about 10-fold lower than on mature T cell compounds the problem of explaining how positive selection can be achieved with receptor affinities undetectable in tolerance or in activation of mature T cells. The main problem with the affinity hypotheses, however, has been that it has been very difficult to design experiments to test them.

The ligand hypotheses get around some of these problems, since they rely on a differences in specificity rather than affinity. These hypotheses also lead to the testable predictions that biochemical differences should be detectable between MHC molecules on thymic epithelium and other cell types and the mature T cells should manifest a detectable reactivity to MHC antigens on thymic epithelium.

Some recent evidence is consistent with these predictions. For example, an anti-class II MHC monoclonal antibody has been described which detects a portion of class II molecules on peripheral cells, but fails to detect the class II molecules of thymic cortical epithelium (Murphy et al. 1989). Weak reactivity of the αβTCR's of peripheral T cells to self-thymic epithelium has also been observed (Marrack et al. 1989). However, a major conceptual problem with ligand hypotheses is that they predict an inefficiency to positive selection. They suggest a limited diversity in the selecting ligand and, therefore, many "holes" should be present in the T cell repertoire not because the appropriate receptors are absent, but because the positively selecting self-MHC element has not been appropriately modified to select these receptors. There is in fact little evidence for such holes in the repertoire in that in most cases where a foreign antigen can be identified which binds to a particular MHC molecule, that complex is immunogenic in vivo.

In summary, although is appears that the mysteries of the thymus are slowly giving way to the constant experimental onslaught, we are not at the end of the quest yet and a number of years of effort remain.

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T Cell Differentiation in Lower Vertebrates

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INTRODUCTION

With all the recent advances in the study of T cell differentiation in mice, it is reasonable to question whether anything can be fetched from the examination of this problem in lower vertebrates. One obvious advantage is the ability to manipulate embryos and to exchange organ primordia (such as the thymus) before blood circulation begins. Thymic transplantaion experiments in birds and amphibians involve less obvious distortion of normal physiology than the mouse models. Another advantage of work with amphibians is the existence of essentially two different lives, that of the tadpole and adult (reviewed by Flajnik et al. 1987). T cell differention must be repeated at metamorphosis when the thymus degenerates and is then repopulated by another wave of immature cells (Du Pasquier and Weiss 1973). Finally, the examination of positive and negative selection events in lower vertebrates is clearly needed to understand the forces which molded the evolution of T cell recognition, and, further, to resolve which aspects are essential and which are accessory.

Lymphocyte heterogeneity (T cells and B cells), associated with the presence of a thymus and spleen, is true of most vertebrates. Collaboration between lymphocytes has been observed down to the level of teleost fish, and the role of the MHC in this collaboration has been demonstrated in anuran amphibians (reviewed by Du Pasquier, 1989). Thus, one can have confidence that immunological discoveries in primitive vertebrates can be extrapolated to the "higher" vertebrates.

A dominant view in the immunological community is that the thymus is involved both in the positive selection of cells with receptors that will later recognize antigen in association with self MHC products, and in negative selection in which cells with receptors that recognize self MHC (plus self antigen) too strongly are physically or functionally deleted (reviewed by von Boehmer et al. 1989). The positive selection has been proposed to be governed by the thymus epithelium, and the negative selection by hematopoietic

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cells residing in the thymus (Zinkernagel et al. 1978; Flajnik et al. 1985; Lo and Sprent, 1986). Data from lower vertebrates will be reviewed with this paradigm in mind.

CHIMERA PRODUCTION

Frog chimeras are produced at the early tail bud stage, one day after fertilization, by cutting embryos at the posterior extent of the pharynx, and exchanging the anterior regions which contain the anlagen (in the endoderm) of the thymuses. The hematopoietic cells are all derived, as determined by experiments with ploidy, MHC, and species markers, from precursors in the dorsal lateral plate and ventral blood island mesoderm of the posterior region (Kau and Turpen 1983; Flajnik et al. 1984a, 1985). Chimeras have been made in which the thymus and hematopoietic cells share the MHC but differ at minor histocompatibility (H) antigens, or that differed at the MHC. The chimeras were produced in many different genetic combinations; there have never been any noticeable differences in mortality in any of these combinations. Graft rejection, MLR, and antibody responses were used to examine T cell selection events in these chimeras after they had undergone metamorphosis.

POSITIVE SELECTION

If absolute positive selection existed in amphibians, it was expected that chimeras with MHC-mismatched thymuses and hematopoietic cells would be unable to produce an IgG (actually, in amphibians IgY) antibody response, similar to frogs that had been thymectomized during embryonic life (Turner and Manning 1974). The results indicated that the ability of the chimeras to make anti-hapten responses was dependent on the MHC haplotypes involved (Flajnik et al. 1985). Some combinations did not, or only very weakly, produce any DNP-specific IgG(Y), although all the animals were capable of producing specific IgM. More commonly, the MHC-mismatched chimeras made IgG(Y) responses, but the kinetics of the response were slower than in controls or MHC-matched combinations. In summary, consistent with the positive selection, MHC-mismatched chimeras were always found to be deficient, either totally or quantitatively, in their IgG responses. Previous studies in which thymectomized animals were implanted with thymuses of different genotypes reached similar conclusions (Du Pasquier and Horton, 1982). The suboptimal antibody responses of the chimeras have been attributed to a lower frequency of T cells capable of interacting with APCs of their own MHC type because the chimeras' T cells had differentiated in an MHC-mismatched thymus.

Other evidence for positive selection was obtained from skin graft rejection studies. MHC-mismatched chimeras were capable of rejecting skin grafts that shared the MHC but were minor H-disparate with the thymus, but other chimeras made tolerant to the same MHC haplotype with an eye anlage graft (i.e. the thymus and hematopoietic cells bore the same MHC antigens) were not able to

reject grafts that differed from the tolerizing eye by minor H antigens. Third party MHC disparate grafts were rejected by all of the chimeras. (demonstrating that T cells were functional in all of the chimeras). This experiment was not only consistent with positive selection, but also indicated that skin graft rejection appears to be MHC-restricted (Silvers et al 1982).

NEGATIVE SELECTION

In contrast to the few studies of positive selection in lower vertebrates, there have been many experiments, owing to the ease of manipulation, examining embryonic tolerance in amphibians and birds. In general (but not always (Volpe 1972), when embryonic anlagen of any tissue are grafted onto other embryos, skin grafts of the same genotype as the embryonic graft are accepted later during the immunocompetent tadpole and adult lives. This is also true when tadpoles are made tolerant to adult skin grafts; after metamorphosis this tolerance is usually stable to another graft of the same genotype as the original tissue (reviewed by Cohen et al. 1985). The famous experiment of Triplett (1962) demonstrating that tolerance to tissue-specific antigens during the embryonic period is learned was not confirmed in later experiments (Rollins-Smith and Cohen 1982; Maeno and Katagiri 1984).

There has been renewed interest in chicken and amphibian embryonic chimeras since the discoveries in mice that the thymus endows *in vivo* but not *in vitro* tolerance upon T cells (von Boehmer and Schubiger 1984; Jenkinson et al 1985; Lo and Sprent 1986). This same basic finding is also true of the embryonic avian and amphibian chimeras: cells reactive to the embryonically-grafted MHC tissue are readily detectable *in vitro*, but *in vivo* tolerance is observed, both to the original grafted tissue and to subsequent grafts of the same genotype (Flajnik et al. 1985; Houssaint et al. 1986; Ohki et al. 1987). Thymectomized animals reconstituted with irradiated allogeneic thymus grafts also exhibit these reactions (Nagata and Cohen 1984; Arnall and Horton 1986).

Graft Rejection and MLR Studies

MHC-mismatched embryonically-produced chimeras remain tolerant to their anterior regions throughout their lives, and accept skin grafts of the anterior region's genotype. Cells reactive to the MHC of the head (and hence the thymus) are nevertheless detectable by MLR (Flajnik et al. 1985). This finding corresponds well with the aforementioned experiments done in mice, and suggests that the reactive cells are downregulated *in vivo*. Studies by Horton and colleauges (1986, 1987) have shown that reactive cells can also be detected to proliferate *in vivo* (to no autoimmune consequence). It is completely unknown how these reactive cells are downregulated *in vivo*. Similar results have been found with chicken allochimeras (Houssaint at al. 1986).

In most systems the thymus is not unique in generating such phenomena. Transplantation of an MHC-mismatched embryonic eye or

wing bud will also make animals tolerant in vivo but not in vitro. MLR tolerance has been observed only when flank mesoderm is transplanted, presumably because this embryonic tissue contains the precursors of all hematopoietic cells (Manning and Botham 1980; Botham and Manning 1980).

When quail wing buds or neural plate is grafted onto chick embryos in ovo, some chimeras hatch and remain healthy for a short period of time. Several weeks later, however, the chimeras reject the transplanted tissue (Kinutani et al. 1985, 1986). This waning of tolerance only occurs when xenogeneic, not allogeneic (MHC-disparate) embryonic tissue is transplanted. If a quail embryonic thymus is grafted along with the wing bud or neural plate, the animal does not reject the transplanted tissue (Ohki et al. 1987). In vitro experiments have not yet been done to determine whether reactive cells are present in the stably-tolerant thymic chimeras (although Houssaint's experiments (1986) have shown the reactive cells are present in her chimeras).

In summary, in vivo tolerance is easily obtained after embryonic transplantation in allogeneic combinations, but cells reactive to the tolerated MHC antigens nevertheless remain in apparently normal numbers. Although more work is necessary, it appears that transplantation of hematopoietic cells during embryonic life will make animals fully tolerant (Davidson,1966; Manning and Botham 1980; Botham and Manning 1980). The work argues that the thymus epithelium (and any other tissue except hematopoietic tissue) is unable to effect deletion of T cells reactive to this tissue. There have been no definitive experiments to determine how these reactive cells are down-regulated in vivo. Some sort of suppression, rather than anergy (Rammensee et al 1989), seems likely since the reactive cells can be revealed in vitro. We have found that the skin of the anterior region in head/body chimeras contains many more lymphocytes (derived from the posterior region) than are found in normal skin. This increase in skin lymphocytes is also true of adult skin grafts used to make animals tolerant during larval life or metamorphosis, but not of isogeneic skin grafts. Whether these lymphocytes are in any way involved in the in vivo tolerance is unknown.

Autoantibody production

MHC-mismatched chimeras were employed with the hope of obtaining antibodies specific for non-class I alloantigens on erythrocytes (Xenopus erythrocytes bear as many class I molecules on the surface as do leukocytes (Flajnik et al 1984b). It was reasoned that such chimeras would be tolerant to the MHC class I antigens (which are always the most potent immunogens upon alloimmunization) of the anterior region, but would be reactive to other alloantigens only found on blood cells. Accordingly, FJ/JJ chimeras (FJ heterozygote anterior region including the thymus/JJ posterior region including all of the hematopoietic cells) were immunized with FF erythrocytes. The chimeras produced agglutinating antisera reactive with the FF erythrocytes within two months after the primary injections. Surprisingly, these

antisera contained immunoprecipitating IgG(Y) antibodies directed to class I molecules encoded by the F strain MHC haplotype (Fig. 1).

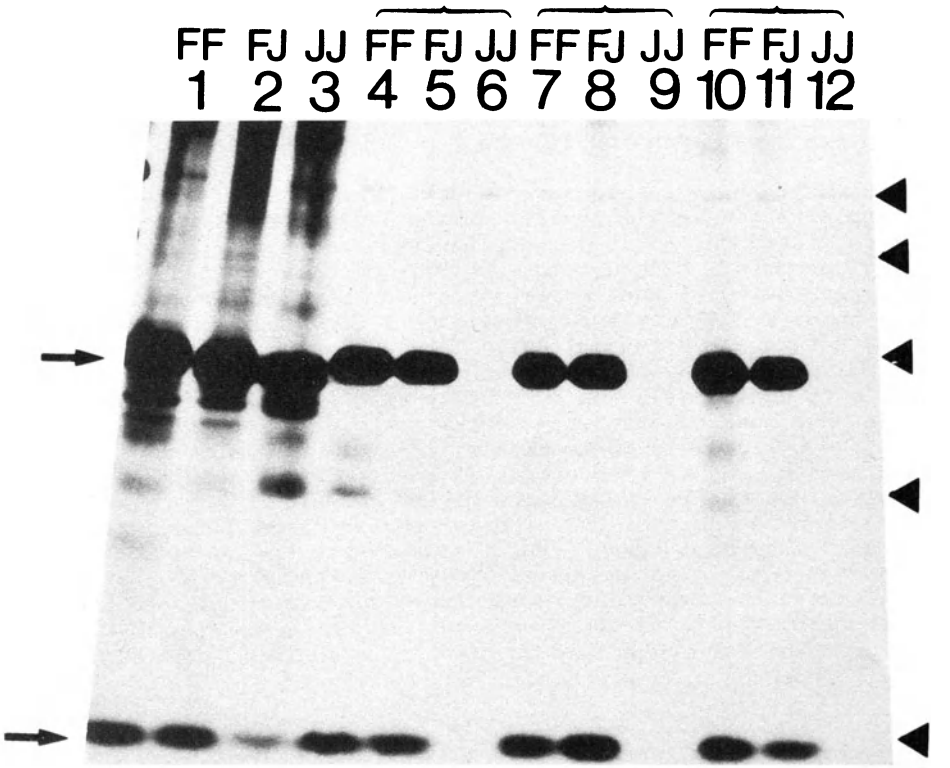

Fig. 1. "Autoantibodies" produced to MHC class I antigens by MHC-mismatched chimeras immunized with erythrocytes of the same MHC as the anterior region. Lanes 1,2,3 display immunoprecipitations with alloantisera produced by normal animals from metabolically-labeled cell lysates of FF, FJ, and JJ animals. Antisera from three FJ/JJ chimeras immunoprecipitate class I molecules from FF, FJ, but not JJ lysates (4,5,6: chimera 1; 7,8,9: chimera 2; 10,11,12: chimera 3). A mouse monoclonal antibody specific for Xenopus IgY was used as an indirect reagent to immunoprecipitate immune complexes. The class I alpha chains and beta2-microglobulin molecules are indicated by arrows on the left. Molecular weight markers (92, 69, 43, 30, 14 kDa) are noted on the right.

These data suggest that the JJ APC of the chimeras processed the FF membrane proteins, presented them in the context of J MHC molecules, and initiated the antibody response. The data also suggest that the chimeras' B cells were not tolerant to the MHC antigens of the head. In these chimeras, the F1 FJ thymus contains JJ homozygous hematopoietic cells (thymic lymphocytes and APCs). It appears that these hematopoietic cells cannot present the thymic (and head) F MHC antigens in the context of J MHC to developing T cells to induce tolerance. Antigen presentation therefore, at least in this system, seems to be different for activation and tolerance. The ability to detect these immunoprecipitating antibodies, like the aforementioned antibody responses to DNP, is dependent on the MHC haplotype combinations employed.

The anti-thymus antibodies appear to be of no autoimmune consequence to the animal. It is possible that these antibodies may somehow be involved in the downregulation of the T cells reactive to the MHC, and may "suppress" the rejection of normal skin grafts or the anterior regions of the chimeras. Experiments to determine whether there are preexisting (i.e. before immunization with erythrocytes) antibodies reactive with the anterior region's MHC are now being carried out. Alternatively, it may be that the epitopes recognized on class I molecules by these alloantisera are different than those found on the class I of the thymus.

ONTOGENY OF MHC EXPRESSION IN XENOPUS

Tadpoles, although immunocompetent, do not express the polymorphic class I molecule on the surface of cells, until prometamorphosis (Flaknik et al. 1986). Class II molecules are expressed, albeit with a different tissue distribution than the adult. The tadpole furnishes a good model to examine T cell selection. Since class I may be expressed by the tadpole thymus epithelium, but not on the surface of hematopoietic cells, it is possible that tadpoles actually select a population of class I-restricted cells without making these cells tolerant. We have recently proposed that such cells may be involved in tissue restructuring at metamorphosis, when class I molecules appear both on newly-arising adult tissues and on tadpole tissues which are to be destroyed (Flajnik and Du Pasquier, 1989).

CONCLUSIONS

It will take a long time to sort out the processes involved in positive and negative selection of the T cells (reviewed by Sprent et al 1988). Experiments over the last two years with transgenic mice (von Boehmer et al. 1989), monoclonal antibody injections (Zuniga-Pflucker et al 1989) and analysis of the normal T cell receptor repertoire in different strains of mice (Blackman et al 1989) have persuaded most of the immunological community that positive selection is true, but the mechanism remains obscure.

It is logical that there should be such a process to direct the selection of T cells for recognition of antigen in association with self MHC and for subset selection. Furthermore, we believe that the extensive MHC polymorphism found in vertebrates may be maintained by positive selection: individuals that are heterozygous at the MHC would have an advantage over homozygotes, not because of the greater "antigen-binding capacity" of a higher number of MHC molecules, but because of the increase in the expression of the potential repertoire.

The frog and chicken have played important roles in our understanding of tolerance, especially in determining how this process occurs during embryonic life. It is encouraging that the paradigm of the thymus epithelium being unable to completely make T cells tolerant arose from several different models at the same time. It is clear that "tolerance" is achieved by several different means, and it is important to determine which of these mechanisms are the most physiologically relevant. It is our belief that the old idea of "no blood-no tolerance" (reviewed by Nossal, 1989) will, in fact, be true, but only when self-reactive cells are clonally deleted. Since all other embryonically-transplanted tissues induce in vivo but not in vitro tolerance of T cells, other mechanisms besides deletion must maintain the non-responsiveness. Thus, these chimeras are choice models to study the generation and maintenance of peripheral tolerance. Two major questions remaining to be answered are how the in vivo reactive cells are downregulated and what, if any, role (blocking factors?) antibody plays in the maintenance of tolerance. We predict that such "autoantibodies" will be detected in T-depleted mice reconstituted with thymic grafts (Lo and Sprent 1986).

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Participation of CD4 and CD8 Accessory Molecules in the Development and Selection of T Lymphocytes

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INTRODUCTION

The contributions of the CD4 and CD8 accessory molecules to T cell receptor (TCR) specificity and thymic selection are matters of considerable interest. The tight correlation between CD4 expression and class II recognition and CD8 expression and class I recognition suggest that the accessory molecules are involved in specific MHC class recognition (Reinherz et al. 1983; Swain 1983). Supporting this notion is the fact that class I-specific responses can be blocked by anti-CD8 and class II by anti-CD4 antibodies (Marrack et al. 1983; Dialynas et al. 1983a). Others have provided data which demonstrate direct binding between accessory molecules and MHC (Doyle and Strominger, 1987).

Whether the accessory molecules function in the receptor-ligand complex to merely enhance avidity, to provide direct signals, or both is not clear. Transfection studies show that cotransfection of CD8 with TCR genes can enhance responsiveness when the target cell expresses low levels of the relevant MHC molecules (Dembic et al. 1987; Gabert et al. 1987). Even transfection of human CD4 into a murine class I-specific hybridoma can enhance responsiveness if the appropriate human class II ligand is transfected into the relevant class I target (Gay et al. 1987). These results suggest that MHC-accessory molecule interactions are due mainly to avidity effects and are not intimately involved in the TCR-MHC complex which results in activation. The fact, however, that $CD4^+$ cells rarely respond to class I antigens on antigen-presenting cells (APC) coexpressing class I and II MHC (and similarly, $CD8^+$ cells fail to respond when class II is coexpressed with class I on the target cell) suggests that the antigen receptor complexes with accessory molecules to determine the relevant interaction. This concept is supported by experiments in which heteroconjugates of anti-CD4 or CD8 with anti-TCR or CD3 are more effective than using anti-receptor antibodies alone for activating T cells (Emmrich et al. 1987; Ledbetter et al. 1988). Finally, there is evidence that CD4 and CD8 are associated with a tyrosine kinase (lck) that mediates auto and CD3ζ phosphorylation as a result of crosslinking the accessory molecules, suggesting a role in intracellular signaling for these molecules (Veillette et al. 1988, 1989).

Given the importance of the coparticipation of the accessory molecules with TCR$\alpha\beta$ in antigen-specific T cell responses, it is appropriate to ask whether these molecules actively participate in the selective processes which occur during development in the thymus. Do these molecules play a role in determining MHC class specificity, lineage commitment, and induction of tolerance to self antigens? Below we

review our studies that demonstrate that the accessory molecules expressed by developing T cells are involved in the interactions that lead to clonal deletions and are required for positive selection into the appropriate T cell lineage.

PARTICIPATION OF CD4 IN CLONAL DELETION

A major breakthrough in the area of thymic selection occurred with the development of antibodies directed toward TCR with defined specificity (Kappler et al. 1987). Through the use of such antibodies, it was shown that Vβ17, which is specific for class II I-E, is expressed on double-positive thymocytes but is absent from single-positive thymocytes and peripheral T cells of I-E-bearing mice. These observations, plus the fact that $V\beta17^+$ cells are deleted in the progeny of $(I\text{-}E^+ \times I\text{-}E^-)F_1$ mice, demonstrate that clonal deletion is an important mechanism for maintaining tolerance to self antigens. In these now-classic studies, Kappler et al. (1987) showed that not only are $V\beta17^+$ cells eliminated from the CD4 subset as might be expected for this class II-specific receptor, but they are also eliminated from the CD8 subset. Two theories could account for this unexpected result of an absence of $V\beta17^+$ $CD4^-8^+$ thymocytes (Fig. 1).

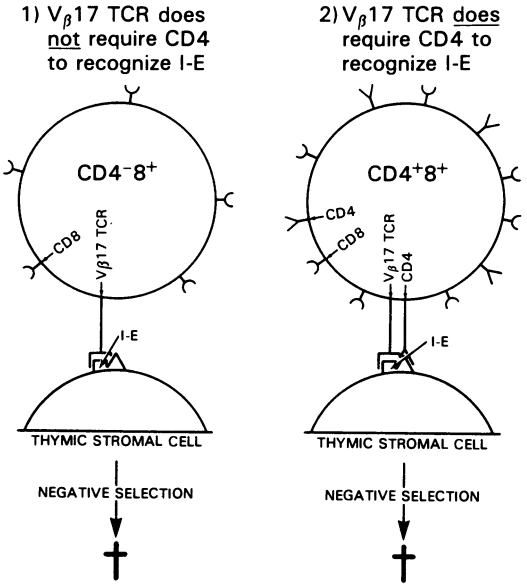

Fig. 1 Two possible models to explain why $V\beta17^+$ thymocytes are eliminated from the $CD4^-8^+$ subset in the $I\text{-}E^+$ C57BR mouse.

1) Clonal deletion could occur at the single-positive stage and, if so, interaction with I-E on the thymic stroma would have to occur in the absence of CD4 accessory molecules. 2) If single-positive thymocytes are derived from double-positive precursors, CD4 would be available at the double-positive stage for interaction of the Vβ17 receptor with I-E. Coparticipation of CD4 with TCR is required for

class II-specific interactions by mature T cells (see Introduction). To distinguish between these two models, anti-CD4 antibodies were used in vivo to block CD4 interactions on developing T cells (Fowlkes et al. 1988). Specifically, C57BR mice which bear I-E and express Vβ17 on $CD4^+8^+$ thymocytes were irradiated and reconstituted with syngeneic bone marrow. From day 9 - 22 after irradiation, these mice were treated daily with the anti-CD4 antibody, GK1.5 (Dialynas et al. 1983b) at a concentration which saturated $CD4^+$ thymocytes in vivo. As seen in Fig. 2, anti-CD4 antibodies prevent the appearance of the $CD4^+8^-$ thymocyte subset (due to a block of positive selection, see below). Since the $CD4^-8^+$ thymocytes do arise in the treated mice, it was possible to assay for Vβ17-bearing cells in this subset. Table 1 shows a representative example of such an experiment. The anti-CD4 treatment caused the appearance of $V\beta17^+$ cells in the $CD4^-8^+$ subset, a population of cells that is normally never observed in C57BR mice.

Table 1. In vivo treatment with anti-CD4 blocks clonal deletion of $V\beta17^+$ thymocytes

Strain	I-E Expression	Antibody Treatment[a]	% of $V\beta17^+$ $CD4^-8^+$ [b]
SJL	-	Control Ab	5.6
		Anti-CD4	5.8
C57BR	+	Control Ab	0.2
		Anti-CD4	5.2

[a] 2 mg every 12 hr of either GK1.5 or isotype-matched (rat IgG2b) control antibody i.p. from 9 - 23 days after irradiation and reconstitution with syngeneic bone marrow

[b] Thymocytes were depleted with anti-CD4 (GK1.5) and J11d (Bruce et al. 1981) plus complement in vitro to enrich for mature T cells and assayed by two-color flow cytometry for Vβ17 and CD8

$CD4^-8^+$ thymocytes occurred at the expected frequency and expressed high levels of Ly1 and no J11d antigen, indicating that the cells had a normal phenotype in the treated mice. The fact that the anti-CD4 treatment blocked clonal deletion and caused the appearance of Vβ17-bearing cells in the $CD4^-8^+$ subset indicated that clonal deletion must have occurred at a $CD4^+8^+$ precursor stage. More relevant to the current discussion, these results indicate that the CD4 accessory molecules on the developing T cell participate in the interaction that leads to clonal deletion. Equivalent results have been obtained from blocking deletion of Vβ6-bearing lymphocytes in mice bearing Mls-1^a with in vivo treatment with anti-CD4 (MacDonald et al. 1988).

$V\beta17^+$ $CD4^-8^+$ cells that appear after anti-CD4 in vivo blocking do not show I-E (self) reactivity in a mixed lymphocyte response (Fowlkes et al. 1988). This observation strengthens the argument that CD4 is required to obtain I-E-specific responses. While these experiments do not delineate the specific interactions or subsequent signals that promote this event, they strongly support a role for CD4 in clonal deletion.

PARTICIPATION OF ACCESSORY MOLECULES IN POSITIVE SELECTION AND MATURATION OF DEVELOPING THYMOCYTES

Recent evidence from TCRαβ transgenic mice suggest that the interaction of a specific TCR with a specific MHC drives the developing T cell to become functionally mature (Teh et al. 1988; Sha et al. 1988). This specific interaction also determines the lineage commitment of that cell; that is, whether it will ultimately be contained in the $CD4^+$ or $CD8^+$ subset. While these results demonstrate a correlation between MHC and accessory molecule expression, they do not directly address whether accessory molecule interactions are required for T cell differentiation. We wanted to provide direct evidence that the accessory molecules participated in the interactions involved in positive selection. Our studies using anti-CD4 to block clonal deletion demonstrate that $CD4^+8^-$ thymocytes do not appear in the presence of the antibody (Fig. 2). We presumed that this failure to generate $CD4^+8^-$ cells is the result of a direct block to an interaction between the accessory molecule and a ligand in the thymic environment which is required for continued differentiation and segregation into the appropriate T cell lineage. An alternative and more trivial explanation is that the antibodies are directly lytic to the appropriate single-positive thymocytes.

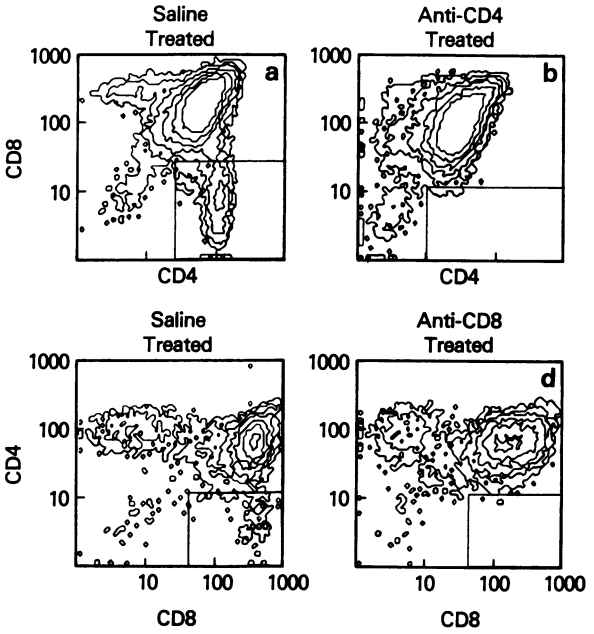

Fig. 2 Effect of in vivo anti-CD4, H129.19 (Pierres et al. 1984) and anti-CD8, 53-6.7 (Ledbetter and Herzenberg, 1979) treatment on thymocyte subsets. Syngeneic BM-reconstituted AKR were treated daily with saline (a, c) or 1 mg of anti-CD4 (b) or anti-CD8 (d). Immunofluorescent staining and two-color flow cytometry were performed as described previously (Fowlkes et al. 1988).

It has been shown previously that in vivo treatment with antibodies of certain isotypes can promote the elimination of cells bearing the specific molecules to which the antibodies are directed. The mechanism of this lytic event is not understood. It appears that rat monoclonal antibodies of the IgG2b isotype result in the elimination of mature peripheral T cells, but antibodies of the IgG2a isotype do not (Qin et al. 1989). Thus, rat IgG2a monoclonal antibody, H129.19 (Pierres et al. 1984), an anti-CD4, and 53-6.7 (Ledbetter and Herzenberg 1979), an anti-CD8, were used to test the effect of these non-deleting antibodies on T cell development (Ramsdell and Fowlkes, 1989). As can be seen in Table 2, mice irradiated and reconstituted with syngeneic bone marrow and treated daily with non-depleting anti-CD4 or -CD8 antibodies from day 9 - 27 fail to generate the corresponding $CD4^+8^-$ or $CD4^-8^+$ single-positive subsets (Fig. 2 and Table 2). Each antibody blocks the appropriate subset but has no effect on the reciprocal subset (Fig. 2).

Table 2 Injection of anti-CD4 or anti-CD8 monoclonal antibodies in vivo blocks the development of the corresponding mature thymocyte subset[a]

	Thymocyte Subset			
Treatment	$CD4^+8^-$	$CD4^-8^+$	$CD4^+8^+$	$CD4^-8^-$
Saline	10.4	3.0	83.8	2.8
anti-CD4	0.4	2.6	92.0	5.0
anti-CD8	10.4	0.3	84.9	4.4

[a] Data are expressed as the percent positive of total thymocytes. Mice were treated as described in the legend for Fig. 2

Although the concentrations of antibody used were able to saturate the $CD4^+8^+$ thymocytes, these cells were not eliminated (Fig. 2). Cell recoveries from antibody- and control-treated mice were comparable. In order to compare the effect of long-term treatment on thymocytes with peripheral T cells, unirradiated adult mice were treated with these non-depleting anti-CD4 or -CD8 antibodies for up to 18 days. Table 3 shows that, while treatment over a prolonged time period fails to eliminate lymph node CD4 or CD8 T cells, it completely blocks the development of the appropriate subset of single-positive thymocytes. These data suggest that the single-positive thymocyte pool is replaced within this time frame. More importantly, they demonstrate that the effect of the antibodies in the thymus is to block development, rather than deplete through lytic effects.

Using a different approach, Zuniga-Pflucker et al. (1989) have shown that $F(ab')_2$ and Fab fragments of anti-CD4 also block the appearance of $CD4^+8^-$ thymocytes. The results with Fab fragments indicate that crosslinking which is required to get auto and CD3ζ phosphorylation (Veillette et al., 1988, 1989) is not required in order to block $CD4^+8^-$ development, arguing that the inhibitory effect of the anti-CD4 in vivo is not a result of inducing this phosphorylation event. Since either anti-CD4 or -CD8 antibody treatment fails to affect the reciprocal subpopulation, it seems unlikely that the antibodies are delivering a negative signal, if single-positive thymocytes actually arise from double-positive precursors. Delivery of a negative signal

Table 3 Anti-CD4 or -CD8 antibodies (of the rat IgG2a isotype) block the development of $CD4^+8^-$ or $CD4^-8^+$ thymocytes without depletion of peripheral T cells[a]

	Lymph Nodes		Thymus	
Treatment	$CD4^+8^-$	$CD4^-8^+$	$CD4^+8^-$	$CD4^-8^+$ [b]
Saline	49.9	24.2	10.6	3.8
Anti-CD4	40.3	32.2	0.4	3.1
Anti-CD8	50.8	17.9	9.6	0.2

[a] Treatment of unirradiated adult AKR mice with antibodies injected daily, i.p., for 15 days

[b] All $CD4^-8^+$ values from thymic staining include only $CD3^+$ cells

at the single-positive stage is more difficult to exclude, but as shown here, the antibodies do not delete single-positive peripheral T cells.

Previous studies using anti-MHC antibodies in vivo demonstrated that maturation to single-positive thymocytes requires an interaction with MHC antigens (class II for $CD4^+8^-$ and class I for $CD4^-8^+$) (Kruisbeek et al. 1985; Marusic-Galesic et al. 1988; Marrack et al. 1988). Thus, it appears that positive selection results from an interaction of TCR on developing thymocytes with polymorphic regions of MHC molecules, which requires the coparticipation of the accessory molecules. Whether the role of CD4 in this interaction is to provide a signal or merely increase the avidity of this interaction is not distinguished by data presented here. The fact that anti-CD4 treatment upregulates expression of CD3 and TCR in neonatal and fetal thymocytes has been used to support the idea that CD4 has a signaling function in ontogeny (McCarthy et al. 1988; Zuniga-Pflucker et al. 1989). Additional evidence for CD4 signaling in development is the demonstration that CD4 is lck-linked and that crosslinking with anti-CD4 promotes phosphorylation as early as CD4 is expressed during fetal ontogeny (A. Kruisbeek and A. Veillette, personal communication).

The actual mechanism of the TCR-MHC interaction which results in the ultimate expression of only one accessory molecule is an important but unresolved issue. The accessory molecules may play no role in this process or, alternately, engagement of one of the accessory molecules into the receptor-MHC complex at the double-positive stage may direct the downregulation of the other accessory molecule (von Boehmer, 1986). Another hypothesis suggests a random turn-off of one of the accessory molecules, followed by positive selection (E. Robey, personal communication).

Thus, several lines of evidence suggest that the CD4/CD8 accessory molecules are involved in the positive selection process. The actual cellular and molecular interactions, intracellular signals, differentiation steps, and function of the accessory molecules in these various events are fascinating aspects of positive selection that are yet to be elucidated.

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Development of the T Cell Repertoire: Contributions of Both TCR-MHC and Accessory Molecule-MHC Interactions

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INTRODUCTION

To examine the issue of thymic selection with experimental systems entirely different from hematopoietic and thymic chimeras, the development of the T cell repertoire has been analyzed under conditions where expression of MHC-encoded gene products is blocked by in vivo mAb treatment during the early post-natal period. Such experiments also serve to re-evaluate in normal mice whether positive selection in the context of thymic MHC indeed forms the basis for the phenomenon of MHC restriction. The initial results demonstrated that blocking of class II-MHC resulted in abrogation of development of $CD4^+CD8^-$ T cells (Kruisbeek et al 1985), and blocking of class I-MHC prevented generation of $CD4^-CD8^+$ T cells (Marusic- Galesic et al 1988). Thus, the impressively strong correlation, in mature T cells, between expression of particular accessory molecules (CD4 or CD8) and MHC-restriction specificity (class II or class I, respectively) (Swain 1983) appears to reflect early selection events. These findings are consistent with the idea that blocking of the appropriate MHC molecules prevents delivery of a positive signal necessary for clonal differentiation.

While these data may reflect a requirement for TCR-MHC interactions, the findings could equally well be explained by postulating that positive selection results entirely from interactions between the CD4 and CD8 accessory molecules (or, rather, "co-receptors", Janeway 1988,1989) and MHC. However, the demonstration that positive selection in the context of thymic MHC occurs in both αβ-TCR transgenic mice (Kisielow et al 1988; Sha et al 1988) as well as in normal mice (Zúñiga-Pflücker et al 1989d; Liao et al 1989; MacDonald et al 1988a) clearly established the requirement for TCR-MHC interactions. Additional experiments presented below support the concept that the selection of the T cell repertoire involves not only TCR-mediated events, but also interactions between co-receptors and their ligands. Furthermore, we address the regulation of development of T cells "escaping" negative selection, and define the negative and positive selecting MHC alleles for Vβ11-expressing T cells in $H\text{-}2^k$ mice.

Participation of CD4 and CD8 molecules in positive selection events

To test the possible contributions of interactions between CD4 or CD8 co-receptors and their ligands to early T cell development, pregnant mice were treated from day 17 of gestation with anti-CD4 mAb (GK1.5) or anti-CD8 (α chain) mAb (2.43) as described (Zúñiga-Pflücker et al 1989a,b), and treatment was continued in the neonatal mice until the age of 2 weeks. Analysis of the thymus at that time (Fig.1A) reveals that expression of most CD4 molecules is completely blocked in anti-CD4-treated mice, and expression of most CD8 molecules is blocked in anti-CD8-treated mice. Yet, most $CD4^+CD8^+$ thymocytes are still present, as visualized by the staining for CD5 vs. CD8 in anti-CD4-, and CD5 vs.CD4 in anti-CD8-treated mice (Fig.1B), as well as by staining, in anti-CD8-treated mice, for CD8-β chain (Lyt3) (Zúñiga-Pflücker et al 1989b).

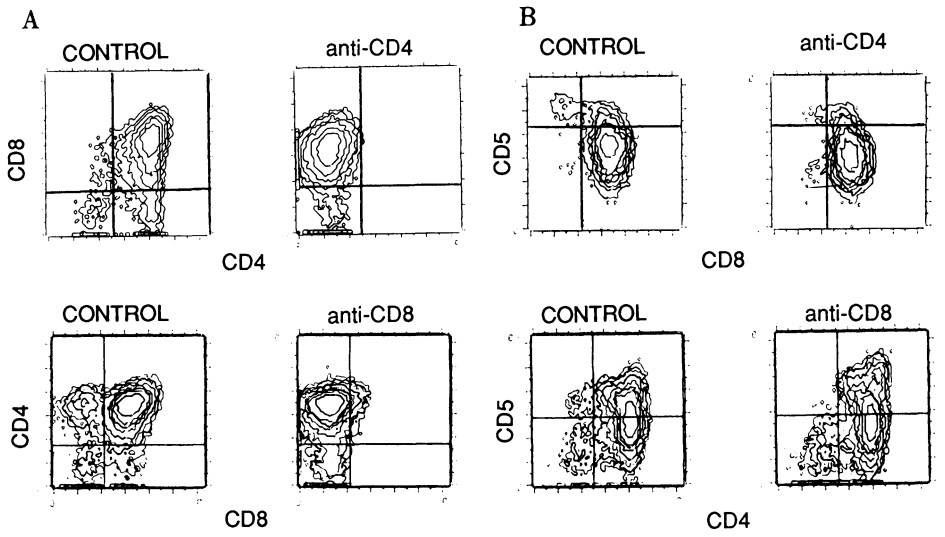

Fig.1. CD4, CD5 and CD8 expression in thymuses from 2-week old mice treated from day 17 of gestation with anti-CD4 (top) or anti-CD8 mAb (bottom)

Nevertheless, it appears that anti-CD4 treatment prevented generation of single positive CD4 cells (as demonstrated by the absence of $CD5^+CD8^-$ cells, Fig.1B and Zúñiga-Pflücker et al 1989a), while anti-CD8 treatment prevented appearence of single positive CD8 cells (i.e., absence of $CD5^+CD4^-$ cells in Fig.1B and Zúñiga-Pflücker et al 1989b). This point is difficult to visualize, given the low numbers of single positive cells in a two-week old mouse

thymus, and we therefore enriched for mature thymocytes by in vivo hydrocortisone treatment. As shown in Fig.2A, only 2 populations of bright CD3+cells are present in a thymus from hydrocortisone-treated mice: $CD3^+CD4^+CD8^-$ and $CD3^+CD4^-CD8^+$. A striking absence of $CD3^+CD4^-$ cells is noted in the anti-CD8-treated mice, demonstrating that, indeed, single positive CD8 cells were not generated. By analogy, the $CD3^+CD8^-$ subset is greatly diminished in the anti-CD4-treated mice, substantiating the blocking of generation of single positive CD4 cells. The specificity of these effects is demonstrated by the fact that anti-CD4 does not affect generation of the single positive CD8 cells, and anti-CD8 does not affect development of CD4 cells. These conclusions are further supported by an analysis of CD5 vs. co-receptor expression (Fig.2B): $CD5^+CD4^-$ cells (corresponding to $CD4^-CD8^+$ T cells) are absent in anti-CD8-treated mice, and $CD5^+CD8^-$ cells (corresponding to $CD4^+CD8^-$ cells) are reduced in anti-CD4-treated mice. Since identical results were obtained with $F(ab')_2$ mAb's (data not shown; Zúñiga-Pflücker et al 1989a,b), these findings demonstrate that the failure to generate the pertinent single positive cells in anti-CD4-or anti-CD8-treated mice is not a reflection of direct removal of these cells, but a consequence of blocked interactions between the co-receptors and their ligands. The observation that double positive thymocytes are still present in anti-CD4-or anti-CD8-treated mice further supports this explanation. It also should be noted that these results can be regarded as a reflection of intra-thymic events, since, at least for anti-CD4, they could be mimicked completely in an in vitro fetal thymus organ culture system (Zúñiga-Pflücker 1989a), with both intact antibodies and $F(ab')_2$ fragments.

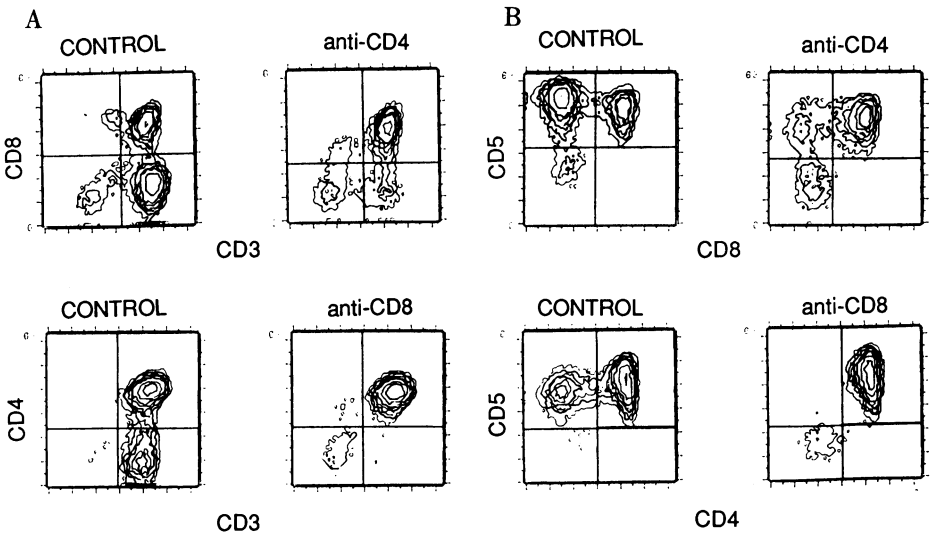

Fig.2. Composition of thymuses from 2-week old mice treated with anti-CD4 (top) or anti-CD8 (bottom) mAb from day 17 of gestation.

The implication of these results is that positive selection of mature T cells is not only a consequence of TCR-MHC interactions, but that CD4-ligand and CD8-ligand interactions contribute to the development of mature T cells as well. This may reflect either a direct requirement (i.e., signals involving CD8 or CD4 are necessary), or an indirect one (i.e., in absence of CD4- or CD8-ligand interactions, effective signalling through the TCR is not possible). The earlier reported findings on abrogated development of single positive CD4 cells in anti-class II treated mice, and of single positive CD8 cells in anti-class I treated mice (see Introduction) may therefore reflect a blockade of both TCR-MHC as well as co-receptor-MHC interactions.

It is not clear in which way interactions involving the CD4 and CD8 molecules participate in selection of the T cell repertoire and, for that matter, in activation of mature T cells. While it is known that CD4 and CD8 can contribute to the total avidity of the interactions between T cells and APC's, models proposing only an avidity role for CD4 and CD8 during development would be unable to explain the bias of the T cell repertoire, i.e., the preferential expression of CD4 on class II- restricted T cells, and of CD8 on class I- restricted T cells. It is therefore more attractive to regard these molecules as signal transducers, and various recent studies are in agreement with such a view: a signal transduction through cross-linking of CD4 or CD8 can be initiated with mAb's to CD4 or CD8 not only in mature T cells (Rudd et al 1988; Veillette et al 1988) but also in $CD4^+CD8^+$ thymocytes (McCarthy et al 1988; Veillette et al 1989); b accumulating evidence suggests that CD4 and CD8 may become physically associated with the TCR during activation of mature T cells (Emmrich et al 1988; Weynand et al 1987; Fazekas de St Groth et al 1986; Janeway 1988, 1989) and have additional regulatory roles (Schrezenmeier and Fleischer 1988); c the specificity of a given TCR dictates whether developing T cells will mature into CD4 or CD8 cells (Teh et al 1988; Sha et al 1988; Scott et al 1989; Berg et al 1989); d clonal deletion of T cells with a class II-restricted receptor involves participation of CD4 (Fowlkes et al 1988; MacDonald et al 1988b), but not of CD8 (Zúñiga-Pflücker et al 1989b). With the findings on signalling in mind, however, any study employing mAb-blocking of CD4/CD8 molecules needs to consider potential side effects of antibody-induced signalling. We consider it unlikely that our findings on blocked development of CD4 cells are due to perturbations caused by Ab-mediated signalling, since non-signalling Fab fragments of anti-CD4 had identical effects to intact anti-CD4 in an in vitro fetal thymus organ culture system, i.e., both blocked development of CD4 cells equally well (Zúñiga-Pflücker et al 1989a). Together, these studies therefore provide formal evidence for the earlier suggestions (von Boehmer 1986; Janeway 1988,1989) that CD4 and CD8 are crucial to positive selection.

TCR-MHC interactions and positive selection in normal mice

In TCR-transgenic mice with a class I-restricted receptor, preferential differentiation of $CD4^-CD8^+$ cells with the complete transgenic receptor occurs, provided the appropriate MHC antigens are available (Teh et al 1988; Sha et al 1988; Scott et al 1989). The reciprocal findings are observed in TCR-transgenic mice with a class II-restricted receptor, in that expression of the restricting class II-elements leads to preferential differentiation of $CD4^+CD8^-$ T cells with both transgenic TCR chains (Berg et al 1989). In our own studies using normal mice, the issue of positive selection was addressed by following the fate of developing TCR-expressing $CD4^-CD8^+$T cells under conditions where one of the main class I-MHC molecules, either H-2K or H-2D,was specifically blocked by in vivo mAb treatment. Indeed, it was found (Zúñiga-Pflücker et al 1989d) that generation of $CD4^-CD8^+$ T cells with a Vβ17 TCR in SJL mice ($H\text{-}2^s$) can be completely blocked by treatment with mAbs specific for K^s, while being unaffected by treatment with anti-D^s mAbs. These data directly demonstrate that generation of $CD4^-CD8^+$ T cells expressing a particular TCR Vβ chain can be correlated with expression of particular class I-MHC antigens, thereby providing evidence for positive selection in normal mice. Together with the demonstration of preferential differentiation of particular Vβ-expressing T cells in the context of thymic MHC in several other normal mouse models (McDonald et al 1988a; Liao et al 1989; Blackman et al 1989), it now appears that a definite role for TCR-MHC interactions in positive selection has been established. Of course, such interactions had earlier been shown to be crucial to negative selection as well (Kappler et al 1987a).

In the present experiments, we address whether positive selection is also responsible for development of $V\beta11^+$T cells "escaping" negative selection, and define the negative and positive selecting elements for Vβ11-expressing T cells. In $H\text{-}2^k$ mouse strains, I-E molecules are potent tolerogens for T cells expressing a TCR with a Vβ11 chain (Bill et al 1989). Confirmation of the idea that I-E molecules are the deleting elements in these mice was obtained through analysis of hydrocortisone-treated thymuses from mice treated from birth with anti-I-E mAb (14-4.4) according to previously described protocols (Kruisbeek et al 1985): substantial numbers of $V\beta11^+$ cells can now be detected (Fig 3 right), at levels comparable to those in $I\text{-}E^-$ mouse strains (Bill et al 1989). Yet, we consistently observed, when analyzing thymuses from control $H\text{-}2^k$ mice after enrichment of mature T cells by hydrocortisone treatment, that low numbers of Vβ11 expressing cells could be detected, particularly in the $CD4^+CD8^-$ subset (Fig.3 left).

We then asked the question whether such $V\beta11^+$ T cells have escaped intra-thymic deletion, or whether they reflect the presence of extra-thymically

generated T cells which may not have been subject to the same tolerizing effects as those operating within the thymus. Fetal thymuses from H-2^k mice were cultured in an in vitro fetal thymus organ culture system as described (Zúñiga-Pflücker et al 1989a), and after 9 days the thymuses were subjected to treatment with J11D mAb plus complement to enrich for mature T cells (Crispe et al 1987).

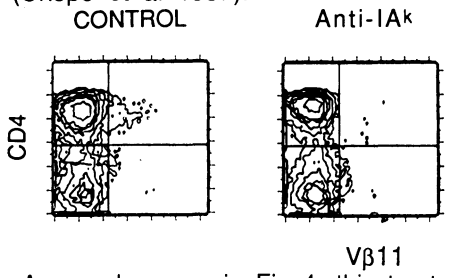

Fig 3
Vβ11 expression in hydrocortisone treated thymus from control, anti-I-A^k or anti-I-E^k treated H-2^k mice

As can be seen in Fig 4, this treatment indeed eliminates all CD4+CD8+ thymocytes (for comparison, see Zúñiga-Pflücker et al 1989a) and thus enriches for single positive mature T cells with an αβ-TCR. Strikingly, these in vitro generated mature T cells also display substantial expression of the Vβ11 TCR (Fig 4 right panel), demonstrating that intra-thymic deletion is not complete. Thus, the Vβ11 cells observed in the H-2^k thymus in vivo may well have originated intra-thymically.

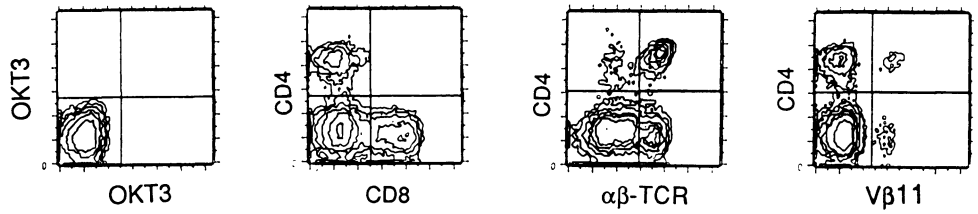

Fig 4. Day 16 fetal thymii from H-2^k mice were cultured for 9 days in an in vitro thymic organ culture system, and flow cytometry analysis was performed after M1/69-plus-C' treatment to enrich for mature T cells.

We finally asked which selecting elements are responsible for the generation of Vβ11 T cells in both anti-I-E treated (Fig 3 right) and control (Fig 3 left) mice. Since development of most CD4+CD8$^-$ T cells is dependent on expression of class II gene products (Kruisbeek et al 1985), two observations favor the idea that the positive selecting element for the Vβ11+CD4+CD8$^-$ subset is I-A. First, there are no class II molecules other than I-A available in anti-I-E- treated H-2^k mice, and substantial numbers of CD4+CD8$^-$ T cells are

generated (Fig 3 right); this argument also applies to H-2^b mice (Bill et al 1989). Second, generation of the small subset of Vβ11+CD4+CD8- cells is completely abrogated in mice treated from birth with anti-I-A mAb (Fig 3 middle panel). These experiments do not resolve which MHC gene products are the positive selecting elements for the Vβ11+CD4-CD8+ subset, but it is likely that the class I-MHC molecules are responsible (Marusic-Galesic et al 1988).

Taken together, the data imply that Vβ11+CD4+CD8- T cells in H-2^k mice which "escape" negative selection do not arise by default, but, instead, are positively selected. Furthermore, the results demonstrate that Vβ11+CD4+CD8- cells are negatively selected on I-E, and positively selected on I-A. It is interesting that only few Vβ11-expressing T cells have reactivity for I-E (Bill et al 1989) while opposite findings were observed with Vβ17a-expressing T cells: I-E can function as a deleting element not only for Vβ11 T cells but also for Vβ17a T cells (Kappler et al 1987a), yet most Vβ17a-expressing T cell hybridomas express reactivity for I-E (Kappler et al 1987b). One could suggest that, as for Vβ11 T cells, the positive selecting element for Vβ17a T cells is I-A, and several previous observations are consistent with this hypothesis. First, many I-E- mouse strains express Vβ17 in the CD4+CD8-subset (Kappler et al 1989); such cells were most likely generated through interactions with I-A (Kruisbeek et al 1985), and it would therefore appear that the positive selecting element for Vβ17a is in fact I-A. This assertion is further supported by the observation that Vβ17a T cells react only to I-E on B cells, and not to I-E on thymic epithelial cells (Marrack and Kappler 1988), and are not "over selected" in thymuses in which I-E expression is restricted to the epithelial components (Marrack et al 1988). While this hypothesis does not explain the high frequency of Vβ17a+T cells that react with allogeneic forms of I-E-plus-B cell peptides (Marrack and Kappler 1988), it does explain why only few Vβ11-expressing T cells have reactivity for I-E (Bill et al 1989).

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Self-Nonself Discrimination by the Immune System

H. von Boehmer, H. Kishi, B. Scott, P. Borgulya, H.S. Teh, and P. Kisielow

INTRODUCTION

The immune system recognizes antigen by sets of very diverse proteins which are anchored in the membrane of lymphocytes. Each lymphocyte has only one type of receptor which differs from that on other lymphocytes (Burnet 1959). The receptor genes are formed by random rearrangement of gene segments in somatic cells (Tonegawa et al 1976). The receptors on B lymphocytes are called antibodies which can be secreted by antigenically stimulated B lymphocytes. T lymphocytes express T cell receptors (TCR) which cannot be secreted and are encoded by a different set of genes (Hedrick et al 1984).

Antigen recognition by T cells differs from that of B cells in that the αβ receptor on T cells has a dual specificity for a foreign antigen as well as a protein, called major histocompatibility complex (MHC) molecule, which presents the foreign antigen to the T cell (Bjorkman et al 1987, Fig. 1). For instance, a peptide derived from a viral protein of a virus infected cell can be bound by MHC molecules inside the cell and transported to the cell surface where it is now recognized by the TCR. This leads to activation of the T cell which destroys the virus infected cell.

There are two classes of MHC molecules: the class I MHC molecules expressed by most somatic cells present peptides which occur in the cytosol. Class II MHC antigens, expressed by B cells, macrophages and epithelial cells, present peptides which occur in lysosomes (Townsend et al 1986). Class I presented peptides are recognized by CD8 and class II presented peptides by CD4 lymphocytes. CD8 and CD4 molecules are expressed on mature T cells in a mutuallly exclusive fashion and bind to nonpolymorphic regions of class I and class II MHC antigens, respectively (Fig. 1). The TCR contacts both the peptide as well as the polymorphic MHC residues (Fig. 1). The MHC polymorphism is extensive, so that most individuals differ in their antigen-presenting MHC molecules.

This scenario of immune recognition poses two questions: first, how does the immune system distinguish self and foreign peptides? Second, how does the immune system learn to distinguish the various antigen-presenting MHC molecules such that it most efficiently interacts with one particular set of MHC molecules, which occur only in one individual?

A partial solution to the former question was proposed by Burnet (1957) and Lederberg (1959) many years ago: their view was that lymphocytes developing from hemopoietic stem cells pass through a stage of differentiation where contact with antigen is lethal rather than stimulatory. In this way, all self reactive, potentially harmful lymphocytes would be deleted, leaving behind a population of cells with receptors for foreign antigens. With regard to the second question, it was argued that lymphocytes which have receptors able to recognize foreign antigens presented by self MHC molecules

pass through a stage of differentiation where binding of the TCR to self MHC molecules, in the absence of the foreign antigen, leads to positive selection (von Boehmer 1986). As a result of both positive and negative selection the mature T cells would be devoid of autoaggressive cells but enriched in T cells able to recognize foreign antigen presented by self MHC molecules.

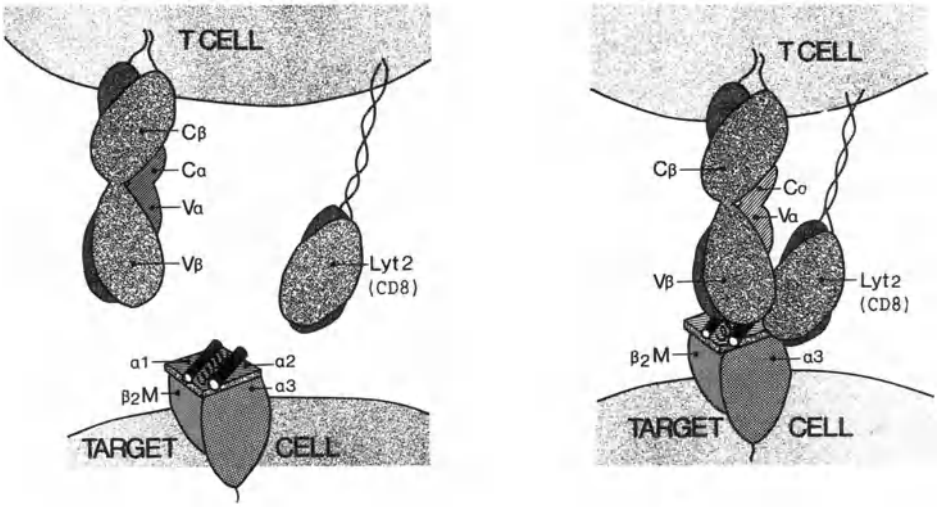

Fig. 1. Antigen recognition by T cells. A peptide (spiral) is bound by a class I MHC molecule between the polymorphic α_1 and α_2 domains. The T cell receptor consisting of α and β polypeptide chains, each consisting of variable and constant domains, binds to the peptide as well as the polymorphic α_1 and α_2 domains. The Lyt2 = CD8 accessory molecule binds to the nonpolymorphic α_3 domain. The crosslinking of the αβ T cell receptor and the CD8 molecule by the same antigen-presenting MHC molecule (right hand side) leads to T cell activation.

These hypothetical selection events are of course very difficult to study in normal animals, which contain probably 10^6 different TCRs. Therefore, we aimed at establishing TCR transgenic mice which express only one receptor. TCR genes were obtained from a T cell, which was specific for a peptide present in male but absent in female animals (HY antigen), and a class I MHC presenting molecule (D^b MHC molecule). The results obtained with such monoclonal TCR transgenic mice show that indeed potentially autoaggressive T cells can be deleted early in the development and that immature T lymphocytes expressing receptors with specificity for self-MHC antigen presenting molecules are selected by the MHC molecules for further maturation.

A mouse with a monoclonal immune system

DNA containing productively rearranged TCR genes, which was isolated from a $CD4^-8^+$ T cell clone specific for HY antigen presented by D^b MHC molecules, was introduced into fertilized eggs and TCR transgenic

mice were born. It turned out that by this procedure most T cells expressed the transgenic receptor but that this was not the only receptor expressed in the transgenic mice; endogenous Vα gene segments could still rearrange and form TCRα genes expressed by many T cells in addition to the transgenes (Teh et al 1988). Because this situation was not ideal for studying negative and positive selection, an additional trick was used to obtain TCR monoclonal mice: by breeding, the transgenes were introduced into a mouse strain with severe combined immune deficiency (scid) because of a defect of the rearrangement process generating receptor genes. T cells from such TCR transgenic scid mice expressed, indeed with very few exceptions, only the transgenic receptor (Scott et al 1989).

Potentially autoaggressive T cells are deleted at an early stage

To find out about the mechanisms of immune tolerance towards self antigens, the thymuses of female and male TCR transgenic scid mice lacking and expressing, respectively, the HY antigen recognized by the TCR, were compared. In the thymus T cells develop from hemopoietic stemm cells and begin to express TCRs as well as CD4 and CD8 molecules. The earliest cells lack TCR as well as CD4 and CD8 molecules and are referred to as $CD4^-8^-$ lymphocytes. At a later stage thymocytes express relatively low levels of TCRs but both CD4 and CD8 molecules, referred to as $CD4^+8^+$ thymocytes, which still cannot be stimulated by antigen. The most mature thymocytes express relatively high levels of TCRs, but either CD4 or CD8 molecules, referred to as $CD4^+8^-$ and $CD4^-8^+$ thymocytes (Table 1).

Table 1. Thymic development

Increasing maturity	Phenotype of thymocytes	Inducible by antigen
↓	$CD4^-8^-$ TCR^-	-
	$CD4^+8^+$ TCR^{+-}	
	$CD4^+8^-$ | > TCR^{++}	+
	$CD4^-8^+$ |	

Analyzing the thymocytes from the female and male TCR transgenic mice it was found that the female thymus contained all three populations of thymocytes reflecting the different stages of differentiation, while the male thymus contained only the most immature $CD4^-8^-$ thymocytes (Kisielow et al 1988). Thus, these experiments supported the notion of Lederberg (1959) that potentially autoaggressive lymphocytes can be deleted at an immature stage, in this case the $CD4^+8^+$ stage of development.

The generation of the most mature T cells requires binding of the TCR to thymic MHC antigens

The positive selection of T cells with a receptor specific for one particular antigen-presenting MHC molecule (in our case a class I D^b MHC molecule) was analyzed in TCR monoclonal mice expressing or

lacking the antigen-presenting D^b MHC molecule, i.e. in H-2^{db} and H-2^d mice, respectively. Here, only female mice lacking HY antigen were analyzed. While the thymus of H-2^{bd} mice contained cells of all three maturation stages, the thymus of H-2^d mice contained only two, namely $CD4^-8^-$ and $CD4^+8^+$ thymocytes (Teh et al 1988, Scott et al 1989, Kisielow et al 1988). These experiments mean that only those immature $CD4^+8^+$ thymocytes whose receptor binds with a certain affinity to antigen-presenting MHC molecules in the thymus differentiate further into mature T cells (Fig. 2).

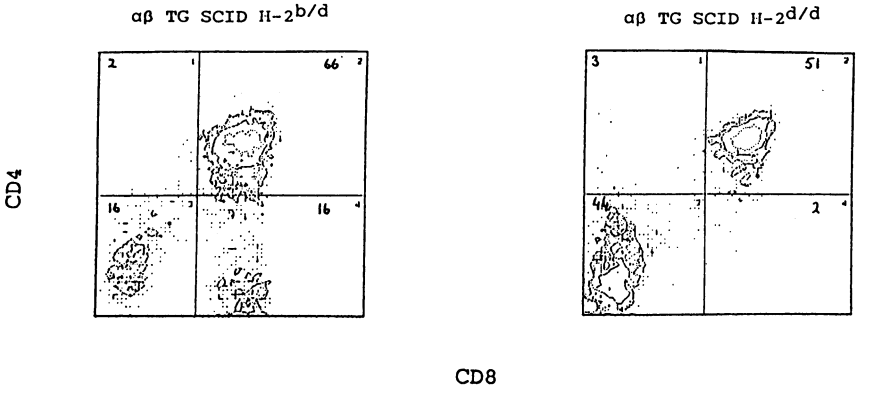

Fig. 2. Analysis of thymocytes from αβ TCR monoclonal (α,β transgenic scid) mice expressing or lacking D^b MHC antigens. The thymocytes are stained by antibodies directed against CD4 (y axis) and CD8 (x axis) accessory molecules and analyzed by the fluoresceine activated cell sorter. The numbers in the quadrants represent the proportions of cells in each quadrant.

The specificity of the TCR determines the CD4/CD8 phenotype of mature T cells

Some experiments support the view that T cells are most strongly stimulated when the TCR on the one hand and the CD4 or CD8 accessory molecules on the other hand are brought into proximity by the same antigen-presenting MHC molecule (Emmrich et al 1986). This means that a T cell with a TCR specific for a class I MHC molecule and a CD4 accessory molecule (binding to a class II MHC molecule) cannot, or only poorly, be activated. Indeed, this constellation is avoided by positive selection in the thymus: the interaction of a TCR with class I MHC molecules in the thymus determines that this receptor is only expressed on mature $CD4^-8^+$ but not $CD4^+8^-$ T cells. This is evident by the fact that the H-2^{bd} thymus of our monoclonal scid mice contained $CD4^-8^-$, $CD4^+8^+$, $CD4^-8^+$ but not $CD4^+8^-$ T cells (Fig. 2). This mechanism avoids that positively selected TCRs are wasted when they are expressed together with inappropriate CD4 or CD8 accessory molecules (Teh et al 1988, Scott et al 1989).

Conclusion

The construction of TCR monoclonal mice has shown in which way the immune system can learn to distinguish self from non-self, which is important both in avoiding auto-immunity as well as selecting a

most efficient immune system. The mechanism of learning appears relatively simple: immature T cells with autoaggressive TCRs are destroyed before they can become autoaggressive. Immature T cells whose receptor binds with a certain affinity to antigen-presenting MHC molecules are selected for further maturation such that the mature T cell contains only cells which are able to recognize foreign antigens presented by self-MHC molecules. The third category, namely immature T cells which do not bind to self antigens, do not mature but die after a short lifespan intrathymically.

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II Cell Developments

Development of B Cells

1. Phylogeny, Stem Cells, Lineages

Development and Regulation of the B Lymphocyte Lineage: An Interpretive Overview

R. A. Phillips

INTRODUCTION

Death is the ultimate fate of all cells in the hematopoietic system, including B lymphocytes. The maintenance of the pool of B lymphocytes requires their continuous production from undifferentiated progenitors (Phillips 1984). While the precise life span of B lymphocytes is unknown, several points are clear. First, the relative number of B lymphocytes in an animal does not change markedly with time. Thus, the number of B cells eliminated each day must approximately equal the number of new B cells produced by differentiation from progenitor cells. Second, there exist long-lived (life span, three weeks) and short-lived lymphocytes (life span, one day), although controversies revolve around the proportion in each category (Freitas et al. 1986). Since an adult mouse makes approximately $3x10^7$ mature B cells per day (Osmond and Park 1987), the size of the short-lived population must be of similar size if the life span of a short-lived B cell is 1 day.

Most cell renewal systems have a structure similar to that outlined in Fig. 1 (Till and McCulloch 1980). In all cell renewal systems, the output of mature cells is maintained by a small number of pluripotent stem cells, capable of both differentiation and self-renewal. Since the number of stem cells remains relatively constant during adult life, the number of divisions resulting in differentiation must equal the number of divisions resulting in self-renewal. To generate large numbers of mature cells, stem cells differentiate into progenitors committed to a specific lineage which then proliferate extensively producing the required number of mature cells. The major mechanism of regulation in cell renewal systems is specific growth factors acting on committed progenitors.

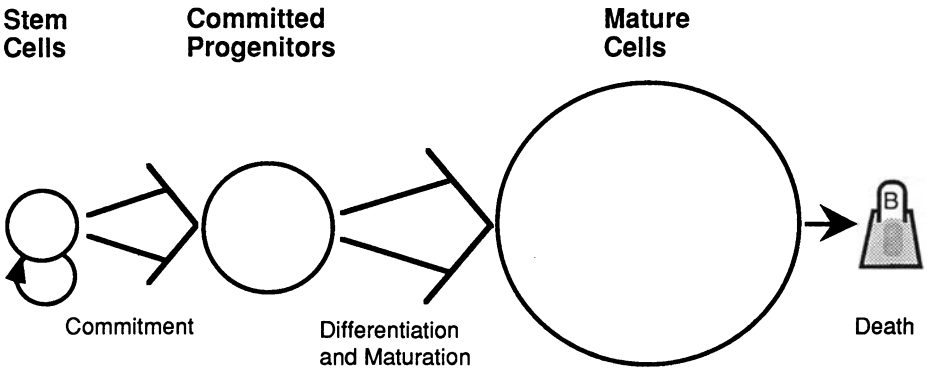

Figure 1. Components of a typical cell renewal system. Each circle represents the size of the pool of cells at that stage of development. Only stem cells have significant self-renewal potential as indicated by the circular arrow.

In the following sections, I will describe the origin, structure, and regulation of the stem cell compartment, and then discuss in detail the stages of development during the generation of B lymphocytes from stem cells. This discussion will present only an interpretive overview of B lymphocyte differentiation. Readers are referred to recent extensive, authoritative reviews by Kincade for details on many aspects of differentiation in this pathway (Kincade 1987; Kincade et al. 1989).

STEM CELLS

Sub-Classes in Stem Cells

There is no universally accepted definition of a stem cell. For the purposes of this presentation, I will use the properties discussed initially by Siminovitch et al. (1963), namely, that stem cells must self-renew, proliferate extensively, differentiate, and respond to regulatory signals. The major difficulty in applying these criteria is to define self-renewal and the amount of proliferation required for a cell to qualify as being a stem cell. In our experiments, we have required that a cell, to be called a stem cell, must either be able to completely reconstitute myeloid and/or lymphoid function in suitable recipients, or form colonies in the spleens of irradiated mice (Phillips 1984). Using these definitions, we have proposed that the stem cell pool in the hematopoietic system can be sub-divided into four sub-classes, as indicated in Fig. 2 (Phillips 1984).

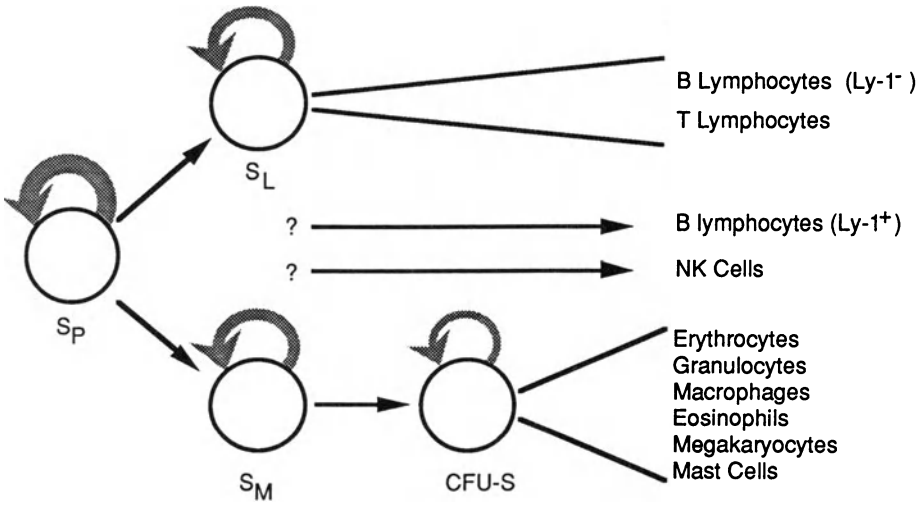

Figure 2. Stem cells of the hematopoietic system. S_P, pluripotent stem cell; S_M, stem cell restricted to myeloid and erythroid differentiation; CFU-S, spleen colony-forming stem cell; S_L, stem cell restricted to lymphoid differentiation. Grey arrows indicate cells with self-renewal potential.

All of the stem cells indicated in this diagram have extensive proliferative and self-renewal capabilities, fulfilling the definitions described above. There is no controversy about the existence of a pluripotent stem cell (S_P) or about the existence of the spleen colony-forming stem cell, CFU-S. The existence of myeloid-restricted, S_M, and lymphoid-restricted, S_L, stem cells has been challenged by some (Snodgrass and Keller 1987). S_M and CFU-S are both restricted to the production of myeloid and erythroid cells; they cannot produce lymphocytes (Abramson et al. 1977; Paige et al. 1979). They differ in two respects. First, a single CFU-S cannot reconstitute hemopoietic function in congenitally anemic recipients (Kitamura et al. 1981), but S_M have this ability (Abramson et al. 1977). Second, S_M cannot form colonies in the spleen; they must first migrate to the bone marrow, where they differentiate into CFU-S.

Several experiments support the existence of a lymphoid-restricted stem cell. The most convincing evidence concerns the ability of cells from long-term bone marrow cultures to reconstitute lymphoid and myeloid function in suitable recipients (Fulop et al. 1989). The stem cells in such cultures can readily reconstitute lymphoid function, but they are markedly deficient in myeloid repopulating ability. In addition, recent studies in our laboratory using retrovirus-marked stem cells have identified lymphoid-restricted stem cells in normal marrow (Fulop et al. 1989).

The origin of some cells in the hematopoietic system remains a mystery. NK cells are clearly part of the hematopoietic lineage, but their origin is unknown. They may arise as part of the lymphoid or the myeloid lineage (Hackett et al. 1986). While some virus-induced tumors have the capacity to differentiate into both B cells and macrophages (Davidson et al. 1988), the only cell with this potential is the pluripotent stem cell, S_P (Fig. 2). No one has yet reported a normal committed progenitor having the capacity to make only B lymphocytes and macrophages.

Another puzzling lineage is the one resulting in the formation of Ly-1$^+$ B cells. These cells arise during ontogeny and are present in newborn animals; they predominate in the peritoneal cavity of adult animals. When bone marrow from an adult animal is transplanted into irradiated recipients or congenitally deficient recipients, Ly-1$^+$ B cells are not produced. However, transplantation of Ly-1$^+$ B cells into such recipients allows a some reconstitution of this sub-population, indicating that Ly-1$^+$ B cells, unlike normal B cells, have limited self-renewal potential (Hayakawa et al. 1986). To this extent, they are similar to the B lineage in chickens, where, in the adult, there are no lymphoid stem cells. Reconstitution of B cell function occurs by self-renewal of existing B lymphocytes (Pink et al. 1985). Whether the failure of bone marrow stem cells to produce Ly-1$^+$ B cells represents an inability of these cells to produce such lymphocytes or a lack of the appropriate stimulatory factors or microenvironment is unknown. It seems likely that Ly-1$^+$ B cells are derived from lymphoid-restricted stem cells during fetal life, but that the factors required for support and stimulation for this pathway are lacking the adult animal.

Ontogeny and Commitment

In the development of both mammalian and avian embryos, hematopoiesis first appears in the extra-embryonic tissues of the yolk sac. This hematopoiesis is restricted to erythropoiesis and myelopoiesis. The origin of stem cells capable of lymphoid differentiation is controversial. In the chicken, there is evidence for both embryonic and extra-embryonic sources of stem cells with lymphoid potential (Dieterlen-Lievre 1987). Ogawa et al. (1988) have found that murine stem cells with lymphoid potential originate in

tissues from the embryo, perhaps in the omentum (Solvason and Kearney 1989). The first detectable lymphoid progenitors appear in the fetal liver (Kincade 1981) and perhaps the placenta (Melchers 1979) in mammals and the bursa in chickens (Le Douarin 1978).

A major unresolved question in the development of stem cells concerns the mechanisms by which stem cells become committed to differentiation, or at least restricted in their differentiation potential. Differentiation of S_P into S_L or S_M likely involves changes in gene expression. The low frequency of stem cells in normal bone marrow precludes the use of standard molecular genetic techniques to approach this question. However, recently reported methods for purifying stem cells (Spangrude et al. 1988) and for preparing cDNA libraries from limited numbers of cells (Belyavsky et al. 1989) may lead to rapid advances in this area.

At the present time, it appears that the "choice" between self-renewal and differentiation, i.e., commitment, is programmed into the cell. Although exposure to external stimuli, such as the microenvironment or factors may influence the commitment of stem cells, the majority of the available evidence supports the stochastic model of differentiation originally proposed by Till et al. (1964). According to this model, during each cell cycle, a stem cell will either differentiate or self-renew. The choice between these two possibilities is programmed internally. External or microenvironmental factors alter this probability slightly, but the probability of self-renewal must never drop below 0.5 or the stem cell pool will ultimately become exhausted. A major challenge for future investigators is to delineate the genetic changes within cells associated with self-renewal and commitment and to unravel the factors which regulate and determine the commitment of multipotent cells to specific lineages of differentiation.

Regulation of Stem Cells

Regardless of the precise mechanism by which stem cells become committed to a specific lineage, both proliferation and differentiation must be carefully regulated. The precise mechanisms of regulation at the stem cell level are unclear, primarily because all assays for stem cells require transplantation into suitable recipients or the use of complicated cell culture systems, which preclude detailed analysis of regulatory mechanisms. The available evidence suggests that cell interactions are the major factors in regulation of the stem cell compartment (Phillips 1984); growth factors bound to the extracellular matrix may play a role in regulating stem cell proliferation or differentiation (Kincade et al. 1989).

Morphological and functional analyses of cells growing in long-term bone marrow cultures indicate that most stem cells exist in direct contact with the adherent stromal cells required in these cultures. Interactions with the hematopoietic microenvironment appear essential for the maintenance and self-renewal of hematopoietic stem cells; they cannot survive separated from stromal cells (Dexter et al. 1984).

The best evidence for cell interactions being involved in the differentiation of hematopoietic stem cells comes from an analysis of mutations affecting hematopoiesis. Mutations at the *W*, *Sl*, and motheaten (*me*) loci all suggest that cell interactions between hematopoietic cells and the microenvironment play an important role in the regulation of differentiation and self-renewal. The well characterized *W* and *Sl* mutations were initially described as dominant spotting mutations, mapping to different chromosomal locations (Russell 1979). Mice homozygous for mutations at either locus have the same phenotype, namely, severe macrocytic anemia, sterility and lack of pigment production

both have subtle effects on lymphopoiesis (Landreth et al. 1984; Dorshkind, personal communication).

The cellular manifestations of these two mutations are best illustrated by studies on CFU-S. Bone marrow from mice homozygous for mutations at the *W* locus lack detectable CFU-S (McCulloch et al. 1964); transplantation of normal bone marrow cells will cure the anemia. In contrast, bone marrow from mice homozygous at the *Sl* locus have normal numbers of CFU-S, but transplanted normal bone marrow cells fail to grow or to produce spleen colonies in *Sl* mutant recipients (McCulloch et al. 1965). Only transplantation of spleen fragments will cure recipients homozygous for mutations at the *Sl* locus. Stem cells in mice with mutations at the *W* locus appear unable to respond to the specific microenvironmental stimuli required by stem cells to maintain normal hematopoiesis. In contrast, mice with mutations at the *Sl* locus appear unable to provide the microenvironmental signal. Two groups have recently shown that the *W* locus codes for the c-*kit* oncogene (Chabot et al. 1988; Geissler et al. 1988). While the function of this oncogene is unknown, it is clear that it codes for a tyrosine kinase membrane receptor on stem cells. An attractive model is that the ligand for this receptor is the product of the *Sl* locus.

Mice homozygous for mutations at the *me* locus have severe hematopoietic problems leading to rapid death (Shultz 1988). Recent evidence suggests that the defect restricts the ability of macrophages to participate in the normal microenvironment (Hayashi et al. 1988). To support myelopoiesis, erythropoiesis and lymphopoiesis, the hematopoietic microenvironment requires both stromal cells and macrophages. The precise nature of the interaction between macrophages and the stromal cells in unclear, but the ability of IL-1 to stimulate the release of numerous growth factors from cloned stromal cell lines may suggest the macrophages regulate factors produced by stromal cells.

THE B LYMPHOCYTE LINEAGE

Stages in the Differentiation Pathway

Table 1 summarizes the properties of cells at the eight known stages of development between the lymphoid-restricted stem cell, S_L, and a mature, antigen-reactive, mature B lymphocyte. The properties shown in Table 1 were selected to allow a unique identification for the cells at each stage of development. These stages are based on analysis of adult murine bone marrow, but a similar scheme applies to known stages of B lymphocyte development in human (Freedman and Nadler 1987). The reader is referred to extensive reviews by Kincade for a detailed summary of the properties and surface markers of cells at various stages of lymphocyte development (Kincade 1987; Kincade et al. 1989).

Mature B cells express both IgM and IgD on their surface, while immature, newly produced B cells express only IgM (Scher et al. 1983). To maintain the precedents set by Kincade (1987) and Osmond (1986), I have restricted the use of the term 'pre-B cell' to cells expressing cytoplasmic μ-chains. Three early stages called 'progenitors of B cells' by Osmond, I have named simply 'pro-B cells' with different adjectives to distinguish the three stages.

Landreth et al. (1981) demonstrated clearly that large pre-B cells differentiate into small pre-B cells and subsequently into immature B lymphocytes. Park and Osmond (1987) defined three pro-B stages prior to the large pre-B cell, based on the presence of the B220

Table 1. Properties of cells at different stages of B lymphocyte development in mouse bone marrow.

Stages: → / Property[a]	S_L	Early Pro-B	$B220^+$ Pro-B	Target Pro-B	Large Pre-B	Small Pre-B	Immature B cell	Mature B cell
Thy-1	lo	lo	lo	–	–	–	–	–
BP-1	–	+	+	+	–	–	–	–
TdT	?	+	+	–	–	–	–	–
B220	–	–	+	+	+	+	+	+
cμ	–	–	–	–	+	+	–	–
sμ	–	–	–	–	–	–	+	+
sδ	–	–	–	–	–	–	–	+
Size[b]	9?	9	10	12	12	8	8	8
V_H[c]	G	G?	DJ?	DJ & VDJ	VDJ	VDJ	VDJ	VDJ
V_L[c]	G	G	G	G	G	VJ	VJ	VJ
A-MuLV Transformation[d]	-	-	+	++	+	-	-	-
No. Divisions[e]	?	1	1	3	1	0	0	N.A.

[a] A '?' indicates property not known or uncertain for that cell type. See text for description of properties.
[b] Median diameter in microns as measured from cells centrifuged onto microscope slides (Osmond et al. 1988). Size of S_L is not known and assumed to be like other stem cells which have sizes similar to lymphocytes.
[c] State of rearrangement of heavy chain genes, V_H, or light chain genes, V_L. G - germline.
[d] Ability of cell to be transformed by Abelson murine leukemia virus.
[e] Number of mitoses to reach next stage of differentiation as calculated by Osmond et al. (1988).
N.A. - not applicable to this discussion.

antigen and expression of TDT; none of these pro-B cells have detectable cytoplasmic μ-chains. The first cell in the pro-B cell compartment, a TDT^+, $B220^-$ cell, I have called an early pro-B cell, since it is the earliest detectable progenitor committed to the B cell lineage. This cell differs from S_L primarily in proliferative ability. An early pro-B cell undergoes only a single division to become converted to the next stage of development (Osmond and Park 1987). S_L, by definition, have extensive proliferative and self-renewal capacity and must differ from the early pro-B cell. The number of stages or divisions involved in the transition from an S_L to an early pro-B is unknown. I have called the next stage a $B220^+$ pro-B cell because it is the first cell in the B lineage to express the B220 surface antigen and the final pro-B stage, the target pro-B cell, since it is the cell most susceptible to transformation by Abelson murine leukemia virus (A-MuLV) (Tidmarsh et al. 1989).

Analysis of lymphopoiesis in the scid mouse (Bosma et al. 1983) also provides clues about the regulation of B lymphocyte development. The bone marrow of scid mice contains no $c\mu^+$ or $s\mu^+$ cells and few $B220^+$ cells (Bosma et al. 1983; Witte et al. 1987). However, the bone marrow contains normal numbers of targets for transformation by A-MuLV (Fulop et al. 1988). Thus, we have suggested that differentiation in the scid mouse occurs normally up to the target pro-B or the large pre-B and that the normal mechanism for eliminating non-productively rearranged cells leads to the rapid elimination of all recognizable B cells, since productive rearrangement is extremely rare in this mouse.

The status of gene rearrangement in the pro-B cell compartment must be considered uncertain at the present time. Joho et al. (1983) found that most of the $B220^+$ cells in normal mouse bone marrow have undergone DJ or VDJ rearrangement of the heavy chain locus. However, Kincade (1987) found a significant proportion of such cells with the heavy chain genes in germline configuration. I have assumed that the heavy chain genes are in the germline configuration in the early pro-B cell, but the presence of TDT expression in these cells may indicate that some early pro-B cells have begun gene rearrangement.

The the scid mouse also provides information about gene rearrangement during B lymphocyte development. The defect in scid mice prevents productive rearrangement of the immunoglobulin heavy and light chains, as well as the chains for the T cell receptor (Malynn et al. 1988; Schuler et al. 1986). Since light chain rearrangement does not occur until the small pre-B stage, a single target pro-B cell with a productive VDJ rearrangement could produce four B cells, each with a different light chain rearrangement. Analysis of "leaky" scid mice by Gibson et al. (1989) has led to a similar conclusion. If one assumes that "leaky" scid phenotype results from a reverse mutation occurring in a target pro-B cell or a large pre-B cell, then functional rearrangement of a heavy chain will likely result in the functional light chains as well. Thus, "leaky" scid mice should have a very restricted heavy chain repertoire and a slightly greater repertoire of light chain V genes. Gibson has observed exactly this phenotype in "leaky" scid mice, and has estimated from his data that a revertant pro-B or pre-B cell produces two to four different B cells.

Kinetics of B Lymphocyte Production

Using detailed stathmokinetic techniques, as well as standard techniques of pulse labeling and surface labeling, Osmond and Park (1987) have carefully estimated the rate of cell production at each stage of development and have estimated the number of divisions which occur within each compartment in the B lymphocyte lineage.

No cell divisions are required to convert a small pre-B cell into a mature B cell. All of expansion occurs prior to the small pre-B stage. Landreth et al. (1981) demonstrated that a single target pro-B cell can differentiate into two large pre-B cells, which in turn undergo one mitosis to produce two small pre-B cells. Thus, a single target pro-B cell will produce four small pre-B cells. Osmond and Park (1987) observed extensive proliferation within the target pro-B population, and Lee et al. (Lee et al. 1989) have shown that this population proliferates extensively in vitro in response to IL-7. Although Osmond and Park estimated that it takes three divisions to convert a target pro-B cell into a large pre-B cell, it is likely that the target pro-B cell responds to IL-7 and is capable of extensive proliferation under some conditions; the value of three divisions should be considered an average number observed in a normal mouse under normal conditions (see below). Relatively little proliferation occurs in the other two stages, only a single division apparently being required to convert an early pro-B into a $B220^+$ pro-B and a $B220^+$ pro-B into a target pro-B.

An interesting outcome of the kinetic analysis is the observation that the production of large pre-B cells greatly exceeds the estimated production of immature B cells (Osmond and Park 1987). These data indicate that perhaps 80% of cells die during the transition of large pre-B cells into immature B cells. Since a significant proportion of gene rearrangements lead to a non-productive immunoglobulin genes, it must be expected that such non-productive cells are eliminated. The mechanism by which this elimination process occurs is not known but it does not appear to act in long-term bone marrow cultures (Witte et al. 1987).

Regulation of B Lymphocyte Production

Many factors appear to regulate proliferation and differentiation in the B lymphocyte lineage; for a review, see Kincade et al. (1989). A factor obtained in the serum of patients and dogs with cyclic neutropenia (Landreth et al. 1985), or from stromal cells (Landreth and Dorshkind 1988), can potentiate the differentiation of early pro-B cells into $B220^+$ pro-B cells; this factor has no detectable ability to stimulate proliferation. IL-7 stimulates the proliferation of target pro-B cells and perhaps large pre-B cells and $B220^+$ pro-B cells (Lee et al. 1989). Several factors, including IL-1 and IL-4, have been implicated in the transition of small pre-B cells to surface Ig^+ B cells (Kincade et al. 1989). King et al. (1988) have recently demonstrated that IL-1 induces the release of IL-4 by stromal cells, and IL-4 enhances the differentiation of small pre-B cells into immature B cells.

The effect of IL-3 on the proliferation and differentiation of cells in the B lineage remains controversial. While there have been reports of IL-3 acting on B lineage cell lines (Palacios and Steinmetz 1985; Rennick et al. 1989), little evidence exists for any effect of IL-3 on normal cells in the B cell lineage. IL-6 interacts with IL-3 on cells in the early stages of the myeloid and erythroid pathway (Ikebuchi et al. 1987). However, there are no reports of IL-6 affecting early stages of B cell development.

Despite information on the effects of various factors acting at different stages during the differentiation of B lymphocytes, little is known about the mechanisms which regulate homeostasis in this pathway. Extensive studies by Fulop et al. (1983) produced the surprising result that depletion of surface Ig+ cells and serum immunoglobulin had no effect on the rate of production of large pre-B cells. This observation is in marked contrast to the erythroid system, where depletion of the number of erythrocytes results in marked stimulation of the erthroid pathway. Non-specific agents transiently stimulate the rate of production of large pre-B cells, probably through activation of macrophages (Fulop and Osmond 1983). It is tempting to speculate that stimulatory agents activate

macrophages to release IL-1, which in turn causes the release of IL-7 and IL-4, resulting in a brief increase in proliferation and differentiation of B cell progenitors.

From the results of the Osmond group and others, one can hypothesize that regulation occurs primarily at the large pre-B and/or target pro-B stages of development. The mouse appears to monitor the size of this population and regulates proliferation to maintain the size of this population, rather than the population of surface Ig^+ B cells or the amount of serum immunoglobulin. Our observations in scid mice are also consistent with this hypothesis. We have observed that transplantation of normal bone marrow into scid mice does not cure the immune deficiency unless the animals are exposed to sub-lethal doses of whole body irradiation (Fulop and Phillips 1986). As mentioned above, scid mice contain a normal number of cells transformable by A-MuLV (Fulop et al. 1988) indicating normal differentiation up to the target pro-B cell stage. If regulation occurs at this stage of development, then the pathway will not be perceived as being deficient, even though the mouse is not producing any functional B lymphocytes. Only following irradiation to reduce the number of target pro-B cells will the system make a homeostatic response and provide the factors required for the stimulation of transplanted S_L stem cells.

THE FUTURE

Many gaps still exist in our knowledge about the differentiation and regulation of B lymphocyte production. The past three years have provided some interesting observations. However, the most exciting advances have involved improvements in technology which will undoubtedly lead by the time of the next International Congress to major advances in our fundamental understanding of B lymphopoiesis. Advances in molecular genetics and in cell separation will allow the elucidation of the genetic changes associated with the differentiation of the B lymphocytes from stem cells. Improved model systems will facilitate studies on early stages of lymphoid development and on the analysis of normal and abnormal lymphopoiesis in humans. With increasing knowledge of the regulation of lymphocyte production and its alteration in disease states will come the ability to design effective interventions in the treatment and prevention of many immunological diseases.

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Control of Expression of Immunoglobulin Genes

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INTRODUCTION

Immunoglobulin genes become activated during the development of B lymphocytes by sequential rearrangement of heavy and light chain genes (Alt et al., 1986). The rearrangement of immunoglobulin genes requires that the VDJ recombinase is present and that the target Ig genes are accessible for recombination (Alt et al., 1987). These events appear to be tightly controlled in a way which leads to allelic and isotypic exclusion of Ig genes (reviewed by Engler and Storb, 1988). Some years ago we began to use transgenic mice to study the mechanisms of allelic and isotypic exclusion (Brinster et al., 1983). This work, as well as the results from many other laboratories, has led to the following conclusions (reviewed by Storb, 1988): There are two levels of control of Ig gene rearrangement; at the early preB cell stage when a correct rearrangement of a heavy chain gene has been made, the further rearrangement of H genes is stopped, perhaps at the level of VD joining, while the VDJ specific recombinase continues to exist and now rearranges κ and/or λ genes. The final stop of the rearrangement mechanism apparently occurs by a shutoff of the recombinase signaled by the assembly of a complete membrane bound Ig molecule, consisting of a μ chain and a κ or λ light chain.

FACTORS WHICH INFLUENCE THE REARRANGEMENT EFFICIENCY OF AN IMMUNOGLOBULIN TEST GENE IN TRANSFECTED CELLS AND TRANSGENIC MICE

A rearrangement test gene, pHRD, containing the mouse immunoglobulin heavy chain (IgH) enhancer and the metallothionein promoter, has previously been shown to rearrange efficiently after transfection into a preB cell line (Engler and Storb, 1987). From this and related experiments in other laboratories (for example Lieber et al., 1987) it was concluded that VDJ recombinase activity is present in precursors of B and T cells, but not in mature B or T cells.

Experiments carried out to determine requirements for Ig gene accessibility showed that pHRD without the metallothionein promoter was efficiently rearranged when transfected into the preB cell line 38B9 (Engler et al., 1989). While several of the transfectants of the promoterless version still showed transcripts of the test gene, apparently initiated from endogenous promoters at

the insertion site, many did not contain detectable transcripts. Overall, there was no correlation between levels of transcription and rearrangement. This independence of rearrangement from transcription is in disagreement with other findings (Blackwell et al., 1986). At present we do not have an explanation for the discrepancy.

When pHRD was transfected into myeloma cells (Ag8.653) no rearrangement was seen, presumably due to the lack of recombinase. In order to determine whether the myeloma cells contained an inhibitor of recombinase, the transfected myeloma cells were fused with the 38B9 preB cells. Under conditions where 100% of preB cells directly transfected with the test gene show rearrangement, none of 24 independent (myeloma + pHRD) x preB cell hybrids rearranged the test gene. However, after prolonged culture, the hybrid cells derived from one of the (myeloma + pHRD) transfectants did rearrange the test gene. The hybrid cells with another pHRD containing myeloma never rearranged the test gene. Thus, either the myeloma cells contain an inhibitor of the VDJ recombinase or the test gene had become completely or partially inaccessible when integrated in the myeloma genome. A distinction between these two possibilities was made with the following experiment: Myeloma cells were fused with preB cells and the stable hybrids were transfected with the rearrangement test gene. In this case all but two of 38 independent transfectants rearranged the test gene very efficiently. These data taken together suggest that myeloma cells do not contain an inhibitor of the VDJ recombinase. Furthermore, integration of a rearrangement target in myeloma cells, despite its being undermethylated and transcribed (Engler et al., 1989) has a high probability of making the test gene inaccessible for rearrangement.

In order to further assess the requirements for rearrangement target accessibility, transgenic mice were produced with the pHRD test gene (Engler et al., 1989). Five independent lines were obtained, each presumably having the transgene integrated at a different chromosomal site. Except for a single individual (see below) none of the transgenic mice rearranged the test gene in the spleen. One of a total of 46 mice rearranged the gene in spleen, thymus and bone marrow, but not in liver and kidney. In this particular mouse the test gene was undermethylated, whereas in all the other mice, including about 40 siblings and offspring from the same transgenic line, the test gene was hypermethylated. We are currently exploring the possibility that the inaccessibility of the test transgene in most of the mice was due to mouse strain related parental imprinting. The original founder mice were (C57BL/6 x SJL)F2, which were subsequently backcrossed to C57BL/6.

These results have led to the following conclusions regarding the accessibility of Ig genes for rearrangement (Table 1): 1) Detectable transcription is not required. 2) A status correlated with undermethylation is required, but not sufficient. 3) Transcription and undermethylation are not sufficient. Unraveling of the additional cofactors required to activate the Ig gene associated chromatin for rearrangement may have to await the cloning of the gene(s) encoding the VDJ recombinase.

Table 1 ACCESSIBILITY OF IG GENES FOR REARRANGEMENT

CELL TYPE	TRANSCRIPTION	TEST GENE UNDERMETHYLATION	RECOMBINASE ACCESSIBILITY		REARRANGEMENT
preB	+ or -	+	+	+	yes
myeloma	+	+	- (or +)	-	no
(myeloma + test gene) X preB	+	+	- (or +)	+	no (yes)
(myeloma X preB) + test gene	+	+	+	+	yes
transgenic	lo	-	-	+	no
	?	+	+	+	yes

FEEDBACK REGULATION OF IMMUNOGLOBULIN GENE REARRANGEMENT

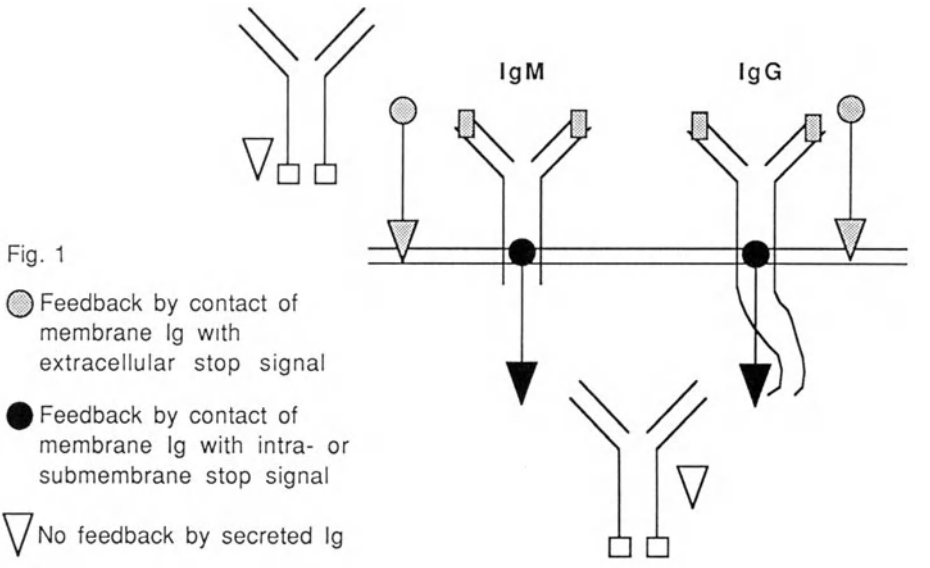
It has been known for a long time that "allelic exclusion" of Ig genes allows for the expression of only one H and L gene in mature antibody producing cells (Pernis et al., 1965; Cebra et al., 1966; Coleclough, 1983). Several mechanisms to exclude the functional activation of more than one allele have been considered. Among them are a regulated and a stochastic model. The regulated model predicts that there is a feedback from a functional Ig gene, or rather its protein product to prevent further rearrangements (Alt et al., 1980; Kwan et al., 1981; Bernard et al., 1981). In contrast, the stochastic model predicts that the chance for a functional rearrangement is low, so that the chance for two functional rearrangements in the same cell is minimal (Walfield et al., 1980; Coleclough et al., 1981). We initiated the use of transgenic mice with immunoglobulin transgenes some years ago to distinguish between these two models (Brinster et al., 1983).

Work from several laboratories has now shown that Ig gene rearrangement is regulated by feedback. A summary of our current knowledge is given in Table 2. At the level of H gene rearrangement the production of membrane bound heavy chains inhibits further rearrangement, perhaps only of VD, but not of DJ (Yancopoulos et al., 1985; Hagman et al., 1989). Both μm and δm, but not μs, have been shown to have this effect (Nussenzweig et al., 1988; Manz et al., 1988; Iglesias et al., 1987). Our own unpublished data with transgenic mice suggest that γ2b may have the same effect. At the level of light chain gene rearrangement membrane bound immunoglobulin containing μ and κ has a feedback effect (Manz et al., 1988). Also, λ2 or λ1 light chains in association

with heavy chains have almost as strong a feedback effect as κ light chains (Hagman et al., 1989; Neuberger et al., 1989; Ritchie et al., 1984). Presumably, the requirement for membrane bound Ig indicates that the feedback signal is produced in association with cellular membranes. It has been postulated that the target in the shutoff of H-gene rearrangement may be transcriptional inactivation of Vh genes (Yancopoulos et al., 1985). The target in the later stage of feedback by a complete Ig molecule may be the VDJ recombinase. A model for feedback inhibition is shown in Fig. 1.

Table 2 SEQUENTIAL IG GENE REARRANGEMENT IN B CELLS

B cell stage	Recombinase	Rearrangement	Feedback	
			Protein	Result
Early pre B	+	V-D-Jh	μm δm γ2b ?	STOP H REARR
			μs γ2b?	NO STOP
Late pre B	+	V-Jκ V-Jλ	κ+μm λ+ H	STOP RECOMBINASE
			κ alone κ + μs	NO STOP
B	-	-		



While it is clear that feedback inhibition occurs as a consequence of the expression of immunoglobulin transgenes, in all the Ig transgenic mice produced so far the inhibition of endogenous Ig gene rearrangement is not complete. Escape from expression is most likely favored by strong antigen driven selection of B cells which express endogenous Ig genes. It is difficult to assess what proportion of developing B cells actually do escape feedback. Table 3 lists a summary of the presumably most common mechanisms for escape from feedback. There is some experimental evidence to support mechanisms #2 and #4. The question of an unregulated B cell lineage will be discussed in the next section. The length of the time span between the creation of a feedback signal and its effect will possibly be learned when the molecular events of the creation of the feedback signal are understood.

Table 3 POSSIBLE MECHANISMS FOR ESCAPE FROM FEEDBACK

1) Time window between feedback signal and stop of VDJ recombinase allows further rearrangement
2) "Unregulated" B cell lineage: unresponsive to feedback at the preB cell stage. Stop of recombinase production later (B cell + antigen?)
3) Sub threshold transgene expression in early preB cells
4) Deletion of transgenes by unequal sister chromatid exchange etc.

REGULATION OF λ GENE EXPRESSION

In plasma cells a clear isotypic exclusion of κ and λ protein production is evident. On the level of the respective genes in these cells, κ-producing cells have rearranged κ genes, but generally have λ genes in germline conformation. Lambda producing plasma cells on the other hand not only have λ gene rearrangements, but almost always also have their κ genes rearranged or deleted by rearrangement of the κ deleting element (KDE) (Moore et al., 1985; Siminovitch et al., 1985). These observations have led to a "sequential model" for κ/λ isotypic exclusion which postulates that κ genes are accessible for rearrangement first, and that λ genes only become accessible after both κ alleles have been nonproductively rearranged or deleted (Hieter et al., 1981). Recent data with κ transgenic mice have challenged this model: these mice were found to have a strong suppression of endogenous κ gene rearrangement, however, in B cells which produced endogenous λ, both λ and κ genes were rearranged (Gollahon et al., 1988). From these and related data a dual lineage model was proposed (Gollahon et al., 1988). Its key feature is the distinction between a κ B cell lineage and a κ/λ lineage. The κ lineage activates κ genes; its Ig gene rearrangement is strictly feedback inhibited at the preB cell stage. The κ/λ lineage activates both κ and λ genes; at the preB cell stage κ and λ gene rearrangement is not susceptible to feedback control.

Some further evidence consistent with this model has been obtained with transgenic mice which carry a λ2 transgene under the control of the heavy chain enhancer (Hagman et al., 1989). These mice show that the λ2 transgene causes feedback inhibition of both κ and heavy chain genes, presumably due to shutoff of the VDJ recombinase as a result of the presence of a complete Ig molecule

consisting of λ2 and an endogenous H chain. In B cell hybridomas produced from these mice the rearrangement of κ genes is greatly suppressed (Table 4). However, the proportion of cells rearranging λ genes is at least as high as in normal mice (Table 4). These cells also rearrange their κ genes (not shown). Since in these mice there is no known selection of λ protein producing B cells, presumably λ and κ gene rearrangement occurs in a relatively fixed number of preB cells, independent of feedback control. Now, that a λ enhancer has been identified (see next section) it may become possible to definitively answer the question of how κ/λ isotypic exclusion is controlled.

Table 4. HYBRIDOMAS OF λ2 TRANSGENIC AND NORMAL MICE*

TRANSGENE	% REARRANGEMENT κ	% REARRANGEMENT λ	% GERMLINE H
+	32	8	43
_	100	~5	<7

*% of hybridomas with a particular genotype

ENHANCERS FOR λ GENES

Mouse λ genes have been mapped within about 200kb of DNA; the gene order is V2-Vx-C2,C4-V1-C3,C1 (Miller et al., 1988; Storb et al., 1989; Carson and Wu, 1989). The distance between V2 or Vx and the C2-C4 cluster is 74 or 55kb respectively, whereas that between V1 and C3-C1 is only about 19kb (Storb et al., 1989). Thus, the distances between the λ subloci are inversely proportional to their frequencies of rearrangement (Eisen and Reilly, 1985).

Since the overall organization of λ genes is similar to that of heavy and κ genes it had been expected that enhancers for λ genes would also be located in the introns between J and C. However, the intron of JCλ1 was found not to have enhancer activity when linked to a β globin reporter gene (Picard and Schaffner, 1984). Extensive searches for enhancer activity within the JCλ1 and JCλ2 introns using a CAT reporter gene and transient transfection or stable transfection with complete λ coding and flanking regions have confirmed and extended the original finding: no enhancer activity could be demonstrated within the J-C introns and about 4kb upstream of the V genes or 2kb 3' of C4 (Hagman et al., 1989; Hagman and Storb, unpublished). We have therefore used a different approach to find potential sites within the λ locus which might have transcriptional enhancer activity. Generally, enhancer regions are hypersensitive when DNA is mildly digested with DNaseI. Using this approach DNaseI hypersensitive sites were found 3kb 5' of both Vλ1 and Vλ2, 8kb 3' of Cλ1 and 8kb and 15kb 3' of Cλ4 (Hagman and Storb, unpublished). The sites upstream of the V genes and downstream of C1 are hypersensitive in all B and T cells. The distant site 3' of C4 is only hypersensitive in B cells, and the C4 proximal site is only hypersensitive in myelomas.

In order to determine whether the hypersensitive site 3' of C1 (hs C1) has enhancer activity, short cloned DNA sequences comprising the hypersensitive site were used in transient and stable transfection assays with CAT and λ genes into a variety of preB, B and plasma cells (Hagman and Storb, unpublished). The activity of this region in cooperation with a Vλ promoter was assayed relative to the activities of the κ and heavy chain enhancers. Compared with the constructs containing the promoter alone, the hsC1 sequence had no or only minimal enhancer activity, whereas the κ and heavy chain enhancers strongly cooperated with a Vλ promoter. Thus, the hypersensitivity of the hsC1 region is apparently not due to the binding of transcriptional activators.

Since the hs sites 3' of C4 appeared to be B cell specific, we isolated new genomic DNA clones for the analysis of these regions (Hagman, Chaplin and Storb, unpublished). The distal site 3' of C4 (hs C_4-2) was found to have strong enhancer activity in a CAT assay (Hagman and Storb, unpublished). The activity was present in either orientation of an hs C_4-2 containing DNA fragment. However, the orientation in which the putative enhancer sequence was closer to the Vλ2 promoter had about 50% higher activity than the opposite orientation. In the former orientation this λ enhancer had about the same activity as a 1 kb fragment containing the heavy chain enhancer.

Thus, a strong enhancer for λ gene transcription is located 3' of the Cλ2-Cλ4 cluster. In vivo, this enhancer presumably cooperates with the Vλ2 and Vλx promoters after rearrangement of these V genes with JCλ2 (JCλ4 is a pseudogene, Miller at al., 1981). It is unlikely to cooperate with Vλ1 since V1 is located at least 100kb 3' of this enhancer (Storb et al., 1989). Furthermore, following a V2/JC3 or V2/JC1 rearrangement this enhancer would be deleted, since the V2 and C3C1 genes are in the same transcriptional orientation (Miller et al., 1988; Storb et al., 1989). Thus, another enhancer is most likely present 3' of Cλ1. In this location it could drive transcription from either the Vλ1 or the Vλ2 promoter after the respective rearrangements. So far, we have not detected another DNaseI hypersensitive site 3' of hs C1 within 20Kb downstream of the Cλ1 gene. It therefore appears that the λ enhancers are some of the most distantly located enhancers found in structural genes.

SUMMARY

Experiments are discussed which deal with the regulation of immunoglobulin gene rearrangement and transcription. Apparently, accessibility of Ig genes for rearrangement does not require detectable transcription, but does require a state which is accompanied by DNA undermethylation. However, undermethylation and transcription of a target gene are not sufficient for rearrangement. After rearrangement of heavy and light chain genes encoding a complete, membrane bound immunoglobulin, the production of the VDJ recombinase apparently ceases; there is no evidence for the presence of an inhibitor of the recombinase in plasma cells. Isotypic exclusion of κ and λ genes may result from the existence of two separate B cell lineages, a strictly

feedback controlled κ lineage and a κ/λ lineage not feedback regulated at the preB cell stage. However, it is not known whether different transactivating factors for κ and λ genes exist.

The mouse λ genes are located within about 200kb of DNA. All are arranged in the same transcriptional orientation with the order V2-Vx-C2C4-V1-C3C1. In contrast to κ and heavy chain enhancers, the λ enhancers are not located within the J-C introns. DNase I hypersensitive sites are found 5' of the V genes and 3' of C4 and C1. A strong enhancer has been located 15kb 3' of C4 by functional assays. Because of the orientation and rearrangement potential of λ genes it is postulated that an analogous enhancer must be found greater than 20 kb 3' of Cλ1.

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Regulation of Immunoglobulin Gene Rearrangement

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INTRODUCTION

The ordered recombination of the immunoglobulin (Ig) heavy (H) and light (L) chain variable (V) region gene segments facilitates the analysis of early B-cell development. In the bone marrow the Ig V_H gene elements are joined in two steps: first a diversity (D) segment recombines to a joining (J_H) segment giving rise to a DJ_H element to which a V_H segment is recombined completing the VDJ_H-V-region gene. Only at this stage the V_L-region gene is assembled by joining a V_L and J_L element. The early B-cell differentiation can therefore be easily followed by monitoring the rearrangement status of the H and L chain genes. Indeed, the analysis of Abelson Murine Leukemia Virus (Abl-MuLV) transformed pre-B-cell lines indicate that rearrangement proceeds step by step through the alleles, thus rarily rearranging two alleles at a time (Alt et al. 1984; Reth et al. 1986). Taking into account only one V_H and V_L allele at least four stages (Fig. 1, stages 2-5) can be discriminated; considering both alleles, a B-cell can maximally run through seven distinct stages (not shown).

RESULTS AND DISCUSSION

Signals preceeding each stage must exist in order to insure stepwise progression through development. These signals open or close a series of genes, many of which are B-cell specific. Evidence for the series of events as shown in Fig. 1 is circumstancial.

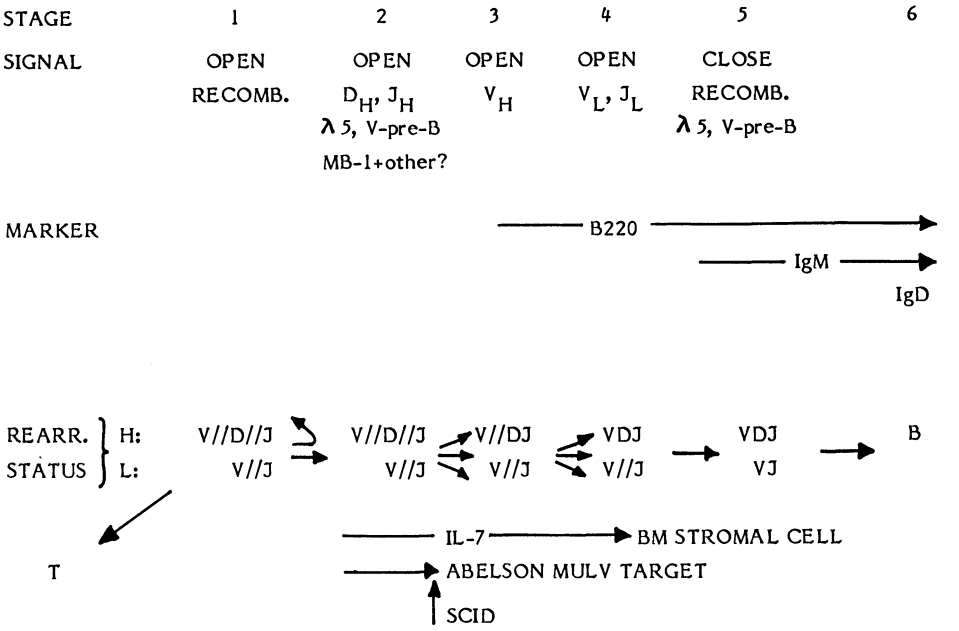

Fig. 1. Scheme of B-cell development.

The "recombinase" is thought to be the same for the B- and the T-cell lineage (Fig. 1). B-cell lines of stage 2 can already rearrange an exogenous substrate (Lieber et al. 1987), although their own loci remain unrearranged in vitro. Since non-lymphoid hematopoetic lines do not express this enzymatic activity, it seems to make sense to ask for the signal to open the recombinase at a common lymphoid precursor stage. The signal to open D_H and J_H to make these sequences available for the recombinase is required as a separate step, since T-cells do not regularly pass through the D to J_H recombination (Kurosawa et al. 1981). Furthermore, it was shown that recombination substrates were more frequently rearranged when selected for expression by a selectable marker, indicating that open chromatin structures (allowing transcription) are better suited for the recombination process (Blackwell and Alt 1984). To place the signal to open the λ5-, V-pre-B- and MB-1-loci (Kudo and Melchers 1987; Sakaguchi and Melchers 1986; Sakaguchi et al. 1988), before recombination has taken place (stage 2), is based on the observation that bone marrow cells of SCID mice, which lack a functional recombinase and which lack B220 positive cells from stage 3 onwards, were positive for λ5 (Schuler et al. 198). The presence of cells of stage 2 in SCID mice is implied by the observation that the target cells of Ab1-MuLV are present in normal numbers (Table 1). MB-1 is expressed in pre-B-cell lines (Sakaguchi et al. 1988). It is

required for membrane anchoring of IgM (Hombach et al. 1988). It probably serves for intracellular signalling and may only be one accessory molecule of several which could be similar to the T3 complex. The T3 complex is a series of 5 proteins necessary to efficiently express the T-cell receptor dimer on the cell surface. The T3 proteins and the product of MB-1 (B34) have a common intracellular aminoacid motif, which indicates similar functions (Reth 1989). To place the open-MB-1-(and others?)-signal at stage 2 is implied by the observation that transgenic IgM is detected on the membrane of embryonal splenocytes 2 days before endogenous IgM can be seen (Lamers et al. 1989). Ab1-MuLV transformed pre-B-cell lines of stage 2 and 3 already express a transgenic Ig on the cell surface (Table 1). Similarly, transfecting a μ gene into Ab1-MuLV pre-B-cell lines of stage 3 (M. Reth pers. communication) or stage 4 (own observation) leads to surface expression for which (in analogy (Hombach et al. 1988), B34 is needed. Since B34 is not found in the T-cell lineage, we propose stage 2 for opening the B34 and similar loci. At stage 3 recombinase is active (Lieber et al. 1987), DJ-Cμ transcription is observed (Reth and Alt 1984). The product of the lambda 5 gene called omega (ω) has homology to a light chain constant region. The product of the V-pre-B-gene is called iota (i) and has homology to V_L. Both chains together form a surrogate light chain where omega is covalently linked to μ (Pillai and Baltimore 1987; 1988). The surrogate light chain and B34 may be needed to allow μ surface expression. Omega (together with iota?) is assumed to detach μ from a protein called heavy chain binding protein (BiP). This protein, known to retain μ in the endoplasmatic reticulum (ER) by binding to the first constant domain of heavy chain, is detached by light chains which then allow secretion of the heavy chains (Hendershot et al. 1987). In analogy, omega (and iota) detach μ from BiP and allow surface expression of μ in pre-B-cell lines. BiP also weakly interacts with Cμ4 of the secretory form of μ (Hendershot et al. 1987). Possibly B34 facilitates μ surface expression by interacting with this domain (M. Reth pers. communication). The interactions of μ with BiP, omega, iota, light chain and B34 are summarized in Fig. 2.

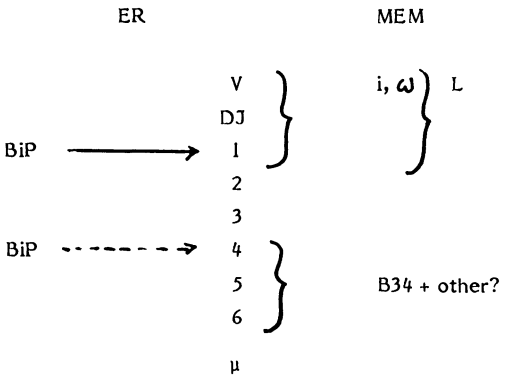

Fig. 2. Known interactions of μ-chain with other proteins.

At stage 3 the V_H-region opens as evidenced by V-region transcripts early in ontogeny (Yancopoulos and Alt 1985) and allows V to DJ recombination. If the VDJ rearrangement leads to an open reading-frame a μ protein is made. Pre-B-cells with intracellular μ have been observed, but so far no surface μ (without conventional light chains) has been detected. We propose that μ surface expression is achieved (as seen in our Abelson lines Table 1), but is below the detection limits in normal pre-B-cells.

Table 1. Frequency of established lines after transformation of pre-B-cells with AMuLV

	surface Ig	normal littermate (cell lines/10^7 cells)	transgenic littermate (cell lines/10^7 cells)	tr/no (%)
μk	+	1090	12	1,6
Scid/μk	+	950	3,3	0,3
Bδ	+	1480	8	1,0
Cδ	+	620	10,0	1,6
Eμ	+	850	0,0	0,0
CμD1	+	620	0,0	0,0
ß-Gal	-	980	1000	102
k 21	-	980	1190	120
Scid	-	630	700	100

At stage 4 a signal is required to open V_L and J_L to make them available to the recombinase. This is the first signal which is known to some extent. The μ chain and more precisely the membrane form of the μ chain containing exons 5 and 6 induce the V_L and J_L recombination in Abelson lines (Reth et al. 1986; Reth et al. 1987). This implies, but does not prove, that surface expression of μ is required for the activation of the L-chain locus. We made transfection experiments into an Abelson pre-B-cell line which was derived from the bone marrow of a Swiss Albino x Balb/C F1 mouse, which was transgenic for μ and Kappa genes (Rusconi and Köhler 1985). This line (33.1^+) continued to rearrange its heavy and light chain locus and in the latter aspect behaved like μ positive Abelson lines (Table 2). It can progress into a more mature B-cell phenotype (stage 5, Fig. 1) as evidenced by subclone $33.1.1^+$ which has low recombination activity and low $\lambda 5$ expression (Table 2). A subline ($3\text{-}1.1^-$), which has lost the transgenic μ and Kappa sequences, had a VDJ^- on both H-chain alleles so that ongoing V_H-rearrangement could not be followed. The V_L-locus was unrearranged and only one V to J_L rearrangement was observed in 42 alleles studied.

Table 2. Characteristics of sublines of the 33-1$^-$ cell line after transfection with wild-type and mutant μ constructs.

subline	μ lysates	μ surface	recombinase activity	λ-5 mRNA	K mRNA	K rearr	K surface
33-1$^+$(μ,K)	nd	nd	1/1320	250	55	6/12	100
33-1-1$^+$(μ,K)	11	66	1/5190	5	100*	0/14	100
33-1-1$^-$	1*	1*	1/542	100*	1.6	1/42	0
3-1-1$^-$(μ)	10	10	1/304	230	29	19/100	0-60
33-1-1$^-$(μD1)	13	30	1/566	125	-	0/146	0

* reference value

Into the 33.1.1$^-$ line a μ gene was transfected.

V_L rearrangement again was induced (Table 2). This demonstrates that loss of μ (Kappa) leads to loss of V_L-rearrangement, re-addition of μ re-initiates V_L-rearrangement. We conclude that in this line only continued presence of μ-chain insures V_L-rearrangement. In one μ expressing subline the recombinase was no longer active. Only in this line a short 0.8 kb Kappa transcript, indicating the availability of the locus for rearrangement, was seen. The other lines lacked this transcript, but showed the mature 1.1 kb Kappa transcript, which originates from a minority of rearranged alleles (Table 2). This indicates that once the Kappa locus is opened, rearrangement proceeds quickly.

In order to test whether surface expression is sufficient to induce V_L-rearrangement, a construct lacking the first constant domain of μ (μD1) was transfected into 33.1-1$^-$ line (Table 2). Although similar intracellular amounts of μ and higher amounts of μD1 were found on the surface, no V_K-rearrangement is observed. Thus, surface expression of μ alone is not sufficient to induce Kappa rearrangement. This points to an active role of the surrogate light chain (iota, omega, Fig. 2) in promoting V_L-rearrangement. Its role cannot be limited to detach μ from BiP and thus allowing surface μ expression. Possibly, association of the surrogate light chain promotes aggregation of μ.

Table 3. Analysis of hybridomas derived from transgenic mouse lines.

mouse line	origin and type of secreted Ig				rearrangement status of endogenous alleles			
	L	H_T+L	H_T+H_E+L	H_E+L	GL	DJ	VDJ	total
sum μ mice	9	29	3	4	6	20	1	27
sum μD1 mice	2	1	18	14	3	11	18	32
littermates	6	0	0	40	0	2	32	44

Results in transgenic mice expressing the μD1-construct are on the whole compatible with a reduced potential of μD1 to induce V_L-rearrangement in vivo. In μ transgenic mice most of splenic B lymphocytes as analysed in hybridoma cell lines express the transgenic μ with endogenous light chains (Table 3, 29 of 45 lines analysed). In contrast, this population of cells is missing in μD1-transgenic mice (Table 3, 1 of 34 lines analysed). That the μD1 gene is not just an unnoticed traveller in B-cell development is seen by a severe (1-5% of normal) B-cell depletion in some of the μD1 transgenic mouse lines. Furthermore, the silent V_H allele is found in 10% of the alleles in an unrearranged configuration, so that VDJ/germ line configurations are observed in hybridomas of μD1 mice, a combination which has never been observed in hybrids of normal mice (Table 3). The observation that the silent allele of μD1 is in a more immature rearrangement configuration, is confirmed by the higher DJ versus VDJ ratio seen in hybridomas of transgenic versus non-transgenic origin. We conclude that in μD1 line only those B cells which managed to express an endogenous μ chain were able to induce V_L-rearrangement. The transgenic μD1 chain alone was unable to induce efficiently V_L-rearrangement.

SUMMARY AND CONCLUSIONS

We propose that transgenic μ- and μD1-expressing early B-cells progress through B-cell development quicker than normal and thereby omit some cell divisions. That could explain the low number of Abelson transformable B-cells (Table 1) and the high frequency of immature V_H-gene configurations in B-cells of H-chain transgenic mice (Table 3). An alternative

explanation would be a feedback inhibition of the transgenic H-chains on the V_H-rearrangement process itself. The low frequency of Abelson transformed B-cell lines must then be explained by a general toxic effect of transgenic heavy chains. Both hypotheses are not mutually exclusive. The transgenic μ, but not the transgenic μD1, is able to induce endogenous V_L-rearrangement (Table 2). This is in agreement with the low B-cell numbers observed in strongly expressing μD1 transgenic mice and the absence of B-cells expressing only μD1 together with endogenous L-chains. The μD1 alone expressing B-cells die at stage 4, only those rare cells expressing in addition a functional, endogenous μ-chain induce V_L-rearrangement and survive. In the transfection experiments (Table 2) a hint for the active participation of the surrogate light chain in the V_L-rearrangement process was observed.

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Isolation and Characterization of Hematopoietic Stem Cells and Progenitors

C.E. Müller-Sieburg

INTRODUCTION

Pluripotent hematopoietic stem cells constantly regenerate all the cells of the myeloid, erythroid, and lymphoid lineages through a complex series of differentiation steps. Since the number of stem cells remains roughly constant through the life span of an adult individual, stem cells are believed to self-renew. How the balance between differentiation and self-renewal is regulated is currently not well understood. The major obstructions to the study of early hematopoietic differentiation are the low frequency of stem cells and progenitors, and the heterogeneity of the hematopoietic tissues (Fig. 1). Many cells, such as T lymphocytes and stromal cells which are abundant in hematopoietic organs, are known to interact and regulate early hematopoietic differentiation through secreted cytokines or cell-cell interactions. Thus, it is important to study the regulation of early hematopoiesis using populations highly enriched for distinct stages of hematolymphoid differentiation to avoid cascade effects.

ASSAYS FOR STEM CELLS AND PRECURSORS

Most assays for stem cells and precursors determine the proliferative and differentiation capacity of these cells by analyzing their progeny. Since cells at early hematopoietic differentiation stages are very infrequent they are often *defined* by specific assays.

Stem Cells

There are few assays that measure pluripotent stem cells accurately, all of which assess repopulation by donor cells in hematologically compromised mice. Some groups have used genetically anemic mice as hosts and have demonstrated repopulation of all lineages by injecting cells from double congenic mice (Mintz et al. 1984; Nakano et al. 1987; Harrison et al. 1988). This elegant animal system has the advantage of avoiding radiation damage to the microenvironment. However, Spangrude et al. (1988) have shown that certain bone marrow derived subpopulation could reconstitute all lineages but, failed to allow the survival of lethally irradiated mice. This finding suggests that this assay may score other hematopoietic progenitors in addition to stem cells.

The radioprotection assay defines pluripotent stem cells by their capacity to protect and completely repopulate all hematopoietic

Many Mature Cells Express Known Cell Surface Markers

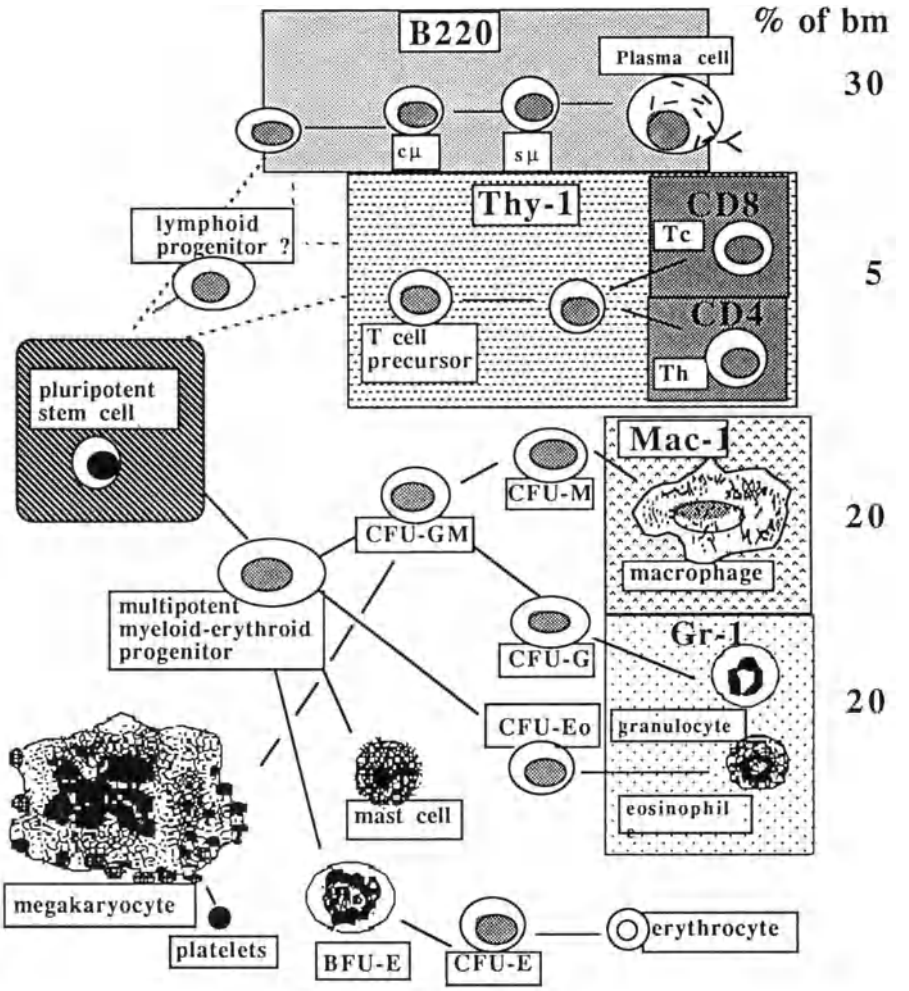

Fig. 1. Differentiation pathways of hematolymphoid cells. Pluripotent stem cells give rise to multipotent myeloid-erythroid progenitors that commit to restricted precursors of the myeloid and erythroid lineages. These precursors are defined primarily through their capacity to give rise to a colony upon stimulation by a growth factor and are called colony forming units (CFU-C). Whether stem cells give rise to a lymphoid progenitor or commit directly to precursors for the T and B lymphocyte lineages is still unresolved. Cell surface markers expressed on mature lymphocytes and myeloid cells can be used to deplete these cells and thus aid in the enrichment of immature cells.

lineages in lethally irradiated animals. Even this assay has limitations. We irradiated C57Bl/6 mice (Ly5.1) with 1140 rads followed by reconstitution with bone marrow cells from C57BL/6-Ly5.2 mice. These congenic mice differ in the Ly5.2 cell surface antigen that is expressed on all hematopoietic lineages with the exception of mature erythrocytes (Scheid and Triglia 1979). We observed survival of a significant number of lethally irradiated animals that showed only minimal donor type repopulation. This phenomenon is particularly apparent when animals are given a low dose of cells (Table 1) and is dependent on injecting the animals with a source of stem cells. Animals that receive stem cell-depleted bone marrow will all die within 2-3 weeks after irradiation (Table 1). It has been previously reported that transplantation damages stem cells (Harrison and Astle 1982) which could give residual radio-resistant host stem cells a repopulation advantage over transplanted stem cells. Why the presence of transplanted stem cells is necessary for survival and repopulation by radio-resistant host stem cells is difficult to surmise. It is conceivable that host stem cells need recovery time after possibly reversible radiation damage. The stem cell-containing Thy-$1^{lo}Lin^-$ population could provide a signal or a cell type necessary for the activation of host stem cells. Regardless of the reason for the repopulation by host stem cells, this observation stresses the importance of determining the level of reconstitution by donor type cells to accurately measure the level of stem cells in a transplanted cell population.

Table 1. Host stem cells can survive lethal irradiation

cells injected[a]	animals surviving[b]	No. of animals reconstituted by at least 4%	30%	50%	80% donor cells[c]
4 x10^4 bone marrow	3/6	3/6	3/6	1/6	1/6
10 Thy-$1^{lo}Lin^-$	4/6	4/6	2/6	1/6	0/6
100 Thy-$1^{lo}Lin^-$	6/6	6/6	5/6	4/6	3/6
5 x 10^4 bone marrow depleted of Thy-1^+cells	0/8	0/8	0/8	0/8	0/8

[a] Adult (≥ 3 month of age) C57Bl/6 mice (Ly5.1) were given 1140 rads and were reconstituted intravenously with congenic C57-Ly5.2 (Ly5.2) cells. Data are from one representative experiment.

[b] Survival was monitored at 4 weeks after injection and remained generally stable for up to 6 months. Data are given as number of surviving mice/ number of injected mice.

[c] Reconstitution was determined 3-6 months past injection on peripheral blood cells by staining with an antibody specific for the Ly5.2 antigen which is expressed on all hematopoietic lineages with the exception of mature erythrocytes (Scheid and Triglia 1979). Co-staining with antibodies specific for B220, or CD-4/CD-8, or Mac-1/Gr-1, showed that myeloid and lymphoid cell lineages were reconstituted to a similar extent.

Progenitors and Precursors

All restricted precursors and progenitors can be assessed *in vivo* by their capacity to give rise to mature progeny. Because progenitors and precursors by definition do not allow survival of lethally

irradiated hosts, it is necessary to co-inject a congenic source of stem cells. In addition, clonogenic myeloid-erythroid progenitors can be enumerated in the spleen colony forming assay (CFU-S) *in vivo,* and *in vitro* in a Interleukin-3 (IL-3) driven colony forming assay (CFU-C) in semi-sold medium (Till and McCulloch 1961; Metcalf 1984). Restricted myeloid precursors also can be counted in the CFU-C assay in the presence of appropriate cytokines. We have assessed the frequency of early B lineage precursors by their capacity to give rise to colonies in limiting dilution in Whitlock-Witte bone marrow cultures (Müller-Sieburg et al. 1986). In these cultures an adherent stromal line supports the proliferation and differentiation of predominantly pre-B cells and B cells (Whitlock and Witte 1982; Whitlock et al. 1988). T cell precursors can be measured *in vivo* by their ability to home to the thymus and differentiate into mature T cells following intravenous injection. Differentiation into T cells as assessed by acquisition of the CD-8 and CD-4 cell surface markers can also be determined following intra-thymic injection of precursor populations (Ezine et al. 1984; Spangrude et al. 1988).

CELL SURFACE MARKERS ON STEM CELLS AND PROGENITORS

Considerable progress has been made in obtaining highly purified populations of hematopoietic stem cells and precursors. Table 2 lists markers that have been used to enrich for pluripotent stem cells. Several groups reported that murine stem cells express the cell surface antigen Thy-1 (Basch and Berman 1982; Hunt 1979). We have previously described (Müller-Sieburg et al. 1986, 1988, 1989) that mouse stem cells could be highly enriched in the 0.1 to 0.3% of normal bone marrow that was characterized by expression of low levels of Thy-1 and lack of high levels of B220, Mac-1, Gr-1, CD-8 and CD-4. We isolated these Thy-1^{lo}Lin$^-$ cells by flow cytometry after staining bone marrow cells with an antibody specific for Thy-1 and a mixture of antibodies specific for the mature markers. The resulting subpopulations are indicated in Fig. 2. This staining pattern reveals also another bone marrow subpopulation, the Thy-1^-Lin$^-$ population. We have previously shown that this population did not contain stem cells but, was enriched for restricted myeloid and B cell precursors. We found most, if not all, precursors that formed colonies in the presence of Granulocyte-Colony Stimulating Factor (G-CSF), Macrophage-CSF, or IL-5 in the Thy-1^-Lin$^-$ population. Il-3 responsive colonies also were contained in the Thy-1^-Lin$^-$ population. In contrast to the Thy-1^{lo}Lin$^-$ cells which gave rise to a high frequency of mixed colonies upon stimulation with IL-3, the Thy-1^-Lin$^-$ population contained only IL-3 responsive precursors restricted to the macrophage and granulocyte lineage (Müller-Sieburg et al. 1988).

The Thy-1^{lo}Lin$^-$ population contained most if not all pluripotent stem cells found in bone marrow and was 300 to 400-fold enriched for stem cells when compared with unseparated bone marrow in the radioprotection assay (Müller-Sieburg et al. 1988). The Thy-1^{lo}Lin$^-$ population was also highly enriched for thymus-homing T cell precursors (Spangrude et al. 1988), B cell precursors that responded to the Whitlock-Witte microenvironment (Whitlock and Witte 1982; Whitlock et al. 1987; Müller-Sieburg et al. 1986), and myeloid-erythroid progenitors (Müller-Sieburg et al. 1986, 1988). The activities of Thy-1^{lo}Lin$^-$ cells are compiled in Table 3.

We had originally published that Thy-1^{lo}Lin$^-$ cells did not express the CD-4 antigen. However, recently we noticed that a subpopulation of the Thy-1^{lo}Lin$^-$ population did express *low* levels of CD-4. The level of CD-4 expression was comparable to that of Thy-1 on the same population and was roughly 10-fold lower than that found on mature T cells. CD-4 expression on the Thy-1^{lo}Lin$^-$ population was revealed by high titer antibodies in flow cytometry or by panning. Panning is known to have an even higher sensitivity than flow cytometry for cells that bear low levels of a cell surface antigen (Nikolic-Zugic and Bevan 1988). If this finding can be extended to human stem cells, it could have implications for the etiology of AIDS.

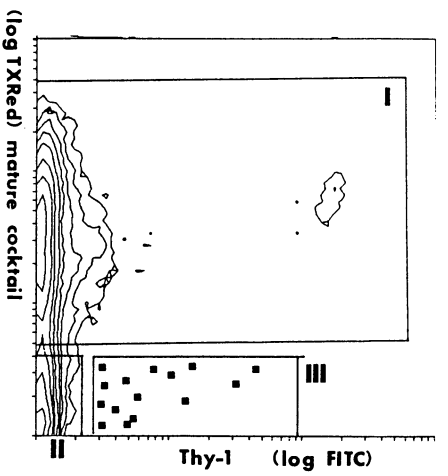

Fig. 2. Precursor and stem cell populations in normal bone marrow defined by cell surface markers. Depicted is the cell sorter profile of cells stained with a cocktail of biotinylated antibodies specific for B220, Mac-1, Gr-1, CD-8 and CD-4, detected with Texas Red conjugated Avidin. (See Fig. 1 for cell distribution of cell surface antigens) Thy-1 was labeled with antibody 31-11, detected with a FITC-conjugated goat anti-rat antibody. Three populations are defined by the staining patterns as indicated by windows. Population I consists predominantly of B cells, pre-B cells, T cells, macrophages, eosinophils, and granulocytes and comprises 75-85% of total bone marrow. Population II, named Thy-1$^-$Lin$^-$ cells, lacks all markers used and represents 15-20% of total bone marrow. Population III, designated Thy-1^{lo}Lin$^-$ cells, make up 0.1-0.3% of bone marrow. For easier identification we have marked with ■ the few events that fall in window III.

From comparing published results it appears that stem cells are similarly enriched in the Thy-1^{lo}Lin$^-$ population and the population that has been isolated by Visser et al. (1984) characterized by high levels of H-2 and binding to WGA. The Thy-1^{lo}Lin$^-$ population has been subdivided recently with the help of an antibody specific for Sca-1 (Spangrude et al. 1988a). Sca-1^+Thy-1^{lo}Lin$^-$ cells were more enriched for stem cells than Thy-1^{lo}Lin$^-$ cells. Coincidentally, cultured totipotent embryonic stem cells also expressed low levels of Thy-1 and the Sca-1 antigen (CMS and M.Moore, unpublished observation).

Table 2. Markers for mouse stem cells

markers	reference
PGP-1	Townbridge et al. 1982
high levels of H-2 bind Wheat germ agglutinin	Visser et al. 1984
Quam-7	Bertonicello et all. 1987
Low levels of Thy-1 absences of B220, Mac-1, Gr-1, CD8, HSA	Basch and Berman 1982; Müller-Sieburg et al. 1986, 1988
Sca-1	Spangrude et al. 1988
Low levels of CD-4 ?	see text

Table 3. Frequency of stem cell and precursor activities in bone marrow populations

activity	Thy-1^{lo}Lin$^-$	Thy-1$^-$Lin$^-$	bone marrow
T cell precursors i.v.	1/600	< 1/10000	1/36000
intra-thymic	1/90	not done	1/5100
B cell precursors[a]	1/15	<1/4000[b]	1/1000
CFU-S	1/31	<1/100000	1/6666
CFU-C IL-3	1/7	1/370	1/666
stem cells	100[c]	<1/ 50000	37000

[a] determined in limiting dilution on the stromal cell line AC3.
[b] The Thy-1$^-$Lin$^-$ population contains B cell precursors that have limited proliferative capacity and do not form colonies in limiting dilution.
[c] Number of cells needed to fully reconstitute 50% of lethally irradiated animals

Table 4. Stem cells in the bone marrow of 5-FU treated mice

source of bone marrow	Number of cells needed to reconstitute 50% of lethally irradiated mice [a]
normal	9×10^4
day 2 post 5-FU	33×10^4
day 4 post 5-FU	10×10^5
day 10 post 5-FU	2×10^4

a Only mice that were ≥ 80% reconstituted by donor cells 12 weeks post injection are included

DISSECTION OF THE THY-1^{lo}LIN^{-} POPULATION

The Thy-1^{lo}Lin^{-} cells appeared homogeneously as small undifferentiated blast cells on morphological examination (Rennick et al. 1987). This population was devoid of restricted myeloid and erythroid precursors that respond to M-CSF, G-CSF, and IL-5 (Müller-Sieburg et al. 1988). B and T cell precursors, myeloid-erythroid progenitors, and stem cells were enriched to a similar extent in the Thy-1^{lo}Lin^{-} population (Table 3). It would be tempting to conclude that the Thy-1^{lo}Lin^{-} cells were a homogeneous population of stem cells. However, the Thy-1^{lo}Lin^{-} population has been phenotypically divided by an antibody specific for Sca-1 (Spangrude et al. 1988). The Sca-1^{+}Thy-1^{lo}Lin^{-} population also contained stem cells, B and T cell precursors and CFU-C (Spangrude et al. 1988; D. Rennick, I.Weissman personal communication) We thus asked whether the various hematopoietic activities found in the Thy-1^{lo}Lin^{-} population could be separated from each other.

We choose the cytotoxic drug 5-Fluorouracil (5-FU) to dissect the hematopoietic activities in the Thy-1^{lo}Lin^{-} population. 5-FU has been described to eliminated predominantly cycling cells but, to spare stem cells (Hodgson and Bradley 1979). The claim that stem cells were not affected by 5-FU was based on determining CFU-S content in bone marrow, an assay which measures stem cells only indirectly (Paige et al. 1981; Van Zant 1984). Thus, we determined stem cell content in bone marrow of mice at 2, 4, and 10 days after injection with a single dose of 5-FU (150 mg/kg mouse) in the radioprotection assay (Table 4). Stem cells were enriched in bone marrow 10 days post injection of 5-FU. We also observed a similar enrichment of CFU-C and B cell precursors. At two days post injection of 5-FU stem cells were actually three fold reduced when compared to normal bone marrow. At that time point IL-3 responsive myeloid-erythroid precursors were strongly reduced (Müller-Sieburg et al. 1988) and B cell precursors which resided only in the Thy-1^{lo}Lin^{-} population were below the level of detection. B cell precursors and myeloid-erythroid progenitors remained repressed at day 4 post injection but, stem cells had recovered close to normal levels (Table 4). Thus, 5-FU separates stem cells from B cell and myeloid precursors. We have verified this finding on the level of isolated Thy-1^{lo}Lin^{-} cells. As with unseparated bone marrow, stem cell activity was close to normal, whereas B cell precursors that responded to the Whitlock-Witte stromal environment, and IL-3 driven CFU-C were strogly reduced. Thus, for the first time stem cell activity has been separated from CFU-C and B cell precursors. We have yet to determine CFU-S and T cell precursor content in day 4 5-FU Thy-1^{lo}Lin^{-} cells. It will also be interesting to assess whether this population can be activated *in vitro* by a combination of cytokines. A population of early myeloid precursors that requires a mixture of IL-3, IL-1, and M-CSF for colony formation has been described (Bertoncello et al. 1987).

In summary, much has been learned about early hematopoietic differentiation by working with highly enriched populations of stem cells and progenitors. While we may not yet have purified stem cells to homogeneity it seems likely that this can be achieved. Perhaps, we have to develop more sensitive and reliable assays for pluripotent hematopoietic stem cells.

More remains to be understood. The significance of expression of low levels of Thy-1 and perhaps CD-4 on stem cells remains to be elucidated. The finding that embryonic stem cells also express the Sca-1 antigen and low levels of Thy-1 suggests that these molecules may be important for cells with extensive differentiation and proliferation capacity. Most importantly, conditions under which stem cells can be induced to self-maintain, without loss of differentiation capacity, have yet to be precisely defined. However, recent progess in culturing Abelson virus transformed early hematolymphoid cells is extremely encouraging (Kimoto et al.1989).

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Structure, Control of Expression and Putative Function of the PreB Cell-Specific Genes V_{preB} and λ_5

A. Kudo, S. Bauer, and F. Melchers

When mouse B lymphocytes develop from progenitors and precursors either during embryogenesis in embryonal blood and fetal liver or during adulthood in bone marrow, Ig gene segments of H and L chain loci are rearranged in temporal order to form complete H and L chain genes from which Ig can be expressed on the surface of B lineage cells (Tonegawa 1983). In fetal liver between day 13 and 16 of gestation B lineage progenitors are induced by contact with stromal cells to four critical divisions in which these Ig gene rearrangements take place (Melchers 1977a, 1977b; Melchers et al. 1975; Paige et al. 1984; Kincade et al. 1981; Whitlock et al. 1985; Dorshkind et al. 1986; Witte et al. 1987; Pietrangeli et al. 1988; Kinashi et al. 1988; Palacios et al. 1989; Kincade et al. 1989; Gisler et al. 1987; Palacios et al. 1984; Palacios and Steinmetz 1985; Palacios et al. 1987; Namen et al. 1988; Melchers et al. 1989). Once preB cells express Ig on their surface they mature between day 16 and 19 of gestation to surface Ig positive mitogen- and antigen-sensitive B cells without much proliferation (Melchers et al. 1989; Osmond 1986). The molecular mechanisms which control the development from progenitors to mature B cells, the critical cell divisions and the Ig gene rearrangements are largely unknown. We have initiated a search for genes and their proteins which are selectively expressed in progenitors and preB cells and which might function to control this development. We have constructed cDNA libraries of mRNAs expressed in a mouse preB lymphoma, 70Z/3, from which sequences were subtracted which are also expressed in a T cell hybridoma. From these libraries four B lineage-specific genes, V_{preB1}, V_{preB2}, λ_5 and mb-1, have so far been isolated and characterized (Sakaguchi and Melchers 1986; Kudo et al. 1987a; Kudo and Melchers 1987; Kudo et al. 1987b; Bauer et al. 1988a; Sakaguchi et al. 1988; Bauer et al. 1988b). V_{preB1}, V_{preB2} and λ_5 are expressed in preB cells, but not in mature B cells, while mb-1 is expressed in preB and B cells, but not in plasma cells. Here we present what we know up to now of the structure of the genes for V_{preB1}, V_{preB2} and λ_5, their pattern of expression and control of transcription and the identification of the corresponding proteins. We speculate about their possible function in the development of B lineage cells.

Structures of the three preB cell-specific genes V_{preB1}, V_{preB2} and λ_5

The cDNA sequences of the three genes expressed in mouse preB cells, and the intron-exon structures of their genomic forms have been elucidated (Sakaguchi and Melchers 1986; Kudo et al. 1987b; Kudo and Melchers 1987). All three genes are located on chromosome 16 which also harbors the λ light chain genes (Kudo et al. 1987a). V_{preB1} and λ_5 are closely linked (Figure 1, top), while V_{preB2} is an unknown distance away from the two others. The leader sequence and the intron

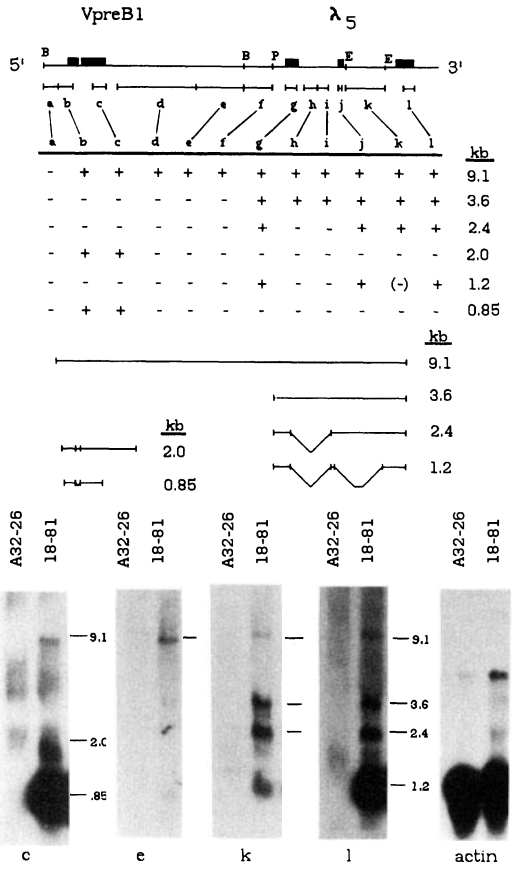


Fig. 1. Northern blot analysis of nuclear transcripts of V_{preB} and λ_5. On top is shown the genomic structure of the V_{preB1}/λ_5 locus on chromosome 16 of the mouse. Two nitrocellulose filters prepared with 10μg nuclear poly(A)-selected RNA from preB cell line 18-81 and T cell hybridoma cell line A32-26 were sequentially hybridized with 12 different probes (from a to l) which were prepared from a genomic DNA clone that spans the V_{preB1} and λ_5 loci as shown at the top of the figure. DNA fragments used are BamHI-BglII 400 bp (a), and BglII-PstI 300 bp (b) from 7pB12-2 (Kudo and Melchers 1987), AccI-EcoRI 220 bp (c) from pZ121 (Kudo and Melchers 1987), KpnI-XbaI 2 kb (d) and XbaI-BamHI 1.2 kb (e) from 7pB12-2, BamHI-PstI 1 kb (f) from 7pB12-1 (Kudo and Melchers 1987), HindIII-StuI 320 bp (g) from pZ183-1a (Kudo et al. 1987b), KpnI-XbaI 340 bp (h), XbaI-BglII 290 bp (i), 34mer oligonucleotide described below (j), EcoRI-EcoRI 1 kb (k) and EcoRI-XhoII 470 bp (l) from 7pB12-1.
The filter was hybridized with an oligolabeled probe (except for probe j, which was end-labelled using γ^{32}P-ATP), washed with 0.1 x SSC, 0.1% SDS at 50°C, exposed to X-ray film. Thereafter, the radiolabelled probe was washed off with 0.1 x SSC, 0.1% SDS at 95°C. The nuclear poly(A) hybridization RNA pattern is shown diagrammatically in the middle of the figure. A + represents hybridization and - represents none. The sizes of the RNA fragments detected are shown as 9.1 kb, 3.6 kb, 2.4 kb, 2.0 kb, 1.2 kb and 0.85 kb and were determined by the use of an RNA molecular weight ladder.

of V_{preB} show sequence homology to V_λ. The 5' part of V_{preB1} shows sequence homology to both V_λ and V_κ L chain sequences, while the 3' part of the second exon of λ_5, the intron between the second and the third exon and the third exon of λ_5 show sequence homology to J_λ and C_λ light chain sequences. The 3' end of V_{preB1}, the first exon of λ_5 as well as the sequences between V_{preB1} and λ_5 show no nucleotide homology to any known DNA sequence in the sequence data banks. The nucleotide sequence of V_{preB2} is 97% identical to V_{preB1}. The few nucleotide differences result in four coding changes in the 142 amino acids of V_{preB}.

In the human there is only one V_{preB} gene. It is found within a cluster of V_λ segments on chromosome 22 (Bauer et al. 1988a). Its 80% overall sequence identity to the mouse V_{preB} sequences is particularly striking in areas corresponding to frameworks 2 and 3 of V gene segments (Bauer et al. 1988b). Similar sequence homologies are likely to exist for other mammalian species, since Southern blots of DNA from calf, rabbit, rat, guinea pig and hamster show bands crosshybridizing under high stringency. Very little restriction fragment length polymorphism is detectable within a species (mouse or man), indicating remarkable conservation of the structure of the V_{preB} genes (D'Hoostelaere et al. 1989). None of the preB specific genes is rearranged during B cell development.

RNA expression in cell lines and normal cells

Northern blot analyses with poly $(A)^+$ RNA, dot blot analysis, and run-on analyses with isolated nuclei of transformed cell lines arrested at various stages of B lineage development, and "in situ" hybridization of normal cells of the B lineage all indicate that the low to medium level expression of V_{preB} and λ_5 is restricted to preB cells. Both V_{preB1} and V_{preB2} are expressed (Figure 2).

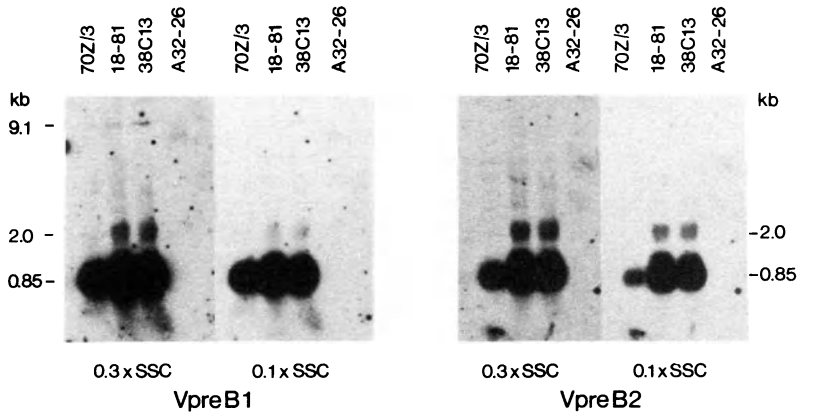


Fig. 2. Northern blot analysis to distinguish V_{preB1} from V_{preB2} transcription. 10 µg nuclear poly(A)-selected RNA from 70Z/3, 18-81 and 38C-13 mouse preB cell lines and from the A32-26 mouse T cell hybridoma was separated on 1.0% agarose gel and transferred to a nitrocellulose filter. The filters were hybridized with the end-labelled 32mer oligonucleotides (positions 928 to 959 in the 3' untranslated region of V_{preB1} and $_2$ [Kudo and Melchers 1987]) specific for V_{preB1} or V_{preB2} genes, washed first with 0.3 x SSC, 0.1% SDS at 50°C, then with 0.1 x SSC, 0.1% SDS at 50°C and exposed to X-ray film (Kodak XS-1) after each wash step.

It is possible that precursors of the B lineage at the stage of first D_H to J_H rearrangements but before differentiation to either myeloid or preB lineage cells (Davidson et al. 1988) already express the genes. Two surface Ig^+ cell lines (300-19, sIg^+ subline; and 38C-13) with preB cell-characteristic markers express the genes (Sakaguchi N, Thalmann P, Davidson W, Pierce J and Melchers F, unpublished observations). All other sIg^+ B cells and plasma cells tested do not express the genes.

Nuclear poly $(A)^+$ RNA from preB cell lines contains several larger molecular weight RNA species, in addition to the mature 0.85 kb mRNA for V_{preB} and 1.2 kb for λ_5 (Figure 1). These larger molecular weight species contain the intron and intergenic sequences indicated in Figure 1, and suggest that V_{preB1} and λ_5 can be transcribed in one 9.1 kb RNA, but more often appears to be transcribed in two separate RNA species, which appear to be spliced in several steps to the mature mRNAs. Nuclear run-on experiments showed that the pre-B cell specificity of RNA expression of the V_{preB} and λ_5 genes is due to transcriptional control mechanisms and not due to post-transcriptional events (Figure 3). These results indicate that V_{preB} and λ_5 can be transcribed from two separate promotors, the preB-specific expression points to a possible existence of a lineage-specific enhancer element; its turn-off in B cells and plasma cells points to possible silencer elements.

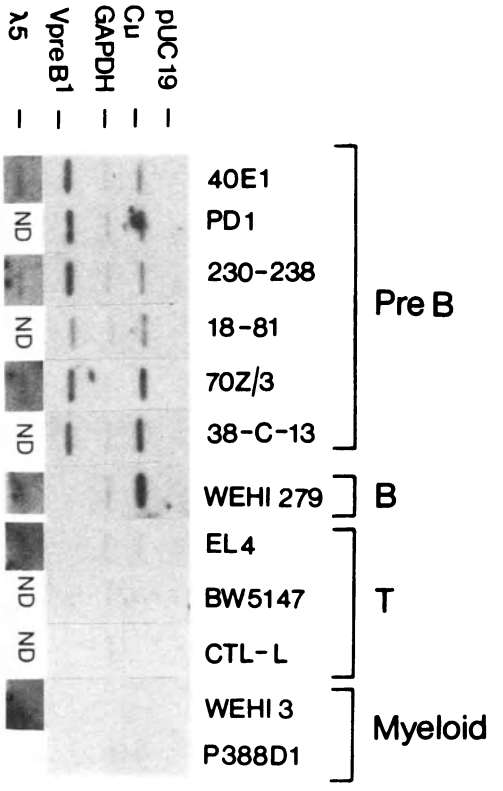


Fig. 3. PreB cell specific transcription of V_{preB} and λ_5 genes. 5µg of linearized, denatured plasmid DNA from: the enhancer region of the IgH chain locus (Cµ), the "housekeeping" gene glyceraldehyde phosphate dehydrogenase (GAPDH), V_{preB1}, λ_5 and pUC19 (plasmid DNA as negative control) were fixed on nitrocellulose and hybridized to ^{32}P-UTP labelled RNA generated in vitro from nuclei isolated from transformed mouse preB cell lines 40E1, PD1, 230-238, 18-81, 70Z/3, 38C-13, the mouse B cell line WEHI279, the mouse T cell lines EL4, BW5147, and CTL-L, and the mouse myeloid cell lines, WEHI3, and P388D1. Results for the λ_5 probe were generated in separate experiments that showed comparable hybridization of ^{32}P-UTP labelled transcripts to the GAPDH probe. ND = not done.

Lineage-specific control of expression of V_{preB1} and λ_5

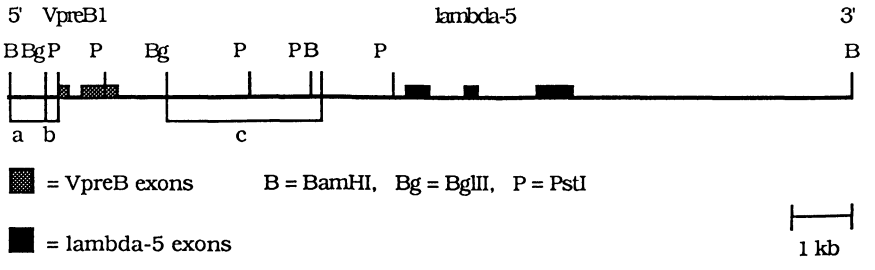
Sequences 5' of V_{preB1} and λ_5, and between V_{preB1} and λ_5 were isolated and combined as possible promoter or enhancer elements with known promoters and enhancers as shown in Table 1. Promoter and enhancer activities were tested with chloramphenicol-acetyl-transferase (CAT) as an indicator gene by transfection into different cell lines, i.e. the preB cell lines 220.8, 18.81, and 38B9, the B cell lines A20.3 and K46, the T cell lymphoma EL-4 and the plasmacytoma J558. The results so far obtained are summarized in Table 1. They define two parts of a promoter element 5' of V_{preB} (a and b), and a region with enhancer activity in between V_{preB1} and λ_5 (c, see map in Table 1), which are required for specific gene expression in preB cell, but not in other cell lines. A promoter region 5' of the λ_5 gene is currently under investigation.

Table 1 Promoter and enhancer activities around the VpreB1 gene

Constructs		CAT activity in cell lines					
Promotor	Enhancer	preB			B		plasma
		220.8	38B9	18-81	A20.3	K46	J558
a+b	c	+	+	+	-	-	-
a+b	$E\mu H$	+	+	+	-	-	-
a+b	SV40		+	+	+	-	-
a+b	-	-	-	-	-	-	-
b	-		-	-	-		-
b	c		-	-	-		-

DNA regions a, b and c were combined with the CAT gene in various constructs as indicated in the table. $E\mu H$ denotes the enhancer region isolated from the Ig-H chain locus, SV40 denotes that from the SV40 virus. The constructs were transfected by the DEAE dextran method (Martenssen & Leandersson, 1987)into the Abelson virus-transformed preB cell lines 220.8, 38B9 and 18-81, B cell lines A20.3 and K46, and the plasmacytoma line J558. CAT assays were done as described by Martenssen & Leandersson (1987). + denotes CAT activity, - no activity.

Genomic map of VpreB1/lambda-5



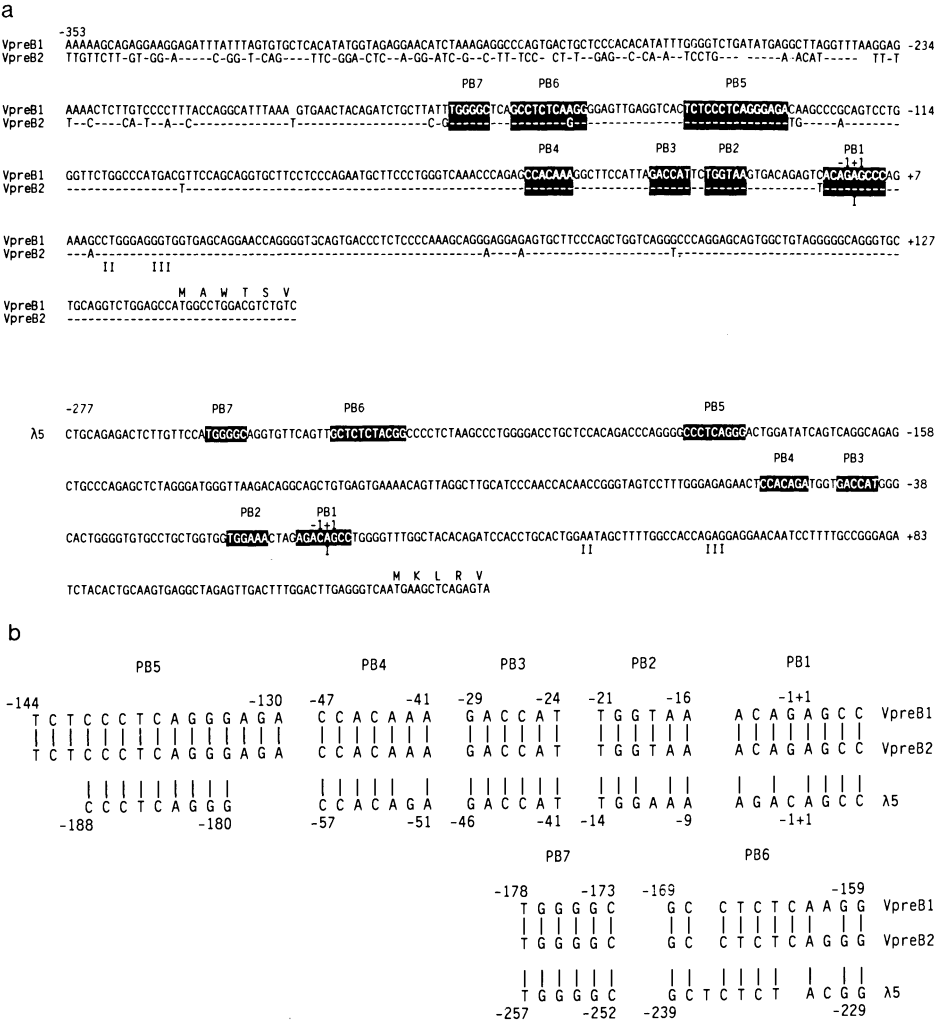


Fig. 4.
a) Genomic nucleotide sequences of 5' region of V_{preB1}, V_{preB2} and λ_5.
Sequencing was performed by the dideoxy chain termination method (Hu and Messing 1982).
Sequences identical between V_{preB1} and V_{preB2} are indicated by dashes (-) for the sequence of V_{preB2}. (+1) shows the transcription start sites $I_{V_{preB}}$ and $I\lambda_5$ which were identified by S1 nuclease protection mapping (unpublished). Two other transcription start sites for both V_{preB} and λ_5, indicated by II and III, are shown below the nucleotide sequence. Sequences identical between V_{preB1}, V_{preB2} and λ_5, and those with strong similarities are boxed and numbered pB1 to 7 (see also b). The putative amino acid sequence is shown above the sequence.
b) Alignment of seven sequences with identity or strong similarity (numbered pB1 to 7) in the regions 5' of the transcription start sites of the V_{preB1}, V_{preB2} and λ_5 genes.

Possible cis-acting elements of preB cell-specific promoter regions of the V_{preB1} and λ_5 genes

The 5' regions next to V_{preB1}, V_{preB2}, and λ_5 were sequenced, and these sequences compared with each other for possible consensus sequences. These sequences, shown in Figure 4, show seven boxes of strong sequence homology or even identity as possible cis-acting elements in the control of the preB cell-specific expression of V_{preB} and λ_5. An analysis of the activity of these cis-acting elements is now under way.

Association of V_{preB} and putative λ_5 protein with μ heavy chain protein in human preB cells

Antibodies raised against human μ heavy chains and antibodies raised against a 24 amino acid-long peptide covering the carboxy terminal part of V_{preB} both precipitate from human preB cell lines, but not from B cells or T cell lines, polypeptides of ~ 70 kd (μH chains), 22 kd (putative λ_5) and 18 kd (V_{preB}) (Figure 5). The precipitation of μH chains, putative λ_5 and V_{preB} by V_{preB}-peptide specific antibodies is inhibited by excess peptide, indicating that the antibodies are V_{preB}-specific, and that the three polypeptides are associated with each other in human preB cells.

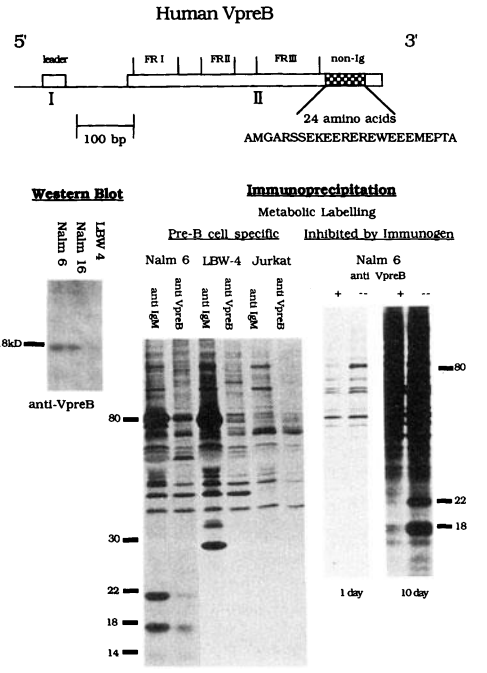


Fig. 5. Characterization of V_{preB} protein. Affinity purified polyclonal rabbit antibody specific for a 24 amino acid peptide from the non-Ig selected domain of the V_{preB} protein (see top of figure) was used in Western blot and immunoprecipitation experiments to show the 18 kD V_{preB} protein in preB cells (Nalm 6, Nalm 16) but not in mature B cells (LBW-4) or T cells (Jurkat). Immunoprecipitation of ^{35}S-methionine labelled total cells lysates from 10^6 cells with antibodies against V_{preB} (anti V_{preB}) or IgM (anti IgM) precipitated the 80 kD μ heavy chain with the 18 kD V_{preB} chain and a 22 kD candidate protein for human λ_5. Immunoprecipitation of all of these bands with the peptide specific anti V_{preB} antibody could be inhibited in the presence (+) of excess unlabelled peptide. The short and long exposure times were necessary to show inhibition of the μ heavy chain (1 day exposure) or the V_{preB} and putative λ_5 proteins (10 day exposure). Western blot and immunoprecipitation experiments were performed using 17.5% PAGE analysis under reducing conditions.

These results support our idea that V_{preB} protein may associate by noncovalent interactions with a λ_5 protein. This association could result in the formation of a light chain-like structure, in which the aminoterminal end of λ_5 and the carboxyterminal end of V_{preB} protrude as two "constant" peptides at a site which corresponds in V_{preB} to the third complementarity-determining region of a variable region of light chains. This might constitute a "constant" binding site for all preB cells (Figure 6). The light chain-like structure V_{preB}/λ_5 then is expected to form a disulphide-bonded association with μ heavy chains in preB cells.

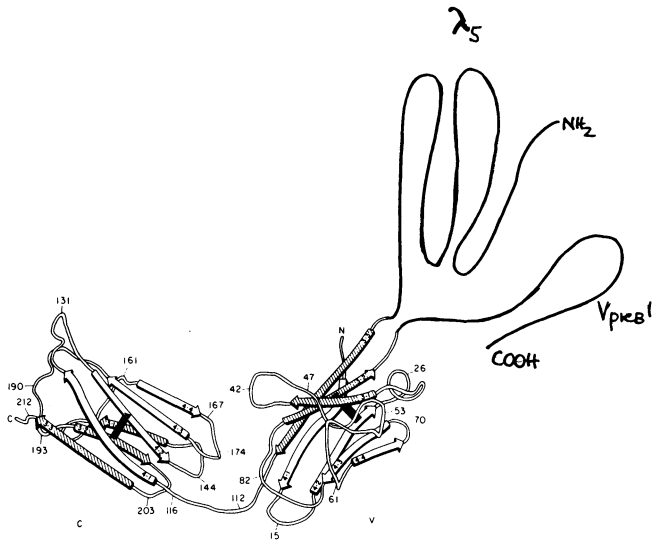


Fig. 6. Proposed structure of associated V_{preB} and λ_5 proteins. It is assumed that the Ig-like portions of V_{preB} and λ_5 fold like the related V and C domains of a light chain similar to that published in "The Immune System", I McConnell, A Munro, H Waldmann, Blackwell Scientific Publications, 2nd edition, Oxford, London, Edinburgh, Boston, Melbourne, Figure 2.5 on p.26. Association between V_{preB} and λ_5 is favored by Ig domain-like interactions between the β-pleated sheet encoded by the J-like sequence of λ_5 with the β-pleated sheets of V_{preB}. The aminoterminal portion of λ_5 and the carboxyterminal portion of V_{preB} form a large protrusion at the site of CD3 of a V-region.

This ternary complex of V_{preB}, λ_5 and μ heavy chains could well appear on the surface of preB cells (Melchers 1977c), may be involved in binding reactions with stromal cells and may signal critical cell divisions and Ig gene rearrangement processes. Details of a model of these controls involving V_{preB1}/λ_5 and μH chains as well as other H chain-like proteins are discussed elsewhere (Melchers et al. 1989).

ACKNOWLEDGEMENTS

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II Cell Developments

Development of B Cells

2. Repertoire Selection

B Cell Differentiation: Development of Antibody Repertoires

F.W. Alt, G. Rathbun, J. Berman, B. Malynn, M. Morrow, T. Logtenberg, and G. Yancopoulos

INTRODUCTION

In mammals, primary B cell differentiation occurs as an antigen-independent process in the liver of the fetus and in the bone marrow of the adult. During these stages, stem cells give rise to precursor B lymphocytes--the cells in which antibody variable region genes are assembled from component germline gene segments (reviewed by Alt et al., 1987). The genes that encode Ig heavy and light chain variable regions are assembled at distinct stages of pre-B cell differentiation. First, heavy chain variable region gene is assembled from component V_H, D and J_H segments and subsequently light chain variable region genes are assembled. Functional heavy and light chains associate to form a complete Ig molecule; the expression of this molecule on the cell surface defines the next major stage of B cell differentiation, the B lymphocyte. Only a single heavy chain and a single light chain are expressed on the surface of any particular clonal population of B cells; this ensures B lymphocyte clones will express a single species of Ig with a unique set of binding specificities. Following generation in primary differentiation organs, B cells migrate to peripheral lymphoid organs such as the spleen. Upon appropriate interaction with a cognate antigen, peripheral B lymphocytes are stimulated to further mature into antibody-secreting cells. During this antigen-driven clonal selection process, additional types of B lineage cells may be generated including cells that have undergone heavy chain class-switching or somatic mutational events. These latter events, although crucial to the diversity of the antigen-binding and effector function repertoires, respectively, will not be considered in this manuscript; these processes have been the subject of several recent reviews (Cuomono, Forster, and Rejewsky, 1987; Lutzker and Alt, 1989). Also, due to space limitations, we will only cite reviews or recent primary references not available in reviews.

MURINE V_H GENE ORGANIZATION

Each germline heavy or light chain variable region gene segment is immediately

flanked by a conserved recombination recognition sequence that consists of a palindromic heptamer and an AT-rich nonamer separated by a spacer. This signal sequence targets the activity of a lymphoid cell-specific recombination system referred to as VDJ recombinase (reviewed by Blackwell and Alt, 1989). The organization of the Ig heavy chain variable region locus is now known in substantial detail (reviewed by Rathbun et al., 1989). There are 4 J_H segments in an approximately 1kb region that lies 7kb upstream of the exons that encode the C_μ constant region gene, the first C_H gene expressed during B cell development. Starting within 1kb of the 5′ J_H segment and spread out over the next approximately 80kb there are at least 12 D segments. The germline V_H gene locus starts upstream of the D locus; the exact linkage has not been elucidated in mice but the most proximal V_H segment is within 200 kb of the J_H segments. The V_H locus contains approximately 200 to perhaps as many as 1000 separate germline V_H segments, depending on the mouse strain (reviewed by Rathbun et al., 1989). The different V_H gene segments have been grouped into families based on greater than 80% relatedness at the nucleotide sequence level; this type of grouping has now allowed the classification of 11 V_H gene families with a few to hundreds of members. The general organization of these families with respect to each other was initially defined by Brodeur and Riblet through a combination of deletion and recombinant inbred strain mapping techniques; this rough outline of V_H organization was extremely important for early studies of the factors affecting V_H utilization (see below). Several groups have determined the organization of these families in more detail over the last years; these studies led to modifications the relative order of the more 5′ gene segments (reviewed by Rathbun et al., 1989; Brodeur et al., 1988). Most studies are consistent with the notion that genes from individual families are relatively clustered, although there is significant interspersion within certain families.

Preferential Rearrangement of J_H-Proximal V_H Gene Segments

With respect to primary repertoire development, an important question was whether there is any relationship between the germline organization of V_H gene families and their utilization for V_H to DJ_H rearrangements. This question was initially pursued by determining which V_H gene segments were used in the V_HDJ_H rearrangements of A-MuLV transformed pre-B cells or fetal liver hybridomas (reviewed by Alt et al., 1987 and by Pearlmutter, 1987). Because heavy chains are expressed before light chains, it was considered that analysis of rearrangements from genomes representing pre-B cells might obviate potential effects of cellular selection, at least those operating on a complete surface Ig molecule. In addition, many A-MuLV transformants represent very early stages of pre-B cell differentiation and actively append V_H gene segments to pre-existing

DJ_H rearrangements in culture. Therefore, these rearrangements could not be influenced by selective mechanisms operating in vivo. The nature of the gene segments employed in such lines was first studied by direct isolation and later by hybridization analyses that utilized V_H family-specific hybridization probes; the latter types of analyses only determine which V_H family is used but usually do not identify members within a family. Studies of this type indicated a strong bias for rearrangement of J_H proximal V_H gene segments and families in pre-B cells. Studies of the A-MuLV transformed lines were particularly informative. In lines derived from BALB/c mice, 20 of 33 characterized V_HDJ_H joins formed at the time of isolation or during growth in culture utilized members of proximal V_H gene families (V_H7183 and V_HQ52); of the proximal rearrangements, 11 utilized the most J_H proximal V_H segment mapped in BALB/c (V_H81X).

There could be several mechanistic and/or functional explanations for the preferential rearrangement of proximal V_H gene segments in BALB/c mice. One possibility is that the rearrangement is strongly influenced by position, with J_H proximal genes having a higher probability of rearrangement. To test this possibility, V_H gene utilization was assayed in subclones of an A-MuLV transformant derived from an NIH/Swiss mouse; this line had no gene very closely related to V_H81X and had members of the V_HQ52 family as the most proximal V_H gene segments. Significantly, of 22 characterized rearrangements, 15 utilized members of the V_HQ52 family, and of these 15, 11 utilized the two V_HQ52 gene segments mapped as most J_H proximal. Therefore, the most simple interpretation of these findings is that the V_H to DJ_H rearrangement process has mechanistic restraints that preferentially, but not exclusively, target proximal V_H gene segments for rearrangements (reviewed by Alt et al., 1987). In this regard, one possible explanation for the observed utilization patterns is that VDJ recombinase initially binds within the J_H region and linearly tracks towards the V_H region leading to more frequent utilization of proximal gene segments. To explain the utilization of upstream V_H gene segments, a correlate of this hypothesis would be that either the recombinase tracks through V_H segments at a relatively high frequency or that it can bind directly to V_H gene segments before association with the J_H region in a tracking independent manner. These findings have important implications with respect to potential physiological significance of this biased rearrangement process as discussed below.

V_H Utilization during Murine B cell Ontongey

There have been a variety of different assays developed to study V_H gene utilization in different populations of B lineage cells. The initial assays relied on the use of transformed counterparts of cells at various stages in the

differentiation pathway; in addition to the A-MuLV transformants described above, hybridomas were utilized by Rajewsky, Coutinho and their colleagues to determine the relative utilization of different V_H gene families in the peripheral B cell populations of the spleen. Another cellular assay to determine V_H gene utilization in different tissues was initially developed by Wu and Paige and also by Kelsoe, Teale and their colleagues; this system was based on the propagation of normal B lineage cells in culture followed by assay of V_H utilization by colony filter hybridization methods. More recently Coutinho, Freitas and their colleagues have begun to exploit the potentially powerful method of determining V_H expression in single cells by direct in situ hybridization techniques. Finally, V_H expression has been assayed in RNA isolated from total lymphoid tissues, either by a Northern blotting V_H expression analysis or by the relative frequency at which particular V_H sequences were isolated from cDNA libraries (reviewed by Alt et al., 1987; Pearlmutter, 1987; Holmberg et al., 1986). In general, these assays have all yielded relatively consistent results; although there are certain situations in which colony assays differed from the results obtained with the other methods (discussed by Yancopoulos et al., 1988).

The utilization of V_H gene families by the major population of splenic lymphocytes appears to correlate directly with the size of the family; thus large families (eg. J558) may be associated with as much as 50% of the spleen μ mRNA whereas as small families (eg. S107) may only contribute less than a few percent. This data can be most easily interpreted to indicate that, in general, all V_H genes are represented at fairly similar frequencies in the repertoire of peripheral B lymphocytes. On the other hand, representation of different V_H gene families in the RNA of fetal liver cells is not directly related to family size; but instead seems to be a function of both family size and, at least with respect to the most J_H proximal families, position on the chromosome. This finding can be explained most easily by assuming that the newly generated V_H repertoire of the fetus directly reflects the rearrangement frequency of particular V_H genes. Thus, rearrangement of a family would be a combined function of chromosomal position (higher rates for J_H proximal genes) combined with the number of members (more individual targets). The conclusion of these types of studies is that an initially biased repertoire generated in differentiating fetal pre-B cells is somehow normalized (randomized) in the peripheral B cells of the adult (reviewed by Alt et al., 1987).

Normalization of the V_H Repertoire

The mechanism by which the initially biased fetal V_H repertoire becomes normalized has been a subject of study by several groups. Several possible, not

mutually exclusive, mechanisms were considered. The first was that there may be programmed changes in the recombination mechanisms such that more distal V_H genes segments become rearranged more frequently later in ontogeny. Such changes in rearrangement frequency would necessarily result from changes in the mechanisms that target V_H to DJ_H rearrangement: for example, the programmed appearance of recombinase entry sites into the upstream region of the locus (discussed by Pearlmutter, 1987; Yancopoulos et al., 1988). Alternatively, a biased primary V_H repertoire may be generated throughout ontogeny with normalization in peripheral tissues resulting from selection mechanisms operating on the biased primary population subsequent to VDJ rearrangement (Yancopoulos et al., 1988). To help distinguish between these possibilities, V_H utilization was determined in cells or tissues representing later stages of ontogeny. Early studies demonstrated that A-MuLV transformed cell lines from adult bone marrow still showed preferential rearrangement of proximal V_H genes suggesting the possibility that mechanism operating subsequent to rearrangement may be more important. This notion has received considerable support from more recent studies by Forster and Rajewsky, Coutinho, Freitas and their colleagues, and Malynn and Alt that demonstrate the repertoire of adult bone marrow B lineage cells is still substantially biased for expression of J_H proximal V_H gene segments. Given that a biased repertoire is generated in primary B cells of the adult, then the question arises as to how this repertoire becomes normalized in peripheral B cells. Although work on this topic is now the subject of extensive investigations, it would appear that selection occurring sometime after the generation of these cells and their transition to the periphery is the major mechanism. Studies of mice with various immune defects (by Malynn, Alt and colleagues) and mice grown in the absence of antigens (by Coutinho and colleagyes), combined with the analysis of various B cell populations (by Rajewsky and Freitas and their colleagues) suggest that T cells and incidental antigens may play a major role in this normalization process.

ORGANIZATION OF THE HUMAN V_H GENE LOCUS

There has been a substantial increase in our knowledge of the organization of the human V_H locus over the last several years. Currently, there are 6 known human V_H gene families termed V_H1 through V_H6 (reviewed by Rathbun et al., 1989). It has been estimated that there are approximately 100 germline V_H gene segments; families range in size from approximately 30 members (V_H3) to a single member (V_H6). When considered with respect to the different V_H subgroups as defined by Kabat and colleagues, there appears to be a relatively good correspondence between the human and murine V_H genes. Human V_H segments are contained within a 2000kb locus. The most proximal known human V_H segment is

the single V_H6 gene; it has been mapped as lying about 85kb from the J_H locus. There are probably more than 20 human D segments contained within the region between the J_H segments and the V_H6 gene; at least one D segment is interspersed with the V_H gene segments but it is not known if it is functional. There appear to be certain differences between the organization of the human and murine V_H locus; the most notable is that as opposed to the relatively clustered organization of murine V_H families, the human V_H genes of different families are highly interspersed. Thus, for any given human V_H gene segment, it is highly likely that neighboring segments will be of different families, whereas for the mouse, neighboring genes are more likely to be members of the same family. Individual studies that have contributed to this picture of the human V_H locus have been reviewed (Rathbun et al., 1989).

Utilization of Human V_H Genes during Fetal Development

Initial analyses of human V_H gene utilization by Perlmutter and colleagues involved isolation of cDNA sequences corresponding to expressed human Ig heavy chain mRNA sequences from a library prepared from human fetal liver RNA. These studies indicated that certain members of the very large human V_H3 family were preferentially utilized; the location of the genes within the human V_H locus has not yet been determined. Subsequently, utilization of human genes in peripheral lymphocytes was estimated by determining which V_H gene segments were expressed by a large panel of Epstein Bar Virus-transformed human B cell lines (reviewed by Logtenberg et al. 1989). These studies demonstrated that utilization of different families occurred in rough proportion to their size; suggesting that the peripheral human V_H repertoire is normalized like that of the mouse. More recently, the relative utilization of human V_H genes in fetal versus adult lymphoid tissues has been compared by Northern blotting analyses as described above for the mouse. In analogy to utilization of proximal V_H genes in the mouse, these studies suggested highly preferential utilization of the J_H-proximal V_H6 gene in fetal as opposed to adult lymphocytes (reviewed by Logtenberg et al., 1989; M. Fougereou, personal communication).

Biased V_H Gene Utilization in CD5-positive B cells and B cell Tumors

Recent studies have demonstrated that clonal outgrowths and tumors derived from the murine lyl^+ B cell subset express a restricted set of V_H genes; these genes do not appear to represent proximal V_H genes (Pennell et al., 1988; Tarlington, et al., 1988; Forster et al., 1988). Human chronic lymphocytic leukemias, which appear to derive from the $CD5^+$ subset, also express a biased V_H repertoire. Initially, Tucker, Blattner and colleagues found that a high

proportion of these tumors express members of the small V_H5 family. More recently, it has been demonstrated that CLLs preferentially express members of the V_H4 and V_H6 family, as well as members of the V_H5 family (discussed by Logtenberg et al., 1989). These studies complement earlier studies of Carson and colleagues that demonstrated biased expression of a κ light chain variable region sequence in CLLs. At present there are several, not mutually exclusive, ways to explain the biased V_H gene expression in the $CD5^+$ tumors. One possibility is that the biased expression patterns simply reflect the expression patterns of the cells from which these tumors derive. An analogy would be the preferential expression of proximal V_H genes by A-MuLV transformed cells; this expression pattern reflects that of normal pre-B cells. Alternatively, the expression of particular V_H genes in $CD5^+$ tumors could be selected in the context of the tumorigenesis process. Several studies have attempted to determine the V_H repertoire of normal $CD5^+$ B cells. In both the mouse (Forster and Rajewsky, peronal communication) and in the human (Logtenberg, unpublished) preliminary studies suggest a significantly biased repertoire in normal cells of this population. In the case of human $CD5^+$ cells, EBV transformed $CD5^+$ chord blood cells appeared to express a distribution of V_H genes quite similar to that observed in the analysis of human CLLs. If $CD5^+$ cells do, indeed, express a biased V_H gene repertoire, then the mechanism by which it arises remains an important question. Once again, such a biased repertoire could arise by cellular selection mechanisms or through biased rearrangement mechanisms. If the latter were the case, then, because biased utilization of relatively J_H distal V_H gene segments is observed, mechanisms distinct from those that lead to biased V_H gene expression in pre-B cells would need to be invoked.

Significance of Biased V_H Gene Expression

It is possible that the preferential expression J_H proximal V_H genes is simply a by-product of the V_H gene rearrangement mechanism that must be overcome by subsequent normalization mechanisms to express a maximally diverse peripheral repertoire. However, several lines of evidence from Kearny, Holmberg and their colleagues suggest that frequently rearranged V_H gene segments may play a special role in the early development of immune repertoires. In this regard, specific mechanisms could have evolved to ensure preferential rearrangement, or, more likely, evolution could have molded proximal V_H genes to encode particular specificities (discussed by Yancopoulos et al., 1988). It is notable that some of the V_H gene segments found preferentially rearranged in human fetal liver are related to V_H7183 segments preferentially rearranged in certain mouse strains. However, to interpret such findings in a functional context, one would need to account for the observation that different strains of mice may preferentially

employ quite different V_H gene segments. The most J_H-proximal human V_H gene segment, V_H6, has a number of features that suggest a potential function for antibodies it encodes (Logtenberg et al., 1989); although again more analyses will be necessary to critically evaluate this possibility. The biased repertoire apparently expressed by $CD5^+$ B cells has been suggested to represent a germline repertoire directed against recurrent pathogens (Forster et al., 1987).

An understanding of the factors involved in the establishment of the primary antibody repertoire will await elucidation of the factors involved in the rearrangement and expression of light chain genes in pre-B cells. The organization of the κ light chain genes has been determined in great detail (reviewed by Zachau, 1989). Unlike the V_H genes many of the V_κ gene segments lie in inverse transcriptional orientation relative to the J locus; therefore, these gene segments must be assembled by inversional recombination. This organization difference may or may not have functional significance. However, preliminary studies of V_κ rearrangement have not indicated the 3′ biases found for V_H genes (P. Gearhart; C. Bona; personal communication). In addition to the inverted organization of certain V_κ gene segments, several factors might affect V_κ utilization including the possibility of secondary rearrangements, selection for pairing with the expressed V_H gene, etc.

ISOLATION OF VDJ RECOMBINASE

A final resolution of the mechanisms involved in the utilization of particular V_H gene segments for rearrangement may await the definition of the enzymatic machinery that carries out this joining. The general mechanism by which VDJ recombinase works is known in outline; it appears that there are a variety of activities required including: recognition of the signal sequences, site-specific endonucleolytic activity at the border of these sequence and the adjacent coding sequences, potential exonucleolytic activity with respect to the coding sequences, potential base addition to coding and/or recognition sequences, potential polymerase activity to fill in 3′ overhangs, and ligase activity (reviewed by Blackwell and Alt; 1989). Whether most of these activities could be accommodated within a single protein or whether there are multiple components is not known. While some of these activities clearly appear to be specific to precursor B and T cells, others may be more generalized. In addition, available evidence suggests that nucleotide addition activity may be carried out, at least in part, by terminal deoxynucleotidyl transferase. Thus, there appear to be, at a minimum, two different lymphoid-specific genes encoding activities important for this system. Differential expression of the TdT activity versus recombinase activity in general appears to lead to the frequent addition of extra bases to heavy as

opposed to light chain variable region joins (discussed by Alt et al., 1987).

A number of strategies have been employed in an attempt to isolate genes that encode VDJ recombinase or that may be involved in directing its activities. Recombination substrate assays have suggested that expression of VDJ recombinase activity generally does not occur in non-lymphoid cells and during lymphocyte differentiation is relatively specific for the precursor stage of B and T cell development (reviewed by Blackwell and Alt., 1989). Based on these findings, one approach to isolate relevant genes has been the use of subtractive hybridization technologies to isolate gene specifically expressed in pre-B and pre-T cells. Several such genes have been isolated; expression of some correlates with recombinase activity (Yancopoulos, Oltz, and Alt, unpublished). However, their relationship, if any, to recombinase or its control remains to be determined. Other studies have taken the approach of isolating genes that encode a signal sequence binding activity. Screening of bacteriophage expression libraries for clones expressing proteins that specifically bind signal sequences led to the isolation of a clone complementary to a pre-B specific mRNA encoding an approximately 80kd protein (H. Sakano, personal communication). On the other hand, a 60kd κ light chain signal sequence binding protein was purified and its sequence used to isolate a corresponding DNA clone; preliminary experiments suggested that this clone may encode activities capable of catalyzing VDJ recombination *in vitro* (T. Honjo personal communication). Finally, a gene capable of transferring VDJ recombinase activity into non-lymphoid cells has been isolated by gene transfer techniques (Schatz and Baltimore, 1988). This gene encodes an approximately 120kd protein that is highly conserved in mammalian evolution and expressed precisely in cells and tissues that have known VDJ recombinase activity (D. Baltimore, personal communication). It is not known whether the latter gene is recombinase itself or a gene that activates recombinase expression. If it is recombinase, then many of the activities described above either would be contained within this single protein or else some would have to be expressed in a non-specific fashion. The relationship between the latter gene and the other two, apparently different, genes isolated based on encoding proteins with signal sequence binding activity remains to be determined. Clearly, remarkable progress has been achieved in the isolation of the gene(s) encoding VDJ recombinase and this will provide a foundation for future studies that should elucidate in more detail the mechanisms that direct its activity.

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Evolution of Immunoglobulin Gene Complexity

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INTRODUCTION

As is evident from other contributions in this volume, considerable progress has been made towards understanding the relationships between immunoglobulin and T cell antigen receptor (TCR) genes at the levels of structure, organization and genetic regulation. From these observations, a remarkable background has emerged and the evolution of these unique, closely related multigene systems now can be interpreted within this context. Such information is of considerable interest in terms of defining the pathways of evolutionary diversification as well as the mechanism(s) for utilization of individual segmental rearranging gene components during B cell ontogeny. Several different approaches currently are being employed to characterize the evolution of antigen receptor genes including: 1) the comparison of genes in closely related mammalian species, 2) defining relationships between molecules serving different functions but sharing common structural features (and presumably a common evolutionary origin) e.g., members of the immunoglobulin gene superfamily and 3) identifying and characterizing structurally and functionally homologous genes in species that occupy distant and critical positions in the evolutionary radiations of vertebrates. Each of these approaches provides different information about the evolutionary process. The last approach has been pursued by our laboratory for the past several years and the results of our recent studies directed at understanding the nature of limited antibody diversity in lower vertebrates, evolution of immunoglobulin heavy chain genes, structure and organization of light chain genes in a primitive vertebrate and relationships between the genomic organization and function of heavy and light chain genes in lower vertebrates are described.

ANTIBODY GENE DIVERSITY

It is accepted generally that lower vertebrate species exhibit restricted antibody diversity (Du Pasquier 1982). Specifically, the affinity of hapten-specific antibody induced in elasmobranchs (Mäkelä and Litman 1980; Shankey and Clem 1980; Litman et al. 1982) and teleosts (Clem and Small 1970) does not increase, even following prolonged courses of immunization. In addition, fine specificity of antihapten antibody elicited in elasmobranchs is indistinguishable between unrelated individuals (Mäkelä and Litman 1980). In an amphibian, Xenopus laevis (African clawed frog), the spectrotypes of antihapten antibody are not as complex as those found in higher vertebrates and are shared by different individual isogeneic animals (Du Pasquier 1982; Wabl and Du Pasquier 1976; Du Pasquier and Wabl 1978). The functional observations that have been made in the lower vertebrate species may be based on the somewhat insensitive assays

that were employed. Spectrotype analyses that have been interpreted to indicate restrictions in Xenopus antibody can be problematic; dominant charge determinant effects arising elsewhere in the molecule may obscure major differences in the structure of the combining sites. Similarly, the use of fine specificity and/or analyses of antibody idiotypes to characterize induced, oligoclonal antibody populations can be complicated. Molecular analyses of DNA encoded gene repertoires and expressed mRNAs (cDNAs) are more easily interpreted with respect to potential antibody diversity.

The earliest point in vertebrate phylogeny where immunoglobulin gene diversity has been characterized is at the level of the elasmobranchs. We previously have reported that the prototypic immunoglobulin genes in Heterodontus (horned shark) are organized in multiple, ~16kb, clusters (defined as the region between the 5' promoter sequence and the polyadenylation signal sequence 3' to the second transmembrane exon [Hinds and Litman 1986; Kokubu et al. 1988b; Kokubu et al. 1988a]) each containing variable (V_H), diversity (D_{H1}, D_{H2}) and joining (J_H) segmental elements linked to a unique constant region ($V\text{-}D_1\text{-}D_2\text{-}J\text{-}C$). Approximately one half of these genes are joined in the germline of nonlymphoid tissue as VD-J-C or VDJ-C (Kokubu et al. 1988b). Other less frequently occurring patterns consisting of only V and J segments, linked V_H pseudogenes and $VD_1\text{-}D_2J$ joined-clusters are found in Heterodontus and Raja, another elasmobranch (unpublished observations). Efforts to detect expression of these germline-joined genes using oligonucleotide probes specific for joined regions, however, have not been successful thus far (unpublished observations).

Both genomic and cDNA libraries have been screened with J_H- and C_H-specific probes in order to detect unique V_H gene families, similar to the studies carried out with Xenopus (see below). At the DNA sequence level, all shark V_H segments, regardless of their genomic organization pattern ($V\text{-}D_1\text{-}D_2\text{-}J$; VD-J, VDJ, etc.), share greater than 70% identity (typically >90%) and thus are members of a single gene family (Kokubu et al. 1988b). Of the eight Heterodontus J_H segments characterized to date, only one exhibits a single replacement difference within the 3' segment of J_H that typically is retained in rearranged genes. Although diversity in V_H framework (FR) and J_H segments is limited, significant variation in the sequences of complementarity determining region (CDR2) segments and D regions has been observed. D segments potentially can be read in all three reading frames; by comparison of cDNA sequences to consensus D sequences, it appears that all three reading frames are utilized (Kokubu et al. 1988b; unpublished observations). The lengths of D segments that are retained vary and show evidence of N region diversity and somatic mutation (or gene conversion) which may play a role in the generation of antibody diversity in this species. Although V_H family diversity in elasmobranchs appears to be limited, the organization of these genes varies appreciably. If gene rearrangements are exclusively intracluster, then combinatorial diversity would be restricted. Some experimental evidence is consistent, however, with intercluster recombination (unpublished observations). As only a single V_H family appears to be present and both somatic rearrangement and mutation may be limited, antibody diversity in Heterodontus is restricted by comparison to mammals and amphibians (see below). These effects may be compensated, in part, by the multiplicity and complex organization of immunoglobulin V_H gene clusters.

These structural observations can be correlated with some of the limited functional information that is available concerning the hapten-specific immune response in elasmobranchs. Specifically, in

mammals, IgM responses fail to exhibit affinity maturation even though these genes undergo somatic mutation (Chua et al. 1987; Kocks and Rajewsky 1989). The failure of elasmobranch and teleost antibody to undergo affinity maturation may be related to the presence of only a single heavy chain class in these species and/or the inability of primary B cells found in these species to interact with antigen presenting and helper T cells leading to further differentiation, hypermutation and increases in antibody affinity (Kocks and Rajewsky 1989). The antigen-dependent B memory cell lineage that may be responsible for affinity maturation, may not be present in these species. Reduced rates of cell division in poikilotherms may be an additional contributing factor (Du Pasquier 1982).

From an overall immunologic standpoint, Xenopus laevis represents the best characterized model of lower vertebrate adaptive immunity where restricted antibody diversity has been described (Du Pasquier et al. 1989). To assess V_H gene complexity, J_H- and C_H- (IgM [Schwager et al. 1988], IgX [Haire et al. 1989], IgY [Amemiya et al. 1989]) specific probes have been used to select rearranged immunoglobulin genes from an adult spleen cDNA library. Over 190 clones have been characterized using an iterative screening procedure, combined with DNA sequencing. Thus far 11 distinct V_H families have been characterized. These gene families differ at least as extensively from one another as do the corresponding V_H gene families in mammals. In addition, J_H and D_H (defined as the sequence region between V_H and J_H) regions are as diverse as seen in mammalian species. Finally, within a single V_H family, CDR diversity is as complex as that in V_H genes of higher vertebrates (unpublished observations). It is unlikely that the high degree of diversity observed in the V_H families arises solely through a somatic mechanism such as gene conversion, since unique Southern blot hybridization patterns of genomic DNA are associated with each V_H gene family. It is most likely that an extensive and diversified antibody gene family is present in Xenopus, and that the somatic recombination of segmental elements generates junctional as well as template independent D region diversity that are typical of the patterns seen in mammals. Similarly, from other studies, it is assumed that combinatorial diversity is equivalent to that observed in mammals (unpublished observations). Thus the immune response repertoire of Xenopus may be restricted, but neither a paucity of antibody genes nor atypical recombination patterns can explain this effect. Evolution of the mechanism for maturing antibody affinity may not have developed until after the divergence of the Sarcopterygii, whose extant early descendants are the lungfishes and amphibians. It is interesting to note that at least three distinct immunoglobulin isotypes (classes) are found in Xenopus laevis (Amemiya et al. 1989). Whether immunoglobulin genes in species found at this phylogenetic level are fully capable of somatic hypermutation remains open to question.

The mammalian-type organization of immunoglobulin V_H genes is encountered first in primitive teleost species. In Elops (ladyfish), a basal teleost species, as many as six independent V_H segments have been detected in a cloned 15kb chromosomal segment. A single C_H gene and a few J_H segments have been detected. The C_H and J_H are linked closely (<3.6kb). Field inversion gel electrophoresis has been used to establish a V_H-C_H linkage distance of <100 kb. In contrast to the single V_H gene family found in elasmobranchs, Elops possesses at least two V_H gene families, each consisting of multiple members (Amemiya and Litman 1989). Presumably other V_H families could be detected by sequencing additional V_H regions of C_H- or J_H-selected clones.

Whereas, cartilaginous fish (e.g. elasmobranchs) possess large numbers of immunoglobulin gene clusters and only a single (or limited number of) V_H family, bony fishes (e.g. teleosts) and higher vertebrate forms possess extended gene loci consisting of tandemly arrayed V_H, J_H and presumably D_H segments that are linked to a single or relatively few constant regions. It is possible that only the tandem-linear array form of gene organization found in more phylogenetically recent species is associated with full utilization of the combinatorial mechanism for generating antibody diversity. The immunoglobulin V_H genes of species that occupy phylogenetic positions intermediate between the cartilaginous fishes and teleosts, i.e. holosteans and chondrosteans, may be organized differently. Although a vertical phylogenetic relationship is not suggested, the immunoglobulin heavy chain locus of avians appears to be most related to the cluster-type genes found in elasmobranchs (Reynaud et al. 1989; J-C. Weill, personal communication). It is likely that the immunoglobulin heavy and light (Reynaud et al. 1985; Reynaud et al. 1987) chain gene loci in avians represent a highly derived adaptation that probably is unique to this vertebrate class.

EVOLUTION OF ANTIGEN RECEPTORS

Immunoglobulin and TCR are homologous at the nucleotide and predicted amino acid sequence levels (Patten et al. 1984). In addition, these antigen receptors utilize the same mechanism(s) for rearrangement of segmental elements (Alt et al. 1986). Immunoglobulin and TCR genes, however, exhibit major functional differences: 1) antibody but not TCR genes can exhibit affinity maturation, 2) TCR genes do not undergo somatic mutation (Kronenberg et al. 1986), 3) the range of antigens recognized by TCR is more limited, 4) TCR, but not immunoglobulin, function in the context of MHC molecules and 5) major differences in the organization and rearrangement patterns of immunoglobulin and TCR have been described (Davis and Bjorkman 1988). Nevertheless, it is likely that these gene families share a common ancestry and it is possible that a gene complex sharing unique properties with both types of antigen receptor may be present in a contemporary representative of a phylogenetically primitive species. Detection and characterization of such a gene complex would shed light on the structure of the ancestral gene(s) and mechanism(s) of its evolutionary diversification.

Heterodontus immunoglobulin heavy chain genes resemble higher vertebrate immunoglobulin in terms of the organization and sequences of C_H exons and the differential splicing mechanism that regulates the transcription of secretory vs. transmembrane immunoglobulin (Kokubu et al. 1988a). The close linkage of segmental elements and the presence of two D segments within an individual Heterodontus immunoglobulin heavy chain gene cluster, however, are more typical of TCRs (Davis and Bjorkman 1988; Kokubu et al. 1988b). In addition, Heterodontus heavy chain and TCR genes lack the regulatory octamer located upstream of the transcription start site of immunoglobulin heavy and light chain genes in all higher vertebrates (Kokubu et al. 1988b). A sequence motif closely resembling the decamer/nonamer sequence found upstream of the TATA box in mammalian ß TCR (Lee and Davis 1988) is located 5' of the initiation codon and 3' of the TATA box in Heterodontus heavy chain genes (unpublished observations). Thus in this primitive elasmobranch, the structures of the recombining gene segments, and the mechanism regulating cell surface expression vs. secretion of the antigen receptor is similar to that associated with immunoglobulin; however, the organization of the segmental elements and certain

aspects of transcriptional regulation are more typical of TCR. The immunoglobulin heavy chain gene system of Heterodontus may reflect properties of the common ancestral gene that gave rise to the different antigen receptors that are associated with lymphoid cells differentiating along B and T lineages.

Based on these and other observations, it is possible to suggest a probable course of the principal events in the evolutionary acquisition of antibody diversity. The initial step in the process most likely involved introduction of a somatic recombination mechanism leading to a segmented antigen receptor gene (Sakano et al. 1979). The linkage distance between the primordial V and J could have been as little as 300-400 bp, distances that now have been shown to be sufficient for functional rearrangement of both heavy (Kokubu et al. 1988b) and light (Shamblott and Litman 1989b; see below) chain genes in Heterodontus. Junctional diversity, defined as the deletion of terminal nucleotides from the coding segments of the rearranged genes, may have preceded N-type diversity. Introduction of an additional D segment(s) would amplify junctional/N diversity further and introduce a limited form of combinatorial diversity, e.g. use of D_1 vs. D_2. Duplication and limited diversification of the linked clusters would result in a pattern of gene organization resembling that seen in Heterodontus. The loss of chromosomal loci containing most gene clusters or successive non-homologous chromosomal exchanges may have represented the initial step(s) in the development of the modern, tandem linear array form of the immunoglobulin V_H gene locus. Duplication of V_H segmental elements, that are chromosomally separated from J_H (and D_H), would increase the potential for unrestricted combinatorial rearrangement. Antibody diversity would be expanded further by diversification of V_H families as well as by the commitment of a cell lineage to antigen-dependent memory function. The latter role would be associated with somatic hypermutation, a highly effective means for modifying the combining site specificity of an antibody molecule. It remains uncertain whether somatic mutation is quantitatively equivalent in lower and higher vertebrate immune systems. At early stages in the evolution of antibodies, lower levels of somatic mutation may have been a factor in the generation of antibody diversity; however, the overall selective advantage to the immune system may differ markedly from that of hypermutation. Expansion in the number and specialized secondary functions associated with immunoglobulin constant regions presumably represented a final stage in the evolutionary diversification of this form of antigen receptor diversity.

LIGHT CHAIN GENES

Thus far, the entire discussion of immune diversity has been restricted to heavy chain genes as these proved to be most readily identifiable across the wide range of phylogenetically significant species that we have studied. In order to characterize immunoglobulin light chain genes in a phylogenetically distant species, such as Heterodontus, spleen cDNA libraries were screened with antibody to Heterodontus immunoglobulin light chain genes and full copy length cDNAs have been sequenced. The predicted amino acid sequences of the light chain constant regions characterized thus far are related to lambda light chains of higher vertebrates. When the entire nucleotide sequence database is searched, the sequences of light chain genes and mammalian ß TCRs are shown to be most homologous, overall (Shamblott and Litman 1989a). It generally is believed that in vertebrate phylogeny, immunity based on recognition by TCRs preceded antibody-

mediated immunity; however, this has not been established unequivocally. Nevertheless, the extensive regions of shared nucleotide sequence identity between Heterodontus and ß TCRs may define essential features of the putative common ancestor of the contemporary TCR and immunoglobulin gene families. These considerations are extremely important in terms of the identification of immunoglobulin-like molecules in species representing the most distant levels of vertebrate evolution, such as the cyclostomes and perhaps even in protochordates.

A homologous, full copy length light chain cDNA probe was used to screen a Heterodontus genomic DNA library and positive clones were isolated and mapped. Light chain variable (V_L), joining (J_L) and constant (C_L) segments are linked closely (<2.7 kb), however, none of the clones examined thus far show evidence of germline V-J joining and thus differ from heavy chain genes in this primitive vertebrate (Shamblott and Litman 1989b).

Southern blot analyses of Heterodontus genomic DNA digested with various restriction endonucleases reveal complex hybridization patterns with light chain probes. In order to estimate the relative numbers of light and heavy chain genes more precisely, duplicate lifts of a genomic library were screened in parallel with C_L- and C_H-specific probes. At identical, moderate stringency hybridization-wash conditions (Litman et al. 1985b), the C_L probe detects only 20% as many unique clones. While these results suggest that there are fewer light chain genes, a large family of light chain genes that is unrelated to the V_L and C_L gene segments may be present. Linkage between heavy and light chain gene clusters is not evident from field inversion gel electrophoretic analyses (Shamblott and Litman, 1989b).

The complete nucleotide sequences of two genomic light chain genes have been determined. 5' and 3' untranslated segments, intervening sequences separating the leader region, V_L-J_L and J_L-C_L, are related closely. The nucleotide sequences of FR1 are identical as are those of FR3; of the three nucleotide differences that occur in FR2, only one results in a replacement substitution. All nucleotide changes in the CDR, are replacement substitutions. The nucleotide sequences of the predicted coding regions of J_L and C_L are identical (Shamblott and Litman 1989b). Identical recombination signal sequences (RSSs) (including the spacer segments) are located 3' of V_L and 5' of J_L. The 12 nucleotide spacer associated with V_L and 23 nucleotide spacer associated with J_L are characteristic of mammalian k vs. λ light chain genes (Tonegawa 1983). V_L and J_L segments, including their respective RSSs, are separated by only 316 nucleotides.

The identification of multiple light chain genomic clones that contain closely linked V_L, J_L and C_L segments is reminiscent of results obtained with Heterodontus immunoglobulin heavy chain genes (Hinds and Litman 1986; Kokubu et al. 1988b). Although fewer light chain genomic and cDNA clones have been characterized, these appear to be even more related than are the V_H genes that can be classified in a single family (Kokubu et al. 1988b; see above). One interpretation for the remarkably close relationship between the coding as well as noncoding regions of different genes is their recent evolution from a common ancestor. It is equally, if not more, probable that these sequence similarities reflect a process of intense gene correction (conversion) that is acting over entire gene loci (Shamblott and Litman 1989b).

The close linkage of V_L and J_L (<400 nucleotides) is similar to the V_H-D_1, D_1-D_2 and D_2-J_H linkage distances reported previously (Kokubu et

al. 1988b). In mammalian antigen receptor systems, variation in the distances between recombining segmental elements may be a factor in the preferential joining of gene segments (Blackwell and Alt 1988; Chou et al. 1987). The similar intersegmental distances observed in Heterodontus light and heavy chain gene families may reflect their presumed common evolutionary origin and/or may lead to preferred recombination with the most immediately proximal partner, while leaving open the possibility for occasional intercluster rearrangement.

The organization of the light chain genes in Heterodontus more closely resembles that of the heavy chain genes found in this species than immunoglobulin genes found in any other higher vertebrate. A typical immunoglobulin regulatory octamer, previously shown to be absent in Heterodontus immunoglobulin heavy chain genes (Kokubu et al. 1988b), is located upstream of the putative transcription start site in Heterodontus light chain genes. The octamer is found in the immunoglobulin heavy and light chain genes of all higher vertebrate species characterized to date, (Falkner et al. 1986; Parslow et al. 1984; Litman et al. 1985a) including amphibians and teleosts (Amemiya and Litman 1989) and appears to be the chief determinant for B cell specificity of both light and heavy chain promoters (Mizushima-Sugano and Roeder 1986;). Its association with both heavy and light chain genes of all more phylogenetically advanced species suggests that the light chain gene system is more modern. Heterodontus may represent the first example where genes encoding different subunits of a single antigen binding heterodimer are regulated through independent mechanisms.

MOLECULAR COEVOLUTION OF IMMUNOGLOBULIN GENE ORGANIZATION

In Heterodontus, heavy and light chain genes are organized similarly; the short distances between the rearranging segmental elements and V-J-C clustering pattern are essentially identical in both systems and contrast markedly with the corresponding tandem array pattern of recombining segmental elements and extreme distances which characterize mammalian immunoglobulin heavy and k light chain gene loci. The organization of the λ locus in mammals resembles that associated with primitive vertebrate immunoglobulin genes (Hinds and Litman 1986; Kokubu et al. 1988b). In an avian, a single functional light chain gene (Reynaud et al. 1985) is the target of extensive gene conversion that utilizes flanking pseudogenes (Reynaud et al. 1987). The heavy chain gene locus is organized and functions similarly; however, multiple D_H segments also can contribute to the generation of diversity (Reynaud et al. 1989; J-C. Weill, personal communication). Thus three distinct patterns of gene organization and mechanisms of diversification are associated with the immunoglobulin genes found in species belonging to different phylogenetic classes. Within a single species, both heavy and light chain genes are organized similarly. It is unlikely that heavy and light chain genes arose independently at different levels of evolution; rather these findings are consistent with the molecular coevolution of heavy and light chain genes as has been suggested for rDNA and other multigene families (Dover and Flavell 1984). The coevolution of immunoglobulin gene organization may involve unique, phylogenetic class-specific adaptations in the recombinase system or in other factors that regulate the joining of segmental elements. Coevolution appears to be taking place within an environment in which the organization of segmental elements relates directly to the principal selection event, i.e. the generation of antibody diversity.

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Are Autoreactive B Cells Deleted?

D. Nemazee and K. Buerki

INTRODUCTION

Understanding the means by which autoreactive lymphocytes are controlled is an essential basis for understanding the mechanisms underlying autoimmunity. Studies of tolerance in the B cell compartment have come to one of three conclusions: (i) B cell tolerance does not exist to a detectible degree (Karray et al, 1986, Rolink et al, 1987); the absence of autoantigen-specific T cell help presumably prevents autoantibody production, (ii) autoreactive B cells are functionally inactivated by interaction with antigen, but persist in the system and are able to bind antigen (Pike et al,1982, Ventkataraman et al, 1977), (iii) autoreactive B cells are eliminated early in development (Sidman and Unanue, 1975, Metcalf and Klinman, 1977). Recent studies using immunoglobulin transgenic mice expressing light and heavy chain genes encoding antibody specific for certain model autoantigens (Goodnow et al, 1988, Nemazee and Buerki, 1989a) have generated data supporting all of these results, and will hopefully lead to a clearer understanding of the limits to self tolerance.

This talk will focus on our recent data obtained using the Tol1 transgenic mouse line expressing the IgM form of the 3-83 antibody, which binds to H-2K molecules of all independent haplotypes tested

except d and f (Ozato et al,1980). In Tol1 mice that are H-2^d, 25-75% of the B cells bear the 3-83 idiotype (Nemazee and Buerki, 1989a), as detected by the rat monoclonal antibody 54.1, which binds to an epitope formed by the combined light and heavy chains of 3-83 (Nemazee,1987). In contrast, F1 crosses of Tol1 mice with H-2^k mice leads to deletion of idiotype-positive B cells and serum IgM (Nemazee and Buerki, 1989a). Subsequent experiments showed that in H-2^d Tol1--->H-2^d/H-2^a radiation bone marrow chimeras, in which irradiated recipients bear the H-2K^k antigen, deletion of the donor anti-H-2K^k-specific B cells occurred in the bone marrow (Nemazee and Buerki, 1989b). This result demonstrates that the B cells need not express the H-2K^k antigen in order to be deleted, but sheds no light on what might occur should autoreactive B cells see autoantigen at a later, post-bone marrow stage of development. The experiments shown here were designed to approach this question.

RESULTS

Experimental design. Tetraparental (allophenic) mice were constructed by aggregation of day 3 embryos of Tol1 X (B10.D2 X DBA/2) [H-2^d] with A/J [H-2^k] embryos, followed by reimplantation in foster mothers (Buerki,1986). It was expected that some of the resulting mice would be chimeras bearing both anti-H-2^k-specific B cells and the H-2K^k antigen. Since the distribution of tissues derived from each parental type would be expected to be random, in some cases little H-2^k would be expressed in the bone marrow, thus allowing the analysis of B cell tolerance to H-2 antigens found in the periphery.

Analysis of Tol1<-->A/J Chimeras. As shown on Table 1, a total of 37 offspring derived from reimplanted embryo aggregates were analyzed. The coat color difference between the two embryo types permitted an estimate of the extent of chimerism in the offspring; only 26 of the 37 mice were true chimeras, bearing tissue of both parental types. Tails were analyzed for the presence or absence of the Tol1 transgenes. Because the locus containing the transgenes was hemizygous, 50% of

Table 1. Summary of Tol1[H-2d]<-->A/J [H-2a] Chimera Data.

Mouse No.	Tg[a]	%A/J[b]	3-83id[c](μg/ml)	Mouse No.	Tg[a]	%A/J[b]	3-83id[c](μg/ml)
374	-	0	0				
376	-	0	0				
381	-	0	0				
421	-	0	0	375	+	0	>100
424	-	0	0	379	+	0	>100
425	-	0	0	380	+	0	>100
433	-	0	0	422	+	0	>100
435	-	0	0	423	+	0	>100
417	-	1	0	432	+	0	>100
426	-	1	0	438	+	0	>100
378	-	2	0	440	+	1	0.5
430	-	5	0	377	+	10	1.0
444	-	5	0	429	+	10	0.5
431	-	10	0	418	+	20	0
442	-	10	0	428	+	20	0.5
439	-	50	0	443	+	20	0.5
419	-	100	0	427	+	30	0
420	-	100	0	436	+	50	0.5
437	-	100	0	445	+	50	1.0

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a Presence of the transgene in tail DNA as determined by blot hybridization as in Nemazee and Buerki (1989a).

b % A/J tissue estimated from coat color pattern.

c 3-83 idiotype concentration was determined as in Nemazee and Buerki (1989a). "0" indicates undetectible levels (<0.1 μg/ml). Titers are accurate to within a factor of two.

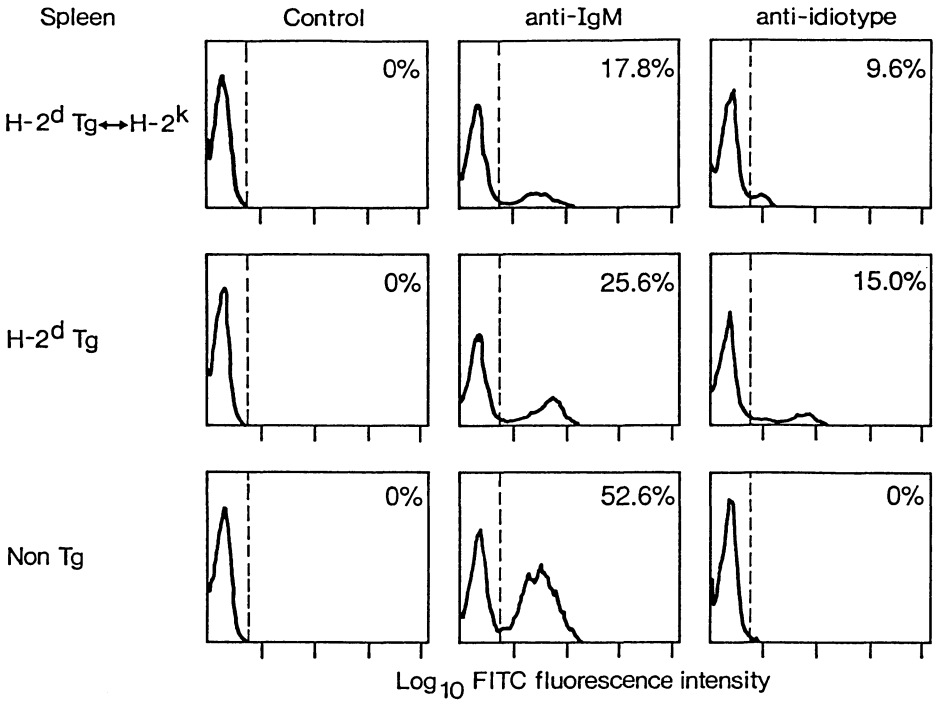

Figure 1. Presence of 3-83 idiotype positive cells in the spleen of Tol1<-->A/J Chimera M377. Mice tested were M377, M379, and M381 in the upper, middle, and lower panels respectively. Results shown were obtained using cells taken by hemisplenectomy at 8 weeks of age. Eight days later the mice were sacrificed and spleen, bone marrow and lymph nodes were tested (not shown). Lymph nodes and spleens taken at the later time point gave similar results to those shown. Immunofluorescence techniques were as in Nemazee and Buerki (1989b).

the mice (excluding the three that were 100% A/J type) were all or part transgenic. That this percentage is in the expected range suggests that there was no selection against transgenic embryos expressing H-2^k antigens in utero.

Transgene-bearing mice that contained A/J tissue had depressed serum levels of the 3-83 idiotype, compared to transgenic mice that lacked A/J tissue. Among the possible explanations for this result are (i) the relative lack of Tol1 type B cells in the chimeras, because of either a low frequency of Tol1 type stem cells or clonal elimination of autoreactive cells, and (ii) the functional inactivation of the anti-H-2^k-specific B cells. To distinguish these possibilities cell surface immunofluorescence analysis was performed. In 5 of 7 Tg Tol1<-->A/J chimeras analyzed, too few Tol1-derived cells were present to assess the fate of the autoreactive anti-H-$2K^k$-specific B cells. However in the remaining two mice, M377 and M440, ~80% of the bone marrow and spleen cells were of the Tol1 type. Surprisingly, high frequencies of 3-83 idiotype-bearing B cells were found in the spleens of these mice, demonstrating the absence of clonal elimination of autoreactive B cells in these mice (Fig 1, Table 2). Importantly, the density of idiotype and IgM on these cells was 3-5 fold lower than in non-chimeric transgenic Tol1 littermate controls (Fig 1, Table 2). The significance of these results is discussed below.

DISCUSSION

The results described here using allophenic mice, suggesting the continued presence of autospecific anti-H-$2K^k$ B cells in the spleen, contrast sharply with the observed clonal deletion of anti-H-$2K^k$ reactive cells in (Tol1 X H-2^k)F1 hybrids (Nemazee and Buerki, 1989a) and Tol1-->H-2^d X H-2^a bone marrow chimeras (Nemazee and Buerki, 1989b), but are reminiscent of the experiments of Goodnow et al (1988) showing clonal anergy of autoreactive anti-lysozyme B cells. In our experiments the same transgenic line (Tol1), with the identical anti-H-2K specificity, was used in both the bone marrow and allophenic

chimeras, yet in one case deletion and in the other anergy of the autoreactive cells was observed. We suggest that the explanation for this difference is that, in the bone marrow chimeras, self-specific B cells encounter antigen at a less mature stage, whereas in the two informative allophenic chimeras (M377 and M440) most anti-H-2K^k B cells encountered antigen at a later stage of development. It is further postulated that B cells encountering antigen at a late stage of development are not eliminated, but functionally inactivated. For this interpretation to be valid it must be assumed that the ca. 20% of H-2K^k-bearing cells in the bone marrows of M377 and M440 either were insufficient in number, or too unevenly distributed, to provide a negative signal to significant numbers of anti-H-2K^k-specific B cells. An interesting alternative hypothesis postulates that a specific "Veto" cell type (Fink et al,1988), which by definition inactivates cells recognizing antigens on the veto cell surface, is required for deletion, and was missing from the H-2K^k-bearing cells of M377 and M440 bone marrow. One would further have to assume in this case that such putative veto cells survived in the 950R-irradiated H-2^d X H-2^a recipients of Tol1 bone marrow, in which anti-H-2K^k B cells were deleted (Nemazee and Buerki, 1989b).

Our evidence suggesting the functional inactivation of autoreactive B cells in the allophenic chimeras is based on two indirect observations: (i) the low titers of 3-83 idiotype in the sera of these mice, which could not be explained quantitatively either by a lack of Tol1-derived B cells or the absorption of idiotype to A/J-derived tissue, and (ii) the low density of surface idiotype and IgM on the surface of splenic B cells, which is similar to what has been seen in anergic B cells in the hen egg lysozyme system (Goodnow et al,1988). Direct functional tests on the low density 3-83 idiotype-positive cells from such chimeras will be required to verify these findings.

Our results hint at the possibility that anergy, rather than deletion, while not the exclusive fate of autoreactive B cells, is the more common one. Recent experiments by Rammensee et al (1989), have

Table 2. Summary of immunofluorescence results with mice M377 and M440.

Mouse No.	% positive* (rel. intensity)@		
	H-2K^k	3-83id	IgM
M 377			
Spleen	N.D.	9.6 (0.23)	17.8 (0.15)
Bone marrow (ungated)	19.1	0.3 (0.40)	2.0 (0.41)
M 440			
Spleen	20.0	7.8 (0.19)	41.0 (0.17)
Bone marrow (gated to exclude myeloid cells)=	19.5	16.8 (N.D.)	28.4 (0.26)

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* Immunofluorescence was performed as in Nemazee and Buerki (1989a).

@ Intensity relative to control non-chimeric H-2^d Tol1 littermate.

= Cells were gated on the basis of 90% light scatter. ~80-90% of viable cells are excluded from analysis by this method.

indicated that auto-specific T cells also can persist in peripheral lymphoid organs. The possible functions of anergic lymphocytes in the physiology of the immune system remain to be explored.

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Self-Tolerance in B-Cells from Different Lines of Lysozyme Double-Transgenic Mice

A. Basten, R.A. Brink, D.Y. Mason, J. Crosbie, and C.C. Goodnow

INTRODUCTION

Self-tolerance like immunity depends on the interaction between antigen and specific membrane receptors on T and B lymphocytes. The question therefore arises as to what factors determine whether this interaction leads to triggering of the cell or its inactivation. Broadly speaking these factors can be divided into two groups consisting of antigen related variables and structural characteristics of the membrane receptors. The latter, in the case of B-cells may include the isotype of membrane immunoglobulin (Ig) which varies depending on the degree of maturation of the B-cells from IgM only to co-expression of IgM and IgD followed by appearance of downstream isotypes such as IgG or IgA. To date, conflicting results have been obtained with respect to the role of IgM and IgD in tolerance induction. On the one hand there are no obvious differences in the signalling properties of IgM and IgD for B-cell activation in vitro or for generation of intracellular second messengers (DeFranco et al. 1987). On the other hand, the selective sensitivity of IgM^+ immature B-cells lacking IgD to tolerance induction in vitro (Sidman and Unaue, 1975; Metcalf et al. 1979; Nossal 1983) has led to the hypothesis that acquisition of membrane IgD confers resistance to tolerance (Vitetta and Uhr, 1977).

In an attempt to define the mechanism of self-tolerance in B-cells more precisely we have developed a transgenic mouse model in which it is possible to examine the antigen and antigen-receptor related variables in a systematic way (Goodnow et al. 1989).

DOUBLE-TRANSGENIC MODEL

The strategy used involved the production of two types of transgenic mice on an inbred C57BL/6 background (Goodnow et al. 1988, Fig. 1). The first parental strain expressed a gene construct encoding hen egg lysozyme under control of either the zinc-inducible mouse metallothionein promoter or the liver specific mouse albumin promoter. The progeny proved to be tolerant in both the T-cell and B-cell compartments irrespective of which construct was used (Goodnow et al. 1988, and Adelstein et al. in preparation).

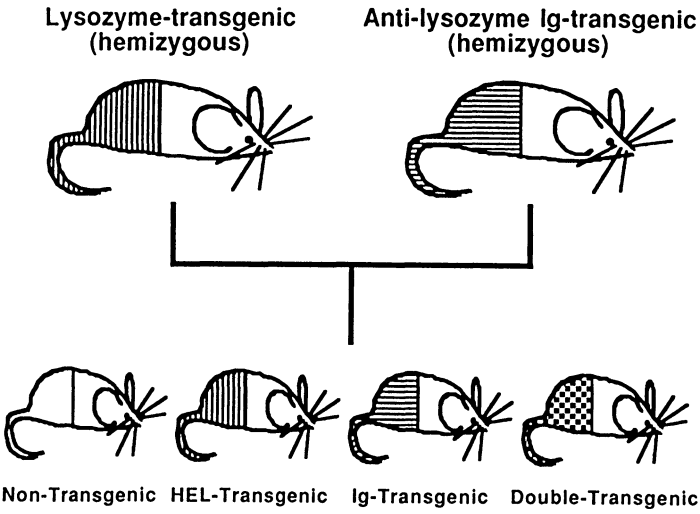

Fig. 1: Schematic diagram of transgenic mouse model of B-cell tolerance. Transgenic mice carrying the gene either for lysozyme or anti-lysozyme antibody were mated to yield 4 possible genotypes which allows each experiment to be internally controlled.

The second parental strain carried rearranged heavy and light chain genes carrying the variable regions of a high affinity anti-lysozyme antibody (HYHEL10) and thus contained very high frequencies of lysozyme specific B-cells in peripheral lymphoid tissue. Since the constant region gene segments were originally obtained from Balb/c genomic DNA (IgH^a allotype), it was possible to distinguish the transgene encoded antibodies from products of the endogenous C57BL6 (IgH^b allotype) heavy chain genes by means of anti-allotypic monoclonal antibodies. In the double-transgenic progeny obtained by mating the two types of parents the lysozyme-specific B-cells encountered lysozyme as a self-antigen, thereby allowing their fate to be followed directly on the FACS by labelling with lysozyme or anti-IgH^a monoclonal antibodies.

Particular attention was paid to the methodology for quantitating transgene encoded anti-lysozyme B-cells to ensure that all of them could be detected particularly in the double-transgenic mice which contained circulating lysozyme capable of binding to the B-cell receptors (Goodnow and Basten 1989). Consequently, two techniques were used for this particular purpose. The first involved incubation of cells with directly biotinylated lysozyme followed by streptavidin-phycoerythrin, while in the second the cells were initially incubated with unlabeled lysozyme followed by a biotinylated anti-lysozyme monoclonal antibody (HYHEL9) which does not compete for binding with the transgene encoded specificity

(HYHEL10). Although the two methods yielded qualitatively similar results in the IgM+IgD Ig-transgenic mice (see below) the sandwich technique resulted in 5-10 fold brighter fluorescence with no increase in background. Moreover B-cell receptor occupany with endogenous lysozyme did not interfere with the sandwich technique which therefore had the advantage of detecting all lysozyme binding B-cells in tolerant double-transgenic mice.

RESULTS

1. IgM+IgD Double-transgenic Mice

The first set of double-transgenic mice were produced by mating lysozyme tolerant mice carrying the lysozyme gene under the control of the metallothionein promoter with Ig-transgenic parents in which the heavy chain transgene included the entire mu-delta constant region locus (Goodnow et al. 1988). The reason for using the complete mu-delta locus was to ensure that B-cell differentiation could take place from expression of IgM alone to co-expression of IgM+IgD. When anti-lysozyme antibody production was measured in the IgM+IgD double-transgenic progeny they were tolerant. However, the anti-lysozyme B-cells were not clonally deleted but rather were functionally altered in the sense that they failed to respond either in vitro to LPS or on adoptive transfer in vivo. Intriguingly, the "silenced" B-cells exhibited 10-20 fold less membrane IgM than those in Ig-transgenic littermates with no change in IgD, a phenotype that was tightly correlated with the tolerant state (Table 1). Furthermore, mature peripheral B-cells could be tolerised as effectively as immature B-cells which see antigen early in development within the bone marrow.

Table 1: Self-tolerance in double-transgenic mice expressing different isotypes

Isotype	Downregulation of membrane Ig.	Tolerance?
IgM+IgD	IgM 10-20 fold IgD no change	Yes
IgM-only	10-20 fold	Yes
IgD-only	3-4 fold	Partial

Immunohistological analysis of the double-transgenic mice and Ig-transgenic controls revealed three key findings: (a) The overall lymphoid architecture was normal, and the differential regulation of transgene-encoded IgM and IgD during B-cell development occurred normally. (b) Lysozyme-binding B-cells were present in the splenic marginal zone and in the follicular mantle zone of Ig-transgenic mice, but were absent from the marginal zone of double-transgenics. (c) Lysozyme-binding B-cells in the double-transgenic mice were located in the follicular mantle zone and expressed high levels of IgD but low IgM. One plausible interpretation of those data would be that self-tolerance may have prevented B-cells in the mantle zone from maturing into IgM^{hi}, IgD^{lo} marginal zone B-cells.

2. IgM Only Double-transgenic Mice

Since IgM but not IgD was downregulated in tolerant IgM+IgD double-transgenic mice, it was possible that the two isotypes could function differently in response to tolerogenic signals. The next step therefore was to determine what happens if IgM or IgD were expressed separately on anti-lysozyme B-cells. Initially, IgM only double-transgenic mice were produced by mating the same lysozyme transgenic parent with Ig-transgenic mice in which the heavy chain gene construct was truncated at the EcoR1 site 3' to C-mu, thus deleting the C-delta exons. In this case the number of high affinity lysozyme-binding B-cells was greatly reduced (approx. 70% reduction relative to the Ig-transgenic littermates), and the density of IgM on the residual lysozyme-binding B-cells was downregulated by 10-20 fold, ie to a similar degree to that observed in the IgM+IgD double-transgenic combination (Table 1). Nevertheless, significant numbers of lysozyme-binding B-cells were still detectable in spleen and lymph node, provided the sensitive sandwich technique described previously was used. Assays employing binding of directly biotinylated lysozyme which is analogous in this context to directly labelled anti-idiotypic antibodies failed to detect these cells, presumably due to blocking of receptors with autologous lysozyme as well as to the greatly reduced receptor density resulting from IgM downregulation.

Taken together these findings indicate that B-cells expressing self-reactive receptors only of the IgM isotype are efficiently silenced in a manner qualitatively identical to silencing of IgM+IgD B-cells. However, the greater magnitude of the decrease in lysozyme binding B-cells observed in tolerant IgM compared with IgM+IgD transgenic mice may point to a specialised function for IgD receptors in allowing persistence of tolerant B-cells. Alternatively, it may simply indicate that a threshold receptor density is required for persistence of follicular mantle zone B-cells and that when IgD is absent tolerance-associated downregulation of IgM results in an insufficient antigen receptor density for the purpose.

3. IgD Only Double-transgenic Mice

In an attempt to distinguish between the above alternatives and to determine whether IgD in the absence of IgM could also transmit a tolerogenic signal to the B-cell, a third set of Ig-transgenic mice was created. For this purpose a construct was prepared in which the heavy chain carried a 12.5 kb internal deletion removing the C-mu exons and bringing the C-delta exons into an equivalent position 3' to the VDJ segments. In the IgD-only mice, IgD was constitutively expressed as the sole antigen receptor on the majority of B-cells from early in B-cell differentiation, and was secreted in place of IgM at the plasma cell stage, resulting in high levels of lysozyme-binding IgD in the serum. When double-transgenic mice were produced by mating with lysozyme transgenic parents, tolerance was 'leaky' and the IgD-only B-cells were silenced to a lesser degree than occurred with B-cells expressing IgM+IgD or IgM alone. Not surprisingly, lysozyme-binding B-cells persisted in IgD-only double-transgenic mice. Moreover, the membrane IgD on these B-cells was only downregulated 3-4 fold compared with the 10-20 reduction in IgM on B-cells expressing both IgM and IgD (Table 1). In other words IgD appears to be capable of mediating the signals involved in B-cell tolerance, albeit much less efficiently than IgM. This difference between membrane IgM and IgD did not appear to be due to variations in affinity, and awaits further elucidation as does the reason why IgD is not downregulated when it is co-expressed on the B-cell membrane with IgM.

DISCUSSION AND CONCLUSIONS

The advantage of the transgenic approach to studying self-tolerance is that the fate of self-reactive lymphocytes can be visualized directly and the many antigen and cellular variables can be analysed systematically in vivo by altering the microinjected constructs. In the current paper the effects of modifying the immunoglobulin construct to generate different isotypes on the B-cell membrane are reported.

In the case of the IgM+IgD double-transgenic mice mature peripheral B-cells were shown to be silenced as effectively as immature B-cells by a non-deletional mechanism, the silenced state being tightly correlated with selective downregulation of membrane IgM with no change in IgD (Goodnow et al; submitted). The susceptibility of IgD^+ mature B-cells to clonal silencing in this model is entirely consistent with earlier reports of unresponsiveness in mature and memory B-cells following in vivo administration of haptens, serum proteins and polysaccharides to conventional mice (Katz et al. 1972; Howard and Mitchison 1975; Metcalf et al. 1979; Parks et al. 1982; Nossal 1983). Furthermore, downregulation of membrane IgM without deletion has been observed previously in vivo in mice given anti-IgM monoclonal antibody from birth (Gause et al. 1987). The particular advantage of the current model is that it provides a mechanism of tolerance which is capable

of silencing self-reactive B-cells at multiple stages of development and can combat the "second wave" of potentially self-reactive B-cells resulting from hypermutation of Ig-genes in mature B-cells (Diamond and Scharff, 1984; Berek and Milstein 1988).

The same tight correlation between the degree of tolerance and the extent of downregulation of membrane Ig was observed in double-transgenic mice containing IgM or IgD only expressing B-cells (Table 1). Thus, even in the partially silenced IgD only double-transgenic mice the level of IgD was reduced 3-4 fold, thereby confirming that functional silencing and downregulation of membrane Ig are intimately related. This association between the two phenomena raises the issues of the relative importance of membrane IgM and IgD in transmitting tolerogenic signals to B-cells and the extent of the 'crosstalk' between them during the silencing process. As mentioned previously, in the IgM+IgD double-transgenic combination, IgM levels were reduced by 20 fold, whereas IgD was apparently unaffected. On the other hand when IgD was not coexpressed with IgM on the B-cell membrane, it did undergo some degree of downregulation and did transmit a negative signal, albeit less efficiently than IgM. In other words, the presence of IgM modifies IgD expression in some way indicating that there may be functional differences between IgM and IgD receptors, but these differences are likely to involve complex interactions between the two receptors and associated signal transduction molecules.

Recently, Nemazee and Burki (1989) have described a different transgenic model of self-tolerance in the B-cell repertoire in which silencing of self-reactive B-cells, although associated with downregulation of membrane IgM appeared to involve clonal deletion rather than functional silencing. The contrasting findings obtained in the two models are intriguing particularly since in the lysozyme system some residual lysozyme binding B-cells were detected in the tolerant IgM only double-transgenic mice. Although the difference could be explicable on technical grounds (see above), the more interesting possibility is that different mechanisms of tolerance are invoked depending on the nature of the self-antigen. In the H-2^k model, the self-antigen is an integral membrane protein expressed at relatively high density by almost all cells in the body. As such, H-2^k/D^k may represent a good model for tolerance mechanisms which deal with B-cells with extremely high avidity for self-antigens. By contrast, lysozyme is a predominantly monomeric soluble antigen and consequently may be much less efficient at crosslinking membrane immunoglobulin, suggesting that the lysozyme model may reflect tolerance mechanisms operating on lower avidity self-reactive B-cells (Goodnow et al. submitted). There may be good physiological reasons for deleting very high avidity self-reactive B-cells, while allowing lower avidity cells to persist in an anergic state. Testing this possibility should be straightforward in both systems, either by converting lysozyme into an integral membrane antigen or by introducing a truncated H-2^k transgene encoding a secreted H-2^k (Arnold et al. 1988).

Acknowledgements

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The Recombination Activating Gene and Lymphoid Differentiation

D. Baltimore, M. Oettinger, and D. Schatz

Lymphoid cell differentiation is like the differentiation of any other cell in the body: a series of events occur that turn an undifferentiated cell into one that carries out a range of specific functions. The most characteristic proteins of lymphoid cells are immunoglobulin (Ig) and the T cell receptor (TCR). These products are formed through a differention event apparently unique to the lymphoid cell series: DNA rearrangement.

Our laboratory is concerned primarily with understanding the particular set of intracellular events that mediate lymphoid differentiation. We would like to know what proteins are involved in controlling the differentiation, how the precise order is determined, and, particularly, how the fascinating process of gene rearrangement that underlies diversity is accomplished.

In this paper, we briefly outline our progress in identifying a gene that is central to the rearrangement process and we fit the expression pattern of that gene into the overall pathway of lymphoid cellular maturation.

The lymphoid gene rearrangement process is a unique event of DNA recombination -- there is no other known recombinational system with all of its peculiar properties. These properties include the site specificity of the recombination, the asymmetric behavior at the coding and signal joints, and the loss and addition of bases at the coding joints. All Ig and TCR gene rearrangements involve the same biochemical and recognition events suggesting that they utilize a common protein (or common proteins) which we will call the recombinase.

To clone the recombinase gene, we established a genomic DNA transfer system that allows for selection of cells that have received a gene that activates recombination (Schatz and Baltimore 1988). Because we do not know just what gene is involved, we call the gene RAG-1 for Recombination Activating Gene. It activates the recombination of V and J segments of an artificial DNA construct implanted in the DNA of a fibroblastic (3T3) cell (Schatz and Baltimore, 1988).

To clone RAG-1, we tagged total genomic DNA with a synthetic DNA oligomer and did serial genomic DNA transfers to identify the fragment containing RAG-1. We cloned a 7.5kb oligomer-marked DNA fragment that consistently tracked with the recombinational activity. We then walked from the marked DNA fragment to the RAG-1

Figure 1

Presence of RAG-1 mRNA in various cell lines and tissues

Positive Lines	Negative Lines
Pre-B (22D6, 38B9, 1-8, 2M3, Nalm 6)	Pre-B/B Transition (70Z/3)
Scid Pre-B	B Cell (WEHI 231, Jurkat, Namalwa, BJAB)
Pre-T (2017)	Myeloma (MPC-11)
	T Cell (RLM-11)
	Erythroid (MEL)
	Macrophage (P388D1)
	3T3
	L cell
	HeLa
	PC12

Positive Tissues	Negative Tissues
Thymus (very high levels)	Spleen
	Brain

Figure 2

Properties of RAG-1

___ Gene encodes a 6.5 kb mRNA making a protein of 1040 (mouse) or 1043 (human) amino acids

___ Protein sequence is ~95% identical between mouse and man

___ No known transcription factor similarities

___ Gene is located on chromosome 11 in man and probably on 2 in the mouse--not coincident with the SCID locus in mouse

___ Gene is detectably expressed only in pre B and pre T cells, not in mature lymphoid cells or in whole brain

___ Both cloned cDNA and genomic DNA will activate V(D)J recombination in 3T3 cells

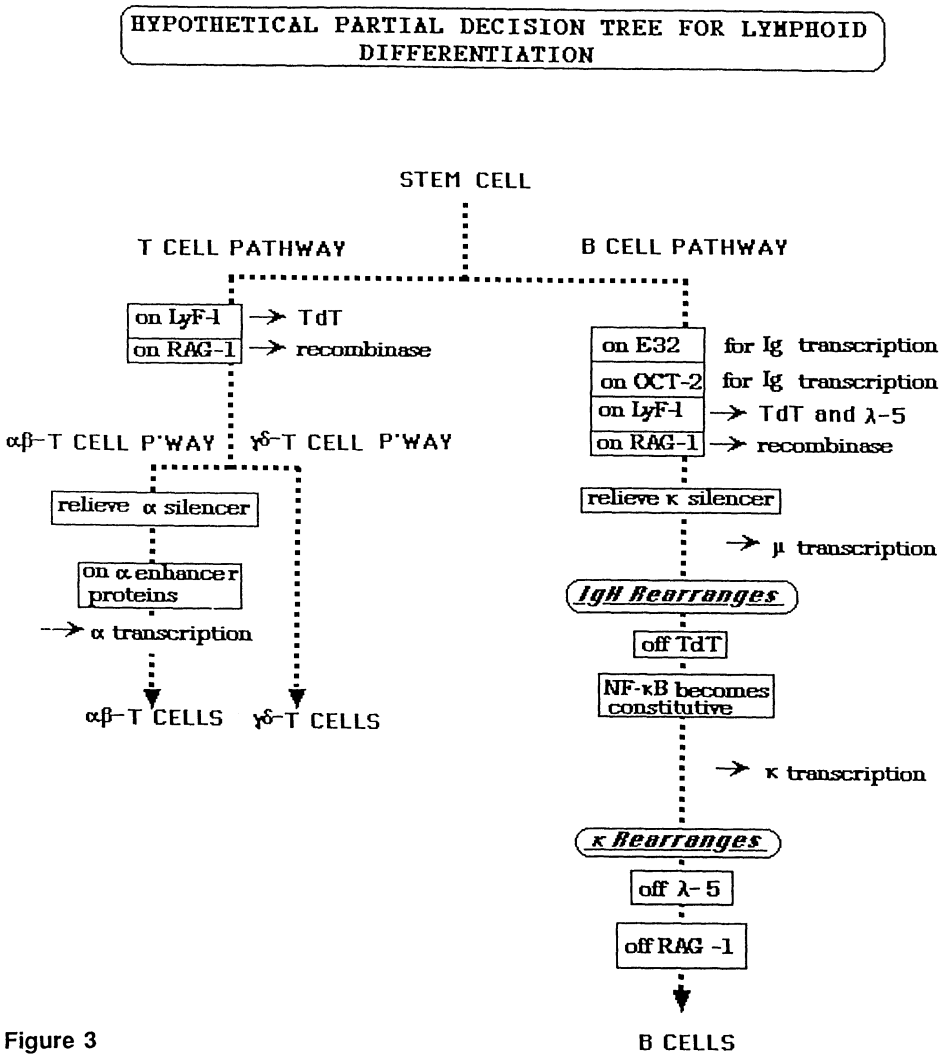
HYPOTHETICAL PARTIAL DECISION TREE FOR LYMPHOID DIFFERENTIATION
STEM CELL
T CELL PATHWAY
B CELL PATHWAY
on LyF-1 → TdT
on RAG-1 → recombinase
αβ-T CELL P'WAY
γδ-T CELL P'WAY
relieve α silencer
on α enhancer proteins
→ α transcription
αβ-T CELLS
γδ-T CELLS
on E32 for Ig transcription
on OCT-2 for Ig transcription
on LyF-1 → TdT and λ-5
on RAG-1 → recombinase
relieve κ silencer
→ μ transcription
IgH Rearranges
off TdT
NF-κB becomes constitutive
→ κ transcription
κ Rearranges
off λ-5
off RAG -1
B CELLS

Figure 3

gene, a distance of some 50kb. We knew that we had reached the vicinity of RAG-1 when the cloned DNA hybridized to new bands in all cells in which recombinational activity had been activated by DNA transfer. We then isolated both human and mouse cDNA clones and sequenced them.

The expression of RAG-1 was checked in a variety of cell lines and tissues (Fig. 1). It was evident that RAG-1 expression is exactly coincident with the recombinational function: all cells and tissues that actively rearrange either Ig or TCR genes express RAG-1. Conversely, no cells -- including mature B and T cells -- that do not actively rearrange Ig or TCR genes have detectable RAG-1 expression using the Northern blot procedure.

The known properties of the RAG-1 gene are summarized in Fig. 2. It is so highly conserved between mouse and human that it is not possible to delineate functionally critical regions. It is not related to any known protein or protein region closely enough to have been picked up in a computer comparison with the data base.

Is RAG-1 the recombinase itself or rather a gene that activates the synthesis of the recombinase? We cannot be sure of the answer at present. If it is an activator then it is likely to be a surface receptor, a transcription factor or a kinase. From its sequence, it is not overtly one of these. Therefore, we tentatively favor the idea that it is the recombinase itself. It is localized to the expected cells and is a large, highly conserved protein that could be imagined to carry out some or all of the multiple reactions involved in recombination. Although it does not map to the SCID locus, the SCID protein apparently is not restricted in its action to lymphoid cells (R. Phillips, personal communication).

The RAG-1 product is just one of many proteins involved in lymphoid cell development. We know only a fraction of these. Each must be controlled in its expression by other proteins, generating a huge number of proteins that appear in ordered succession and allow stem cells to become lymphocytes. Figure 3 shows a flow sheet incorporating many of the factors now known to play a role in lymphoid differentiation. There are presumbly many others; we probably now know only a few percent of the proteins that act along this one developmental pathway, though it is perhaps as well understood as any.

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Repertoire Selection in B Cell Subpopulations

I. Förster, H. Gu, and K. Rajewsky

INTRODUCTION

In this paper we discuss two different aspects of repertoire selection in the B cell compartment.

The first part deals with B cell development and addresses the question whether the V gene repertoire expressed in mature, peripheral B cells is different from that expressed in early B cells. Differences in the utilization of particular V_H gene families have been described comparing B cells from neonatal with those of adult mice (reviewed in Alt et al., 1987) and also pre B and B cells in the bone marrow with splenic B cells (Malynn et al., 1987; A. Freitas, personal communication). In these studies an overrepresentation of the most D proximal V_H gene family, 7183, over V_H gene families located more 5' on the V_H gene locus was seen among V_H genes expressed in neonatal and bone marrow B cells. Using amplified cDNA libraries we show that a similar shift in V_H gene utilization can be observed comparing newly generated δ^- with long-lived δ^+ B cells in adult mice.

In the second part we concentrate on repertoire selection within the Ly1 B cell subpopulation. One of the main characteristics of Ly1 B cells is that these cells appear to be generated during the first weeks of life and are then propagated as mature B cells over the lifetime of the animal (Herzenberg et al., 1986; Rajewsky et al., 1987; Hayakawa and Hardy, 1988). Eventually, the long-term propagation of Ly1 B cells leads to the development of chronic B cell leukemias (B-CLL). With respect to these unique growth properties and to earlier findings that the antibody repertoire of Ly1 B cells seems to be restricted (reviewed in Hayakawa and Hardy, 1988) we studied the V gene repertoire of Ly1 B cells during different stages of ontogeny. We show that Ly1 B cells of neonatal as well as aged mice express a similar, selected set of germline V region genes.

SELECTION OF NEWLY GENERATED B CELLS INTO THE STABLE, PERIPHERAL B CELL POOL

The bone marrow of mice contains two populations of B cells: $B220^{dull}\mu^+\delta^-$ and $B220^{bright}\mu^+\delta^+$ cells. By measuring the lifespans of these B cell subsets we found that most of the δ^- B cells in the bone marrow have a rapid turn-over whereas most of the δ^+ B cells are stable, long-lived cells (Förster et al., 1989). In Fig. 1 a

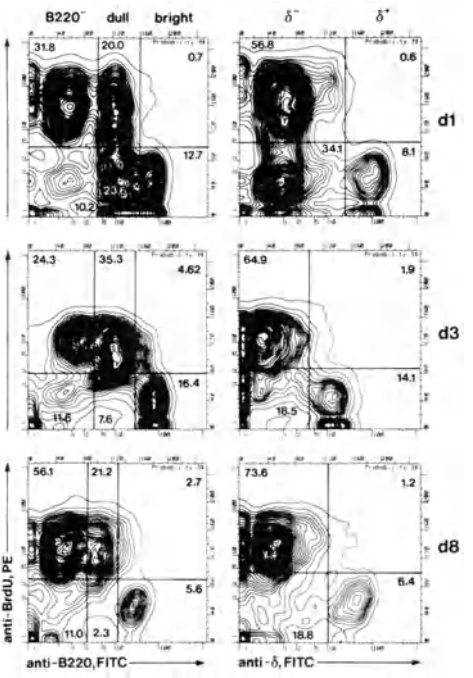

Figure 1: Flow cytometric analysis of bone marrow cells from adult BALB/c mice which were fed with BrdU for either 1, 3 or 8 days. Staining was done with anti-BrdU (PE) and anti-B220FITC or goat-anti-δFITC, respectively. Percentages of cells within the different fluorescence windows are indicated and refer to cells in the "lymphocyte-gate" (taken from Förster et al., 1989, with modifications).

flow cytometric analysis of bone marrow cells from BALB/c mice which were fed with the thymidine analogue 5-bromo-2'-deoxyuridine (BrdU) in their drinking water for 1, 3 or 8 days is shown. The cells were first stained on the surface with antibodies directed against B220 or δ, then fixed and, after denaturation of the DNA, stained with anti-BrdU (see Förster et al., 1989). While B220$^-$ and B220^{dull} cells are labeled with BrdU within 1-3 days, most of the B220$^{bright}\delta^+$ B cells remain unlabeled even after a labeling period of 8 days. These data indicate that most $\mu^+\delta^-$ B cells (which are included in the B220^{dull} population (Förster et al., 1989)) do not mature into δ^+ B cells in situ. Probably, these cells leave the bone marrow and either die within a short time or are selected into the pool of long-lived δ^+ B cells which may then reenter the bone marrow.

On the basis of these findings we were interested to see whether the V gene repertoire expressed in newly generated $\mu^+\delta^-$ B cells is different from that expressed in long-lived $\mu^+\delta^+$ cells. For this purpose, we analyzed the representation of V_H genes belonging to the 7183 or J558 V_H gene families in δ^- and δ^+ bone marrow B cells. $\mu^+\delta^-$ and $\mu^+\delta^+$ B cells were isolated from the bone marrow of normal adult BALB/c mice by fluorescence-activated cell sorting (FACS). cDNA was prepared from total cellular RNA of the sorted cells using a cμ-specific primer, tailed with poly-dG and then amplified with cμ- and poly-dC-primers using the polymerase chain reaction (see Rajewsky et al., 1989). As determined by cytoplasmic staining, the contamination of plasma cells within the sorted cell populations was less than 1 in 2-5x10^4. The amplified cDNA was used to construct cDNA libraries which were then hybridized with 7183 or J558 specific probes (Table 1). We found that $\mu^+\delta^-$ bone marrow B cells show a similar overrepresentation of 7183 V_H genes as has been described for neonatal and pre B cells. In contrast, a J558/7183 ratio typical of peripheral B cells of BALB/c mice (2.5) can be seen in the δ^+ B

cell population. This repertoire shift indicates that a selective step is involved in the transition from newly generated δ^- to stable δ^+ B cells.

Table 1: Representation of J558 and 7183 V_H genes in δ^+ and δ^- bone marrow B cells.

	No. of positive clones [b]		
	V186.2 (J558)	VH81X (7183)	J558/7183
$\mu^+\delta^-$	24	22	1.1
$\mu^+\delta^+$	30	12	2.5

[a] $\mu^+\delta^-$ and $\mu^+\delta^+$ cells were sorted from the bone marrow of 14-week-old BALB/c mice

[b] Amplified cDNA libraries were prepared from the RNA of $\approx 1x10^5$ cells. From each library a total of 110 clones were hybridized with the V186.2 or VH81X probe. Some of the clones might have contained only DJ rearrangements.

REPERTOIRE SELECTION IN THE LY1 B CELL SUBPOPULATION

In a first series of experiments we had analyzed the V gene repertoire of Ly1 B cells from aged mice. These cells originated from 6-10-month-old CB.20 mice and had been propagated in allotype-congenic BALB/c mice over a period of 9 months (Förster et al., 1988). The V region genes expressed in 17 hybridomas which were derived from Ly1 B cells of two different mice were identified by direct mRNA sequencing. The result of the sequencing analysis demonstrated that the Ly1 B cells i) had undergone substantial clonal expansion and ii) expressed a highly selected set of germline V_H and V_κ genes . In addition, the Ly1 B cell lymphoma B16.2 isolated in our lab expressed the same V_H gene (belonging to the J558 V_H gene family) as 3 out of 12 clonally independent Ly1 B cell hybridomas analyzed (Förster et al., 1988).

At about the same time when we sequenced the V region genes expressed in long-term selected Ly1 B cells, Haughton's group analyzed the V genes expressed in 10 Ly1 B cell lymphomas belonging to the CH-series (Pennell et al., 1988). A comparison of their and our result is depicted in Table 2. Remarkably, 6 out of 10 CH-lymphomas expressed identical V_H and/or V_κ genes as 8 out of 12 independent Ly1 B cell hybridomas and one Ly1 B cell lymphoma isolated in our lab. This finding indicated that the Ly1 B lymphoma cells had been selected at some stage according to the same principles as the "normal" Ly1 B cells isolated from aged mice.

We were then interested to see whether the predominance of particular V genes within the Ly1 B cell population could already be observed early in ontogeny. For this purpose amplified cDNA libraries were constructed from Ly1 B cells isolated by FACS from the peritoneum or spleen of 4-week- or 4-day-old mice, respectively, using the same method as described above. In this case we focussed on the analysis of cDNA clones containing V_H genes of the J558 V_H gene family as identified by hybridization with a J558-specific probe. A comparison of V_H genes identified in the cDNA libraries and

Table 2: Comparison of Ly1 B cell lymphomas and hybridomas expressing identical V region genes

CH-lymphomas (Pennell et al., 1988)	Ly1 B cell hybridomas/lymphoma[a] (Förster et al., 1988)	sequence identity[b]
CH12	3B9PC/5D6S	V_H
CH27	1B1S	V_κ
CH32		(1 nucl.diff.)
	3B11PC/4A9S/4F5S/6C7S	V_H, J_H, V_κ, J_κ
CH10	1A1PC	V_κ, J_κ
CH31	3D11PC	V_κ, J_κ
	12F10S	V_κ
	7E9S	V_κ
CH34	lymphoma B16.2	V_κ, J_κ (1 nucl.diff.)

[a] Hybridomas separated by "/" were shown to be clonally related
[b] Sequence variations at the VD and DJ borders have not been taken into account

those expressed in the Ly1 B cell lines described above is schematically shown in Fig. 2. Surprisingly, 7 out of 11 cDNA clones from 4-week-old mice were either very similar (more than 95% homologous) or identical to V_H sequences of the original sequence collection. Two other sequences ("G") were identical to each other, joined to the same D and J_H elements, but differed in N-sequences. Even more striking is the finding that 2 out of 6 cDNA clones analyzed from 4-day-old mice contained V_H genes which had been found expressed in Ly1 B cell hybridomas as well as Ly1 B cell lymphomas ("E" in CH10 and CH31; "C" in B16.2). In addition, the V_H gene found in another cDNA clone out of this set differed in only 3 nucleotides from the V_H gene designated "E". Looking at cDNA libraries from Ly1-negative B cells of 4-week- and 4-day-old mice we found that some of the V_H genes predominantly expressed in Ly1 B cells ("C", and genes similar to "B"; see Figure 2) are also overrepresented in Ly1-negative B cells (data not shown). The V_H genes designated "E" and "F" have so far only been identified in Ly1 B cells. Another V_H gene which is found selectively expressed in Ly1 B cells is VCP12 which is often rearranged in B cells specific for bromelain-treated mouse red blood cells (Reininger et al., 1988). However, the overrepresentation of this gene is only seen in mice more than 2-4 weeks of age (Andrade et al., 1989; Pennell et al., 1989).

The above data demonstrate that the predominance of particular V genes in the Ly1 B cell population can be seen as early as day 4 of life. Conservation of CDR3 regions in several of the cDNA clones containing the same V_H genes suggests that antigenic selection is at least in part responsible for the selective V gene expression (data not shown). However, it can not be excluded that preferential V gene rearrangement is also involved in this process.

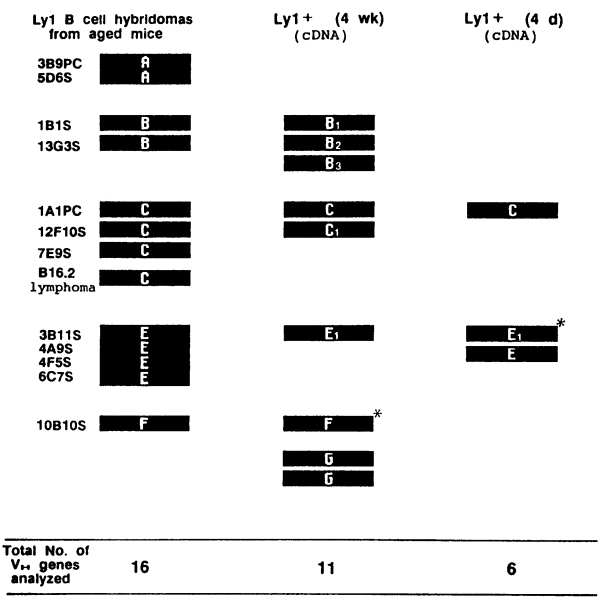

Figure 2: Comparison of J558 V_H genes expressed in long-term selected Ly1 B cells and those expressed in Ly1 B cells of 4-week- and 4-day-old mice. The nos. on the bottom indicate the total no. of cell lines/cDNA clones analyzed in each group. The different characters represent particular V_H genes out of the J558 family. B_{1-3}, C_1 and E_1 stand for V_H genes which are more than 95% homologous to B, C or E, respectively, but probably represent different germline genes rather than somatic mutants ("E_1" is identical to the germline gene V_3 (see Förster et al., 1988) and differs from "E" in 4 nucleotides). In the cDNA clones marked with "*" we found a single nucleotide difference to "E_1" or "F", respectively.

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II Cell Developments

Memory

Evolutionary and Somatic Immunological Memory

K. Rajewsky

INTRODUCTION

In this introduction to the symposium on immunological memory I give an outline of the topic as I see it and as it is further developed in the contributions of John Kearney, Leonore Herzenberg, Martin Weigert, Norman Klinman, Peter Beverley, Kyoko Hayakawa and Ian MacLennan. A few old and new facts are reviewed and unresolved problems pointed out. I discuss some work from my own laboratory in this context. With respect to the literature I refer the reader in most instances to the other contributions and give only a few references here.

The term immunological memory describes the fact that the immune system is able to react with increased efficiency to antigens encountered in the past. Depending on whether antigenic experience has occured in the history of the species or of an individual, one can distinguish two forms of immunological memory which I designate evolutionary and somatic memory. Evolutionary memory predisposes the individual to efficient defense *before* contact with an antigen (e.g. a common environmental pathogen) and thus falls into the category of "natural" immunity. In contrast, somatic memory develops as a consequence of antigenic contact during lifetime and thus reflects "acquired" immunity.

EVOLUTIONARY IMMUNOLOGICAL MEMORY

There are indications that the immune system has evolved a special regulatory mechanism which establishes predominant expression of antibodies with specificity against common environmental pathogens before contact of the individual with those pathogens. J.F. Kearney and his collaborators (this volume) suggest on the basis of their experimental evidence that this mechanism is based on idiotypic interactions between B lymphocytes. Here the hypothesis is that a germ line encoded idiotypic network (Jerne 1974) has evolved which is self-stimulatory and initiates the expansion of cells expressing a restricted repertoire of antibody specificities involved in natural defense.

This repertoire selection may largely occur in a special compartment of the immune system, namely that of Ly1 B cells. These cells may represent a distinct B cell lineage and are described in more detail by Leonore Herzenberg (this volume) whose laboratory has established many of the main features of the Ly1 B compartment in the mouse.

Various properties of Ly1 B cells make them good candidates as carriers of "natural" immunity as defined above: Despite being a minor B cell subset, Ly1 B cells produce a large fraction of serum antibodies and appear to express a selected set of antibody specificities which include specificities for certain bacterial as well as self antigens. A system of natural defense should be targeted at structures which pathogens are unable to change. Such structures would include epitopes which microbes have evolved in the course of their adaptation to the host. This would prevent escape of the pathogens from immune defense through mutation, a necessary condition for germ line encoded natural immunity. Epitopes of that nature would often reflect microbial mimicry of structural elements of the host. Natural defense may thus necessarily involve self-reactivity, and the latter may then in turn be used to drive the system (Kocks and Rajewsky 1989). Idiotypic control of natural immunity would represent a special case of this general principle.

A molecular analysis of antibody V regions expressed in the Ly1 B compartment, carried out in my laboratory (Förster et al. 1988; Rajewsky et al. 1989; I.Förster, H. Gu and K.R., this volume) revealed what would be expected for an evolutionarily selected, restricted repertoire: predominant expression of certain V genes and V gene combinations from very early in ontogeny; marked clonal expansion; and, at least in a pre-immune situation, virtual absence of somatic antibody mutants.

Thus, an attractive hypothesis has emerged of how evolutionary immunological memory is expressed in ontogeny as a (germ line encoded) mechanism of natural defense. This hypothesis awaits its functional test.

SOMATIC IMMUNOLOGICAL MEMORY

Concepts

Somatic immunological memory is the key feature of acquired immunity in response to antigens encountered in ontogeny. It typically develops in the T cell compartment and in T cell dependent antibody responses where it has been extensively studied at both the T and particularly the B cell level. Secondary antibody responses, a read-out of somatic memory, tend to be larger than primary responses, and the antibodies produced have a higher average affinity for the antigen - a phenomenon called affinity maturation.

What is the cellular and molecular basis of the memory state? Various concepts have been put forward:

1) Expansion of pre-existing, antigen-specific cells. This possibility, exemplified for B cells in the upper part of Fig. 1, relates to the classical clonal selection theory (Burnet 1959). There is no question that cell proliferation is involved in the generation of B cell memory; however, as I shall discuss below, the expansion of the pre-existing antigen-specific cells is accompanied by profound changes in their receptor repertoire and other properties of the cells, which leads us to the next point.
2) Generation of memory cells, distinct from naive cells. This notion is supported by strong experimental evidence and is a

main subject of this symposium (see also below).

3) Changed equilibrium of the cellular system. Rather than ascribing memory to certain sets of cells or special cell types, the network hypothesis (Jerne 1974) interprets memory as a change in the equilibrium of interacting cells. Here, the essential feature is not the proliferation or differentiation of particular cell clones, but rather changes in the frequencies of receptor specificities in a network of interacting receptors. Memory of this kind could rely, like memory in our minds, on the preservation of an "internal image" of the external world - antibody or T cell receptor variable regions mimicking epitopes of the antigen.

Although various forms of network control may well contribute to the generation and propagation of somatic immunological memory in both the T and the B cell compartment (see Eichmann et al. 1983; Colle et al. 1988), information on this point is indirect and fragmentary. Therefore, the present dicussion focusses on the generation, selection and properties of memory cells.

Distinctive Properties of Memory Cells

The literature ascribes a variety of distinctive properties to memory cells:

i) Distinct phenotype in terms of surface markers, which may correlate with distinct activation requirements, homing patterns etc.. These issues are discussed in this symposium by P. Beverley, K. Hayakawa, L. Herzenberg and N. Klinman for T and B cells. In the case of memory B cells (see also Yefenov et al. 1986) differences in activation requirements as compared to naive B cells (e.g. increased sensitivity to antigenic triggering) could partly or largely reflect changes of the receptor antibody itself, since memory cell maturation is accompanied by class switching and selection of high affinity somatic mutants (see ii). This important point needs further investigation at the level of molecular biology.

ii) Distinct receptor repertoire, generated by somatic hypermutation. This point, also discussed by M. Weigert and N. Klinman in this symposium, applies to B, not T memory cells and is put into the context of affinity maturation further below.

iii) Longevity. Somatic immunological memory can be astonishingly long-lived. An impressive example is the measles epidemic on the Faroe islands in 1846. Almost the entire population of the islands suffered from the infection, except a few old people who were the only ones infected by the measles virus earlier - 65 years before (Panum 1847; quoted by Jerne 1966). Can this long persistence of memory be ascribed to long-lived memory *cells*? While there is clear evidence that *clones* of memory B cells can persist in vivo over long periods of time (Askonas and Williamson 1972; Berek et al. 1987), the issue of the long-lived, resting memory cell (Gowans and Uhr 1966; Strober 1972) is still controversial. Upon transfer of memory cells into syngeneic, irradiated recipients, memory function of B cells disappears within a matter of weeks, if antigen is not given along with the cells (Celada 1967; Askonas et al. 1972; Gray and Skarvall 1988). This has been taken as an indication that longer-lived memory may depend upon continuous stimulation of memory B cells (and/or T helper cells - the experiments do not rigorously distinguish this) by persistent antigen. However, the transfer of lymphoid cells into irradiated recipients leads to massive general cell proliferation and may therefore not be

representative of what happens in the intact animal. Recent experiments by P. Vieira in my laboratory have shown that the blockade of T helper cell function in mice by anti-CD4 antibodies at a dose which completely blocks the induction of memory as well as of primary and secondary responses, leaves established B cell memory intact over a period of at least 6 weeks (P. Vieira and K.R., to be published). Consequently, the memory cells present in the animals either do not require continuous antigenic stimulation for their persistence at all, or the mechanism of stimulation differs from that operating in memory cell generation and the recruitment of memory cells into the secondary response. Thus, the life time of resting memory cells may be longer in the intact animal than in an irradiated, reconstituted host and the unquestionable phenomenon that antigen persistence influences the persistence of the memory state (live vaccines usually confer longer-lasting immunity than killed ones) could partly or largely reflect recruitment of naive cells into the memory pool.

iv) Distinct cell lineage. The above considerations are compatible with the view that memory cells are generated from "naive" cells in a special differentiation pathway; a different pathway could lead to the recruitment of the same cells into a primary response. In contrast to this view, N. Klinman and his colleagues propose that memory cells develop from precursor cells which differ from those responsible for the production of primary antibody responses and may represent a separate B cell lineage. The evidence supporting this provocative hypothesis is discussed by N. Klinman in this symposium.

Affinity Maturation in Memory B Cells

Affinity maturation of antibodies (Jerne 1951; Steiner and Eisen 1967; reviewed by Siskind and Benacerraf 1969) accompanies the generation of B cell memory as its key feature. Two basic concepts attempting to explain affinity maturation are depicted in Fig. 1.

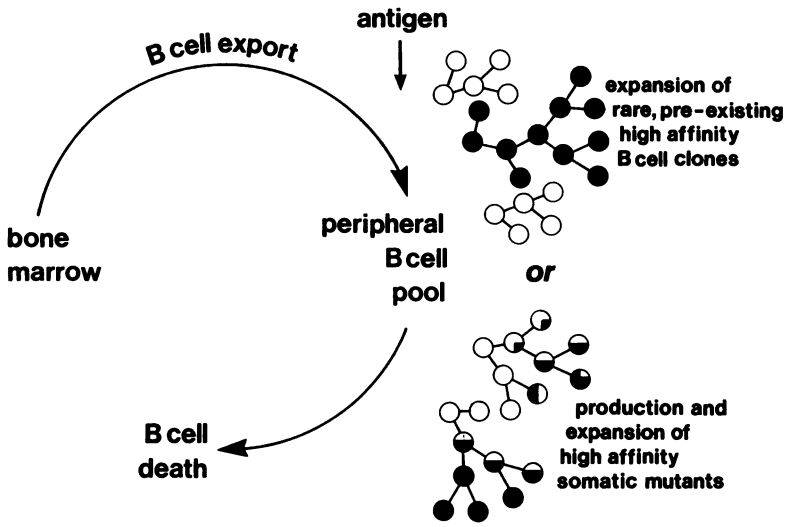

Figure 1: Models of affinity maturation

On the one hand, antigen may selectively expand rare, pre-existing B cells expressing antibodies with high affinity for the antigen. As the concentration of antigen decreases with time, selection for such cells will become more and more stringent (see Siskind and Benacerraf 1969; Fig. 1, upper part). Alternatively, somatic antibody mutants could be generated within the B cell clones initially triggered by antigen, and the best fitting mutants subsequently selected (Fig. 1, lower part).

It seems clear by now that the latter model, that of a rapid process of somatic evolution, describes the situation adequately, at least in the case of T cell dependent B cell memory development in the mouse (reviewed by Kocks and Rajewsky 1989)[1]. The antibody V regions expressed by memory B cells invariably carry somatic point mutations, often in large numbers. Some of these mutations are clearly selected; others, however, cannot be subject to selection by ligands binding to the antibody, like base exchanges not resulting in amino acid replacements. Mutations of the latter type appear in the population because of a very high mutation rate, estimated to be in the order of $1x10^{-3}$/base pair/cell generation. Through the molecular analysis of antibody V regions expressed by clonally related B cells from individual mice, first performed by M. Weigert and his colleagues, genealogical trees such as depicted in the lower part of Fig. 1 could be reconstructed on paper, indicating that somatic mutations are introduced stepwise in the course of clonal proliferation. We have taken a further step and reconstructed such a genealogical tree experimentally, using methods of gene technology. This directly demonstrated affinity maturation through somatic mutation of a germ line encoded precursor antibody. The experiment also exemplified stepwise affinity maturation in the course of clonal expansion (Kocks and Rajewsky 1988).

Taking this together with evidence indicating onset of somatic hypermutation after antigenic stimulation (Manser and Gefter 1986; Griffiths et al. 1984), a picture emerges in which the antibody repertoire in memory B cells is generated following two main principles: 1) Rapid generation of somatic antibody mutants with a wide range of specificities within the initially responding B cell clones, through a mechanism of largely random somatic hypermutation, and 2) efficient selection of the best-fitting mutants into the memory B cell pool.

We have argued elsewhere (Rajewsky et al. 1989) that the main physiological function of this process may be related to the key requirement of acquired immunity, namely prompt adaptation to any given immune stimulus. (This differs from what is required in natural immunity; see above). The rapid generation of somatic antibody mutants may predispose the immune system to deal effi-

[1] It is often forgotten that Burnet (1959) included the modification of clonal patterns by somatic mutation into the clonal selection theory. To quote from his text (p.80): "The ... postulate, that active sites on cell surface or globulin molecule can be modified to a wider reactivity by somatic mutation, provides the chief agent to allow change in antibody character as immunization proceeds. If the primary postulates of the clonal selection theory are accepted, such a result of somatic mutation is as much in order as any other."

ciently with microbial mutants which often accumulate rapidly in the course of an infection.

The somatic hypermutation mechanism involved in the generation of memory B cells also represents a major physiological problem for the organism which in general tries to avoid somatic mutation of what has been carefully selected in evolution. Although hypermutation is targeted to rearranged V region genes, a problem inherent in the mechanism remains, namely that autoreactive antibody mutants may arise by accident. Considering the extent of somatic mutation in memory B cell generation and the evidence that antibody mutants with changed specificity can indeed be isolated from the memory B cell compartment (Siekevitz et al. 1987), a stringent control preventing auto-aggression through somatic antibody mutants is clearly required. It is presumably for this reason that somatic hypermutation appears to be limited to a particular phase of B cell differentiation (see Siekevitz et al. 1987) and also to a particular anatomical microenvironment, as discussed by I. MacLennan in this symposium. N. Klinman suggests that in this phase of differentiation B cells are exquisitely susceptible to tolerance induction. The absence of somatic hypermutation in T helper cells which drive B cells into the memory pathway may be another safeguard against the expansion of autoaggressive B cell mutants. However, as M. Weigert points out in his contribution to this symposium, such mutants do seem to play a major role in certain autoimmune diseases. The mechanism of this dysregulation is at this point a matter of speculation.

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Development of the B Cell Repertoire and the Ability to Respond to Bacteria Associated Antigens

J. Kearney

INTRODUCTION

The acquisition of the B cell repertoire in mice follows a reproducible and ordered developmental sequence. This is reflected by, (i) early predominant use of particular V_H and V_L genes (Yancopoulos et al. 1984; Perlmutter et al. 1985; LeJeune et al. 1982; Lawler et al. 1989; Hilson et al. 1989), (ii) a B cell repertoire which is characterized by a predominance of multispecific autoreactive IgM antibodies (Holmberg et al. 1984; Vakil and Kearney 1986), and (iii) the predominance of a particular subset of B cells expressing the Ly1 antigen early but not later in development.

In functional studies, it has also been shown by many investigators that there is a reproducible and ordered development of the ability to respond to different antigens. This aspect of development of B cells has been frequently reviewed, and one of the most striking aspects is that the ability to produce B cells responsive to many bacteria associated antigens occurs relatively late in ontogeny in mice (Sigal and Klinman 1978). This occurs also in humans, and has the clinically important sequela that infants until 2-3 years of age are susceptible to various infections with encapsulated organisms. The polysaccharide determinants in the capsules of these organisms have the ability to induce protective antibody of the IgM class in adult animals so that the inability to respond in a T independent manner to these kinds of antigens in the newborn has far reaching consequences (Peltola et al. 1977; Pincus et al. 1982).

The protective IgM antibodies induced by polysaccharide antigens are generally highly restricted in the V_H and V_L combinations used. This results in not only a restricted set of B cell clones that respond to the particular polysaccharide antigen but also expression of a dominant idiotype by these clones. This has been shown previously in several mouse models (Sigal and Klinman 1978; Blomberg et al. 1972); and more recently studies by Scott et al. 1989, have shown that antibodies induced by Hemophilus influenzae (Hib-Ps) polysaccharide are remarkably conserved between individuals and show evidence for expression of one particular VH_{III} family and several V_L genes.

We are faced, therefore, with the problem that the earliest appearing B cells that develop within the mouse do not appear to produce protective antibodies to bacterial antigens and instead appear to be multispecific, interactive, and to bind to a number of laboratory haptens (Vakil and Kearney 1986). In this symposium, I will develop the hypothesis that this early multireactive set of B cells is necessary to induce the selection and/or expansion of clones of later appearing B cells that have the ability to make protective responses to bacterial antigens. In this respect, the early appearing B cells,

by virtue of their idiotypic interactions with later appearing B cells, appear to imprint on the developing immune system the ability to make these protective responses. This kind of B cell "memory" is built into the immune system and does not rely on prior activation by antigen. In fact, inappropriate intervention with antigen in these early stages of development appears to interfere with the normal developmental sequence of acquisition of the B cell repertoire, as will be discussed later.

INTERFERENCE WITH THE DEVELOPMENT OF NATURAL IMMUNITY

In previous studies it has been shown that there is extensive connectivity between early B cells (Holmberg et al. 1984). This connectivity has been shown to involve antibody responses to a number of bacterial capsule or cell wall-associated antigens (Vakil and Kearney 1986). The connectivity can be depicted as a cascade-like set of reactions with those initiating the connected set belonging to the early autoreactive set of B cells. It was shown initially that positive effects on phosphorylcholine (PC) and $\alpha 1 \rightarrow 3$ dextran (DEX) specific clones were produced by the introduction of an IgM antibody with anti-idiotype activity to the predominant idiotypes T15 and J558, characteristic of the anti-PC and anti-DEX responses in BALB/c mice, respectively (Vakil and Kearney 1986). These results suggested that the observed augmentation of the anti-PC and DEX responses was due to the superimposition of the activity of the neonatally isolated anti-Id antibodies on a process that is occurring during normal development. A corollary to this experiment involved the functional deletion of a subset of neonatal B cells by the introduction of an IgG anti-Id antibody which reacted with a large number of perinatal IgM hybridomas, including those in the cascade interacting with the anti-PC and DEX clones. Although this antibody did not react directly with PC or DEX as shown by in vitro binding experiments, in utero or perinatal treatment with this antibody produced long lasting and severe depression of the responses to PC and DEX (Vakil et al. 1986). Again, these experiments suggest a vital role for the early appearing set of B cells in establishment of the adult repertoire to PC and DEX. The establishment and dominance of the particular clones therefore result from a germline encoded network which enhances the development of those clones of cells responsible for natural immunity to organisms expressing these antigens.

If as proposed, this germline encoded network interacts through V regions of immunoglobulin receptors, then other ways of interfering with the signalling that is proposed to occur can be tested. Injection of exogenous TI5 idiotype expressed in association with different isotypes and also of a non-antigen binding but TI5 idiotype positive variant to mice at a stage when these interactions are occurring, severely inhibits the development of these TI5 positive anti-PC responses. These results add further support to the role of idiotypes in the selection and expansion of the dominant clones. The mechanism of these interactions is not clear, but may involve a selection process in which a signal delivered to an idiotype bearing receptor on a newly emerging B cell provides for the entrance of that B cell into a pool of cells which can be selected further by exposure to antigens into antibody forming cells. In the absence of this encounter, this newly emerging B cell may die. The various manipulations described above, including deletion of a set of B cells that deliver such signals and blockade of these signals by introduction of idiotype, results in the failure of such B cells to enter

the functional adult B cell pool and an accompanying deficiency in the ability to respond to PC and DEX.

ENHANCEMENT OF GERMLINE ANTIBODY RESPONSES BY PERINATAL INTERVENTION

The above outline of experiments demonstrating the fundamental importance of connectivity also suggests ways of enhancing the development of the normally delayed responses to these bacterial antigens.

The obvious approach would at first appear to be the administration of a suitable vaccine to the developing mammal at a time when these clones are appearing and provide the hypothetical selection signal. In such an experiment a pneumococcal vaccine, heat-killed streptococcus pneumoniae (R36A) was given to two-day-old mice, which were then rechallenged as adults. The perinatal treatment with the vaccine increased the overall anti-PC response by six-fold, but did not expand the TI5 or dominant idiotype response. Indeed the serum from rechallenged mice did not contain protective antibody, as does serum from normal R36A immunized mice. Furthermore, mice treated at day 2 with R36A vaccine and rechallenged with DEX as adults showed almost complete ablation of their ability to make antibodies to DEX. These experiments show that the strategy of vaccination with PC containing organisms does not result in protective immunity, and furthermore, presumably because of the connectiviness of the early emerging B cells, inhibits the development of later appearing clones of B cells responsive to the polysaccharide DEX.

In view of our model whereby a set of multispecific B cells is normally responsible for the development of PC and DEX clones expressing the dominant idiotypes, another strategy was suggested whereby the early appearing clones could be activated by deliberate immunization, and in this way augment their normal role in selection of the B cell clones of appropriate specificities. Since many of these clones of early appearing multispecific cells bind oxazalone (Vakil and Kearney 1986), this hapten coupled to heat-killed Brucella abortus was administered once to two-day-old mice. These mice, when challenged as adults with the R36A, also made large amounts of anti-PC antibody, however, in this case most of the antibody expressed the dominant TI5 idiotype. These experiments suggest the use of an alternative strategy for enhancement of normally late developing anti-PC and anti-polysaccharide responses; one that manipulates the normal mechanisms proposed for development of natural germline encoded antibody responses which convey protection to a variety of pathogenic organisms.

DEVELOPMENTAL ORIGIN OF B CELLS INVOLVED IN IDIOTYPIC INTERACTIONS

As suggested in the introduction to this Symposium on memory by Dr. Klaus Rajewsky, it appears that the subset of Ly1 B cells which predominates early in development is responsible for the initiation and maintenance of what he has termed evolutionary memory. The immunobiology and functional capacity of this subset of B cells will be covered in more depth by Dr. Leonore Herzenberg in the next section, however, I will conclude this section with a brief description of experiments in which we have tested the idea that Ly1 B cells

are derived from mesenchymal tissue in the developing fetus (Holub et al. 1971; Dux et al. 1977; Dieterlen-Lievre 1987; Kubai and Auerbach 1983).

The basic principle in these experiments was to test the potential of a variety of fetal primordia to reconstitute lymphoid cells in recipient scid mice (Bosma et al. 1983). Spleen, thymus, omentum, liver and bone from F1 (C57B1/6 x BALB/c mice) were grafted under the kidney capsule of scid mice. The mice were then sacrificed four months later and their lymphoid tissues, including peritoneal cavity, analyzed for the content and phenotype of lymphoid cells. It was found that only liver and omentum reconstituted lymphoid cells. In the case of the liver transplant, both T and B cells of donor origin appeared in the scid host recipient. In the peritoneal cavity both liver and omentum gave rise to Ly1 B cells, and again liver but not omentum transplants gave rise to a population of CD5 positive T cells (Kearney et al. 1989). The reconstitution was also evident in serum where donor derived IgM and varying levels of IgG isotypes were detected, with the liver reconstituted scid mice producing more of the T cell dependent isotype IgG_1 than the omentum transplanted mice which produced mostly IgG_3 (Solvason et al. 1989).

SUMMARY

We have shown that large numbers of IgM antibodies from hybridomas constructed from fetal and neonatal liver and spleen are interconnective and reactive to self antigens, including defined immunoglobulin idiotypes. Experiments were described where manipulation of the developing B cell repertoire either by deletion or positive expansion of certain clones resulted in permanent effects on the ability of these adult animals to respond with germline encoded responses to certain antigens such as PC and DEX.

With respect to the origin of the B cells involved in these early networks, 50-60% of B lineage cells at this stage are $Ly1^+$, and we have shown that fetal omentum as well as liver is a source of $Ly1^+$ B cells. This reactivity towards self idiotypes appears to be a critical feature of the normal early B cell repertoire and is involved in the establishment of the natural or "evolutionary" memory referred to in the introduction to this symposium.

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B Cell Lineages and Immunologic Memory

L. A. Herzenberg and A. Stall

Immunologic memory was originally defined in terms of the *in situ* anamnestic response that occurs when an individual re-encounters an antigenic stimulus. As the development of *in vitro* and adoptive transfer methods enabled identification of the cells participating in memory responses, however, a subtle redefinition occurred and immunologic memory came largely to mean the presence of such "memory" cells in a stimulated animal, regardless of whether they were detected *in situ* (by production of an anamnestic response), in vitro, or in a secondary host.

The importance of the ambiguity introduced by this redefinition became clear in later studies which showed that the presence of memory B cells and T cells is necessary but not sufficient for an anamnestic response. These findings, reviewed comprehensively in the first volume of Annual Reviews of Immunology (1), demonstrate the existence of an "epitope-specific" regulatory system that often blocks the *in situ* expression of memory cells whose activity is readily demonstrated in adoptive and in vitro assays. The cellular basis for this epitope-specific regulation has yet to be defined; however, the cells involved clearly "remember" previous antigenic stimulation. Thus, the immune system must contain at least one more functional memory cell than is currently recognized.

The recognition of a second B cell lineage introduces another level of complexity to the definition of immunologic memory, particularly with respect to memory B cells. Until the recent discovery of the Ly-1 B cell lineage, all antibody responses were thought to derive from (what we now call) conventional B cells, which rearrange and differentiate continually from Ig negative progenitors in the bone marrow and are relatively short-lived unless they encounter antigen and are triggered to differentiate further to long-lived memory B cells. By and large, these are the self-replenishing, easily transferred memory cells that produce the high affinity IgG1 and IgG2a anti-hapten responses (e.g., anti DNP, anti NP) from which so much of our information about B cell memory development and expression is derived.

Ly-1 B lineage cells have certain properties in common with these memory cells in that they are also long-lived and self replenishing; however, as the evidence summarized below demonstrates, the Ly-1 B cells are phenotypically and functionally distinct from conventional B cells (2-5). Furtherrmore, they have a unique developmental pathway that results in their repertoire being defined early in life and maintained thereafter without the influx of newly rearranged Ig genes (5-7).

B Cell Lineage Characteristics

	Ly-1 B	Conventional B
Ontogeny	Arise first	Arise later
Main location	Serosal Cavities (peritoneal, pleural)	Lymphoid Organs
Adult population	Self-replenishing (no new entrants)	Constantly being renewed
Adult source for adoptive transfer	Mature Ly-1 B (PerC)	Ig- progenitors (BM, spleen)
Developmental regulation	Feedback inhibition by mature Ly-1 B	unknown
Development impaired	Xid (CBA/N)	me ^{v} (motheaten) μ transgenic
Phenotype:		
IgM	high	low
IgD	low	high
B220/6B2	low	high
CD5 (Ly-1)	low	negative
CD11a (MAC-1)	positive (only in PerC)	negative
CD23 (Fce R)	negative	positive
Size	large	small
Density	low	high
Function:		
Serum IgM, IgG3	+++	+
IgG1	+	+++
IgG2a, IgG2b	+-++	++-+++
IgM autoantibody	+++	+(?)
IgM anti bacterial ab	+++	+-+++
Anti hapten, anti protein	+(?)	+++

Studies demonstrating that the presence of Ly-1 B cells in animals over 6 weeks of age blocks the subsequent differentiation of Ly-1 B cells from Ig negative progenitors have recently been published (6,7) and are summarized below. These findings distinguish Ly-1 B lineage cells from conventional B cells in that conventional B cells continue to differentiate from Ig negative progenitors throughout life, regardless of the status (presence/absence) of the current Ly-1 B population.

Feedback Inhibition of Ly-1 B Development*

Neonatal Animals Treated with Anti Igh-6b§

Igh allotype	Depleted Ly-1 B cells	Remaining Ly-1 B Cells	Recovery of depleted Ly-1 B#
a/b	b allotype only	a allotype	No
b/b	all	none	Yes
b/b (+ a/a)	all	a allotype Ly-1 B from congenic donor	No

*Summary based on data shown in next table.

§ Mice were injected with 2 mg of anti-Igh-6b during the first 4 weeks of life. No Igh-6b positive cells were detected for the first 6 weeks of age.

Peritoneal cells were analysed from mice up to 8 months of age. Conventional B cells are depleted and always completely recover,.

Introduction of Mature Ly-1 B Cells Blocks Endogenous Ly-1 B Cell Development

C.B-17 Neonatal Treatment	BALB/c Cells Injected *	B Cells Recovered (x10 ^{6}) Ly-1 Lineage Host	Ly-1 Lineage Donor	Conventional Host	Conventional Donor
Igh^b	Igh^a	Igh^b	Igh^a	Igh^b	Igh^a
Anti Igh-6b	Unsorted PerC	0.1	2.1	1.2	<0.1
Anti Igh-6b	FACS Ly-1 $^+$B	0.1	2.5	0.8	<0.1
Anti Igh-6b	Ly-1 B Clone (BCL-85)	<0.1	6.5	0.5	<0.1
Anti Igh-6b	None	2.6	--	1.6	--
None	None	3.0	--	1.5	--

* Injected i.p. on day 3 following birth.

The demonstration that *de novo* Ly-1 B development normally terminates within the first two months of life has signifcant consequences for the life-long composition of the Ly-1 B repertoire. In effect, the maximal breadth of this repertoire is established during the neonatal development period. Furthermore, the predominant antibody specificities within the repertoire mainly appear to be established at this time (although later selective pressures could expand or shrink individual components throughout life).

John Kearney, speaking in this symposium, has presented a more detailed view of the kinds of forces that initially shape the Ly-1 B repertoire and perhaps the repertoire of other (as yet unspecified) populations of long-lived neonatal B cells. The long term changes in immune responsiveness that occur when Kearney creates conditions that interfere with the normal neonatal repertoire development pattern confirm the importance of this initial phase of repertoire development throughout life.

Development of Ly-1 B Cell Repertoire

Pups (< 4 weeks)

- Self-replenishing Ly-1 B cells develop from Ig^- precursors
- Repertoire potential is shaped

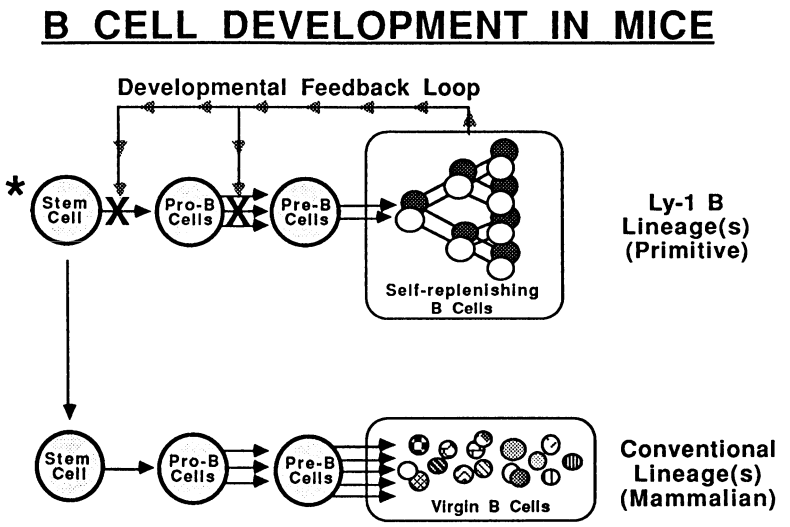
Adolescents (4-8 weeks)

- Feedback blocks new Ly-1 B development
- Ig^- precursors disappear
- Repertoire potential is fixed

Adults (> 8 weeks)

- Individual clones expand or are deleted
- Repertoire becomes progressively more restricted

The evidence summarized above demonstrates that Ly-1 B cells development tends to resemble the B cell developmental patterns described for birds more closely than B cell development patterns previously recognized in mammals (8,9). This suggests that the Ly-1 B lineage represents the most primitive layer of the mammalian immune system. This layer, we believe, has been retained throughout evolution because it provides a basic immuno-protective capability (a kind of immunologic memory?) that allows the operation of the more highly flexible (but perhaps somewhat more cumbersome) mammalian immune mechanisms that evolved later. Thus we currently view the immune system as consisting of the two basic layers shown in the diagram below. The first of these layers contains the self-replenishing Ly-1 B lineage cells and perhaps contains other primitive immunocytes (e.g., gamma/deltat T cells). The second layer, in contrast, contains cells deriving from more evolutionarily mature stem cells that continue to differentiate to immunocompetant cells (conventional B and T) throughout life.



Acknowledgement

Many investigators have contributed either directly or indirectly to the studies described here. Nearly all of the summarized work has been published, either by our laboratory or by others. In a publication of greater length, we would carefully credit these findings; however, in the interests of brevity, we have chosen to omit most of these references. Instead, we refer the reader to recent reviews (1-5) that provide a more comprehensive (and hence more useful) guide to the Ly-1 B lineage literature and to the investigators who have built it.

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The Influence of Somatic Mutation on Clonal Expansion

M.J. Shlomchik, S. Litwin, and M. Weigert

INTRODUCTION

The key feature of the process of somatic diversification is that mutations accumulate sequentially during clonal expansion. This was first recognized from the hierarchy of shared mutations in antibodies from clonally related lymphocytes (McKean et al. 1984) and supported by the finding that the frequency of mutations increases during the immune response to antigen (Berek et al. 1985). Although the mechanism of mutation is still a mystery, one consequence is to introduce discrete point mutations into expressed V genes. This is readily observed among antibodies at initial stages of diversification when expressed V genes can differ from their germline counterparts by single base changes (Weigert et al. 1970) and among clonally related antibodies where siblings differ from each other by single base changes (for example, Radic et al. 1989). Thus the high frequency of mutations in V genes of antibodies from the secondary response to antigen is likely due to the sequential accumulation of point mutations.

This feature has two important implications. One is that the mutation rate must be high. Whereas our original findings of single point mutations in mouse λ-chain could have been explained by standard rates with selection upon mutants (Weigert et al. 1970), high frequencies of independent point mutations in a V gene can only be explained by high mutation rates (Weigert 1986). We have estimated these rates to be in the range, 10^{-3}/bp/div (McKean et al. 1984). The second implication is that since diversification is gradual, positive selection can act during clonal expansion. Drastic diversification mechanisms such as gene conversion or V gene replacement that introduce multiple substitutions in one lymphocyte division do not provide V gene substrates upon which selection can act. This is because multiple substitutions are likely to change rather than modify a specificity and are likely to include substitutions that destroy antibody structure.

As mutations can occur throughout V genes we must consider not just selection upon mutations that modify specificity, but also mutations that destroy antibody structure. Mutations which modify specificity lead to amino acid replacements (R mutations) in the parts of V genes that code for the complementarity determining regions (CDRs), whereas R mutations in regions that determine antibody structure, the framework regions (FRs), have the potential of influencing antibody structure. Rcdr mutations may be positively selected for by antigen if they lead to enhanced binding; Rfr mutations may be lost during clonal expansion. In this article we assess the extent to which cells are lost during clonal expansion because of negative mutations. These estimates allow us to put certain limits on the rate of somatic mutation, to determine the effect of negative mutations on the rate of clonal expansion and to lay the foundation for a precise method for determining whether positive selection has occurred during the expansion of a clone.

METHODS AND RESULTS

We have used two approaches to estimate the fraction of R mutations which are subject to negative selection. The first method categorizes each residue according to patterns of variability found in a large database of protein sequences (Kabat et al. 1983). The pattern of variability is used to infer the fraction of mutations at each residue which will be selected against during clonal expansion (see below). The second method utilizes nucleotide sequence data from hybridomas generated during secondary or chronic immune responses in which numerous somatic mutations have been identified. In the absence of selection, the ratio of replacement to silent mutations (R/S) should be 2.9 (Jukes and King 1979). In a population of B cells subject to negative selection, the relative loss of R mutations --as measured by R/S ratios below 2.9-- is an indication of the fraction of R mutations selected against.

In each approach, we assume that only residues in the FRs are subject to negative selection. These regions are relatively more conserved than CDRs and are thought to encode the generic framework on which the CDR loops are anchored. Presumably, a large fraction of R mutations in these regions could adversely affect antibody function by disrupting the basic protein structure. Clearly, some residues in CDRs could play a similar role and/or be absolutely required for binding to a particular antigen. For either of these two reasons, such residues could be selected against. In addition, concomitant positive selection, which should be negligible in FRs, will be substantial in CDRs. As we do not know for a given response which residues in CDRs will be subject to positive or negative selection, we cannot analyze negative selection in CDRs. By confining our attention to the FRs, we therefore underestimate the effect of negative selection on clonal growth (see below). The fact that FRs are three times bigger than CDRs (Kabat et al. 1983) should minimize the adverse consequences to our analysis of only considering FRs.

Protein Sequence Analysis

We used the mouse V_K sequences collected by Kabat et al. (1983), since this is the largest collection of homologous sequences of one type from one species. We chose to assess only one gene locus in order not to overlook constraints on variability which result from the particular function of this locus (vs. constraints common to all "immunoglobulin fold" type genes). This database is a heterogenous collection of V region protein sequences derived from plasmacytomas, hybridomas, and a few germline gene nucleotide sequences. The V region sequences include examples from most of the homologous murine V_K groups, as defined by Potter et al. (1982). Thus, the database includes both germline and somatically derived variability. As detailed below, our analysis essentially considers the union of these two sources of variability, thus the somatic variation included in the database of Kabat et al. (1983) is fully accounted for and not swamped by the inherent germline variation.

We began by comparing the consensus sequence to all other sequences. Each FR residue was classified as invariant, conservative or nonconservative, based on this comparison. The classifications were defined as follows: Invariant residues had, among the collected sequences, either no or one deviation from the consensus sequence. Conservative residues had only substitutions which were conservative relative to consensus, i.e. with dissimilarity values less than random substitution dissimilarity according to the criteria of Grantham (1974). Nonconservative residues had at least one nonconservative deviation from the consensus according to the same criteria.

The categorization was then used to calculate the fraction of R mutations "allowed". (Here we assume that the database is large enough that failure to observe certain changes is not because of

small sample size but instead because such residues have been eliminated by negative selection and are thus not "allowed"). For each invariant residue, we assumed that no R mutations are allowed. For each nonconservative residue, we assumed that any R mutation is allowed, even those not explicitly seen in the database. For each conservative residue, we assumed that only those residues found in the database are allowed. In the latter case, to determine the fraction of R mutations allowed, we began with all of the codons for the consensus amino acid and determined the fraction of single base changes from these which lead to allowed amino acids. No adjustment for codon usage was made. Table 1 summarizes the results of this analysis. From these considerations, we calculate that about 1/2 of all R mutations in FRs will be eliminated by negative selection. At random, in the absence of negative selection, the ratio of R to S mutations should be 2.9. Thus, this analysis of protein sequences predicts a ratio of about 1.5.

Nucleotide Sequence Analysis

We used nucleotide sequences from hybridomas generated in the secondary response to a variety of foreign antigens, and in the autoimmune response to self-IgG (rheumatoid factor (RF)) and DNA (Table 2). The use of these sequences allows us to consider only somatically generated variability. Table 2 summarizes the number of R and S type somatic mutations in the FR's of the V region sequences from these various responses. The average R/S ratio is 1.4; note that the ratios are fairly consistent from clone to clone and among the various antigen specificities, with a range of 0.8 to 2.0.

Effect of Negative Selection on Clonal Expansion

The elimination of some cells with deleterious R mutations in FR residues will result in slower clonal expansion rates in clones which are undergoing mutation than in nonmutating clones. The estimate that 1/2 of such R's in FR's would be eliminated allows us to model clonal expansion under negative selection at various mutation rates. Figure 1 shows a plot of cell number vs. generation at different mutation rates. At the rate estimated for secondary immune responses of 10^{-3}/bp/div, the clonal burst size is about 125 cells after 10 generations, as opposed to about 1000 cells at a rate of 10^{-5}/bp/div.

Table 1. Prediction of the extent of negative selection in framework regions using the mouse V_κ sequences in Kabat et. al.[a]

Categories of positions in FRs:

invariant: virtually always the same amino acid
conservative[b]: only conservative changes from the most common amino acid
nonconservative: at least one nonconservative change from the most common amino acid

Number in each category:

invariant:	21 positions
conservative:	27 positions
nonconservative:	31 positions

Assume, for:

invariant:	0% of R mutations "allowed"
conservative:	25% of R mutations "allowed" (based on allowing only those R's which have been observed in known sequences.)
nonconservative:	100% of R mutations "allowed"

Thus, the fraction of R's allowed is a weighted average:

$$\frac{(21 \times 0.0) + (27 \times 0.25) + (31 \times 1.0)}{79} = 0.48$$

Expected value of R/S in FRs is thus ~0.5 x 2.9 or ~1.5.

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[a] Sequence data from Kabat et al. (1983).
[b] Criteria for conservative and nonconservative amino acid substitutions are from R. Grantham (1974). Mutations were regarded as conservative if they led to amino acid substitutions with dissimilarities lower than the average structural dissimilarity for random substitution (Padlan 1977).

Table 2. Replacement/silent (R/S) ratios of somatic mutations in framework regions.

Specificity		Mutations R	S	R/S
Influenza Hemagglutinin[a] Clones	a	10	6	1.7
	b	1	1	1.0
	c	6	4	1.5
	d	10	5	2.0
Influenza Hemagglutinin[b]		14	9	1.6
Influenza Hemagglutinin[c]		20	16	1.2
Rheumatoid Factor[d]		19	12	1.6
Rheumatoid Factor[e]		9	11	0.8
DNA[f]		8	8	1.0
Phosphocholine[g]		25	14	1.8
4-hydroxy-3 nitrophenyl[h]		18	9	2.0
4-hydroxy-3 nitrophenyl[i]		15	12	1.2
Total		155	107	1.4

[a] Clarke et al. (1985).
[b] Hüppi et al. in preparation.
[c] Caton et al. (1986).
[d] Shlomchik et al. (1987a).
[e] H. Shan in preparation.
[f] Shlomchik et al. (1987b), Radic et al. (in press), and Shlomchik et al. submitted.
[g] Levy et al. (1989).
[h] Cumano and Rajewsky (1986).
[i] Blier and Bothwell (1987).

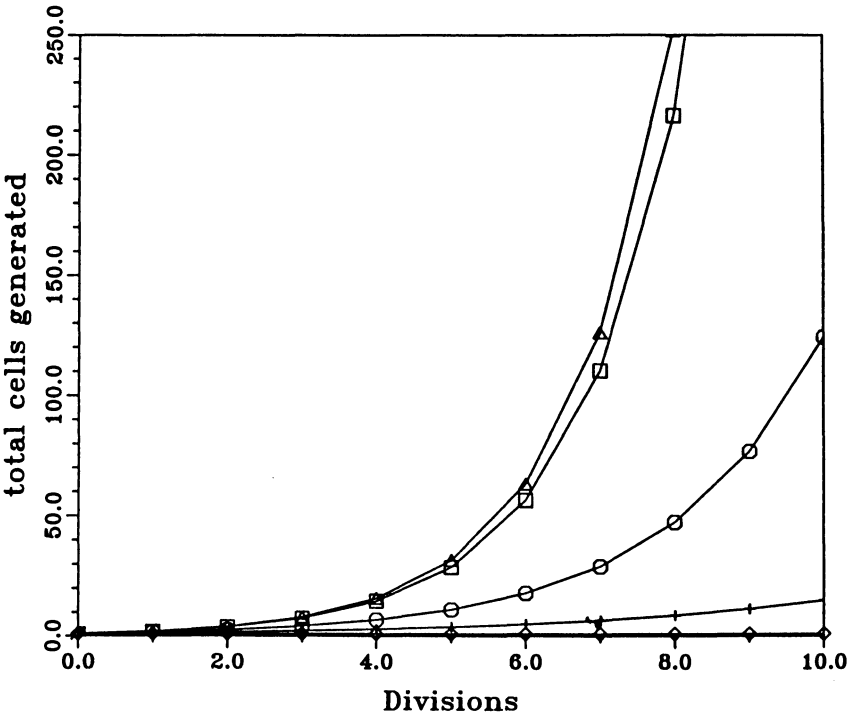

Fig. 1. Clonal expansion with only framework mutations at rates (top to bottom): 10^{-5}, 10^{-4}, 10^{-3}, $2x10^{-3}$, $5x10^{-3}$ mutations per base pair per division. This simulation was done within the scope of a more general model which also allows a variety of CDR mutations. These, however, were switched off for the present analysis. In this simulation, 3/4 of mutations occur in the framework region, in turn 3/4 of these are replacement mutations, and finally, of these 1/2 are lethal (see text). Thus, 9/32 of mutations are framework lethal. Each new cell receives a Poisson distributed number of mutations, (mean r, as above), according to the algorithm in Newman and Odell (1971) for generating Poisson deviates. Each mutation is tested for lethality by comparison of a uniform random deviate to 9/32, e.g., if a cell receives three mutations then three independent uniform deviates are compared to 9/32, all three must exceed 9/32 for the cell to be counted as viable.

Statistical Detection of Positive Selection

Quantitating the extent of negative selection in FRs also allows us to develop a more powerful test for the presence of positive selection. In contrast to negative selection, positive selection could result in an excess of R mutations in CDRs. Mutations which confer a binding advantage to cells which harbor them are expected to be fixed in the specific B cell population, whereas nonselectable silent mutations will accumulate in the population as a function of the underlying mutation rate. Thus, a statistically significant excess of R mutations in CDRs is evidence for positive selection. The random ratio of R to S in CDRs is about 2.9 (although individual genes may vary). A binomial probability model can be used to test for significant deviations from this expected value; however, because the expected value at random is so high (0.75) and the absolute number of CDR mutations is usually low, this test lacks statistical power. A somewhat more sensitive test would consider the number of Rcdr in relation to all other types of mutations. This assumes that the random rate in all regions of the V gene is similar. (The rate does appear to be similar enough for the purposes of this analysis, as judged by the distribution of silent mutations, our unpublished observations.) However, selection against Rfr--the most common type of mutation--could lead to a serious underestimate of the total number of mutations which a particular clonal lineage has sustained. If this is not corrected for, a false positive conclusion could be reached. Since the rate of elimination of Rfr (1/2) is consistent and known, a reasonable correction is possible and a more sensitive and accurate test for positive selection can be performed. In particular, we correct the total number of mutations in a cell or clone in question as follows:

$$\text{Total} = \text{All S mutations} + (2 \times \text{Rfr}) + \text{Rcdr}.$$

We then determine the p value of the distribution of the mutations, with the null hypothesis being that the number of Rcdr could have been generated at random given the total (corrected) number of mutations and the expected mean fraction of Rcdr (=0.19). The latter number is simply the product of the target size (0.25) and the fraction of R mutations at random (0.75). The 0.75 parameter could be adjusted for an individual gene according to the exact sequence of the CDRs. This method has been described in brief by us and used to show that the distribution of Rcdr in a clonally related set of anti-DNA hybridomas was non-random (Shlomchik et al. 1987b).

DISCUSSION

By two separate analyses of known sequences we have arrived at similar estimates of the fraction of R mutations in FRs which are eliminated during clonal expansion and selection, i.e. 1/2. While these two analyses are assessments of the same inherent process, they are independent in several important ways. First, the databases used were nonoverlapping; none of the nucleotide sequences was present in the database of Kabat et al. (1983). Moreover, the nucleotide sequence analysis included heavy chains and light chains, whereas the protein analysis included only light chains. Second, they are based on two different sets of assumptions. The protein analysis is based on a set of rules concerning how observed variability reflects potential variability; however, the analysis is independent of assumptions about the rate or pattern of mutation. On the other hand, the nucleotide sequence approach assumes that, in the absence of selection, the R/S ratio in FRs is 2.9. That the two estimates agree suggests that the assumptions made were indeed reasonable. The R/S ratios were remarkably consistent, regardless of V region gene or antigen specificity, suggesting that selection against certain Rfr is a general phenomenon pertaining to antibody structure and is not significantly affected by the particular specificity of the antibody.

Quantitation of the relative paucity of R mutations among antibodies from B cells which have been subjected to in vivo selection makes no assumptions about the reasons for the selection against certain R mutations. Assuming that affinity is the critical parameter, the approach of mutating invariant residues in vitro and assessing the effect on affinity for hapten antigens has been used (Sharon 1988; Simon and Rajewsky in preparation). In the few cases tested, it was found that although affinity could be destroyed by some changes, certain nonconservative changes had only a small (i.e. two-fold) negative effect. These results could be used to support the idea that negative selection may apply to fewer mutations than we have estimated (Shlomchik et al. 1987b). However, it is not clear that affinity for antigen is the sole parameter on which selection acts, nor is it clear that even small decreases in affinity are irrelevant to selection. Ability to transmit signals, resistance to proteolysis, ability to associate with other Ig chains, and efficiency of folding are just a few obvious functions aside from affinity on which selection could act. Indeed, that in vitro changes of (in vivo) invariant residues has little effect on affinity strongly suggests that other factors are involved in negative selection.

Knowledge of the extent of negative selection has implications for understanding clonal dynamics during the immune response. As shown in Fig. 1, when mutation rates are high and 1/2 of Rfr are eliminated by selection, clonal growth rates are markedly slowed and burst size reduced. This degree of negative selection puts an upper limit on mutation; as shown in Fig. 1, even rates of $2x10^{-3}$/bp/div are too high to allow for significant clonal expansion.

The effects of negative selection on the distribution of Rfr is based on data mainly gathered late in the immune response. To assess the effects of negative selection on clonal growth rate, we have assumed in Fig. 1 that negative selection operates continuously throughout the diversification stage of B lymphocyte clonal expansion. However, little data giving a time course of the distribution of Rfr is available. Thus, it is possible that the efficiency or "tolerance" of the process could vary during an immune response and clonal growth rates could be transiently faster or slower than we have estimated. We do know, however, that the ultimate effect of the selection is to eliminate 1/2 of Rfr. As a significant proportion of the nucleotide sequence data comes from 21 day responses and the R/S ratio in these is 1.4, it seems likely that the average efficiency of the process is reached by 21 days. Furthermore, it is likely that the process of negative selection occurs prior to 21 days since in one study of 7 and 13 day responses to PC, the framework R/S were already reduced in both cases to 1.8 (Levy et al. 1989). Regardless, in continuously mutating clones, burst sizes at 21 days should conform to our estimates as shown in Fig. 1.

The mutation rate clearly will have an impact on clonal growth rates. Moreover, the mutation rate falls dramatically late during clonal expansion (Siekevitz et al. 1987; Shlomchik et al. 1987a, 1987b) as suggested by the shape of clonal trees. This event of mutation stopping may be stochastic (Shlomchik et al. 1987a, 1987b). Thus, lineages of the same clone which have and have not stopped mutating will coexist during the course of an immune response. Those lineages in which mutation has stopped will expand at a faster rate than sibling lineages which continue to mutate, and this selective advantage may explain why cells from nonmutating lineages seem to dominate late immune responses.

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Self-Nonself Discrimination by Precursors of Memory B Cells

P.-J. Linton and N.R. Klinman

INTRODUCTION

Nucleic acid sequence analyses have greatly increased our understanding of the molecular mechanisms responsible for the generation of secondary antibodies. From these studies it has been concluded that: a) although secondary antibodies appear to be derived from the same V region gene segment pools and combinatorial associations as primary antibodies, primary and secondary responses are often dominated by clonotypes using different V genes (McKean et al. 1984; Berek et al. 1985; Cumano and Rajewsky 1986; Manser et al. 1987); and b) secondary but not primary antibodies are characterized by the accumulation of numerous V region somatic mutations particularly those that yield amino acid replacements in complementarity determining regions (CDRs), thus evidencing antigen selection of favorable mutations (McKean et al. 1984; Berek et al. 1985; Cumano and Rajewsky 1986; Manser et al. 1987; Malipiero et al. 1987). At the cellular level these findings pose several intriguing questions. From the standpoint of recognition of the universe of non-self antigens, how do B cells whose V regions are rare or absent in primary responses become dominant in secondary responses? From the standpoint of non-recognition of the universe of self antigens, how is the repertoire of new secondary specificities resulting from somatic mutations purged of reactivity to self antigens?

Differences Between Precursors to Secondary B cells and Primary AFC Precursors

Recently we have reported that primary antibody forming cell (AFC) clones and secondary B cells derive largely from separate precursor cell subpopulations (Linton and Klinman 1986; Linton et al. 1988; Klinman and Linton 1989; Linton et al. 1989). This conclusion was based on the following findings: 1) A subpopulation representing 10-15% of Ia positive precursor cells could be isolated reproducibly from total splenic B cells of non-immune mice on the basis of their

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low expression of the cell surface antigen recognized by the monoclonal antibody J11D ($J11D^{lo}$), which is a characteristic of secondary B cells as well (Bruce et al. 1981; Raychaudhuri and Cancro 1985). 2) SCID mice repopulated with helper T cells from carrier-primed mice and $J11D^{lo}$ Ia positive precursor cells from non-immune mice produced poor primary serum antibody responses but vigorous secondary serum antibody responses. 3) SCID mice repopulated with helper T cells from carrier-primed mice and $J11D^{hi}$ Ia positive precursor cells from non-immune mice produced vigorous primary serum antibody responses but little or no secondary serum antibody responses. 4) The majority of responsive fragment cultures from spleens repopulated with limiting numbers of $J11D^{lo}$ cells produced antibodies only after two or more in vitro stimulations. 5) Almost all responsive fragment cultures of spleens repopulated with $J11D^{hi}$ cells produced antibodies after a single in vitro stimulation. 6) In fragment culture, the majority of anti-(4-hydroxy-3-nitrophenyl)acetyl (NP) antibodies produced by $J11D^{lo}$ precursor cells isolated from Igh^{b} mice bore the κ light chain, which is also the dominant phenotype found late in anti-NP secondary responses of Igh^{b} mice (Makela and Karjalainen 1977; Reth et al. 1979; Cumano and Rajewski 1986). 7) In fragment culture the majority of anti-NP antibodies produced by $J11D^{hi}$ precursor cells isolated from Igh^{b} mice had the λ light chain, which is the dominant phenotype found in primary anti-NP responses of Igh^{b} mice (Cumano and Rajewsky 1986; Makela and Karjalainen 1977; Reth et al 1979; Stashenko and Klinman 1980; Riley and Klinman 1985). 8) Whereas no hybridomas were generated from the spleen cells of SCID mice repopulated with $J11D^{lo}$ precursor cells that were immunized only once with NP, numerous hybridomas were generated if these mice were boosted. 9) A high frequency of hybridomas could be generated from the spleen cells of SCID mice that were immunized only once after repopulation with $J11D^{hi}$ precursor cells; however, few hybridomas could be generated after boosting these mice. 10) Sequence analysis of the λ chain RNA of NP-specific hybridomas prepared from the spleen cells of SCID mice that had been repopulated with $J11D^{lo}$ precursor cells and boosted 8 days after primary immunization, evidenced the accumulation of multiple somatic mutations.

Differences Between Precursors to Secondary B cells, and Secondary B cells.

Because secondary B cells and precursors to secondary B cells share certain repertoire characteristics, and both express low levels of the surface antigen recognized by the J11D monoclonal antibody, it is conceivable that the findings observed for the $J11D^{lo}$ precursor cells of non-immune mice may have been due to responses of contaminating secondary B cells. These cells may be present in the spleens of non-immune mice by virtue of having been stimulated by cross-reacting environmental antigens. However, the following results would argue against this; a) The surface immunoglobulins expressed by the non-immune $J11D^{lo}$ precursors were IgM and IgD whereas secondary B cells often express other isotypes (Black et al. 1978; Teale et al. 1981; Linton et al. 1989). b) Unlike secondary

B cells, which generate vigorous AFC responses following a single immunization (Klinman 1972), most J11D^{lo} precursor cells from non-immune mice require both primary and secondary stimulation to yield AFC (Linton et al. 1988; Klinman and Linton 1989; Linton and Klinman 1989). c) Somatic mutations in the λ chains of hybridomas obtained from the spleen cells of SCID mice repopulated with J11D^{lo} precursors required several days to accumulate and did not preferentially encode amino acid replacements in CDRs as would be anticipated for secondary B cells.

Recognition of Non-Self by Precursors to Secondary B cells.

Determinant recognition and clonotype representation in the primary vs secondary response to a given antigen falls into three broad classifications. In some instances, the clonotype or clonotype families that dominate the primary response also dominate the secondary response (Malipiero et al. 1987). On the other end of the spectrum, clonotypes can be found to dominate secondary responses that are rare or absent in primary responses (McKean et al. 1984), and, in some instances, determinants appear to be recognized by secondary B cells that are not recognized by the primary repertoire (Duran and Metcalf 1987). Between overlap and nonoverlap of specificities is a class of responses in which the clonotypes found to be dominant in the primary response constitute a minority in the secondary response and vice versa (Manser et al. 1984; Berek et al. 1985; Cumano and Rajewsky 1986). This category of responses is exemplified by the response of Igh^{b} mice to NP wherein κ bearing antibodies are dominant in the secondary response and λ bearing antibodies are dominant in the primary response (Makela and Karjalainen 1977; Reth et al. 1979; Stashenko and Klinman 1980; Riley and Klinman 1985; Cumano and Rajewsky 1986).

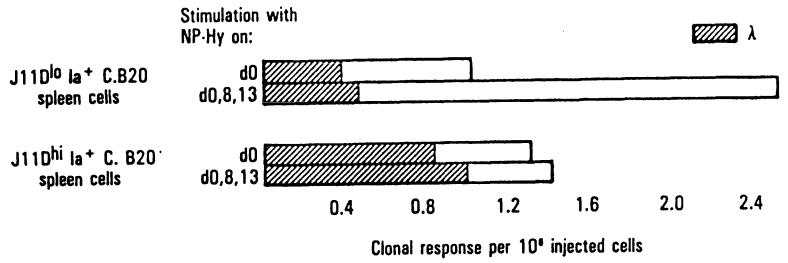

Fig. 1. *In vitro* clonal responses to single vs multiple stimulation with NP-Hy. Fragment cultures containing transferred Hy-primed T_H and either J11D^{lo} or J11D^{hi} Ia positive spleen cells from non-immune C.B20 mice were stimulated with NP-Hy on day 0 or days 0, 8, and 13 of culture. The antigen was present for 48hr. Specific antibody was detected by ELISA in culture fluids collected at 3-4 day intervals. The hatched bars represent the proportion of responses bearing λ light chains.

As shown in Fig. 1, the difference in the phenotype of the response produced by NP specific primary vs secondary B cells of Igh^b mice was reflected in the phenotype of $J11D^{hi}$ primary AFC precursors vs $J11D^{lo}$ precursors to secondary B cells isolated from the spleens of non-immune Igh^b mice. Close inspection of these findings reveals several disparities in the responses of $J11D^{hi}$ primary AFC precursors vis a vis responses of $J11D^{lo}$ precursors to secondary B cells. These disparities exemplify a variety of molecular and cellular mechanisms that together could potentially account for the differences in primary vs secondary repertoire expression.

The simplest explanation for differences in the repertoires of primary vs secondary B cells would be that V region clonotypes may be distributed disproportionately among the two relevant precursor cell populations in non-immune mice. Evidence that this may be the case is seen in the higher absolute frequency of λ bearing $J11D^{hi}$ than $J11D^{lo}$ NP specific precursors. This finding is even more impressive in light of the higher overall frequency of NP responsive $J11D^{lo}$ precursors. In comparison to the representation of λ bearing B cells among all B cells and in most other responses, the frequency of λ bearing NP specific primary AFC precursors in Igh^b mice is inordinately high. It is possible that this, and possibly other peculiarities in repertoire distribution among primary AFC precursors, does not apply to other B cell subpopulations such as precursors to secondary B cells. If this is the case it might imply separate origins for these precursor cell subsets during bone marrow B cell development.

Although the frequency of λ bearing $J11D^{lo}$ NP specific precursors to secondary B cells is relatively low, it is important to note that the same λ bearing clonotypes that dominate the primary response do participate in the secondary response. Thus, λ bearing $J11D^{lo}$ precursors do generate secondary B cells and do accumulate somatic mutations (Reth et al. 1979; Cumano and Rajewsky 1986; Blier and Bothwell 1987; Linton et al. 1989). Indeed, presumably because of their relatively high affinity, λ bearing antibodies can predominate early in secondary serum antibody responses (Makela and Karjalainen 1977; Cumano and Rajewsky 1986; Blier and Bothwell 1987). Thus, while the proportionate representation of given V regions may differ, $J11D^{hi}$ and $J11D^{lo}$ primary precursors can express identical clonotypes.

The overall frequency of $J11D^{lo}$ precursors from Igh^b mice that produced κ bearing NP specific antibodies was five fold higher than the frequency of similar responses obtainable on a per B cell basis from $J11D^{hi}$ precursor cells (Fig. 1). This difference in repertoire distribution between the two precursor cell subsets, like the difference in λ bearing NP responsive cells, could reflect differences in V region utilization during their bone marrow development. However, there are two other interesting potential explanations for the greater frequency of NP responsive κ bearing $J11D^{lo}$ cells. Although the frequency of κ bearing mature primary AFC precursors in Igh^b mice is low, the frequency of precursors that

yield κ bearing NP specific AFC clones is relatively high in bone marrow B cell precursors isolated as surface immunoglobulin negative cells (Riley and Klinman 1985). Since the diminution in frequency of these precursors as B cells mature affects disproportionately relatively high affinity precursors, this phenomenon has been attributed to tolerance induction by cross-reaction to self antigen during the maturation of κ bearing precursors (Riley and Klinman 1985). By this reasoning, it is possible that the higher absolute frequency of κ bearing J11D^{lo} precursors may in part reflect a relative resistance to tolerance induction of precursors to secondary B cells during their maturation.

Another potential explanation for the higher frequency of κ bearing NP responsive J11D^{lo} precursors would be that the affinity requisites for stimulation of precursors to secondary B cells may be lower than those for primary AFC precursors, thus enabling the participation of substantially more precursors by the inclusion of those with relatively low affinities. This explanation would also be consistent with responses to 2,4-dinitrophenol (DNP) spleen cells of BALB/c mice, in which a 2-3 fold greater frequency was observed for J11D^{lo} than J11D^{hi} precursor cells (Klinman and Linton 1989; Linton et al. 1989). Upon close inspection, several parameters of the responsiveness of J11D^{lo} vs J11D^{hi} precursor cells appear to be consistent with both lower affinity and less specific triggering of J11D^{lo} precursors. First, responses of J11D^{lo} precursors specific for DNP almost totally overlap with those specific for the cross-reactive antigen 2,4,6-trinitrophenol (TNP) in that the frequency of fragment cultures responding after primary stimulation with either TNP conjugated to hemocyanin (Hy) or DNP-Hy was almost equivalent to the frequency of fragments responding to a mixture of both antigens. Only a small percentage of DNP or TNP responsive J11D^{hi} precursors could be cross-reactively stimulated which is consistent with the idea that antigenic stimulation of primary AFC precursors is highly specific (Klinman et al. 1973). Further evidence in support of the proposed lower affinity threshold for the stimulation of the J11D^{lo} precursors, was the finding that several NP specific hybridoma antibodies prepared from spleen cells of SCID mice repopulated with J11D^{lo} precursors displayed low affinity as did many monoclonal antibodies produced in fragment cultures from the progeny of J11D^{lo} precursors.

What then are the consequences of the findings that: 1) the differences in the repertoires of the primary vs secondary antibody response in part may be reflective of the differences in the distribution of V region clonotypes among their respective J11D^{hi} and J11D^{lo} precursors in non-immune mice, and 2) the affinity requisites and specificity of triggering of J11D^{lo} precursor cells may be lower than those of J11D^{hi} primary AFC precursor cells. Since J11D^{lo} precursors represent only 10-15% of B cells, both of these phenomenon could serve to expand the spectrum of clonotypes that can participate in secondary responses. Indeed, as others have reported, high affinity secondary antibodies are often the product of antigen selection of favorable somatic mutations among clonotypes

of precursors that were initially low affinity (Berek et al. 1985; Manser et al. 1984; Manser et al. 1987).

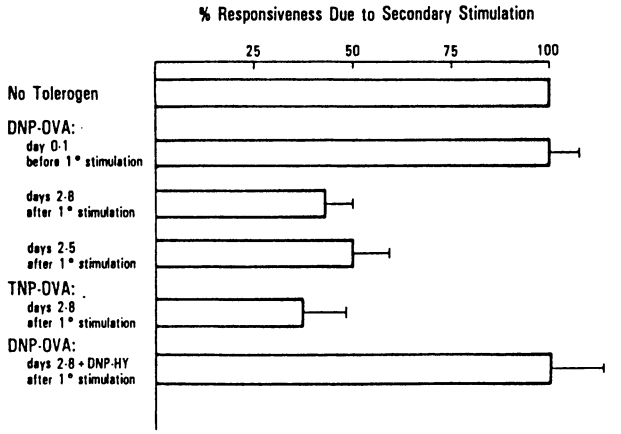

Fig. 2 The inhibition of secondary B cell generation by homologous and cross-reactive tolerogens. Fragment cultures containing transferred Hy primed T_H and $J11D^{lo}$ precursors from non-immune BALB/c mice were stimulated with DNP-Hy at 10^{-6}M hapten for 48 hrs on day 0 (background responses), or day 0 and 8 (total secondary responses). Those cultures incubated with tolerogen prior to primary antigenic stimulation had DNP-Hy added at day 1 for 48 hrs. Tolerogen, DNP-OVA or TNP-OVA at 10^{-6}M hapten, was added to culture on the days indicated. In addition to the day 0 and 8 antigenic stimulation, some cultures had DNP-Hy incubated along with the tolerogen on days 2-8. The percent responsiveness was determined as the fraction of total secondary responses and standard error of the mean after subtracting the background responses (10-20% of total).

The Avoidance of Self Recognition by Memory B cells

The accumulation of somatic mutations, which is a random process "guided" by antigenic selection, could also generate new anti-self specificities. Thus, it may be necessary that, during the generation of these potentially harmful progeny there would be a "window" of tolerance susceptibility. In order to examine tolerance susceptibility of the $J11D^{lo}$ precursors and their progeny, fragment cultures containing these cells were stimulated by the standard protocol with DNP conjugated to the cognate carrier for T_H, Hy. A tolerogen, DNP conjugated to the non-cognate carrier ovalbumin (OVA), was added at various times in culture. The findings from these studies (Figure 2) indicate that the primary $J11D^{lo}$ splenic precursors to secondary B cells are not tolerance susceptible. However, as early as 2-5 days following primary antigenic stimulation and prior to a secondary stimulation, hapten presented on a non-recognized carrier has a marked inhibitory effect on the

capacity of the progeny of J11D^{lo} precursors to yield antibody responses following a second antigenic challenge. As shown in Figure 2, the generation of secondary B cells can also be readily inhibited by a cross-reacting antigen TNP-OVA. This finding differs markedly from previous studies demonstrating that tolerance induction of neonatal or immature bone marrow B cells is highly specific (Metcalf and Klinman 1976; Metcalf and Klinman 1977; Riley and Klinman 1986; Teale and Klinman 1980). Importantly, the inactivation of newly generating secondary B cells can be bypassed by continued antigenic stimulation in the presence of the cognate antigen. Thus, the interaction of newly generating secondary B cells with antigen appears stimulatory rather than inhibitory so long as T_H recognition is possible.

Worth mentioning are preliminary findings indicating that the progenitors to tertiary, quaternary, etc., responses are also found in the J11D^{lo} subpopulation of precursor cells as evidenced by a small increase in the number of responding precursors upon a third and fourth stimulation of fragment cultures containing J11D^{lo} cells. This raises some very interesting questions concerning the generation of tertiary, quaternary, etc., B cells and their susceptibility to tolerance induction. For instance, are tertiary and quatenary precursor cells generated from progenitor pools separate from precursors that give rise to secondary B cells, or do the same J11D^{lo} precursor cells that give rise to secondary B cells also give rise to tertiary progenitors etc?. Since the accumulation of somatic mutations appears to "build" upon pre-existing mutations (McKean et al. 1984; Berek et al. 1985), the latter mode of generating tertiary and quaternary precursor cells seems more likely. If precursors to tertiary B cells are only tolerized as they mature to tertiary B cells, as is the case with precursors to both primary and secondary AFC precursors, then newly generated secondary B cells within a given clone may be tolerized without affecting the subsequent generation of tertiary B cells derived from the same progenitors. Theoretically, these precursor cells would be able to continue to somatically mutate and in some instances may lose self recognition.

CONCLUSIONS

Although precursor cells that can give rise to secondary B cells exist as a small subpopulation, their clonotype repertoire responsive to foreign antigens may be greatly expanded vis a vis primary AFC precursors by: a) the presence of potential anti-self specificities, b) shifts in clonotype distribution particularly with respect to lower representation of predominant primary specificities, and c) the incorporation into responses of cells with relatively low affinity receptors. Superimposed upon this is the random occurrence of somatic mutations after stimulation which antigen then selects for higher affinity responses. Throughout this process specificities may be confronted that recognize self antigens; however, these can be eliminated by a tolerance mechanism as secondary B cells mature. Taken together, in the course of the

generation of secondary B cells a selective advantage would accrue to precursors expressing variable regions that include somatic mutations that yield a higher affinity for the stimulatory non-self antigen whereas precursors that recognize a self-constituent would be selectively disfavored.

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Identification of Human Naive and Memory T Cells

P.C.L. Beverley, M. Merkenschlager, and D.L. Wallace

INTRODUCTION

The specificity and memory of immune responses sets them apart from most other physiological responses but while specificity can be well explained by the clonal distribution of B and T cell receptors, immunological memory still poses problems.
During an antibody response isotype switching and changes in affinity take place so that secondary (memory) responses differ from the primary response. The molecular genetic mechanisms underlying these events in B cells have been well studied. Recent data also suggests that maintenance of B cell memory is antigen dependent (Gray and Skarvall 1988). In contrast in T cell responses neither changes in isotype or affinity have been described during the development of effector or memory cells nor is it known whether maintenance of T cell memory, which is considered to be long lived, is antigen dependent. It is thus of particular interest to determine how T memory cells differ from unprimed cells and how this affects the nature of primary and memory T cell responses.

HUMAN T CELL HETEROGENEITY

The initial separation of T cells into largely non-overlapping CD4 and CD8 subsets with distinct properties and genetic restriction has been confirmed and extended in many laboratories. While co-expression of the CD4 and CD8 antigens on peripheral T cells has been described (Blue et al. 1985) the importance of this remains uncertain and there is little evidence for interconvertion between CD4 and CD8 cells. Thus these two subpopulations may be considered to be stable sublineages of T cells, presumably originating from a thymic double positive precursor.

With the development of many monoclonal antibodies (mAbs) against human leucocytes it has become apparent that there is considerable further antigenic heterogeneity within CD4 and CD8 but the significance of this is less clear. There are several possibilities. Phenotypic differences might indicate further distinct sublineages, might identify stages of maturation, be related to cell cycle or be expressed as a consequence of the local environment in blood or lymphoid tissue. For some antigens the picture remains unclear but we shall argue that expression of different isoforms of CD45 is closely linked to maturation of T cells in both the CD4 and CD8 sublineages.

CD45

The CD45 (leucocyte common or T200) antigen is a complex of high molecular weight polypeptides expressed on all leucocytes. There is a

single CD45 gene but several isoforms can be produced by alternative splicing of exons near the N-terminus of the molecule. In man four different types of antibody to CD45 have been identified. Conventional CD45 mAbs bind to all isoforms while one mAb against exon B immunoprecipitates three polypeptides. CD45RA mAbs against exon A react with 220 and 205kDa polypeptides while the CD45RO mAb UCHL1 reacts only with the 180kDa polypeptide (Streuli et al. 1987). Among T cells CD45RA and CD45RO mAbs identify largely non-overlapping subpopulations (Morimoto et al. 1985, Smith et al. 1986).

Function of the CD45RA and CD45RO subsets of CD4 cells.

The functions of these two subsets of cells have been studied in a number of laboratories(Morimoto et al. 1985, Tedder et al. 1985, Smith et al. 1986). Many of these data relate to CD4 and the results can be summarised as follows. Both subsets respond equally in mixed lymphocyte cultures (MLC) and to phytohaemagglutinin or pokeweed mitogen (PWM), but only the CD45RO population responds to soluble antigens. The CD45RA population responds better in the auto-MLR than do CD45RO cells. Only the CD45RO population can provide help for PWM driven immunoglobulin synthesis or a specific antibody response (Beverley et al. 1986). In the PWM model CD45RA cells have been shown to induce suppression.

These data can be interpreted either as indicating two sublineages within CD4 or as separating unprimed (CD45RA) from memory (CD45RO) cells. If the former is correct, the lack of antigen specific reactivity to recall antigens is puzzling. Our own attempts to reveal antigen specific responses of CD45RA cells by limiting dilution, with or without addition of cytokines and removal of CD8 cells failed to do so (Merkenschlager et al. 1988). These experiments showed a large difference between CD45RA and CD45R0 cells in the precursor frequency for responses to soluble recall antigens but no difference in frequency of alloreactive precursors.

Function of the CD45RA and CD45RO subsets of CD8 cells.

There is much less data available with regard to the function of CD8 subsets detected by CD45RA and RO mAbs. However we have recently shown that when T cells are separated into CD45RA+ and CD45R0+ cells, both subsets can generate allo-specific cytotoxic cells (CTL) while only UCHL1+ cells (CD45RO) generate EBV specific restricted CTL (Merkenschlager and Beverley 1989). Limiting dilution analysis of CTL precursor (CTLp) frequency gave straight lines in the Poison analysis suggesting that no other cell type was required for generation of CTL from CTLp. The frequency of CTLp among CD45RO T cells varied between 1:16,000 and 1:49,000 while in the CD45RA population it was <1:100,000. We further showed that in bulk cultures purified CD8+ UCHL1+ cells alone were able to generate CTL when stimulated with autologous EBV lymphoblastoid cell lines (EBV-LCL). This is in line with earlier data suggesting that CD8 cells alone are capable of inducing regression of autologous EBV infected B cells (Crawford et al. 1983) and strongly suggests that CD8+UCHL1+ cells are a memory cytotoxic population.

It is not at present clear how this data fits with reports that allospecific CTL and CTLp express CD28 while suppressor cells are CD11b+ (Yamada et al. 1985). It remains to be determined whether EBV specific CTL express CD28 and whether CD8+CD11b+ T cells contain both CD45RA+ and CD45R0+ subsets. If these exist they might represent naive and memory suppressor cells.

MATURATION OF T CELLS.

Since it appears that in both the CD4 and CD8 sublineages, responses to recall antigens are located within the CD45R0 subpopulation, we and others have suggested that CD45R0 cells may represent a memory T cell population while CD45RA are naive or virgin cells (Fig. 1). This hypothesis is supported by the observation that when CD45RA cells are stimulated in vitro with mitogens or alloantigens CD45R0 is rapidly expressed and there is a decline in the expression of CD45RA (Akbar et al. 1988). Others have reported that Il-2 dependent T cells are also CD45RA- and UCHL1+ (Serra et al. 1988). Whether CD45RA continues to be expressed on some T cells after activation is still a matter of debate though it has been reported that after Con A activation of T cells, there is a transient increase in expression of CD45RA which then decreases but does not disappear by 21 days (Takeuchi et al. 1989). These cells apparently maintain distinct functional properties, leading to the suggestion that the expression of different CD45 isoforms is not solely a marker for T cell differentiation. However, others have reported that CD45RA cells stimulated with PHA and maintained in IL-2 acquire the ability to provide help (Clement et al. 1988), so that the functional capabilities of CD45R0+ cells derived from the CD45RA pool may depend on the mode of activation.

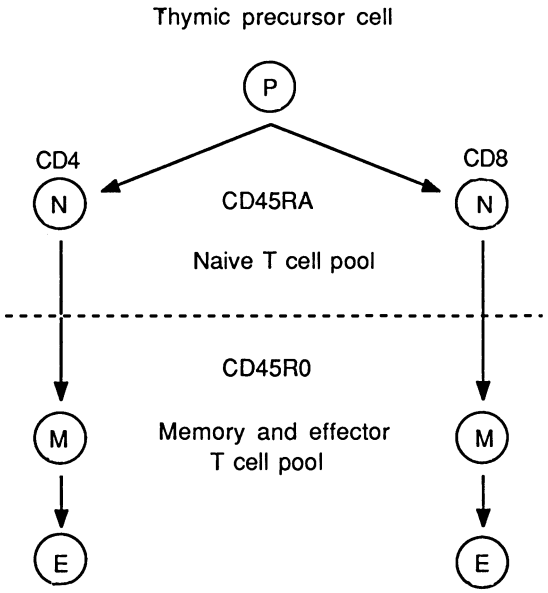

Fig. 1. Scheme showing the maturation of CD4 and CD8 peripheral T cells from a thymic double positive precursor to CD45RA+ naive and CD45R0+ memory and effector cells

That there is a relationship between expression of different isoforms of CD45 and maturation of peripheral T cells is supported by data from the rat. Results obtained in this species have shown that CD4+ helper cells for a secondary antibody response antibody lack the antigen detected by the Ox-22 mAb but derive from naive precursors which are Ox-22+ (Powrie and Mason 1988). Ox-22 is directed against a determinant on the B exon of CD45 and is therefore not directly analogous to CD45RA mAbs. However, in both species expression of high molecular weight CD45 isoforms appears to be a property of naive, and low molecular weight

isoforms of memory T cells. In the mouse the situation is less clear at present (see below).

Interestingly recent data on the ontogeny of T cell subsets in man shows that among both TCR αβ and γδ T cells, the majority are CD45RA+ at birth but there is a gradual increase in the proportion of CD45RO+ cells to reach adult levels by approximately 16 years of age (Hayward et al. 1989). If the expression of CD45RO is accepted as a marker for memory these data can be taken to indicate the development of a pool of primed TCR γδ cells as well as αβ cells.

PROPERTIES OF NAIVE AND MEMORY T CELLS.

We have chosen to use mAbs to CD45 isoforms to separate T cells with high expression of CD45RA or RO because this allows isolation of either population by negative selection, so that the cells do not carry over bound mAb into the in vitro cultures. However other mAbs have been used to achieve separation of functionally similar subpopulations (Sanders et al. 1988). Thus it has become clear that naive and memory T cells differ not only in expression of CD45 isoforms but show major quantitative differences in the expression of several other surface antigens (Table 1). We have confirmed these results by double staining of unseparated T cells or by restaining negatively selected CD45RA and RO cells (Wallace, unpublished data)

Table 1. Phenotype and function of naive and memory T cells

	Naive cells	Memory cells	Activated cells
Phenotype			
CD45R	++	-	-or+
UCHL1	-	++	++
CD29 (VLA β chain)	-	++	++
CD44 (Pgp-1)	+	++	++
CD18/CD11a (LFA-1)	+	++	++
CD2	+	++	++
CD58 (LFA-3)	+	++	+++
CD54 (ICAM-1)	+/-	+	+++
CD25 (Il-2R)	-	+/-	+++
MHC class 2	-	+/-	+++
Function			
Response to recall antigens	-	++	
Response to alloantigens	++	++	
Response to mitogens	++	++	
Helper activity	-	++	
Cytokine production			
IL-2	++	++	
IL-4	-	+	
IFN-γ	+/-	++	

Several of the antigens showing differences in expression between CD45RA and R0 cells have been shown to be important in cell-cell interaction. Thus CD2, LFA-3, LFA-1 and ICAM-1 have all been shown to

play a role in adhesion (Shaw et al. 1986, Springer et al. 1987). CD2 is also capable of transmitting an activation signal. It is perhaps not surprising that several groups have reported that CD45RO cells respond well to CD2 mAbs while CD45RA cells do not (Huet et al. 1988, Sanders et al. 1988), although there is one report showing the converse (Morimoto et al. 1988). The mechanism underlying this remains unclear since CD2 mAbs induce a rise in intracellular free calcium ion concentration in both subsets and in our hands CD45RA cells can respond to CD3 mAbs(Morimoto et al. 1988 and Wallace, unpublished data).

While the difference in expression of these adhesion molecules on CD45RA and RO cells is not very large, both subsets show a much larger increase of LFA-3 and ICAM-1 following activation in vitro (unpublished data). The peak of expression occurs at 2-3 days following PHA stimulation in either subset. This suggests that while the (lower) expression of these molecules on resting T cells may play a role in the initial events in T cell activation, the much higher levels expressed following activation, which decline as cell proliferation decreases late in a PHA response, play a role either in maintenance of cell proliferation or in effector function or in determining the migration pattern of the cells. There is evidence for the latter two roles in the ability of mAbs to adhesion molecules to block cytotoxicity and in the observation that CD45RO cells adhere preferentially to endothelium and accumulate in sites of inflammation (Cavender et al. 1988, Pizalis et al. 1987).

If LFA-3 and ICAM-1 can be considered as activation antigens, this raises the question whether CD45RO cells should be judged to be activated. Additional evidence that this might be the case is the slightly larger cell size of this population and the expression of low levels of CD25 and MHC 2 antigens on these cells (Buckle and Hogg 1989). Whether this indicates that T memory cells are continually antigen driven, as may be the case for B cells (Gray and Skarvall 1988), remains to be determined.

As well as the well characterised adhesion molecules discussed above,other molecules such as CD29 and CD44 show increased expression on CD45RO T cells (Sanders et al. 1988). CD29 is one of the β chains of the integrin family of molecules which play a role in platelet adhesion while CD44 has been suggested to be an addressin. Both these molecules may therefore play a role in T cell migration. While rodent data on the phenotypes of naive and memory cells is less extensive than in man, increased expression of Pgp-1, the murine homologue of CD44, has been shown to characterise memory T cells in at least some strains of mice (Budd et al. 1987).

HETEROGENEITY OF MEMORY CELLS.

In the mouse two distinct categories of CD4 T cells clones have been described (Mossmann et al. 1986), differing in their lymphokine production. Predominantly T_H1 or T_H2 clones are obtained by different immunisation schedules. The two types of clone can be identified phenotypically by the 16A mAb which is against the B exon of CD45. This mAb was produced by immunisation with a T_H2 (IL-4 producing) T cell clone and stains T_H2 but not T_H1 clones (Bottomly et al. 1989). Paradoxically the mAb separates among fresh T cells a 16A high subset which produces mainly Il-2, from 16A low cells which produce IL-2 and Il-4. The latter result is similar to earlier findings with the Ox-22 mAb in that Ox-22+ cells produce more IL-2 and Ox-22- cells are helpers

for a recall antibody response(Arthur and Mason 1986), but Ox-22+ and Ox-22- cells have a precusor-product relationship and are not distinct sublineages of T cells as has been suggested for the 16A high and low cells. However it has not yet been tested whether 16A high cells lose the antigen on stimulation.

In man further phenotypic heterogeneity of CD45RO cells can be detected with several mAbs but it is not yet clear whether the further subsets separated for example, with Leu8 or CD29, represent sublineages or further steps in maturation. But such subsets isolated from fresh T cells differ considerably in cytokine production (Salmon et al. 1988, Hedlund et al. 1989).Human T cell clones are also heterogeneous with respect to cytokine production (Paliard et al. 1988) though two broad categories, differing in cytolytic activity and the spectrum of cytokines produced, have been described (Rotteveel et al. 1988). It is not yet clear how different the different types are related to the subsets of cells detected in freshly isolated T cells.

CONCLUSION

In man heterogeneity of peripheral T cells detected by mAbs to different isoforms of CD45 is probably most closely related to the maturation state of the cell, both in the CD4 and CD8 lineages (Fig. 1). The tyrosine phosphatase activity of the cytoplasmic domain (Charbonneau et al. 1988) and the association at the cell surface of CD45 with other molecules such as CD2 (Schraven et al. 1988), suggest mechanisms by which CD45 may regulate the function of different T cells. It is not yet clear whether heterogeneity detected with some other subset markers such as Leu 8, indicates further stages in T cell maturation or stable sublineages within CD4 or CD8. The existence of such further sublineages is indicated by murine data demonstrating two major categories of CD4 T cell clones.

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Characterization of the Secondary Antibody Response Using Purified Memory B and T Cells

K. Hayakawa and R.R. Hardy

INTRODUCTION

Multicolor immunofluorescence analysis of antigen binding cells allows us to detect and purify memory B cells in a highly specific manner. We use the fluorescent protein PE (phycoerythrin) as an antigen in order to reveal antigen binding cells (PE^{+++}) (Hayakawa, 1987) and simultaneously permit analysis of the expression of other cell surface molecules. In addition, we can resolve a memory T cell subset which provides help for antibody secretion in the secondary response from the total $CD4^+$ T cell population by expression of particular determinants (Hayakawa, 1988). Using this approach, we find that the secondary antibody response by long-term memory B and T cells can be uniquely characterized as follows.

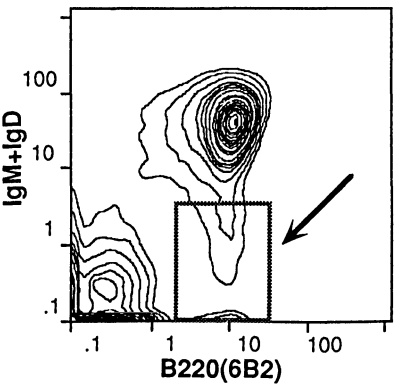
MEMORY B CELLS

Expression of the 6B2 epitope of B220 distinguishes between antibody secreting cells and mature memory B cells.

Several antigens show differential expression between antibody secreting cells and memory B cells (Hardy, 1984). Most such determinants are found on antibody secreting cells (but not on the memory B cells) due to their induction following cell activation. In comparison, expression of the 6B2 epitope of the B220 molecule is unique. An epitope of CD45R (B220-6B2; 6B2 in brief) is expressed on memory B cells at the level of resting B cells but at reduced or negligible levels on antibody secreting cells.

The 6B2 epitope recognized by the antibody RA3-6B2 (Coffman, 1982) is expressed on the surface of IgM^+ B cells but not on the other classes of lymphocytes. Non-specific activation of B cells results in decreased 6B2 expression by most B cells, and in fact, specific antibody secreting cells are enriched in the $B220\text{-}6B2^{low/-}$ fraction after antigen priming (data not shown). In contrast, we find that memory B cells long after antigen priming are $6B2^{++}$. Furthermore, a large part of such memory B cells are immunoglobulin isotype-switched cells as Black et al. showed previously (Black, 1977). Thus, sorting of $6B2^+(IgM,IgD)^-$ cells, constituting only a small fraction (1-2%) of spleen cells (shown in Fig. 1), enriches for IgG memory B cells which, when appropriately stimulated, secrete high affinity antibody, typical of the secondary antibody response.

Figure 1



Antigen binding memory B cells are enriched in the 6B2$^+$(IgM, IgD)$^-$ cell fraction.

Since memory B cells express surface immunoglobulin, they can be directly revealed as antigen binding cells. The fluorescent antigenic protein, phycoerythrin (PE), is useful for this purpose. Combined with the fact that a large portion of memory B cells are already immunoglobulin isotype-switched cells (retaining 6B2 expression as a B cell marker), we can highly purify memory B cells away from primary virgin B cells in long-term primed mice.

Figure 2

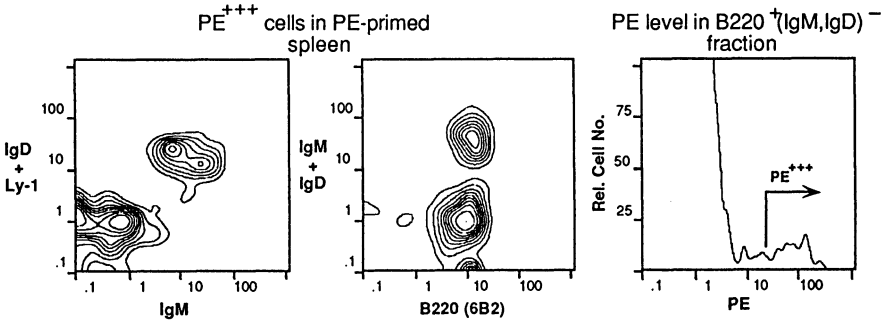


Fig. 2 demonstrates that antigen (PE) binding cells are indeed enriched in the 6B2$^+$(IgM,IgD)$^-$ population. Their affinity for antigen is apparently high since despite the fact that the amount of surface immunoglobulin is lower than that of IgM$^+$ B cells, the average PE brightness in this cell fraction is the highest compared with other cell fractions. These PE^{+++} cells comprise only 1-2 % of the 6B2$^+$(IgM,IgD)$^-$ cell fraction (providing considerable enrichment, Fig. 2) and since they are only detected in PE primed mice, they are clearly induced by antigen priming.

Memory B cells present antigen to T cells without further accessory cells.

PE binding cells in the 6B2$^+$(IgM,IgD)$^-$ cell population are functional memory B cells since they begin to secrete antibody only when incubated with PE-specific T cells. They are highly purified isotype-switched memory B cells: as few as 50-500 PE^{+++} cells can be used to elicit detectable IgG (IgG_1) secretion in an *in vitro* culture system (Table 1).

Table 1 Antigen presentation by memory B cells to memory T cells in the secondary antibody response

PE^{+++}6B2$^+$(IgM,IgD)$^-$ cells (500/well)	CD4$^+$ (Ia$^-$) T cells ($1x10^5$/well)	IgG1 anti-PE (ug/ml)
+	(–)	<0.01
+	PE primed	6.60
+	DNP-KLH primed	0.01

Since further addition of antigen (beyond the amount already bound to PE-pulsed memory B cells) is not required for initiation of the antibody response, this must mean that memory B

cells are efficient in presenting antigen to helper T cells. The antigen "pulse" of memory B cells occurs rapidly and does not require a high antigen concentration when compared to the primary antibody response which is initiated by different antigen presenting cells (macrophages).

Secretion of antibody from memory B cells is largely dependent on antigen binding to cell surface immunoglobulin.

By restricting the response to the 6B2$^+$(IgM,IgD)$^-$ cell fraction as a memory B cell source, we can study how memory B cells are activated to secrete immunoglobulin and how this process differs from naive B cells. Table 2 demonstrates that "by-stander" activation occurs with memory B cells (as with naive B) in consequence of T cell activation; however, it is further dependent on antigen binding (cells must be antigen pulsed). Thus, antigen is required for induction of secretion from memory B cells under conditions of the normal secondary antibody response.

Table 2 Bystander antibody secretion from memory B cells is largely dependent on antigen binding.

Co-culture						
DNP-KLH primed spleen			PE primed spleen		IgG1 (ng/ml)	
Ag Pulse	memory* B cells	CD4$^+$ T	Ag Pulse	memory* B cells	anti-DNP	anti-PE
DNP-KLH	+	+	PE	+	2190	240
DNP-KLH	+	+	–	+	2160	<3

* 6B2$^+$(IgM,IgD)$^-$ cells

MEMORY T CELLS

Memory helper T cells for the secondary antibody response are restricted to the 6C10$^+$ CD4$^+$ T cell subsets.

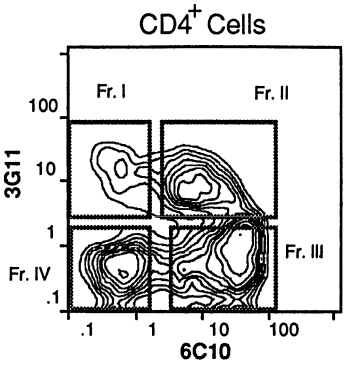
It has been known for some time that the T cells which help antibody secretion are CD4$^+$. As was found with B cells, CD4$^+$ T cells can also be fractionated into subpopulations by cell surface molecule expression. Among the four CD4$^+$ T cell subsets defined by 6C10 and 3G11 expression (Fig. 3), 3G11$^-$ cells appear to be enriched for more mature cells (Hayakawa, 1988; Hayakawa and Hardy, mss. in prep.). In particular, the Fr III cells generated under normal environmental conditions express high levels of Pgp-1, lack CD45R-DNL1.9 and secrete IL-4 (without IL-2) following Con A activation.

We found that memory helper T cells for the antibody secretion are restricted to the CD4$^+$ subsets which can respond to B cell accessory function: the 6C10$^+$ subsets, Fr II and Fr III. In older mice long after priming, memory function was finally restricted to the 3G11$^-$ Fr III, probably due to further maturation of Fr II cells. Fig. 3 illustrates such CD4$^+$ subsets in old (PE primed) mice. After restimulation with antigen (by pulsed memory B cells) memory T cells produce lymphokines which are involved in the proliferation and differentiation of memory B cells. One of these is IL-4 which may play a role in the increase efficiency of antigen presentation by B cells (increase of Ia expression) and also in the

Figure 3

Helper memory T cells in old mice are restricted to Fr III

CD4+ T cell fractions	anti-PE antibody IgG1 (ug/ml)	IgG2a (U./ml)
unseparated	7.3	380
6C10+	13.0	135
6C10−	<0.1	<0.2
6C10+ Fr II	<0.1	<0.2
6C10+ Fr III	17.0	240



proliferation of memory B cells. Thus, the memory T cells for antibody secretion seem to be characterized as cells prepared for B cell antigen presentation and also capable of providing factors which enhance memory B cell function.

SUMMARY

The generation of memory B cells and T cells is associated with an alteration of cell surface molecule expression permitting their enrichment and isolation. High affinity immunoglobulin expression on memory B cells allows B cells to efficiently present antigen to memory helper T cells. Memory T cells, induced following the primary response, respond preferentially to antigen presentation by B cells and thereupon secrete lymphokine(s) appropriate for B cells. Finally, antibody is secreted from the progeny of memory B cells which initially bound antigen. These observation would explain why a low dose of antigen initiates the specific, rapid and high magnitude of response characterized as the secondary immune response.

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The Production and Selection of Memory B Cells in Follicles

I.C.M. MacLennan, Y.-J. Liu, D.E. Joshua, and D. Gray

THE NATURE OF MEMORY B CELL CLONES AND THEIR DEPENDENCE ON ANTIGEN FOR SURVIVAL

The recruitment of virgin B cells into responses to T cell-dependent antigens has been found to be restricted to periods immediately following antigen administration. The established phase of these responses, on the other hand, is maintained by activation of persistent memory B cell clones (Gray et al. 1986, MacLennan and Gray 1986). Memory B cell clones which come to dominate T cell-dependent responses appear to be able to survive throughout the life of mice and rats (Askonas et al. 1970, Gray et al. 1986). They can be transferred to syngeneic or congenic hosts where they again can dominate responses to the same antigen. Following transfer it is necessary to administer antigen to the recipient to initiate antibody production (Askonas and Williamson 1972, Siekevitz et al. 1987). Also, although transferred B cell memory will persist in the absence of antigen for a little over a month, after this period a response from the donor cells can no longer be elicited (Gray and Skarvall 1988).

Memory B cells which are not in cell cycle can be found both in the recirculating B cell pool (Strober 1975) and among the static B cells of the marginal zones of the spleen (Liu et al. 1988). Available evidence indicates that neither of these populations of memory cells will survive for more than a few weeks unless they are reactivated by antigen. As T cell-dependent antibody responses can last for months or even years, it follows that both recirculating and marginal zone memory cells are being generated from proliferating precursors throughout the response; i.e. a memory B cell clone must have some of its members in cell cycle if it is to survive and dominate antibody responses for months or years.

The blasts which are required to maintain B cell memory must also continue to produce plasmablasts, if antibody production is to be maintained. Plasmablasts migrate either to the bone marrow (Benner et al. 1981) or the lamina propria of the gut (Gowans and Knight 1965), depending upon the lymphoid tissue where they are generated. Plasma cells in these sites have a life-span in the order of a month (Ho et al. 1986). It follows that they also must be renewed in antibody responses which continue for many months.

The B cell follicles, which are found in all secondary lymphoid tissues, provide the most likely site for the B cell proliferation 2 which is required to sustain memory B cell clones. In responses to hapten-protein antigens hapten-specific B blasts can be found in the follicles of secondary lymphoid organs for some months following antigen administration (MacLennan et al. 1988). It seems probable that activation of these blasts is dependent upon antigen held on follicular dendritic cells (FDC). FDC have been shown to retain antigen for protracted periods (Tew and Mandel 1979). There is some conceptual difficulty in accepting that antigen could persist on follicular dendritic cells for years. Consequently the possibility that responses may be maintained by anti-idiotype rather than antigen has been raised. Established T cell-dependent antibody responses, however, can not be transferred with lymphocytes unless the recipient is given antigen. Consequently, if such an idiotype:anti-idiotype network does serve to maintain long-term antibody production, it is destroyed if the microenvironment sustaining B blasts is disrupted.

AFFINITY MATURATION OF T CELL-DEPENDENT ANTIBODY RESPONSES

As T cell-dependent antibody responses progress the average affinity of the antibody produced increases. This, in most instances is associated with somatic mutation in rearranged immunoglobulin V region genes (Gearhart et al. 1981, Bothwell et al. 1981, Tonegawa 1983, Griffiths et al 1984). Somatic mutation occurs selectively in immunoglobulin heavy and light chain V region genes implying they are the target for a process of hypermutation. Analysis of the time of appearance of mutations in the rearranged immunoglobulin V region genes indicates that they first appear shortly after the onset of antibody production. The number of mutations increases rapidly over the next 2 or 3 weeks (Berek et al. 1985). Transferred memory B cell clones, however, can undergo extensive proliferation in the recipient without further V region mutation (Siekovitz et al. 1987).

These data indicate that somatic mutation is occurring at times when germinal centres are present within follicles. This and other features of germinal centres have led to the hypothesis that the proposed V region hypermutation mechanism is activated in centroblasts within germinal centres (MacLennan and Gray 1986).

GERMINAL CENTRE FORMATION

Germinal centres arise within B cell follicles during T cell-dependent antibody responses. They start to develop shortly after the onset of antibody production. Their formation is antigen-dependent (Thorbecke et al. 1975) and in most circumstances appears to require T cell-help (Jacobsen et al.1974).

There are three distinct phases to the follicular response to T cell-dependent antigens. Resting follicles comprise a rich network of FDCs; the spaces between the FDC being filled with small recirculating B cells. The appearance of B blast cells within the FDC network marks the start of the follicular response. These blasts, which express immunoglobulin on their surface, proliferate rapidly until they come to fill the spaces in the FDC network. The small recirculating lymphocytes in this way are displaced to the edge of the follicle to form the follicular mantle.

When the B blasts have reached confluence in the follicle centre, a process which takes approximately 2 days, the second stage of the follicular reaction begins. This stage is associated with the appearance of classical germinal centres. The B blasts give rise to centroblasts which accumulate at one pole of the follicle (i.e. adjacent to but not within the FDC network). The area occupied by these cells is termed the dark zone of the germinal centre. Centroblasts show a number of phenotypic differences from the follicular B blasts. In the present context their failure to express immunoglobulin is perhaps most significant. At this time the number of B blasts within the follicle centre diminishes markedly and the FDC network fills with non-cycling cells known as centrocytes. Labelling studies with tritiated thymidine (3HTdr) show that up to half the centrobasts incorporate this agent into their DNA during a 30 minute pulse labelling period in vivo (Fliedner et al. 1964). Few centrocytes are labelled at this stage. After administering 3HTdr for 24 hours, however, more than 90 per cent of centrocytes are labelled. These data imply that although centrocytes are not in cell cycle they are continually being replaced from centroblasts. Many of the centrocytes die in situ. This can be deduced by the finding of nuclear fragments within local macrophages. After 24 hours' 3HTdr infusion these nuclear fragments are also labelled. Some centrocytes, however, survive to become memory B cells (Klaus et al. 1980, Coico et al 1983).

During the transition from centroblast to centrocyte immunoglobulin again becomes expressed at the cell surface. It seems possible that the high death rate among centrocytes in vivo is associated with selection of these cells on the basis of their affinity for antigen localized on FDC. Evidence in favour of such a selection process has been obtained by analysis of germinal centre cells isolated from human tonsil (Table, Liu et al. 1990). Although these cells show good viability after isolation at $4^{o}C$, they begin to kill themselves by apoptosis (Wyllie et al. 1984) if they are cultured at $37^{o}C$ (Table). They can be prevented from entering apoptosis, however, if they are cultured with anti-immunoglobulin conjugated to sheep red cells. Rescue from apoptosis is more impressive if the CD40 antibody G28-5 (Clarke and Ledbetter 1986) is also added to the cultures (Table). None of the following prevented these cells entering apoptosis: soluble anti-immunoglobulin, interleukins 1, 2, 4, 5 or 6, mixed leukocyte culture supernatant, gamma or alpha interferon, CD23 antibody.

The second phase of the follicular response declines during the third week after antigen administration By the fourth week centroblasts and centrocytes are no longer apparent, but B blasts can still be found in the heart of the FDC network. At this stage the volume occupied by the blasts is less than a tenth of that at the height of the follicular reaction. These foci of B blasts can be identified for several months following antigen administration (MacLennan et al. 1988). Centroblasts and centrocytes are only found during stage 2 of the follicular reaction. As this stage is effectively over 3 weeks after antigen administration these cells cannot be the direct source of memory B cells and plasma cells which are generated in the later stages of the response. It is likely that the chronic B blasts of stage 3 of the follicular response are derived from cells which have participated in germinal centre reactions.

Table
Self destruction of isolated germinal centre cells is prevented by activation through their surface receptors for antigen and CD40

B cell fraction (1)	Additives to culture	Per cent cells viable after culture at 37 C for:	
Small resting (2)	none	96-99	60-81
Germinal centre cell-depleted (3)	none	87-95	68-90
Germinal centre cell-enriched (4)	none	26-14	0
Germinal centre cell-enriched	anti-Ig SRBC	82-70	27-19
Germinal centre cell-enriched	CD40	87-63	55-23
Germinal centre cell-enriched	CD40 + anti-Ig SRBC	92-80	82-68

(1) B cells isolated from human tonsil cell suspensions by removing T cells by two cycles of rosetting with AET-modified Sheep red blood cells (SRBC) and separating these on Ficoll-Paque.
(2) Cells of the B cell fraction which penetrated a 60 per cent Percoll gradient.
(3) Cells of the B cell fraction not penetrating a 60 per cent Percoll gradient and not rosetting with CD38-coated SRBC. CD38 is expressed by all germinal centre B lineage cells but not by follicular mantle or most extra-follicular B cells.
(4) Cells of the B cell fraction not penetrating a 60 per cent Percoll gradient which did not rosette with either IgD-coated or CD39-coated SRBC. CD39 is expressed on tonsil B cells outside germinal centres.

THE PRECURSORS OF CENTROBLASTS AND CHRONIC FOLLICULAR B BLASTS

There is evidence that recirculating memory B cells but not virgin cells can be activated by antigen pre-localized on follicular dendritic cells (Gray 1988, MacLennan et al. 1989). The ease with which B blasts, which occupy antigen-retaining sites on FDC, are displaced by recirculating memory cells remains to be determined. While memory B cells can give rise to stage 3 follicular B blasts it is not clear if they are capable of generating centroblasts and centrocytes. Comparison of the magnitude of germinal centre reactions during primary B cell responses in conditions where T cell help is not limiting with that in secondary B cell responses suggests that they may not. In these experiments the response to hapten-protein conjugate in rats primed with the protein-carrier was compared to that in rats primed with carrier but also boosted with hapten-carrier before tertiary immunization with hapten-carrier. It was found that the peak germinal centre volume in the rats undergoing the primary anti-hapten response reached a median of 4.2 per cent of the splenic volume (range 3-5.6). In the rats re-immunized with hapten carrier the median germinal centre volume only reached 1.2 per cent (range 0.9-1.8) (MacLennan et al. 1988). Further work is required to clarify this point.

SUMMARY AND CONCLUSIONS

This brief review has discused the maintenance both of long term antibody production and B cell memory during the established phase of T cell-dependent antibody responses. It is concluded that both rely on chronic activation of memory B cell clones by antigen retained on follicular dendritic cells. Indirect evidence is cited which suggests that a mechanism resulting in hypermutation in rearranged immunoglobulin V region genes is activated in centroblasts in germinal centres. It is shown that centrocytes the progeny of centroblasts kill themselves by apoptosis unless they can be activated through their receptors for antigen. This provides a mechanism by which B cells with high affinity for antigen are selected.

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II Cell Developments

Neoplastic Transformation of Lymphoid Cells and Myeloid Cells

Tumors of Lymphoid and Myeloid Cells: Introduction

M. Potter

INTRODUCTION

This symposium on tumors of lymphoid and myeloid cells (leukemias and lymphomas) focuses primarily on the issue of pathogenetic mechanisms. Essentially neoplastic development is a series of genetic and biochemical changes that begin in a normal cell and culminate in the formation of an autonomously growing cell. A prevailing concept is that neoplastic transformation results from multiple changes, the majority of which are generally considered to be mutations (alterations in the genomic structure of DNA). The gene targets of these mutational processes are called protooncogenes. There is growing evidence that supports the mutational theory. Most often only a single but highly consistent mutation has been found, rather than a series or a program of mutations. Well studied examples of these are the retroviral insertions and chromosomal rearrangements that target on the c-myc proto-oncogene in B cell tumors. The consistency with which the single mutations have been found in some systems has raised the possibility that second and more mutational changes can be found. However there is currently a paucity of evidence of multiple mutations in the natural history of a single tumor, but this may well be due to the limited number of genes examined.

There should be some caution though in attempting to explain all of neoplastic development and progression in mutational terms. The development of several of different tumors in fixed tissues provide some evidence for the role of adaptive changes in tumorigenesis (Rubin 1985; Farber 1984). Most of these are caused by the disruption of the organization of fixed tissues and the ensuing adaptation of cells to new conditions. Essentially the underlying concept of adaptation is that genes inappropriate to the stage of development or even differentiation are activated. Physiological adaptation is more difficult to assess in hematopoietic tissues where the cells normally are only loosly attached to other cells and continuously expended and replaced. But there may be forms of physiological adaptation that occur in these populations that we do not recognize easily. Conditions that would enhance the survival of cells in face of the mechanisms that normally lead to elimination and cell death present one possible abnormal phenotype. For example there is considerable evidence that certain hematopoietic cells can be immortalized in vitro without acquiring the ability to grow autonomously when returned to the intact animal. Similar phenotypes in neoplastic cells however may be caused by mutational rather than adaptive changes; the bcl-2 activation by the t(14;18) translocation in follicular lymphomas may be such as example. Possibly these mutations might begin a process of adaptation.

ACTIVE AND PASSIVE FORMS OF TUMOR INDUCTION

As is well known there are a great number of different kinds of leukemias and lymphomas and these are related to each other by their origin from the pluripotential hematopoietic stem cell. There are probably certain common features in all hematopoietic tumors. Since it is not possible to discuss the tumors of all of the lineages in depth this symposium will concentrate on model systems involving tumors of B and T lymphocytes where a very extensive number of pathogenetic studies are currently being carried out. It is relevant to the general discussion to recognize two general kinds of tumor induction processes relevant to the B and T lymphocytic lineages which are for the sake of discussion referred to as 'active' and 'passive' modes of tumors formation. Active meaning the basic genetic changes are generated in the cells during tumorigenesis; passive meaning the oncogenic mutations are from another source are supplied to the cells.

ACTIVE FORMS OF TUMOR DEVELOPMENT

In the 'active' forms of tumorigenesis an inducing agent and/or a set of promoting conditions initiate and drive the process of tumor progression (Table 1). The 'active' forms of tumorigenesis include the familiar experimental model systems where mutagenic agents such as the aromatic polycyclic hydrocarbons or nitrosourea compounds are used as inducing agents. A second common experimental system is tumor formation in chickens, mice or cats that express endogenous retroviruses or acquire them by infections. It is thought that tumors arise in these animals chiefly by the process of insertional mutagenesis resulting from the integration of retroviruses into host genes. The most important group (which includes many of the common neoplasms in man) however consists of the so called 'spontaneous' tumors where triggering agents are not yet identified. A fourth group is comprised of various tumor formation systems where the putative inducing agents are not known to be mutagenic (Table 1). In this group of pathogenetic processes the agents may be inducing special conditions such as immunodeficiency or hyperactivation of target cells or other cell types that facilitate or promote the formation of tumors. In all of these 'active' forms, mutations must be generated directly or indirectly by the inducing agents, or arise (in spontaneous tumors) by naturally occurring processes. A remarkable feature of the active hematopoietic forms of tumorigenesis is the ability of certain agents to induce tumors in a specific differentiation lineage.

The phenomenon of tumor promotion is well known in the carcinogenesis literature but it is still questionable whether there is a correlate in the hematopoietic tumors. The classical model of initiation and promotion is epidermal carcinogenesis in mice. In this system mutagenic carcinogens can be applied to the skin in doses that are non-tumorigenic. This step initiates the process of carcinogenesis. The striking features of initiation are first persistence of the initiated state and second the apparant lack of effect of the initiated state on normal epidermal proliferation. Berenblum and Shubik (1949) showed many years ago that the initiated state can persist for as long as a year in the mouse. There is now considerable evidence that base substitution mutations in *ras* genes may be initiating

ACTIVE FORMS OF B- AND T-CELL TUMORIGENESIS

Form	Oncogenes	Examples*	Ref
SPONTANEOUS (no known causative agent)	bcr-abl	Acute B-cell leukemia	a
	bcl-2	Follicular lymphoma	b
	c-myc	Sporadic Burkitt's lymphoma	c
	c-myc	Immunocytoma (rats)	d
	none so far	Chronic lymphocytic leukemia	
		Multiple Myeloma	
IN RETROVIRUS INFECTED HOSTS (insertional mutagenesis)	c-myc	Bursal Lymphoma (chickens)	e
	c-myc, Pim-1	AKR thymic lymphomas (mice)	f
	c-myc	T-cell leukemias (cats)	g
IN VIRUS INFECTED HOSTS (insertional mutagenesis is not the mechanism	c-myc	Endemic Burkitt's lymphoma (EBV, Falciparum Malaria)	c
	c-myc	HIV B-cell lymphomas	h
IN HOSTS TREATED WITH NON-MUTAGENIC AGENTS	c-myc	Pristane-induced plasmacytomas	i
IN HOSTS TREATED WITH MUTAGENIC AGENTS		Carcinogen (APC) induced T-cell leukemias (mice)	j
	K-ras, N-ras	X-ray and MNU induced thymic lymphomas (mice)	k

Table 1. Footnotes

This is not an exhaustive list. APC=aromatic polycyclichydrocarbons, MNU=methyl nitrosourea a: Clark et al 1987; Chan et al b: Yunis et al 1987. c: Neri et al 1988 d: Bazin et al 1988 e: Hayward et al 1981 f: Cuypers et al 1984 g: Neil et al; h: Subar et al 1988; Potter 1972; j: Ishizaka and Lilly 1987; k: Diamond et al 1988

mutations in the mouse epidermis (Quintilla et al 1986; Roop et al 1986). Non-mutagenic factors such as the phorbol esters can stimulate initiated mouse epidermal cells to proliferate abnormally into papillomas. This raises the possibility that some potential oncogenic mutations in hematopoietic cells could also remain latent until appropriately influences permit them to proliferate.

The promoting action of phorbol requires the continual presence of the agent. It has been suggested that there are counterparts of promotion in hematopoietic tumor formation. For example in endemic Burkitt's lymphoma, Falciparum malaria infections may act as a general stimulant to B-cell proliferation (Weidanz 1982; Playfair 1982 and Kataaha et al 1984). The pristane induced oil granulomatous tissue may provide a promoting microenvironment for plasma cell proliferation by supplying growth factors or reactive oxygen intermediates. It might also be argued that the bursal and thymic microenvironments serve as promoting influences for the development of tumors by supplying unique sources of natural growth stimulating factors.

In many experimental model systems the genotype, sex and age of the host frequently play a major determining role in tumor development. In systems employing genetically defined animals such as inbred strains of mice rats and chickens differences in susceptibility and resistance to developing certain kinds of tumors have been observed. These strain differences suggest that allelomorphic forms of genes that are not deleterious to the host may influence the course of tumor progression. This of course remains to be proven as susceptibility and resistance genes potentially can act at two levels. First they may participate in the processing of inducing agents or secondly they may act in the target cell itself. In the first case, susceptibility/resistance genes may only be influential for specific inducing agents. In the second case such genes might act in concert with oncogenes to influence the response of the target cell. The nature of susceptibility and resistance genes is not yet known.

'PASSIVE' FORMS OF TUMORIGENESIS

In the 'passive' forms of tumorigenesis preformed oncogenes are introduced into cells by a variety of methods such as transfection, injection or infection by retroviruses (Table 2). These oncogenes are not only regulated by unnatural promoters and enhancers but they may also have originated as mutations in different cell types e.g. viral oncogenes from a chicken, cat or mouse sarcomas may be utilized to induce B-cell or T-cell tumors. In many of the passive forms of B- and T-cell tumorigenesis the oncogenes are introduced in multiple copies or are transcribed excessively. The 'dose' of the oncogene and oncoprotein is greatly elevated, and this has been shown to affect transformation in vitro (Kovary et al 1989).

MIXED FORMS OF TUMORIGENESIS

There are also mixed forms of tumorigenesis in which the preformed oncogene requires specific conditions to be effective (Table 3). One example is rapid plasmacytoma induction by transforming retroviruses in BALB/cAn mice that depends upon pristane conditioning. In these systems the preformed oncogenes introduced by retroviral vectors are ineffective in untreated mice. The dose of pristane needed for priming is very low approaching non-plasmacytomagenic levels. In other situations the inducing agent such as a large dose of pristane, MoMuLV, or a chemical carcinogen directly or indirectly induces a mutation (by chromosomal rearrangement, retroviral insertion, base substitution or deletion) that cooperates with a preformed oncogene.

PASSIVE FORMS OF B- AND T-CELL TUMORIGENESIS

Form	Tumor	Ref
IN TRANSGENIC MICE		
Eμ-c-myc	Pre-B hyperplasia, clonal B-cell tumors	a
Eμ-N-myc	B-cell lymphomas	b
Eμ-v-abl	Plasmacytomas	c
Eμ-bcl-2	Follicular center hyperplasia	d
Eμ-Pim-1-LTR	T-cell lymphomas (low incidence)	e
BY INFECTION WITH TRANSFORMING RETROVIRUSES		
Abelson virus [abl]	Pre-B lymphoma (mouse)	f
cas NS1 virus [cbl]	Pro-Pre-B lymphoma (mouse)	g
REV-T (csv) [rel]	Mature B lymphomas (chicken)	h
HBI [myc]	B lymphomas (chicken)	i
BY TRANSFECTION INTO EBV IMMORTALIZED CELLS		
SV-Myc 2,3 [myc]	B lymphoma	j
SVN-N-ras [ras]	Ig-secreting lymphoma	k

Table 2. Footnotes

a: Adams et al 1985 b: Dildrop et al 1988 c: Harris et al 1988 d: McDonnell et al 1989 e: van Lohuizen et al 1989 f: Risser et al 1978 g: Langdom et al 1989 h: Barth and Hamphries 1988 i: Enrietto et al 1983 j: Lombardi et al 1987 k: Seremetis et al 1989.

MIXED FORMS OF B-CELL AND T-CELL TUMORIGENESIS

INDUCING AGENT	TRANSFORMING RETROVIRUS	TUMOR	REF
Pristane	J-3* [v-raf+v-myc]	Plasmacytoma	a
Pristane	RIM [Ha-ras + Eμ-myc]	Plasmacytoma	b
Pristane	YC-Leuk [A-raf + v-myc]	Plasmacytoma	c
Pristane [1.0 ml]	3611 [v-raf-1]	Plasmacytoma (low incidence)	d
Pristane [chromosomal translocation activates myc	AbMuLV [abl]	Plasmacytoma (low incidence)	e

INDUCING AGENT	TRANSGENIC MOUSE	TUMOR	REF
MoMuLV [insertion into c-myc, N-myc	EμPim-1-LTR	Thymic lymphoma	f
Ethylnitroso-urea	EμPim-1-LTR	T-cell lymphoma	g

Table 3. Footnotes

a: Potter et al 1987, Troppmair et al 1989 b: Clynes et al c: Potter M, et al unpublished d: Ohno S, et al 1984 e: Lohuizen et al 1989 f: Breuer et al 1989

PROLIFERATIVE ACTIVITES IN T AND B LYMPHOCYTES

The B- and T- lymphocytic lineages differ from other lineages in the hematopoietic system by having multiple proliferative compartments. These extended forms of proliferative activity apparently evolved for the development of highly specialized clones of cells and immunological memory. The most complex of these is B-cell development (Fig 1).

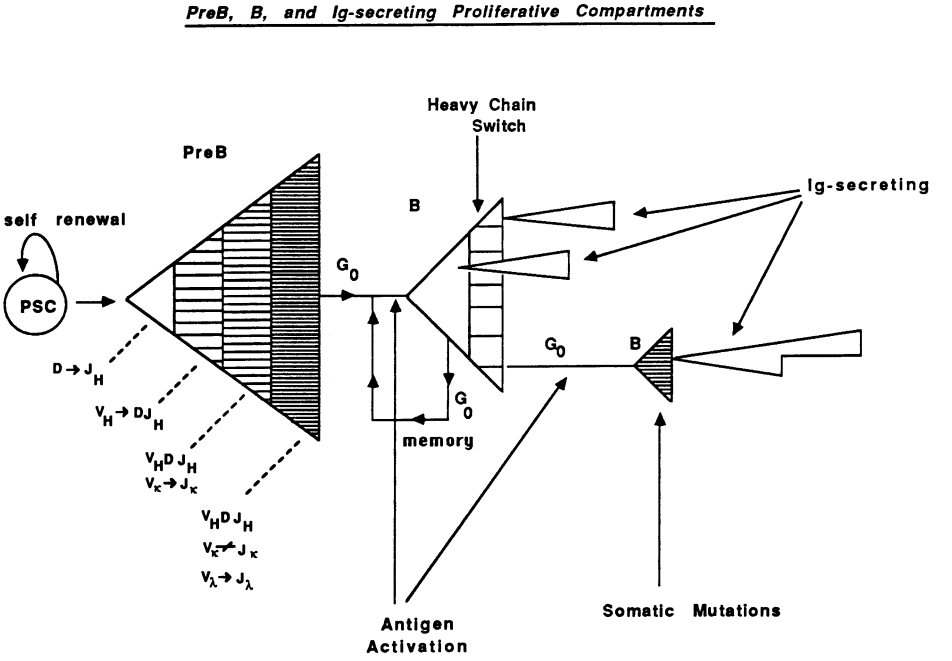

Fig 1. Scheme of B-cell development showing the proliferative compartments which are depicted as various sized triangles. There are three main stages of B-cell development, Pre-B, B and Ig-secreting. In the Pre-B and B-cell stages clonotype and subclonotypes develop through V-D-J rearrangements (in Pre-B), heavy chain switching and somatic mutations in the B cell stage. Ig-secreting populations arise from different populations of B-cells. As cells leave one proliferative compartment they enter Go phase of mitosis. Proliferation is followed by quiescence in which the cells are mitotically inactive. Thus B-cells must be reactivated to bring them from a Go phase of the mitotic cycle into G1.

The process of activation of B-cells from a Go state to a burst of mitotic activity in a clone is a complex process and probably requires multiple signals from antigen, cooperating T-cells and accessory cells. T-cells and macrophages are thought to transmit their signals to B-cells via polypeptide growth factors. B and T lymphocytes have multiple cell surface receptors (Clark and Ledbetter 1989) and respond a variety of different exogenous growth factors even during the course of a single round of mitosis (Crabtree 1989;

Dutton and Swain 1987; Loughnan and Nossal 1989; Lernhardt and Melchers 1987; Neckers and Cossman 1983). Further there is evidence in B-cell differentiation that the activation of one pathway may trigger the expression of a second receptor and hence open a new pathway that leads to the activation of an additional set of genes (Fig.2). Dysregulation of proliferative activities in B- and T-cells may require multiple mutations that affect different pathways.

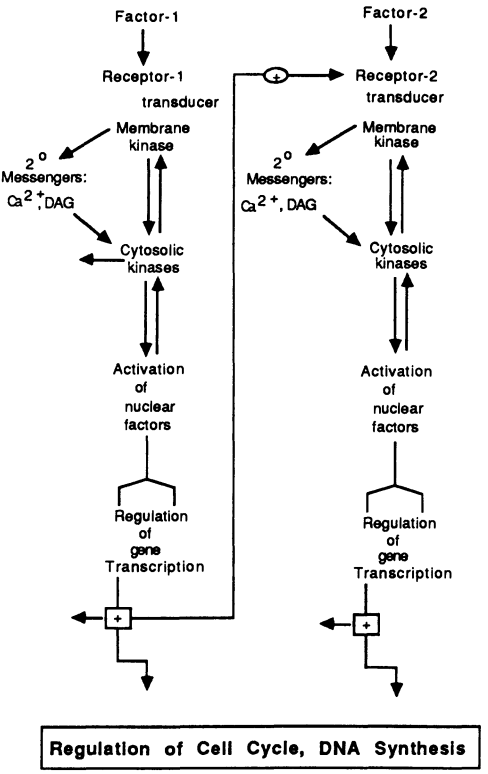

Fig 2. Hypothetical scheme depicting pathways that lead to the activation of genes essential for driving the cell through the mitotic cycle. Pathway-1 regulates the transcription of a gene that codes for a receptor that opens up pathway 2 and leads to the activation of a new set of genes.

COOPERATION OF ONCOGENES

It has been well established that neoplastic transformation of fibroblasts in vitro depends upon the cooperative activities of at least two oncogenes (Land et al 1983a,1983 b;Weinberg 1985). These oncogenes fall into two classes each of which has multiple members. One oncogene from each class is required for transformation. C-myc, E1A, PolyLgT, p53 are in one class and ras, mos, src, raf and abl are in the second class. When fibroblasts are transfected with one of the oncogenes from one group they fail to become transformed but the addition of an oncogene from the other group results in

complete transformation. A challenging problem in oncogenesis is to explain the biological basis of oncogenes gene cooperation chiefly because this is one of the most supportive sources of evidence for the multiple mutational concept of neoplastic development.

The possibility that the products of some of the genes operate in different growth regulatory pathways is revealed by recent data. Waslyk et al (1988) have provided evidence suggesting that src, MT, ras, mos, raf, and fos proto-oncogene products are components of pathway(s) that terminally activate the transcription factor gene PEA-1 (a homologue of c-jun and AP1). Suggestively from this work the PEA-1 oncoprotein may regulate a different set of genes than does the c-myc oncoprotein.

The proliferation of normal hematopoietic cells is thought to depend upon exogenously supplied growth factors. An exception may be the autocrine production of IL-2 by certain T-cells (Crabtree 1989). Heldin et al (1987) and Hunter (1987) have pointed out that the constitutive activation of pathways that transmit such signals would relieve the requirements for an exogenous source of these factors. Thus the neoplastic transformation of B-and T- cells could largely depend on the constitutive activation of growth regulatory pathways. There is increasing evidence that many of the genes that control the components of these pathways can be activated by mutational mechanisms; i.e. the mutation dysregulates transcription or produces an oncoprotein that is irreversibly active (deVos 1988, Franz et al 1989).

The different groups of cooperating oncogenes may represent components of separate pathways that are required for traversal through the mitotic cycle. A concept that has been discussed for a number of years is that the completion of the mitotic cycle is controlled in steps most of which occur in the G1 phase of the cycle in mammalian cells (Pardee et al 1986). In mammalian cells two general phases of the G_1 period of the cell cycle are competence and progression as originally defined by Pledger et al 1977.

Competence pathways are activated to bring cells from a resting state to enter the G1 phase of the cycle (see Pardee et al 1986). These pathways can be initiated by short exposures to exogenous inducing agents. In the absence of progression factors the preparation for mitosis will not proceed. Furthermore cells that are not competent do not respond to progression factors. Competent cells are stimulated to complete further steps in preparation for DNA synthesis and cell division by separate types of factors that operate through different channels (progression pathways).

The association of bursts of elevated c-myc transcription and translation occur when resting B-cells are activated to enter into the cell cycle (Dean et al 1986, Klemsz et al 1989). Dysregulation of c-myc transcription results in sustained high levels of myc activity throughout the cell cycle. If high levels of c-myc transcription and translation are essential for rendering the cells competent to enter the mitotic cycle then the high constitutive levels of myc biosynthesis in cells carrying mutant myc genes would favor the reentry of cells just completing a mitotic division to begin a new cycle rather than becoming resting cells. It is interesting to

note that the major forms of active tumorigenesis that involve c-myc are B and T cells which enter and reenter the mitotic cycle. Myc mutations may be particularly important in the later stages of B cell development where cells such as plasma cells and memory cells are under pressure to become quiescent(Go).

A general concept is that progression through the mitotic cycle is impeded by relatively strong down-regulating factors. Support for this hypothesis comes from the evidence of 'suppressor' or 'anti-

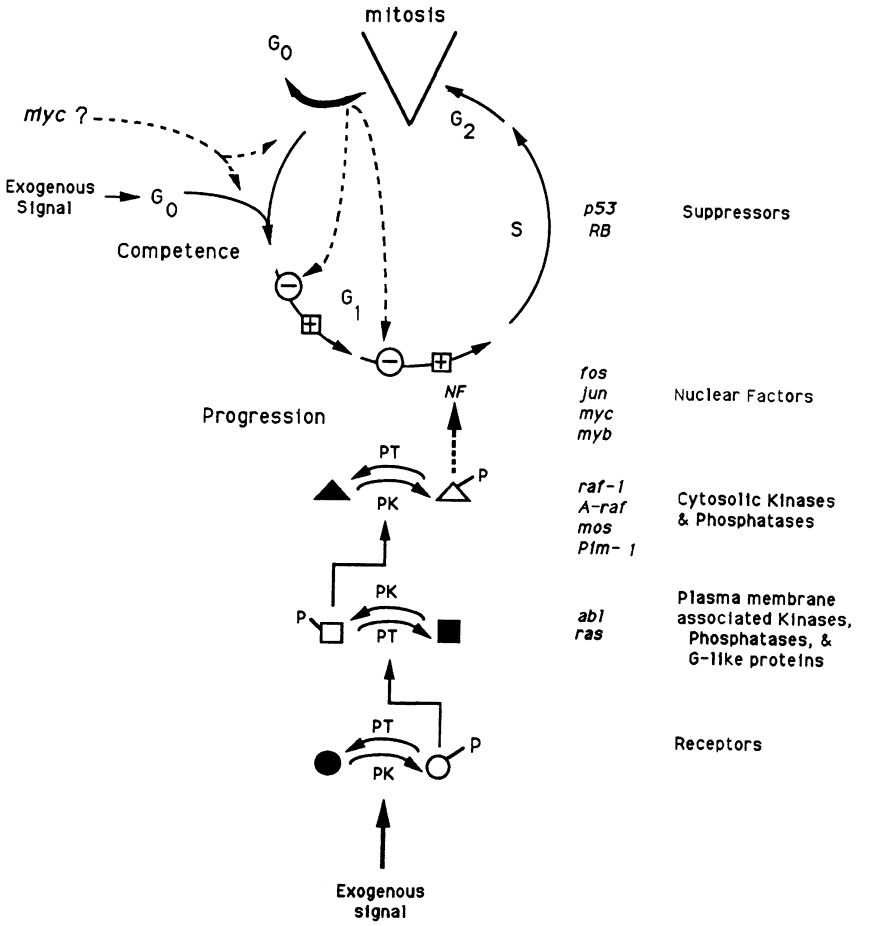

Fig 3. Hypothetical simplified relationship of growth regulatory pathways to the mitotic cycle. The scheme is biased and showed regulatory sites only in G0 and G1. Other regulatory sites have been proposed in G2 by Lernhardt and Melchers 1987. Three control points are depicted by dotted lines. The first in the upper left shows a control point in G0. Dysregulation of c-myc may facilitate the reentry into the cycle. The second control is the suppressive effect on mitosis possibly exerted by such genes as p53 or RB. The third pathway (progression) is shown as one pathway but may actually be several. The arrows in G_1 with + and - boxes symbolize genes with positive and negative regulatory sites.

oncogenes' whose natural function appears to be inhibition of mitotic cycling; e.g.the RB and p53 genes(Klein 1988; Green M.R. 1989). When the products of these genes are no longer active (through deletion or base substitution mutations), the inhibition of cell division is presumably relieved. A simplified hypothetical scheme showing some possible relationships of growth regulatory pathways to cell cycle is shown in Fig 3).

MOUSE PLASMACYTOMAS

The mouse plasmacytoma system provides illustrative and supportive evidence for oncogene cooperation. There are both 'active' and 'passive' forms of plasmacytomagenesis. In the 'active' system various substances such as solid plastics or paraffin oils (e.g. pristane) are introduced intraperitoneally to BALB/cAn mice. Approximately 60% of these mice develop a plasmacytoma with a mean latent period of 7 months. Virtually 100% of these plasmacytomas have some kind of mutation (chromosomal rearrangements) affecting the transcription of the c-myc oncogene. In sharp contrast to these highly consistent mutations in c-myc in mouse plasmacytomas only a few potential cooperating mutations have been identified. Two plasmacytomas have transpositions of intracisternal-A particle sequences in the c-mos gene (Cohen et al 1983; Gattoni-celli et al 1983) Konrad Huppi in my laboratory has carried out an extensive search for ras mutations without success. Despite this lack of evidence, additional oncogenic mutations are nonetheless thought to exist but remain to be identified.

Plasmacytomas can also be induced by various passive mechanisms Tables 2 and 3). This evidence does not identify the gene targets in 'active' plasmacytomagenesis but does implicate possible pathways in which genetic lesions may be sought. In our laboratory we have been inducing plasmacytomas by infecting pristane treated mice with retroviruses that have Moloney helpers and which carry various defective transforming elements (Tables 2 and 3). The protocol which we now use as standard is to inject 3-4 week old BALB/cAn mice with 0.2ml pristane and to give them the virus the next day. Strikingly when the viruses are injected in the absence of pristane they have not produced plasmacytomas.

Viruses containing only Eμ-myc or v-myc do not produce plasmacytomas. We originally reported that the J-3 virus constructed by Ulf Rapp at the NCI was plasmacytomagenic. The original J-3 was derived from J-2 by deleting a segment in the 5' end of the v-raf and this deletion caused the formation of a frame-shift mutation in v-raf so that it became non-functional. Most of the stocks of J-3 virus used in the study previously reported (Potter et al 1987) had been passaged in culture. We have recently repeated this work using the original J-3 virus and compared it to the passaged virus used in the reported study (Potter et al 1987) and have now found that the original J-3 is non-plasmacytomagenic. Jacob Troppmair and Ulf Rapp have cloned out the passaged J3 virus (named J-3*) and have shown that it has undergone further deletions that remove more bases in the gag and 5' end of v-raf (Troppmair et al in preparation). This corrects the frameshift mutation and permits the transcription of the minimal transforming unit of the v-raf. Thus the plasmacytoma-

genic J-3* virus contains two functional cooperating oncogenes. We have also found that a virus containing a mutant A-raf and v-myc is highly plasmacytomagenic.

Transforming retroviruses carrying v-myc+ v-Ha-ras or v-raf-1 or A-raf have been able to induce high incidences of plasmacytomas in pristane treated mice. In experiments where v-abl, v-Ha-ras or v-raf are given alone a low but substantial incidence of plasmacytomas can be induced. The ability of these genes to cooperate with mutant c-myc genes suggests that a multi-component pathway in which the gene operates is implicated in plasmacytoma development. Future work will hopefully tell us how these pathways operate and should lead to an understanding of how dysregulation of pathways can lead to autonomous proliferation. Rapid plasmacytoma induction by virus containing v-myc and other oncogenes provides a method for testing a number of genes for cooperative oncogenic activity in vivo. Proof of the multiple mutational origin of neoplasia however remains to be confirmed for most if not all of the active forms of B- and T- cell tumorigenesis.

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The Ig/myc Translocation in Burkitt's Lymphoma Is a Rate Limiting Step in Tumor Development with Multiple Phenotypic Consequences

G. Klein

Reciprocal translocation between the c-myc carrying chromosome and one of the three Ig loci is a regular feature of certain B-cell derived tumors in three species: Burkitt lymphomas (BL) in humans, induced plasmacytomas in Balb/c mice (MPC), and the spontaneous immunocytomas (RIC) of the Louvain rat (for review see Klein and Klein 1985). I know of no other example in the cancer field where equally homologous oncogene activation events are associated with tumors of different species and of different natural histories, that have their cell linage derivation as the only common denominator.

In this brief review I shall discuss the possible reasons for the extraordinary penetrance of the Ig/myc translocations, as reflected by their virtually uniform presence in the three tumors, to the exclusion of other B-cell derived neoplasms. I shall argue that this is due to multiple phenotypic effects by which the translocations facilitate the uncontrolled growth of their carriers. The constitutive activation of the Ig-juxtaposed myc gene prevents the translocation carrying cells from leaving the cycling compartment when they are programmed to become resting virgin or memory cells and have already switched some of their phenotypic markers in accordance with that program. In the case of the BL cell, the resulting phenotype can be referred to as a "resting cell that is not resting". I shall argue that cells of this phenotype are relatively unavailable for T-cell mediated rejection.

The immunological and non-immunological consequences of the Ig/myc juxtaposition will be discussed in this order.

Non-immunological consequences of the Ig/myc translocation

The translocation brings the protooncogene c-myc that appears to play some as yet undefined role in the control of cell proliferation, under the regulatory influence of a constitutively expressed immunoglobulin gene. The localization of the breakpoints within the Ig-loci corresponds to the target sites of recombinases that participate in the physiological rearrangement of immunoglobulin genes during B-cell differentiation, suggesting that the illegitimate, non-homologous recombination with another chromosome is an accident of the normal rearrangement process. The translocation leads to the constitutive activation of the myc gene as reflected in high mRNA and protein levels.

The function of the myc-protein is unknown, but there is suggestive evidence that it may stimulate DNA replication (Claesson et al, 1987). All proliferating cells express c-myc. Cells that enter G_0 as part of their life cycle or in connection with terminal differentiation, down regulate myc, as a rule. Introduction of myc-expressing constructs can inhibit the differentiation of such cells (Dmitrovsky et al 1986, Prochownik et al 1986, Coppola et al 1986, Larsson et al). The oncogenic role of retrovirally activated c-myc in avian leukosis (Hayward et al,

1981) and of the Ig/myc juxtaposition by chromosomal translocation may depend on a similar block.

B-lymphocyte differentiation, as reflected by Ig secretion is compatible with continued cell division. There are two points in the life cycle of the B lymphocyte where the cell is programmed to rest in G_0: after the rearrangement of the Ig-genes, as a mature but virgin B-cell, and after prior clonal expansion, as a long lived memory cell. We have previously suggested (Klein and Klein, 1985) that the Ig/myc translocation prevents the B-cell from leaving the cycling pool at one or both of these points.

Facsimile experiments have confirmed the oncogenic potential of myc-expressing constructs for pre-B and B-cells. Infection of pristane treated Balb/c mice with a retrovirally activated myc construct (J3) has dramatically reduced the latency period of plasmacytoma development. Tumors that expressed the v-myc insert had no translocations (Potter et al 1987). Emu-myc transgenic mice develop pre-B or B-lymphomas in up to 90% (Adams et al, 1985). We have recently found that the introduction of helper-free Abelson virus into such transgenics leeds to the appearance of plasmacytomas that lack the translocations (Sugiyama et al, submitted for publication). Oligo- or monoclonal tumors appeared in all these facsimile experiments, however, indicating that at least one additional event was required for full tumorigenicity. Activation of another oncogene or loss of a suppressor gene may be involved.

What is the translocation prone stage in B-cell development?

This question was the subject of much speculation, mainly deduced for the breakpoints within the Ig-loci and the stage in B-cell development when the physiological DNA rearrangement of the same region was known or was presumed to occur. We have recently obtained some unexpected new information that may be relevant to this question, at least as far as the typical IgH/myc (8;14) translocation in sporadic BL is concerned. This translocation moves the coding exons of c-myc into an IgH switch region. Smu is most frequently involved, as a rule. This has led to the suggestion that the translocation may arise from a mistake at the time of an IgH class switch. Most of the BLs produce IgM, however. This implies that the translocation has occurred in cells that have not yet engaged in switch recombination.

We have recently found (Altiok et al, in press) a series of new 14q+ markers that have occurred in separately propagated sublines of an EBV-transformed pro-B-cell line, FLEB-14. This line was originally diploid and all its Ig-loci were in the germ line configuration (Katamine et al, 1984). A 14q+ marker has appeared in 5 of 7 independently maintained sublines, during periods of culturing that varied between 4 and 36 months (Altiok et al, in press, Otsu et al, 1987). Each translocation arose by a break in the Smu region, but the exact breakpoint was different in each line. The transposed chromosome piece has been derived from a different chromosome in each line. Chromosomes 6, 16 and 18 served as the donors in three lines. The 14q+ markers of the other two sublines were different from each other and from the three others but the origin of the transposed piece could not be identified. Two lines remained diploid.

In the single thoroughly analysed case (Otsu et al, 1987) the transposed chromosome 6 derived sequences could not be identified with any known gene. We have nevertheless assumed that all five translocations may have conveyed some in vitro growth advantage, since their carrier cell has

overgrown the diploid progenitor. This impression was further corroborated by finding that one of the sublines, FLEB 14-7, has duplicated its 14q+ marker in the course of serial culturing and has lost the normal chromosome 14 homologue.

Similar 14q+ markers have been very infrequently seen in EBV transformed B-cell lines and then only in a small minority of the cells (Altiok et al, in press). Our observations therefore suggest that the pro-B cell may provide a more translocation prone stage than the mature B-cell. The chromatin structure of the Ig-loci opens up for transcription in this cell, as signalled by the appearance of sterile Cmu transcripts. This opening may be a prerequisite for both legitimate and illegitimate DNA rearrangements in the course of B-cell differentiation (Alt et al, 1986).

The translocations detected in the FLEB-14 sublines did not involve chromosome 8. It is unlikely, however, that a myc-IgH juxtaposition would convey a major selective advantage on an EBV-transformed cell that already expresses c-myc at a high level, like all other proliferating cells. The situation is quite different in vivo where the myc/Ig juxtaposition would prevent the B-cell from leaving the cycling compartment at a point where it is programmed to do so, as already discussed. This reasoning implies that many translocations may occur in pro-B cells that are never seen, since they do not confer a selective advantage on the cell. The breaks on the donor chromosome may occur more or less at random. We could not identify any of the breakpoints in the FLEB 14 sublines with known fragile sites. A random break is also in line with the extensive evidence for wide variation in the location of the breakpoint on the myc-carrying chromosome in BLs and MPCs (Cory, 1986, Magrath, in press). The break can affect intron 1 or the non-coding first exon within the c-myc gene, but the coding 2d and 3d exons always remain intact, in consistence with the postulated role of the myc protein in the oncogenic change. Outside breakpoints can be at either the 5′ or the 3′ end of myc quite close or relatively far from the gene itself.

The random break of the myc carrying chromosome is probably an accident of prolonged cell division. EBV and chronic malaria may be responsible for the chronic stimulation of cell division in BL. In MPC, a foreign body granuloma must be induced in a highly susceptible mouse strain, usually Balb/c, and Abelson virus accelerates tumor development. The third IgH/myc carrying tumor, the spontaneous immunocytoma of the Louvain rat (RIC) arises in most cases from the small minority of IgE producing cells. The translocation juxtaposes c-myc to the Sepsilon region in the majority of the cases, although unusual receptor sites have been found as well. The breakpoints on the c-myc-carrying chromosome are more clustered than in BL and in MPC (Pear et al, 1988 a, 1988 b). The expansion of the IgE producing B-cell minority is probably due to hypersensitization to parasite antigens.

The typical translocations that affect the heavy chain locus and the variant translocations that juxtapose c-myc to one of the light chain loci differ in their geometry both in the human and the murine system. This may merely reflect the orientation of the participating genes on the chromosome. The IgH cluster is oriented head down, whereas myc, kappa and lambda are head up. Reciprocal exchanges between terminal chromosome segments must give rise to the existing configurations. It is more remarkable that the murine kappa/myc variant translocation breaks the chromosome at a distance of at least 92 kb distal to the 3′ end of

myc, at a sequence designated as pvt-1 (Cory, 1986). The functional involvement of this sequence and its relationship to myc is obscure, but it emphasizes the theme of random breakage still further.

If it is correct that the BL, MPC and RIC associated translocations arise from the joining of a recombination prone site in an Ig-locus and a random break on the partner chromosome that may involve any terminal chromosome segment, the exclusive involvement of c-myc in the three tumors becomes even more puzzling. The explanation may be found in the extraordinary selective advantage provided by the myc/Ig juxtaposition. We shall proceed to consider its immunological effects.

Immunological consequences of the Ig/myc juxtaposition

This is only known for the BL system. The following consideration is therefore entirely restricted to that tumor.

Comparisons between BL lines and EBV transformed B-cell lines of normal origin (LCLs), derived from the same individual, have shown a marked difference in their sensitivity to the lytic effect of cytotoxic T-cells (CTLs) (Rooney et al, 1984, 1986). The BLs were partially or wholly resistant to CTLs, generated by repeated stimulation with autologous EBV transformed B-cells, that killed the corresponding LCL.

At first, it was thought that the CTL sensitivity is exclusively a function of EBV-antigen expression. There is a major and regular difference between BL and LCL lines in this aspect. LCLs express at least seven virally encoded antigens: the nuclear antigens EBNA-1-6 and the membrane antigen LMP. BL cells express only EBNA-1 (Rooney et al, 1986, Rowe et al, 1987). This is due to a host cell dependent difference in viral gene regulation, associated with a difference in viral DNA methylation (Masucci et al, in press, Ernberg et al, in press). Rescue of the BL associated virus into normal B-cells resulted in the expression of all seven antigens in the derived LCLs.

These findings are consistent with the idea that BL cells express fewer EBV encoded proteins. This is not the whole picture, however, BL-cells are also less sensitive to ordinary allospecific CTLs, directed against certain MHC class I antigens. The allospecific T-cell clones were generated by stimulating the PBL of EBV seronegative donors who lacked immunological memory to EBV antigens (Torsteinsdottir et al, 1988). Both EBV negative and EBV-converted BLs were resistant to this allokilling, whereas LCLs of the same donor were sensitive. EBV antigens can obviously not be held responsible for this differential sensitivity. The difference could be explained by the down regulation of certain HLA class I specificities, HLA-A11 in particular, in the BL cell (Torsteinsdottir et al, 1988, Masucci et al, 1989). Other class I specificities were not affected. Down-regulation of HLA-A11 was characteristic for the typical BL phenotype. It was found in all six BL lines tested that had been derived from A11 positive donors. Switching the BL cell to a more LCL-like phenotype, as indicated by the appearance of immunoblast-associated activation markers has led to the parallel upregulation of HLA-A11 and of LMP (Masucci et al, 1987). HLA-A11 could be also up-regulated in some BL lines by combined treatment with IFN-gamma and TNF-alpha (Avila-Carino et al, 1988).

BL cells also differ from LCLs in a third, immunologically relevant aspect. The cell adhesion molecules LFA-1, ICAM-1 and LFA-3 are expressed at very low levels on BL cells but they are coordinately up-regu-

lated when the cells become more LCL-like. They are highly expressed on the LCLs (Gregory et al, 1988). These adhesion molecules are known to play an important role in cell mediated cytotoxicity, by stabilising the cell-to-cell contact and probably also by contributing to the functional activation of the T-cell.

Three different phenotype associated features of the BL cell may thus contribute to its resistance to CTL-mediated lysis: a) down regulation of six of the seven EBV-encoded antigens expressed in EBV-transformed cells of normal origin; b) lack or low expression of certain MHC class I polymorphic specificities; and c) insufficient expression of adhesion molecules required for the effector-target cell interactions.

How is it possible that all these changes are coincidental in the same target cell? There are two main possibilities: i) they are due to immunoselection, that has occurred during the growth of the tumor in vivo; ii) they are characteristic for the normal progenitor cell of the tumor.

The first alternative is possible, but not highly likely. EBV negative and EBV carrying BLs have the same phenotype in vivo and initially in vitro as well, although the EBV positive BLs tend to shift to a more LCL like phenotype during prolonged passage in culture (Rowe et al, 1987). They also show the same down-regulation of some HLA class I and adhesion molecules. Both histopathological and clinical evidence indicate, however, that EBV-positive, but not EBV negative BLs evoke relatively strong immune responses in vivo. It is therefore rather unlikely that surveillance could have shaped the same drastically altered phenotype in both cases and with such regularity.

According to the second alternative, these are the vestiges of the normal precursor cell or, more precisely, the normal phenotypic "window" that has been fixed by the translocation. Early electron microscopic studies have shown that BL cells resembled resting B-cells while LCLs corresponded to activated immunoblasts (Nilsson et al, 1987). It was also noted that BL cells tend to grow as single cell suspensions and have a smooth surface, whereas LCLs grow in large clumps and have villous surfaces. The B-cell marker expression of the two cell types is also radically different. BL cells are CALLA (CD10) and BLA (CD77) positive and lack activation markers whereas LCLs show the opposite pattern (Rowe et al, 1987, Ehlin-Henriksson et al, 1987). The closest normal counterpart of the BL cell was identified as a germinal center centroblast (Gregory et al, 1987). The phenotype of the centrocytic and memory cell populations has not been determined, however. They are directly derived from the centroblastic cells, as they move towards the resting stage. It is possible that they are also CALLA and BLA positive, activation marker negative as the BL cells, since centroblastic and centrocytic lymphomas have a corresponding phenotype (Sakthivel et al, 1989).

The idea that BL cells are "resting cells that are not resting" (Klein G, in press) is consistent with the myc/Ig translocation scenario. If the translocation occurs at the pro-B cell stage as postulated, but its tumorigenic effects are only expressed later when the cell is programmed to leave the cycling compartment and rest in G_0, the phenotypic switch of the proliferating blast to the resting cell phenotype may proceed according to the program, even though the cell is unable to stop dividing, because it cannot down-regulate myc.

The homeostatic control that prevails on resting B-cells must be radica-

lly different from the regulation of the immunoblasts. Virgin or memory B-cells can only be triggered by a specific activation complex, composed of appropriately processed and presented antigen, helper factors and lymphokines. This strong dependence on positive signals may have obvicted any need for negative host control. The immunoblast is a very different cell. It proliferates in response to antigen and lymphokine stimulation. Its expansion must be closely monitored by the host, as indicated by the fact that the size of the B-cell pool cannot be increased by hyperimmunization. This may be the reason why EBV transformed cells are non-tumorigenic in immunodefective mice and only rarely give rise to lymphopoliferative disease in severely immunosuppressed patients. It is also consistent with our finding that a major switch of a BL cell to an LCL-like phenotype may be accompanied by the loss of tumorigenicity, in spite of the continued presence of the Ig/myc translocation and constitutive c-myc expression (Torsteinsdottir et al, 1989).

Is it conceivable that the three immune escape facilitating features of the BL cell, the down-regulation of certain HLA class I specificities, EBV-encoded antigens and adhesion molecules, are also characteristic for their normal progenitor? There is no direct information on this question, but some indirect evidence may be relevant.

The most suggestive point concerns EBV latency (Klein G, in press). We have recently found (Gratama et al, 1988) that bone marrow transplantation eradicates the EBV-population of seropositive persons. If the marrow donor is seronegative, the recipient becomes seronegative and may remain so for long periods of time until newly infected with another viral substrain. If the donor is seropositive, his virus totally replaces the original viral substrain. This suggests that the viral reservoir, responsible for the lifelong persistence of EBV, is in the hemopoetic compartment. It is also in line with the finding (Lewin et al, 1987) that directly growing EBV-carrying LCL could be established from the small, heavy B-cell fraction of healthy EBV seropositives, after the separation of blood lymphocytes on density gradients. This suggests that the virus may persist in small resting B-cells. The EBV studies thus lead us to the same potential BL progenitor cell as the analysis of the myc/Ig translocation scenario, even though it proceeds through an independent chain of experiments and reasoning.

The viral gene expression of the EBV carrying small lymphocyte is not known, but it is intriguing to speculate that it may correspond to the BL cell, its inferred neoplastic counterpart. If so, it may express EBNA 1 but not EBNA 2 - 6 and LMP. This may help EBV to persist in a latent form where it escapes immune surveillance.

A scenario of this type would make much sense from the point of view of viral strategy. It is known that EBNA-positive B-blasts appear in the peripheral blood, during the acute phase of mononucleosis. They can enter mitosis, possibly under the stimulus of the autocrine B-cell growth factor/BCGF receptor circuit detected in the LCLs (Gordon et al, 1985). After a few weeks, both non specific and specific cytotoxic T-cells appear (Rickinson AB, 1987). Concomitantly, the EBNA positive blasts disappear from the blood. EBV transformed B-blasts are probably good CTL targets, as indicated by their ability to trigger the proliferation of autologous cytotoxic T-cells in vitro. Among the virally encoded antigens expressed on LCLs, EBNA-2, EBNA-3 and LMP are likely targets for the rejection response.

After convalescence and in healthy seropositives, EBV-carrying cells can

be regularly grown into lines from the blood if 10^6 or more cells are explanted. If the latently EBV-infected small B-lymphocytes express only EBNA 1 as I am postulating, this protein and its peptide products may be less immunogenic than EBNA 2 - 6 and LMP. However this may be, the down-regulation of most viral proteins in the resting cell may be an important feature of viral persistence. The corresponding antigenic down-regulation in the malignant counterpart would help the EBV carrying BL cell to escape rejection. This would be simply another consequence of the "suspended resting cell phenotype", or, in other words, an indirect consequence of the translocation that would increase the growth advantage of the BL cell in vivo.

It is more difficult to speculate about the question whether the down-regulation of certain HLA antigens and of adhesion molecules is a feature of normal resting B-cells. Our preliminary evidence suggests that resting B and T-cells may express less HLA - A11 than the corresponding blasts, while other class I antigens are equally expressed on both (Torsteinsdottir et al, 1988). If T-cells play a regulatory role in preventing the overexpansion of the B-cell pool and if certain class I interactions play a part in this, some class I molecules may have to be selectively down-regulated on the memory cells to prevent "immunological amnesia".

In conclusion the high penetrance of the Ig/myc translocation in BL and, probably, also in MPC and RIC may be due to its ability to contribute to the tumorigenic phenotype in more than one way. The findings and derived scenarios summarized in this article suggest that it may act at an immunological and non immunological level, at least as far as BL is concerned. The subordination of myc to the regulation of a constitutively expressed Ig locus incapacitates the programmed exit of the cell from the cycling compartment. Concomitantly, the phenotypic freezing of the cell at a stage corresponding to a resting B-cell endows it with at least three different features that may help it to escape immune rejection: down-regulation of a) EBV encoded antigens, b) certain HLA class I polymorphic specificities and c) adhesion molecules.

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The Development of B-Lymphomas in the Chicken

E.H. Humphries

A few years ago, only a single B-cell tumor was known to develop in the chicken. Avian leukosis virus (ALV) induced a bursal-dependent B-lymphoma. The tumor appeared in 4 to 6 month old birds that had been infected on the day of hatch with ALV. We have recently described the development of two other B-cell lymphomas in the chicken. Their biological features and the molecular nature of the initial oncogenic events indicate that these tumors are distinct from each other and from the ALV-induced bursal-dependent lymphoma. It appears that the three tumors develop using different genetic pathways. In this report, I will discuss the biological differences between these tumors.

The original bursal-dependent B-lymphoma, at one time called lymphoid leukosis (Cooper *et al* 1968 and Purchase and Payne 1984), is an extended stochastic disease process that involves at least three distinct genetic events (Fig. 1). ALV infection of 1 day old chicks results in extensive viral integration within several hematopoietic tissues including bursal lymphocytes (Baba and Humphries 1986). During this period of viral integration, numerous preneoplastic lesions appear within the bursa (Baba and Humphries 1985). It has been proposed that these lesions, called transformed follicles, appear as a result of ALV integration within and dysregulation of the c-*myc* locus (Neiman *et al* 1985). The transformed follicles appear normal in size, have poorly defined cortical-medullary compartments and are filled with blasting B lymphocytes. The majority of these follicles persist without

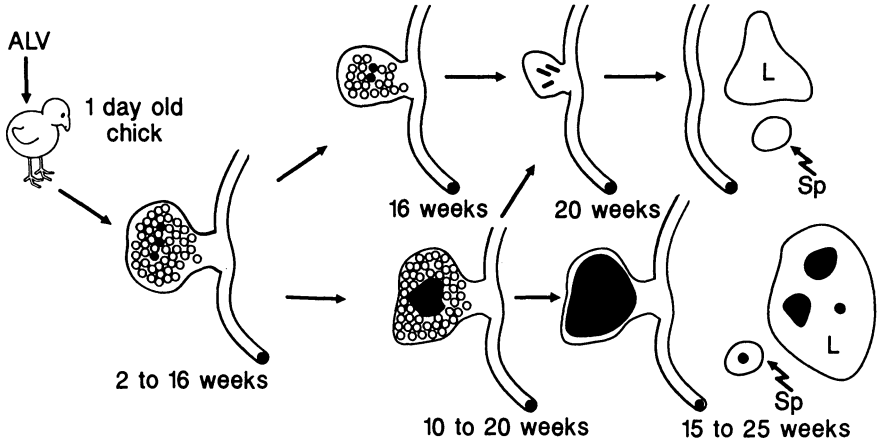

Fig. 1. A model for development of ALV-induced *myc*-driven lymphoma.

noticeable alteration until involution of the bursa at approximately 16 weeks of age. A small proportion of these follicles progress to form clonally dominant primary bursal tumors that express elevated levels of c-*myc* (Hayward *et al* 1981). During tumor formation, the follicle expands in size, becomes highly vascularized and eventually approaches or surpasses the mass of a normal bursa (Fig. 2A). The genetic alteration that leads to this tumor progression has not been identified but may involve quantitative or qualitative changes in the expression of c-*myc* and/or additional retroviral-mediated insertional mutagenesis (Clurman and Hayward 1989). Both the preneoplastic lesion and the primary tumor are dependent upon the functional integrity of the bursal environment (Purchase and Payne 1984). Treatment of chickens with testosterone at this stage of tumor development results in elimination of the primary tumor. Additional tumor progression occurs with the appearance of metastatic disease. Survival of the tumor in the liver or spleen signifies a genetic alteration that results in bursal-independent, testosterone-resistant disease and eventual death of the chicken. Metastatic disease occurs in stochastic fashion and is not a necessary consequence of primary tumor development.

In contrast to the ALV-induced *myc*-driven lymphoma, a more rapid tumor develops when embryonated eggs are infected with ALV at day 9 or 10 of embryogenesis (Kantor and Hayward 1988, Pizer and Humphries 1989a). Death normally occurs between 4 and 6 weeks after infection. Tumor development follows insertion of the ALV provirus within the c-*myb* locus and elevated expression of *myb* RNA. Three distinct insertion sites have been located (Fig. 3) predicting that 3 different *myb* proteins are synthesized, 2 of which are *gag-myb* fusion products. Two of these proteins have been observed by Western analysis of tumor extracts (Pizer and Humphries 1989b). ALV integration within either the c-*myb* locus or the c-*myc* locus depends upon the time of infection and would appear to be a reflection of relative frequencies of distinct target cells. Distinct stages of tumor development have not been observed. Recent data indicate, however, that development of the *myb* positive lymphoma does not necessarily result in death of the bird. While only 15% of the

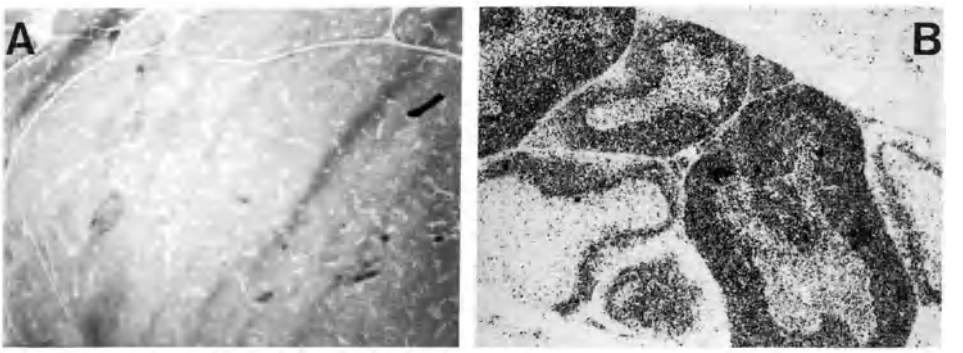

Fig. 2. Two distinct ALV-induced primary tumors. A. An 8 micron frozen section of a bursal tumor bearing an ALV insertion within the c-*myc* locus isolated from a 15 week old chicken and stained with hematoxylin and eosin. B. An 8 micron frozen section of a bursal tumor isolated from a 2 week old chick infected *in ovo* with ALV and stained with α-*myb* monoclonal antibodies (Pizer 1990).

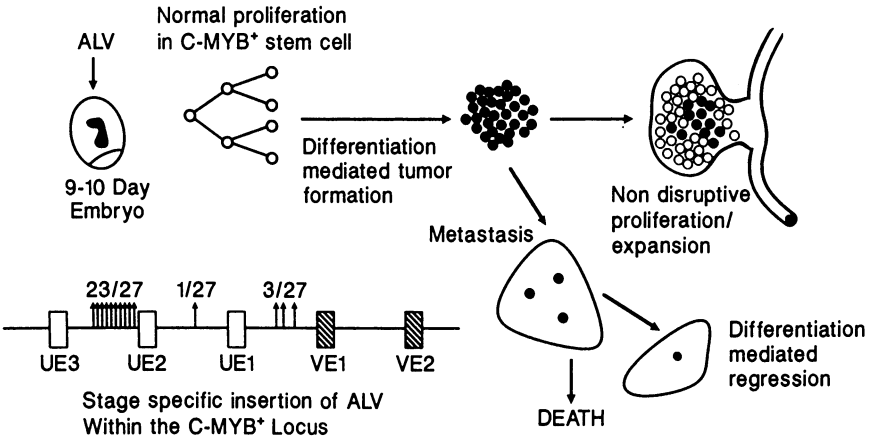

Fig. 3. A model for development of ALV-induced *myb*-driven lymphoma.

chicks die, tumor can be detected in approximately 75% of the infected birds (Pizer 1990). The pattern of tumor spread within the bursa contrasts sharply with that of the *myc*-driven lymphoma. Staining of early bursal tumors with α-*myb* monoclonal antibodies suggests the tumor spreads via cell migration from one follicle to another (Fig. 2B). As follicles fill with *myb* positive cells, the tumor spills over into additional follicles. This phase of bursal tumor spread fills the bursa and results ultimately in a clonal (or pauci-clonal) tumor as judged by ALV integration sites. Strikingly, both the size of the bursa and the follicular structure are normal in appearance. This metastatic behavior, as well as the appearance of tumor in the liver by 3 weeks, suggests that the *myb*-driven tumor in the bursa is independent of the bursal environment. Staining of tumor-bearing liver with α-*myb* monoclonal antibodies identified extensive tumor masses in which only cells at the periphery of the mass expressed *myb* protein. This result suggests

Table 1. REV-T(CSV) *in vitro* B-cell transformation

Age of chick[a]	Bursal titer[b]	IgM$^+$ clones[c]	Splenic titer[b]	IgM$^+$ clones
1 week	1×10^4	15/15	2×10^3	5/8
2 weeks	6×10^3	15/16	4×10^3	11/17
3 weeks	2×10^3	ND	6×10^3	19/20

[a] The age of the chick at the time of sacrifice. Bursal and splenic mononuclear cells were isolated by Ficoll gradient centrifugation.

[b] 10^7 cells were infected with different dilutions of REV-T(CSV) and 10^5 cells plated per microtiter well. The titer was determined from the number of wells showing clonal growth.

[c] Clones were transferred 3 times *in vitro* and assayed for IgM using immunoperoxidase and an α-IgM monoclonal antibody.

TABLE 2. A comparison of three avian B-cell lymphomas

Property	*myc*-driven	*myb*-driven	v-*rel*-driven
Time until death	4 to 6 months	4 to 6 weeks	7 to 10 days
Mortality rate[a]	50%	15%	100%
Presence of Preneoplasia	Yes	Unlikely	No
Growth and location of the primary tumor	Bursal; clonal; develops from expansion of a single follicle	Bursal; clonal; develops from tumor cells migrating within the bursa	Probably bursal; oligoclonal; no distinct development
Microenvironment required for tumor proliferation	Bursa required for both preneoplasia & primary tumor	Unknown	No
Presence of metastatic tumor	Variable: approx. 50%[a]	Always present	Always present
Evidence for Tumor regression	Yes until metastases	Yes	No
In vitro Proliferative Capacity	Demonstrated following *in vivo* tumor transfer	Not reported but very poor at best.	Probably greater than 95% efficient[b]
Specific target Cell	Yes; bursal stem cell (?)	Yes; pre-bursal stem cell (?)	No; multiple targets

[a] Data from this laboratory using Hyline SC chickens.
[b] The vast majority of REV-T(CSV) infected B-cells grow as clones *in vitro*.

that the proliferative capacity within the tumor mass may vary.

A third B-cell lymphoma develops following infection with the reticuloendotheliosis strain T-chicken syncytial virus complex [REV-T(CSV)] (Barth and Humphries 1989a & b). Chicks die 7 to 14 days after infection with REV-T(CSV) from extensive metastatic disease in the spleen and liver. The short duration of the disease suggests it is unlikely that distinct stages of tumor are present. Small lesions isolated from the liver 7 days after infection give rise to immortal cell lines. The capacity for *in vitro* proliferation expressed only shortly after appearance of the tumor marks the most dramatic feature that distinguishes the v-*rel* lymphoma from either the *myc* or *myb*-driven lymphomas. This property suggests not only that tumor progression plays no role in tumor development but also that the tumor is bursal-independent requiring no microenvironmental conditioning post v-*rel* expression for tumor growth. Based upon this observation, an *in vitro* B-cell transformation assay has been developed (Table 1). Greater than 95% of the bursal clones isolated and 75% of the splenic clones were IgM^+ indicating that B-cell

transformation occurred efficiently *in vitro* (Barth and Humphries 1989). All clones tested were capable of prolonged proliferation.

In summary, the three B-lymphomas described here, initiated by expression of *myc*, *myb*, or v-*rel*, are biologically distinct (Table 2). In contrast, it has not yet been possible to distinguish the tumors at the cellular level. All appear to share common surface markers, synthesize IgM and have fully diversified light chain genes. The continued examination of the biological properties of these tumors as well as an in depth comparative analysis at the molecular level ought to provide the identification and characterization of distinctive genes that play crucial functions in B-cell proliferation, differentiation and lymphoma development. Current experiments are designed to identify genes (i) that are activated by expression of v-*rel* and (ii) that participate in progression from preneoplasia to neoplasia in the c-*myc*-driven tumor.

I thank Dr. J. Uhr for continued support and thoughtful criticism throughout this work. This work is supported by Public Service Awards CA32295 and CA41450 from the National Institutes of Health.

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Bcl-2α Encodes a Novel Small Molecular Weight GTP Binding Protein

S. Haldar, Ch. Beatty, and C.M. Croce

INTRODUCTION

After antigen or mitogen stimulation of B-cells, a well-established signal transduction pathway via inositol phospholipid hydrolysis is triggered by an intermediary G-protein whose identity is still unknown (Gold 1987; Harnett 1988). Normal resting B-cells do not express bcl-2 (Reed et al. 1987), the gene involved in follicular lymphoma (Tsujimoto and Croce 1986; Tsujimoto et al. 1985). Transcription of bcl-2 is induced, however, following mitogenic stimulation (Reed et al. 1987). A recent report (Tsujimoto et al. 1987) on the localization of bcl-2 in the inner surface of the cell membrane suggests the possibility that this gene may play a role in transductionn of mitogenic signals.

In addition to well-known large molecular weight heteromeric G-proteins (Gilman 1987), the existence of multiple G-proteins with M_r values about 20,000-30,000 daltons, tentatively designated as small M_r G-proteins, has been documented very recently (Barbacid 1987; Madaule and Axel 1985); Chardin and Tavitian 1986; Lowe et al. 1987; Haubruck et al. 1987; Touchot et al. 1987; Russ Price et al. 1988; Ohmori et al. 1989; Matsui et al. 1988; Kawata et al 1988). This small M_r G-protein group includes H-ras, rho, ral, R-ras, ypt1, rab2 and arf gene products (Barbacid 1987; Madaule and Axel 1985); Chardin and Tavitian 1986; Lowe et al. 1987; Haubruck et al. 1987; Tuchot et al. 1987; Russ Price et al. 1988). Several hematopoietic cells and brain tissue have been found to be good sources of these small M_r proteins (Ohmori et al. 1989; Matsui et al. 1988; Kawata et al 1988). The amino acid sequences deduced from these proteins demonstrated weak homololgies (30-33%) among those having GTP binding consensus sites. For homololgy searches, a list was compiled containing recently published amino acid sequences of low molecular mass G-proteins. Computer analysis revealed approximately 30% similarity btween bcl-2α and several members of the small M_r G-protein family viz, rho, ral, smg-21, smg-25A (Ohmori et al. 1989; Matsui et al. 1988; Kawata et al 1988) and H-ras. Interestingly, like other small M_r G-proteins, bcl-2α also exhibited a conserved significant homololgy to the GDP/GTP binding region and tryptic cleavage site (see Fig. 1).

Since the bcl-2α protein is on the inner surface of the cell membrane (Tsujimoto et al. 1987) and the expression of bcl-2 correlates with the induction of B cells to proliferate (Reed et al. 1987), we wondered if bcl-2α encodes a novel small M_r G-protein. Thus we investigated GTP binding activity, under denaturing conditions, in the cellular extracts isolated from human pre-B-cells such as 697 and 380, and mouse pre-B-cells

viz, ABC-1, LS8-T2 and ABE that have been shown to express bcl-2 (Negrini et al. 1987). The large M_r G-proteins can be differentiated from the small M_r ones by assay of GTP binding under denaturing conditions, since only small M_r G-proteins exhibited GTP binding under these conditions (Lapetina and Reep 1987). The cellular extracts, after being transferred to nitrocellulose filter, were incubated with [α-^{32}P]GTP. After autoradiography we detected a 26-kD protein bound GTP in all the pre-B cell lines we tested (Fig. 2B, lanes 11,12; Fig. 2D, lanes 10-12). To confirm this finding, we determined if a protein of a similar molecular weight would bind GTP in NIH-3T3 cells expressing a transfected bcl-2α gene (B$_3$,B$_4$ clones). As shown in Fig. 3A (lanes 17,18), a 26-kD protein in bcl-α tranfected NIH 3T3 clones possesses GTP binding ability.

To test the specificity of GTP binding we performed competitive inhibition studies using the cold competitors guanosine 5'-[γ-thio] triphosphate (GTPγS), GTP, ATP and ADP respectively. Among these nucleotides GTPγS at 1,000 nM concentration was more effective than GTP in abrogating [α-^{32}P] GTP binding in human pre-B-cells as shown in Fig. 2B (lanes 15,16). Identical effects of guanine nucleotides in abrogating the extent of GTP binding have also been observed in mouse pre-B cells as well as in bcl-2 transfected NIH 3T3 cell (not shown). Adenin nucleotides such a ATP and ADP were ineffective in preventing GTP binding (Fig. 2A, lans 13, 14). The dose response curve of inhibition of [α-^{32}P] binding by guanine nucleotides revealed 1,000 μM to be optimal for complete abolition of GTP binding (not shown). In summary, these results indicate that a 26 kD protein specifically binds GTP in cells expressing bcl-2 transcripts.

Experiments were performed to determine whether this GTP-binding 26 kD protein is capable of reacting with anti-peptide antibodies (S006 and S010) raised against different epitopes chosen from the bcl-2 open reading frame (see Fig.2A). For this purpose, replica Western blot filters carrying lysates were reacted with the anti-peptide antibodies (S006 and S010). Both the antibodies (S006 and S010) specifically react with a 26 kD protein in human pre-B-cells 697 and 380 (Fig. 2B, lanes 3-6), in mouse pre-B-cells ABC-1,, LS8-T8, ABE (Fig. 2D, lanes 4-6), and in bcl-2 transfected NIH 3T3 cells (Fig. 3A, lane 6). The specificities of the antibodies are indicated by the abolition of the 26 kD band when the blots were co-incubated with antibodies and respective peptides (Fig. 3A, lane 4) (data for S010 antibody not shown). In addition, specificities of the antibodies were confirmed by the recognition of the β-gal/bcl-2α fusion protein expressed in E. coli carrying a plasmid containing the bcl-2α gene (see Fig 3). As shown in Fig. 3, the β-gal/bcl-2 fusion protein appeared in three bands. The upper band corresponds in size to the intact protein, and the lower bands are probably the result of proteolytic cleavage as observed earlier (Tsujimoto et al. 1987) (data for S010 antibody not shown). Both antibodies (S006 and S010) also immunoprecipitated a 26-kD protein from [^{35}S] methionine metabolically labeled NIH 3T3 cells expressing transfected bcl-2 (Fig. 3A, lanes 13,14). These data indicate that the S006 and S010 antibodies are specific for the bcl-2α protein.

We also investigated whether the binding of the anti-peptide antibodies to their respective epitopes exerts an effect on the GTP binding ability of the

26-kD protein. Interestingly, 5 µg of S006 antibody raised against an epitope close to the N-terminus of bcl-2α was capable of inhibiting [α-^{32}P]GTP binding to the extent of 50% as compared with preimmune IgG. The same concentration (5 µg) of S010 antibody (raised against a different epitope, amino acid position 98-114) was ineffective in inhibition as seen in Fig. 2C. The inhibitory effect of antibody S006 was consistently observed in all members of our panel of bcl-2 expressing cells (data not shown).

We also investigated whether the antibody binding to bcl-2α protein is inhibited by prior incubation of the protein with saturating amounts of guanine nucleotides. The results (as shown in Fig. 3A, lanes 7-12) clearly show that pre-incubation with guanine nucleotides exerted a partial inhibitory effect on antibody binding to the bcl-2α protein. Adenine nucleotides were without effect, indicating the guanine nucleotide specificity of the bcl-2 protein.

The simplest explanation of the partial inhibitory effect of S006 antibody on GTP binding and vice versa is that their respective binding sites partially overlap with each other. In the case of ras p21, antibody raised against the GTP binding domain near N-terminus completely abolished GTP binding and conversely saturating amounts of guanine nucleotides (1,000 µM) completely inhibited antibody binding to the ras protein (Barbacid 1987).

To convincingly assess the GTP binding ability of the bcl-2α protein, we immunoprecipitated the unlabeled bcl-2α protein from cellular extracts of 697,380 (human pre-B), ABC-1, LS8-T2, ABE (mouse pre-B) and Jurkat (human T) cell lines. Subsequently, the proteins were transferred to nitrocellulose filters and the filters were subjected to [α-^{32}P] GTP binding assay. Fig. 2E clearly demonstrates that a 26-kD protein immunoprecipitated by specific peptide antibody (S006) can bind (α-^{32}P]GTP in all the above cell lines except Jurkat. The negative result in Jurkat cells can be explained by the very low expression of bcl-2 in these cells; a similar observation was made in the case of the lymphoblastoid B-cell line GM-1500. In fact, immunoprecipitation and immunoblotting experiments showed only traces of bcl-2 protein in both of these cells (data not shown). We were, however, unable to show GTP binding activity of the isolated β-gal/bcl-2 fusion protein used in Western blots; a similar observation was made in the case of the human glutathione-S-transferase fused with the β-gal gene (Chow 1988).

The GTP binding ability of immunoprecipitated bcl-2α protein led us to determine whether the bcl-2 protein has autophosphorylating activity. Accordingly, isolated membranes from several human and mouse pre-B-cells were incubated with [γ-^{32}P] GTP followed by immunoprecipitation with the S006 antibody. Immunoprecipitation of ^{32}P-labeled, 26 kD protein from different cell membrane preparations (Fig. 2B, lanes 9,10; Fig. 2D, lanes 7-9) following [γ-^{32}P] GTP incubation confirms the localization of the bcl-2α in membrane as observed earlier (Tsujimoto et al. 1987) and shows that it might have autophosphorylation activity. The possibility of GTP-specific autophosphorylation of bcl-2 is promising since anti-peptide antibodies could not immunoprecipitate any 26-kd protein from [γ-^{32}P]ATP-labeled

membrane (not shown). Moreover, lack of GTP-specific kinase activity (Shih et al. 1980) excludes the possibility of bcl-2 being a substrate for contaminating protein kinase. In ras p21, the Threonine (Thr) at amino acid position 59 is known to be the phosphate receptor (Barbacid 1987). Similarly, bcl-2α has a Thr residue at amino acid position 56 which might be involved in autophophorylation.

Bcl-2α protein was purified to near homogeneity by gel filtration and ion exchange chromatography from the particulate fraction of human pre-B-cell 380 cells which carry a t(14;18) chromosome translocation (Tsujimoto and Croce 1986). To determine whether purified bcl-2α possesses GTPase activity, we electroeluted the protein band which corresponded to [α-^{32}P]GTP binding. This electroeluted protein recognized bcl-2 antibody as evidenced by Western blot (Fig. 4C, lane 2), and showed low GTPase activity similar to ras p21 product and to some of the other small M_r G-proteins (Ohmori et al. 1989). Thus purified bcl-2α possesses GTP binding as well as GTPase activity (Fig. 4).

Preliminary characterization of G-proteins responsible for receptor-effector coupling often rely on bacterial toxins which inhibit this coupling by transferring ADP-ribose moiety from NAD+ molecule to the α-subunit of a large M_r G-protein (Gilman 1987). Several large M_r proteins have also been found to be insensitive to the bacterial toxins containing ADP-ribosyltransferase activity, thus proving that these G-proteins do not constitute an ADP-ribose acceptor (Gilman 1987). To test whether bcl-2α constitutes an ADP-ribose acceptor, we performed ADP-ribosylation experiments with several bacterial toxins such as pertussis, cholera and botulinum type D containing ADP-ribosyltransferase activity in the membrane preparations of human pre-B cells as well as in purified fractions. This experiment indicated the ability of these toxins to transfer the ADP-ribose moiety from a [^{32}P] NAD+ molecule to any 26-kD protein (not shown). This observation led us to the conclusion that bcl-2α might, like the ras oncogene, lack an ADP-ribose acceptor.

Despite little sequence homology other than the GDP/GTP binding region, bcl-2 and ras proteins behave in similar fashion in many physiological and biochemical aspects. Both of them are toxin insensitive, small M_r G-proteins attached to the inner surface of the cell membrane with autophosphorylation activity (Barbacid 1987) and can cooperate with c-myc in cell transformation (Barbacid 1987; Vaux et al. 1988). In the case of bcl-2, however, the mechanism of activation is still unclear. One possibility is that, following antigen or mitogen stimulation the bcl-2 protein is activated by nucleotide exchange; then the activated bcl-2 protein may interact with some other protein in the signal transduction pathway leading to cell proliferation. Dissection of the role of bcl-2 in the regulation of B-cell proliferation will be an important step in understanding its role in the pathogenesis of B-cell neoplasia.

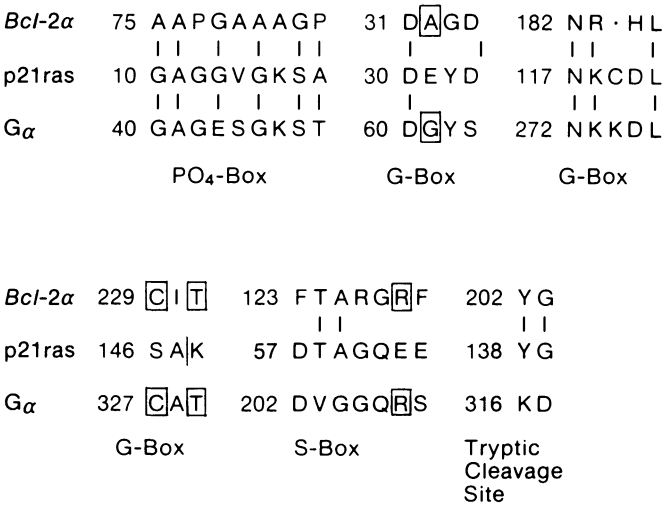
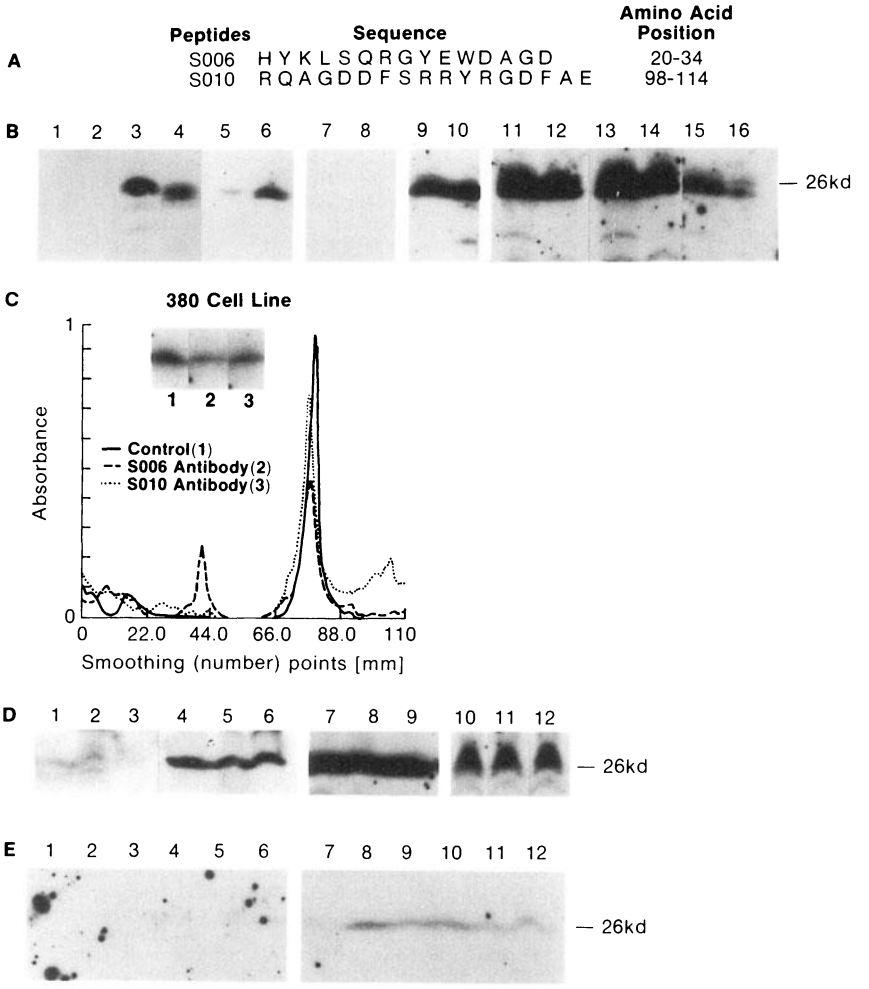


Fig. 1. Sequence similarity between bcl-2α, ras p21 and Gα. Gα denotes a structural model of large M_r G-protein α subunits (adapted from Holbrook and Kim 1989). G-Box: residues required for guanosine binding; PO_4 Box: residues required for binding phosphates; S Box: residues involved in conformational switch mechanism. Computer analysis was carried out using University of Wisconsin genetic analysis software package.

Fig. 2. A) Amino acid sequence of peptide immunogen. Two peptides, S006 (amino acid position 20-34) and S010 (amino acid position 98-14), were chosen from open reading frame of the nucleotide sequence (Tsujimoto and Croce 1986) of the bcl-2 gene to raise polyclonal antiody. B) Lanes 1-6, Western blot of cellular proteins extracted from human pre-B cells 380 and 697. Lanes 1,3 and 6, 380 cells with pre-immune, S006 and S010 IgG respectively. Lanes 8 and 10, immunoprecipitation of [γ-^{32}P]GTP labeled membrane with pre-immune and S006 IgG. Lanes 7 and 9, 380 cell membrane with pre-immune and S006 IgG, respectively. Lanes 8 and 10, 697 cells against pre-immune and S006 IgG respectively. Lanes 11-16, [α-^{32}P]GTP binding to 380 and 697 cell lysate without any nucleotide pre-incubation. Lanes 15-16, 380 lysate pre-incubatd with 1,000 nM ATP and ADP, respectively. Lanes 15-16, 380 lysate pre-incubation with 1,000 nM GTP and GTPγS, respectively. C) Inhibition of [α-^{32}P] GTP binding by peptide antibody S006. Spectrophotometric scanning of the autoradiograph was done at 516 nm in a Beckman DU-70 spectrophotometer. D) Lanes 1-6, Western blot of cellular proteins isolated from several mouse pre-B cell lines. Lanes 1-3, ABC-1, LS8-T8 and ABE with preimmune serum, respectively. Lanes 4-6, same cell extracts with peptide antibody (S006). Lanes 7-9, immunoprecipitation of [γ-^{32}P] GTP labeled membrane isolated from ABC-1, LS8-T8 and ABE, respectively. Lanes 10-12, [α-^{32}P] GTP binding to cellular protein extracted from ABC-1, LS8-T2 and ABE cells respectively. E) [α-^{32}P] GTP binding to immunoprecipitated bcl-2α from human and mouse pre-B cells. Lanes 1-6, preimmune serum; lanes 7-12, S006 antibody. Lanes 1 and 7, Jurkat cells. Lanes 2 and 8, 380 cells. Lanes 3 and 9, 697 cells. Lanes 4 and 10, ABC-1 cells. Lanes 5 and 11, LS8-T2 cells. Lanes 6 and 12, ABE cells.



Methods: Equal amounts of protein (50 µg) were fractionated with 15% SDS-PAGE and transferred to nitrocellulose sheet (0.45 µm) for Western blot (Vaux et al. 1988). [α^{32}P] GTP binding was carried out according to the method of Lapetina and Reep (1987). For competitive binding studies, transfer blots were preincubated for 15 minutes with 1,000 nM ATP, ADP, TGPγS and GTP following incubation with [α-^{32}P] GTP (Amersham; 3,000 Ci/mmol). To study the inhibitory effect of antibody on GTP binding transfer blots were first incubated with antibody and then subjected to GTP binding. Immunoprecipitation and [γ-^{32}P] GTP (Amersham; 3,706 Bq/mmol) labeling were done by established methods (Shih et al. 1980; Murthy et al. 1986). For GTP binding studies on immunoprecipitatd bcl-2α protein, immunoprecipition was carried out without any radioisotope labeling followed by transfer of immunoprcipitated protein to nitrocellulose filter.

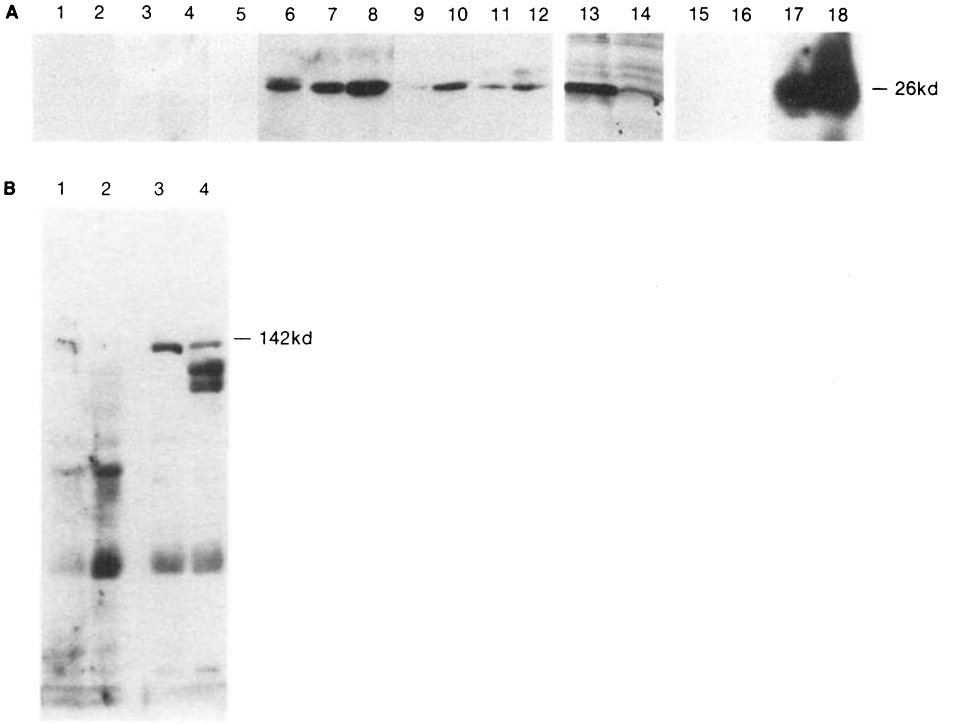


Fig. 3. A) Lanes 1-12, immunoblot analysis of cellular proteins iolated from bcl-2 transfected NIH-3T3 cells (B4 clones) as well as control NIH 3T3 cells without bcl-2 (490 SV-1): effect of different adenine and guanine nucleotides on antibody binding to bcl-2α protein. Lanes 1-2, cellular extract from 490 SV-1 and B-4 clones, respectively, incubated with preimmune IgG; lanes 3-4, 490 SV-1 and B-4 cell lysate incubated with S006 antibody and S006 peptide; lanes 5-6, 490 SV-1 and B-4 cellular proteins incubated with S006 antibody only; lanes 7-12, Western blot using the S006 antibody after preincubating B-4 cell lysate with 1,000 μM ADP, ATP, GTPγS, GDP, GTP and GPP (NH)p, respectively. Lanes 13-14, immunoprecipitation of [^{35}S] methionine labeled bcl-2α protein from B-4 clone by S006 and S010 antibody, respectively; preimmune serum also reacted with upper non-specific bands but not with the 26 kD protein (data now shown). Lanes 15-18, [α-^{32}P] GTP binding to cellular proteins isolated from 490 SV-1, 490 SV-4 (control) and B-3, B-4 (bcl-2 transfected clones); lanes 15-16, 490 SV-1 and 490 SV-4 respectively. Lanes 17-18, B-3 and B-4 clones respectively. B) Lanes 1-4, immunoblot analysis of proteins isolated from β-gal/bcl-2α fusion construct. Lanes 1 and 3, cell lysate extracted before isopropylthio β-galactoside (IPTG) induction and blotted against preimmune and S006 antibody, respectively; lanes 2 and 4, cell lysate extracted after IPTG induction and blotted with preimmune and S006 IgG, respectively.

Methods: B-3 and B-4 clones were NIH 3T3 fibroblasts transfectd with bcl-2 DNA under the control of an SV40 promoter by calcium phosphate precipitation technique, whereas 490 SV-1 and 490 SV-4 are respective control NIH

3T3 cells without bcl-2 (Gorman 1985). Metabolic labeling of these cells was carried out with [^{35}S] methionine (50 µCi/ml; Amersham 800 Ci/mmol) in methionine-free DME medium for 16 hrs. For both Western blot and GTP binding studies, subconfluent cultures were extracted with lysis buffer without trypsinization. Lysate from β-gal/bcl-2α fusion gene construct was prepared by the method of Huynh (1985).

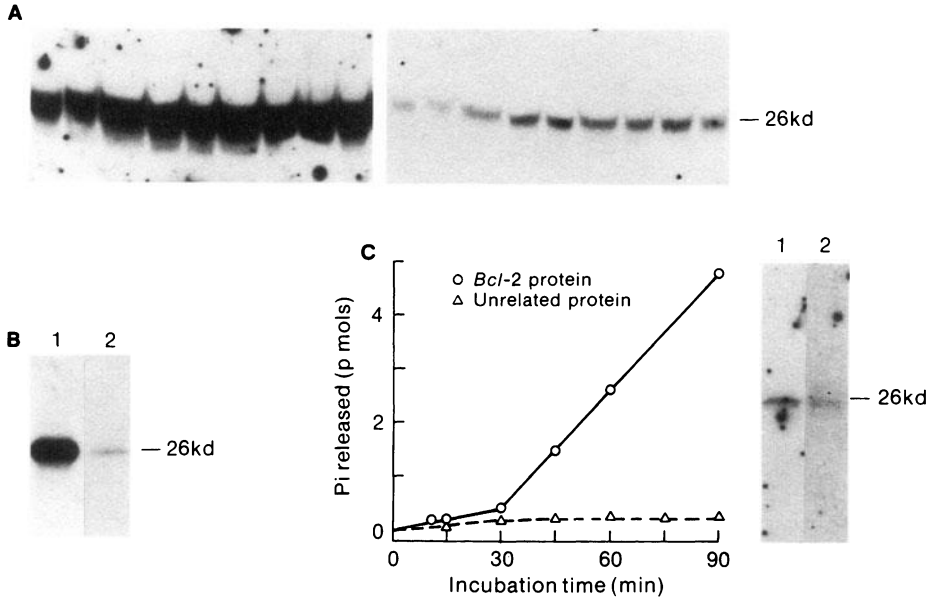


Fig. 4. A) [α-^{32}P] GTP binding to different fractions eluted from Ultrogen AcA-44 column. Left panel represents 16 hrs. exposure, whereas right panel is indicative of 1 hr. exposure of the same blot. Note that shorter exposure also reveals a single protein band of 26-kD which binds GTP. The autoradiograph is representative of fractions no. 21-29 eluted from Ultrogel AcA-44 column. Besides these fractions, 16-20 and 30-34 also showed [α-^{32}P] GTP binding activity by the same molecular weight protein (data not shown). B) Western blot and [α-^{32}P] GTP binding of purified bcl-2 protein. Lane 1, GTP binding; lane 2, immunoblot with S006 antibody. Pooled fractions showed GTP as well as antibody binding after Ultrogel AcA-44 and hybroxyapatite column were further purified by Q-sepharose column. An aliquot of the purified protein was subjected to the above studies. C) Electroeluted 26-kD protein binds GTP, recognizes peptide antibody against bcl-2 and possesses slow GTPase activity. Left panel represents GTPase activity of electroeluted bcl-2α protein. 100 ng of protein was incubated with [γ^{32}P] GTP (Amersham; 10 Ci/mmol) for different time periods as shown in the graph, and Pi released was measured in liquid scintillation counter. Protein purified to near homogeneity was electroeluted using Biorad electroelutor (Model 422). Lanes 1 and 2 of right panel showed GTP binding and antibody binding (S006), respectively. Assay of GTPase activity was carried out by the method of Ohmori (1989). Purification of bcl-2 protein was done by the method of Ohmori (1989) with modifications.

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Role of the Bcl-2 Proto-Oncogene in Neoplasia

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Specific chromosomal translocations are found in distinct neoplasms but not their normal cellular counterparts, implicating them in the origin or maintenance of malignancy. The first interchromosomal translocation to be well characterized juxtaposed a known cellular oncogene, c-myc, with the immunoglobulin (Ig) locus in Burkitt lymphoma and mouse plasmacytomas (Dalla-Favera 1982; Taub 1982; Sneng-Ong 1982; Adams 1983; Marcu 1983). A second generation of translocations in lymphoid neoplasms juxtaposes a known gene on one side (Ig or T cell receptor, TCR) with a new putative proto-oncogene on the other. The quintessential example of this is the t(14;18) of follicular lymphoma, a neoplasm of IgM/IgD bearing follicular center cells. The technical approach to these second generation breakpoints is to identify an Ig or TCR rearrangement that is atypical and use this to clone the breakpoint and search for a new gene on the far side (Bakhshi 1985; Cleary 1985; Tsujimoto 1985).

Despite the mature B cell phenotype of follicular lymphoma the t(14;18) appears to occur early in pre B cell development in that the breakpoints at the derivative (der) 14 chromosome are at a joining (J) region while the der (18) is at a diversity (D) region (Bakhshi 1987). Provocatively, ~ 70% of breakpoints on 18 occur within a 150 bp major breakpoint region (MBR); but no conserved heptamer-spacer-nonamer motifs are found there (Bakhshi 1987). However, the MBR proved to be within the 3' untranslated region of a new gene, Bcl-2. Moreover, the fact that the translocation occurred early in development suggested it would prove of prospective importance in generating malignant cells.

Our studies concerning the normal regulation of the Bcl-2 gene indicate that its expression is associated with B cell activation and proliferation. Bcl-2 is located on chromosome segment 18q21.3 in a telomere to centromere orientation (Seto 1988). The Bcl-2 gene is a 3 exon gene with an untranslated first exon. It has two promoter regions. P1 is GC rich with multiple SP1 sites, is used predominantly, while P2 has a classical TATA and CCAAT-box and a motif homologous with the immunoglobulin octamer and SV40 decamer. Bcl-2 is an enormous gene with an approximately 350 Kb Intron II that divides the protein encoding regions of exons II and III. The Bcl-2 gene encodes a 25 Kd membrane-associated intracellular protein whose precise biochemical role is still under investigation.

The molecular consequence of translocation moves the Bcl-2 gene to the distal tip of chromosome 14 in the same transcriptional

orientation as the Ig H chain locus, and gives rise to a series of chimeric RNAs (Fig 1)(Cleary 1986; Seto 1988). This does not interrupt the protein encoding region, although somatic mutation can occur within this area. While pre B cells possess high levels of Bcl-2 mRNA, mature B cells have very low levels. In contrast, t(14;18) bearing B cells have inappropriately elevated levels of the fusion RNA (Fig 1)(Graninger 1987). The quantity of the 25 Kd Bcl-2 protein parallels these RNA levels.

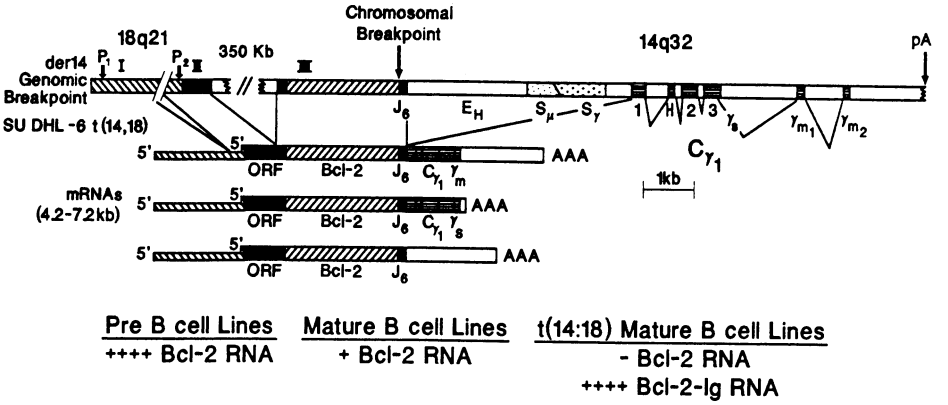


Fig. 1 Bcl-2-Ig Fusion Gene. The t(14;18) introduces Bcl-2 into the Ig locus in the same transcriptional orientation. This generates a series of (4.2-7.2 Kb) fusion RNAs. Relative levels of steady state RNA levels are indicated.

A major question still remained as to whether newly discovered genes found at site of interchromosomal translocation would prove to be transforming in a prospective fashion. We have taken several approacnes to examining the functional role of Bcl-2. We used the N2 series of retroviral vectors from Dr. Eli Gilboa which possess a NeoR gene for selection, and inserted either the human or murine cDNA under the control of the SV40 promoter. These integrated vectors highly expressed Bcl-2 RNA and protein. In collaboration with Dr. Riccardo Dalla-Favera's lab we placed the human Bcl-2, N2-H-Bcl-2, into lymphoblastoid lines (EBV immortalized B cells) and assessed classic measurements of oncogenesis: clonogenicity and tumorigenicity (Nunez 1989a). Lymphoblastoid lines infected with N2-H-Bcl-2 as compared to the parental N2 virus demonstrated a consistent 3-fold improvement in clonogenicity within soft agar. However, N2-H-Bcl-2 was insufficient as a single agent to confer tumorigenicity to lymphoblastoid cell lines. Consequently, we next examined Bcl-2's ability to complement other cellular oncogenes, such as myc. We introduced either the N2 or the N2-H-Bcl-2 virus into lymphoblastoid lines already possessing a deregulated myc gene carried by an EBV vector. The addition of deregulated Bcl-2

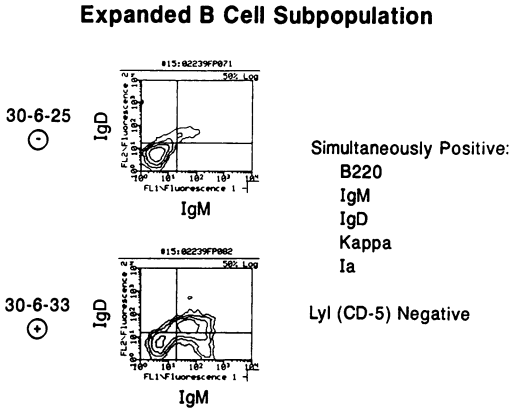
increased the frequency and shortened the latency of tumor formation in immunodeficient nude mice (Nunez 1989a). Thus Bcl-2 appeared to have a subtle yet consistent effect upon B cell growth and neoplasia.

These findings prompted us to examine whether Bcl-2 itself might be directly involved in a growth factor pathway. To approach this, we placed the murine N2-M-Bcl-2 vector into a variety of interleukin dependent cell lines to determine if overproduced Bcl-2 would spare the need for a specific receptor/ligand complex. No long-term growth factor independent lines resulted from deregulated Bcl-2 in IL-3, IL-2, or IL-6 requiring cell lines (Nunez 1989b). However, a remarkable short-term response was witnessed with a pro B lymphocyte line, FL 5.12, established from fetal liver by Dr. John McKearn. Bcl-2 conferred a death sparing effect to these cells. FL 5.12 is entirely dependent upon IL-3 for its survival and proliferation (London 1987). Over 95% of FL 5.12 or N2 infected FL 5.12 is dead within 72 hours following IL-3 deprivation. However, 60-95% of N2-M-Bcl-2 infected cells are still viable 72 hrs status post deprivation. Yet, these cells are not proliferating, and acridine orange studies to assess cell cycle demonstrate they are resting in Go. Subsequent examinations of serial time points following IL-3 deprivation and assessment of numerous parameters of cell survival and proliferation provided further insights. The predominant effect of deregulated Bcl-2 is to delay the onset of death and to modestly augment viable cell proliferation and cell division in the first 48 hrs after factor removal. Bcl-2's effects were not limited to prolymphocytes, but also spared the death of promyeloid (Vaux 1988) and mast cell lines deprived of IL-3 (Nunez 1989b). Moreover, Bcl-2's effects are not restricted to the IL-3, IL-3 receptor signal transduction pathway in that IL-4 and GM-CSF adapted FDC-P1 cells consistently showed the effect of Bcl-2 upon survival. However, some lineage and/or growth factor pathway restriction does exist for Bcl-2's effects. Neither IL-2 dependent T cell lines, HT-2 or CTLL-2, nor the IL-6 dependent MH60 myeloma line showed any effect (Nunez 1989b). This provided some of the first evidence that Bcl-2's role in promoting tumors might relate to enhancing the survival of cells by perhaps interfering with a cell death pathway.

The most unrestricted tests of Bcl-2's transforming ability was to place it into the germline of mice to observe its effects upon all lineages. We generated minigene constructs that recreated the pathologic consequence of the t(14;18)(McDonnell 1989). We utilized the normal 5' genomic flank of Bcl-2 including the promoter region to confer any tissue and stage specificity located there. The enormous 350 Kb intron II prompted the use of a short portion of cDNA bearing the ExonII/Exon III juncture. The genomic 3' flank of Bcl-2 followed and the Ig heavy chain including the enhancer and C_γ region was introduced at the MBR exactly as it occurs in lymphomas. Two Bcl-2-Ig constructs bearing either the normal or somatically mutated Bcl-2 produced similar results. Both constructs were expressed effectively as transgenes in a lymphoid pattern and both thymus and spleen demonstrated substantial amount of the 25 Kd Bcl-2 product (McDonnell 1989).

Bcl-2-Ig transgenic mice uniformly developed polyclonal lymphoproliferation of an expanded follicular center B cell compartment. Transgenics demonstrated hyperplasia of splenic

follicles and contiguous nodules would coalesce into large geographic regions of white pulp. The predominant expansion of cells was in the peripheral B cell zone of the follicle. Two color flow cytometry indicated that the expanded population was simultaneously positive for B220, IgM, IgD, K, and Ia, but negative for CD5, (Ly1) (Fig 2)(McDonnell 1989). Overall the absolute number of splenic B cells was increased approximately 4-fold at 9-12 weeks of age. Lymph nodes showed similar changes and the percentage of B cells in the bone marrow was markedly elevated. Despite the marked lymphoproliferation and histology, the B cells at this timepoint are still polyclonal as assessed by endogenous Ig gene configuration.



Absolute Number of Splenic B Cells x 10^6

	# Mice	B220	IgM
Bcl-2-Ig Transgenic	11	168 ± 47	136 ± 36
Normal Littermates	11	43 ± 9	40 ± 8
		p < .02	p < .02

Fig. 2. Bcl-2-Ig Transgene expands a B cell Subpopulation. Two color fluorescence contour plots of IgM and IgD are shown for a normal control littermate 30-6-25 and a transgenic 30-6-33. Absolute numbers of splenic B cells at 9-12 wks of age are indicated.

A most remarkable property of the Bcl-2-Ig transgenic mice is that they possess a relatively immortalized B cell population. Splenocytes from normal non-transgenic littermates die off rapidly in culture, while in marked contrast ~ 10% of transgenic splenocytes demonstrate extended survival in vitro. Transgenic splenocytes will survive for over 3 months in simple liquid culture with only 5% FCS. Flow cytometry examination reveals that the surviving cells are the mature B cells bearing B220, IgM/IgD, K, and Ia. However, these B cells are not proliferating but 99% are in Go. While resting these B cells will traverse the cell cycle and proliferate given appropriate stimuli such as LPS (McDonnell 1989).

Thus the Bcl-2-Ig transgene is selectively expanding a distinct subpopulation of IgM, IgD resting B cells that demonstrate extended

survival. In addition these transgenics are confirming a prospective role for the t(14:18) and the Bcl-2 Ig fusion gene in the pathogenesis of follicular lymphoma. Moreover, they provide a prospective animal model to identify the 2° oncogenes capable of complementing Bcl-2 in the progression from an indolent follicular lymphoproliferation to a monoclonal high grade neoplasm.

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Transgenic Mice as Models for the Development of Haemopoietic Neoplasia

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INTRODUCTION

Transgenic mice are powerful tools for dissecting the multi-step process of neoplastic transformation (Cory and Adams, 1988). Focussing our attention on the genetic basis of haemopoietic neoplasia, we have constructed a series of transgenic lines harboring oncogenes suspected of playing a role in the aetiology of specific lymphomas or leukaemias. We targeted haemopoietic cells for expression of the trans-oncogenes by linking them to the well-studied immunoglobulin heavy chain enhancer (Eμ), which can function *in vivo* not only in B lymphoid cells (Adams et al, 1985) but also in T cells (Grosschedl et al, 1984) and, from our unpublished results, in at least certain myeloid cells.

The impetus for these studies was the desire to test directly *in vivo* whether a deregulated *myc* gene was tumorigenic for B lymphoid cells, an hypothesis strongly supported by the demonstration in our laboratory and elsewhere (reviewed by Cory, 1986) that the chromosome translocation typifying most human Burkitt lymphomas and murine plasmacytomas reflects a recombination event juxtaposing the c-*myc* proto-oncogene with the immunoglobulin heavy chain (IgH) gene locus. As reported at the Sixth International Congress (Cory et al, 1986), constitutive *myc* expression is indeed lymphomagenic: transgenic mice carrying an otherwise normal c-*myc* gene linked to the IgH enhancer (Eμ) are doomed to develop a disseminated clonal pre-B or B lymphoma and associated lymphoblastic leukaemia (Adams et al, 1985). Detailed analysis of the tumour-prone line has revealed that the disease involves at least two stages: a benign polyclonal overproliferation of large, cycling pre-B cells in the bone marrow and spleen (Langdon et al, 1986), followed by development of a clonal neoplasm manifested in the lymph nodes and/or thymus (Harris et al, 1988). We ascribe the first phase solely to the action of constitutive *myc* expression and the second to the synergistic action of *myc* and additional, somatically activated, oncogenes (see below).

The other oncogenes we have tested in the transgenic context are of various classes. We have established lines harboring N-*myc* which, like c-*myc*, encodes a nuclear DNA-binding protein; N-*ras*, which encodes a cytoplasmic protein considered to be a signal transducer; *abl*, a member of the tyrosine kinase family and *bcl*-2, which encodes a cytoplasmic protein of unknown function. As summarised below, each trans-oncogene engenders a characteristic disease and the tumorigenic process appears to involve acquisition of additional tumor-promoting gene(s). In certain tumors, the culpable collaborating genes have been identified, but in others they remain unknown.

B-LYMPHOMAGENESIS IN Eμ-*myc* MICE

The Preneoplastic Phase

Young healthy Eμ-*myc* mice contain no transplantable malignant cells but their pre-B and B cells are uniformly large and most (perhaps all) are in cycle (Langdon et al, 1986). Thus, constitutive c-*myc* expression may prevent B lymphoid cells from entering the G_0 state. The process of B lymphoid differentiation in the mice is clearly abnormal, because pre-B cells are elevated 4- to 5- fold and Ig-bearing cells slightly depressed. Moreover, although the mice are immunocompetent, fewer Ig-bearing cells respond immunologically, perhaps because the cells are not fully mature (Vaux et al, 1987). We therefore proposed that c-*myc* expression in lymphoid cells regulates the balance between self-renewal and differentiation, constitutive expression promoting self-renewal at the expense of maturation but not imposing a complete block on differentiation (Langdon et al, 1986).

Since *myc* is considered a nuclear transducer of signals from growth factor receptors, it is somewhat surprising that the growth properties of pre-neoplastic Eμ-*myc* pre-B cells are very similar to those of normal pre-B cells. In liquid culture, they die rapidly unless provided with a stromal feeder layer (Langdon et al, 1988), and they fail to grow as colonies in agar (Dyall-Smith and Cory, 1988; Alexander et al, 1989a). Thus the cells appear to retain their absolute dependence on growth factors and do not appear to be immortal, despite the commonly held notion that *myc* is an immortalising gene. Nevertheless, as in the transgenic mice, the Eμ-*myc* lymphocytes growing on feeder layers are large and at least 2.5 times as many as normal are in cycle. Thus the *myc*-driven cells appear either to remain in cycle or to die (Langdon et al, 1988).

Search for the Somatic Alterations that Generate Spontaneous Eμ-*myc* Tumors

The kinetics of tumour onset in Eμ-*myc* mice suggests that the rate at which the cycling, benign pre-B cells spontaneously convert to malignancy is about 10^{-10} per cell per generation (Harris et al, 1988). It is thus possible that the tumorigenic process requires more than one somatic mutation. Indeed, *in vitro*, at least two stages were apparent. After prolonged culture on stromal cells (14-20 weeks), Eμ-*myc* pre-B cells started to grow to 10-fold higher densities but were not tumorigenic; eventually, however, a tumorigenic clone emerged (Langdon et al, 1988).

Somatic alteration of the *myc* transgene could be invoked as a step contributing to tumorigenesis in the Eμ-*myc* mice. In human Burkitt lymphomas, *myc* exon 1 mutations have been implicated in tumorigenesis, since the *myc* gene has not only been translocated to an immunoglobulin locus but usually also mutated near the 3′ end of exon 1 (Pelicci et al, 1986), a region where *myc* transcription is attenuated (Bentley and Groudine, 1988, Nepveu and Marcu, 1986) and translation of a larger *myc* polypeptide initiates (Hann et al, 1988). To determine whether exon 1 mutations are necessary, we utilised the polymerase chain reaction to analyse *myc* mRNA sequences from the exon 1 region in five Eμ-*myc* B lymphoid tumors. No exon 1 mutations were found; hence its alteration

is not necessary to render *myc* tumorigenic. Moreover, no structural rearrangement of the Eμ-*myc* gene could be detected in any of 20 tumors and in only two cases was there any amplification. We conclude (Webb, Barri, Cory & Adams, submitted) that somatic mutation of the Eμ-*myc* transgene is unlikely to account for the onset of tumours in Eμ-*myc* mice.

On the hypothesis that tumour onset requires activation of an independent oncogene, we screened 14 Eμ-*myc* lymphoma DNAs for genes capable of transforming NIH-3T3 fibroblasts to grow as fibrosarcomas in nude mice. Fibrosarcomas with a particularly rapid onset were provoked by DNA from two lymphomas. These lymphomas were found to carry a mutated N-*ras* or K-*ras* gene (Alexander et al, 1989b), the mutation in each case involving amino acid 61, one of the three residues most frequently implicated in *ras* activation (Barbacid, 1987). The mutated N-*ras* gene was cloned into a retroviral vector and shown to transform pre-B cells from Eμ-*myc* mouse bone marrow. Thus a spontaneous *ras* mutation contributed to the development of two of the Eμ-*myc* tumours, but the collaborating genes responsible for the majority of the tumours do not register in the fibroblast assay and remain to be identified. The relevant genes might prove to include anti-oncogenes as well as oncogenes.

Eμ-*myc* Mice as Tools for Testing Oncogene Cooperativity

While the gene(s) responsible for most spontaneous Eμ-*myc* lymphomas remain elusive, we have shown that certain oncogenes can act in synergy with c-*myc* to transform B lymphoid cells. Lymphoma development was greatly accelerated by infecting newborn Eμ-*myc* transgenic animals with helper virus-free stocks of viruses carrying either the v-H-*ras* or the v-*raf* oncogene (Langdon et al, 1989). *In vitro* infection of bone marrow cells from Eμ-*myc* mice with the same viruses (Alexander et al, 1989a) confirmed and extended the *in vivc* work. The v-H-*ras* and v-*raf* viruses both generated a rapid polyclonal expansion of Eμ-*myc* pre-B cells in culture. The infected transgenic cells were autonomous, cloned efficiently in agar, and grew as tumours in nude mice. With normal bone marrow, most cells infected with a v-*raf* or v-H-*ras* virus cloned poorly in agar and were rarely malignant. These results imply that both v-H-*ras* and v-*raf* can collude with constitutive *myc* expression to transform B lymphoid cells. They leave open the issue as to whether v-H-*ras* + *myc* or v-*raf* + *myc* is sufficient to transform a B lymphoid cell or whether still further genetic change is required.

Surprisingly, no synergy could be detected by infecting Eμ-*myc* cells with Abelson virus, either *in vivo* or *in vitro* (Dyall-Smith and Cory, 1988; Alexander et al, 1989a; Langdon et al, 1989). These results appear to indicate that v-*abl* cannot collaborate with c-*myc* to transform pre-B cells, although they may act together at a later stage (see below).

Insertional Mutagenic Approach for Identifying Cooperating Oncogenes

Since retroviruses that lack an oncogene can contribute to tumorigenesis by chance insertion near critical cellular genes (Bishop, 1987), we are exploiting Moloney leukaemia virus to identify genes able to collaborate with deregulated *myc* expression to produce tumours. The same approach has been used by Berns and his colleagues (Van Lohuizen et al, 1989) to identify genes which accelerate T lymphoma onset in transgenic mice

harboring an activated *pim*-1 gene, the gene originally identified as a preferred proviral insertion site in T lymphomas of viral aetiology (Cuypers et al, 1984). Strikingly, Eμ-*myc* mice infected as newborns with Moloney virus were all moribund before any uninfected transgenic littermate became ill. Moreover, all the induced Eμ-*myc* tumours were B lymphoid - in conventional mice this virus produces only T cell tumours. Hence the tumours must have arisen by synergy between Eμ-*myc* expression and Moloney virus insertion.

Two strategies are being used to screen the virus-induced Eμ-*myc* tumours for proviral insertions near a collaborating oncogene. The first entails analysis of the tumour DNAs for rearrangement of oncogenes known to contribute to lymphoid tumorigenesis, e.g. *cbl*, *bcl*-2, *pim*-1, *raf* and *abl*. Somewhat surprisingly a substantial proportion (19%) of the B lymphomas bear proviruses near *pim*-1. Thus, *pim*-1 can contribute to B as well as T cell neoplasia. The second approach is to determine whether any proviral insertion sites are common to several tumours. Already one such site has been identified (W. Alexander et al, unpublished results). The DNA flanking a provirus cloned from one tumour proved to be rearranged in at least 27% of the other tumours. This locus, which we have provisionally designated *emi*-1 (Eμ-*myc* Moloney virus insertion region-1), is transcribed at higher levels in the tumours harboring a rearrangement than in those which lack one. The *emi*-1 gene will be characterised by cloning and sequencing the cDNA. This analysis will also clarify its relationship, if any, to c-*bic* (Clurman et al, 1989) and c-*bim* (A. Berns, personal communication), proviral insertion regions recently implicated in collaborating with *myc* in B lymphoid tumours.

In summary, Eμ-*myc* mice have become a paradigm for analysing the molecular basis of neoplastic progression. They have demonstrated that lymphomagenesis *in vivo* or *in vitro* can involve collaboration between oncogenes, an hypothesis widely accepted but previously studied mainly only in fibroblasts and primary kidney cells (Land et al, 1983; Ruley, 1983). They will continue to be an ideal test-bed for determining which genes can collaborate with *myc* to transform B lineage cells and whether certain combinations are effective only at specific differentiation stages.

N-*myc* TRANSGENIC MICE AND THE CONTROL OF *myc* EXPRESSION

The vertebrate genome contains several *myc*-related genes. One of these, N-*myc*, is expressed in lymphoid cells only at early (pre-B) stages of differentiation (Zimmerman et al, 1986), unlike c-*myc*, which is expressed in proliferating cells of all stages. The elevated expression and frequent amplification of N-*myc* in certain advanced cases of human neuroblastoma, retinoblastoma and small cell lung carcinoma suggested that its abnormal expression was instrumental in their progression (reviewed by Alt et al, 1986), but it had never been associated with haematological tumours.

Significantly, we have found that N-*myc* affects lymphocytes very similarly to c-*myc*. Eμ-N-*myc* mice succumb to clonal pre-B or B cell lymphomas with similar kinetics to Eμ-c-*myc* mice and the bone marrow of

pre-lymphomatous mice contains abnormally high numbers of pre-B cells (Rosenbaum et al, 1989). N-*myc* also appears to be lymphomagenic for T cells, because certain T lymphoid tumours induced in Eμ-*pim*-1 mice by Moloney virus exhibit proviral insertions near N-*myc* as well as c-*myc* (Van Lohuizen et al, 1989). These results firmly establish N-*myc* as a bona fide oncogene and point to the need to reassess its role in human haematological malignancies.

Like the c-*myc* gene (Leder et al, 1983; Adams et al, 1985; Alexander et al, 1987), N-*myc* appears to be subject to negative feedback regulation. Although N-*myc* is normally expressed in pre-B lymphocytes, the normal N-*myc* alleles are silent in Eμ-N-*myc* pre-B lymphomas (Rosenbaum et al, 1989). Moreover, c-*myc* is also silent in these tumours, indicating that N-*myc* can completely supplant c-*myc* function and suggesting that N-*myc* and c-*myc* can regulate one another. In support of this hypothesis, the endogenous c-*myc* and N-*myc* genes are also silent in Eμ-c-*myc* tumours. Thus N-*myc* and c-*myc* appear to be subject to cross-regulation as well as auto-regulation. Alt and his colleagues have independently developed Eμ-N-*myc* mice and reached similar conclusions (Dildrop et al, 1989).

Eμ-v-*abl* TRANSGENIC MICE DEVELOP PLASMACYTOMAS HARBORING *myc* ALTERATIONS

Abelson murine leukaemia virus, which bears the v-*abl* oncogene, generates pre-B and T lymphomas and accelerates development of plasmacytomas in BALB/c mice also treated with the mineral oil pristane (Whitlock and Witte, 1985). The pattern of lymphomagenesis is strongly influenced by the route of virus administration and the helper virus used. To study the transforming potential of v-*abl* for lymphoid cells in the absence of such complicating variables, we developed several strains of mice carrying the *gag-abl* gene from Abelson virus linked to the SV40 promoter and IgH enhancer (Eμ-v-*abl* strains). Although their lympho-haemopoietic development was apparently normal, three strains proved to have a remarkable predisposition to develop plasmacytomas. About 65% of the mice became terminally ill within 12 months with tumours diagnosed as plasmacytomas by cellular morphology, the presence of clonal rearranged heavy and light chain genes and high levels of clonal immunoglobulin (usually IgA or IgG) in their serum (Rosenbaum, Harris, Bath, McNeall, Webb, Adams and Cory, submitted).

Strikingly, at least 80% of the plasmacytomas contained a rearranged c-*myc* gene and half of these seemed to result from linkage of *myc* to the Ig Cα gene. These data strongly suggest that v-*abl* and *myc* co-operate in the transformation of plasma cells. Indeed, the rate-limiting step for the onset of Eμ-v-*abl* plasmacytomas may well be the chance of a *myc* translocation occurring in a single member of the susceptible population. Our data complement earlier observations (Ohno et al, 1984) that the plasmacytomas induced by Abelson virus in pristane-treated BALB/c mice carry *myc* translocations.

In view of the predilection of Abelson virus for pre-B lymphomagenesis why have no lymphomas appeared in three independent Eμ-v-*abl* lines? The transgene is clearly capable of generating such tumours, because they do

arise in a fourth Eμ-v-*abl* line we are currently analysing. The hypothesis we favor to reconcile this paradox is that v-*abl* induction of pre-B lymphomas requires its activity within a very early cell type, one that is infectable by Abelson virus but in which the IgH enhancer, and hence an Eμ-transgene, is usually inactive. (Presumably the transgene in the lymphoma-prone Eμ-v-*abl* line is activated abnormally early by some influence of the flanking DNA). The model posits that the typical Abelson virus pre-B lymphoma results from the transformed cell continuing to differentiate, at least with respect to IgH rearrangement. To account for the paucity of Abelson transformants that are generated either *in vivo* or *in vitro* from Eμ-*myc* bone marrow (Dyall-Smith and Cory, 1988; Alexander et al, 1989a; Langdon et al, 1989), despite its elevated pre-B cell content (Langdon et al, 1986), the model also postulates that v-*abl* is relatively ineffective as a transforming agent for bona fide pre-B cells. Intriguingly, recent data generated with sorted cell populations also suggest that the major Abelson target is more primitive than a pre-B cell (Tidmarsh et al, 1989).

Eμ-N-*ras* MICE DEVELOP T LYMPHOMAS AND HISTIOCYTIC SARCOMAS BUT NO B LYMPHOID TUMOURS

Although the closely related H-*ras*, N-*ras* and K-*ras* genes are widely expressed and activated by similar mutations, it is N-*ras* that is mutated most frequently in haemopoietic tumours (Diamond et al, 1988; Farr et al, 1988; Seremetis et al, 1989). We have produced two strains of mice harboring a human N-*ras* cDNA mutated at codon 12 and coupled to the immunoglobulin heavy chain enhancer (Eμ) and the SV40 promoter. Unexpectedly, no mice from either line have developed any B lymphoid tumours, despite evidence of transgene expression in B lymphoid cells. They become ill instead with thymic T lymphomas and/or histiocytic sarcomas predominantly located in the spleen and liver (Harris et al, in preparation). The histiocytic tumours have provisionally been classified as macrophage tumours on the basis of morphology, phagocytic activity, immunohistochemical detection of the Mac-1 marker and lack of rearranged genes for immunoglobulin or T cell receptors. These data imply that, when it is the first oncogenic event, a mutated N-*ras* gene potentiates tumorigenesis more effectively in T lymphoid cells or macrophages than in B lymphoid cells. We suspect that the absence of B lymphoid tumours means that B cell development has been altered in these mice, an issue currently being addressed.

Eμ-*bcl*-2 PROVOKES A B CELL EXCESS SYNDROME

The *bcl*-2 gene was discovered by virtue of its fusion to the IgH locus in human follicular cell lymphomas carrying the 14;18 chromosome translocation (Tsujimoto et al, 1984). While clearly a candidate oncogene, the biological activity of *bcl*-2 was unknown. The first clue to its function was our observation (Vaux et al, 1988) that immortal but factor-dependent cell lines infected with a *bcl*-2-bearing retrovirus remained viable after removal of growth factor, although they did not proliferate. This finding suggested that *bcl*-2 represents a novel type of gene that confers a "survival" rather than proliferative function.

To explore the oncogenic potential of *bcl*-2 and characterise its activity *in vivo*, we have developed strains of mice expressing *bcl*-2 under the control of the IgH enhancer and either an SV40 or V_H promoter (our unpublished results). Intriguingly, spleen cells from such mice also exhibit the ability to survive exposure to adverse growth conditions: when plated *in vitro* in medium containing serum but no lymphokines, the transgenic cells survived far longer than cells from normal littermates. Korsmeyer and his colleagues have also observed this phenomenon in their strains of *bcl*-2 transgenic mice (McDonnell et al, 1989). Intriguingly, the *bcl*-2 mice exhibit limited splenomegaly, increased numbers of lymphocytes in the peripheral blood and an increased frequency of sIg^+ B lymphoid cells in haemopoietic tissues, particularly the bone marrow. The small size of these cells suggests that they are not actively proliferating.

The data from *bcl*-2 mice are consistent with the hypothesis that constitutive *bcl*-2 expression confers on the typical G_0 lymphocyte a greatly increased lifespan *in vivo*, perhaps because the cell is now resistant to conditions (such as a dearth of factors) that normally promote its demise. Thus the role of *bcl*-2 in oncogenesis may simply be to promote the longevity of the B lymphocyte clone bearing the 14;18 translocation and hence increase its chance of acquiring another activated oncogene, or of forfeiting an anti-oncogene. Significantly, sporadic haemopoietic tumours are beginning to occur in the Eμ-*bcl*-2 colony. Detailed characterisation of the tumours and the lymphoproliferative syndrome will be major endeavours in coming months.

FUTURE DIRECTIONS

Clearly the availability of these unique animal models for various haemopoietic malignancies will be an invaluable resource for further analysing both the function of particular oncogenes and the pathway to haemopoietic malignancy. A particularly fascinating prospect is the opportunity they present for cross-breeding and therefore analysing oncogene co-operation *in vivo*.

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Tumor Suppression in Somatic Cell Hybrids Between Burkitt's Lymphoma Cells and EBV-Immortalized Lymphoblastoid Cells

J. Wolf, M. Pawlita, J. Bullerdick, and H. zur Hausen

SOMATIC CELL HYBRIDS IN THE ANALYSIS OF TUMORIGENESIS

Suppression of the tumorigenic phenotype has been demonstrated for a wide spectrum of murine and human tumor cells fused with normal fibroblasts (Harris 1988). These observations have led to the hypothesis that loss of specific genetic information which is present in the nontumorigenic fusion partner, but apparently absent in the malignant one, represents an essential event in malignant transformation. Since tumor suppression could also be demonstrated in hybrids containing activated oncogenes, a recessive nature of these genes was proposed (Sager 1986). Therefore, analysis of tumorsuppression in somatic cell hybrids may represent the experimental counterpart to transfection experiments in which introduction of two oncogenes caused malignant transformation (Land et al. 1983) and which therefore suggested dominance of activated oncogenes.

While the view that loss of genetic information plays a role in the development of tumors of epithelial and mesenchymic origin has been stressed even more by the finding that specific alleles are consistently lost in several of these malignancies (Ponder 1988), this mechanism has not been implicated in models for the development of hematopoietic and lymphatic malignancies. Molecular analysis of the specific chromosomal translocations of human lymphomas and leukemias suggested transcriptional deregulation (prototype: c-myc deregulation in Burkitt's lymphoma) or expression of an altered oncogene product (prototype: c-abl-bcr hybrid transcript in chronic myelogenous leukemia) to be essential for malignant transformation in these diseases (Croce 1986).

In agreement with this concept suppression of the tumorigenic phenotype up to now has not been demonstrated for human lymphoma/lymphocyte hybrids. On the contrary, a malignant phenotype has been reported in murine T-lymphoma/lymphocyte hybrids (Hays et al. 1986; Uno et al. 1987).

Assuming that experimental artefacts including chromosomal instability were the reason for these results rather than dominance of the malignant phenotype and that loss of genetic information may also be involved in hematopoietic and lymphatic malignancies, we intended to demonstrate suppression of tumorigenicity in human lymphoma/lymphocyte hybrids.

ESTABLISHMENT OF SOMATIC CELL HYBRIDS BETWEEN A MALIGNANT BURKITT'S LYMPHOMA CELL LINE AND A NONMALIGNANT LYMPHOBLASTOID CELL LINE

Selection of Cell lines

Burkitt's lymphoma provides several experimental advantages for the analysis of tumor suppression by somatic cell fusion. Firstly, well characterized cell lines are available. Secondly, by using Epstein-Barr virus (EBV) immortalized lymphoblastoid cell lines, it is possible to fuse BL cells with a nonmalignant fusion partner of the same differentiation lineage, also originating from the same individual, i.e. providing the same genetic background. We selected the following cell lines, which were established and kindly provided by G.Lenoir (Lenoir et al 1985):

Table 1. Parental cell lines

	BL 60	IARC 277
origin:	Burkitt's lymphoma of a 4 years old Northafrican female	EBV immortalized B-lymphocytes of the same patient
EBV:	positive	positive
chromosomal translocation:	t (8;22)	no translocation
in vitro phenotype:	no clumping in suspension culture	clumping
in vivo phenotype:	highly tumorigenic in nude mice	non tumorigenic

Experimental Procedure

As shown in figure 1, a 'universal fuser' subline of BL 60 was constructed by first introducing a neomycin resistance plasmid and subsequent cultivation in 6-thioguanine to select for hypoxanthine-guanine-phosphoribosyltransferase deficient($HGPRT^-$) mutants. To minimize genetic damage of the BL60 cell line, $HGPRT^-$ mutants were selected without preceding mutagenization.
The nontumorigenic fusion partner was not manipulated at all to avoid selection for tumorigenic segregants.

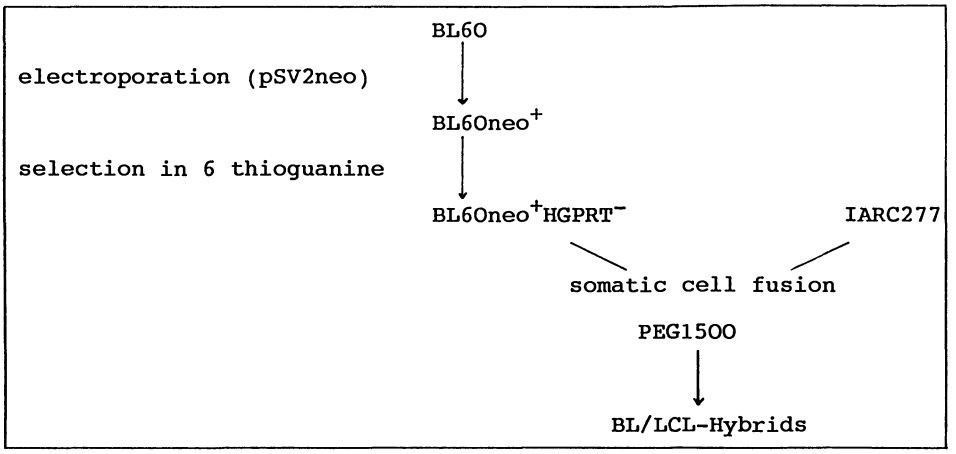

Fig. 1. Experimental procedure

Results

Seven BL60/IARC 277 hybrid clones were analysed in detail (manuscript in preparation). They demonstrate a near tetraploid karyotype (modal counts:82-93) including one copy of the chromosome $8q^+$ involved in the specific (8;22) translocation of BL 60 versus 3 normal copies of the chromosome 8. In the variant (8;22) translocation of BL60 immunoglobulin light chain genes translocate from one chromosome 22 to one chromosome 8 ($8q^+$) where they are localized distal to the c-myc gene, whose deregulated expression pattern is thought to contribute to the malignant phenotype. All 7 hybrid clones exhibited the phenotype of the parental lymphoblastoid IARC 277 cell line by forming large clumps in suspension culture and were not tumorigenic in nude mice. However, despite suppression of tumorigenicity they demonstrated the deregulated c-myc expression pattern of the parental Burkitt's lymphoma cell line, i.e. highly abundant transcripts originating nearly exclusively from chromosome $8q^+$ (see table 2).

Table 2. Comparison of parental and hybrid cell lines

	BL60	Hybrid Clones	IARC277
EBV:	+	+	+
C-myc deregulation:	+	+	-
tumorigenicity:	+	-	-

IMPLICATIONS FOR THE PATHOGENESIS OF ENDEMIC BURKITT'S LYMPHOMA

Endemic Burkitt's lymphoma is the most common malignancy of children in certain areas of equatorial Africa and New Guinea (de The 1982). Seroepidemiological studies (Henle et al. 1969) as well as the detection of the viral DNA in the tumor (zur Hausen et al. 1970) have provided strong evidence for an etiological role of Epstein-Barr virus (EBV) infection in the development of endemic BL. In addition specific chromosomal translocations are consistently found in BL which involve the cellular oncogene c-myc and immunoglobulin genes. Deregulated expression of the c-myc gene, caused by these translocations, is thought to contribute to the malignant BL phenotype (Leder et al. 1983). According to these findings EBV-infection and c-myc deregulation represent the critical events in the proposed scenarios for the development of endemic BL (Klein 1979; Lenoir and Bornkamm 1987). This view was strongly supported by Lombardi et al. (1987), who reported that EBV immortalization and subsequent introduction of a constitutively expressed c-myc gene were sufficient for the malignant transformation of human B-lymphocytes.
This concept of endemic BL as a malignancy caused by two oncogenic functions is questioned by our finding which demonstrates that the malignant phenotype of a Burkitt's lymphoma cell line can be suppressed by fusion with an EBV-immortalized B-lymphoblastoid cell line despite the continued deregulation of the c-myc gene.
Therefore our results suggest that in analogy to results obtained by the analysis of somatic cell hybrids established with tumors of epithelial and mesenchymic origin, absence of specific cellular gene functions may be considered also in the pathogenesis of endemic BL.

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II Cell Developments

Primary Immunodeficiencies

Primary Immunodeficiencies: Current Findings and Concepts

M. Seligmann

INTRODUCTION

Human primary immunodeficiencies (ID) represent true experiments of nature. In depth study of such patients led to important insights on the physiology of the immune system. Conversely, some discoveries in the field of fundamental immunology have led to beneficial outcome for patients. This kind of back and forward game is what makes clinical immunology exciting. During the past few years, the pathogenesis of several primary ID has been specified with increasing precision. In this field of immunology, as in many others, molecular biology has provided most of these new insights.

SEVERE COMBINED IMMUNODEFICIENCIES (SCID) IN MEN AND MICE

Human SCID's are characterized by a profound deficiency in both B and T cell functions. Infants with these disorders usually dye early in life, unless successfully treated by bone marrow transplantation, from overwhelming infections, particularly those caused by opportunistic microorganisms. Several distinct diseases are grouped under the term of SCID and only some of these will be briefly discussed here.

SCID with alymphocytosis was originally described by Swiss pediatricians. It is an autosomal recessive disease with virtual absence of both mature B and T cells, without any defect of other hematopoïetic lineages. This led to the hypothesis that the basic defect could lie at the level of a lymphoid stem cell, although there is no convincing evidence in favour of the existence of such a cell. The discovery by M. Bosma (this volume) of a murine model with striking analogies with this Swiss type of human SCID strongly argued against this hypothesis.

Murine SCID results from a spontaneous autosomal recessive mutation in CB 17 mice with failure to develop mature T or B lymphocytes. The study of Abelson transformants and of spontaneous thymomas in such mice indicated (Schuler et al. 1986) that both B and T lineage cells made highly aberrant rearrangements and suggested that the SCID mutation affects the V(D)J recombination activity. Recent studies (Malynn et al. 1988 ; Lieber et al. 1988) showed that this mutation more precisely impairs the correct joining of coding segment ends generated by a normal endonucleolytic activity of recombinase. Both pre-B and pre-T lines share the same recombinational defect(s), thus confirming that identical V(D)J recombination activity operates in both

lineages. SCID mutants provide therefore a useful model for dissecting the recombination activity. These mice have also allowed the description of a new pathway of reticuloendothelial system activation : Unanue showed that, in spite of the absence of lymphocytes, SCID mice infected with *listeria* do express high numbers of activated macrophages and that interferon γ, produced by NK cells, plays a major role in this process. Xenogeneic transplantation into SCID mice of either human peripheral blood leucocytes (Mosier et al. 1988) or of human fetal liver, thymus or lymph node cells (McCune et al. 1988) provides a remarkable tool for the study of human immune response to pathogenic agents (such as HIV) and vaccines and for direct analysis of human physiological or pathological hematolymphoid differentiation.

Another interesting human disease is **SCID with presence of mature B cells**. It is most often an X-linked disease and RFLP studies on informative pedigrees have located the defective gene on Xq13 (De Saint Basile et al. 1989). B cells in these patients appear potentially functional although ME Conley (this volume) showed that the defective gene is indeed transcribed in B lymphocytes.

Two combined ID result from genetic defects of enzymes involved in the purine metabolism : adenosine deaminase (ADA) and purine nucleoside phosphorylase (PNP) deficiencies.

ADA deficiency represents approximately half of the autosomal recessive cases of SCID. The molecular defect consists in most cases of point mutations within the gene encoding ADA on chromosome 22, that abolish enzyme activity. It may also consist in a deletion of the first exon and promoter region of the ADA gene (Markert et al. 1988). Why does a deficiency of an ubiquitous enzyme result in a deleterious effect only in lymphoid cells ? The explanation is that the absence of ADA leads to accumulation within the lymphoid cells of toxic metabolites of the purine (dATP) and methylation (s-adenosyl homocysteine) pathways. The limited clinical benefit of treatment of such patients by transfusions with irradiated normal red cells, providing a nonselective form of ADA replacement, led Hershfield et al. (1987) to use polyethylene glycol-modified ADA (PEG-ADA) as treatment for ADA deficiency. PEG-ADA extends the half-life and reduces the immunogenicity of the missing gene product. This treatment results in clinical improvement, along with an increase in circulating T lymphocyte numbers and proliferative responses to mitogens. Such kind of therapy has potential application to a wide range of inherited disorders. ADA deficiency is theoretically a very good candidate for gene therapy since bone marrow cells are readily available and since the ADA gene has been cloned and its expression is not subject to complex regulation. *In vitro* expression has been achieved through defective retrovirus vectors. *In vivo* application remains however uncertain since it needs access to stem cells with high renewal capacity.

PNP deficiency is an autosomal recessive disease. The defective gene has been located on chromosome 9. As in ADA deficiency, ID results from accumulation of toxic metabolites (dGTP) to which mostly activated T cells are

sensitive. This accounts for immunologic differences between ADA and PNP deficiencies, the latter beeing a less severe ID, impairing mainly cellular immunity.

BONE MARROW TRANSPLANTATION (BMT)

HLA-identical BMT into SCID patients does not lead to graft versus host reaction and is therefore successful in the absence of preparative regimens. When the patient is still young and not severely infected, it results presently is close to 100 % cure. Preparative myeloablative and immunosuppressive regimens are necessary only in variants involving more than the lymphoid lineage, such as reticular dysgenesis or Omenn's syndrome. In the X-linked form of SCID with B cells, BMT results in split chimerism between donor T cells and host B cells. Although carrying the abnormal gene, the host B cells are able to provide satisfactory humoral immunity in the presence of the engrafted donor T cells.

When an HLA-identical donor is not available, attempts to transplant fetal liver have been successful in only 10 % of cases. The development of methods (such as lectin columns, E rosette depletion or monoclonal antibodies) allowing *in vitro* removal of marrow mature T cells, in order to prevent graft versus host disease, has led to use **haplotype-disparate BMT** for correction of SCID (reviewed in O'Reilly et al. 1989). Such transplants are presently successful in 60 % of ADA positive patients. In patients with ADA deficiency, the incidence of graft failure is much higher, irrespective of the T-cell-depletion technique used. When a successful reconstitution of immune function occurs, the development of T cell numbers and functions is delayed, as compared to HLA-identical BMT. Even in SCID with B cells, B cell immunodeficiency persists in around 50 % of patients, usually associated with the absence of detectable donor B cells ; this biological paradox remains yet unexplained. Such patients should of course receive long term substitutive treatment by immunoglobulins. The early occurrence, in some instances, of B cell lymphomas, usually EBV-positive and of host origin, represents a vexing problem. Some progress has been made towards the elucidation of the cellular mechanisms responsible for graft-host tolerance. Tolerance of donor T cells towards host MHC antigens is indeed achieved in SCID patients after haplotype-disparate BMT. There is no evidence in favour of suppression and one therefore deals with either deletion or inactivation of host-reactive clones. The engrafted donor T cells can recognize foreign antigens in the context of both host or donor, but not third-party, MHC molecules. The significance of this restriction pattern is still unclear.

Immunodeficiencies other than SCID can be cured by BMT. Indications for BMT in chronic granulomatous disease and congenital agranulocytosis become questionable in view of the availability of adapted antibiotics and hematopoietic growth factors. In order to get engraftment in patients affected with any ID other than SCID, conditioning regimens are necessary even in HLA-identical situation. Such BMT are presently successful in up to 80 % of patients with the Wiskott-Aldrich syndrome (F Rosen, this volume) and in a high proportion of patients with other ID with cell membrane abnormalities

such as HLA class II deficiency or leucocyte adhesion deficiency. In HLA-non identical situation, the difficulty relies on the double risk of graft rejection and of graft versus host disease. A combination of marrow T cell depletion and of specific immunosuppression is therefore required. The use of antibodies to LFA-1 to achieve such immunosuppression has been successful in more than half of the cases (A Fischer, this volume).

IMMUNODEFICIENCIES WITH CELL MEMBRANE ABNORMALITIES

Several of these ID are characterized by a defective cell to cell interaction.

MHC class II deficiency (Griscelli et al. 1989 ; B Mach, this volume) is an autosomal recessive disease featured by combined cellular and humoral immunodeficiency, with impairement of antigen presentation and cognitive functions. Circulating lymphocyte numbers are normal ; CD4 T cells are decreased. There is a lack of delayed cutaneous hypersensitivity and of *in vitro* T cell responses to antigens. Serum Ig levels are usually decreased and antibody responses to most antigens are severely impaired. A puzzling observation is that T cells of these patients acquire an apparently normal capacity to distinguish self from non self. A possible explanation of this finding resides in the relatively preserved expression of class II molecules on thymic cells, especially during fetal life. Molecular biology studies showed no abnormality within the MHC class II genes and suggested involvement of a transacting regulatory factor. The defect lies in a specific protein, RF-X, that binds to the HLA class II promoter.

Another prototype of ID due to defective cell to cell interaction is **leucocyte adhesion deficiency** (Fischer et al. 1988 ; A Fischer, this volume). In the recent years, a family of proteins -the integrins- that are involved in cell adhesion has been recognized. Amongst integrins, the leucocyte adhesion proteins LFA-1, Mac-1 and p150, 95 were shown to play a major role in the function of both phagocytic cells and lymphocytes. Much of our understanding of the structure and function of these proteins stem from the recognition of a rare inherited ID, the leucocyte adhesion deficiency which is characterized by a defective expression of the β95 chain common to these 3 leucocyte adhesion proteins. The study of patients affected with this disease has led to important insights on the biochemistry of the α and β subunits of the adhesion proteins, their synthesis and assembly and on the characterization of leucocyte adhesion pathways. These studies allowed identification of the LFA-1 ligand, ICAM-1 and led to the use of specific monoclonal antibodies as immunosuppressive agents (as outlined above for BMT) and as anti-inflammatory reagents (as illustrated by the beneficial action of antibodies to the β chain on the size of experimental myocardial infarction in dogs).

CD3 deficiency is a rare familial defect that is of great interest to immunologists. Opposite to the T cell receptor (TCR) chains, the CD3 polypeptide chains possess a long intracytoplasmic tail and probably act as tranducers of the ligand-binding signals to the intracellular compartment. Two spanish patients were studied in details by Alarcon et al. (1988) because most

of their T cells showed very low expression of the TCR-CD3 complex. Functional analysis showed a severely impaired proliferative response to allo antigens, tetanus toxoid and mitogens via the TCR-CD3 pathway, whereas proliferation via the alternative CD2 pathway was normal. CD3 deficiency is characterized by a low intracellular expression of the CD3-ζ chain and impaired association with the other chains of the complex. This defect prevents the transport to cell surface of the incomplete complex, since the final addition of the ζ chain to the complex plays a crucial role in this process. The basic defect is presumably a mutation in the CD3-ζ chain.

T cell activation deficiencies represent a subgroup of ID characterized by phenotypically normal T lymphocytes that appear poorly functional both *in vitro* and *in vivo*. Very few patients with this kind of defect have yet been reported but many will probably follow in the near future. Chatila et al. (1989) have recently described a case with defective coupling of the antigen receptor to the signal transducing G protein machinery, more precisely involving the IP_3 second messenger pathway that mobilizes intracellular stores of calcium. The defect appeared restricted to the T cell lineage and may thus involve a T cell specific component of the signal transduction system. Kersey et al. (1977) and Gehrz et al. (1980) had reported two cases of children in whom the severely impaired proliferative responses of T lymphocytes returned to normal following stimulation with calcium ionophore. It is of interest that neutrophil chemotaxis was defective in at least one of these patients, since a defect of calcium transport would likely involve cell lines other than lymphocytes.

Another ID that may involve defective T cell activation is the Wiskott-Aldrich syndrome (F Rosen, this volume). In this X-linked recessive disorder, characterized by eczema, thrombocytopenia and recurrent infections, the defective gene is on the short arm of the X chromosome. A surface glycoprotein, named sialophorin or CD43, that is encoded on chromosome 16 and is involved in an alternative T cell activation pathway, appears to be defective in patients with this disease.

HUMORAL IMMUNODEFICIENCIES

X-linked agammaglobulinemia (XLA) is the prototype of pure antibody deficiency. The defect lies within the B cell itself. In most instances, no circulating B lymphocytes are found and there is a maturation block in pre-B cell to B cell differentiation. In a few patients, the arrest of development occurs at the stage of immature B cells. In some such cases, Schwaber et al. (1988) showed that there is production of truncated μ chains composed of DJ-C, resulting from failure of V_H gene rearrangement. Fusion of B cells from such a patient with mouse myeloma cells resulted in hybrid cells producing full length chains with V_H of human origin. This failure of V_H gene recombination does probably not apply to all cases of XLA since Max Cooper's group found, in EBV lines from some patients, full length glycosylated intracytoplasmic μ chains, with V_H antigenic determinants. The XLA gene defect has been located to the long arm of the X chromosome and the defective chromosome is

preferentially lyonized, permitting carrier detection (ME Conley, this volume). X-linked hypogammaglobulinemia associated to growth hormone deficiency is a very rare disorder genetically distinct from XLA (Notarangelo et al. abstract 51-27).

Immunoglobulin deficiency with increased IgM ("the hyper IgM syndrome") represents a cluster of diseases featured by a failure of isotype switching. In the most studied variety, that shows X-linked inheritance, there is no argument in favour of an intrinsic B cell defect. Genomic switch regions are normal and the γ gene is in germ-line configuration. Mayer et al. (1986) showed, in some patients, that the defect could be corrected *in vitro* by a T cell line from a patient with Sezary syndrome ("Switch T cell").

A further group of humoral ID is that of **IgG subclass deficiencies**. When the deficiency deals with the minor IgG subclasses, the total IgG level may be within normal limits and diagnosis requires careful evaluation of the level of all IgG subclasses. A minor subset of these subjects have deletions in the 14q32 band, as first observed by Lefranc et al. (1982) in an inbred community of tunisian berberes. Migone et al. (1984) described large deletions in a subject with IgA1, IgG2 and IgG4 complete deficiency and another with IgA1, IgG2, IgG4 and IgE deficiency. These authors favoured the hypothesis of an unequal homologous cross-over. A striking point is that none of these subjects with large chromosomal deletions, detected through screening of blood bank donors, were infected, except for a recently described case of IgG1 deficiency (Smith et al. 1989) in whom the maternal haplotype contained a deletion encompassing Cγ1, Cα1 and Cγ2, whereas the deletion on the paternal haplotype was confined to the Cγ1 gene. In contrast, patients with IgG subclass deficiencies and recurrent infections display no genomic abnormalities and patients with partial IgG2 deficiency often fail to make antibodies to polysaccharide antigens.

Selective IgA deficiency involves both IgA1 and IgA2 subclasses in serum as well as secretions, and is often associated to IgG subclass deficiencies. B lymphocytes are normal and the defect appears to be a failure of IgA-bearing lymphocytes to differentiate into plasma cells. Many of these subjects are asymptomatic, whereas others suffer from respiratory and/or digestive tract infections. IgA deficiency is often found in family members of patients with so called common variable immunodeficiency.

Common variable immunodeficiency (CVID) is the most common primary ID in adults. It is still a kind of waste basket of syndromes whose primary expression is a defect in antibody production. We have observed striking spontaneous variations of serum immunoglobulin class and subclass levels in some adults with CVID. Table 1 shows some of the data in 3 such patients.

Table 1. Spontaneous variations in Ig levels in some patients with CVID

Dates	IgA	IgM	IgG	IgG1	IgG2	IgG3	IgG4
Mr. WO							
1981	0	2.2	10	**8.2**	0.07	1.68	<0.005
1982	0	**3.2**	5.6	**3.4**	0.01	1.4	<0.005
1985	0	1.2	4.8	4.2	0.5	1.1	0.03
Mrs LE							
1980	0	**0.19**	**1.47**	**1.3**	0.09	**0.09**	<0.01
1983	0	**0.86**	**7.5**	**6.75**	<0.05	**0.67**	<0.01
1984	0	**4.16**	6.1	5.4	<0.05	0.55	<0.01
1985	0	0.5	3.3	2.6	<0.05	0.5	<0.01
Mrs CO							
1985	**<0.1**	**<0.1**	**5.6**	4.9	0.2	**1.15**	0.05
1986	0.43	0.5	7.6	5.8	0.25	2.5	0.17
1987	**1.7**	**1.45**	**12.3**	6.3	0.4	**6**	0.16
1988	0.35	0.40	10.1	5.3	0.45	2.7	0.23

Mr. WO, 33 years old, suffered from upper respiratory tract infections when splenomegaly and moderately enlarged lymph nodes were discovered in 1981. He had at that time IgA, IgG2 and IgG4 deficiency. One year later the IgG1 (and total IgG) showed an important decrease, whereas IgG3 remained normal and IgM was high. During the following years, under substitutive treatment by intravenous IgG, the pattern remained roughly unchanged, except for some decrease in IgM level.

Conversely, Mrs. LE, 35 years old, who suffered from severe respiratory tract infections since childhood, converted from panhypogammaglobulinemia in 1980, at the time of diagnosis, to IgA, IgG2 and IgG4 deficiency with near normal levels of IgG1 and IgG3 in 1983 ; at that time substitutive treatment had been interrupted since 9 months because of a severe shock with IgG1 antibodies to IgA. During the following years and in the absence of treatment by γ globulins, the pattern remained roughly unchanged, except for important spontaneous variations in IgM level.

Mrs. CO, 57 years old, suffered from rheumatoid arthritis since 1973 and mild respiratory tract infections. At the time of initial examination, in 1985, Ig deficiency involved predominantly IgA, IgM, IgG2 and IgG4. Antibody responses to both tetanus toxoid and pneumococcal vaccine were satisfactory. During the following years, in the absence of any substitutive treatment by immunoglobulins, striking spontaneous variations were noted for the levels of IgG3 ranging from 1 to 6 mg, IgA ranging from undetectable to 1.7 mg and IgM ranging from undetectable to 1.45 mg, with concommittant appearance of positive test for rheumatoid factor.

These yet unreported findings have obvious practical (difficulties for diagnosis and decisions about substitutive treatment) and theoretical implications. Immunoregulatory defects may indeed constitute an important pathogenic

mechanism in CVID, as well as IgA deficiency. Analysis of some affected individuals suggest either a deficit in helper T cell function or excessive suppressor activity.

Studies of individuals with IgA deficiency have revealed the frequent association with certain HLA haplotypes, such as A1, B8, DR3 (Hammarstrom and Smith 1983) and MHC supratypes with mainly a null C4A gene product (Wilton et al. 1985). In studies at the genomic level, Schaffer et al. (1989) recently showed a high incidence of particular RFLPs or deletions of MHC class III genes in both IgA deficiency and CVID, supporting the hypothesis that both disorders are related and that a gene defect located in this region of chromosome 6 may predispose to these diseases. In this context, I may recall an anecdotal report (Seligmann et al. 1979) of a young man with homozygous C2 deficiency associated with CVID. There was a very high degree of consanguinity since his parents were brother and sister ; the proband was heterozygous for A10, B 18, i.e. the phenotype usually associated with C2 deficiency. In addition to unusual susceptibility to infections, many complement deficiencies are associated with autoimmune conditions. A significant number of autoimmune disorders such as pernicious anemia, autoimmune hemolytic anemia, systemic lupus, etc... can indeed be associated to many of the IDs discussed above, especially CVID.

OTHER IMMUNODEFICIENCY DISEASES

Amongst the many other primary ID (WHO*) two disorders are of special interest : ataxia telangiectasia and the X-linked lymphoproliferative syndrome.

Ataxia telangiectasia (AT) is an autosomal recessive complex disorder characterized by progressive cerebellar ataxia, telangiectasia and endocrine abnormalities associated to partial cellular and humoral immunodeficiency. This syndrome is related to a repair defect in DNA since patient's DNA is hypersensitive to radiation. A genetic analysis of 31 families allowed Gatti et al. (1988) to localize a gene to 11q22-23 in at least the main of the complementation groups defined by fusion experiments. The most intriguing abnormality in AT relies on the clustering of chromosomal breaks on bands where genes of the Ig supergene family (especially TCR and IgH genes) are located. These breaks lead to translocation and inversion through illegitimate rearrangements. In 10 % of patients, at a median age of 13 years, non malignant T cell clones are detected with such abnormalities that may lead to gene fusion. These clones may remain clinically silent, although later occurrence of T cell leukemias is not uncommon. This 3 step cytogenetic evolution represents a remarkable model of oncogenesis. Patients with AT show indeed a 100 fold increased incidence of cancers of various origins, including lymphoid malignancies. Patients with other ID, such as the Wiskott-Aldrich syndrome and CVID, are also prone to such lymphoid malignancies.

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* Primary immunodeficiency diseases. Report of a WHO sponsored meeting. in : Immunodeficiency Reviews (1989) 1:173-205.

Possible mechanisms include defective immunosurveillance, chronic overstimulation of antigen-responsive cells and defective immune responses to oncogenic viruses. The latter possibility is illustrated by the so called **X-linked lymphoproliferative syndrome** (XLP), with unusual response to EBV (reviewed in Sullivan and Voda 1989). This syndrome is manifested by severe or fatal infectious mononucleosis, bone marrow aplasia, B cell lymphoma and acquired immunodeficiency. Males who survive acute EBV infection demonstrate global cellular immune defects, whereas no immunological abnormality can be demonstrated prior to EBV infection. The immunopathology of XLP may be due to uncontrolled alloreactive T cell responses triggered by EBV-transformed B cells or, alternatively, to abnormal resistance of these B cells to cytotoxicity. The gene defect has been located to Xq26-q27.

CHROMOSOME LOCATION AND PRENATAL DIAGNOSIS

The known chromosomal map location of several ID is given in Table 2.

Table 2. Chromosome map location

X-linked chronic granulomatous disease	Xp21.1
Wiskott-Aldrich syndrome	Xp11
X-linked agammaglobulinemia	Xq21.3-22
X-linked severe combined immunodeficiency	Xq13
X-linked hyper IgM syndrome	Xq29
X-linked lymphoproliferative syndrome	Xq26-27
Adenosine deaminase deficiency	20q13-ter
Purine nucleoside phosphorylase deficiency	14q13.1
Kappa chain deficiency	2p12
Ig heavy chain deletion	14q32.3
Ataxia telangiectasia	11q22.3

It is worth noting that six ID-related genes are located on the X chromosome although these gene loci are dispersed among different X regions. It is tempting to speculate on the existence of a gene family on the human X chromosome that would be involved in lymphoid differentiation, similarly to the XLR genes in mice, one of which appears to be linked to the gene defining the xid mutation of the CBA/N immunodeficient mouse.

These recent advances in the precise mapping of various ID have created opportunities for prenatal diagnosis. At present, in addition to the use of immunological methods or biochemical methods on cord blood and of cytogenetic analysis (for AT), techniques of in situ hybridization, RFLP and PCR can be used to establish diagnosis prenataly. The RFLP method may be useful in informative families to detect carriers, as well as the study of patterns of X chromosome inactivation (ME Conley, this volume).

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X Chromosome Inactivation Analysis: A New Tool To Examine X-Linked Immunodeficiencies

M.E. Conley and J.M. Puck

INTRODUCTION

Immunologists have long been intrigued by the striking number of immunodeficiencies inherited on the X chromosome. In man, there are five well described X-linked disorders of lymphocyte proliferation or differentiation: X-linked agammaglobulinemia (XLA), X-linked severe combined immunodeficiency (XSCID), Wiskott-Aldrich syndrome (WAS), X-linked lymphoproliferative syndrome (XLP) and X-linked hyper-IgM syndrome. For each of these disorders, atypical families have been described, leaving open the possibility that there are in fact additional X-linked immunodeficiencies that have not yet been well characterized. Although there are at least preliminary mapping data for each of these disorders (Table 1) the absent or abnormal gene product has not yet been identified for any one of them.

We have taken an indirect approach to studying the nature of these X-linked gene defects. Based on the observation that the obligate carriers of these disorders are normal by all immunologic criteria, we hypothesized that the failure of these women to show signs of their gene defect could be attributed to selection against the mutant X as the active X in the cell lineages primarily affected by the gene defects (Conley 1986, 1988c). If this were true, then analysis of X chromosome inactivation patterns in various cell lines from obligate carriers could be used to address several important questions. Can carrier detection be provided for the sisters, aunts and cousins of affected males? In what cell lineages can the gene product be found? At what point in cell differentiation is the gene product expressed? What is the relationship of the atypical X-linked immunodeficiencies to the typical?

TECHNIQUES USED TO EVALUATE X CHROMOSOME INACTIVATION PATTERNS

Early in embryogenesis of the female, random inactivation of one of the two X chromosomes occurs in each somatic cell and is transmitted to all descendants of that cell (Lyon 1966). The mechanism by which inactivation is initiated is

TABLE I

Disorder	Localization	References
WAS	Xp 11 - 11.3	Peacocke 1987; Kwan 1989
XSCID	Xq 13.1 - 21.1	deSaint Basile 1987; Puck 1989
XLA	Xq 21.3 - 22	Kwan 1986; Mensink 1986; Malcolm 1987
XLP	Xq 24 - 27	Skare 1987
Hyper IgM	Xq 24 - 27	Mensink 1987

unknown but it is maintained, at least in part, by methylation of certain cytosine residues (Yen 1986). As a result, normal females appear to be mosaics; that is, approximately half of their cells have the maternally derived X as the active X and half have the paternally derived X as the active X. The exceptions to this mosaicism indicate origin from a single cell, as in a monoclonal malignancy (Fialkow 1972), or preferential survival or proliferation of cells having one X active over cells having the other X active.

An X-linked gene defect need not cause profound defects in proliferation or survival to result in non-random X chromosome inactivation in a particular cell lineage. For example, obligate carriers of HPRT deficiency, Lesch-Nyhan syndrome, demonstrate preferential use of the normal, non-mutant X as the active X in T cells (Kamatani 1984), although males with HPRT deficiency have normal numbers of T cells and normal T cell function (Allison 1975).

Several techniques have been used to evaluate patterns of X chromosome inactivation. Glucose 6-phosphate dehydrogenase (G6PD), which is a housekeeping gene inherited on the X chromosome gives rise to a protein polymorphisms that can be detected by starch gel electrophoresis (Fialkow 1972). If a woman is heterozygous for G6PD, then on the average half of the cells in any tissue will contain the A allele and half will have the B allele. This technique has the advantage that it can be used to study X inactivation in non-nucleated cells, such as platelets and red blood cells. However, only about 35% of black women and less than 1% of white women are heterozygous for G6PD, and there are no other common X-linked protein polymorphisms that are expressed in most cells, making this technique uninformative in most women.

A second technique to analyze X chromosome inactivation patterns depends on the differences in methylation between the active and inactive X (Vogelstein 1985). DNA samples from the cells of interest are digested with two restriction enzymes. The first reveals a restriction fragment length polymorphism (RFLP) for which the woman in question is heterozygous, and the second digests DNA that has not been methylated. This technique also requires

the woman being studied to be heterozygous for one of the two specific RFLPs that are associated with variable methylation.

The technique that we have developed, which can be used in any woman, includes two steps. In the first step the active X is separated from the inactive X in a series of human/hamster somatic cell hybrids (Puck 1987; Conley 1988c). The human cells of interest are fused to a chinese hamster cell line, RJK88. This cell line, which has a deletion of the X-linked enzyme hypoxanthine phosphoribosyl transferase (HPRT), forms human/hamster hybrids efficiently but the hybrids tend to lose human chromosomes. However, if the hybrids are grown in selective medium they must retain the active human X to provide HPRT. In the second step, an X-linked RFLP for which the woman in question is heterozygous is used to distinguish between the two X chromosome in each hybrid. In the normal woman, or in a cell line not affected by the gene defect, about half of the hybrids retain the maternally derived X and half retain the paternally derived X. Figure 1 shows an example of hybrid analysis in a carrier of X-linked agammaglobulinemia. The T cells demonstrate random X chromosome inactivation whereas the B cells show selective use of a single X as the active X.

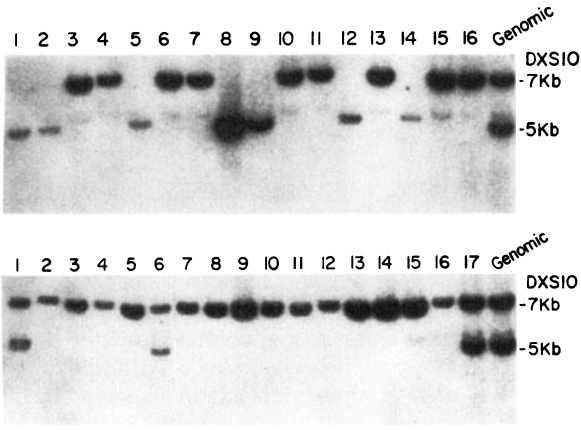

Fig. 1 Evaluation of X chromosome inactivation patterns in T cells (top) and B cells from a carrier of XLA. DNA from a series of human/hamster hybrids was digested with Taq 1 and analyzed with the probe 36B-2. One allele detected with this probe is a 5kb fragment and the other is a 7kb fragment. On both blots the genomic DNA is shown at the right. Of the 16 T cell hybrids, those in lanes 3,4,6,7,10,11,13,15 and 16 contain the X bearing the 7kb fragment. Those in lanes 1,2,5,12, and 14 contain the X bearing the 5kb fragment. Of the 17 B cell hybrids, those in lanes 1, 6, and 17 contain both X chromosomes; the remaining 14 hybrids all contain the X bearing the 7kb fragment (Conley, 1988b). Reproduced with permission of C. V. Mosby Company.

APPLICATIONS OF X CHROMOSOME INACTIVATION ANALYSIS

Carrier Detection: X-linked severe combined immunodeficiency is characterized by markedly reduced numbers of T cells, normal to elevated numbers of B cells, hypogammaglobulinemia and absent proliferative responses to mitogens (Gelfand 1983, Conley, manuscript submitted). Studies from our laboratory and from others have shown that T cells from obligate carriers of XSCID exhibit preferential use of the normal, non-mutant X as the active X (Puck 1987; Goodship 1988). Thus, in families with known XSCID, analysis of X chromosome inactivation patterns in T cells from women at risk can provide a mechanism of carrier detection. In addition to giving these women valuable information, carrier detection increases the number of individuals who are informative for mapping studies (Puck 1989).

In time, linkage analysis with flanking RFLP markers will be used for prenatal diagnosis and carrier detection in families with known XSCID if DNA is available from affected or non-affected family members. However, the gene for XSCID, like all other lethal X-linked disorders (Haldane 1935), has a high rate of spontaneous mutation. In a male child with severe combined immunodeficiency, and no family history of a similar disorder, it is not currently possible to distinguish an autosomal recessive form of the disease from a new mutation of the XSCID gene. Because new mutations are silent in females the boy's mother and sisters may be at risk of being carriers. Evaluation of X chromosome inactivation patterns will always be useful in this situation. We have used the hybrid technique to examine X chromosome inactivation patterns in T cells from 16 women who have had sons but no other family members with severe combined immunodeficiency (Conley, manuscript submitted). T cell hybrids from 7 of these women demonstrated non-random X chromosome inactivation, indicating that these 7 women were carriers of XSCID.

Cell target of the gene defect: Patients with X-linked agammaglobulinemia have normal numbers of T cell, normal proliferative responses to T cell mitogens but profound hypogammaglobulinemia and markedly reduced numbers of B cells (Conley 1985). However, pre-B cells can be found in the bone marrow in approximately normal numbers (Pearl 1978). One might ascribe the failure of pre-B cells to differentiate into B cells to a defect in the bone marrow microenvironment or to the absence of a required growth factor. To address this problem we analyzed patterns of X chromosome inactivation in several cell lineages in two obligate carriers of XLA who were also heterozygous for G6PD. Although T cells, monocytes and granulocytes from both women exhibited random X chromosome inactivation there was exclusive use of a single X as the active X in the B cells (Conley 1986). Similar results were obtained using both methylation and hybrid analysis (Fearon 1987; Conley 1988b). These results suggest, but do not prove, that the XLA gene product is expressed only in cells of the B cell lineage.

The cell target of the Wiskott-Aldrich gene is not as clear. This syndrome is characterized by thrombocytopenia, eczema and failure to make antibody to carbohydrate antigens (Cooper 1968). Affected patients have both decreased production and survival of platelets (Ochs 1980). Cells in other lineages are usually present in normal numbers but may demonstrate variable functional defects. Proliferative responses to T cell mitogens may be decreased and delayed hypersensitivity skin testing usually reveals anergy.

Two studies in 1980 demonstrated non-random X chromosome inactivation in platelets, granulocytes, monocytes, T cells and B cells from obligate carriers of Wiskott-Aldrich syndrome (Gealy 1980; Prchal 1980). In the Gealy experiment there was exclusive use of the normal X in platelets and T cells but there was a small proportion of the monocytes, granulocytes and T cell depleted lymphocytes (B cells) that contained the mutant X as the active X. In the Prchal study there was exclusive use of the normal X as the active X in all five cell lineages. Recently studies from Fearon et al. (1988) and Greer et al. (1989), using methylation analysis, confirmed non-random X chromosome inactivation in neutrophils, monocytes, T cells and B cells from obligate carriers of Wiskott-Aldrich syndrome. In contrast, cells from skin biopsies from obligate carriers exhibited random X chromosome inactivation (Fearon 1988). Our studies, using hybrid analysis, indicate that although there is preferential use of the normal X as the active X in T cells, a small percentage of T cells, approximately 5%, use the mutant X as the active X (Puck, manuscript submitted).

The above studies suggest that the Wiskott-Aldrich gene product is expressed in most hematopoetic cell lineages, including platelets, phagocytes and lymphocytes, and that the defective gene product results in a relative disadvantage in proliferation and/or survival of these cells.

Point in differentiation at which the gene product is expressed: As noted above, in addition to having profound T cell defects, patients with X-linked severe combined immunodeficiency have hypogammaglobulinemia, although they have normal or increased B cell numbers. To determine whether the hypogammaglobulinemia could be explained by expression of the XSCID gene product in B cells as well as T cells, we examined patterns of X chromosome inactivation in EBV expanded B cells from obligate carriers of XSCID (Conley 1988a). B cell hybrids from 9 obligate carriers of XSCID all exhibited preferential use of the same X that was used as the active X in T cell hybrids. However, in several experiments there were a small number of B cell hybrids that had the mutant X as the active X.

One possible explanation for the rare B cell hybrids that used the mutant X as the active X is that the B cell population used to make the hybrids was contaminated with a small number of cells from a cell lineage not affected by the gene defect, for example monocytes. However, this explanation seems unlikely because B cell hybrids from obligate carriers of X-linked

agammaglobulinemia, made using the same techniques, had fewer than 1% hybrids containing the mutant X as the active X (Conley 1988b).

A second explanation for the occasional B cell hybrid that had the mutant X as the active X is that the gene defect causing XSCID results in a relative disadvantage in B cell proliferation or survival but not a complete block. If this were the case, then one would expect the B cells that had undergone the most cycles of replication, B cells committed to IgG or IgA production, to be the least likely to show exceptions. To test this hypothesis T cell depleted lymphocytes from three carriers of XSCID were separated into B cells with high concentrations of surface IgM, a population previously shown to represent immature B cells (Conley 1985), and B cells that were negative for surface IgM, that is, B cells committed to IgG or IgA production. In all three experiments there was random X chromosome inactivation in the least mature B cell population and selective use of the non-mutant X in the most mature B cell population (Conley 1988a). These results indicate that a mutant XSCID gene product is detrimental to B lineage cells but the deleterious effect is seen only in mature B cells.

Atypical X-linked Immunodeficiencies: Several forms of atypical X-linked agammaglobulinemia have been reported. In some families affected males have higher concentrations of serum immunoglobulins or more B cells than would be expected in typical XLA (Goldblum 1974; Leickley 1986). In 1980 a family with XLA and coincident growth hormone deficiency was reported (Fleisher 1980). We have recently evaluated a second family with this combination of disorders. In each of these situations one might ask whether the disorder was an allelic variant of typical XLA or an unrelated gene defect with similar immunologic findings. Evaluation of X chromosome inactivation patterns can provide two approaches to this question. First, if the atypical disorder is an allelic variant of the typical disorder then the cell lineages demonstrating non-random X chromosome inactivation in obligate carriers should be the same in both. Second, allelic variants of a disorder should map to the same locus on the X chromosome. Analysis of X chromosome inactivation patterns in women at risk of being carriers in atypical families will increase the number of individuals that can be included in mapping studies.

We have studied three families in which some of the affected males had higher concentrations of serum immunoglobulins than expected in typical XLA and one family in which the affected males had both XLA and growth hormone deficiency. In all four families the B cells from obligate carriers showed preferential use of the normal, non-mutant X as the active X (Conley 1988b; Conley, manuscript in preparation). In none of the families did mapping studies rule out linkage to the locus for typical XLA at Xq21.3-22. These findings are compatible with these atypical forms of XLA being allelic variants of the typical form.

SUMMARY

In the last five years there has been significant progress toward the localization and identification of the genes involved in the X-linked immunodeficiencies. Mapping studies have already become clinically useful in that it is now possible to provide prenatal diagnosis and carrier detection for many families with well characterized immunodeficiencies. However, it is very difficult to isolate and clone a gene based only on localization of that gene to a particular point on a chromosome. Additional approaches that help characterize the nature of the defective gene are clearly valuable. Analysis of X chromosome inactivation patterns in three X-linked immunodeficiencies; X-linked agammaglobulinemia, X-linked severe combined immunodeficiency and Wiskott-Aldrich syndrome have indicated the cell lineages in which the defective gene product is expressed. The gene for XLA is most likely expressed only in B lineage cells, the gene for XSCID is detrimental to proliferation or survival of both T cells and B cells; while the Wiskott-Aldrich gene is expressed in platelets, granulocytes, and monocytes as well as T and B cells. The point in differentiation at which the gene product is expressed in a particular cell lineage may also be clarified by examination of X chromosome inactivation patterns. Prior to that point, cells from an obligate carrier should demonstrate random X chromosome inactivation, whereas selective use of the normal X as the active X will be seen after that point. Finally, analysis of X chromosome inactivation patterns in carriers of atypical X-linked immunodeficiencies should help elucidate the relationship of these disorders to typical X-linked immunodeficiencies and by so doing extend the definition of the typical disorders.

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Nature of the Defect in the Scid Mouse Mutant

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Early B and T lymphocyte differentiation appears to be arrested in C.B-17/Icr scid/scid mice (Bosma et al. 1983), a mutant mouse strain homozygous for an autosomal recessive mutation (scid) on chromosome 16 (Bosma et al. 1989). Though developing B and T lymphocytes in these mice (hereafter referred to as scid mice) reach the stage at which immunoglobulin heavy (Igh) chain genes and T cell receptor (TCR) β and γ genes become transcriptionally active (Schuler et al. 1988), cells with Igh or TCRβ and TCRγ rearrangements have not been directly detected in scid lymphopoietic tissues (Schuler et al. 1986). The only indication that early scid lymphocytes attempt Igh or TCR rearrangements has come from findings of abnormal antigen receptor gene rearrangements in transformed scid lymphocytes (Schuler et al. 1986; Lieber et al. 1988; Malynn et al. 1988; Hendrickson et al. 1988; Kim et al. 1988) and in early B cells recovered from long-term cultures of scid bone marrow cells (Okazaki et al. 1988). For example, infection of scid bone marrow cells with Abelson murine leukemia virus (A-MuLV) results in the recovery of transformed pre-B cells with abnormal Igh rearrangements, even though such cells cannot be detected by fluorescent activated cell sorter (FACS) analysis of scid bone marrow cells. To explain the apparent paucity of these cells and their abnormal Igh rearrangements, it was hypothesized (Schuler et al. 1986) that they die prematurely as a result of a defective recombinase system; transformation would thus serve to immortalize early scid lymphocytes before they die. This hypothesis recently received added support from more detailed analyses showing that transformed, immature scid lymphocytes have an active, but abnormal VDJ recombinase activity which is unable to catalyze with appreciable frequency the formation of functional V(D)J coding joints (Lieber et al. 1988; Malynn et al. 1988).

What has been experimentally missing in support of the above hypothesis is direct evidence that the various scid cell lines are truly representative of developing scid lymphocytes. Such evidence is presented in the first part of this report, where we summarize our recent results with μ and μκ transgenic scid mice (Fried et al. 1989). We show that introduction of a functionally rearranged μ transgene alone or both a μ and κ (μκ) transgene into the scid mouse genome enables "arrested" scid pro-B cells in the bone marrow to differentiate further into pre-B cells, or into pre-B and more mature B cells in the case of the μκ transgenic scid mice. These results support the hypothesis that scid severely impairs the assembly of functional Ig genes and thereby prevents immature scid B cells from differentiating into more mature B cells.

In the second part of this report we present direct in vivo evidence for ongoing rearrangement of antigen receptor genes in scid mice. We have found that TCRδ rearrangements can be readily detected in DNA of whole scid thymus and in DNA of scid thymocyte hybridomas in the apparent absence of TCRγ and TCRβ rearrangements (Carroll and Bosma 1989). What is puzzling is that a common set of TCRδ recombination fragments (6.6, 5.4 and 4.3 kb) seems to be present in all scid mice in addition to various other size fragments seen in the thymocyte hybridomas. As discussed

later, these findings raise several questions about the nature of the scid defect and the possible recombination requirements of different TCR loci.

Ig transgenic scid mice

The introduction of functionally rearranged μ and μκ gene constructs (transgenes) into the scid mouse genome was accomplished by means of selective genetic crosses with two previously established transgenic mouse strains; M54, a μ transgenic strain (Grosschedl et al. 1984) and 207-4, a μκ transgene strain (Storb et al. 1986). The transgenes (Tg) were first crossed onto a constructed mouse stock (Epstein et al. 1986) having an Ig light chain locus (Igl-1^b) distinct from the Igl-1^a locus of M54, 207-4 and scid mice. As Igl-1 is closely linked to scid (Bosma et al. 1989) and as Igl-1^a and Igl-1^b can be distinguished on the basis of a restriction fragment length polymorphism (Arp et al. 1982), we used Igl-1^a as a positive marker for scid in the subsequent crossing of the Tg onto the scid mouse background. Each of the Tg segregated independently of Igl-1.

Ig transgenic scid mice along with appropriate control mice were produced by backcrossing mice heterozygous for the Tg (Tg-μ/+ or Tg-μκ/+) and for scid (scid/+) to scid homozygotes (scid/scid). This resulted in four classes of progeny: 1) non-transgenic scid mice (+/+, scid/Igl-1^a/scid Igl-1^a); 2) transgenic scid mice (Tg/+, scid Igl-1^a/scid Igl-1^a); 3) transgenic heterozygous scid mice (Tg/+, scid Igl-1^a/+ Igl-1^b); and 4) non-transgenic heterozygous scid mice (+/+, scid Igl-1^a/+ Igl-1^b). Classes 1, 3 and 4 served as controls for class 2. The classes were identified by dot blot hybridization of tail DNA to pBR322, which scored for the presence or absence of prokaryotic vector sequences flanking the μ and μκ transgenes, and by hybridization of Kpn 1/Eco R1 restricted tail DNA to pCλ-4E, which is specific for Igl-1 and allowed us to distinguish Igl-1^a/Igl-1^a scid homozygotes from Igl-1^a/Igl-1^b scid heterozygotes.

To evaluate the affect of the μ transgene on B cell differentiation, bone marrow and spleen cells of μ transgenic scid mice and control littermates were examined by multiparameter fluorescence activated cell sorter (FACS) analysis. Three cell surface markers were scored; B220, Thy-1.2 and IgM. This enabled us to distinguish and identify putative pro-B cells ($B220^+$ Thy-1.2^{lo} IgM^-) (Tidmarsh et al. 1989), pre-B cells ($B220^+$ Thy-1.2^- IgM^-) and more mature B cells ($B220^+$ Thy-1.2^- IgM^+). Our results (Fried et al. 1989) are summarized in Table 1. As indicated, non-transgenic scid mice (class 1) show normal frequencies of pro-B cells in their bone marrow, but lack detectable pre-B cells. In contrast, the bone marrow of μ transgenic scid mice (class 2) contains normal levels of both pro-B and pre-B cells, indicating that the μ transgene permits scid pro-B cells to differentiate into pre-B cells. Not surprisingly, more mature B cells do not appear in μ transgenic scid mice. This step would require the productive rearrangement of endogenous Ig light chain genes and is presumably precluded by the defective scid recombinase system. Indeed, Western blot analysis of bone marrow lysates from μ transgenic scid mice has shown κ light chains to be absent, though κ chains can be readily detected in the bone marrow of control mice (classes 3 and 4); all classes except non-transgenic scid mice have been found to contain μ chains (Fried et al. 1989).

Table 1. Frequencies of distinct B lineage cells in bone marrow and spleen of scid/scid and scid/+ littermates with and without a μ transgene.

		Bone Marrow			Spleen
Class	Genotype	$B220^+$ $Thy\text{-}1.2^{lo}$ IgM^-	$B220^+$ $Thy\text{-}1.2^-$ IgM^-	$B220^+$ $Thy\text{-}1.2^-$ IgM^+	$B220^+$ $Thy\text{-}1.2^-$ IgM^+
1	+/+, scid $Igl\text{-}1^a$/scid $Igl\text{-}1^a$	3.3±0.4	0.3±0.2	-	-
2	Tg-μ/+, scid $Igl\text{-}1^a$/scid $Igl\text{-}1^a$	3.6±0.3	5.7±0.9	-	-
3	Tg-μ/+, scid $Igl\text{-}1^a$/+ $Igl\text{-}1^b$	2.6±0.3	6.3±0.6	4.1±1.0	23.6±2.2
4	+/+, scid $Igl\text{-}1^a$/+ $Igl\text{-}1^b$	2.6±0.4	5.5±1.6	5.2±2.0	43.5±3.5

Frequencies of distinct B lineage cell populations are given as percentage of total nucleated cells analyzed (30,000 to 100,000 cells per analysis); values were obtained by integration of multiparameter FACS analyses and represent the average values (± SEM) of four to seven mice of each genotype. The absence of detectable cells of the indicated phenotypes is indicated with a (-). Cells were stained as previously described (Hayakawa et al. 1985) using phycoerythrin/anti-IgM (331.12), fluorescein/anti-Thy-1.2 (30H12) and allophycocyanin/anti-B220 (RA3-6B2). (The monoclonal antibodies, 331.12, 30H12 and RA3-6B2 are described in Kincade et al. 1981, Ledbetter and Herzenberg 1979, and in Coffman and Weissman 1983, respectively). FACS analysis was carried out with a dual laser dye laser FACStar^{PLUS} equipped with filters for four color analysis (triple immunofluorescence plus propidium iodide staining). Forward and large angle light scatter gates were set to exclude non-lymphoid cells and debris. Dead cells were excluded from the analysis by propidium iodide staining.

Table 2 summarizes the FACS results obtained with μκ transgenic scid mice and control mice. B cells ($B220^+$ IgM^+) are clearly evident in the bone marrow of μκ transgenic scid mice (class 2), though the frequency of such cells is 2-3-fold less than that of the controls. Very few B cells are found in the spleen (<1% of the cells analyzed) and none have been detected in the lymph nodes or peritoneal cavity (data not shown). Further, most μκ transgenic scid mice lack detectable serum IgM. The basis for this relative absence of peripheral B cells and Ig-secreting cells is not clear. Interestingly, μκ transgenic scid B cells do appear capable of differentiating into Ig-secreting cells. Repeated immunization of serum IgM negative μκ transgenic scid mice with a phosphorylcholine containing immunogen (R36A), to which transgene encoded μκ molecules specifically bind, results in the appearance of serum IgM bearing the transgene allotype (our unpublished results).

Table 2. Frequencies of early ($B220^+$ IgM^-) and late ($B220^+$ IgM^+) B lineage cells in bone marrow and spleen of scid/scid and scid/+ littermates with and without the μκ transgene.

		Bone Marrow		Spleen
Class	Genotype	$B220^+$ IgM^-	$B220^+$ IgM^+	$B220^+$ IgM^+
1	+/+, scid Igl-1^a/scid Igl-1^a	3.7±0.7	-	-
2	Tg-μκ/+, scid Igl-1^a/scid Igl-1^a	2.1±0.3	3.4±1.1	0.2±0.02
3	Tg-μκ/+, scid Igl-1^a/+ Igl-1^b	3.7±1.4	7.2±3.0	24.1±0.8
4	+/+, scid Igl-1^a/+ Igl-1^b	9.1±2.2	5.4±1.7	41.0±2.6

See legend of Table 1 for explanation of values and Material and Methods.

In conclusion, the introduction of μ and μκ transgenes into the scid mouse genome removes in stepwise fashion the developmental "arrest" in the differentiation of B lineage scid lymphocytes: scid pro-B cells differentiate into pre-B cells in the presence of a functional μ gene, and further, into B cells in the presence of a functional μ and κ gene. These findings strongly suggest that scid interferes with the functional assembly of Ig heavy and light chain genes and provide further support for the hypothesis that scid specifically impairs the VDJ recombinase system.

Rearrangement of TCRδ genes in scid thymocytes

Although it is clear that developing scid thymocytes reach the stage at which TCRγ and TCRβ genes become transcriptionally active, rearrangement of these genes cannot be directly detected in DNA of whole scid thymus (Schuler et al. 1988) or in DNA of scid thymocyte hybridomas (Carroll and Bosma 1989). However, rearrangement of the recently described TCRδ locus is readily detectable in scid thymocyte DNA (Carroll and Bosma 1989).

The TCRδ locus was characterized by Chien et al. 1987a, 1987b and Elliott et al. 1988, and consists of two D and two J elements and at least six V genes. As illustrated in Figure 1, a common set of recombination fragments is revealed when scid thymocyte DNA is hybridized with a probe (p3′Jδ1) specific for a region 3′ of Jδ1. In addition to a p3′Jδ1-hybridizing germline fragment of 7.5 kb, three smaller fragments are seen, including one of 6.6 kb that possibly corresponds to a Dδ2-Jδ1 rearrangement commonly seen in thymocyte DNA from neonatal normal mice (Chien et al. 1987a). The thymocytes of newborn and adult scid mice show the same apparent set of TCRδ recombination fragments; whereas the two sets of TCRδ fragments from newborn and adult normal mice (C.B-17+/+) differ greatly from the set seen in scid mice and slightly from one another with the adult mice lacking the prominent 6.6 kb fragment.

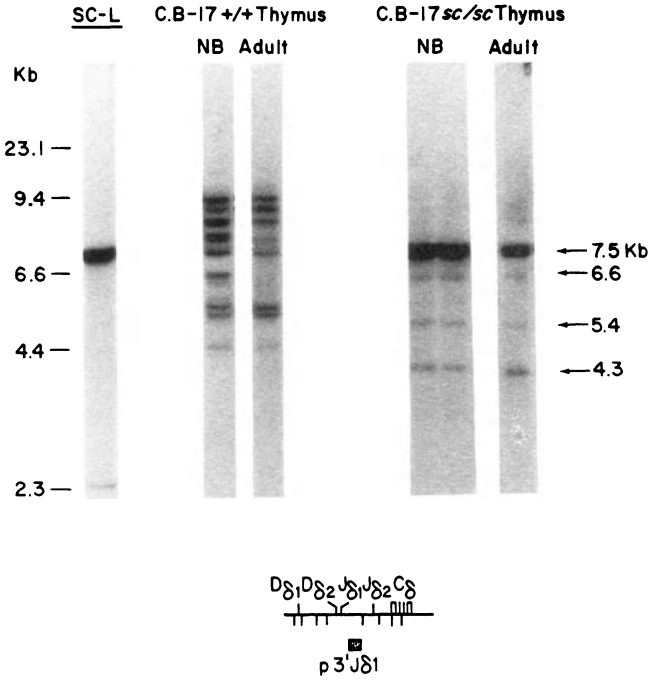

Figure 1. Southern blot analysis of Eco R1 restricted DNA from scid liver (SC-L), and from individual preparations of normal (C.B-17+/+) and scid (C.B-17sc/sc) newborn (NB) and young adult thymus. The DNA was hybridized with p3'Jδ1, a 2 kb Sac I fragment obtained from Y. Chien. A map of the TCR δ locus and p3'Jδ1 (derived from Chien et al. 1987) is shown below the Figure. (Reprinted by permission from Current Topics in Microbiology and Immunology 152:64, 1989, courtesy of Springer-Verlag, Berlin)

To evaluate the status of TCRδ, TCRγ and TCRβ genes in individual cells, scid thymocytes from young adult mice were fused with a non-expressing TCRα/β variant of the BW5147 thymic lymphoma, kindly supplied by W. Born of the National Jewish Hospital, Denver, CO (both TCRδ alleles are deleted in BW5147). Twenty-three of these hybridomas were cloned and Eco RI restricted DNA from each clone was sequentially hybridized to p3'Jδ1, pJβ2 and pCγ2. Representative results are illustrated in Figure 2. Various size TCRδ recombination fragments are seen in addition to the 6.6, 5.4 and 4.3 kb fragments characteristic of whole scid thymus (Figure 3A). The smaller recombination fragment in SA1 appears abnormal as it is too small to contain an upstream Jδ1 coding sequence. In two clones only one TCRδ allele is present (5A6 and SA8) and one clone has lost both TCRδ alleles (SA3). Figure 3B and 3C indicate that TCRβ2 and TCRγ scid genes remain in germline configuration. The only additional TCR fragments detected are those apparently contributed by BW5147, including a 3.9 kb TCRβ fragment (Figure 3B) and three TCRγ fragments of 6.6., 16 and 22 kb (Figure 3C). We cannot presently exclude that a TCRγ rearrangement at one scid allele could have been masked by the 16 or 22 kb TCRγ fragments of BW5147.

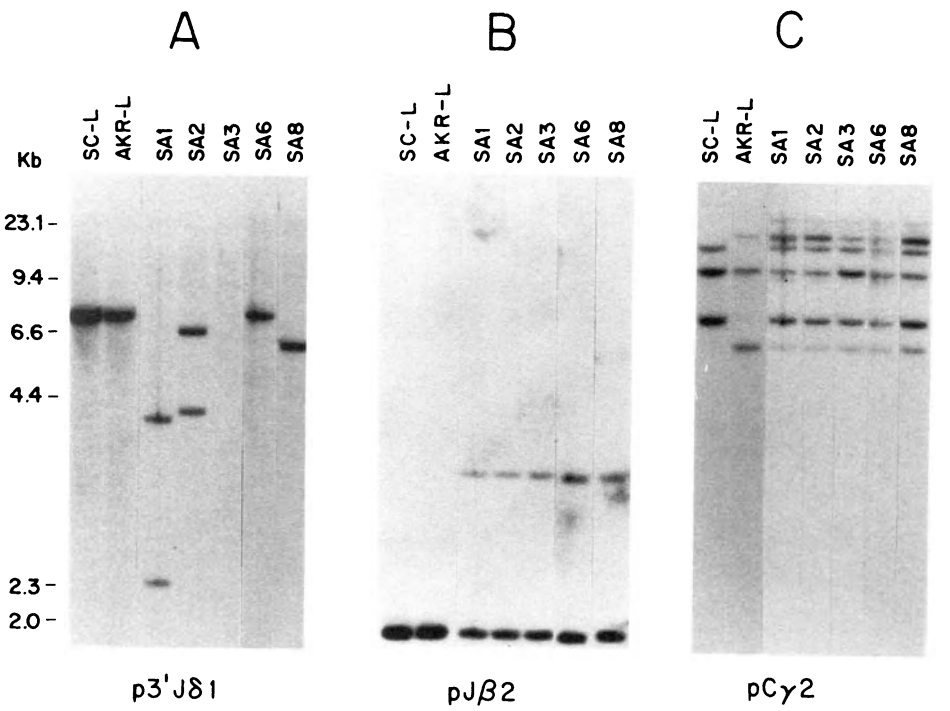

Figure 2. Southern blot analysis of Eco R1 restricted DNA of scid liver (SC-L), AKR liver (AKR-L) and several scid thymocyte hybridomas (designated SA). The blot was sequentially hybridized with p3'Jδ1 (Chien et al. 1987) pJβ2 (Malissen et al. 1986) and pCγ2 (Iwamoto et al. 1984). The AKR-derived BW5147 fusion partner contained one TCRβ fragment of 3.9 kb and three TCRγ fragments of 6.6, 16 and 22 kb. (Reprinted by permission from Current Topics in Microbiology and Immunology 152:65, 1989, courtesy of Springer-Verlag, Berlin)

The status of the scid TCR loci analyzed to date can be summarized as follows: The majority of scid thymocyte hybridomas (17/23) showed TCRδ rearrangements. A few of these rearrangements (4/21) appeared grossly abnormal. None of the examined hybridomas had detectable rearrangements of TCRγ (10/10 analyzed) or TCRβ (18/18 analyzed). In contrast, most control thymocyte hybridomas of age-matched C.B-17+/+ mice showed TCRγ and TCRβ rearrangements and deletion of both TCRδ alleles, the latter presumably resulting from rearrangement of the TCRα locus as TCRδ genes are located within the TCRα locus between Vα and Jα elements; rearrangement of these elements results in the deletion of the TCRδ locus (Chien et al. 1987a).

According to our working hypothesis, attempted rearrangement of scid TCR (or Ig) genes results in premature cell death due to the inability of the scid VDJ recombinase system to join broken chromosome ends bearing V, D or J coding segments or due to non-VDJ mediated recombination of the broken chromosome ends (Lieber et al. 1988). The latter event would yield non-functional TCR recombination fragments of random and abnormal size. How then does one explain the non-random and predominate set of TCRδ recombination fragments and the apparent absence of TCRγ and TCRβ recombination fragments in DNA of whole scid thymus? One explanation assumes a low frequency of functional TCR rearrangements in developing scid thymocytes despite their impaired VDJ recombinase system. Accordingly, cells that make a functional rearrangement

(e.g., Dδ to Jδ or Vδ to Dδ) would survive and accumulate. However, the chance of such cells making a second functional rearrangement to form a fully assembled productive TCRδ gene would be highly improbable, and assuming that this is necessary before TCRγ and TCRβ rearrangements can occur would explain the absence of the latter. Alternatively, the scid VDJ recombination system may not be equally impaired at all stages of T cell development. For example, the effect of scid may not be fully manifest in the early stages of T cell development at which TCRδ genes begin to undergo rearrangement. This could result in a high frequency of normal VDδ or DJδ recombination joints. Finally, one could suppose that certain spatial or structural features of the TCRδ locus allow the scid VDJ recombinase system to recombine TCRδ genes more frequently and successfully than TCRγ or TCRβ genes. This explanation would not require a strictly ordered expression of TCRδ, TCRγ and TCRβ genes. Whether one of the above explanations is likely to be valid clearly awaits the results of ongoing analyses and future experiments.

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The Wiskott-Aldrich Syndrome and CD43

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INTRODUCTION

The Wiskott-Aldrich Syndrome (WAS) is a rare X-linked immunodeficiency. Affected males have thrombocytopenia, eczema and recurrent bacterial infections. The gene for WAS maps to Xp11.1 by the use of restriction fragment length polymorphism (Kwan 1988). The number of platelets in the blood of these children is markedly decreased and the platelets are abnormally small. The size of the platelets has been noted to increase following splenectomy (Lum 1980). Cell-mediated immunity progressively declines with age but the number and phenotype of the blood lymphocytes is generally normal. Like the platelets, the blood lymphocytes are also small and their size increases following splenectomy. By scanning electron microscopy these blood elements have a characteristically abnormal appearance (Kenney 1986) and this has proved useful in the perinatal and prenatal diagnosis of WAS. The serum immunoglobulins are abnormal in that the concentrations of IgA and IgE are elevated and the concentration of IgM is decreased; IgG is normal. Affected males do not respond to polysaccharide antigens whereas the response to protein antigens is normal (Ayoub 1968; Blaese 1968). Several affected males have received successful bone marrow transplants from histoidentical siblings following lethal doses of irradiation or busulfan (Parkman 1978). Their immunological function and platelet number and function is subsequently normal. For reasons, which are unclear, it has been very difficult to transplant patients with WAS with haploidentical marrow; this procedure has succeeded in only a few instances. In some cases recipients have been rendered partial T cell chimaeras, without correction of the hematological abnormalities, but the eczema disappeared, serum immunoglobulin concentrations became normal and a normal response to polysaccharide antigens was noted (Parkman 1978).

THE ABNORMALITY IN CD43

The abnormality in the size of the lymphocytes prompted an examination of the cell membranes from lysates of radioiodinated cells. A band of 115,000 daltons was identified under both reducing and non-reducing conditions in normal lymphocyte membranes but not in preparations from lymphocytes of a male with WAS (Parkman 1981). The protein thus first identified was called gpL115. By virtue of its adherence to wheat germ lectin and peanut lectin gpL115 was purified; it was found to have a very high content of sialic acid and was therefore named sialophorin (Remold-O'Donnell 1984, 1986, 1987). Several monoclonal antibodies to sialophorin were generated; it subsequently became apparent that several investigators had monoclonal antibodies to this same glycoprotein; it was assigned the designation CD43 at the Oxford workshop.

CD43 is present not only in the membranes of both CD4 and CD8 subsets of T lymphocytes but also is found in the membranes of mononuclear phagocytes, a portion of B lymphocytes, neutrophils, natural killer cells and platelets. However CD43 is not present on erythrocytes nor on any other cell type thus far tested (e.g. fibroblasts, glioblasts or rhabdomyoblasts) (Remold-O'Donnell 1987; Mentzer 1987).

CELL ACTIVATION BY ANTI-CD43

A monclonal antibody (L10) to CD43 activates and induces proliferation of blood mononuclear cells (Mentzer 1987). The increased incorporation of tritiated thymidine into T lymphocytes depends on the presence of mononuclear phagocytes and the integrity of the monoclonal antibody (i.e. the F(ab')2 fragments are 50-fold less efffective). The stimulation appears to be independent of the CD3-Ti complex and can be induced in mutants of HBP-ALL cells that are defective in the T cell antigen receptor at the level of transcription/assembly and are therefore unresponsive to other T cell agonistic antibodies (Silverman 1989). L10 induces increased secretion of interleukin-2 (Axelsson 1988) and an increase in intracellular calcium, diacylglycerol and inositol phosphates (Silverman 1989).

L10 also increases the adhesiveness of blood monocytes and causes homotypic adhesion of these cells. This requires magnesium ions and LFA-1. Moreover, L10 increases hydrogen peroxide production in mononuclear phagocytes challenged with PMA. This action of L10 thus resembles that of interferon-gamma (Nong 1989).

CLONING OF CD43

cDNA clones of CD43 have been obtained from T cell libraries (Pallant; Shelley 1989). They reveal that CD43 is composed of 400 amino acid residues. The amino-terminal 235 residues form a mucin-like extracellular domain followed by a putative 23 residue transmembrane region and a 123 residue carboxy-terminal intracellular domain; the signal sequence is presumed to contain 19 residues. There is one site for attachment of an N-linked oligosaccharide at an asparagine residue at position 251, sixteen residues from the transmembrane region. The extracellular domain of 235 residues contains 46 serines and 47 threonines; it appears that 84 of these 93 residues are O-glycosylated with sialylated Gal-GalNAc side chains. More complex O-linked saccharides are present on activated T lymphocytes (Piller 1988). Residues 135 to 224 form a 90 amino acid stretch containing five repeats of 18 residues.

135	I	T	T	N	S	P	E	T	S	S	R	T	S	G	A	P	V	T
153	T	A	A	S	S	L	E	T	S	R	G	T	S	G	P	P	L	T
171	M	A	T	V	S	L	E	T	S	K	G	T	S	G	P	P	V	T
189	M	A	T	D	S	L	E	T	S	T	G	T	T	G	P	P	V	T
207	M	T	T	G	S	L	E	P	S	S	G	A	S	G	P	Q	V	S

The function of the stretch containing these repeating elements is not known at the present time.

A genomic clone containing the entire human sialophorin gene reveals that it is unusual in that a single exon encodes the extracellular, transmembrane and intracellular regions. A single intron of 378

base pairs interrupts the sequence specifying the 5' untranslated regions of the two sialophorin mRNAs derived from the gene. These mRNAs differ principally in the length of their 3' untranslated regions, depending on the use of a single polyadenylation signal in the case of the shorter 1.9 kilobase mRNA or the use of a cluster of five overlapping polyadenylation signals located 2285 bases further downstream in the case of the longer 4.3 kilobase mRNA (Shelley 1989b).

As the gene encoding CD43 maps to chromosome 16 at band p11.2, the defect in CD43 observed in the WAS cannot be the primary defect (Pallant; Shelley 1989).

TRANSFECTION OF COS CELLS

Transfected COS cells express CD43 as a broad band with a range of Mr from 100,000 to 135,000 (Shelley 1989). It is tempting to speculate that the faulty expression of CD43 on COS cells could be corrected by the as yet unknown gene on the X chromosome. It appears preliminarily that the biosynthesis of CD43 is normal in WAS lymphocytes but that it is abnormally susceptible to proteolytic cleavage. The reason for this is not clear at the present time and thus the primary defect in the WAS remains a puzzling enigma.

DISCUSSION

A unique aspect of the WAS is the failure of affected males to respond to polysaccharide antigens. There are few B lymphocytes that bear surface idiotypes for polysaccharide antigens and they mature late in B cell development. The response of these B lymphocytes requires T cell help that is elicited in a non-specific manner, that is independent of the T cell antigen receptor. Polysaccharides are neither processed nor presented in the manner of protein antigens. It remains to be determined if CD43 is a non-specific receptor for polysaccharides, which is capable of transducing a message so as to elicit non-specific T cell help.

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The Leukocyte Adhesion Deficiency

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INTRODUCTION

Impaired chemotaxis of phagocytic cells has been observed in a group of children who were suffering from recurrent bacterial infections (Anderson et al. 1987 ; Fischer et al. 1988). It has been later recognized that a membrane glycoprotein was missing on patients leukocytes. This defect was identified as a lack of the leukocyte adhesion molecules LFA-1, Mac-1 and p150,95 (Springer et al. 1984).

The leukocyte adhesion molecules are α-ß heterodimers (CD11a-CD18, CD11b-CD18, CD11c-CD18) sharing the 95 KD, ß-subunit. They belong to the integrins family characterized by the following properties : heterodimeric membrane glycoproteins involved in cell adhesion, divalent cation-dependency of the function, binding to RGD ligands for some of them. There are at least three subgroups i.e. 1) the VLA molecules expressing the ß1 subunit, 2) the leukocyte adhesion proteins (ß2) and 3) the GpIIbIIIa and the vitronectin receptor (ß3). The ß1, 2 and 3 subunits have a partial homology.

The LFA-1 molecule is expressed on all leukocytes while Mac-1 is expressed on phagocytic cells and on large granular lymphocytes, p150,95 is expressed in addition on some cytotoxic T lymphocytes. Two LFA-1 ligands have recently been identified as ICAM-1 and ICAM-2 molecules. ICAM-1 is widely distributed and its expression is regulated by several cytokines. Mac-1 binds iC3b coated particles, fibrinogen and factor X (Kishimoto et al. 1989).

THE LEUKOCYTE ADHESION DEFICIENCY DISEASE (LAD)

At least 62 patients have been found throughout the world to suffer from the LAD (A. Fischer et al. 1988). They can be phenotypically distinguished into two groups : the severe phenotype corresponding to undetectable levels of leukocyte adhesion proteins expression and the moderate phenotype characterized by a residual membrane expression (1 to 10 % of normal).

Indolent, necrotic infections of skin and soft tissues are the clinical hallmark of the disease. Such infections may disseminate to organs and provoke death. Bacterias and fungi are the main organisms although severe viral infections have occurred in a limited number of patients. Infectious sites are devoid of granulocytes and of monocytes contrasting with extreme leukocytosis up to 100,000/µl.

Transfusion of normal granulocytes are followed by migration to infectious sites (Bowen et al. 1982). The disease is very often associated with a delayed loss of umbilical cord, the mechanism of which being not well understood.

The natural outcome of the disease is poor since most (80 %) of the patients with the severe phenotype have died before the age of 2, patients with the moderate phenotype have a longer life expectancy. However actuarial survival curve shows that 75 % of them do not live more than 35 years.

LAD is inherited as an autosomal recessive trait. The incidence of consanguinity is high. Patients have been found in several parts of the world.

Structural defect

Immunofluorescence studies with specific monoclonal antibodies and immunoprecipitation of 125I-labelled membrane glycoproteins show either a complete absence of LFA-1, Mac-1 and p150,95 or a residual expression (1 to 10 % of normal levels) on patients'leukocytes. Agents shown to upregulate the expression of these proteins either by mobilization of granule reserves (fMLP, PMA) or by enhancing gene expression (TNF, interferon gamma) fail to induce or to augment membrane expression.

Biosynthetic analysis performed either on EBV-transformed B cells or activated T cells have revealed in all cases a normal synthesis of the LFA-1 α subunit while the ß subunit is either present or not (Anderson et al. 1985 ; Lisowska-Grospierre et al. 1986). In the severe phenotype, αß complexes are not produced and there is no processing of the subunits that are not glycosylated. The alpha subunit and if present the ß subunit tend to be rapidly degradated. In accordance with the biochemical studies, ß subunit mRNA is either detected in normal amounts, in reduced amounts or absent (Kishimoto et al. 1987 ; Dana et al. 1988 ; Dimanche et al. 1988). In one case of the moderate phenotype, we observed that ß subunit mRNA was found at a reduced level but could be upregulated by PMA, TNF γ and IFN γ, however this did not result in increased ß membrane expression (Dimanche et al. 1989). No anomaly of the ß subunit gene has been found by Southern blot analysis (Kishimoto et al. 1989).

It is very likely that in most, if not all cases, the LAD genetic defect relies in the ß subunit gene. Indeed, transcription and biosynthesis of the subunit is often impaired while the α subunits are normally synthetized. In addition, the LAD is inherited as an autosomal recessive disease although involving the three leukocyte adhesion molecules. Finally Marlin et al. (1986) have shown by complementation studies using human X mouse hybrids that the α subunit of patients'cells associates with the mouse ß subunit leading to a functional hybrid molecule.

The heterogeneity of "mutation" is underlined by the different anomalies found (see Table I). The causal mutation has however not been characterized except in one family (see below). One cannot entirely rule out the possibility of cis or trans regulatory defects in the cases with no or low levels of ß subunits mRNA. Since the ß subunit gene has been mapped in the 21q22.3 region, linkage studies using RFLP might be of help (Gardner et al. 1988) as well as patients ß subunit cDNA sequencing. In one family, Kishimoto et al. (1989) have elegantly characterized the mutation resulting in an aberrantly small ß subunit precursor. They have shown by using S1 nuclease protection method that there was a 90 nucleotide deletion in the ß subunit mRNA. By amplification of the abnormal mRNA they have identified that the missing nucleotides corresponded to an exon expressed in the extracellular domain.
Sequencing of patients'genomic ß subunit DNA gave evidence for a single point mutation in the 5' splice site of that exon, causing aberrant RNA splicing. The low level of membrane expression was the consequence of residual normal RNA splicing.

Table I. Heterogeneity of LAD

ß subunit mRNA expression	ß subunit precursor	∝-ß membrane expression	phenotype
None	None	None	None
Low	None	None	Severe
Low	Low	None or low	Severe or moderate
Normal	detectable (normal size)	None or low	severe or moderate
Normal	Aberrantly large	None	Severe
Normal	Aberrantly small	Low	Moderate

Functional consequences of LAD

The characterization of LAD has brought much insight into the functions of the leukocyte integrins (Anderson et al. 1987). As suspected from patients' in vivo observations, the defective expression of LFA-1, Mac-1 and p150,95 results in adhesion failure of granulocytes and monocytes. Consequently spreading and chemotaxis are defective. These anomalies account for the in vivo lack of mobilization of phagocytic cells towards infectious sites, adhesion to endothelial cells being impaired. Antibody-dependent cellular cytotoxicity mediated by granulocytes is also defective (Kohl et al. 1986). iC3b-coated particles also fail to induce phagocytosis and the oxidative burst. Adhesion-independent responses of phagocytic cells such as oxidative burst induction by soluble stimuli are preserved.

Lymphocyte functions are less markedly impaired than phagocytic cell functions in LAD. T and B cell differentiation occur normally. There is possibly a moderate anomaly in the homing of lymphocytes to lymphoid organs. Indeed, we found in the post mortem examination in one case a normal architecture of the thymus but an immature aspect of lymph nodes and spleen (unpublished data). This indicates that the LFA-1 independent pathway of adhesion of lymphocytes to endothelial cells may compensate for the absence of the LFA-1 dependent mechanisms (Dustin et al. 1988). Dustin et al. (1988) have demonstrated that LFA-1(-) lymphocytes can bind to endothelial cells. Similar findings have been obtained by Pals et al. (1988).

Activation of LFA-1(-) T lymphocytes by soluble anti CD3 monoclonal antibodies has been reported to be defective, the suggestion of an absent LFA-1 mediated transducing signal being put forward (Van Noesel et al. 1988). In other instances, however such activation was found normal. Antigen-induced in vitro and in vivo (delayed type hypersensitivity) responses are present, indicating that memory T cells which exhibit high levels of adhesion molecule expression (CD2...) are functional. Two T cell functions have however been found more consistently impaired. Cytotoxic T cell activity (CTL) (and Natural Killer activity) is abnormal although repeated in vitro stimulations of LFA-1(-) T cells can led to CTL generation (Mentzer et al. 1987). Helper T activity for antibody production has been found partially defective in the severe type of LAD since no antibody responses were observed in vivo following immunizations. It was shown by in vitro studies that the expression of LFA-1 at the T cell surface (but not at the B cell surface) was determinant (Fischer et al. 1986).

Both CTL and helper defects appear to be the consequence of poor T cell binding to targets. The adhesion defect is not complete and residual adhesion can be blocked by an anti CD2 antibody. Interestingly enough, non specifically activated LFA-1(-) T cells normally bind to B cells. This adhesion process is not exclusively CD2 dependent since adhesion is partially

present following CD2 modulation. VLA molecules (VLA4) may account for this adhesion since the latter is blocked by anti CD29 (VLA ß) antibody (Mazerolles et al. 1988 and unpublished data). The presence of both an LFA-1 independent adhesion pathway mediated by CD2 and the possible compensatory effect of other adhesion molecules such as VLA4 may explain why LAD has more limited consequences on T lymphocyte functions as compared to phagocytic cell functions.

Table II. Functional consequences of LAD

1. Phagocytic cells
. Impaired cell adhesion spreading and mobility
. Defective iC3b coated particles-induced respiratory burst
. Defective antibody dependent cell cytotoxicity
2. T lymphocytes
. Suboptimal T cell activation
. Partially impaired cytotoxic activity and helper activity for antibody production
. Normal in vivo delayed-type hypersensitivity
. Normal T. cell differentiation
3. NK cells
. Defective Natural Killer activity

Therapy of LAD

LAD is a life threatening disease. Antibiotics and granuloctye transfusions may temporarily control severe infections. The only curative procedure available so far is allogeneic bone marrow transplantation. This procedure has been found efficient in 5 out of 6 patients (A. Fischer et al. 1983 ; F. Le Deist et al. 1989), both using HLA identical and non identical donors. This results in stable mixed chimerism between LFA-1(-) and LFA-1(+) leukocytes. The presence of approximately 20 % normal cells appears to be enough to be protective against infections.
The success of HLA non identical bone marrow transplantation in 3/3 patients was remarkable because it is in striking contrast to the poor results of HLA non identical BMT in other clinical settings, except SCID, mainly because of graft rejection. It was thus thought that the absence of LFA-1 expression on T lymphocytes may avoid graft rejection by precluding adhesion to donor marrow cells. Based on this observation, monoclonal anti LFA-1 antibody was infused to recipients of MHC non identical BMT. This procedure was found efficient in the mice (Ferrara et al. 1988) and partially in men (A. Fischer et al. 1986). To date, 42 children with various inherited diseases have received an HLA non identical T cell depleted marrow in corporation with an anti LFA-1 α subunit monoclonal antibody (0.2 mg/kg x 10 days). Engraftment rate was 74 % (contrasting to 12 % in an historical control group) and survival rate 50.5 % (contrasting to 5 % in the control group).

Further application of such antibody specific for leukocyte integrins as a way to inhibit adhesion between effector T cells and target can be envisaged. It is interesting to note that an anti ICAM-1 antibody has been successfully used to prevent kidney graft rejection in monkeys (Cosimi et al. 1989). Since leukocyte integrins are major components of inflammation through their role in leukocyte mobilization, anti CD18 or anti Mac-1 antibodies are potent agents to reduce inflammation (Simpson et al. 1988 ; Vedder et al. 1989). Thus, the characterization of the LAD has been a major advance in the understanding biology of leukocyte integrins and has opened doors for new approaches for the control of immune responses and inflammation.

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Genotypic Studies of MHC Class III Genes in Individuals with IgA Deficiency and Common Variable Immunodeficiency

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INTRODUCTION

IgA deficiency (IgA-D) and common variable immunodeficiency (CVID) are heritable disorders both of which feature an arrest in B cell differentiation. IgA-D is characterized by a normal number of immature IgA-bearing B cells which fail to undergo differentiation into IgA-secreting plasma cells (Conley and Cooper 1981). Similarly, CVID usually features a normal number of B cells, exhibiting a normal distribution of Ig isotypes, which fail to undergo terminal differentiation into antibody-secreting plasma cells (Cooper et al. 1971; Preud'Homme et al. 1973). The causes for the B cell differentiation arrests in IgA-D and CVID are not understood.

IgA-D and CVID have sometimes been identified in immediate relatives (Wollheim et al. 1964) suggesting a common genetic origin for these two immunodeficiencies. In search of a common genetic basis for these disorders, we have focused on the major histocompatibility complex (MHC) of genes on the short arm of chromosome six for two reasons. First, these genes encode molecules that play a major role in antigen presentation and T and B cell interactions. Second, studies by Dawkins and associates (see Wilton et al. 1985) indicate that IgA-D patients often have extended MHC haplotypes which include C4A null alleles (C4AQO). We have evaluated RFLP's of the MHC class III genes for C4, 21-hydroxylase (OH), and C2 and HLA haplotypes of patients with CVID and IgA-D in comparison to healthy control individuals. The results suppport the hypothesis of a common genetic basis for the two immunodeficiencies. They further suggest that CVID and IgA-D individuals are homozygous for a recessive gene within the MHC class III region which predisposes to the development of these disorders.

METHODS

Thirty four healthy individuals, 19 CVID patients and 16 IgA-D patients were included in the study. All immunodeficient individuals were Caucasian and their clinical, immunologic, and serologic parameters have been reported (Schaffer et al. 1989). First degree relatives of 12 of the CVID and 10 of the IgA-D patients were also included in the study in order to define the MHC haplotypes of the patients. DNA extraction, restriction digestion, electrophoresis and Southern blotting were performed as described previously (Schaffer et al. 1989). The Taq I, Hind III,

and Bam H1 RFLP's were used to evaluate C4 gene deletions, duplications, and C4B gene length (Carroll et al. 1985, Yu and Campbell, 1987). The 21-hydroxylase (OH) genes were analyzed by using the Taq I RFLP (White et al. 1985). The C4 and 21-OH DNA probes utilized were provided by Dr. David Chaplin (Washington Univ., St. Louis, MO). Four RFLP's of the C2 gene were analyzed by using Sst I, Bam H1, or Taq I restricted genomic DNA and cDNA and genomic probes (Zhu et al. 1989; Horiuchi et al. 1989). A 300 bp genomic probe for C2 (Bentley et al. 1985) was provided by Dr. Duncan Campbell (Oxford Univ., U.K.). HLA-A, B, and DR typing was carried out by the microdroplet lymphocytotoxicity assay (Terasaki et al. 1978). Differences in the frequency of study parameters between the control and the patient populations were assessed by the chi-square test with correction for continuity.

RESULTS AND DISCUSSION

Results of Southern blot analyses of the C4, 21-OH, and C2 genes are summarized in Fig. 1. The most significant finding was that 32% (6/19, $p < 0.02$) of CVID and 31% (5/16, $p < 0.02$) of IgA-D patients had heterozygous deletions of C4A and 21-OH A genes. In the control group only one individual (3%) was found to have heterozygous deletion of the C4A and 21-OH A genes.

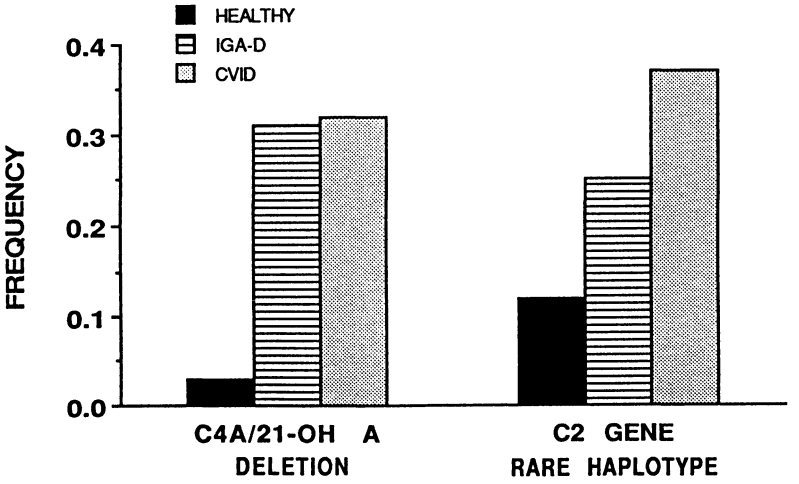

Fig. 1. Frequency of C4A/21-OH A deletion and of rare C2 gene haplotypes among 19 CVID, 16 IgA-D, and 34 healthy individuals.

Five C2 gene haplotypes, characterized by unique combinations of restriction polymorphic fragments, were observed. The common C2a haplotype was present in 93% of the chromosomes of the healthy controls. An increased frequency of rare C2 gene haplotypes was observed in immunodeficient individuals (Fig.1) although the difference from the controls was not statistically significant.

Family studies demonstrated that C4A/21-OH A gene deletion in 5 CVID individuals was associated with the HLA-A1, B8, DR3 haplotype. Similarly, this haplotype was associated with C4A/21-OH A deletion in the 4 IgA-D individuals for whom family members were available for study. In addition, in 3 IgA-D and 2 CVID patients for whom family members were available, the C2b rare gene haplotype was associated with the HLA-B44, DR7 haplotype. Further family studies, although incomplete at present, indicate that the majority of the IgA-D and CVID patients share a small number of HLA haplotypes associated with MHC class III gene RFLP's. These results strongly suggest a common genetic basis for the two disorders.

Based on an analysis of 29 independent HLA supratypes from 17 IgA-D patients, Wilton et al. (1985) proposed a recessive mode of inheritence for an MHC-associated and relatively frequently occurring gene located between the C4A and C4B genes. The penetrance of this putative gene was proposed to be determined by another factor not linked to the MHC. On the basis of our phenotypic and genetic studies, we conclude the IgA-D and CVID are related disorders. Our data support the hypothesis that a recessive gene, probably located between the C4A and C2 genes, endows susceptibility to both IgA-D and CVID. Development of immunodeficiency may depend on environmental (Gilhus et al. 1982) or additional genetic factor(s).

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Mapping of Two IgCH Gene Deletions in a Patient with IgG1 Deficiency

C.I.E. Smith and L. Hammarström

SUMMARY

A preliminary mapping of the IgCH gene deletions was performed in a patient with IgG1 deficiency. In contrast to previous hypotheses, we demonstrated that in none of the deletions was there a breakpoint located in the switch γ1 region.

INTRODUCTION

Immunodeficiencies have taught us much about the *normal* immune system. However, one disadvantage with immunodeficiencies is that they in most cases are likely to be heterogeneous and that we lack the tools to perform a proper subdivision. Individuals with homozygous gene deletions constitute one important exception, since they have defined types of defects. Thus, the study of individuals with IgCH gene deletions has made it possible to investigate the effect of a complete lack of particular isotype(s). The first individuals with IgCH gene deletions were described by Lefranc et al. in 1982. In total, 66 or 45 different single forms of deletions could occur in the IgCH region, the former figure including pseudogenes. To date, eight different deletion haplotypes have been found (Smith et al. 1989). However, only four of these haplotypes have been shown to exist in a homozygous form whereas three of the homozygous deletions, listed in Table 1, occurred as a result of the inheritance of two different deletion haplotypes. In this report we describe the preliminary mapping of two IgCH deletions in a patient with IgG1 deficiency. It has previously been hypothesized that switch (S) regions are involved in the deletion event (Lefranc et al. 1982; Migone et al. 1985). However, we demonstrate that in none of the haplotypes was the breakpoint located in the Sγ1 region.

Table 1. Homozygous IgCH deletions in humans

No of deleted genes◊	Homozygous deletion*	Reference
1	γ1	Smith et al. 1989
1	α1	Lefranc et al. 1983
1	γ2	Bottaro et al. 1989
3	α1-γ2-γ4	Migone et al. 1985
4	γ1-α1-γ2-γ4	Lefranc et al. 1982
4	α1-γ2-γ4-ε§	Migone et al. 1984; Chaabani et al. 1985
4	γ2-γ4-ε-α2	Bottaro et al. 1989

◊Pseudogenes are not included
*Deletions were ordered according to J_H proximity
§One deletion does (Chaabani et al.) and one does not encompass ψε1

MATERIAL & METHODS

Patient: The patient with IgG1 deficiency has previously been described in detail (Smith et al. 1989). In brief, the patient is prone to respiratory tract infections and has asthma. She is the first individual with a homozygous IgCH gene deletion not originating from the Mediterranean area. The maternal deletion was found to encompass Cγ1-Cψε1-Cα1-Cψγ-Cγ2, whereas the paternal deletion was confined to Cγ1.

Analysis of DNA: Southern blotting analysis was performed essentially as previously described (Smith and Hammarström 1984). Probes were obtained from cosmids cos Ig1, cos Ig6, cos Ig8 or cos Ig9 (Flanagan and Rabbitts 1982). The cosmid clones were kindly provided by T.H. Rabbitts. The Sx-γ3 probe was obtained from P. Sideras. The Sx region is located 5' of the S region and has been shown to be transcriptionally active (P. Sideras et al., manuscript in preparation). The restriction map is based upon the results of Flanagan and Rabbitts (1982).

RESULTS

The figures 1 and 2 depict results obtained with probes identifying restriction fragments in the Cγ3-Cγ1 region. The probe used in figure 1 was obtained from region upstream of the switch region, Sγ3, but has been demonstrated to cross-hybridize with Sx-γ1. The probe used in figure 2 also cross-hybridizes with fragments from other IgCH regions. The analysis demonstrates that the homozygous deletion encompassed the Sγ1 region as well as Sx-γ1. The blot also demonstrate that the corresponding band intensities in the parents were approximately half the intensity of normal controls, showing that they carry a heterozygous deletion. Preliminary results using the adjacent *Bam*HI-*Bam*HI fragment as a probe indicate that the homozygous deletion also encompassed this fragment (data not shown). The deletion would then extend >13 kb Cγ3 proximal of Cγ1.

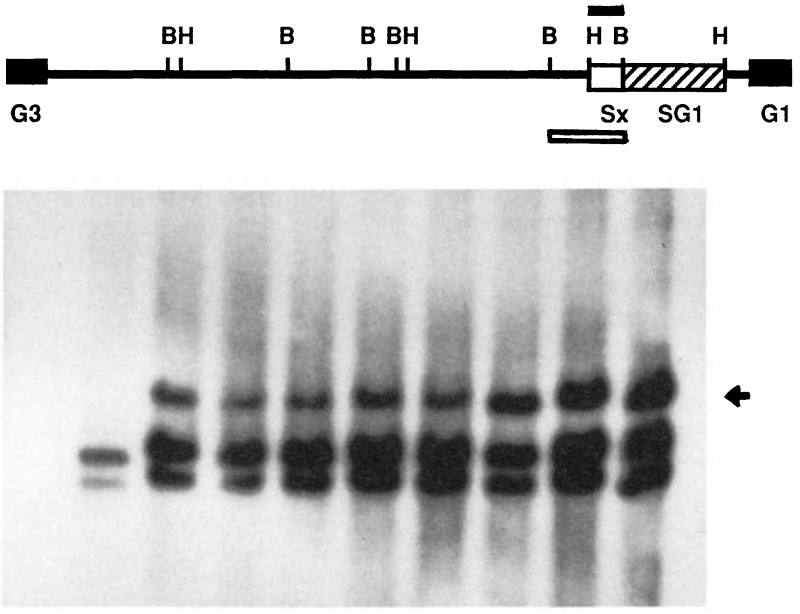

Fig. 1. Southern hybridization of a Sx-γ3 probe to genomic *Bam*HI digested DNA from (lanes from left) - lane 1 proband, lane 2 mother, lane 3 father, lanes 7-9 healthy controls. The Sx-γ1 region corresponding to the probe is indicated with a filled bar above the restriction map. The *Bam*HI fragment (≃2 kb) containing this sequence is indicated as an open bar below the restriction map and with an arrow in the blot.

The paternal deletion did not seem to involve the *Hin*dIII fragment approximately 5 kb Cψε1 proximal of Cγ1 (data not shown). *Hin*dIII digested DNA, hybridized with a *Bam*HI-*Bam*HI fragment (Fig. 2), resulted in one ≃ 9 kb fragment (the fragment is located above the two γ1 fragments). This band corresponds to a fragment located between the Cγ2 and the Cγ4 genes ≃ 3 kb from the Cγ2 gene. Comparison of the intensity of this band in the proband and her parents with that of normal controls indicated that the maternal deletion did not encompass this fragment.

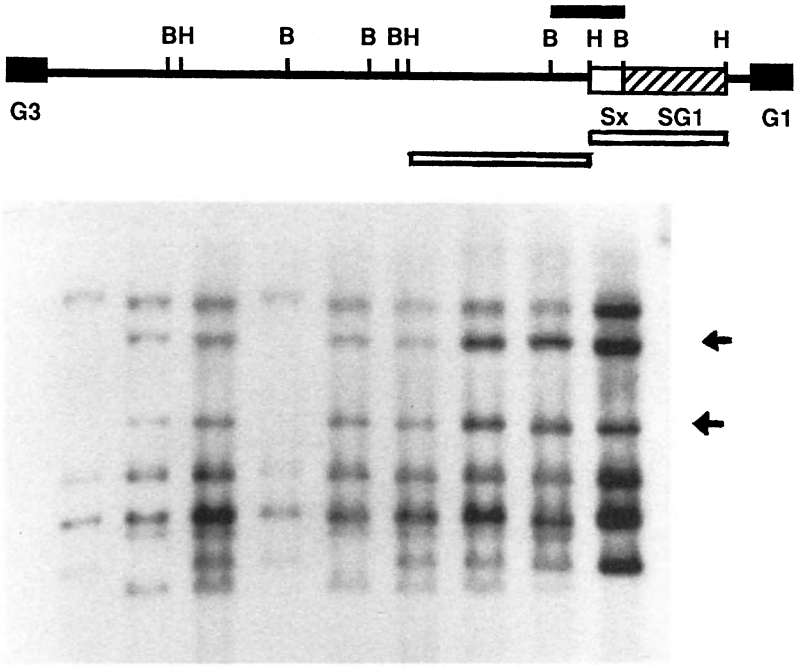

Fig. 2. Southern hybridization of a probe from cos Ig1 (*Bam*HI-*Bam*HI fragment indicated with a filled bar above the restriction map) to *Hin*dIII digested DNA from (lanes from left) - lanes 1 and 4 proband, lanes 2 and 5 mother, lanes 3 and 6 father and lanes 7-9 healthy controls. The two *Hin*dIII fragments (≃5.5 and 7 kb) containing probe sequences are indicated as open bars below the restriction map and are also identified with arrows in the blot.

DISCUSSION

To date, we are not aware of any published report on the isolation of the breakpoints in patients with IgCH gene deletions. However, the breakpoints in a patient with a Cγ1-Cψε1-Cα1-Cψγ-Cγ2-Cγ4 deletion has recently been identified (M.-P. Lefranc, pers. comm.). We found that the breakpoints in our Swedish patient were not located in the Sγ1 region and similar results were obtained by M.-P. Lefranc et al. (pers. comm.). Although we have recently observed a non-related variable immunodeficiency patient with a heterozygous Cγ1 deletion (P. Olsson et al., manuscript in preparation), only three haplotyes with Cγ1 deletions encompassing different IgCH genes have sofar been identified. The isolation of the breakpoints in our patients may make it possible to gain further insight into the deletion process.

As has previously been discussed (Smith et al. 1989) it is noteworthy that our patient is the only individual with an IgCH gene deletion, sofar described, with a definite increase in infection rate. However, as can be seen in Table 1, only two of the homozygous deletions encompass $C\gamma 1$. Thus, it could be argued that the most J_H-proximal IgCH genes ($C\mu$, $C\gamma 3$ and $C\gamma 1$) are the most important genes for the immune defense. The future identification of individuals with homozygous $C\mu$ and $C\gamma 3$ deletions is necessary in order to prove this hypothesis.

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III Effector Phases

Cell Cooperation

Cellular Cooperation in Antibody Production

W.E. Paul

Cellular interactions are a prominent aspect of immune responses both in vivo and in vitro. In particular, antibody production in response to immunization with most proteins depends upon the "helper" function of T cells. Despite the fact that T cell help for antibody production has been recognized for over two decades, several enigmas exist regarding the means through which it is mediated. In this Introduction to the Symposium on Cell Cooperation, I review some of the major issues that underlay the interaction between immunocompetent cells, with particular reference to the interaction between T cells and B cells that is involved in antibody production. I also point out that this one central aspect of immunity represents in microcosm the two dominant themes of immunological science, recognition and regulation. I have suggested that the great successes of the first century of immunology have been in understanding the cellular, molecular and genetic basis of antigen recognition. The field of immune regulation, although already well launched, is the one that we may expect to dominate the second century of our science (Paul 1987).

Initial Description of T Cell/B Cell Cooperation

That cellular interaction is a key to antibody production was shown in the pioneering experiments of Claman and his colleagues in 1966 (Claman et al 1966). These experiments demonstrated that irradiated mice could only mount antibody responses to sheep erythrocytes if they received both thymus and bone marrow cells from normal donors, suggesting that both cell types were required for antibody production. Mitchell and Miller (1968) showed that the bone marrow was the source of precursors of antibody producing cells while the thymus provided cells that mediated a critical helper function. At the same time, Mitchison (1967) had developed a cooperation model in which cells from donors primed to a hapten (4-hyroxy-3-iodo-5-nitrophenacetyl [NIP]) on the carrier ovalbumin (OVA) were transferred to sublethally irradiated recipients together with cells from a second donor primed to bovine serum albumin (BSA). He demonstrated that in such circumstances, both NIP-OVA and NIP-BSA could elicit a striking secondary anti-NIP antibody response; it was essential for such secondary responses that the hapten and the carrier be physically linked to one another. Raff (1970) then showed that the carrier-specific cells expressed the Thy 1 antigen, implying they were T cells, while the hapten-specific cells lacked Thy 1, indicating they were the source of B cells. These experiments established the existence of a cellular interaction in antibody production in which T cells mediated a specialized but poorly understood helper function while B cells acted as precursors of antibody producing cells.

T Cell-B Cell Interactions Are "Histocompatibility Restricted"

At first, it appeared as if the interaction between T and B cells might be a unidirectional one in which the T cells bound antigen and prepared and/ or presented it for the B cell. However, the demonstration of the phenomenon of "histocompatibility restriction" in cellular interactions led to a realization that the interaction was more complex, involving an active role for the B cells (and for other cells acting as "antigen-presenting cells" [APC]). Katz, Hamaoka and Benacerraf (1973) showed that T cell help for antibody responses required that the donors of the T and B cells possessed the same allelic form of a gene in the major histocompatibility complex (MHC). This work paralleled comparable work on the antigen-presenting action of macrophages, in which Rosenthal and Shevach (1973) demonstrated that the action of macrophages in T cell activation required that the interacting cell types be derived from donors that expressed a common form of an MHC molecule. It was logical to infer from the similarity in requirements of the two interactions that they might reflect a commonality of function. When T cell antigen recognition was examined in detail, it became clear that T cells did not recognize antigenic epitopes displayed on the surface of globular proteins but rather that they recognized linear determinants that could be found in peptides derived from the proteins (for review see Unanue 1984). Thus, two notions became established : 1) T cells and B cells did not recognize the same epitopes or, indeed, epitopes that existed on a single molecule *at the same time;* 2) a process of antigen preparation and presentation was essential for the "creation" of the peptides that were recognized by the T cells. As the function of MHC class I and class II molecules as "receptors" for antigen-derived peptides came to be recognized, it was clear that B cells were not simply passive participants in the interaction between T cells and B cells. Rather, like other APC, they presented antigen, in the form of a complex of an MHC molecule, usually a class II molecule, and a peptide derived from the native or "nominal" antigen, to T cells. This led to our current view that helper function represents a mutual interaction between T cells and B cells in which the B cell acts as an APC, providing critical receptor-transmitted triggering stimuli for the T cell (as well as "accessory cell function"). The T cell, in its turn, causes the B cell to become activated, to divide and to develop into an antibody producing cell. I should further point out that this description is that of the type of cellular interaction often referred to as a "cognate" T cell- B cell interaction, which has the essential properties described here: the B and T cell epitopes must be found on or derived from the same molecule and the B cell must express the antigen/class II complex on its surface. T cells may also aid B cells to respond through a "bystander" mechanism, which very likely involves lymphokine production and usually the stimulation of B cells that are already activated.

The view of cognate T cell- B cell interactions that has been developed to this point is largely dominated and illuminated by our understanding of the recognition events involved in the interaction. What is still not clear is how this interaction leads to the activation of the two cell types. Indeed, both the T cell activation

and the subsequent T cell-aided B cell response depend on stimuli that are only incompletely understood.

T Cell Activation in Cell Cooperation Models

Let us first consider the activation of the T cell. T cells from primed donors can be stimulated by a variety of antigen-presenting cells that express class II MHC molecules. Among these are macrophages, dendritic cells, epidermal Langerhan's cells and activated B cells. There is evidence that resting B cells can stimulate T cells that have previously been activated (Ashwell et al 1984) but they appear less efficient than activated B cells or other APC. Whether resting B cells can act as efficient APC for activation of resting T cells is not certain and thus whether the T cell- B cell interaction leading to help can be a primary event or must always be preceded by prior T cell or B cell activation remains to be established.

Great progress has been made in analyzing the chemical processes set in motion by engagement of the T cell receptor complex. Nonetheless, recognition of the antigen-MHC cell surface determinant is only one aspect of the activation process. Apart from the function of auxiliary molecules, such as CD4, there appears to be a need for an additional signal provided by the APC; often this is described as accessory cell function. Indeed, recent work indicates that the stimulation of many T_{H1} cell lines (i.e.-IL-2/IFNγ-secreting T cells) with antigen/class II complexes on fixed cells induces a state of peripheral immunologic unresponsiveness (Mueller, Jenkins and Schwartz 1989). Fixed cells lack the ability to provide accessory function; the addition of suitable accessory cells prevents induction of unresponsiveness. For some rodent T cells and thymocytes, accessory signals may be provided by interleukin-1 (IL-1). However, IL-1 does not seem to mediate this function for the majority of T cells, including long term T cell lines of the T_{H1}-type and IL-1 does not prevent induction of unresponsiveness in T_{H1} cells stimulated with antigen/class II complexes on fixed presenting cells. Determining the molecular basis of accessory cell function is a key to further progress in T cell activation in general and to an understanding of cellular interactions.

Cell Cooperation is Critical for B Cell Responses to Thymus-Dependent Antigens

It has been customary to categorize antigens into three broad classes, often referred to as thymus-dependent (TD), type 1 thymus-independent (TI-1), and type 2 thymus-independent (TI-2) antigens (Mosier et al 1977). TD antigens generally fail to elicit any antibody responses in *nu/nu* mice; they are usually protein antigens that appear to be completely dependent upon T cell help to generate antibody responses. TI-1 and TI-2 antigens are often referred to simply as Type 1 and Type 2 antigens since responses to them are not truly independent of T cell influence. They do elicit antibody responses in *nu/nu* mice but in vitro antibody responses to these antigens are either dependent upon or enhanced by action of T cell products. Type 1 antigens are

substances with intrinsic mitogenic activity such as hapten derivatives of lipopolysaccharide (LPS); the prototypic type 2 antigens are macromolecules with repeating antigenic determinants such as polysaccharides and hapten polysaccharide conjugates. Type 2 antigens are clearly capable of cross-linking cell surface Ig, which has been shown to lead to a set of receptor-mediated cellular biochemical events marked by hydrolysis of polyphosphoinositides with generation of inositol phosphates and diacylglycerol. However, it seems unlikely that this pathway of B cell activation is important in the response of B cells to most TD antigens, since these molecules do not generally bear repeating epitopes and should fail to cross-link receptors unless they become aggregated. (In those cases where globular proteins bear repetitive epitopes, receptor crosslinkage-mediated activation may enhance the response of the B cells to T cell help). In general, it appears that TD antigens depend upon the intimate cell cooperation effects described here to elicit responses. In these responses, the major function of the B cell receptor is to bind antigen and thus to concentrate that antigen upon those B cells that can produce antibody specific for the immunogen, if they receive T cell help. Receptor-bound antigen is endocytosed and a portion of it appears to enter an intracellular pathway characterized by degradation to peptides, association with class II MHC molecules and expression of the peptide/class II complex on the cell surface, creating the "antigenic target" for specific T cells. The receptor thus makes antigen-presentation a specific function of antigen-binding B cells and gives to intimate or "cognate" cellular interactions the information necessary for the elicitation of specific antibody responses (Lanzevecchia, 1985).

T Cell-Derived Lymphokines: Production and Action

T cells that recognize and respond to antigen/MHC epitopes on the B cell surface can stimulate the activation, growth and differentiation of the B cell. How they do this remains a major unsolved aspect of B cell responses. Part of the action of T cells is certainly mediated by the lymphokines that these cells produce. T cells make a series of lymphokines several of which are known to act on B cells. Among these are interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-2 (IL-2) and interferon γ (IFNγ). IL-4 is known to act on resting B cells to induce class II MHC molecules and to increase expression of FcεRII (CD23). It is a potent co-stimulant of the growth of B cells and acts as a specific switch factor for IgG1 and IgE. IL-5 in the mouse is a B cell differentiation factor; much of this action appears to be directed to $CD5^+$ B cells. IL-6 is a striking stimulant of Ig secretion by B cells. IL-2 can act to regulate the growth of B cells and can stimulate B cell Ig secretion. IFNγ has many functions in the immune system including regulation of production of IgG2a antibodies and inhibition of many of the functions that IL-4 exerts on B cells.

The antigen-specific interactions of T cells and B cells are marked by the formation of a physical association between the two cell types. Furthermore, T cell recognition of antigen/MHC epitopes in these interactions results in a morphologic reorganization of the T cell so

that the Golgi apparatus and the microtubule organizing center are found in the pole of the T cell that is in apposition to the antigen-presenting B cell (Kupfer, Swain and Singer 1987). This has suggested that the T cell may preferentially secrete proteins into the limited space between the T and B cell. Indeed, it has been shown that the mouse T cell line D10 will preferentially secrete IL-4 from the face of the cell at which its surface receptors have been cross-linked (Poo, Conrad and Janeway 1988). This would indicate that lymphokine secretion is directional and that high concentrations may be achieved in the "synapse" that links the two cell types.

Nonetheless, it seems unlikely that the lymphokines that have already been well characterized and whose genes have been molecularly cloned can be entirely responsible for the activation of resting B cells, particularly those whose receptors have not been cross-linked by a multivalent antigen. One must either postulate unrecognized functions mediated by a combination of lymphokines; the existence of other lymphokines that have B cell activation capability; or the delivery of a signal through some other route. B cell activation factors have been reported by several groups. These factors appear to act upon resting B cells and to cause them to become activated and to be capable of proliferating in vitro. Thus, far the detailed characterization and purification of B cell activation factors has been particularly difficult so that their biological properties have not been evaluated in detail. The functional significance of B cell activation factors remains to be established.

Antigen-Specific T Cell Helper Factors

An alternative view is that the T cell delivers some other type of stimulation to the B cell. Indeed, the point has been made by many students of cell cooperation that physical interaction with T cells elicits responses in B cells that cannot be accounted by any of the known lymphokine products of T cells (Vitetta et al 1987). Evidence has been presented that cross-linkage of cell surface class II molecules on B cells may lead to translocation of protein kinase C to the nuclear compartment (Chen et al 1987), suggesting that class II molecules may have the capacity to act as signal generating molecules in a pathway that may have several novel features. T cell receptors or specific soluble factors that bind to antigen/class II complexes on the B cell may elicit the activation of resting B cells through this signalling pathway.

Antigen-specific T cell factors capable of mediating helper or suppressor function have been reported in many laboratories (For review, see Tada 1984). Such factors could be major mediators of the action of T cells and may lead to the creation of unique signals capable of activating resting B cells that are unresponsive to any of the well recognized stimulants currently available. Unfortunately, relatively little progress has been made in the last several years in chemically characterizing and purifying these factors. Indeed, the rapid movement of the lymphokine field and the slow movement in the "specific factor" area has led to considerable skepticism regarding the significance of much of the work using soluble antigen-specific

T cell factors. It is thus particularly notable that Guy and colleagues (1989) have observed that several cloned T cell lines produce an antigen-specific factor that replaces specific T cells in the stimulation of in vitro secondary IgG anti-hapten antibody responses. These factors act in an antigen-specific/histocompatibility-restricted manner, analogous to the specificity of the T cells from which they are derived. In order for the factor to be active, it was necessary that the hapten be linked to the carrier for which the T cells were specific, just as is required for the action of the T cells themselves. What is particularly striking about this report is that factors derived from T cells that utilize a Vβ8-encoded T cell receptor polypeptide chain express an antigen (F23) known to be associated with T cell receptor Vβ8-derived chains. By contrast, antigen-specific helper supernatants from $F23^-$ T cells lack the F23-determinant.

If this report can be generalized, it would imply that a major aspect of T cell/B cell interaction, perhaps the B cell activation phase, may be mediated by the binding of T cell receptors or their soluble products to B cell surface molecules with consequent signal transduction. Clearly, the detailed examination of this issue together with an exploration of the B cell activation factors will be a major theme in the study of T cell-driven B cell responses.

How Does T Cell Activation Regulate the Type of Immune Response that Occurs

The interaction of T cells with B cells and with other cells of the immune system is not only the key to the occurrence of thymus-dependent immune responses but is critical to the type of immune response that ensues. It is well known that different types of infectious agents elicit responses that are quite different from one another. Infections with helminths and several other parasites elicit responses dominated by the production of relatively large amounts of IgE antibodies and, in some instances, IgG1 antibodies. By contrast, viral infections of mice are marked by striking induction of cytotoxic T cell responses and the IgG antibody that is produced is usually of the IgG2a class (Coutelier et al 1988). The recent subdivision of long term lines of mouse T cells into two major populations, designated T_{H1} and T_{H2}, that produce distinct panels of lymphokines (Mosmann and Coffman 1989) has suggested that the control of different types of immune responses may operate at the level of what type of T cell response is elicited. To this end, one of the most important goals of current research on T cell activation is to delineate the types of stimuli that elicit T cells that preferentially produce IL-4 (the prototypic T_{H2} lymphokine) or IL-2 and IFN γ (the prototypic T_{H1} lymphokines). Work in my laboratory has demonstrated that forms of immunization that favor IgE production (i.e., infection with *Nippostrongylus brasiliensis* or immunization with low doses of antigen on alum) are characterized by vigorous production of IL-4 when T cells are stimulated in vitro with anti-CD3 antibodies (Conrad et al 1989). These results support the association of production of a set of lymphokines and the elicitation of a specific type of immune response. One promising approach to understanding how such specific responses

are favored is the examination of the distinctive stimulatory properties of different types of antigen-presenting cells as well as the significance of the physical form of the antigen and the nature of the microenvironment in which it is concentrated.

An alternative possibility is that amplification mechanisms may exist that preferentially enhance one type of response over another. We have observed that long term IL-3-dependent murine mast cell lines are capable of producing many of the same lymphokines produced by T_{H2} cells, including IL-4, IL-5, IL-3, GM-CSF and IL-6 (Plaut et al 1989). These lymphokines are not produced constitutively but rather require the activation of the mast cells which can be achieved either by calcium ionophores or, on mast cells sensitized with IgE anti-DNP antibodies, through cross-linkage with a multivalent DNP-protein conjugate or an anti-IgE antibody. With the recognition that long term mast cell lines produce lymphokines in response to cross-linkage of $Fc\varepsilon$ receptors, we turned to the examination of whether normal splenic cell populations contained cells that could produce T_{H2}-type lymphokines in response to Fc receptor cross-linkage. We observed that mice infected with *N. brasiliensis* or injected with anti-IgD antibodies possess a population of spleen cells that lack either B cell or T cell markers but which are vigorous producers of IL-4, but not IL-2 or IFNγ, when stimulated with polymerized IgE or IgG (Ben-Sasson et al 1989). Splenic non-B, non-T cells from untreated, pathogen-free mice produce a meager Il-4 response to those stimuli; if treated with IL-3, these cells make a strong response. An analysis of this effect indicates that IL-3 acts on this cell population to cause differentiation into cells capable of producing IL-4 upon appropriate stimulation (LeGros et al 1989).

It is possible that non-B, non-T cells may be recruited by initial activation of T cells and their production of lymphokines characteristic of T_{H2} cells could strongly amplify the effects of T cells that normally participate in anti-parasite and allergic responses. Non-B, non-T cells might also mediate certain types of help, particularly when they are sensitized with antibodies of the same specificity as those represented by receptors of the B cells. This might allow B cells to cross-link cell surface Fc receptors and lead to local production of lymphokines. An alternate role for non-B, non-T cells is in the production of lymphokines and subsequent induction of cellular inflammatory responses at the site of deposition of immune complexes.

Conclusion

Cellular interactions constitute the most sophisticated mechanisms available to the immune system for regulating the level of antibody production in immune responses to T-dependent antigens. They allow the play of the full range of T cell products on the resting B cell and because of the high local concentrations and the opportunity for synergy among different lymphokines, effects may be achieved that are difficult to mimic in any single cell system. Nonetheless, the mechanism through which helper T cells cause the activation of the antigen-specific resting B cell has still not been resolved. The

analysis of the events that underlie the regulatory processes in cognate T cell-B cell interactions should provide the knowledge needed to develop effective strategies for immune intervention in a variety of pathologic conditions and may offer insights for drug discovery programs.

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Clonal Deletion of Autoreactive T Lymphocytes: Insights from Neonatal Ontogeny

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KINETICS OF CLONAL DELETION OF AUTOREACTIVE T CELLS DURING ONTOGENY

It is now well established that intrathymic clonal deletion of autoreactive cells is an important mechanism for maintaining tolerance to self antigens (Kappler et al. 1987, 1988; MacDonald et al. 1988b). However it is not evident when (or how) certain tissue-specific self antigens can be presented within the thymus, nor is it clear how clonal deletion actually occurs. In an effort to begin addressing these issues, we have investigated the clonal deletion of 3 self antigens that are preferentially recognized by T cell receptors (TCR) bearing distinct β-chain variable (V_β) domains (Table 1).

Table 1. TCR V_β domains (and corresponding self antigens) analyzed in this study

V_β	Reactivity		References
	MHC	Antigen	
3	class II (I-E)	Mls-2^a	Pullen et al. 1988; Fry and Matis 1988
6	class II (I-E)	Mls-1^a	MacDonald et al. 1988b; Kanagawa et al. 1989
8	———	———	———
11	I-E	?	Tomonari et al. 1988; Bill et al. 1989

The congenic strain Balb.D2.Mls^a (Festenstein and Berumen 1984) expresses the minor antigens Mls-1^a and Mls-2^a as well as the class II MHC gene I-E^d. Thus $V_{\beta3}^+$, $V_{\beta6}^+$ and $V_{\beta11}^+$ mature T cells are eliminated by clonal deletion in these mice, but not in C57BL/6 (Mls-1^b, Mls-2^b, I-E^-) controls. We recently observed that $V_{\beta6}^+$ cells with a mature ($CD4^+8^-$)phenotype persisted in the thymus of neonatal Balb.D2.Mls^a mice for several days (Schneider et al. 1989). It was therefore of interest to compare $V_{\beta6}^+$ with other autoreactive TCR V_β domains in these animals. The results (Table 2) indicate that $V_{\beta11}^+$ $CD4^+$ thymocytes are present in even greater numbers (relative to adult C57BL/6 controls) than $V_{\beta6}^+$ T cells at 4 days after birth; in striking contrast $V_{\beta3}^+$ $CD4^+$ cells are completely absent at this time. A

kinetic analysis of the expression of these autoreactive V_β (as well as a $V_{\beta 8}$ control not subject to deletion) is shown in Table 2. It can be seen that $V_{\beta 6}^+$ and $V_{\beta 11}^+$ $CD4^+$ cells persist for 1-2 weeks in thymus, whereas $V_{\beta 3}^+$ $CD4^+$ cells are not detected at any time tested. Similar results were obtained for lymph node (data not shown).

Table 2 Differential kinetics of deletion of autoreactive TCR V_β domains among mature ($CD4^+8^-$) thymocytes[a]

V_β domain	Self antigen	Balb.D2.Mls[a]				C57BL/6
		Day 4	Day 7	Day 12	Adult (6wk)	Adult
3	Mls-2[a]	0.2	0.9	0.3	n.d.[b]	3.4
6	Mls-1[a]	4.0	2.7	0	n.d.	7.1
8	____	11.7	12.7	13.1	17.3	16.1
11	?	9.2	7.8	4.1	0.9	4.7

[a] CD8-depleted thymocytes were stained with mAbs KJ25 (anti-$V_{\beta 3}$), 44-22-1 (anti-$V_{\beta 6}$), KJ16 (anti-$V_{\beta 8.1/8.2}$), or RR3-15 (anti-$V_{\beta 11}$) followed by fluorescent goat anti-rat Ig. Data are expressed as percent positive cells (following background subtraction and normalization to total $CD4^+8^-$ subset).

[b] Not done.

Several important points emerge from this analysis. First the fact that some autoreactive V_β but not others are deleted early in development indicates that limitations in self antigen expression (and/or accessibility to the thymus) are responsible for the observed heterogeneity rather than some intrinsic defect in thymic antigen presentation. Second the parallel kinetics of $V_{\beta 6}$ and $V_{\beta 11}$ expression observed in thymus and lymph node suggests that «autoreactive» cells in the periphery emigrate from thymus but do not expand, even in the presence of putative self antigens.

FAILURE OF AUTOREACTIVE TCR TO EXPAND FOLLOWING NEONATAL THYMECTOMY

In view of the presence of significant numbers of potentially autoreactive T cells expressing $V_{\beta 6}$ and $V_{\beta 11}$ in the lymph nodes of young Balb.D2.Mls[a] mice, we decided to investigate the effect of neonatal thymectomy on the subsequent fate of these cells. As shown in Table 3, day 3 thymectomy did not significantly affect the proportion of $V_{\beta 6}^+$ or $V_{\beta 11}^+$ $CD4^+$ cells measured in spleen 8-12 wks later (compared to control mice), although the fraction of $CD3^+$ cells was reduced in the thymectomized group. Similar results were obtained when thymectomy was performed on day 6 (data not shown).

Table 3 Failure of postnatal thymectomy to induce expansion of autoreactive TCR V_β in Balb.D2.Mls^a mice

Treatment[a]	% $CD3^+$	$V_{\beta 6}$	$V_{\beta 8}$	$V_{\beta 11}$
Control	86.7 ± 2.4	0.2 ± 0.1[b]	14.3 ± 1.5	0.8
Day 3 Tx	45.7 ± 14.5	1.0 ± 0.5	13.3 ± 2.8	1.9 ± 1.0

[a] Nylon wool purified spleen cells (depleted of $CD8^+$ cells) were analyzed 8-12 weeks after day 3 thymectomy.

[b] Mean ± SD for 3-4 individual mice (normalized to total $CD3^+$ cells).

The failure of neonatal thymectomy to maintain (or increase) the levels of potentially autoreactive ($V_{\beta 6}^+$ or $V_{\beta 11}^+$) T cells in adult Balb.D2.Mls^a mice suggests that the expansion of these cells is actively inhibited, as opposed to simply diluted by the continuous production (in the normal thymus) of new T cells lacking these V_β domains. The mechanism underlying this apparent inability of peripheralized $V_{\beta 6}^+$ or $V_{\beta 11}^+$ T cells to proliferate requires further study, but may be related to recent reports suggesting that extrathymic contact of $V_{\beta 6}^+$ T cells with Mls-1^a antigens leads to clonal anergy (Qin et al. 1989; Rammensee et al. 1989).

Early postnatal (day 3) thymectomy has been shown to result in an increased occurrence of autoimmune disease (Nishizuka and Sakakura 1969). In this context, it is somewhat surprising that neither $V_{\beta 3}^+$, $V_{\beta 6}^+$ or $V_{\beta 11}^+$ «autoreactive» T cells were expanded in thymectomized Balb.D2.Mls^a mice. It may be that the self antigens recognized by these TCR (Mls-2^a, Mls-1^a and ?) do not readily induce autoimmunity, consistent with the relative inefficiency of the Mls antigens to provoke graft rejection (Festenstein 1973). Furthermore Balb/c mice are relatively unsusceptible to autoimmunity provoked by early postnatal thymectomy (Tung et al. 1987). In more susceptible strains such as $(C57BL/6 \times A/J)_{F1}$, expansion of some «autoreactive» V_β has recently been observed following thymectomy (K Tung Personal communication). Thus it appears that early thymectomy is in itself insufficient to allow expansion of potentially autoreactive TCR V_β and that other genetic factors (perhaps controlling the expression of autoantigens) play an important role in this process.

PROGRAMMED DEATH AS A MECHANISM OF CLONAL DELETION

Thymocytes are acutely sensitive to glucocorticoid-induced cell death in vivo, and this phenomenon has been shown to involve activation of an endogenous endonuclease (Wyllie 1980; Cohen and Duke 1984). Recent studies indicate that triggering of TCR (via monoclonal anti-CD3 antibodies) likewise leads to thymocyte death and DNA fragmentation (frequently referred to as apoptosis) both in vitro and in vivo (Smith et al. 1989; Shi et al. 1989). In this context, we were intrigued by our previous observation that $V_{\beta 6}^+$ $CD4^+$ thymocytes in neonatal Balb.D2 Mls^a mice expressed lower levels of TCR than age-matched (or adult) Balb/c controls (Schneider et al. 1989). Such a finding would be consistent with TCR down-regulation as a result of encounter with self (Mls-1^a) antigens.

In order to pursue this issue further, we isolated neonatal (day 4) $CD8^-$ thymocytes from Balb.D2.Mls^a mice and put them in short-term culture. The results (Table 4) demonstrate that the proportion of viable $V_{\beta 6}^+$ cells is selectively decreased in overnight cultures kept at 37°C. Furthermore, death of $V_{\beta 6}^+$ cells could be blocked by inhibitors of macromolecular (RNA and protein) synthesis, as is the case for glucocorticoid-induced thymocyte death (Cohen and Duke 1984).

Table 4 Selective death in vitro of $CD4^+$ $V_{\beta 6}^+$ neonatal thymocytes : requirement for RNA and protein synthesis

Incubation[a]	$V_{\beta 6}$	$V_{\beta 8}$
4°C	4.1 ± 0.3[b]	11.6 ± 0.9
37°C	1.9 ± 0.4	10.7 ± 1.3
37°C + cycloheximide	3.8 ± 0.6	11.7 ± 0.4
37°C + actinomycin D	3.7 ± 0.2	11.1 ± 0.1

[a] $CD8^-$ thymocytes from 4 day-old Balb.D2.Mls^a mice were incubated (1.5×10^6/ml) for 20h at 4°C or 37 C in normal tissue culture medium supplemented with cycloheximide (50µg/ml) or actinomycin D (5µg/ml) where indicated. Recovered viable cells were stained with anti-$V_{\beta 6}$ or anti-$V_{\beta 8}$ mAbs.

[b] Mean ± SD of 3 independent experiments (normalized to total $CD4^+8^-$ subset)

Taken together, our data provide suggestive evidence that autoreactive thymocytes are deleted by a mechanism that is similar (or identical) to apoptosis. Furthermore they indicate that clonal deletion can occur at a relatively late ($CD4^+8^-$) stage of development. However we do not mean to imply that a similar mechanism is not operative at other (earlier) developmental stages. Indeed apoptosis induced by glucocorticoids (Ceredig et al. 1983) or by anti-TCR antibodies (Smith et al. 1989) affects mainly immature $CD4^+8^+$ thymocytes, and there is presumptive evidence that the $CD4^+8^+$ subset is a principal target of clonal deletion in both normal and transgenic mice (Kisielow et al. 1988; Fowlkes et al. 1988; MacDonald et al. 1988a). Thus we would prefer to conclude that programmed cell death can occur at various stages of T cell development, up to and including at least some cells with a mature phenotype.

CONCLUDING REMARKS

Clonal deletion of autoreactive T cells in the thymus is a major (Kappler et al. 1987; 1988; MacDonald et al. 1988b) but not unique (Qin et al. 1989; Rammensee et al. 1989) mechanism for the maintenance of tolerance to self antigens. We show here that the kinetics of deletion of mature ($CD4^+$) T cells expressing 3 independent TCR V_β domains (reactive with 3 distinct but poorly defined self antigens) shows remarkable heterogeneity in the early neonatal period. It is therefore highly likely (although not possible to test directly) that these 3 self antigens are encoded by genes (Mls-1^a, Mls-2^a, and ?) that are differentially regulated during ontogeny. Alternatively, it is possible that their gene products, although simultaneously present, have differential access to the thymus or to processing pathways necessary for their presentation in that organ. A more precise molecular definition of the Mls (and other minor antigen) gene products will be required to address this issue.

The mechanism of clonal deletion has also been explored for $CD4^+$ cells expressing one particular autoreactive TCR V_β ($V_{\beta 6}$) that disappeared very rapidly in the early postnatal period. Evidence is presented that many of these cells undergo rapid (and selective) cell death in vitro that is dependent upon RNA and protein synthesis. These observations are consistent with the phenomenon of programmed cell death (apoptosis) seen in glucocorticoid-induced thymocyte death, where activation of an endogenous endonuclease (and/or other as yet undefined proteins) is required to trigger the lethal event. Based on data summarized elsewhere (MacDonald et al. 1989), it seems likely that apoptosis of developing thymocytes results from contact with MHC class II-expressing cells of hematopoietic origin. Whether such contact occurs in vivo (i.e prior to thymocyte isolation) or during overnight culture in our model system remains to be determined.

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Developmental Arrest of T Cell Receptor (TcR) Negative SCID Thymocytes and Release in the Presence of TcR^+ Cells

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INTRODUCTION

Upon entering the thymus, progenitor T cells, lacking CD4, CD8, and T cell receptor (TcR), differentiate into mature, functional thymocytes which express TcR in conjunction with either CD4 or CD8. The forces driving thymocyte maturation have remained largely uncharacterized. In particular, the role of the TcR in this process has not been examined. In order to assess the role of TcR expression in T cell development, we have examined thymocyte differentiation in the presence and absence of intrathymic TcR^+ cells in immunodeficient C.B-17/scid mice (Bosma et al., 1983.)

Phenotyping of SCID Thymocytes

Mice expressing the scid genetic mutation exhibit a severe immune defect due to defective recombination enzymes that cannot function to productively rearrange genes encoding the antigen receptors of T and B cells (Bosma, et al., 1983; Schuler, et al., 1986; Malynn, et al., 1988; Lieber, et al., 1988) As a result, SCID mice are severely deficient in TcR-bearing thymocytes. Thus we began our assessment of T cell differentiation in the absence of TcR expression by phenotyping thymocytes from young adult SCID mice. Although only 0.7-2 X 10^6, were obtained from individual SCID thymi, these cells were $Thy1^+$, and as expected, failed to express surface TcR as they were $CD3^-$ (Table 1). Interestingly, SCID thymocyte populations were enriched in large, $CD5^{lo}$, $IL\text{-}2R^+$, and $Ly24^{bright}$ cells (Table 1). Moreover, TcR^- SCID thymocytes failed to express either CD4 or CD8 accessory molecules (Table 1), which confirms and extends the findings of others (Habu, et al., 1987). Thus, SCID thymocytes were $Thy1.2^+TcR^-IL\text{-}2R^+CD5^{lo}CD4^-CD8^-$ and therefore closely resembled early progenitor thymocytes present in normal mice which have been shown to give rise to CD4 and/or CD8-bearing cells (reviewed by Scollay et al, 1988). However, SCID $Thy1.2^+TcR^-IL\text{-}2R^+CD5^{lo}CD4^-CD8^-$ thymocytes were arrested in their differentiation as indicated by the absence of any $CD4^+$ or $CD8^+$ cells in SCID thymi.

Table 1. Cell Surface Phenotype of Thymocytes from Young SCID Mice

Antigen	C.B-17 +/+	C.B-17 scid/scid*
Thy1.2$^+$	98#	94
CD3$^+$	66	0
CD5 bright	26	<1
IL-2R$^+$	1	80
Ly24 bright	6	23
CD4+8$^+$	97	2
Size	470&	594&

*Pool of thymi from four SCID mice
#Percent cells staining positively with experimental antibody relative to background staining with negative control antibody.
&Mean light scatter intensity in arbitrary units.

Thymocytes from "Leaky" SCID Mice

In order to determine if the absence of TcR$^+$ cells in SCID thymi was responsible for the arrest in SCID thymocyte maturation, we made use of the fact that a small percentage of SCID mice contain some receptor positive T and/or B cells ("leaky" SCID mice), resulting from rare productive gene rearrangement in a particular lymphocyte clone or clones (Habu et al., 1987, Bosma et al., 1988). Thus we evaluated CD4 and CD8 expression in unmanipulated SCID mice whose thymi possessed spontaneously generated TcR$^+$ thymocytes. These studies revealed that all SCID mice which possessed intrathymic TcR$^+$ cells, also possessed CD4$^+$ and/or CD8$^+$ thymocytes. Furthermore, when we comprehensively examined all our data from a total of 67 SCID thymi, (summarized in Table 2), we observed an interesting relationship between CD4/CD8 expression and TcR expression. In Table 2, all possible combinations of CD4/CD8 and TcR expression are considered in thymi derived from SCID mice that are either less than 7 months old or greater than 7 months old as the frequency of "leaky" SCID mice increases with age (Bosma et al., 1988). The vast majority of SCID mice, and in fact all the mice less than 7 months of age possessed thymi that could be categorized as SCID Type 1 (lacking both TcR$^+$ and CD4$^+$/CD8$^+$ cells), or SCID Type 2 (containing both TcR$^+$ and CD4$^+$/CD8$^+$ thymocytes). SCID Type 3 thymi (containing CD4$^+$/CD8$^+$ cells without detectable numbers of TcR$^+$ cells) were rarely observed and were only found in SCID mice greater than 7 months of age. Interestingly, we never detected SCID Type 4 thymi (containing TcR$^+$, in the absence of CD4$^+$/CD8$^+$ cells), thus the presence of TcR$^+$ cells was always accompanied by the presence of CD4$^+$/CD8$^+$ cells (Table 2). Thus, a striking relationship exists between the presence of TcR$^+$ thymocytes and the generation of CD4$^+$/CD8$^+$ SCID thymocytes, with the only apparent exceptions found in older SCID mice (SCID Type 3) that might well have previously possessed CD3$^+$/TcR$^+$ cells.

Table 2. Relationship Between TcR^+ and $CD4^+/CD8^+$ SCID Thymocytes

SCID Type	Thymi Containing Thymocytes Expressing: TcR	CD4/CD8	Age <7mos	>7mos
1	-	-	49*	7
2	+	+	5	2
3	-	+	0	4
4	+	-	0	0

*Number of mice based on individual and pooled thymi, in which analysis by Poisson statistics has revealed a 10% frequency of SCID mice possessing thymi containing CD4/CD8-expressing cells and TcR^+ thymocytes.

Interestingly, although expression of TcR by SCID thymocytes was always accompanied be the presence of CD4/CD8-bearing SCID thymocytes, those cells bearing CD4/CD8 were not necessarily themselves TcR^+ (data not shown). Consequently, CD4/CD8 expression by an individual SCID thymocyte was not dependent on that cell itself being TcR^+, but rather expression of CD4 and CD8 by SCID thymocytes appeared to be dependent upon the presence of TcR^+ cells within that same thymus.

Introduction of TcR^+ Cells into SCID Thymi

That the emergence of $CD4^+/CD8^+$ thymocytes is dependent upon the the intrathymic presence of TcR^+ cells was confirmed by an independent experimental approach in which we introduced normal AKR bone marrow cells (which eventually give rise to intrathymic TcR^+ cells) into TcR^- SCID mice. Since thymocytes of AKR origin express the Thy1.1 and Lyt2.1 alleles, thymocytes of exogenous AKR origin within the SCID thymus are distinguishable from endogenous SCID thymocytes that express the Thy1.2 and Lyt2.2 alleles.

We examined both the AKR and SCID thymocytes populating the SCID thymi in 37 AKR->SCID mice. The thymi from such mice contained, on the average, $41x10^6$ cells of which 85% were of AKR origin. The percentage of SCID thymocytes in the chimeric thymi was highly variable, ranging between 1% and 95% with a mean value of 15%. However, in all of these chimeras, the SCID thymus was found to support the normal differentiation of the exogenous $Thy1.2^-$ AKR thymocytes since the expected subsets of AKR thymocytes ($CD4^-8^-$, $CD4^+8^+$, $CD4^+8^-$, $CD4^-8^+$and TcR^+ cells) were present in the chimeric animals (data not shown). Yet, despite the presence of TcR^+ AKR thymocytes in the SCID thymus, the $Thy1.2^+$ SCID thymocytes remained TcR^-, indicating that the presence of the normal exogenous AKR thymocytes did not correct the scid genetic defect (Table 3). However, unlike unmanipulated TcR^- SCID thymi, SCID thymi that possessed TcR^+ AKR thymocytes clearly possessed SCID $Thy1.2^+$ thymocytes which expressed the SCID allele of CD8 (Lyt2.2) (Table 3). Moreover, CD4 was also expressed on a subpopulation of SCID $CD8^+$ thymocytes (Table 3). These results demonstrate that the addition of normal bone marrow stem

cells, able to give rise to TcR^+ thymocytes, can promote the differentiation of $CD4^-8^-$ SCID thymocytes into $CD4^+/CD8^+$ thymocytes.

Table 3. Cell surface phenotype of SCID thymocytes from AKR->SCID chimeras*.

	BALB/c	AKR->SCID Chimera:		
		#1	#2	#3
% $Thy1.2^+$ Thymocytes	99	20	95	2
% $Thy\ 1.2^+$ Cells that are:				
$Thy1.1^-CD3^+$	35	<1	<1	<1
$Thy1.1^-Lyt2.2^+$	93	84	99	4
$Lyt2.2^+CD4^+$	95	62	99	92

*Results of 3 color flow cytometry in which $Thy1.2^+$ SCID thymocytes were assessed for expression of CD3, Lyt2.2, and CD4.

Thus, the present study suggests that the failure of $CD4^+/CD8^+$ to arise in TcR^- SCID mice is due to the lack of critical differentiation signals, that in normal mice presumably would be supplied be TcR^+ thymocytes. In this regard our data demonstrates that $CD4^-8^-$ TcR^- SCID thymocytes can be released from their developmental arrest and can be induced to differentiate into $CD4^+/CD8^+$ TcR^- cells either in the presence of TcR^+ SCID thymocytes that fortuitously arise in rare ("leaky") SCID mice or by the transfer of normal bone marrow stem cells that ultimately give rise to TcR^+ thymocytes.

Implications of Current Findings for Normal T Cell Ontogeny

On a broader level, we would like to suggest that these findings have important implications for the process of normal thymocyte differentiation. During ontogeny, the earliest arising TcR^+ thymocytes distinctively express the ($\gamma\delta$) form of the TcR (Bluestone et al., 1987; Pardoll, et al., 1987). Interestingly, these cells do not express either CD4 or CD8, and develop prior to the emergence of $TcR(\alpha\beta)^+$ thymocytes (Bluestone et al., 1987, Pardoll et al., 1987). The role of these cells is controversial but our data indicate that these cells may play a critical function in promoting the entry of immature $CD4^-8^-$ cells into the $CD4^+/CD8^+/TcR(\alpha\beta)^+$ lineage of thymocytes. Several mechanisms might be evoked to explain how these $TcR(\gamma\delta)$, once activated, might regulate thymocyte differentiation. One manner in which these cells might exert their influence on thymocyte maturation, would be through the release of cytokines, which could directly interact with $CD4^-8^-TcR^-$ thymocytes and promote their differentiation into $CD4^+8^+$ cells. On the other hand, activated $TcR(\gamma\delta)$ cells might serve to activate the thymic stroma, leading to the release of release factors from the thymic stroma, which would ultimately be responsible for promoting the entry of $CD4^-8^-TcR^-$ thymocytes into the CD4/CD8 differentiation pathway. The recent finding that $TcR(\gamma\delta)$ cells can recognize Class I determinants and release a variety of cytokines is consistent with either of these mechanisms (Matis et al., 1987; Bluestone et al., 1988, Marusic-Galesic, et al., 1988; Tentori, et al., 1988). Moreover, in recent

immunohistologic staining studies with antibodies able to distinguish different thymic stromal cells (ER-TR antibodies: Van Vliet, et al., 1984), we found that the thymic stroma of TcR^- SCID mice was not fully mature (W. Van Ewijk, personal communication). Interestingly, this aberrant thymic development was reversed in AKR->SCID mice, suggesting that the presence of normal TcR^+ cells is essential to the maturation of the thymic stroma. Thus, we would propose that the reason TcR^- SCID thymocytes fail to progress beyond the $CD4^-8^-$ stage of differentiation is because they are deprived of critical signals produced by TcR^+ cells.

CONCLUSION

In conclusion, these findings demonstrate that although thymocytes from unmanipulated TcR^- SCID mice are arrested in their development, they can be induced to differentiate in the presence of endogenous or exogenous TcR^+ thymocytes. Finally these studies have important implications for normal thymocyte maturation and the function of the early arising $TcR(\gamma\delta)$ thymocytes.

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Costimulatory Interactions Determine Proliferation Versus Induction of Clonal Anergy During Type I CD4 + T-Cell Stimulation

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INTRODUCTION

Work in our laboratory has recently focused on two aspects of T-cell responsiveness: a) the biochemical signalling events occurring upon antigen stimulation that are responsible for the initiation of lymphokine secretion and cell division, and b) the ability of a partial subset of these signals to induce an alternative pattern of activation leading to clonal anergy. Beyond the potential benefits to the understanding of T-cell physiology, this research has established an *in vitro* model of T-cell tolerance that may have important correlates to the maintenance of self-tolerance *in vivo*. Discovery of the signalling events that determine the destiny of a T cell encountering antigen (either self or non-self) may both provide clues to the etiology of autoimmune or immunodeficiency diseases as well as provide a basis for the specific suppression of responses to transplanted tissues.

Clonal anergy or *unresponsiveness* in a Type I CD4+ T-cell clone is best characterized as an inability to proliferate in response to antigen due to a failure to produce interleukin 2 (IL-2) (Jenkins and Schwartz 1987, Jenkins et al. 1987; Quill and Schwartz 1987). T cells enter this unresponsive state following exposure to antigen in the absence of an accessory cell-derived costimulatory signal. Such stimulation can occur during incubation of T cells with peptide antigen in the presence of paraformaldehyde or 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (ECDI) fixed syngeneic antigen-presenting cells (APC). In these cultures, the fixed APC remained capable of presenting the antigen and occupying the T-cell antigen receptor (TCR); however, fixation of the APC destroyed their ability to provide a costimulatory signal. Evidence that costimulation represents an activity independent of T-cell receptor occupancy was confirmed by the observation that normal accessory cells (that fail to bear the relevant Ia molecule), when added to these cultures, are capable of preventing the induction of the unresponsive state (Jenkins et al. 1988, Ashwell et al. 1988). We have since determined that stimulation of these T cells with concanavalin A (Con A) or an anti-CD3 monoclonal antibody in the absence of a potent source of costimulatory signals also results in the development of the anergic state (Mueller et al. 1989, Jenkins et al. 1989).

BIOCHEMISTRY DURING EARLY ACTIVATION OF TYPE I CD4+ T CELLS

An examination of biochemical activation events during normal stimulation versus stimulation in the absence of good costimulation might be expected to uncover the identity of the costimulatory pathway, as well as establish those biochemical pathways necessary for the induction of the unresponsive state. Therefore, we undertook a series of experiments to characterize the activity of several well-defined biochemical pathways following activation of our T cells.

Table 1. Alternative Responses to T-Cell Stimulation

Events Associated with Activation	*Biological Response Induced*	
	Clonal Expansion	*Clonal Inactivation*
inositol phosphate generation	++	++
mobilization of calcium ions	++	++
diacylglycerol production	ND[1]	++
CD3-gamma serine phosphorylation	++	++
CD3-zeta tyrosine phosphorylation	+	+
blastogenesis	++	++
IL-2 production	++	-
IL-3/GM-CSF production	++	+
IFN-gamma production	++	+
increased IL-2R p55 expression	++	+
proliferation	++	-

[1] not determined

Table I is a summary of our results (Jenkins and Schwartz 1987, Jenkins et al. 1987, 1988, 1989; Mueller et al. 1989). During stimulation with antigen in the presence of effective accessory cells, T cells generated free inositol phosphates (IP) including 1,4,5-inositol trisphosphate within several minutes. In addition, we observed increases in intracellular free calcium ions ($[Ca^{2+}]_i$) in these clones upon stimulation with antigen and syngeneic APC. We also looked at phosphorylation of the CD3 complex for evidence of protein kinase activity following stimulation and found substantial antigen-induced gamma chain phosphorylation, as well as a modest degree of zeta chain phosphorylation, consistent with the activation of both protein kinase C (PKC) and a protein tyrosine kinase, respectively, following T-cell stimulation.

Using these same assays we have also examined T cells stimulated in the absence of a good source of costimulatory activity (Table 1). Under these conditions, we can demonstrate that the T cells become functionally inactivated. Unfortunately, our experiments did not define the costimulatory signalling pathway; however, a number of interesting observations were made regarding the role of calcium and PKC in the regulation of IL-2 production. We initially observed that stimulation of T cells with peptide antigen and ECDI-fixed APC induced a rise in $[Ca^{+2}]_i$ in a significant proportion of T cells. Surprisingly, assays for IP production under similar conditions demonstrated a significant defect in the fixed APC. It became clear later, however, that fixation of the APC results in a reduction in the IP generation in part because of a defect in conjugate formation, because sedimentation of the culture into a pellet at the initiation of stimulation significantly enhanced IP production. Such a defect is not inconsistent with the calcium data as the calcium measurements were performed by flow cytometry only on T cells in a conjugate with an APC. Taking into account this deficiency in conjugate formation, there still remained a defect in antigen presentation by fixed APC (as measured by IP generation). Because this defect could be overcome with the addition of more antigen, it seemed likely that it was related to the destruction of MHC molecules by the ECDI treatment. The functional requirement for costimulation, on the other hand, could not be overcome by the addition of more antigen. Thus, the costimulatory signalling pathway does not impinge directly upon the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2).

Several other pieces of evidence support this conclusion. Stimulation with Con A or anti-CD3 antibody in the absence of accessory cells induced significant IP production (as well as a rise in $[Ca^{+2}]_i$; unpublished observations, D.L.M.). Furthermore, the addition of costimulators during the Con A incubation had no effect on the IP response, although the addition was required to induce a strong proliferative response. Finally, the addition of normal allogeneic accessory cells (incapable of occupying the TCR for lack of the appropriate Ia molecule) during the incubation with the peptide antigen and fixed syngeneic APC also had no effect on the IP response, although proliferation was now induced. Taken together, these results suggest that the loss of costimulatory activity following APC fixation is a defect independent of their reduced ability to occupy the TCR.

We additionally examined protein kinase activities during the induction of anergy. Gamma chain phosphorylation occurred in the absence of normal accessory cells, and its level correlated well with the level of IP production observed under similar conditions. Again, the addition of normal accessory cells with their costimulatory activity had no effect on the level of PKC activity achieved with stimulation. Finally, the activity of the protein tyrosine kinase responsible for zeta chain phosphorylation (assayed at 45 minutes) was increased following incubation with Con A in the absence of accessory cells, and its activity was unaffected by accessory cell addition.

Overall, our experiments have documented that the hydrolysis of PIP_2 with production of IP, and mobilization of intracellular calcium ions, as well as the activation of PKC occurs following stimulation of T cells with antigen in either the presence or absence of the costimulatory signal. During the induction of anergy, the IP-initiated increase in $[Ca^{+2}]_i$ appears to be important. EGTA blocks the induction ; furthermore, treatment of purified T cells with the calcium ionophore, ionomycin, induces the unresponsive state at doses which raise $[Ca^{2+}]_i$ to levels normally observed during full activation (Jenkins et al. 1987). Since the induction of clonal anergy can be inhibited by cycloheximide (Quill and Schwartz 1987), the results suggest that increases in intracellular calcium concentration unaccompanied by the costimulatory signal initiate the synthesis of a protein capable of inhibiting subsequent IL-2 production in response to TCR occupancy. One can also conclude from these data that TCR-mediated mobilization of calcium ions and PKC activation alone are insufficient signals to discriminate between the two functional outcomes of T-cell stimulation, namely, IL-2 gene activation and clonal expansion versus a failure to induce IL-2 production and functional inactivation of the clone. It is the costimulatory signal which appears to provide the information required by the cell to choose between these fates.

COSTIMULATORY INTERACTIONS DURING INITIATION OF T CELL PROLIFERATION

Antigen Responses

Previous work has established that the costimulatory signal must be delivered at close range, possibly through contact between the T cell and the costimulator (Jenkins et al. 1988). Therefore, its delivery should be sensitive to the density of the cells providing the signal during the course of an immune response. We have utilized this characteristic to examine the independence of the costimulatory activity from the effects of TCR occupancy in a number of experiments. In Table 2 we show the relationship between receptor occupancy, the delivery of the costimulatory signal, and the development of an IL-2 dependent proliferative response in cultures of varying APC density (Mueller et al. 1989). In the presence of an abundance of APC (syngeneic APC:T cell ratio of *10:1*), stimulation of the T cells with the lowest dose of antigen capable of eliciting the maximal proliferative response (antigen concentration of *1X*) resulted in only low IP production (13% of the maximum). Increased concentrations of antigen (*100X*) induced significantly greater rates of IP generation and increased production of IL-2, but proliferation remained on plateau and did not improve. With the antigen concentration held constant at *100X*, a reduction in the number of accessory cells in the culture to a syngeneic APC:T cell ratio of *1:1* resulted in a depression of all the parameters tested (as expected); however, the relationship between the different assays changed. Proliferation fell to 44% of the maximal response, due to a significant reduction in the production of IL-2 (now undetectable). Simultaneously, the level of IP production decreased to 41%,

consistent with a significant reduction in the level of TCR signalling associated with the decrease in APC. Is it surprising that the proliferative response was significantly reduced despite the reasonably high levels of IP production that persisted (given that we showed in this experiment that maximal proliferation can occur in association with very small levels of IP production)? No, because a reduction in the syngeneic APC number does not result simply in a decrease in TCR occupancy, but also in a reduction in costimulation. In support of this, the addition of a high density of allogeneic accessory cells resulted in increased IL-2 production and the proliferative response again rose to the plateau level, without affecting the level of IP generated (Table 2). We suggest that these additional accessory cells provide an influx of costimulatory activity necessary to achieve the improved response. These experiments demonstrate that the quantity of costimulatory activity present during an immune reaction can have an important effect on the level of T-cell proliferation realized in the response.

Table 2. Relationship between IP generation, proliferation, and IL-2 production

Stimulation		*T-cell Response*		
Antigen concentration [1]	*APC:T cell ratio* [2]	*Total IP %max* [3]	*Proliferation %max* [4]	*IL-2 %max* [5]
1X	*10:1 syngeneic*	*13*	*96*	*2*
100X	*10:1 syngeneic*	*100*	*90*	*100*
100X	*1:1 syngeneic*	*41*	*44*	*0*
100X	*1:1 syngeneic+ 10:1 allogeneic*	*30*	*101*	*9*

[1]peptide antigen DASP at 0.1 μM used as 1X concentration.
[2]syngeneic B10.A or allogeneic B10 T-depleted spleen cells used as accessory cells; T cell was A.E7 at 5 x 10^4 per culture for proliferation and IL-2 production; 5 x 10^5 for inositol phosphate assay.
[3]Maximum IP generation 3,209 cpm at 10 μM DASP (background 72 cpm) at 30 minutes of culture.
[4]Maximum proliferation 194,350 cpm at 1 μM DASP (background 469 cpm) at 64 hours of culture.
[5]Maximum CTLL proliferation (to a 50% A.E7 supernatant taken at 20 hours of culture) 22,455 cpm at 10 μM DASP stimulation of A.E7 (background 257 cpm). Data compiled from a separate experiment.

Mitogen Responses

TCR occupancy alone cannot generate the signals necessary to fully activate the IL-2 gene and induce T-cell proliferation, despite the observed increases in $[Ca^{+2}]_i$ and PKC activity. Yet, treatment of carefully purified T cells with ionomycin and phorbol 12-myristate 13-acetate (PMA) results in a high level of T cell proliferation. Likewise, stimulation with anti-CD3 antibody coated to a plate almost invariably induces some IL-2 production and proliferation. Finally, an occasional proliferative response to Con A can also be observed with these purified cells.

Several recent experiments have suggested that even these "accessory cell-independent" responses are dependent on the delivery of a costimulatory signal through a cell-cell interaction (Mueller et al. 1989a). First, enhanced proliferation can always be demonstrated with the addition of accessory cells, suggesting a synergistic effect of costimulation. Furthermore, the addition of paraformaldehyde-fixed spleen cells to cultures of purified T cells stimulated with ionomycin and PMA markedly reduces the proliferation induced by the chemicals, suggesting a sort of "cold-target inhibition" in which the fixed cells with poor costimulatory activity compete for interactions with the T cells and effectively inhibit the delivery of the costimulatory signal by non-fixed cells. Finally, our attempts to induce single T cells to divide in response to ionomycin and PMA have always failed, despite the demonstration that these cells are capable of dividing upon the addition of exogenous IL-2.

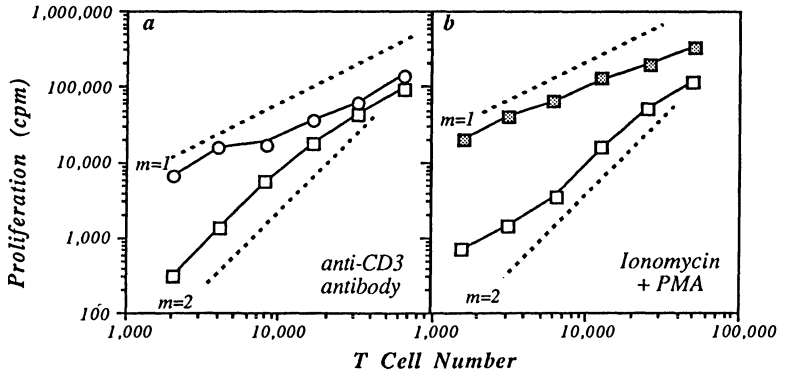

Fig. 1. Cell-cell interactions are required for proliferation. Titration of the purified T cell clone A.E7 (see Mueller et al. 1989 for details of cell preparations) in the presence of either *anti*-CD3 monoclonal antibody 145-2C11 (1 μg/ml coated to the plate) or *b*) ionomycin 0.5 μM plus PMA 10 ng/ml. Additions to the T cells were none (□), or rIL-2 200 u/ml (○), or 10^4 accessory cells (B cell tumor line LS 102.9; ▨). Theoretical slopes of 1 and 2 are shown.

To test the requirement for cell-cell interactions in several of these responses we utilized a system of T-cell titrations. In this experiment, T cells are added at varying densities and stimulated with either ionomycin and PMA or anti-CD3 antibody (coated to the plate). At low T-cell density, one can expect proliferation to these stimuli to be directly proportional to the T-cell number only if the response is independent of a cell-cell interaction within the culture. If, on the other hand, the response of the T cells is dependent on cells interacting to deliver a costimulatory signal, low density cultures should demonstrate depressed proliferation secondary to a reduction in the frequency of such interactions. Since the frequency of a cell-cell interaction will vary as the square of the T-cell number, plotting the log of the T-cell number versus the log of the thymidine incorporation (cpm) would reveal a slope of 2; responses independent of cell interactions would show a slope of 1 (Coppleson and Michie 1966).

Figure 1*a* demonstrates the effect of T-cell density on proliferation of purified T cells to anti-CD3 monoclonal antibody coated to the bottom of a flat-bottom microwell. Low density T cells respond poorly to anti-CD3 stimulation, presumably as a result of low IL-2 production, because the addition of exogenous IL-2 significantly enhances the response. Note that the slope of the plot approaches 2, while proliferation in the presence of exogenous IL-2 has a slope of approximately 1. Similar results are obtained with ionomycin and PMA stimulation (Fig. 1*b*). Titration of the T cells in the presence of a constant density of the B cell tumor line LS 102.9, however, results in a slope of about 1, and demonstrates that satisfying the requirement for an interaction with an accessory cell allows a maximal response.

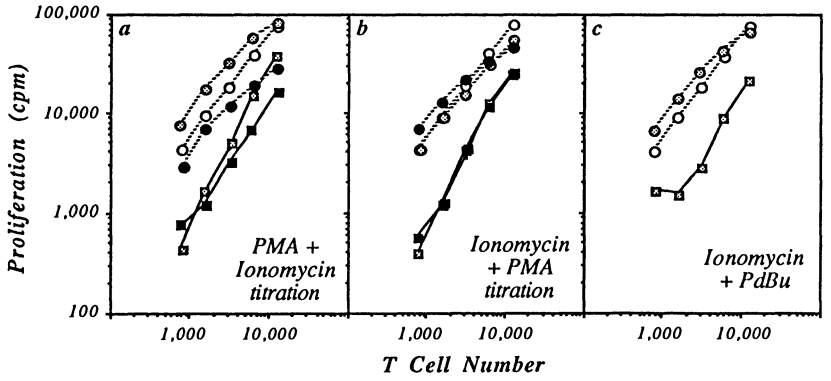

Fig. 2. Titrations of ionomycin and phorbol ester. Proliferation of A.E7 in either the presence (circles) or absence (squares) of rIL-2 50 u/ml. Open circles (o) represent rIL-2 alone. *a*) PMA 10 ng/ml plus ionomycin 0.25 μM (o □) or 1 μM (● ■). *b*) Ionomycin 0.5 μM plus PMA 3 ng/ml (o □) or 30 ng/ml (● ■). *c*) Ionomycin 0.5 μM and phorbol dibutyrate (PdBu) 10 ng/ml (o □).

Further experiments were carried out to examine this failure of ionomycin and PMA treatment to induce proliferation independent of costimulatory interactions. Titrations of the ionomycin in the presence of PMA (Fig. 2*a*) resulted in a density dependence to the proliferation at all the doses tested; reducing the concentration of ionomycin did not improve the response at low T-cell density. Comparisons of the proliferation observed in the presence of exogenous IL-2 did document inhibitory effects of the ionomycin with increasing dose, but this seemed to be most apparent at higher T-cell densities. In fact, the addition of 0.25 μM ionomycin and PMA at low T-cell density enhanced the response to IL-2. Titration of the PMA (Fig. 2*b*) revealed that the IL-2 response enhancement at low T-cell density was a function of the PMA concentration. Again, varying the PMA concentration had no effect on the density-dependence of the ionomycin- and PMA-induced proliferation. Finally, substitution of phorbol dibutyrate (PdBu) for PMA (Fig. 2*c*) did not change the result; proliferation still required a cell-cell interaction.

These experiments demonstrate that the density effect is not simply toxicity related to T cells growing at low density. The results also appear to rule out a simple nutritive effect at low density as it would seem unlikely that both purified accessory cells and IL-2 could substitute for a nutritive factor found in excess at high cell density, but deficient at low density. They instead suggest that a cell interaction is required prior to proliferation in response to anti-CD3 or ionomycin and PMA. The identity of the cell providing the costimulation in these responses (in the absence of added accessory cells) is not yet certain. We are in the process of growing out the T cell clone in the absence of APC (from limiting dilution) and plan to assess their ability to proliferate in the absence of accessory cells. One piece of data suggesting that it may be a T cell-T cell interaction responsible for the delivery of the costimulatory signal, rather than the effects of a contaminating accessory cell, is the finding that the Type II CD4+ T cell clone D10.G4 (cultivated in the complete absence of accessory cells) appears capable of enhancing the proliferative response of the Type I clone A.E7 to Con A or anti-CD3 antibody (unpublished observation, D.L.M.). The effect is independent of IL-4 and IL-1 and appears to be a costimulatory signal from the Type II cell to the Type I cell. Thus, at least the Type II T-cell population appears capable of expressing some costimulatory activity upon activation.

CONCLUSION

Type I CD4+ T cells require a costimulatory signal to proliferate in response to antigen; furthermore, stimulation in the absence of this signal induces anergy to later stimulation. The biochemical data show no role for the costimulatory signal in either the rise in $[Ca^{2+}]_i$ or PKC activation observed following stimulation of the TCR. Taken together, the data suggest that an increase in $[Ca^{+2}]_i$ and PKC activation are insufficient biochemical events to fully activate the IL-2 gene. The costimulatory signal must utilize a unique signal transduction pathway acting in

parallel with the activation events occurring subsequent to TCR occupancy to induce the production of IL-2 and expansion of the clone, as well as prevent the induction of unresponsiveness. This effect of costimulation on the proliferative potential of a T cell suggests a regulatory role for antigen-presenting cells in determining the outcome of TCR occupancy.

It is possible that similar mechanisms could operate in the development and/or maintenance of self-tolerance. Self-antigens associated with the MHC of poor costimulators (e.g. epithelial elements that express Ia) might induce anergy in the periphery rather than clonal expansion (Stein and Stadecker 1987; Gaspari et al. 1988; Markmann et al. 1988) Limiting delivery of costimulatory signals to situations where bone marrow-derived cells play a role in antigen presentation could provide one means of controlling responses to self-antigens not expressed in the thymus. On the other hand, interference with the delivery of costimulatory signals, e.g. by suppressor cells, could constitute a form of feed-back inhibition capable of regulating responses to self-antigens expressed on good costimulators (Salgame et al. 1989). Finally, tolerance induction to self-antigen by clonal deletion in the thymus is likely to proceed through the same calcium pathway that is responsible for the induction of clonal anergy. In this case the immature T cell might be deleted because it lacks the receptor for the costimulatory signal, and only the TCR-mediated signalling events take place upon encountering self-antigen on thymic APC. Such TCR signalling has been shown to induce calcium-dependent apoptosis in thymocytes (Smith et al. 1989).

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Signal Requirements for the Primary Activation of Murine CD8 T Lymphocytes

H. Wagner and K. Heeg

INTRODUCTION

Primary activation of resting murine T cells consists of two distinctly regulated phases termed competence and progression. Competence signals are delivered to resting T cells by antigen presented via professional antigen presenting cells (APC) such as dendritic cells (DC), thereby inducing receptors for a variety of polypeptides often referred to as lymphokines. Binding of autocrine or paracrine secreted growth promoting lymphokines then causes progression, i.e. growth and differentiation into effector T lymphocytes.

Lymphokine production is a property of activated T cells, the principal producers being the CD4 helper/inducer T cell subset (TH). The induction of lymphokine gene transcription and subsequent secretion depends upon T cell receptor (TCR) mediated T cell activation, and it is this signal pathway which appears to be blocked by the immunsuppressive drug cyclosporin A (Bunjes et al, 1981; Krönke et al, 1984). Cloned murine CD4 T cell lines produce given combinations of lymphokines: TH-1 cells secrete Il-2, IFN-y and lymphotoxin (LT), while TH-2 cells produce Il-4, Il-5 and Il-6 (Cherwinski et al, 1987). There is now good evidence that each lymphokine exerts multiple functions (pleiotropy) dependant on the respective target cell. It becomes also clear, that there exists redundancy, i.e. more than one lymphokine may mediate identical functions (Mosmann and Coffmann, (1987).

In view of this complexicity of lymphokine biology, and in view of the possibility that inbetween the areas of cell contact between APC's and antigenreactive T cells high concentrations of lymphokines may accumulate not yet accesible for detection, we ressorted to a simplicistic model system to analyse the signal requirements for the primary activation of CD8 T responder cells. First we used solid phase anti TCR monocl. antibodies (Mab) to efficiently initiate TCR mediated activation via crosslinking of TCR structures. Physiologically, TCR recognise conjugates of

antigencic peptides and MHC molecules which probably cause weak TCR-crosslinking. Therefore additional inductive signals are necessary, as provided by interaction of accessory molecules such as CD4, CD8 and CD11 with their respective ligands. Our approach enabled us to bypass this aspect of APC-T responder cell interaction and thus to concentrate on the activation requirements of one cell type, i.e. resting CD8 responder T cell. In addition, we plated resting CD8 T cells at limiting dilution, or at least in low cell density cultures. Thus the liklihood of contaminating cells and/or heterogeneous activation states within the limited number of purified CD8 T cells became reduced. Note that at low cell densities TCR crosslinking of CD8 T cells does not yield in dedectable lymphokine gene transcription and secretion. It follows, that under these conditions the cascade of activation events ought to be strictly dependant on the availability of lymphokines added from without.

Here we discuss (1), that in cultures devoid of APC's, Il-6 causes Il-2 responsiveness of Il-2 R^+ CD8 T cells. We show, that Il-4 promotes clonal growth of CD8 T cells equally well as Il-2. In synergy both growth pathways bypass the immunsuppresive effect of cyclosporin A. Finally we provide evidence, that at the clonal level resting CD8 T cells have the option to produce lymphokines such as Il-2, and/or to be cytolytic; the decision being dependant on the nature of the T cell stimuli.

TECHNICAL ASPECTS

Resting responder CD8 T cells were prepared in a three step protocoll from nylon-wool nonadherent lymph node cells subjected (1) to negative selection by treatment with a cocktail of anti-IA, anti-IE and anti-L3T4 Mab plus complement, (2) a selection on the basis of cell density, and (3) out of the high-density (ϱ = > 1.076 kg/l) cells CD8 T cells were positive selected using an Epics V cell sorter. Anti-Vß8 1, 2, 3 Mab F 23.1 or anti-CD3 Mab (hybridoma 145-2 C11) were purified from ascites and covalently coupled to CNBr-activated sepharose 4 B (Pharmacia) (0,44 mg protein/ml to 0,1 g beads). Human rec. Il-2 was provided by Biogen, Geneve, murine rec. Il-4, human rec. Il-1-α , rec. Il-1-ß and rec. Il-6 was from Immunex Corp. Seattle. The culture conditions have been described (Schmidtberger et al, 1988, Miethke et al, 1988).

RESULTS AND DISKUSSION

(1) Interleukin 6 (Il-6) acts as competence factor by promoting sensitivity to Il-2.

The ability of Il-2 to promote cell growth (clonal expansion) of CD8 T cells expressing high affinity Il-2 receptors (Il-2 R) is well recognised. It also appears to be accepted, that triggering of TCR mediated signalling via APC's, mitogens, or anti-TCR solid phase Mab directly causes expression of high affinity Il-2 R which in turn conveys responsiveness to Il-2 (Smith, 1984). Our results oppose this view. Using the mitogen Con A, or anti-TCR solid phase Mab to trigger under limiting cell densities resting CD8 T cells, we have defined a state of preactivation in which responder CD8 T cells can express Il-2 R, but are still refractory to Il-2. Responsiveness is brought about by an accessory factor initially semipurified (Hardt et al, 1987) from the macrophage cell line P 388 D-1 and operationally termed Il-2 R inducing factor (RIF) (Hardt et al, 1985). None of the purified rec. lymphokines available such as Il-1, Il-3, Il-4 IFN-y and TNF-α exhibited RIF activity. With one notable exception, i.e. Il-6. As it turns out (Miethke et al, 1989) it is Il-6 which confers Il-2 responsiveness to resting CD8 T cells triggered by crosslinking of their TCR-structures. In addition anti Il-6 Mab neutralise RIF activity within supernatants of the macrophage cells P 388 D-1. Low concentrations (0.1 - 1.0 ng/ml) of rec. Il-6 already exhibit maximal activity, and its function is restricted to the initiation phase of the culture. In the presence of Il-2 the ensuing cell proliferation is entirely Il-2 driven, i.e. can be abolished by anti Il-2 R Mab. Interestingly, when resting CD8 T cells are triggered by solid phase anti TCR Mab, the expression of Il-2 R becomes greatly upregulated by rec. Il-2 added. One can positivly select such Il-R^+ CD8 T cells and yet these Il-2 R^+ CD8 T cells remain refractory to the growth promoting effect of Il-2, unless Il-6 has been added within the first 24^h of the culture. Previous studies had demonstrated that Il-6 restors the proliferation of accessory cell depleted mouse T cells in response to the lectin Con A (Uytenhove et al, 1988). Since anti Il-2 R Mab blocked cell proliferation, and since the proliferating cells produced Il-2 it was concluded that the prime function of Il-6 is to cause Il-2 gene transcription with subsequent secretion of this growth factor (Garman et al, 1987). While in our model system we confirmed these results only in cultures plated at high cell densities ($>$ x 10^4 cells per culture), in low cell density cultures ($<$ x 10^3 cells per culture) the function of Il-6 was only to act as competence factor; i.e. to convey Il-2 responsiveness to those preactivated CD8 T cells, which had been triggered by anti TCR Mab.

(2) Synergy between Il-4 and Il-2 conveys resistance to cyclosporin A during primary in vitro activation of murine CD8 cytotoxic T cell (CTL) precursors.

Originally described as lymphokine acting on B cells, Il-4 is now recognised to exert pleiotropic effects on a variety of cells of the haemopoetic lineage (Paul and Ohara, 1987). As with resting CD8 T cells we observed, in line with others (Widmer and Grabstein, 1987), that Il-4 acts as autonomous growth factor, the signal pathway of which being independant to that used by Il-2 (Miethke et al, 1988). However at the cellular level both progression pathways appear to be interconnected (Bubeck et al, 1989, Heeg et al, 1989). First, in combination Il-4 and Il-2 greatly augment the proliferative and differentiative (cytolytic) responses generated. Second, at low concentrations Il-4 synergizes with Il-2. Third, Il-4 inhibits the decay of Il-2 responsiveness, presumably by upregulating the expression of high-affine Il-2 R. Interestingly, Il-4 acts only as growth factor upon continuous crosslinking of TCR structures via solid phase Mab or mitogens. Upon primary activation of class I MHC-reactive CD8 CTL-precursors in C57BL.6 anti-bm 1 murine mixed lymphocyte reactions (MLR), Il-4 not only induces cell growth but selectively augments in a dose dependent fashion the lytic activity of differentiating CTL. The differentiative action of Il-4 acts late and within 24^{h} prior to the 51 Cr-cytotoxicity assay. Thus besides its action as growth factor one of the pleiotropic effects of Il-4 is to function as cytotoxic T cell differentiation factor (CTDF).

The immunsuppressive drug cyclosporin A (CsA) affects primary T cell activation by blocking TCR mediated signalling of lymphokine gene transcription such as that of Il-2 and of Il-4. In addition CsA blocks not the expression of Il-2 R but the acquisition of sensitivity to Il-2 and Il-4 during primary activation of antigen reactive, resting CD8 T cells (Heeg et al, 1988, Bubeck et al, 1989). As mentioned, the Il-4 and Il-2 dependent signal pathways synergise with each other and thus appear to be connected intracellularly. This might explain our unexpected finding, that in the presence of Il-2 and Il-4, the CsA mediated immune suppression is selective in the sense that highly cytotoxic CD8 T cell become generated in primary MLR's in the virtual absence of cell proliferation (Heeg et al, 1988, Bubeck et al, 1989). Obviously, cell growth (clonal expansion) appears to be CsA sensitive, while the Il-4 driven differentiation of antigen specific CTL is CsA resistant.

(3) Lymphokine secretion and expression of cytolytic activity by CD8 cells.

Even though activated CD4 T cells are the prime source of lymphokines, it is clear, that also activated antigen reactive CD8 T cells produce lymphokines such as Il-2. (Singer et al, 1987, Heeg et al, 1987). In analysing clonally developing CD8 T cells responding either to Con A, allogencic class I MHC antigens, or to TNP conjugated syngencic MHC antigens, we observed a clear cut segregation of those colonies producing Il-2 from those exhibiting cytolytic activity (Heeg et al, 1987, Schmitt et al, 1988). In fact, both functions appeared to exclude each other; bifunctional clones being the exception. However an exception was noted. If resting CD8 T cells were first induced with the mitogen Con A, and subsequently clonal growth was sustained in the absence of Con A, than the great majority of clonally developing colonies were bifunctional, i.e. secreted Il-2 and exhibited cytolytic functions as well. Yet, upon addition of Con A in the second step culture, and subsequent clonal expansion by growth factors such as Il-2 clonal segregation of both functions reappeared. Obviously resting CD8 T cells have the potential to be bifunctional, and it is the nature of the T cell stimuli used for activation which determines the set of final functions expressed (to be published).

In conclusion, the results discussed point out, that during primary activation of murine CD8 T cells

(1) Il-6 primarily acts as competence factor by conveying Il-2 reponsiveness to Il-2 R^+ CD8 T cells

(2) both Il-2 and Il-4 function as growth factors, the CTDF function of the latter being remarkable

(3) it is the nature of T cell stimulus, which determines the set of functions (Il-2 secretion versus cytolytic potential) in clonally developing CD8 T cells.

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Contact with Helper T Cells Does Not Limit the Induction of Resting B Cell Differentiation

M. H. Julius and K. Louste

INTRODUCTION

The induction of an antigen specific, primary, humoral immune response, in vivo, likely requires that helper T lymphocytes contact resting B cells (Sprent 1978a; Sprent 1978b). This cellular interaction is mediated by three classes of polymorphic molecules: the T cell receptor for antigen (TCR), the B cell receptor for antigen (mIg), and molecules encoded by the major histocompatibility complex (MHC) of the B cell. The function of T helper cells is restricted by the MHC of the B cell (Andersson et al. 1980; Jones and Janeway 1981; Julius et al. 1982a; Julius et al. 1982b), that is, the ligand for TCR is a composite of an antigen derived peptide and the polymorphic region of MHC class II (or I) expressed on the B cell surface. Therefore, the specificity of humoral immune responses is preserved through selection of B cells by native antigen.

Ligation and crosslinking of mIg using antibodies or antigen results in a series of progressive activation signals which in themselves are not sufficient for the induction of B cell differentiation (Cambier and Ransom 1987; Cambier et al. 1987). Concomitantly, mIg mediates the internalization of native antigen, resulting in its partial degradation within endosomes. It is likely that the peptides generated are ultimately associated with newly synthesized intracellular MHC molecules destined for the B cell plasma membrane (Berzofsky et al. 1988). The B cell, which now expresses membrane associated determinants which satisfy TCR specificity, can focus the interaction with T helper cells. The combined effect of signals generated through mIg crosslinking and those resulting from interaction with T cells is the induction of B cell growth and differentiation.

Model systems established in vitro have enabled a further parsing of the events and their sequence which comprise T cell dependent B cell activation. Primarily, studies have focused on establishing whether the membrane molecules which have evolved to mediate antigen specific T-B interaction are also transducers of obligatory cellular activation signals. Using antigen derivitized antibody fragments, "bridge molecules" can be generated which allow conjugate formation between histoincompatible T and B cells, in vitro. Depending on the specificity of antibody used in the bridge, the effects of varying combinations of membrane molecules on the respective lymphocytes which are included/excluded in the conjugate formation can be assessed. The data from a number of laboratories suggest that neither mIg ligation, nor the interaction of TCR with MHC class II/antigen composites on the B cell surface are required for the induction of a resting B cell to synthesize DNA or to differentiate into a high rate immunoglobulin secreting cell (Owens 1988; Julius and Rammenssee 1988a; Julius et al.

1988b; Thompson et al. 1985; Leclercq et al. 1987). The obligatory requirement was found to be crosslinking of TCR, which minimally, results in lymphokine production. In addition, with two exceptions (Leclercq et al. 1987; Leclercq et al. 1984), reported studies indicate that the histoincomptable T and B cells needed to be co-cultured.

Two potentially alternative explanations for the necessity of co-culturing T and B cells are immediately obvious. Either the interaction action of non polymorphic determinants on the respective lymphocytes provide essential activation signals, or limited production of T cell derived soluble mediators of B cell activation require that the cells be in close proximity. The results presented here support the latter.

RESULTS AND DISCUSSION

The following series of experiments were designed to determine whether contact between histoincompatible Th and resting B cells is necessary for the induction of B cell differentiation to high rate Ig secretion. The initial work was done using a clone of Th, T94. As previously described (Julius and Rammenssee 1988a; Julius et al. 1988b), this Th was able to support the differentiation of co-cultured histoincompatible resting B cells when activated by crosslinking its TCR either directly, using monoclonal anti-V_8-8 antibodies, or indirectly, using monoclonal anti-CD3-epsilon antibodies. In these circumstances, T94 produces detectable IL-4 and IL-5. Neither IL-4 nor IL-5, alone, or in any combination we have tested support the differentiation of resting B cells (data not shown). Thus, either some as yet unidentified soluble mediator or combination of mediators must be supporting B cell differentiation in previously described histoincompatible contact (HIC) cultures (Julius and Rammenssee 1988a; Julius et al. 1988b). Alternatively, Th-B cell contact, mediated by nonpolymorphic membrane molecules, is providing necessary activation signals.

To assess whether Th-B cell contact was obligatory, we established histoincompatible non-contact (HINC) cultures using the Transwell culture system (Costar). As shown in Fig. 1, the architecture of the transwell enables co-culture of physically separated Th and B cells. In all of the following experiments, Th were cultured in the lower chamber with stimulating anti- TCR / CD3 antibodies, and density fractionated B cells were cultured in the upper chamber. When graded numbers of T94 are added to 4 x 10^6 irradiated splenic B cells and stimulated with 10 ng/ml of anti-V_8-8 antibody a robust induction of resting B cell differentiation ensues in the upper chamber within 100 hours of culture (Fig. 2). Similar results were obtained when 10 ng/ml of anti-CD3-epsilon antibody was used (not shown). This proves that T94 mediated resting B cell differentiation does not require Th-B cell contact.

Fig. 1

The transwell

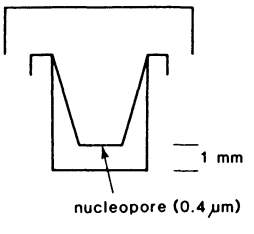
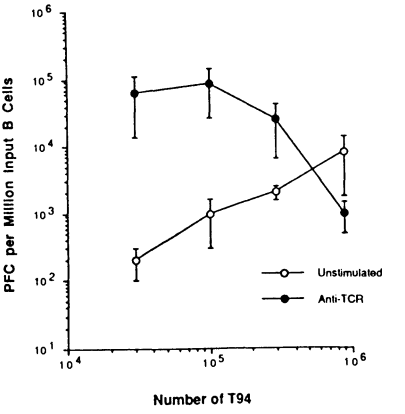


Fig. 2

Transactivation of Resting B Cells

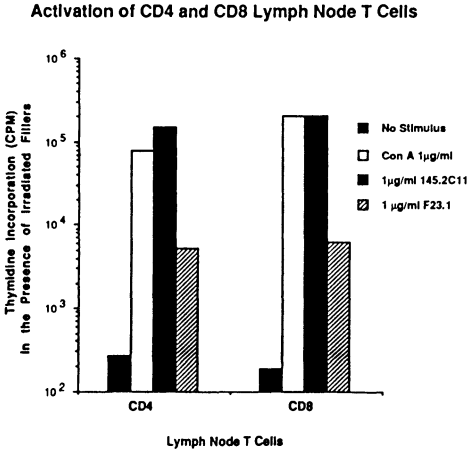


T94 alone produces all of the necessary factors supporting resting B cell differentiation. This is not clear from data shown in Fig. 2 since irradiated splenic B cells were added to the lower chamber. T94 can be activated by soluble or deliberately plastic coated anti-TCR/CD3 antibodies in the absence of fillers (not shown). The addition of fillers in this experiment (Fig. 2) was to allow direct comparison with later experiments using immediately ex vivo lymphnode T cells. The latter cannot be stimulated by anti-TCR/ CD3 antibodies in the absence of fillers. Since $F(ab')_2$ fragments of anti-TCR/CD3 antibodies are ineffective, the fillers likely serve as a source of Fc receptors which having adsorbed antibody can efficiently crosslink TCR/CD3. Whether other filler effects play a role in this culture system is unclear. If so, at a minimum they are not obligatory for T94 mediated B cell activation.

The efficiency of B cell induction by T94 in HINC cultures is 30-40% of that observed in HIC cultures. In addition, only 30% of our Th clones efficiently transactivate resting B cells. Those clones which do not, not only produce levels of IL-4 and IL-5 comparable to T94 but function as efficiently as T94 in HIC cultures. Moreover, other investigators using clones of Th which do not produce IL-4 or IL-5 have demonstrated the efficient activation of resting B cells in HIC cultures (Owens 1988). Taken together these data support the existence of an as yet uncharacterized T cell derived lymphokine, or lymphokine combination, produced in variable quantities by different Th clones. However, care must be taken to exclude trivial explanations such as, dysregulated gene expression in long term propagated Th clones or their contamination with as yet undetectable mycoplasma. Toward this end, we have repeated our transactivation experiments using immediately ex vivo lymphnode T cells.

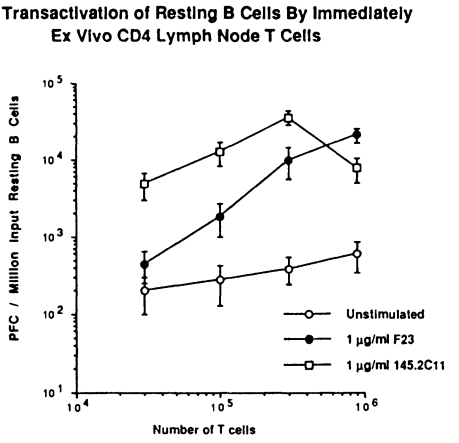
Lymphnode T cells were purified using the cell passage system devised by Sci-Can Diagnostics. Populations of CD4 and CD8 single positive T cells were then prepared by negative selection using anti-CD4 and anti-CD8 antibodies and complement. The purity of resulting populations was <1% Ig positive and >95% positive for Thy 1.2, CD3-epsilon and CD4 or CD8, as assessed by immuno-fluoresence staining. In addition, approximately 20% of CD4 and CD8 T cells expressed V_β-8 determinants. As shown in Fig. 3, comparable levels of DNA synthesis from 40-46 hrs of culture were induced when 2.5 x 10^5 CD4 or CD8 T cells were cultured with either 1 μg/ml each of ConA, anti-CD3-epsilon, or anti-V_β-8 antibody. These inductions were dependent on co-culture with irradiated splenic B cells.

Fig. 3



The results presented in Fig. 4 demonstrate that immediately ex vivo, CD4 positive lymphnode T cells are able to transactivate resting B cells within 100 hours of culture. Again, the induction depended on

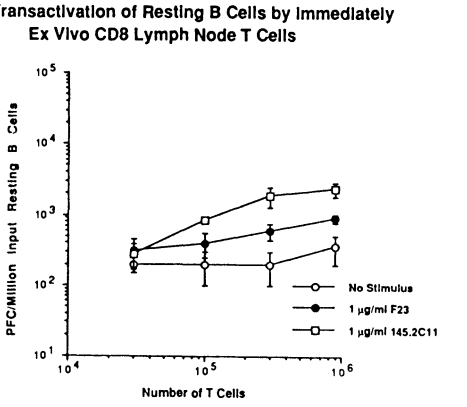
Fig. 4



the presence of fillers in the lower chamber and the presence of 1 µg/µl of anti-TCR/CD3 antibody. Since the efficiency of transactivation of resting B cells (Fig. 4) is within 35% of that for transactivation of activated B cells (Fig. 6), it is unlikely that these results are due to B cell blasts contaiminating resting B cell populations. These data obviate the concerns that previous transactivation results obtained using cloned Th were due to their dysregulated physiology or to non-T cell derived contaminants. Moreover, they support the notion that induction of resting B cell differentiation into high rate Ig secreting cells can be achieved with soluble T cell derived products.

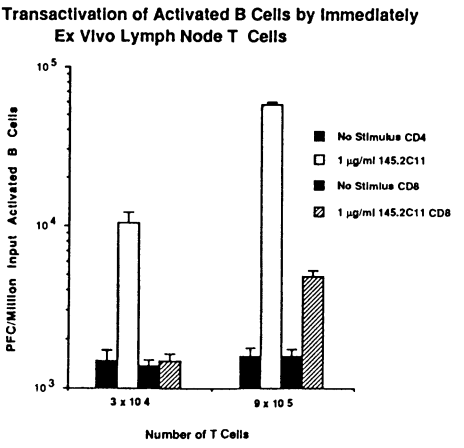
At best, the B cell response supported by immediately ex vivo CD4 positive T cells was 30-40% of the maximum response induced by optimal numbers of T94 (Compare Fig. 2 and Fig. 4). This is perhaps not surprising given the potential heterogeneity of the ex vivo T cells with respect to the quality of lymphokines produced. More striking is the ineffectiveness of CD8 positive T cells in HINC cultures (Fig. 5). The relative inefficiency of CD8 positive T cells was also noted when attempting to transactivate low buoyant density, activated splenic B cells (Fig. 6). High numbers of CD8 positive T cells were necessary to obtain even marginal transactivation of either resting or activated B cells (Fig. 5 and Fig. 6). At these T cell numbers, it is difficult to rule out the possibility that contaminating CD4 positive T cells are mediating Th activity. Notwithstanding this potential caveat, it is clear that patterns of lymphokine production by populations of immediately ex vivo CD4 and CD8 positive T cells are distinct.

Fig. 5



Recent work has established that helper T cells cannot be categorized into subclasses based on patterns of lymphokine secretion (Kelso and Gough 1988). Moreover, no qualitative differences in lymphokine production were noted between CD4 and CD8 positive alloreactive clones (Kelso and Gough 1988). However, two observations in these studies merit further comment. CD4 clones produced on the average 6-10 fold more of the lymphokines analyzed (GMCSF, IL-3 and IL-2) than CD8 clones; and very few of the clones were observed to produce IL-4. Quantitative differences in lymphokine production may indeed

Fig. 6



characterize functionally distinct subsets of T cells. Further, the molecular environment which supports T cell activation can change which T cells are selected and or their pattern of lymphokine production (Gajewski et al. 1989). In our studies, the use of anti-TCR/CD3 antibodies normalizes the potential effects that antigen recognition in the context of MHC class I or class II may have on the quality/quantity of lymphokines produced by CD8 and CD4 positive T cells, respectively. One interpretation of differences observed between CD4 and CD8 positive T cells is that mechanisms of thymic selection during their ontogeny from double positive progenitors also play a direct role in determining their available lymphokine repertoire.

In summary, we conclude that T cell dependent induction of resting B cell differentiation does not require T-B contact. In physiological circumstances T helper cells likely do contact the resting B cells they help and it is this interaction which ensures specificity of the response. However, it appears that signals delivered through those membrane associated molecules which mediate cognate Th-B interaction are not obligatory. Finally, the enhanced helper capacity of CD4 positive T cells suggests that T cell function is related to antigen specificity.

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Clonal Expansion and Differentiation to Effector Function in Normal CD4 T Cell Subpopulations

K. Bottomly, M. Luqman, J. Murray, J. West, A. Woods, and S. Carding

INTRODUCTION

The immune response to infection has two major effector mechanisms, usually referred to as humoral and cell-mediated immunity. These two effector mechanisms are both driven by CD4 T cells. This raises the question of whether all immune responses derive from the action of a single functional class of CD4 T cells, or whether CD4 T cells are functionally heterogeneous. Early studies in animals suggested that humoral and cell-mediated immunity could be dissociated, (Asherton 1962; Battisto 1962; Crowle 1966; Bretscher 1983) but it could not be determined if this reflects the action of functionally distinct CD4 T cells. More recent studies have addressed the question of CD4 T cell functional heterogeneity in two ways: separation of CD4 T cell subpopulations on the basis of differential cell surface expression of molecules detected by monoclonal antibodies, (Reinherz 1982; Morimoto 1985; Arthur 1986) and the production of cloned CD4 T cell lines of distinct function (Kim 1985; Mosmann 1986). Studies in rats and humans have utilized monoclonal antibodies that react with restricted forms of CD45, the leukocyte common antigen (Smith 1986; Sanders 1988; Spickett 1983). These studies have led to the suggestion that CD45 isoforms are differentially expressed on virgin and memory CD4 T cells (Smith 1986; Tedder 1985; Powrie 1989). A very different approach in the murine system has revealed functional heterogeneity amongst CD4 T cell cloned lines (Cherwinski 1987; Cher 1987; Kim 1985; Stout 1989), one set (Th2) producing the lymphokines IL-4 and IL-5 and particularly specializing in the activation of B cells secreting IgE (Snapper 1987; Coffman 1986; Maggi 1988; Del Prete 1988), while the other (Th1) produces IL-2, IFNγ, and LT/TNFß, and specializing in macrophage activation. In vivo studies of infectious disease and of the immune responses to specific antigens have supported the existence of comparable CD4 T cell populations in vivo(Heinzel 1989; Locksley 1987 Hayakawa 1988; Schoenbeck 1989; Carding 1989).

These data appear on the surface to be consistent if non-overlapping; however, further studies have pointed to anomalies between the studies carried out in mice and those performed in other species. In particular, human and mouse CD4 T cell clones often make cytokines typical of both Th1 and Th2 subsets, and thus appear to violate the patterns observed for cloned T cell lines. This raises the possibility that the subpopulations of cloned T cell lines described in the mouse are in vitro culture artifacts or, alternatively, extreme forms not normally observed in vivo. To examine the differences between the various species, and to determine whether normal CD4 T cells in the mouse belong to functionally distinct subpopulations secreting distinct cytokines, we have produced a monoclonal antibody that subsets CD4 T cells into two distinct populations. Using this antibody, we have examined the capabilities of CD4 T çells in each population, and the developmental relationships of these two populations.

The monoclonal antibody, 16A, was initially prepared by immunization with cloned CD4 T cell lines of one functional type, and was selected for its ability to distinguish Th1 and Th2 cloned lines. This antibody also divided CD4 T cells from normal mice into two distinct subpopulations (Bottomly 1989). Interestingly, this antibody, like the antibodies that subset human and rat CD4 T cells, reacts with a restricted set of isoforms of the CD45 molecule. The fact that CD45 isoforms correlates with functional heterogeneity in these three species strongly suggests a direct involvement of CD45 itself in functional heterogeneity. This finding superficially suggests that mouse, rat and human CD4 T cell subsets may all be comparable, and that CD45 isoforms may correlate similarly in the three species. While these conclusions may be correct, they can not be decisively proven using the antibodies currently available. The anti-human CD45 antibody 2H4 reacts with a high molecular weight form of CD45 that is lost upon T cell activation, revealing reactivity with antibody UCHL1, directed at the lowest molecular weight form(Akbar 1988; Sanders 1988). The anti-rat antibody, OX22, and the anti-mouse antibody, 16A, also react with high molecular weight forms of CD45 that are lost in memory cells in the rat (Powrie 1989). However, 2H4 reactivity depends on the first variable exon of CD45, whereas OX22 and 16A depend on the second variable exon for reactivity, and are thus distinct(Spickett 1983;Barclay 1987;Johnson 1989). Furthermore, there is insufficient data to state that OX22 and 16A see analogous CD45 isoforms. The complexity of CD45 makes the identification of isoforms using antibodies a difficult problem.

Our initial functional studies with antibody 16A showed that CD4 T cells from normal mice could be accurately subdivided into stable populations using this antibody. The 16A high density population produces IL-2 and IFNγ upon activation with T cell mitogens in short term culture, while the 16A low density population produces IL-4 and IL-5 and provides excellent helper T cell function in polyclonal responses. Thus, it appeared that 16A subsets normal CD4 T cells into Th1-like and Th2-like T cell subsets.

In an attempt to synthesize the data in the three species, certain models of CD4 T cell functional and phenotypic heterogeneity have been proposed. We have used antibody 16A to examine these hypotheses using normal mouse CD4 T cells. This analysis has led us to a new, and more appropriate, model of CD4 T cell heterogeneity and its development in the course of an immune response.

The first model is based on studies carried out in humans, showing that 2H4 positive T cells are naive, while UCHL1 T cells are memory cells. To examine if 16A separates cells comparably, we have immunized mice with a protein antigen, keyhole limpet hemocyanin (KLH), and then fractionated them on the basis of reactivity with 16A. 16A low T cells make IL-4 and IL-5 in response to antigen and help B cells to produce antibody. They thus appear to be comparable to Th2 cells. 16A high T cells make IL-2 and IFNγ, and proliferate extensively, in response to antigen; they act as helper T cells only at high concentrations of antigen; they would appear to be comparable to Th1 cells. These results show that priming has happened in both T cell populations (Table 1), and that the major proliferative response to recall antigen is in the 16A high population. This result is in conflict with the simple model that 16A high cells are virgin T cells, while 16A low T cells are memory T cells. In any case, these data raise questions about the simple model that 16A high cells rapidly switch to a 16A low phenotype upon priming.
A second model, suggested by these data, is that 16A is a true marker of functional phenotype. In this model, CD4 T cells develop as either 16A

high cells destined to produce IL-2 and IFNγ, or 16A low cells destined to produce IL-4 and IL-5 upon immunization. To examine this question, 16A high CD4 T cells have been prepared from KLH-immunized or from normal mice, and stimulated with antigen or mitogens. The initial production of cytokines mimicked that found with mitogenic lectins. However, when longer term cultures or cloned T cell lines were derived from these populations, it was noted that all the cloned 16A high cells switch to production of IL-4 and IL-5. This could reflect overgrowth of 16A low T cells, but this seems unlikely, since both the short term cultures of 16A high and cloned lines derived from them consistently switch to IL4 production. Thus, it appears that some 16A high cells give rise to 16A low cells.

A third model, consistent with the results just presented, is that CD4 T cells start having one phenotype and function, and mature into cells having a different phenotype and function. Several studies have suggested that naive T cells produce IL-2 upon activation, and thus are equivalent to Th1 cells. Upon activation, these cells switch to IL-4 production and resemble Th2. One difficulty with this model is that it predicts that all persistent immune responses should shift from cell-mediated immunity such as DTH, a function of Th1 cells, to humoral immunity, predominantly a function of Th2 cells. A further problem with this proposal is that 16A high CD4 T cells, while they make some IFNγ early in the response to antigen, do not persist in this response. The 16A low cells, which make IL-4 in response to antigen, also make large amounts of IFNγ later in the response. Furthermore, many cloned lines have been described in mouse and human that make both IL-4 and IFNγ, and we have derived many such cloned lines from both 16A high and 16A low precursors. Thus, it seems unlikely that 16A low cells are the in vivo equivalent of Th2 cells that derived from the 16A high equivalent of Th1 cells. A further difficulty with this proposition is that cloned CD4 T cells of the two different functional phenotypes are unlike these normal populations in their expression of 16A itself. Th2 clones, whose behavior most closely resemble 16A low CD4 Tcells are actually intermediate in their expression of 16A, while Th1 cloned T cell lines are usually completely negative in their expression of the determinant recognized by 16A.

TABLE 1
16A DEFINES TWO SUBSETS OF MEMORY T CELL

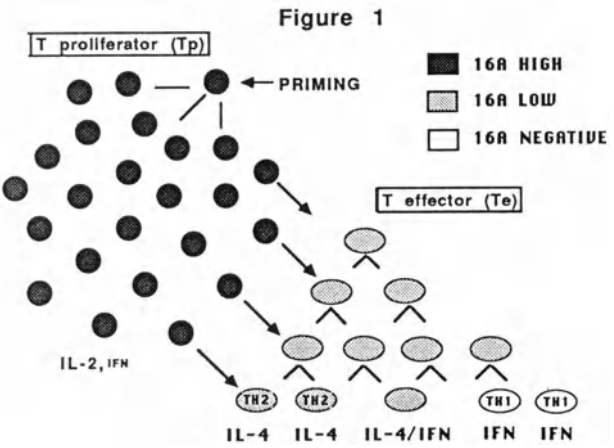
		Cytokine Release	
	Ag	IL-2	IL-4
KLH-PRIMED CD4 T CELLS			
16A high density	KLH	+++	-
16A low density	KLH	+/-	+++
UNPRIMED CD4 T CELLS			
16A high density	KLH	-	-
16A low density	KLH	-	-

These studies, taken together, have suggested a new and more complex hypothesis to account for all of the data on CD4 T cell heterogeneity. This hypothesis is shown schematically in Fig.1. We propose that CD4 T cells can be thought of as falling into two populations, a 16A high proliferative population (Tp) capable of undergoing clonal expansion driven by autocrine secretion of IL-2, and an effector population (Te) that derives from the progeny of clonal expansion. This effector population would include a range of CD4 T cells with Th1 and Th2 clones forming its extremes. Maturation from the clonally expanding population (Tp) to the effector population (Te) would occur during clonal expansion, and would be accompanied by a decreased expression of the CD45 isoform(s) recognized by 16A. This effector population would totally lose 16A reactivity in differentiating into the normal T cell equivalent of Th1, or retain 16A at a reduced level in differentiating into the normal equivalent of Th2. Some cells of intermediate phenotype would also be present in this population. The importance of these cells in immunity is not known, nor is their position in the development of Th1-like and Th2-like CD4 T cells.

This model leaves open several critical questions. The first, of course, is whether the model is accurate. The data that led to our proposal are summarized above. Second, one would like to know what drives the differentiation of proliferative, 16A high CD4 T cells to effector, 16A low CD4 T cells. Third, one would like to know how cells in this population mature into the equivalent of Th1 and Th2 cells, and to what extent intermediate forms exist in vivo. This question is of great importance in protective immunity, as shown by studies of leischmaniasis and leprosy.

We have previously proposed that this critical distinction may be controlled by ligand density(Bottomly 1988). Ligand density could control CD4 T cell subset activation or differentiation if quantitative differences in signals could drive uncommitted CD4 effector T cells to one or the other subpopulation. Alternatively, since B cells can generate

Figure 1



very high ligand densities under certain circumstances, it may be that accessory molecules expressed by B cells as opposed to macrophages controls the CD4 T cell subset activated by antigen. In any case, it is clear that much remains to be learned about the heterogeneity of CD4 T cells and the development of this heterogeneity. Nevertheless, there is already much data from in vivo immune responses to suggest that the major branching point in the immune response, the generation of humoral or cell-mediated immunity, may be regulated at the level of CD4 T cell subset activation. Thus, it is clearly of the utmost importance to understand these populations and their development, and to gain control over the process.

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III Effector Phases

Cytokines and Their Receptors

Structure and Function of Lymphokines and Their Receptors

A. Shimizu, T. Kinashi, Y. Ishida, and T. Honjo

INTRODUCTION

Generally speaking, information exchange between different cells is mediated by soluble effector molecules as well as by direct contact. Soluble effector molecules include a variety of chemical substances. Classical neurotransmitters like acetylcholine and adrenaline, and peptide hormones like insulin and glucagon are examples. Cytokines, lymphokines and monokines all belong to protein mediators of the information network within the immune system and sometimes between the immune system and other compartments like the nervous system. The information transfer takes place often in a cascade of, or with synergy between, different mediators. Conversely, the same information can be translated in different ways by receivers;thus a single mediator can transduce many different signals.

Such a complicated information network system alone was enough to mislead many immunologists to propose many different mediators, each responsible for one aspect of diverse biological phenomena that take place when different cells interact. In addition, a dogma that an immunological signal mediator has to be specific to each message, which probably arose from the impressive specificity of the antigen receptor, made the confusion worse. In the first five International Immunology Congresses before Toronto we have seen a growing number of cytokines claimed to be responsible for each biological phenomenon. This era is called B.C., as it was Before Cloning.

In the last International Congress of Immunology in Toronto, we began to see a better view of cytokines and lymphokines. Cloning of IL-1 cDNA and production of recombinant IL-1 clearly demonstrated that a single lymphokine can have multiple effects on multiple target cells. However, IL-1 cDNA cloning was not enough to make this rule generally accepted. Subsequent cloning of IL-4 and IL-5 cDNAs paved the way to the acceptance of the general idea that a lymphokine has multiple functions and multiple targets. Cloning of lymphokine cDNA certainly brought us to the A.D. era, as it was After DNA, and we can now discuss the structure and function of lymphokines and their receptors with a universal language *i.e.* chemistry.

LYMPHOKINES HAVE MULTIPLE TARGETS AND MULTIPLE FUNCTIONS

During past three years many laboratories have been involved in structural and biological characterization of lymphokines using recombinant or highly purified materials mostly in *in vitro* systems.In this symposium characterizations of two new lymphokines, IL-6 by

Kishimoto *et al.* and IL-7 by Hensey *et al.*, were reported. It became clear that every single lymphokine has a variety of activities that are apparently unrelated with each other (Table 1). The targets of these lymphokines are also widely distributed among different cell lineages including not only lymphoid but also other hematopoietic cells (Table 1, also reviewed by Paul, 1989). In some cases, different factors stimulate growth of a specific lineage cell (redundancy of activities). All these results made it difficult to name the factor by their activities, functions or target cell lineages like B cell growth factor or B cell stimulatory factor.

Table 1. Lymphokines have multiple targets and multiple functions

Lymphokines	Functions	Targets
IL-1 α,β	Induction of IL-2 receptor	T cells
	Differentiation of hematopoietic cells	Hematopoietic cells
IL-2	T cell growth (TCGF)	T cells
	Stimulation of Ig secretion	B cells
INFγ	Induction of MHC class I and II molecules (Immune interferon)	B cells
	Ig class switching	B cells
	Macrophage activation	Macrophages
IL-3	Stimulation of growth differentiation of hematopoietic precursors (Multi-CSF)	Hematopoietic precursors
	Mast cell growth	Mast cells
IL-4	B cell activation (BSF-1, BCGF-I)	B cells
	Ig class switching	B cells
	T cell growth	T cells
	Mast cell growth	Mast cells
	Macrophage activation	Macrophages
	Stimulation of hematopoietic progenitors	Pro B and Pro T cells
IL-5	B cell growth (TRF, BCGF-II)	B cells
	Growth and differentiation of eosinophils (EoDF)	Eosinophils
IL-6	Plasmacyte growth (Myeloma GF)	Plasmacytes
	Ig secretion (BSF-2)	B cells
	T cell growth	T cells
	Induction of acute phase proteins	Hepatocytes
IL-7	Pre B cell growth	Pre B cells
	Thymocyte proliferation	Thymocytes

One interesting aspects of lymphokine functions is their effects on hematopoietic progenitor cells. Recent studies on early stages of hematopoietic or lymphoid cell differentiation revealed that progenitor cells are induced to differentiate into various cell lineages by complex stimuli through the direct contact with stroma cells and by soluble factors produced by stroma cells or themselves. Some lymphokines such as IL-1[1], IL-3, IL-4, IL-5 and IL-7 are shown to involve in such early progenitor growth and differentiation. For

[1] Abbreviations: IL-; interleukin-, Ig; immunoglobulin

example, Kinashi *et al* (1989) found that there was IL-4 dependent stage at early differentiation into B and myeloid cells (Fig. 1). This result suggests that IL-4 might be involved in the early stage of B and myeloid cell differentiation at least *in vitro*. Palacios and his colleagues (Takeda *et al.*, 1989) have shown that IL-7 dramatically enhance differentiation of pro B cells into B cells in their *in vitro* system. All these data suggest that the lymphokine mediates not only the information transfer between mature cells but also developmental signals in early hematopoietic progenitor cells. However, we do not have strong evidence that IL-3 and IL-7 are produced in the bone marrow. The origin and role of these factors *in vivo* still remains to be clarified.

Another important aspect on lymphokine functions is species variations As most of the analyses of lymphokine activities were carried out using the mouse system, it is important to know wether these activities are conserved in the human system, especially for clinical application of lymphokines. One of the controversial lymphokines is IL-5. However, Enokihara *et al.*, (1989) have shown that human IL-5 has the eosinophil colony-forming activity. The B cell stimulatory activity of human IL-5 was reported by Benson and Bertolini at this conference. These findings suggest that general functions of IL-5 are conserved between the species. It might be important to point out that the difference could arise in part from difference in target cells for *in vitro* experiments. Usually murine target cells are spleen cells and human target cells are peripheral blood cells.

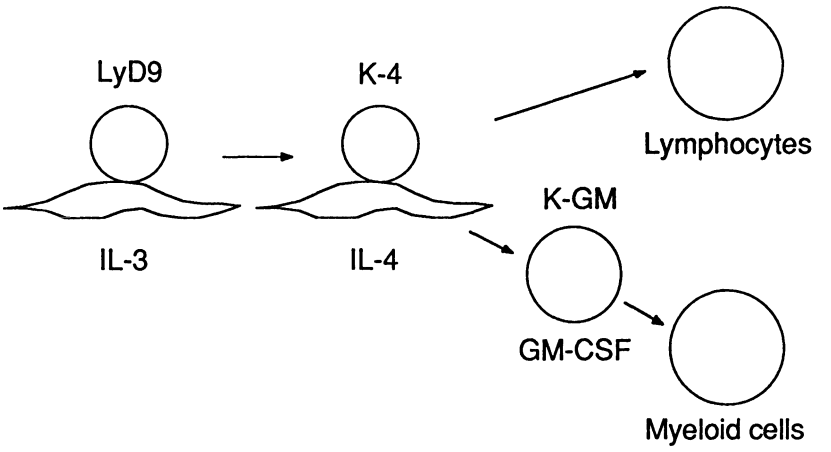

Fig. 1. IL-4 is required for differentiation of a progenitor cell line into B cells and myeloid cells.

NOT ALL THE *IN VITRO* FUNCTIONS MANIFEST *IN VIVO*

Since the *in vitro* assay system for a variety of lymphokine activities are artificially designed to augment a particular activity, it is important to test whether all these activities do manifest *in vivo*.

Continuous administrations of a lymphokine is one way to look at the *in vivo* effect but this is not always easy because of very short half lives of lymphokines *in vivo*.

In order to overcome such difficulties, several laboratories made transgenic mice which produce lymphokines under the control of constitutive promoters like those of MHC class I antigen and Ig genes. These investigations revealed rather unexpected findings. For example, double transgenic mice of IL-2/IL-2 receptor light chain (Tac antigen, p55) did not show antigen-nonspecific expansion of T cells which was expected from the T cell growth factor activity *in vitro*. By contrast, the mice showed the reduced T cell function and enormous expansion of NK cells (Fig. 2., Ishida et al., in press). Although ordinary T cells of this transgenic mice express both p55 and p75 chains of the IL-2 receptor, they did not proliferate in response to IL-2 (about 50 pM) in the serum. The results could be explained by the following three possibilities; (1) ordinary T cells may require additional signals for their proliferation, (2) ordinary T cells may have a strong feedback system for proliferation which is missing in NK cells, and (3) ordinary T cells may require another growth factor for their proliferation *in vivo*. Another transgenic mice containing the IL-6 gene under the control of Ig promoter-enhancer (Suematsu *et al.*, in press) showed expected and unexpected symptoms common to this transgenic mice. It is interesting to note that all the IL-6 transgenic mice showed polyclonal plasmacytosis which is consistent with the myeloma growth factor activity shown *in vitro*. However the Ig isotype produced was restricted to IgG1 by unknown reasons.

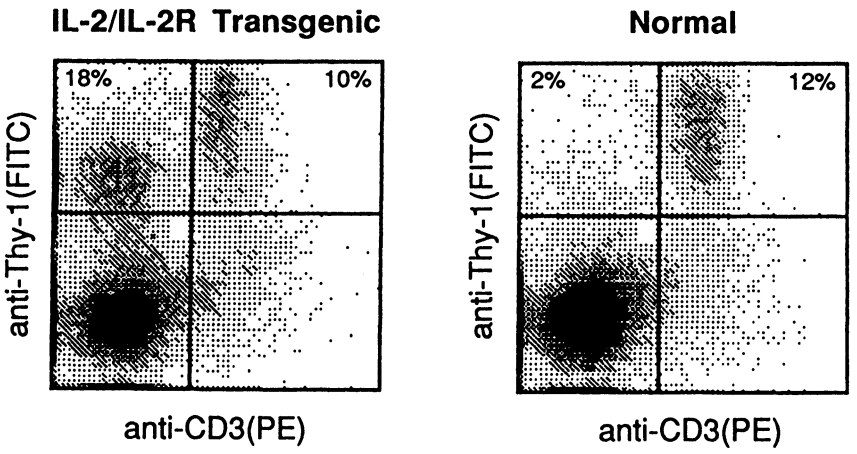

Fig. 2. Expansion of $Thy1^+/CD3^-$ spleen cells in IL-2/IL-2 receptor light chain transgenic mouse (Taken from Ishida *et al.*, in press).

Another unique approach was taken by Tepper *et al.* (1989) to analyze *in vivo* functions of lymphokines. They first transfected various kinds of tumor cell line with the IL-4 gene. They transplanted the IL-4-

producing tumor cells to normal and nude mice, and found that the IL-4-producing tumors were rejected probably by the defence mechanism of the host animals regardless of normal or nude mice. The efficiency of the rejection was well correlated with the amounts of IL-4 produced by the tumor cells although there was no difference in the growth rate or other characters of the tumor cells *in vitro*. It appears that IL-4 induces the tumor-suppressing activity by activating not only T cells but also whole defence-committed cells which could be brought together to the site of tumors. Such whole body reactions to lymphokines are almost unexpected from activities characterized *in vitro* and impossible to be analyzed *in vitro*.

T CELLS MAY PRODUCE A VARIETY OF LYMPHOKINES BY DIFFERENT STIMULI

To understand the regulation of the information transfer network, it is obviously important to know the regulation of lymphokine synthesis. It is an attractive idea that a certain combination of lymphokines produced is associated with the function of the T cell subset.

Mosmann *et al.* (1986) reported that murine helper T cells were classified into two groups by combination of lymphokines produced. Th1 that produces IL-2 and INFγ mediates delayed type hypersensitivity and Th2 that produces IL-4 and IL-5 stimulates antibody production. Similar studies on human T cell lines, however, did not support such classification. Noma *et al.* (1989) studied lymphokine production profiles of human T cells transformed by HTLV-I infection, and found no obvious grouping by the lymphokine production. Most interesting observations were obtained by their study of four cell lines that had been established from the same patient at different times but shown to be clonally related by the profile of T cell receptor rearrangement. These four related T cell clones had different profiles of lymphokine production; one produced IL-2 and another produced IL-4 as shown in Table 2. These results suggest that the lymphokine production profile of a particular human T cell line may not be a stable phenotype but convertible by different of stimuli (Fig. 3). It remains to be seen whether rodents and primates employ different strategies for regulation of lymphokine production or the difference is related with procedures for establishing T cell clones.

Table 2. Production of lymphokines in clonally related ATL cell lines

			Expression of lymphokine mRNA						
Cell lines	Dates of establishment	Growth factor	IL-1 α	IL-1 β	IL-2	IL-3	IL-4	IL-5	IL-6
ED40515	1984/05/15	IL-2	−	−	−	−	−	−	−
ED40515A	1984/05/15	IL-2	−	−	+	NT[a]	−	−	−
ED40810	1984/08/10	None	+	−	−	−	+	+	++
ED41214B	1984/12/14	None	−	−	−	−	−	−	++

[a] NT; not tested. Data were taken from Noma *et al.* (1989).

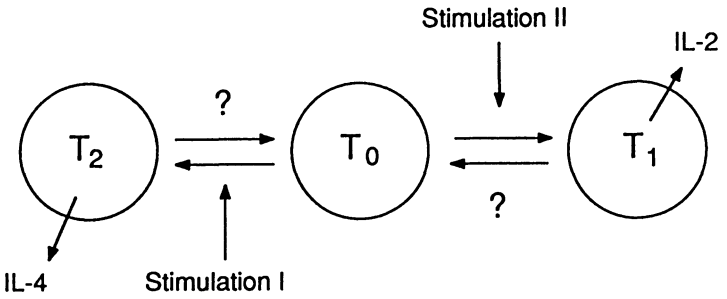

Fig. 3. Profile of lymphokine production may be convertible by different stimuli.

TWO CHAIN RECEPTORS MAY BE MORE COMMON

At the last International Congress of Immunology we had the structural information of only the L chain of the IL-2 receptor (Leonard *et al.*, 1984; Nikaido *et al.*, 1984). We now know the primary structures of receptors for IL-1, the H chain of IL-2, IL-6 and INFγ by cloning of their cDNAs (Yamasaki *et al.*, 1988; Aguet *et al.*, 1988; Sims *et al.*, 1988; Hatakeyama *et al.*, 1989). Structural features of the cloned lymphokine receptors are listed in Table 3. Cell surface receptors can be grouped into several types by their strategies for signal transduction such as ion channel (acetylcholine receptor), tyrosine kinase (CSF-I receptor, EGF receptor) and those linked to G proteins (adrenaline receptor). Receptors for the lymphokines, however, do not seem to belong to any of such categories but form a unique family, *i.e.* two chain receptors.

Table 3. Structure of lymphokine receptors

Receptors	Chains	Size (kD)	Affinity species	Structure
IL-1 R	one(?)	80	one(?)	Ig supper gene family / short tail
IL-2 R	two	55(L)	two	Complement receptor-like repeats / short tail (L chain)
		70(H)		Kinase in cytoplasmic domain(?) (H chain)
IL-6 R	two(?)	65	two	Ig supper gene family / short tail
INFγ R	one(?)	90	one(?)	Unrelated to known receptors

Many lymphokine receptors have two affinity states (high-affinity; $Kd \approx 10^{-11}M$ and low-affinity; $Kd \approx 10^{-8} \sim 10^{-9}M$). Molecular mechanism(s) to make two different affinity states has been an interesting question.

Chemical cross-linking experiments also suggest that many lymphokine receptors may have two polypeptide chains. The best studied lymphokine receptor is the IL-2 receptor. Soon after cloning of cDNA for the L chain of the IL-2 receptor, it became clear that at least one more chain was required for high-affinity binding (Sabe *et al.*, 1984; Kondo *et al.*, 1986a). When human L chain cDNA was expressed in CTLL cells expressing murine high-affinity sites, the human L chain was freely associated with the endogenous murine H chain. Competitive blocking antibodies against human or mouse light chain alone could not change the total number of high-affinity binding sites whereas the simultaneous addition of both antibodies completely abolished high affinity binding of mouse T cell line expressing the endogenous and transfected human light chains. The HIEI antibody, which immunoprecipitates the human L chain but do not block IL-2 binding, blocked the high-affinity binding. To explain these observations the affinity conversion model was proposed (Kondo *et al.*, 1986b; Shimizu *et al.*, 1986). Subsequent identification of the H chain (p75) by chemical cross-linking to IL-2 (Sharon *et al.*, 1986; Tsudo *et al.*, 1986; Teshigawara *et al.*, 1987) led to a hypothesis that a preformed heterodimer of the L and H chains serves as the high affinity receptor. According to this hypothesis an excess number of L chains have not any biological significance. Recent careful kinetic analyses by Saito *et al.*(1988), however, have shown that a larger number of L chains accelerate IL-2 binding to the high-affinity sites without changing the total number of high-affinity binding sites which is usually equal to the number of the H chain (Fig. 4). This finding contradicts with the preformed heterodimer model which predicts the same association kinetics when the number of the high-affinity site is the same, and consistent with the affinity conversion model which assumes that IL-2 first binds to the L chain and then this complex binds with the heavy chain to form a ternary complex (Fig. 5).

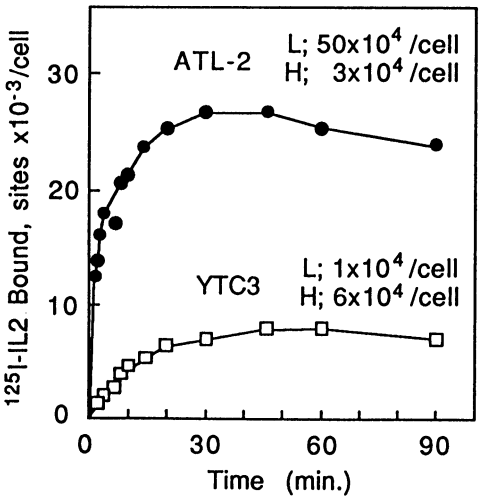

Fig. 4. A large number of light chains accelerate IL-2 binding to the high-affinity sites (Taken from Saito *et al.*, 1988).

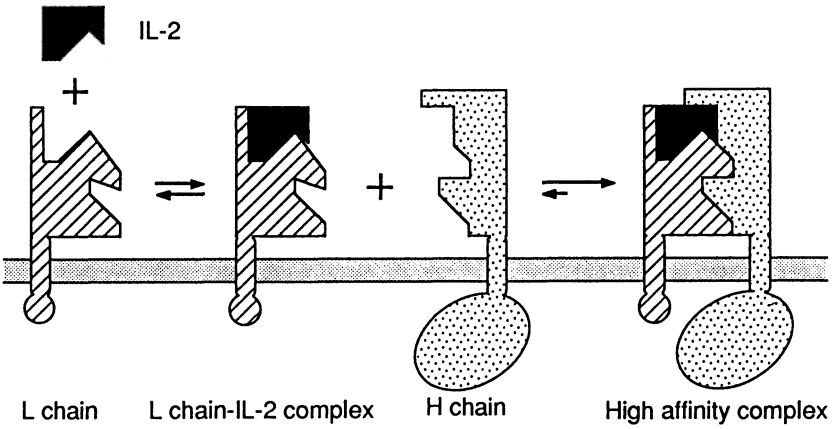

Fig. 5. Affinity conversion model

More recently, Hatakeyama *et al.* (1989) have cloned the H chain of the IL-2 receptor by screening COS cells transfected by cDNA library with a monoclonal antibody which could block the IL-2 binding to the H chain. The H chain cDNA encodes the receptor with the intermediate-affinity when transfected into a murine T cell line. The high-affinity binding receptor was expressed by co-transfection of the T cell line with the H and L chain cDNA. Unexpectedly, however, the transfection of COS cells by the H chain cDNA alone did not express the receptor that binds IL-2 but did express the epitope that was recognized by the monoclonal antibody which could block the binding of IL-2 to the H chain, indicating that either another molecule(s) or modification(s) specific to T cells is required for IL-2 binding of the H chain. IL-2 binding to the H chain itself does not seem to be absolutely necessary for the formation of the high-affinity binding.

Several other lymphokine receptors also consists of two chains: Receptors for IL-1 and IL-6 which have the immunoglobulin like domains and belong to the immunoglobulin super gene family have short cytoplasmic tails and are likely to have second chains responsible for the signal transduction. In fact, a candidate of such molecule is suggested for the IL-6 receptor by Kishimoto and his colleagues. The number of receptors which consist of two polypeptide chains is growing. Cloning of cDNA encoding IL-4 receptor was also reported from two groups at this International Congress of Immunology.

FUTURE PROSPECTS OF LYMPHOKINE RESEARCH

More Informations of *in Vivo* Lymphokine Function Are Necessary.

As described in the first and second sections, lymphokine functions *in vivo* are sometimes unexpected and unpredictable from *in vitro* studies. Although extensive analyses of their *in vitro* activities revealed new aspects of their targets and functions, we still have limited

informations about lymphokine functions in a whole body. Approaches to investigate such functions may not be easy but deeply demanded. Depletion of lymphokine-producing cells by introducing toxinic genes driven by lymphokine gene promoters as a transgene or destroying the lymphokine gene by homologous recombination using embryonic stem cells might be very useful for such analyses.

Clinical Application Should Be Done with an Extreme Care and a Good Knowledge of *in Vivo* Effects.

As *in vivo* functions of lymphokines are often unexpected and unpredictable from *in vitro* studies, clinical applications of them should be done with an extreme care of possible side effects. If we are able to avoid unpleasant side effects by precise targeting , lymphokines could be most powerful and promising drugs because of their strong and diverse activities. A drug delivery system that allows focused administration to target site(s) such as malignant tumors should be developed to fully utilize the effect of lymphokines.

Rapid Progress of Studies on Regulation of Lymphokine Expression Is Foreseen.

Regulation of lymphokine gene expression is essential for understanding of the immune response. Though we did not describe about such studies in this review, both *cis*-acting control element like NFkB binding site (Lowenthal *et al.*, in press) and *trans*-acting factors like interferon regulating factors (Miyamoto *et al.*, 1988) have been already identified. Rapid progress in this field will provide us with valuable and important informations about ordinate or competitive regulation of many lymphokine genes.

Structural Studies on Lymphokines and Their Receptors Will Facilitate Discovery of Antagonists and Agonists of Lymphokines.

A large scale production of pure lymphokines using recombinant DNA technology made it possible to analyze the three dimensional structure of lymphokines by X-ray crystalography. Such approach to elucidation of the IL-2 structure was initiated (Brandhuber *et al.*, 1987). Elucidation of the three dimensional structure of the lymphokine and its receptor will help us to design antagonists or agonists. A primitive trial using soluble form of the IL-2 receptor L chain showed immune suppressive activities *in vitro* (Kondo *et al.*,1988).

More Informations on Molecular Mechanisms of Signal Transduction Are Required.

Although we now know the structure of several lymphokine receptors, little is known about their signal transduction mechanism. Most lymphokine receptors (ligand binding molecules) do not seem to have obvious catalytic domains. We might be able to identify another yet unknown type of mechanism for lymphokine signal transduction.

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Heterogeneity of Mouse Helper T Cells and Cross-Regulation of TH1 and TH2 Clones

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INTRODUCTION

The immune response against most antigens or infections shows a characteristic pattern of effector functions, typical of that antigen. For many pathogens, the immune response is appropriate, i.e. the effector functions are capable of neutralising or eliminating the infection. Examples include the induction of antibodies to neutralise toxins, cytotoxic T lymphocytes (CTLs) to kill virus-infected cells, and Delayed Type Hypersensitivity against intracellular pathogens. The mechanisms controlling this immune class regulation have been difficult to determine until recently, when two functionally different types of T cell were defined according to their different patterns of cytokine synthesis. These two T cell types appear to be responsible for at least part of immune class regulation.

THE TH1 AND TH2 CYTOKINE SECRETION PHENOTYPES

In a large panel of long-term mouse TH clones, two major types of T cell could be distinguished, each with a distinctive cytokine secretion pattern (Mosmann et al., 1986a; Cherwinski et al., 1987) (Table 1). TH1 but not TH2 clones, in response to antigen stimulation, produce IL2, IFNγ and LT, whereas only TH2 clones express IL4, IL5, IL6, P600, and Cytokine Synthesis Inhibitory Factor (CSIF, see below) (Mosmann et al., 1986a; Cherwinski et al., 1987; Brown et al., 1988) (Table 1). In addition to these type-specific cytokines, both TH1 and TH2 clones express granulocyte-macrophage colony stimulating factor (GM-CSF), tumour necrosis factor (TNF), met-enkephalin, IL3 and three other proteins characterised mainly as cDNA clones, P500, H400 and TY5 (Brown et al., 1988). We have recently determined that cytotoxic T lymphocyte (CTL) clones can express the full range of TH1 cytokines, but not the TH2-specific cytokines (T.A.T. Fong and T.R. Mosmann, unpublished).

Functions of TH1 and TH2 clones.

The cytokine secretion patterns of TH1 and TH2 clones are major determinants of the functions of the clones. The work of several groups (reviewed in Mosmann and Coffman, 1989; Coffman et al., 1988)) suggests that TH1 clones can help B cells effectively, but only if their characteristic cytokines are present at the correct concentrations (low IFNγ and high IL2 levels), whereas the presence of excess IFNγ and LT probably inhibits B cell responses. We have recently shown (T.A.T. Fong and T.R. Mosmann, submitted). that the TH1-specific cytokine IFNγ contributes to the swelling and vascular leakage of the Delayed Type Hypersensitivity (DTH) reaction

induced by TH1 clones (Cher and Mosmann, 1987). TH1 clones also induce several other effector functions that are suitable for attacking intracellular parasites such as viruses, intracellular bacteria and protozoans (reviewed in (Mosmann and Coffman, 1989).

Table 1 Cytokine patterns of mouse T cell clones.

	CTL	TH1	TH2
Interferon γ	++	++	-
Interleukin 2	+/-	++	-
Lymphotoxin	+	++	-
GM-CSF	++	++	+
Tumour necrosis factor	+	++	+
TY5	++	++	+
P500	++	++	+
H400		++	+
Interleukin 3	+	++	++
Met-Enkephalin	+	+	++
Interleukin 4	-	-	++
Interleukin 5	-	-	++
Interleukin 6	-	-	++
P600	-	-	++
Cytokine synthesis inhibitor (CSIF)		-	++

TH2 clones are normally excellent helpers for antibody production by B cells, due in large part to the synthesis of IL4, IL5, and IL6, which are all effective B cell growth and differentiation factors (Coffman et al., 1988; Vitetta et al., 1988). TH2 clones are especially good helpers for IgE responses, since IL4 induces a switch to IgE production in activated B cells (reviewed in (Coffman et al., 1988). TH2 cells can also contribute to eosinophilia, due to the production of IL5 (Coffman et al., 1989) and the proliferation of mast cells, due to the production of IL3 and IL4 (Mosmann et al., 1986b).

Evidence for TH1 and TH2 patterns in vivo.

Several features of general immune responses suggest the involvement of TH1 and TH2 cytokine secretion phenotypes (reviewed in (Mosmann, Coffman, 1989). Infestation with some helminth parasites results in very high IL4-dependent IgE levels (Finkelman et al., 1988), accompanied by IL5-dependent eosinophilia (Coffman et al., 1989). The spleen and lymph node cells produce less IL2 and IFNγ, and much more IL4 and IL5 than normal (N.E. Street and T.R. Mosmann, unpublished). All of these effects are consistent with the selective activation of a strong TH2 response. Even more striking evidence for TH1 and TH2 involvement is seen in mouse and human responses against *Leishmania* parasites. The TH2-like response of Balb/c mice is associated with severe, generalized disease, whereas the TH1-like C57Bl/6 response is associated with a contained, local infection and cure (Heinzel et al., 1989; Scott et al., 1988). These results demonstrate that the choice of a TH1 or TH2 bias to the immune response can be crucially important in combatting different infections.

EVIDENCE FOR OTHER TH TYPES

Cytokine secretion patterns of freshly isolated spleen cells.

The discussion above has been based on the TH1 and TH2 cytokine secretion phenotypes defined by long-term mouse T cell clones. Evidence from normal T cell populations, human T cell clones and short-term mouse T cell clones has suggested that there are additional cytokine secretion phenotypes, and that the TH1/TH2 phenotypes may represent late or final stages in the differentiation of helper T cells. Normal mouse spleen cells produce large amounts of IL2, but little IFNγ, IL4, IL5, GM-CSF or IL3, unless immunized strongly (Table 2). The ability to produce IFNγ, IL4 and IL5 is increased in the memory cell population relative to the unstimulated, virgin population (Budd et al., 1987), (R.C. Budd, J.H. Schumacher and T.R. Mosmann, unpublished).

Table 2. Comparison of Cytokine Secretion Patterns of T Cell Clones and Splenocytes.

Cells	Cytokine/10^6cells[a]					
	IL2 (SU/ml)	IFNγ (ng/ml)	GM-CSF (ng/ml)	IL3 (ng/ml)	IL4 (SU/ml)	IL5 (ng/ml)
TH1 (Average of 9)	2188.9	722.6			<0.3	<0.01
TH2 (Average of 10)	<0.4	<0.1			4096.7	40.50
TH1 (HDK-1)	23.9	1923.78	9.162	52.70	<0.6	<0.008
TH2 (D10)	0.4	<0.08	6.561	241.40	19291.0	229.290
BALB/C Spleen						
Non-Immune $CD8^-$	70.7	0.27	<0.008	0.10	2.1	0.022
Nippostrongylus $CD8^-$	132.4	0.99	0.044	7.74	485.6	0.405
Brucella abortus $CD8^-$	258.9	2.86	<0.008	0.25	7.5	0.035

[a] T cell clones or $CD8^-$ splenocytes were stimulated *in vitro* with Con A for 24 hours and the secreted cytokines measured as described previously (Mosmann and Fong, 1989).

Identification of additional types of TH cell by limiting dilution cloning.

Since TH1 and TH2 cloning frequencies can vary widely from one experiment to another, we carried out limiting dilution cloning experiments using cells from mice that had been immunized under conditions likely to induce very strong TH1-like or TH2-like responses. We reasoned that the immune response of these mice might be "locked" in either the TH1 or TH2 direction, and so the resultant cloning patterns might be more predictable.

Figure 1 shows the cytokine secretion patterns of such clones, assessed over a 70-day period. The results are displayed as the ratio of IL4/IL2 plotted versus IL5/IFNγ. This method results in the segregation of long-term clones into two distinct regions of the plot (TH1 clones in the lower left, TH2 in the upper right). Among short-term clones derived from strongly immunized mice, clones expressing distinct TH1 or TH2 patterns were detected. In addition, many clones displayed cytokine secretion phenotypes intermediate between the TH1 and TH2 patterns. The proportion of

recognizable TH1 and TH2 clones at early times in culture was greatly increased by immunization of the mice from which the responder and stimulator cells were derived; *Brucella abortus* immunization resulted in the isolation of exclusively TH1 clones, whereas infection with *Nippostrongylus brasiliensis* resulted in a strong trend towards the isolation of TH2 clones (Fig. 1). These results were confirmed by detailed examination of the data, which showed that by day 70, the clones from Ba-immunized mice were typically TH1, with strong synthesis of IFNγ and IL2, and no detectable IL4 or IL5. Clones from Nb-immunized mice were more mixed, but several TH2 clones could be detected even at early times, and these were stable on repeated stimulation.

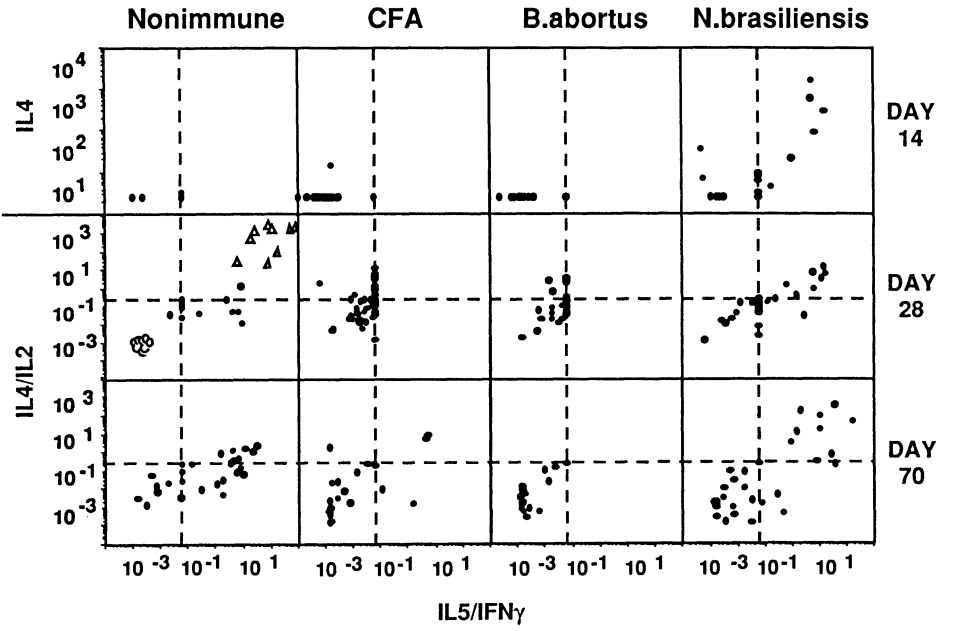

Figure 1. Cytokine secretion patterns of short-term TH clones. Clones were stimulated at 14, 28 and 70 days with Con A, and the cytokines in 24-hour supernatants were measured. The dashed lines show the ratios of the threshold values of the assays, i.e. a clone producing neither IL2 nor IL4 would be found on the horizontal line. The cytokine secretion patterns of long-term TH1 and TH2 clones are shown for comparison in the 28-day non-immune panel. o - TH1 clones, ▲ - TH2 clones.

These results are consistent with a model in which TH1 and TH2 phenotypes represent late stages in helper T cell differentiation, with their precursors expressing different cytokine secretion patterns. The simplest interpretation of the results with normal spleen cells (Table 2) and short-term in vitro clones (Fig. 1) is that many spleen cells (designated THp) secrete only IL2 when first activated, and that these then differentiate into an intermediate cell, TH0, that can secrete both TH1 and TH2 cytokines. In

response to further stimulation, this cell can differentiate into either TH1 or TH2 cells, depending on the nature of the antigenic stimulus and/or the accessory molecules present during the stimulation. This model is also compatible with data showing that freshly isolated spleen cells from unimmunized mice require a short period of in vitro stimulation before IL4 and IL5 can be produced at substantial levels (Swain et al., 1988).

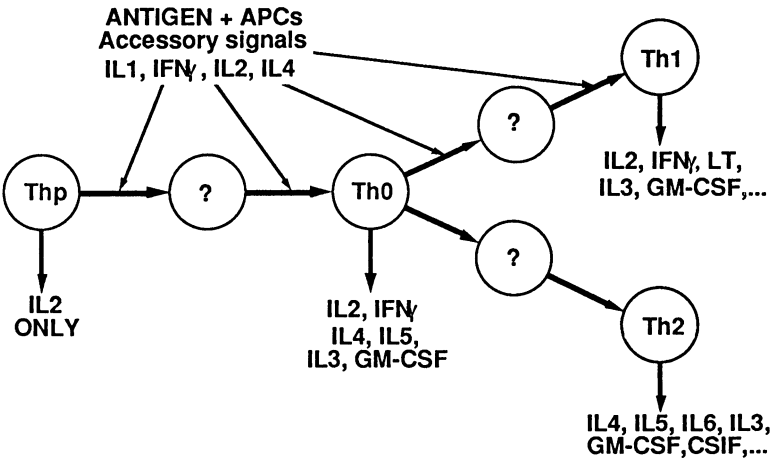

Figure 2. Possible relationships of TH subpopulations.

CROSS-REGULATION OF TH1 AND TH2 CELLS.

During many immune responses, DTH and antibody production appear to be reciprocally regulated (Parish, 1972). Strong immune responses influence simultaneous responses against other antigens, in the same direction as the major response. One possible explanation for these results is that TH1 and TH2 cells reciprocally inhibit the growth and/or function of the other type. Some of the cytokines responsible are now known: IFNγ inhibits the growth of TH2 cells (Fernandez-Botran et al., 1988; Gajewski and Fitch, 1988); and IL4 is a better growth factor for TH2 than TH1 cells (Fernandez-Botran et al., 1988).

Cytokine Synthesis Inhibitory Factor.

We have recently found that TH2 supernatants contain activities that inhibit TH1 proliferation and cytokine synthesis. The Cytokine Synthesis Inhibitory Factor (CSIF) is secreted by antigen- or lectin-induced TH2 but not TH1 clones (Table 3). The absence of CSIF in TH1 supernatants was confirmed by the demonstration that TH1 supernatants neither inhibited the synthesis of IFNγ nor prevented the activity of TH2-derived CSIF (Table 3). CSIF can inhibit the production of IL2, IL3, LT/TNF, IFNγ and GM-CSF by TH1 cells responding to antigen and APCs, but does not inhibit the

ability of antigen-stimulated TH1 cells to proliferate in response to IL2. TH2 cytokine synthesis is not significantly affected by CSIF.

Table 3. TH2 but not TH1 Supernatants Inhibit Synthesis of IFNγ by TH1 cells

Test Supernatants[a] (1.6% v/v)		IFNγ (% of control)[b] Supernatant only	Supernatant + CSIF
TH2:	D10	22	
	MB2-1	24	
	M411-2	36	
	M411-6	25	
TH1:	HDK1	91	24
	LB2-1	93	22
	MD13-5	91	37
	M264-37	103	31
	Control	100	33

[a] TH1 samples were depleted of IFNγ on a monoclonal anti-IFNγ column.

[b] The amount of IFNγ synthesized by antigen-stimulated HDK1 (TH1) cells in the presence or absence of various T cell supernatants was measured.

The action of CSIF may be indirect, since anti-T3 or lectin stimulation of TH1 cells in the absence of APCs induced IFNγ synthesis that was not inhibitable by CSIF. The kinetics of action of CSIF are also consistent with indirect action. Other cytokines also influence the synthesis of IFNγ, as shown in Table 4.

Table 4. Effects of CSIF, IL2, IL4 and TGFβ on IFNγ synthesis by a TH1 clone.

Addition to culture	IFNγ secretion (% of control) - CSIF	+ CSIF
None	100.0 ± 5.9	20.6 ± 4.8
anti-IL2	60.6 ± 6.0	15.9 ± 2.7
anti-IL2 + IL4	97.1 ± 13.4	15.4 ± 2.4
anti-IL2 + IL2	395.5 ± 19.1	49.1 ± 3.6
Rabbit IgG	116.1 ± 5.5	25.4 ± 3.3
anti-TGFβ	140.3 ± 1.6	40.1 ± 0.5
TGFβ	77.5 ± 8.0	17.0 ± 1.1

IL2 and IL4 both enhance IFNγ synthesis, whereas TGFβ is inhibitory. CSIF appears to act independently of these other cytokines, since the inhibitory effects of CSIF were seen in the presence or absence of IL2, IL4 or TGFβ. Biochemical characterization, monoclonal antibodies and recombinant or purified cytokines were used to show that CSIF is distinct from IL1, IL2, IL3, IL4, IL5, IL6, IL7, IFNγ, GM-CSF, TGFβ, TNF, LT, and P40 (D.F. Fiorentino, M.W. Bond and T.R. Mosmann, submitted).

The existence and function of CSIF have recently been confirmed by the isolation of a cDNA clone that expresses CSIF activity when transfected into the monkey COS7 cell

line (Fig. 3). The activity of the recombinant material appears to be similar that of natural mouse CSIF, and preliminary sequence information confirms that the cDNA clone is different from any known TH2 product, and may represent a novel cytokine.

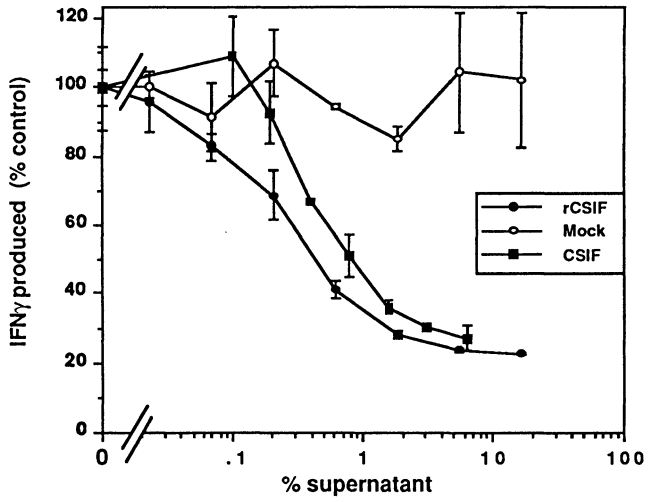

Fig. 3. Comparison of natural and recombinant CSIF. TH1 cells were stimulated with antigen + APCs, in the presence of dilutions of natural CSIF, or supernatants from COS cells transfected with the cDNA clone for CSIF, or mock-transfected COS cells.

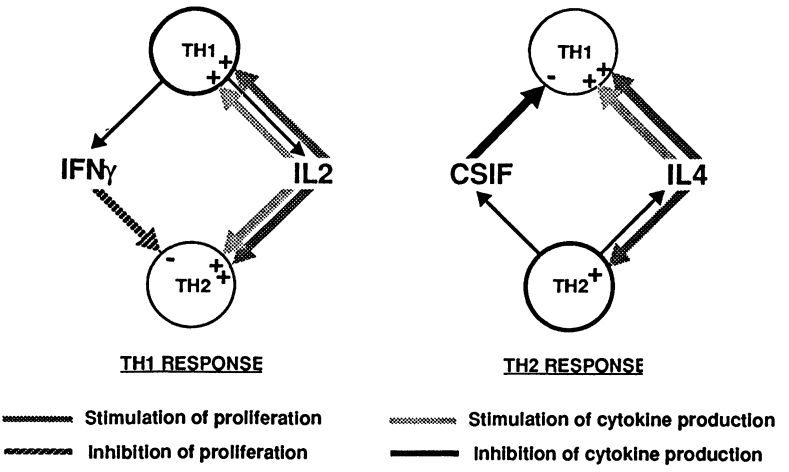

Figure 4. Model of inter-regulation of TH1 and TH2 cells.

The activity of CSIF suggests that this cytokine may be an important regulator of TH1 function (Fig. 4). Since TH1 activation leads to synthesis of IFNγ, which suppresses TH2 proliferation, it is also likely that CSIF will indirectly enhance the growth of TH2 cells. Thus CSIF may be an important component of the networks that control immune class regulation, and may be useful in modifying the class of immune response induced by infections and vaccines. Future studies are aimed at understanding the roles of IFNγ and CSIF in cross-regulation of TH types, and also searching for additional mediators of cross-regulation.

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Interleukin 2 Receptor Targeted Immunotherapy – Update and Attempt at Synthesis

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INTRODUCTION

The adverse effects of currently used immunosuppressive agents and lack of their invariable success in preventing/treating graft rejection or autoimmune diseases, warrant a search for novel therapeutic regimens. Antigen induced lymphocyte activation leads to the induction of cell surface molecules, which are absent in the resting state. One early activation marker, the Interleukin 2 receptor (IL-2R), is expressed transiently on T cells, certain B lymphocytes, macrophages and dendritic cells (reviewed by Diamantstein and Osawa 1986). The interaction between IL-2R and its ligand IL-2, is critical in the cascade leading to expansion of the respective T cell clones. The accruing data from both experimental and clinical studies suggest that IL-2R represents a potential target for specific immunosuppression without detrimental effects upon host normal defense mechanisms. Recent findings on the structure of IL-2R itself and the therapeutic applications of anti IL-2R directed modalities will be discussed in this chapter.

THE IL-2R STRUCTURE: A TARGET FOR IMMUNOSUPPRESIVE THERAPY

Three classes of IL-2R defined by their affinity to IL-2 have been characterized. The **α-chain** (p55 subunit; in humans the **Tac antigen**) and the **β-chain** (p75 subunit) entities bind IL-2 with low ($K_d = 10^{-8}$ M) and intermediate ($K_d = 10^{-9}$ M) affinity, respectively (Robb et al 1984, Sharon et al 1986). The latter apparently contains two distinct but homologous β1 and β2 glycoproteins (Herrmann and Diamantstein 1988). The high affinity IL-2R ($K_d = 10^{-11}$ M) corresponds to a membrane heterodimer composed of noncovalently associated α- and β-chains (Teshigawara et al 1987). The growth signal seems to be delivered by the β-chain of the receptor. However, non lymphoid cells, e.g. L-cells (mouse fibroblast cell line), transfected with both the α- and β-chain encoding genes express intermediate affinity IL-2 binding sites (Hatakeyama et al. 1989). Thus, either constitutive cell membrane component(s) distinct from the α- and β-chain glycoproteins may participate in the formation of functional IL-2R or alternatively, in contrast to the lymphoid cell membrane, non lymphoid cells, e.g. L-cells, may comprise membrane constituents which do not allow formation of the high affinity IL-2 receptor. Indeed, additional complexes of > 70 KD which may constitute a putative gamma chain of high affinity IL-2R have been identified (Herrmann and Diamantstein 1987, Saragovi and Malek 1987).

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Data supporting the second possibility are yet lacking. Theoretically, IL-2R may serve as a target for immunosuppressive therapy by using: a) monoclonal antibodies (mAb) directed against the α- or β-chain, b) toxins, conjugated to the anti α- or β-chain mAb, c) chimeric recombinant anti α- or β-chain mAb toxins, d) IL-2 toxin conjugates, e) chimeric IL-2 toxins (Diphteria toxin or Pseudomonas exotoxin), or f) IL-2 antagonists.

IMMUNOTHERAPY WITH ANTI IL-2R MABS

Most of the experimental and clinical trials utilized mAbs directed against the α-chain of the IL-2R complex. Their efficacy has been tested in a variety of systems including local or systemic GvHD, DTH, allograft rejection and autoimmune diseases (reviewed by Diamantstein and Osawa 1986, Diamantstein et al 1987, Kupiec-Weglinski et al 1988, Diamantstein et al 1989). Recent findings from the latter two will be emphasized.

Allograft Rejection in Experimental Animals

Kirkman et al (1985) first demonstrated that a short term treatment of mice with IL-2R mAb (M7/20, IgM) following transplantation, significantly prolongs the survival or even results in the acceptance of H-2 mismatched cardiac allografts in some strain combinations. This study and the report of Granstein et al (1986) on the additive effect of M7/20 and low dose irradiation on murine skin grafts, prompted comprehensive mechanistic studies in rats, which were followed by the use of anti IL-2R mAbs in subhuman primates and in human.

Two families of Berlin (ART) and Oxford (NDS) produced IL-2R mAbs have been utilized with considerable success in rats to combat acute rejection of heart, kidney, liver, small bowel, pancreatic islets and peripheral nerve allografts (reviewed by Diamantstein et al 1987 and by Kupiec-Weglinski et al 1988). As the interactions between mAbs themselves and feedback with the host immune repertoire has become more appreciated, a cogent picture of the principles for the optimal IL-2R mAb therapeutic strategies has emerged.

The cellular targeting pattern is critical (Kupiec-Weglinski et al 1989), as individual IN VITRO characteristics of IL-2R mAbs do not correlate with their IN VIVO efficacy. Thus, mouse anti rat OX39 and ART-75 mAbs are ineffectual IN VIVO despite displaying the highest affinity and having the largest number of binding sites/target cell, respectively. Although the most effective mAb (ART-18) identifies an epitope identical to or overlapping with the functional IL-2 binding domain, treatment with ART-65, a mAb that affects neither IL-2 binding nor IL-2 driven growth IN VITRO, abrogates acute rejection as well. Moreover, simultaneous targeting of two functionally distinct epitopes may be strongly synergistic (ART-18 + ART-65), additive (ART-75 + ART-35), or may not improve further graft survival at all (ART-18 + OX39). Similarly, anti human IL-2R mAb 1-HT4-4H3, which cross-reacts with Con A activated primate blasts was inoperative IN VIVO, whereas administration of anti Tac, an anti α-chain mAb of the same isotype and similar binding parameters significantly improved renal graft survival in cynomolgus monkeys. All these observations stress the importance of mapping of IL-2R epitope clusters.

The discrete and distinct epitope specificities may also contribute to the non-random biodistribution of IL-2R mAbs (Kupiec-Weglinski, Mariani et al). Antibody "effective" in combating rejection (ART-18) penetrates selectively and specifically host lymphoid tissues and the graft itself, whereas the one which lacks biological activity (OX39) is retained in the circulation. Two points need to be stressed in the conclusions from these studies. Firstly, the heavy glycosylation of the α-chain along with non-random epitopic map arrangement may mask certain areas of IL-2R with resultant selective access of mAbs to different epitopes IN VIVO. Moreover, as the three-dimensional structure of the epitope occurs on both related and unrelated molecules, mAb may be captured IN VIVO before reaching the related targets and/or some epitopes may be more accessible than others for exogenously applied mAb. Secondly, the high blood levels of mAb may not necessarily translate into its high efficacy IN VIVO; this may not be a reliable marker of therapeutic applicability of IL-2R mAbs following transplantation.

Synergy between IL-2R mAbs and cyclosporine (CsA) is of interest because of its potential clinical applicability. Originally described by Diamantstein et al (1986) in rat, recipients of cardiac allografts became a well documented biological phenomenon (reviewed by Kupiec-Weglinski et al 1988a). As noted by immunoperoxidase studies, Ia+ cells and activated macrophages are abundant in cardiac allografts despite ART-18 treatment. Additionally, although IL-2R+ cells are diminished, the cells elaborating IL-2, IFN-gamma and TNF-α are frequent; these probably contribute to the eventual graft loss (heart) or its poor function (kidney, pancreatic islets) in the animals conditioned with mAb alone. As CsA induces transplantation unresponsiveness and greatly depresses cytokine production on its own without affecting IL-2R expression per se, combined treatment with ART-18 and subtherapeutic doses CsA (one-tenth of the standard dose) prevents/reverses acute rejection and produces often permanent graft acceptance with much improved function. Adjunctive CsA contributes to the development of transplantation tolerance in the induction phase of graft survival, presumably by preventing activation and proliferation of alloreactive lymphocytes and activation of macrophages. The lack of dramatic prolongation of kidney allografts in monkeys given anti-Tac in conjunction with low dose CsA (Reed et al 1989) may be related to the relatively lower efficacy of IL-2R mAbs in the uremic state (Ueda, Tilney, unpublished).

Allograft Rejection and Graft versus Host Disease (GvHD) in Humans

Antibodies against human α- and β-chain of IL-2R have been produced and characterized. However, based on the documented reports only few of them, all against the IL-2 binding domain of the α-chain subunit, have been utilized in organ allograft recipients. Recently, such antibodies have been shown to be effective in the treatment of acute corticosteroid resistant GvHD in humans (Hervé et al, 1988). In 1987, Soulillou et al reported that treatment of renal transplant patients with anti human IL-2R mAb 33B3.1 (rat IgG2a), in association with prednizone and azathioprine was efficient in preventing early graft loss. A subsequent randomized trial has confirmed the prophylactic efficacy of 33B3.1 (Soulillou and Jacques). Although mAb induced a 40 % drop in circulating CD4 and CD8 cells, both clinical and biological tolerance were excel-

lent. However, in contrast to rodents, IL-2R mAb was much less efficient in reversing the ongoing rejection.
A randomized trial of renal transplant patients treated with anti Tac (mouse IgG2a) in conjunction with CsA and prednizone is currently underway in Boston (Kirkman et al 1989). This therapy reduces the frequency of early rejection episodes and delays the onset of those which do occur, as compared to controls. As the rejection crises were reversed with OKT3, the use of anti Tac does not preclude subsequent treatment with other mAbs. These data correlate with the report of Friend et al (1988) describing a small group of patients treated with the mAb Campath-6 (rat IgG2b) following liver transplantation who remained free of early rejection. However, anti Tac therapy failed in renal patients given also azathioprine and prednisone. Thus, in contrast to the Nantes series, anti Tac without concomitant CsA is ineffectual. Additionally, unlike 33B3.1, anti Tac did not diminish numbers of the circulating IL-2R+ cells despite continuous mAb treatment. However, the presence or accessibility of epitopes on the same IL-2R subunit differed from patient to patient. It is unclear whether these differences reflect important regulatory subset distribution or have a genetic basis. The early clinical data on the combined anti Tac + low dose CsA treatment are relatively encouraging.

Spontaneously Developing Autoimmune Diseases

The hypothesis that antigen activated IL-2R+ lymphocytes initiate a programmed set of events that culminate in T cell dependent autoimmune diseases has been tested in spontaneously developing autoimmune states, using IL-2R mAbs as probes. In 1987, Hahn et al cured BB rats of freshly manifested spontaneous diabetes, resembling human diabetes type I by a short course of ART-18 plus subtherapeutic doses of CsA. The majority of otherwise diabetic rats remained normoglycemic and maintained B cell volume density and increased pancreatic insulin content throughout. Moreover, the glucose tolerance of successfully treated animals was comparable to that of normoglycemic controls long after the therapy was discontinued. Although ART-18 greatly reduced IL-2R expression, the metabolic success of the therapy correlated with the depletion of Ia+ lymphocytes (Hahn et al 1988). Thus, reduction of IL-2R+ cells alone is not sufficient to interrupt the cascade of events leading to the autoimmune destruction of insulin producing beta cells. More recently, marked prolongation of allogeneic islet graft survival in diabetic BB rats conditioned with ART-18 plus low dose CsA has been reported (Hahn et al 1989). That IL-2R targeted therapy provides a discrete method of mitigating autoimmunity has been also confirmed by Kelley et al (1988) who showed that treatment with M7/20 mAb protected NZB x NZW hybrid mice from the development of lupus nephritis and suppressed diabetic insulitis in NOD mice. Similarly, Weetman et al (1988) demonstrated that the induction of autoimmune thyroiditis in neonatally thymectomized rats depends on the presence of IL-2R+ rather than Ia+ thyroid follicular cells and ART-18 plus low dose CsA therapy prevented the disease.

Mechanisms of action of anti α-chain mAb

Although all these studies provide evidence for the important role of IL-2R+ cells in graft rejection and certain autoimmune states, there is little consensus as to the mode of action of IL-2R mAbs IN

VIVO. Terminal C- component fixation is critical for the beneficial effects of anti mouse M7/20 (Kelley et al 1987), whereas anti human AHT-54 and AHT-107, mAbs of distinct epitope specificities but the same isotype, may act synergistically via potentiation of C3 activation (Diamantstein et al, unpublished). Alternatively, modulation or alteration of IL-2R have been implicated in the effect of anti rat NDS-61 (Tellides et al 1989). In contrast, competitive inhibition of functional IL-2 binding appears to contribute to the effects of anti Tac and 33B3.1 in primates and human, respectively (Reed et al 1989; Soulillou and Jacques).

Much work on the mechanistic aspects of IL-2R targeted therapies has been done in the rat cardiac allograft model employing mAbs of the ART family. Prolongation of graft survival depends on the elimination of IL-2R+ target cells as unequivocally has been shown by Tanaka et al, who compared IN VITRO/IN VIVO effects of ART-18 and its F (ab)/F(ab')2 fragments. The differential efficacy of ART-18 isotype switch variants to affect rejection supports the notion of antigen reactive cell clearance which probably occurs either via ADCC or opsonization, but not via C- mediated lysis of the cells (Stünkel et al 1989). The divergent effects of IL-2R mAbs in rodents upon cellular and humoral components of host immunity and their interactions with the IFN-gamma network, raised the hypothesis that the TH1 but not the TH2 subset of CD4 cells is the prime target of IL-2R targeted therapy (Diamantstein et al 1988).

The unexpected observation of Kupiec-Weglinski et al (1986) on the sparing of donor specific T suppressor cells (Ts) in allografted rats undergoing ART-18 therapy has been expanded. Thus, sparing of phenotypically distinct Ts following treatment with mAbs defining functionally distinct IL-2R epitopes (CD8 in ART-18 and CD4 in ART-65 treated hosts) may indeed contribute to the IN VIVO synergy between both mAbs (Di Stefano et al 1988). Similarly, specific suppressor cells counteract autoaggressive processes in long-term normoglycemic BB rats and in those cured from freshly manifested diabetes by ART-18 therapy (Hahn et al., unpublished). Moreover, the mAbs of ART family do not inhibit the development of functional Ts generated in MLC (Tanaka et al, submitted). All these observations suggest that Ts either express IL-2R that utilize β-chain alone, and/or Ts differentiate in the presence of cytokine(s) other than IL-2.

Host immunization represents the major drawback of IL-2R mAb treatment. Thus, a gradual increase in the anti mouse Ig antibody titer interfering with anti α-chain mAb binding to the receptor may play a decisive role in the relatively low efficacy of anti α-chain mAb in monkeys (Reed et al 1989). Similarly, almost all patients treated with 33B3.1 developed IgM and IgG antibodies, which coincided with the drop of mAb serum concentration; however, no direct correlation between 33B3.1 level and the onset of graft rejection was noted (Soulillou and Jacques). As the anti-idiotype response preceeded the anti-isotype response and did not correlate with the time of rejection, anti mouse Ig antibody presumably operates through opsonization of 33B3.1 rather than direct inhibition of the idiotype binding site on the target cells.

IMMUNOTHERAPY WITH OTHER IL-2R DIRECTED MODALITIES

Recently, new IL-2 toxin hybrids have been produced in E.coli through genetic fusions of cDNA encoding the human IL-2 fused to the modified forms of either Diphtheria toxin (DT) or Pseudomonas exotoxin (PE-40) which lack the sequences encoding the cell recognition domains. It is believed that such chimeric toxins should be more effective than IL-2R mAbs as IL-2 is endocytosed and binds to its receptor with multi-fold higher affinity than mAb. Additionally, IL-2 toxins should be potentially more effective in the intoxication process, as it is thought that high and intermediate affinity (β-chain), but not low affinity (α-chain) IL-2R are endocytosed.

IN VITRO, IL-2-DT or IL-2-PE40 are toxic for cells expressing the high affinity IL-2R (α- and β-chain), but in contrast to anti IL-2R α-chain mAbs, they should be also toxic for NK and some resting CD8+ cells expressing constitutively the β-chain of the IL-2R. Administration of IL-2-DT IN VIVO selectively eliminates IL-2R+ CD4 and CD8 cells and results in a dose dependent suppression of murine DTH (Kelley et al 1988a). Short term therapy with IL-2-DT compound prevented acute rejection of murine pancreatic islets (Pankewycz et al 1989) and cardiac allografts (Kirkman et al 1989a). However, in cardiac allografted rats IL-2-DT treatment was ineffectual on its own and only moderately effective when combined with subtherapeutic doses of CsA (Kupiec-Weglinski, unpublished).

IL-2-PE40 suppressed CTL activity in MLR, and if given IN VIVO, extended the survival of cardiac allografts in mice, with 100 % graft acceptance in some strains at high doses (Lorberboum-Galski et al 1989). IL-2-PE40 proved effective in rats by delaying the onset of adjuvant arthritis and modifying the magnitude of residual arthritis as evaluated by clinical, histological and radiographic criteria (Case et al 1989). Similar to anti IL-2R α-chain mAbs, IL-2-PE40 suppresses DTH but not antibody formation in mice, counteracts acute rejection of allogeneic pancreatic islets in rats as well as the destruction of syngeneic islet cells when grafted into diabetic BB-rats (Diamantstein et al, submitted). However, IL-2-PE40 signifcantly diminished the number of NK as well as of resting CD8+ lymphocytes. Similar to treatment with IL-2R mAb, the inhibition of DTH by IL-2-PE40 was reversable by application of IFN-gamma (Diamantstein et al, submitted).

A recent report of Chaundhary et al (1989) demonstrated that a chimeric toxin, anti α-chain (Fc)-PE40, in which the variable regions of anti α-chain mAb join PE40, is toxic IN VITRO to IL-2R+ (but not IL-2R-) human cell lines. The production of this compound, several times more active on a molecular basis as compared to anti α-chain mAb chemically linked to PE, raises hopes that active recombinant immunotoxins with other antibodies can be created. Finally, IL-2 antagonists, such as IL-2-Asp^{20} substituted with Lys or Phe^{124} deleted have been prepared (Collins et al, 1988). Such IL-2 antagonists interact with the α- but not with the β-chain. They inhibit binding of IL-2 to the α-chain, thereby converting high affinity to intermediate affinity IL-2R. If these early IN VITRO and IN VIVO data on the efficious and safe use of chimeric toxins and other genetically engineered materials can be used reproducibly in large animal models, a real alternative to IL-2R mAbs to target IL-2R+ cells in humans may emerge.

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Molecular Analysis of the Interleukin-2 Receptor Complex: Expression of the Human α and β Chain cDNAs

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INTRODUCTION

Lymphokines, a class of soluble mediators for intercellular communications are known to play a key role in the regulation of the immune system through interaction with specific receptor(s) on the target cells. Interleukin-2 (IL-2), the first of a series of lymphokines to be discovered and completely characterized, plays a key role in the antigen-specific clonal proliferation of T lymphocytes (T cells). Expression of IL-2 and functional, high-affinity IL-2 receptor (IL-2R) complex is induced in the antigen-activated T cells. IL-2 also acts on other cell types such as B lymphocytes (B cells), macrophages, natural killer cells (NK cells), immature thymocytes and neural cells such as oligodendrocytes (for reviews, see Smith, 1984, 1988; Taniguchi et al., 1986; Merrill, 1987). To date, little has been known about the mechanism(s) of signal transduction in the IL-2 system. The receptor for IL-2 is unique in that the ligand binds to at least two distinct membrane components, giving rise to the expression of high-, intermediate- and low-affinity IL-2R forms with the respective dissociation constants (Kds) of about 10^{-11}M, 10^{-9}M and 10^{-8}M (Robb et al., 1984; Smith, 1988). Following the initial expression studies for the clones human IL-2Rα cDNA, it became evident that IL-2Rα constitutes the low-affinity form and it participates in the formation of the functional, high-affinity IL-2R in association with a specific membrane component(s) of lymphoid cells (Hatakeyama et al., 1985; Kondo et al., 1986; Robb, 1986). Subsequently, such a component was identified to be a novel IL-2 receptor chain, termed IL-2Rβ chain (IL-2Rβ or p70-75) (Sharon et al., 1986; Tsudo et al., 1986; Teshigawara et al., 1987; Dukovich et al., 1987). Thus the two IL-2R components, the α and β chain, form the heterodimeric high-affinity IL-2R via noncovalent interactions, whereas the β chain alone constitutes the intermediate-affinity IL-2R. Experimental evidence suggests that IL-2Rβ chain is the main component in driving the IL-2-mediated signal transduction (for review, see Smith, 1988). As an essential step to gain further insight on the molecular basis of the functional (i.e. high-affinity) IL-2R and on the mechanism of IL-2 mediated signal transduction, we have isolated the cDNAs encoding the human IL-2Rβ chain.

ISOLATION OF THE cDNA ENCODING THE IL-2Rβ CHAIN

Poly A^+-RNA was isolated from human cell line YT (Yodoi et al., 1986) and a cDNA library was prepared by a standard procedure (Seed, 1987). The plasmid DNA representing 5.6×10^6 independent colonies were prepared by the standard procedure and the DNA was transfected into COS cells by the standard DEAE dextran procedures (Seed and Aruffo, 1987).

The transfected cells were then treated with the cocktail of Mik-β 1 and -β 2 antibodies (400-fold diluted ascites for each antibody both of which recognize the human IL-2Rβ (Tsudo et al., 1989) and subjected to the standard panning procedure. After the panning, Hirt extract was prepared by the standard procedure (Seed and Aruffo, 1987) and the recovered plasmids were introduced into E. coli. Those bacterial colonies were fused with COS cells by the standard protoplast fusion procedures, followed by the second cycle of panning (Seed and Aruffo, 1987). After four rounds of panning, the enriched plasmid DNAs were analyzed by picking up 30 bacterial colonies arbitrarily. Of these, only 7 colonies contained plasmids from which cDNA inserts can be excised by the restriction enzyme XhoI. The vector-derived XhoI sites are located at the both ends of the cDNA (Seed, 1987) and all other plasmids had lost such cleavage sites due to DNA rearrangements. All of the 7 colonies were derived from the same mRNA, as confirmed by the conventional restriction enzyme cleavage analysis and DNA blot analysis. Of these, one plasmid, termed pIL-2Rβ 30 contained longer cDNA than other 6 plasmids which were turned out to be derived from the same clone (designated pIL-2Rβ 9). In this procedure, therefore, we isolated two independent cDNA clones, pIL-2Rβ 9 and pIL-2Rβ 30; each of the expression products specially reacted with the antibodies (Hatakeyama et al., 1989).

DEDUCED PRIMARY STRUCTURE OF THE IL-2Rβ CHAIN AND COMPARISON WITH THE IL-2Rα CHAIN

The complete nucleotide sequence of the cloned cDNAs were determined (Hatakeyama et al., 1989). The cDNA contains a large open reading frame that encodes a protein consisting of 551 amino acids (a.a.). No significant homology with other known proteins was found in the Protein Sequence Database (National Biomedical Research Foundation, Washington, D.C.) or in our own data base for the recently published sequences. Thus, IL-2Rα chain and IL-2Rβ chain are structurally unrelated (Leonard et al., 1984; Nikaido et al., 1984; Cosman et al., 1984; Hatakeyama et al., 1989). The mature form of the IL-2Rβ chain appears to consists of 525 a.a. of which 214 a.a., 25 a.a. and 286 a.a. constitute the extracellular, transmembrane and cytoplasmic regions, respectively (Figure 1). The extracellular region contains 8 cysteine residues of which 5 residues are found in the N-terminal half and they are interspaced rather periodically by 9-12 amino acids. Presumably, disulfide linkages between the cysteine residues impart a stable configuration for ligand binding. Interestingly, the predicted number of amino acid (a.a.) within the extracellular region of the IL-2Rβ chain (214 a.a.) is similar in number to that of the IL-2Rα chain (219 a.a.) (Fig. 1). Such size similarity may be significant in considering the conformation of the heterodimeric receptor complex that is quite unique for this receptor, as both α and β chains each binds distinct sites of the same IL-2 molecule (Collins et al., 1988). The cytoplasmic region of the β chain is far larger than that of the α chain, which is only 13 a.a. long. The consensus sequences of tyrosine kinase (Gly-x-Gly-x-x-Gly) (Hanks et al., 1988) are absent in the β chain. The primary structure of this region revealed an interesting feature; a rather strong bias for certain characteristic amino acids. This region is rich in proline (24/286) and serine (30/286) residues. The proline-rich structure may impart a non-globular conformation to this region that may be important in coupling of the receptor molecule with other signal transducer(s). The predominant serine residues may be the major target for phosphorylation, which could also modulate the receptor function (Fig. 1). In addition, the cytoplamsic region is notably biased for negatively charged amino acids. Such a bias is

particularly notable in the middle portion (a.a. 345-390) of the cytoplasmic region (Fig. 1). The role of the cytoplasmic region of the IL-2Rβ has to be examined by using systems in which the cDNA-directed IL-2Rβ allows IL-2-mediated growth signal transduction.

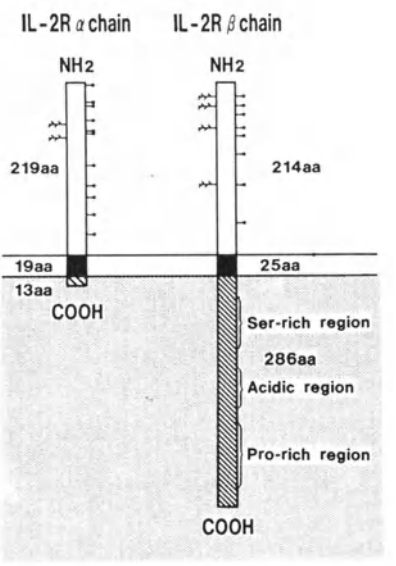

Fig. 1. Structure of the human IL-2Rα and β chains. Symbols on the right and left sides of each column represent the positions of Cystein residues and N-glycosylation sites, respectively. For the details of the IL-2Rβ chain cytoplasmic region, see Hatakeyama et al.(1989).

EXPRESSION OF THE IL-2Rβ CHAIN cDNA IN VARIOUS HOST CELLS

A series of cDNA expression studies were carried out in order to examine if the cDNA product binds IL-2 and indeed manifests the properties of the native IL-2Rβ chain that have been demonstrated and/or suggested in previous studies. A cDNA expression plasmid was constructed in which expression of the cDNA spanning the entire coding region was directed by the mouse lck gene promoter (pLCKRβ) (Hatakeyama et al., 1989). The plasmid pLCKRβ was introduced into the human T cell leukemia Jurkat, which is known to be devoid of surface molecules that bind human IL-2. Stable transformant clones expressing the cDNA product were obtained, as judged by FACS analysis. In addition, we also introduced the same gene into the Jurkat transformant clone, Jα-5, which expresses the transfected, human IL-2Rα chain cDNA. Thus, the resulting transformants was found to express both α and β chains (Hatakeyama et al., 1989). When the IL-2 binding studies were carried out with ^{125}I-labeled recombinant human IL-2, the following binding profiles were obtained by Scatchard plot analyses. Actually, Jurkat clone, expressing the β chain cDNA displayed intermediate-affinity to IL-2 with an estimated Kd value of ~10^{9}M.

Furthermore, the Jurkat clone expressing both α and β chains displayed both high- and low- affinity receptors with respective Kd values of $\sim 10^{-11}$ M and $\sim 10^{-8}$M, as summarized in Fig. 2. The high-affinity IL-2R complex was completely abolished following treatment of the cells by Mik-β1 antibody (Hatakeyama et al., 1989). In contrast to the IL-2Rβ expressed on Jurkat T cell line and a mouse T lymphoma line, EL-4, the IL-2Rβ expressed on non-lymphoid cells such as NIH3T3, L929 and COS cells failed to display significant IL-2 binding ability (Hatakeyama et al., 1989; S. Minamoto, unpublished data). However, expression of both α and β chain in mouse fibroblast cells resulted in the appearance of a new form of IL-2R with a Kd value of $\sim 10^{-10}$M (S. Minamoto, unpublished data). These data suggest that another lymphoid-specific component(s) is involved in maintaining a functional conformation for IL-2Rβ in the absence of IL-2Rα. However, α and β chains can associate with each other on fibroblast cells and create IL-2R complex with increased affinity to IL-2 (Fig. 2).

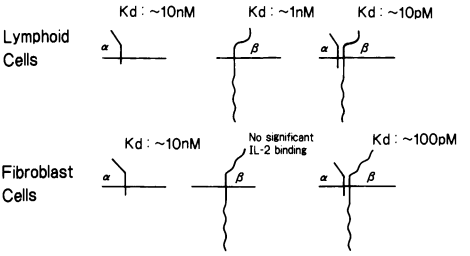

Fig. 2. Expression of the various forms of the IL-2R in lymphoid and fibroblast cells. See text and Hatakeyama et al.(1989) for the details.

PERSPECTIVES

We reported the isolation, characterization and expression of a cDNA encoding the human IL-2Rβ chain. The availability of the cDNAs for IL-2 (Taniguchi et al., 1983), IL-2α chain (Leonard et al., 1984; Nikaido et al., 1984; Cosman et al., 1984) and IL-2Rβ chain (Hatakeyama et al., 1989) will make it possible to gain further insights of the signal transduction mechanism in IL-2 system. The functional IL-2 receptor complex is unique in that two structurally distinct membrane components, the IL-2Rα and IL-2Rβ chains, both bind IL-2 independently. Our preliminary data suggest that IL-2 binding to both α and β chains is important for the IL-2-mediated growth signal transduction (T. Doi et al., manuscript in preparation). At present, little is known about the cascade of biochemical events triggered by cytokines interacting with their homologous receptors. Our findings on the structure of the IL-2Rβ chain demonstrate the presence of a large cytoplasmic region which appears to be involved in driving the IL-2 signal pathway(s). The availability of the expression system in which the cDNA-encoded β chain can deliver growth signal (T. Doi et al., manuscript in preparation) will allow us to dissect further the functional domains of the receptor. Finally, evidence for dysregulation of the IL-2Rβ gene in cellular transformation has been obtained recently (T. Kono, unpublished data). The possibility that the aberrant IL-2Rβ gene expression is involved in the generation of various leukemic cells is to be examined further.

ACKNOWLEDGMENT

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Interleukin 6 and Its Receptor in Immune Regulation

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INTRODUCTION

Several factors have been shown to be involved in the regulation of B cell responses into antibody producing cells. The cDNAs for three interleukins, IL-4, IL-5 and IL-6, have been molecularly cloned and functions of these molecules in the activation, proliferation and differentiation of B cells were confirmed utilizing recombinant molecules (Kishimoto and Hirano 1988). However, the studies with recombinant molecules also showed that the functions of these molecules are not restricted to B lineage cells but have a wide variety of biological activities on various tissues and cells. One of the typical examples of multifunctional interleukins is IL-6 (Hirano and Kishimoto in press).

IL-6 was originally identified as a T cell-derived lymphokine which induced the final maturation of B cells into antibody producing cells (Hirano *et al* 1985, 1986). However, subsequent studies demonstrated that it acted not only on B cells but also on hematopoietic stem cells and hepatocytes and induced hematopoiesis as well as acute phase reactions. As summarized in Fig. 1, it was also shown to act on T cells, nerve cells, keratinocytes, renal mesangial cells, megakaryocytes and myeloma/plasmacytoma cells. Since antibody production, hematopoiesis and acute phase reactions are three major responses against infection, inflammation and tissue injuries, IL-6 may have a central role in host-defense mechanisms. On the other hand, IL-6 has two edges and the deregulation of the IL-6 gene expression was shown to be involved in various diseases as summarized in Table 1.

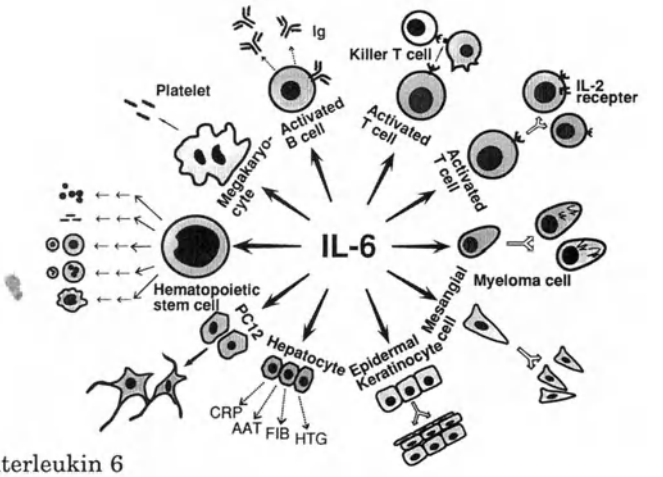

Fig. 1. Multifunctions of interleukin 6

Table 1. Interleukin 6 and diseases

Cardiac myxoma (Hirano *et al* 1987)
Rheumatoid arthritis (Hirano *et al* 1988)
Castleman's disease (Yoshizaki *et al* in press)
Plasmacytoma and myeloma (Nordan and Potter 1986, Kawano *et al* 1988)
Lennert's T cell lymphoma (Shimizu *et al* 1988)
Renal cell carcinoma (Miki *et al* in press)
Mesangio-proliferative glomerulonephritis (Horii *et al* submitted)

THE BIOLOGY OF IL-6

i) Regulation of antibody response.

The experiments with recombinant IL-6 (rIL-6) confirmed that IL-6 functions as B cell differentiation factor *in vitro* as well as *in vivo*. The addition of 1 to 10 ng/ml of rIL-6 could augment pokeweed mitogen (PWM)-induced IgM, IgG and IgA production in human peripheral mononuclear cells *in vitro*. Anti-IL-6 antibody could inhibit PWM-induced Ig-production, indicating that IL-6 is one of the essential factor for B cells to produce immunoglobulin (Muraguchi *et al* 1988). rIL-6 could also enhance IgA synthesis in freshly isolated murine Peyer's patch B cells (Beagley *et al* 1989). The effect of IL-6 on IgA induction was greater than that of IL-5. Isotype-switching was not a mechanism for this marked increase in IgA synthesis, since membrane IgA-negative B cells were not induced to secrete IgA by rIL-6. The persistent infusion of rIL-6 by diffusion pump augmented *in vivo* anti-SRBC antibody production more than 30 fold in SRBC-primed mice, indicating that IL-6 could enhance antigen-specific antibody response *in vivo* (Takatsuki *et al*, submitted).

IL-6 could also induce growth and differentiation of T cells. As will be described, IL-6 receptors were shown to be expressed on activated as well as resting T cells, indicating that IL-6 could function as growth and differentiation factor as well as activation factor. IL-6 promoted the growth of mitogen-stimulated thymocytes and peripheral T cells (Lotz *et al* 1988, Le *et al* 1988, Helle *et al* 1988). IL-6 was also shown to induce the differentiation of cytotoxic T cells in the presence of IL-2 from murine as well as human thymocytes and splenic T cells (Takai *et al* 1988, Okada *et al* 1988). Furthermore, it was found that IL-6 could promote the growth and differentiation of double negative T progenitor cells into double positive or $CD4^+$ thymocytes in thymus. (Nakano, unpublished data).

ii) Effect of IL-6 on hematopoiesis

It was reported that IL-3 and IL-6 acted synergistically to support the formation of multilineage blast cell colonies in murine spleen cell cultures (Ikebuchi *et al* 1987). Our subsequent studies demonstrated that short-term liquid culture of murine non-adherent bone marrow cells with IL-6 and IL-3 was found to increase the number of CFU-S approximately 5-fold. When $2x10^5$ non-adherent bone marrow cells were transplanted to lethally irradiated recipients, the survival rate at day 30 was only 20%. However, when these cells were pre-cultured with IL-6 plus IL-3 before transplantation, the survival rate was raised to 90% (Okano *et al* in press).

Recently, Ishibashi *et al* (in press a) found that rIL-6 induced the maturation of megakaryocytes *in vitro*. It promoted marked increments in megakaryocytic size and acetylcholinesterase activity, a marker enzyme of the lineage. IL-6 also induced a significant shift foward higher ploidy classes. Our following studies confirmed that IL-6 is a potent thrombopoietic factor *in vivo* in mice (Ishibashi *et al* in press b, Suematsu *et al* in press).

iii) Transgenic mice with the human IL-6 gene

The study with human myeloma cells isolated from patients with multiple myelomas demonstrated that IL-6 is an autocrine growth factor for human myeloma cells; i) myeloma cells produce IL-6, ii) myeloma cells express IL-6 receptors, iii) rIL-6 augments *in vitro* growth of myeloma cells and iv) anti-IL-6 antibody inhibits the spontaneous growth of myeloma cells (Kawano *et al* 1988). The result suggested that deregulation of the IL-6 gene expression may be responsible for the oncogenesis of human multiple myelomas.

In order to test this possibility, we prepared transgenic mice with the human IL-6 gene conjugated with the immunoglobulin enhancer for the constitutive expression of IL-6 in B lineage cells (Suematsu *et al* in press). In all 10 separate founder mice, we could detect a marked increase in serum IgG_1 and enlargement of spleen, thymus and lymph nodes. All mice died within several months and the histology of lymph nodes showed a massive infiltration of plasma cells. However, Southern blot analysis of DNAs from lymph nodes or spleen did not show any monoclonal or oligoclonal rearrangements of the Ig-heavy chain genes. Furthermore, plasma cells were not transplantable to syngeneic recipients. Therefore, this is not a typical monoclonal plasmacytoma but rather γ_1-plasmacytosis. The result suggests that certain secondary changes, such as the c-*myc* gene activation, are required for the generation of monoclonal, transplatable plasmacytomas.

The other changes consistently observed in Eμ-IL-6 transgenic mice were increase in mature megakaryocytes in bone marrows and mesangial proliferative glomerulonephritis. Subsequent studies revealed that IL-6 functions as thrombopoietin as described and IL-6 is also shown to be a potent growth factor for kidney mesangial cells (Horii *et al*, submitted). Increase in the IL-6 level in urine of patients with mesangial proliferative glomerulonephritis was observed, suggesting the involvement of the deregulation of the IL-6 gene expression in this disease.

IL-6 RECEPTOR

i) The regulation of its expression

As expected from a pleiotropic function of IL-6, its receptor (IL-6R) is expressed on various tissues and cells (Taga *et al* 1987) as summarized in Table 2. In normal B cells, resting B cells do not express IL-6R, but IgD-negative activated B-blast cells were found to express the receptors, when examined with anti-IL-6R monoclonal antibodies (Fig. 2). The result is in agreement with the previous report that surface IgD negative B cells could be led to a final maturation stage to produce immunoglobulins. In contrast to B cells, IL-6R was detected on non-stimulated $CD4^+/CD8^-$ as well as $CD4^-/CD8^+$ T cells. The level of IL-6R on both T cell subpopulations was not significantly changed after mitogen-stimulation.

Table 2. Expression of IL-6 receptor on a variety of cells

cells	number of receptor/cell
EBV-transformed B cell lines	200 - 3,000
Burkitt's lymphoma cell lines	not detectable
myeloma cells and cell lines	100 - 20,000
hepatoma cell lines	2,000 - 3,000
myeloid leukemia cell lines	2,000 - 3,000
rat pheochromocytoma cell line (PC12)	~ 1,200
resting B cells	not detectable
activated B cells	~ 500
resting T cells	~ 300

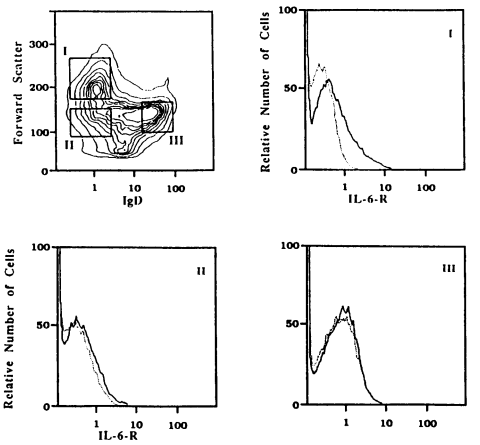

Fig. 2. Immunofluorescent analysis of IL-6R on activated B cells. B cells purified from tonsilar mononuclear cells were cultured with PWM for 4 days. Level of surface IgD and forward scatter were expressed in the upper left panel. IgD negative large (I), IgD negative small (II), and IgD positive small (III) cells were analyzed for IL-6R expression using phycocyanine-conjugated MT18 monoclonal antibody. Dotted lines represent staining without PC-MT18.

ii) Molecular structure of IL-6 receptor

As depicted in Table 2, the number of IL-6R expressed on various cells is extremely low, in the range of 10^2 and 10^3. Therefore, we employed high efficiency COS cell expression vector, CDM8 for the molecular cloning of the receptor and the cells which expressed IL-6R were detected by fluorescein-labelled IL-6. The cDNA encoding IL-6R was molecularly cloned and the nucleotide sequence of the IL-6R cDNA was determined (Yamasaki *et al* 1988). There is a single open reading frame, in which the initiator ATG is followed by 467 codons before the termination at triplet TAG. A hydropathy plot of the deduced amino acid sequence indicated that IL-6R had a single transmembrane domain and its intracytoplasmic portion consisted of 82 amino acids. The IL-6R lacks tyrosine kinase domains, unlike some other growth factor receptors, although IL-6 has been found to be a potent growth factor for myeloma/plasmacytoma cells. Intracytoplasmic portion of IL-6R does not include any unique structure for the signal transduction.

The comparison of the deduced amino acid sequence of IL-6R demonstrated the homology with several members of the Ig-superfamily. The IL-6R sequence between position 20 and 110 fulfills the criteria proposed by Williams and Barclay (1988) for the

constant 2 (C2) set of Ig-superfamily. Interestingly, the receptors for polypeptide growth factors, such as PDGF, CSF-1, IL-1 and IL-6 could then be grouped in Ig-superfamily. However, the deletion of Ig-like domain did not affect the binding of IL-6, indicating that Ig-like domain is not involved in the ligand binding. The structure of IL-6R was schematically summarized in Fig. 3.

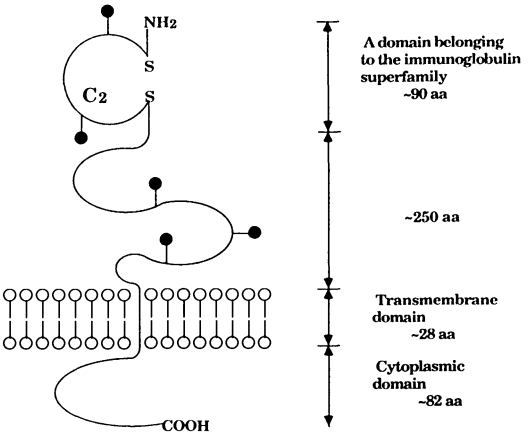

Fig. 3. Schematic model of human IL-6R

iii) IL-6 stimulation triggers the association of 80k IL-6R and a second non-ligand binding chain, gp130

As described, an intracytoplasmic portion of IL-6R does not include any unique structure for the signal transduction. In order to elucidate the possible presence of a signal transducing molecule associated with the IL-6R, IL-6R molecule was precipitated under a non-dissociating condition with a monoclonal anti-IL-6R antibody, MT18 (Taga *et al* in press). MT18 monoclonal antibody precipitated 80k IL-6R from a human plasmacytoma cell line, U266 under mild lysis conditions (1% digitonin buffer). Another polypeptide chain with a Mr of 130k was coprecipitated only when the cells were incubated with IL-6 at 37°C for 30 min. before digitonin-lysis (Fig. 4a). The result suggested that IL-6 triggered the association of the 80k IL-6R with a cellular 130k molecule. This 130k molecule was further demonstrated to be a glycoprotein by endoglycosidase treatment and thus called gp130.

The transfection of human IL-6R cDNA into the murine B lymphoma line M12 which has no detectable number of IL-6R, demonstrated that human 80k IL-6R can associate with murine gp130. Although M12 cells showed no responsiveness to IL-6, M12 cells expressing human IL-6R responded to IL-6 with a decrease in their growth. The result indicated that M12 cells expressed a murine gp130 homologue but did not bind IL-6 nor respond to IL-6, confirming that gp130 does not have ligand-binding property by itself.

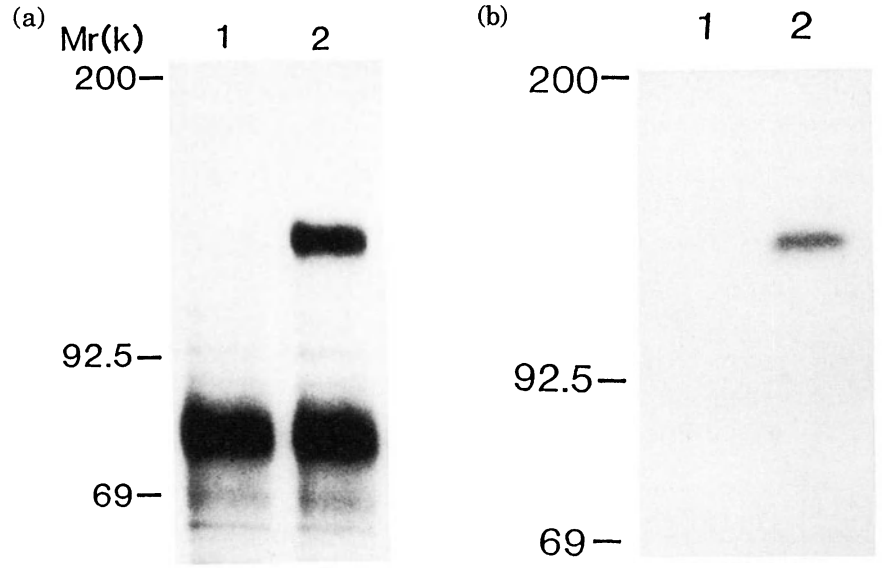

Fig. 4. Association of IL-6R and gp130. (a) Metabolically labeled U266 myeloma cells were incubated with (lane 2) or without (lane 1) IL-6 at 37°C for 30 min. After digitonin lysis, immunoprecipitates were analyzed on SDS-PAGE under reducing conditions. (b) Surface-iodinated M12 cells were incubated with (lane 2) or without (lane 1) IL-6 at 37°C for 30 min in the presence of COS7 culture supernatant containing soluble IL-6R.

iv) An intracytoplasmic portion of IL-6R is not required for the signal transduction

A cDNA of IL-6R or a mutant cDNA, IL-6RΔIC, which did not have its intracytoplasmic portion, was transfected into the murine myeloid leukemia cell line, M1 and transformants were obtained. Anti-IL-6R antibody, MT18, could coprecipitated IL-6R or the mutant IL-6R lacking its intracytoplasmic portion with a murine gp130 homologue when transfectants were preincubated with IL-6, indicating that intracytoplasmic portion of IL-6R was not required for the interaction of IL-6R with a gp130 moleule.

Both M1-transfectants, M1IL-6R and M1IL-6RΔIC, expressed 20 - 40 times higher density of IL-6 binding sites compared to the parental M1 cells. It made both the transfectants more sensitive to IL-6 in their growth inhibition, indicating that human IL-6R on murine M1 cells could transduce the IL-6 signal even with a truncated intracytoplasmic region.

In order to confirm that the intracytoplasmic portion of IL-6R is not required for the signal transduction, we prepared soluble IL-6R without intracytoplasmic and transmembrane portions and asked whether the complex of soluble receptor and IL-6 could bind with gp130 and transduce the signals. The soluble IL-6R was mixed with a surface iodinated murine B lymphoma cell line, M12, which did not have IL-6R but expressed a murine gp130 homologue, and incubated with or without IL-6, then digitonin-lysis and immunoprecipitation with anti-IL-6R antibody were processed. As shown in Fig. 4b, gp130 was detected on SDS-PAGE from the cells incubated with the soluble IL-6R and IL-6. The co-immunoprecipitation of gp130 with the soluble IL-

6R was not observed in the absence of IL-6. Thus, these results confirmed that the soluble IL-6R could associate with gp130 in the presence of IL-6.

When M1 cells were incubated with the soluble IL-6R in the presence of IL-6, the soluble receptor could augment the sensitivity of M1 cells to IL-6 in their growth inhibition. However, without IL-6 the soluble IL-6R did not show any inhibitory effect on M1 cells. The result indicated that the complex of the soluble IL-6R and IL-6 could transduce the signals by binding with gp130.

SUMMARY - A NOVEL MECHANISM(S) OF THE IL-6 MEDIATED SIGNALS -

We have demonstrated that 80k IL-6R is the only molecule involved in ligand binding and that IL-6R associates with a possible signal transducer, gp130 in the presence of IL-6. The observations indicate that the IL-6R system is composed of two functional chains; a ligand-binding 80k IL-6R and a non-ligand binding but signal transducing gp130. The binding of IL-6 with IL-6R may induce a certain allosteric change of the receptor molecule at its extracellular portion, which may trigger the association with gp130 as schematically shown in Fig. 5. Since gp130 is expressed on various cells which do not express IL-6R, it might function as a signal transducer for other cytokines. If this is the case, the IL-6R system might be more generalized for other cytokine systems. Molecular cloning of gp130 will provide useful informations on an unique mechanism of the signal transduction through cytokine receptors.

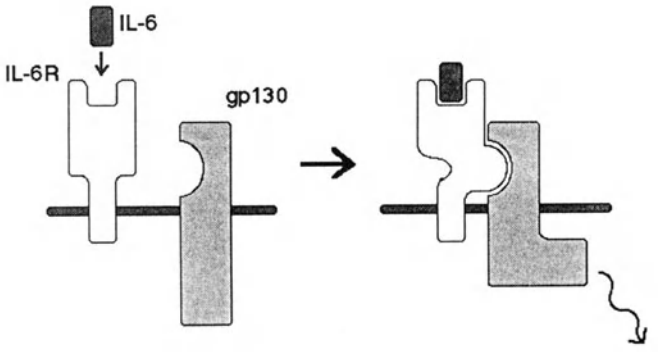

Fig. 5. Schematic model of IL-6R and gp130.

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The Molecular and Cellular Biology of the IFNγ Receptor

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It is now well recognized that interferon-gamma (IFNγ) plays a pivotal role in promoting host defense. It exerts its effects either through nonimmunologic or immunologic mechanisms. In the former case, IFNγ resembles the other two members of the IFN family (IFNα and IFBß) by its ability to induce antiviral and antiproliferative activities in a variety of normal and neoplastic cells. However, IFNγ is distinguished from IFNα/ß by its numerous and potent immunomodulatory activities. During the past 10 years, work from several laboratories has established that IFNγ plays a major role in modulating expression of Class I and Class II antigens of the major histocompatibility complex. It has also been identified as one of the primary lymphokines responsible for activating or otherwise regulating a variety of macrophage functional activities such as the capacity to nonspecifically kill a variety of intracellular and extracellular parasites and neoplastic cells. IFNγ is now known to regulate B cell responses in either a positive or negative manner depending upon the state of cellular differentiation. Finally, IFNγ regulates the production and secretion of other immunomodulatory cytokines such as IL-2, tumor necrosis factor, and indirectly, IL-1.

IFNγ induces these pleiotropic biologic responses by interacting with a specific receptor at the target cell surface. During the past several years, a number of laboratories, including our own, have demonstrated IFNγ receptors on a wide variety of human and murine cell types (reviewed in Langer and Pestka, 1988). Using radioligand binding and chemical cross-linking techniques, murine and human IFNγ receptors have been generally characterized as 70-100 kDa, single chain polypeptides which are expressed in only modest numbers on cell surfaces (500-20,000 sites/cell) but which bind their respective ligands in a species specific manner with moderately high affinity ($1x10^9$ - $1x10^{10}$ M^{-1} at 4°C). Under physiologic conditions, these receptors were found to direct the internalization and eventual degradation of ligand. Moreover, in many cell types (such as macrophages and fibroblasts), IFNγ receptor recycling back to the cell surface was observed. Expression of this receptor shows little tissue specificity. It is present on a wide variety of hematopoietic cells (such as mononuclear phagocytes, polymorphonuclear leukocytes, B cells, many T cells, platelets, but not erythrocytes), somatic cells (such as epithelial and endothelial cells) and primary and cultured tumor cells. Although these earlier studies unequivocally established the existence of IFNγ-specific cell surface receptors, they left unanswered the question of whether more than one IFNγ receptor existed and provided little insight into how this protein functioned. In this manuscript we summarize work performed in our laboratories over the past four years that has helped to fully elucidate the structure of the human and murine IFNγ receptors and better define the role that these proteins play in inducing cellular responses.

Purification of the Human IFNγ Receptor from Placenta

Four years ago, we set out to isolate and characterize the human IFNγ receptor in an attempt to unequivocally determine whether IFNγ receptors on different cell types were related. Part of this goal was achieved two years ago when we succeeded in purifying the human IFNγ receptor on a preparative scale from placenta (Calderon et al., 1988). We specifically chose to work with human placenta because we found that placental membranes expressed extremely high levels of IFNγ receptor. Radioligand binding data indicated that placental membranes expressed 2.0-5.9 x 10^{12} receptors/μg membrane protein, a value that was 13-163 times higher than the amount of receptor expressed on a typical cultured cell line such as U937. This observation meant that a single 400 gm human placenta supplied as much receptor (60-200 μg) as a thousand liter culture of U937 (10^{12} cells). We also established an assay capable of rapidly quantitating the soluble IFNγ receptor. The assay we eventually developed was based on the ability of polyethylene glycol to selectively precipitate ^{125}I-labeled human IFNγ only when it was in complex with soluble IFNγ receptor. Using this assay we showed that (1) soluble IFNγ receptors bound radioligand in a specific, reversible and saturable manner and (2) the IFNγ receptor could be quantitatively recovered from detergent solubilized placental membranes in fully active form.

After completing these two preliminary studies we then focused our efforts on purifying the receptor. We initially used a two step isolation protocol that involved sequential affinity chromatography of solubilized placental membrane on Sepharose columns containing immobilized wheat germ agglutinin (WGA) and purified recombinant human IFNγ. This protocol resulted in the 5800 fold purification (theoretical purification =8300x) of 20 μg of active receptor with an overall yield of 25%.

Purified receptor was injected into mice to prepare receptor-specific monoclonal antibodies. Hybridoma supernatants were screened for the ability to block the specific binding of ^{125}I-IFNγ to placental membranes (Sheehan et al., 1988). Ten cultures were identified that inhibited binding between 10 and 100%. Three hybridomas (denoted GIR-208, GIR-301, and GIR-94) were cloned and purified monoclonal antibodies produced. Purified GIR-208 (an IgG1) and GIR-301 (an IgA) completely inhibited the binding of radioligand to either soluble or membrane-associated forms of the placental IFNγ receptor. Epitope mapping analysis revealed that the two antibodies reacted with similar epitopes on the IFNγ receptor that were similar to or linked to the receptor's ligand binding site. In contrast, GIR-94 displayed only marginal (10%) receptor blocking activity and did not compete with either GIR-208 or GIR-301 for receptor binding. Thus, the three antibodies identified at least two distinct epitopes on the extracellular domain of the placental IFNγ receptor.

We now utilized these reagents to firmly establish that the placental membrane protein we had purified was indeed the IFNγ receptor. When analyzed by SDS-PAGE (either under reducing or nonreducing conditions) and silver staining the purified IFNγ receptor migrated as a single modestly diffuse component, of Mr 90 kDa. Unequivocal identification of this component as the IFNγ receptor was accomplished by Western blot analysis using either

labeled IFNγ or labeled receptor specific monoclonal antibody as the developing reagent. In either case, the same 90 kDa modestly diffuse component was again identified. The Western blot patterns were specific because (1) ligand blotting was inhibited if the electrotransfers were pretreated with either excess unlabeled human IFNγ or GIR-208 (unlabeled murine IFNγ and irrelevant isotype matched IgG were not inhibitory), and (2) identical patterns were obtained using any of the three receptor-specific monoclonal antibodies while no bands were detected using irrelevant isotype matched IgG controls. Moreover, both ligand and antibody blotting patterns were substantially reduced or eliminated entirely if the receptor was subjected to SDS-PAGE under reducing conditions. These results thus confirmed that the 90 kDa single chain polypeptide isolated from placenta was the IFNγ receptor and indicated that the receptor contained at least one critical intrachain disulfide bond.

The availability of high affinity, receptor-specific monoclonal antibodies permitted us to improve our purification and detection protocols. Currently we purify the receptor by chromatography on GIR-208 Sepharose and subsequently on WGA-Sepharose. The receptor is now detected with a double monoclonal antibody (GIR-208 and GIR-94) ELISA. These modifications have allowed us to purify larger quantities of receptor (50-200 μg) in less time (3 days) and have provided sufficient material for protein sequencing studies.

During the course of these studies, we observed that certain purified receptor preparations contained a second component of Mr 55 kDa that reacted with all 3 receptor-specific monoclonal antibodies. Subsequent experiments revealed that the 55 kDa component represented a proteolytic breakdown product of the IFNγ receptor (Calderon et al., 1989). An identical 55 kDa fragment could be intentionally generated by trypsin treatment of purified, 90 kDa receptor preparations. Moreover, the intact receptor, the spontaneously produced-fragment, and the trypsin generated-fragment could be equally reconstituted into liposomes and thereby confer upon the particles the ability to react with ligand and receptor specific monoclonal antibodies. These results indicated that the 55 kDa fragment consisted of the entire extracellular and transmembrane receptor domains, and a small portion of the intracellular domain. The data also suggested that the transmembrane domain of the receptor was located in the approximate center of the molecule.

Immunochemical Analysis of IFNγ Receptors on Different Cells

Although the previous experiments helped to elucidate the structure of the IFNγ receptor on placental membranes, they did not answer the question of whether IFNγ receptors on different cells were similar. We chose to initially address this issue immunochemically. We first examined whether ^{125}I-IFNγ could bind to cells that were pretreated with either GIR-208 or GIR-301 (Sheehan et al., 1988, Khurana Hershey and Schreiber, 1989a). The antibodies blocked 100% of the binding of radioligand to all cells tested in our laboratory to date. These include mononuclear phagocytes (freshly isolated peripheral blood monocytes, U937 and THP-1), peripheral blood T cells, fibroblasts (WISH), epithelial-like adenocarcinoma cells (COLO-205), and the HepG2 hepatoma cell line. Moreover, GIR-208 and GIR-301 also blocked induction of IFNγ-dependent biologic responses in these cells. These experiments have been performed on peripheral blood monocytes (IP10 induction, Fc

receptor induction), THP-1 (MHC Class II induction), and WISH cells (antiviral activity).

The second approach was to conduct an immunohistochemical analysis of the IFNγ receptor on primary human tissues and cells (Luquette et al., 1989). Using immunoperoxidase and immunofluorescence techniques, the three receptor-specific monoclonal antibodies specifically stained nearly all cells in tissues and peripheral blood. A notable exception were erythrocytes where no staining was detected. It was also noteworthy that certain specialized nonimmune cell types, such as the placental syncycial trophoblasts, vascular endothelial cells, nerve cells, and epidermal cells of the skin displayed extremely intense specific staining indicating that they expressed far greater amounts of IFNγ receptor than the cells of the immune system. Taken together, this data indicates that IFNγ receptors on different cells are antigenically related and directly documents the nearly ubiquitous expression of IFNγ receptors on the surface of primary normal cells.

Structural Analysis of IFNγ receptors on Different Cells.

We next sought to determine whether IFNγ receptors on different cell types were structurally related. To approach this question, we established a biosynthetic labeling and immunoprecipitation system for the IFNγ receptor (Khurana and Schreiber, 1989a). These studies revealed that mature IFNγ receptors on COLO-205 and HepG2 displayed molecular masses of 90 kDa and were structurally indistinguishable from the IFNγ receptor of placenta. Using pulse-chase analysis and extrinsic labeling techniques on untreated- or tunicamycin-treated COLO-205, we demonstrated that the IFNγ receptor is post-translationally modified as it matures into the 90 kDa form that is exclusively expressed on the cell surface. These experiments revealed that N-linked oligosaccharides contribute 20 kDa to the apparent molecular weight of the mature receptor and also indicated that mature forms of the receptor carry some other form of post-translational modification possibly O-linked oligosaccharides. As determined by Western blot experiments, glycosylation was not required for expression of the receptor's ligand binding activity or for maintenance of the GIR-94, GIR-208, or GIR-301 epitopes. When similar analyses were performed on other cell types, a minor degree of molecular weight heterogeneity was observed. In this experiment metabolically labeled receptor from untreated COLO-205 migrated predominantly as a 90 kDa component. A minor second component of Mr 75 kDa was also observed. This component was identified as the penultimate receptor biosynthetic intermediate that contained high mannose N-linked oligosaccharides which had not yet been converted to complex oligosaccharides. In contrast, the fully mature ^{35}S-labeled IFNγ receptors from U937 and BeWo-30 cells (a human cultured trophoblast cell line) displayed molecular weights of 95, and 85 kDa respectively. However, immunoprecipitates from all three cell types commonly displayed the 75 kDa component. This observation suggested that the molecular weight differences might be attributable to differences in post-translational processing. To validate this hypothesis, cells were treated with tunicamycin before and during the metabolic labeling phase of the experiment. Under these conditions all three cells produced a common receptor protein that displayed an Mr of 65 kDa. These results thus demonstrated that IFNγ receptors on different cells are structurally similar and differ only in their content of N-linked oligosaccharides.

Cloning and Expression of the Human and Murine IFNγ Receptor cDNAs.

Our ability to purify reasonably large quantities of the human IFNγ receptor allowed us to conduct amino acid sequence analysis on the protein. We obtained sequence from three regions of the molecule; the N-terminus (26 residues) and two internal regions contained in Lysine C-derived receptor peptides of 14 and 19 amino acids. Based on these sequences, we generated a family of synthetic oligonucleotides and probed a human placental library prepared in the λgt10 vector. This screening procedure eventually identified a family of three clones that hybridized to an oligonucleotide based on the 19 amino acid Lysine C peptide. While we were sequencing our longest clone, a paper by Aguet et.al. (1988) appeared that reported the cloning and characterization of a human IFNγ receptor cDNA isolated from a Raji cell λgt11 expression library. A comparison of the two cDNA sequences indicated that we had indeed isolated a full length 2.1 Kb receptor clone that differed from the sequence of Aguet et.al. by 7 nucleotides (Gray et al., 1989a). Only one nucleotide difference occurred in the coding region and did not result in an amino acid alteration. Two of the nucleotide differences were found to be a result of genetic polymorphism in the human IFNγ receptor gene. The cDNAs encode a protein of 489 amino acids that (based on our amino acid sequence data) contains a 17 amino acid signal sequence and a single hydrophobic transmembrane domain located in the approximate middle of the molecule. The 229 amino acid extracellular domain of the receptor contains 5 potential N-linked glycosylation sites and 2 serine/threonine rich regions which may serve as acceptor sites for addition of O-linked oligosaccharides. The 223 amino acid intracellular domain contains an unusually high content of serine and threonine resides. The latter constitute 23% of the amino acids in this portion of the molecule. No sequence homology between the human IFNγ receptor and any other protein was found. Using a ^{32}P-labeled 480 base EcoRI fragment derived from the 5' end of our cDNA, we performed Northern blot analysis on mRNA derived from a number of tissues and cells. Only a single 2.3 Kb transcript was identified. Moreover, Southern blot analysis of human genomic DNA revealed the presence of only a single human IFNγ receptor gene. This data thus supports the concept of a single type of IFNγ receptor on different cells.

It has been difficult to obtain stable expression of the human IFNγ receptor cDNA in mammalian cells. Aguet et al. (1988) also reported this difficulty but eventually succeeded in obtaining stable, but low level expression of the human IFNγ receptor in murine cells using the entire human IFNγ receptor gene. Although the transfected murine cells acquired the ability to bind human IFNγ, they did not respond to it. This important observation indicated that the transfected murine cells either lacked sufficient quantities of the human receptor to manifest a biologic response or that formation of a functionally active human IFNγ receptor in heterologous cells required the presence of a second human component. The latter possibility had been originally suggested by Jung et. al. (1987) on the basis of experiments using human-rodent somatic cell hybrids.

In an attempt to resolve this issue, we decided to isolate and express the cDNA encoding the murine IFNγ receptor. We recently isolated a full length murine IFNγ receptor cDNA by screening a

murine T cell hybridoma library prepared in λgt10 with probes prepared from the human IFNγ receptor cDNA (Gray et al., 1989b). The 2065 base cDNA encodes a polypeptide of 477 amino acids that shows a molecular organization that is similar to its human counterpart. It consists of a 26 amino acid signal sequence and nearly equivalent sized intracellular and extracellular domains of 228 and 203 amino acids respectively that are separated by a single transmembrane domain. The extracellular domain contains 5 potential N-linked glycosylation sites and the intracellular domain is serine/threonine rich. However, despite their similar molecular structure, the human and murine proteins are only 52% homologous at the primary sequence level. While it is not surprising that the extracellular domains of the two proteins show only modest (50%) homology (because they bind, in a species specific manner, to ligands that are only 40% homologous), it is quite unusual to find such little sequence identity (55%) in intracellular domains of identical membrane proteins from different species. This observation suggests that the receptor may have the capacity to interact with intracellular components in a species-specific manner.

This hypothesis has been strengthened by our recent studies using human fibroblasts (293 cells) stably transfected with the murine IFNγ receptor cDNA. Stable transfectants were first selected for neomycin resistance and then were sorted for their ability to bind murine IFNγ using flow cytometry. Biosynthetic labeling analysis of the transfected cells indicated that they produced a normal form of the murine protein which migrated as a 90 kDa component on SDS-PAGE and was indistinguishable from metabolically labeled natural forms of the receptor present on murine EL-4 cells. Moreover, this molecular weight is consistant with the Mr observed for a purified preparation of the murine IFNγ receptor reported by Basu et.al. (1988). The recombinant murine receptor displayed normal ligand binding activity. As determined by Scatchard analysis, the sorted transfected human cells expressed 530,000 recombinant murine IFNγ receptors/cell while nontransfected cells showed no specific binding of the murine ligand. Thus, unlike its human counterpart, the murine IFNγ receptor could be stably expressed at high levels in heterologous cells. The Ka for murine IFNγ displayed by the recombinant murine IFNγ receptor on 293 cells ($1.0 \times 10^9\ M^{-1}$) was indistinguishable from the Ka exhibited by natural murine IFNγ receptors on authentic murine cells ($1.7 \times 10^9\ M^{-1}$). Murine receptors on human 293 cells also directed the internalization and degradation of the murine ligand in an appropriate manner. However, despite high level muriné IFNγ receptor expression, the transfected human cells remained unresponsive to the murine ligand. Whereas the cells responded to human ligand at inputs of 10-1000 units/ml with increased expression of MHC Class I antigens and induction of antiviral activity, they showed no response to murine IFNγ even at inputs of 10,000 units/ml. This data thus demonstrates that the 90 kDa IFNγ-binding protein is both necessary and sufficient to confer species-specific ligand binding activity to cells. However, it also strongly supports the concept that generation of a functionally active IFNγ receptor requires that participation of at least one additional species-specific component. We are currently attempting to identify this other component(s) and elucidate its mechanism of action.

Conclusion

Figure 1 depicts our current concept of the human and murine IFNγ receptors. They are both single chain glycoproteins that consist of polypeptide cores of 52 and 48 kDa respectively which are heavily glycosylated to form mature molecules of Mr approximately 90 kDa. Only the fully mature form of the receptor is the only form expressed at the plasma membrane and exhibits a minor degree of molecular weight heterogeneity due to cell-specific differences

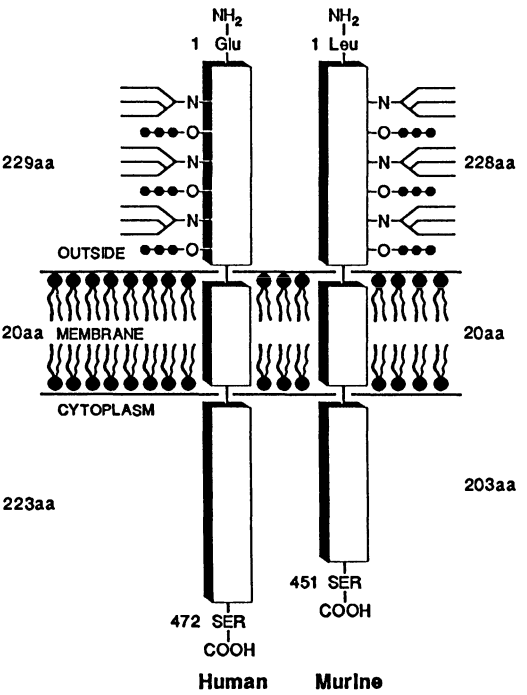

Figure 1. Schematic representation of the human and murine IFNγ receptors.

in N-linked glycosylation. The receptor is encoded by a single gene that gives rise to one major receptor transcript which thereby directs the synthesis of a single receptor protein that is widely expressed on hematopoietic and somatic cells. The receptor is an integral membrane protein that is divided into nearly equal sized extra and intracellular domains. The intracellular domain is particularly rich in serine and threonine residues making it an excellent target for phosphorylation by serine/threonine-specific kinases. In fact, we have recently found that the IFNγ receptor is indeed phosphorylated following its interaction with ligand at the cell surface. (Khurana Hershey and Schreiber, 1989b). Finally, our data supports the concept that formation of a functionally active receptor (i.e. one that can effect a cellular response) requires at least two species specific components: the 90 kDa IFNγ binding protein and a second, as yet undefined, component.

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The Regulation of Lymphopoiesis by Interleukin (Ch.S. Henney)

III Effector Phases

Signal Transduction in Hematopoietic Cells

Membrane Immunoglobulins Are Associated with a Family of Membrane Phosphoproteins

J.C. Cambier and K.S. Campbell

INTRODUCTION

The immune response of B lymphocytes is regulated by a variety of soluble and cell-associated species including antigen, T cell-associated ligands such as CD4 and αβTcr, and a variety of lymphokines and other cytokines. These agents regulate B cell function via interaction with cell surface receptors which transduce information across the plasma membrane. Signal transduction involves receptor mediated activation of intracellular enzymes leading to generation of information carrying "second messenger" molecules.

Clearly the best defined of B cell receptors is membrane immunoglobulin, two isotypes of which (mIgM and mIgD) are expressed on most peripheral B cells (Hardy, et al, 1982). These receptors contain transmembrane regions and carboxylterminal cytoplasmic tails which presumably play a central role in transmembrane signal transduction. Ligand binding to mIg leads to rapid, GTP-dependent hydrolysis of phosphatidylinositol 4,5-bisphosphate by a phospholipase C generating diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (InsP3) second messenger molecules (Cambier and Ransom, 1987; Harnett and Klaus, 1988; Gold et al, 1987). The GTP-dependence of this response suggests that a GTP-binding protein may be involved in coupling mIg to PLC. Although the molecular basis by which mIg molecules are coupled to presumptive G-proteins is obscure, some insight is provided by concepts developed in other G-protein coupled receptor systems. Receptors of this type constitute a family of proteins which are structurally similar, containing seven membrane spanning regions with cytoplasmic loops of varying lengths and a large carboxylterminal cytoplasmic tail (Neer and Clapham, 1988). The muscarinic receptor is one example of the G-protein coupled receptor family which, like mIg, physiologically stimulates PLC. Available evidence indicates that multiple loops and the carboxylterminal tail are involved in receptor coupling to G-proteins.

Membrane immunoglobulin M and D molecules have two α-helical, transmembrane spanning regions of 26 amino acids each and short carboxylterminal cytoplasmic tails consisting of three amino acids, KVK in sequence (Cheng et al, 1982; Rogers et al, 1980). The transmembrane and KVK cytoplasmic tail are highly conserved among various isotypes and across species barriers. Membrane IgG molecules exhibit a 25 amino acid cytoplasmic tail beginning at the membrane with the KVK motif (Yamawaki-Kataoka et al, 1982). Obviously the structure of cytoplasmic regions of mIg molecules is in stark contrast to those of members of the G-protein coupled receptor family. Membrane IgM and IgD molecules seemingly contain insufficient cytoplasmic and transmembrane structure to allow direct interaction with a G-protein.

An alternative mechanism for receptor coupling to PLC is illustrated by the T lymphocyte antigen receptor. This receptor is composed of a heterodimeric antigen/MHC binding subunit analogous to mIg in that both chains (α and β) span the membrane once and have a short, 5 amino acid cytoplasmic tail (Clevers et al, 1988; Allison and Lanier, 1987). This heterodimeric receptor is non-covalently associated with the CD3 complex, consisting of noncovalently associated γ, δ and ε chains, and disulfide bonded homo- and heterodimers of ζ and η chains (Clevers et al, 1988; Allison and Lanier, 1987). The component chains of the T cell antigen receptor-transducer complex range in molecular weight from 16-26kDa. It is unclear which (if any) of these chains is involved in G-protein interaction during signal transduction.

Based on structural considerations, it seems likely the mIg molecules transduce signals via a secondary transducer complex analogous to that associated with the T cell antigen

receptor. Despite years of study, however, such a complex, though suggested by a number of reports (Haustein and Von der Ahe, 1986; Koch and Haustein, 1983; Sidman et al, 1980; Rosenspire and Choi, 1982) has not been reproducibly observed. The strongest support for a mIg-associated complex has come from recent studies by Hombach et al (1988) and Sakaguchi et al (1988) which indicate that mIgM is noncovalently associated with a protein termed MB-1 or B34 which has a molecular weight of ~ 30,000 Da. This protein, which has been cloned by Sakaguchi, has a single transmembrane spanning region, (22 AA) a large extracellular domain (137AA), and a smaller intracellular domain (61 AA). Available evidence suggests that this molecule is required for transport of mIgM to the cell surface. Its role in signal transduction is unknown.

We have recently begun a series of studies which utilize a novel strategy in efforts to define potential mIg-associated proteins. The approach utilized was inspired by observations (Cambier et al, 1988) that crosslinking of mIgM molecules can lead to the subsequent inability of mIgD molecules to transduce signals leading to Ca^{++} mobilization (or vice versa). This phenomenon appears to reflect receptor desensitization (Sibley and Lefkowitz, 1985). Desensitization occurs in a number of other receptor systems and is generally mediated by receptor phosphorylation. Since mIg molecules contain no cytoplasmic phosphate acceptor sites, we hypothesized that if mIg desensitization is mediated by phosphorylation, the target of that phosphorylation event must be the presumptive transducer complex. Therefore, we thought that it might be possible to visualize mIg-associated proteins based on radioactive phosphate labeling.

Here we describe the use of this approach to define three mIg-associated phosphoproteins. These proteins, which have molecular weights of 32/33, 34 and 37 kDa, occur in specific association with mIgM and mIgD but are not associated with MHC class I or class II molecules or CD3. They are glycoproteins which exist predominantly as disulfide-linked dimers. They are inducibly phosphorylated and directly precipitable with anti-phosphotyrosine antibodies. Finally, the lowest molecular weight subunit differs slightly in electrophoretic migration between mIgD- and mIgM-associated forms.

RESULTS AND DISCUSSION

Coprecipitation of mIg and ^{32}P-phosphoproteins from flouride and vanadate stimulated B cells.

We first explored the possibility that phosphoproteins can be found in association with mIg from cells which were stimulated with aluminum fluoride (ALF_4^-) or vanadate, which have been shown previously to activate G-proteins (Blackmore et al, 1985; Kanaho et al, 1988). B cells were isolated from spleens of BDF1 mice by depletion of erythrocytes with Gey's solution and T cells with anti-Thy 1.2 antibodies (H013.4 and T24/30) and complement. Small and intermediate density B cells (ρ = 1.066-1.092) were isolated by centrifugation through discontinuous Percoll gradients. Cells were labeled with [^{32}P]-ATP using an adaptation of the methods of Mire et al (1985). Briefly, cells were cultured 60 minutes in phosphate free medium before being washed and suspended (4 x 10^7 cells/350 µl) in a permeabilization buffer consisting of 10 mM $MnCl_2$, 20 mM MgAcetate, 1 mM β-mercaptoethanol, Ca^{++} buffered to 200 nM with EGTA, 40 mM HEPES (pH 6.9), and 285 µg α-lysophosphatidylcholine/ml. After allowing cells to become permeabilized for 2 minutes on ice, 150 µCi [γ^{32}P]-ATP and appropriate stimuli were added. After an appropriate period of incubation at 37°C, cells were centrifuged (5000 rpm) for 30 seconds and supernatant was removed. Pelleted cells were then lysed using a lysis buffer containing 1% digitonin (Sigma, purified according to the method of Oettgen, et al., 1986), phosphatase inhibitors (0.4 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 0.4 mM EDTA, and 10 mM sodium fluoride), 150 mM NaCl, 10 mM Tris (pH 7.3) and protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 µg/ml α-1-antitrypsin) (all from Sigma). The cell lysate was clarified by centrifugation, and digitonin soluble material was immunoprecipitated using monoclonal antibodies coupled to Sepharose 4B beads. Immune complexes were eluted by boiling in SDS-PAGE sample buffer. SDS-PAGE analysis was performed as described by Laemlli (1970) using 10-15% polyacrylamide.

In the first experiment, cells were labeled in the absence of added stimuli or with stimulation by ALF_4^- or sodium orthovanadate for 30 minutes. Digitonin lysates were precipitated using the monoclonal rat anti-mouse kappa chain antibody 187.1 (Yelton et al, 1981) or anti-MHC class I antibody M1/42.398 (Springer, 1980) and immunoprecipitates were analyzed using 15% acrylamide gels under reducing conditions. Shown in figure 1 are autoradiographs of the gels which demonstrate ALF_4^- and vanadate induction of phosphorylation of three predominant mIg-associated proteins with molecular weights of 33, 34 and 37kDa. Note that heavy and light chains (60-70 kDa and 23-26 kDa) are not phosphorylated and that the association of these proteins is specific for mIg, i.e. they are not precipitated with MHC class I molecules. The molecular weight of the smallest of these phosphoproteins is similar to the 33-34 kDa protein, designated B34, observed in association with mIgM by Hombach et al (1988).

We then conducted a more careful analysis of the specificity of the association of pp33, pp34 and pp37 with mIg. The experiment was conducted as that presented in figure 1 except that all cells were stimulated with ALF_4^- for 30 min. As shown in figure 2, the family of phosphoproteins coprecipitated only with mIg. Using the anti-mouse kappa chain antibody, 187.1, they were not precipitated by antibodies specific for rat kappa chain (RG7/90.1 gift from Dr. T. Springer), CD3-ϵ chain (145-2C11, Leo et al, 1987) or MHC class I (M1/42.398) or class II (D3.137.5.7. a gift from Dr. S. Tonkonogy) antibodies. Note that anti-CD3ϵ precipitated lower molecular weight phosphoproteins, presumably CD3γ chains from contaminating T cells. Clearly, anti-CD3ϵ does not crossreact with the complex of pp33, pp34 and pp37 or molecules associated with the complex.

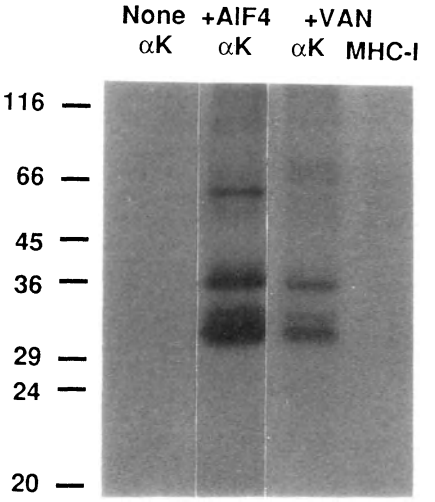

Figure 1 - **Coprecipitation of phosphoproteins with mIg from fluoride and orthovanadate stimulated B cells.** B cells were isolated, phosphate depleted, permeabilized, and incubated for 30 minutes at 37°C with [^{32}P]-ATP, either without added stimulus (None) or with aluminum fluoride (ALF_4^-, 10 μM $AlCl_3$ and 30 mM NaF) or sodium orthovanadate (VAN, 400 μM). Cells were lysed in 1% digitonin and the detergent soluble fraction was immunoprecipitated with monoclonal rat anti-mouse kappa antibody (187.1,) or rat anti-mouse MHC class I antibody (M1/42.398,). Immunoprecipitates were defined using 15% vertical slab SDS-PAGE (15%) under reducing conditions and autoradiography. Methods are described in detail in the text. Phosphoprotein bands of 33, 34, and 37kD were specifically immunoprecipitated with mIg from aluminum fluoride and orthovanadate stimulated cell lysates.

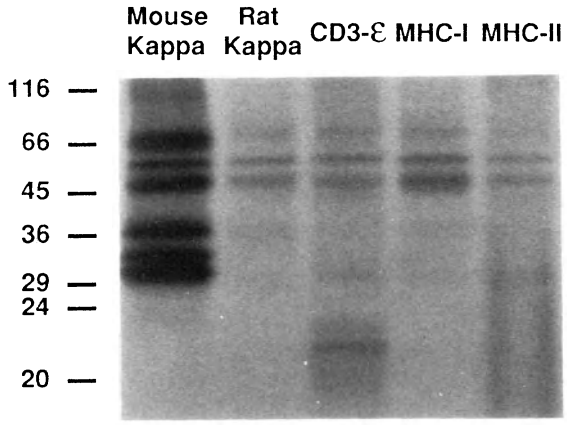

Figure 2 - **The phosphoprotein complex is specifically associated with mIg.** Permeabilized B cells were labeled in the presence of aluminum fluoride as in figure 1. Digitonin cell lysates were immunoprecipitated with monoclonal antibodies directed toward mouse kappa chain (187.1), rat kappa chain (RG7/90.1), CD3-ε (145-2C11), MHC class I (M1/42.398, reacts with all H2K), or MHC class II (D3.137.5.7, reacts with $I\alpha^{b/d}$). Immunoprecipitates were analyzed by SDS-PAGE (15%) under reducing conditions and autoradiography. The phosphoprotein complex (pp33, pp34, and pp37) is specifically coimmunoprecipitated with anti-mouse kappa antibody in this experiment.

Biochemical Characteristics of the mIg-Associated Phosphoprotein Complex

We subsequently explored the nature of the intermolecular associations among mIg, pp33, pp34 and pp37 by assessing stability of the complex in various detergents. We observed that association of the phosphoproteins with mIg was disrupted in 0.5% Triton X-100 indicating that this association was non-covalent (data not shown). We also analyzed the phosphoprotein complex by 2-dimensional nonreducing/reducing gels. As shown in figure 3, the labeled components of the complex fell off of the diagonal. These data indicate that complexes consist of disulfide-bonded dimers of the pp33, pp34 and pp37 polypeptides. Unfortunately, the gel system used (10% acrylamide) did not allow us to distinguish between association of these molecules as homo- or heterodimers. In conclusion, mIg is noncovalently associated with disulfide-linked dimeric structures containing phosphorylated subunits of 33, 34 and 37 kDa.

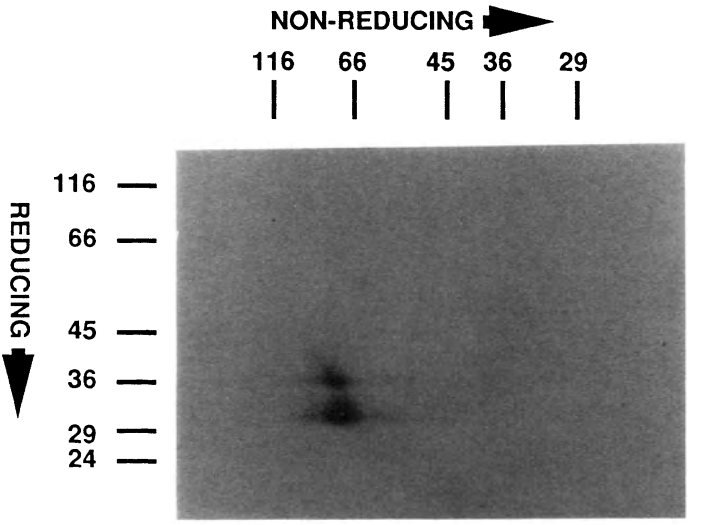

Figure 3 - **The mIg-associated phosphoprotein complex consists of disulfide-linked dimers.** Permeabilized B cells were labeled in the presence of aluminum fluoride, lysed, and immunoprecipitated with monoclonal anti-mouse kappa chain (187.1) as in figure 1. Immunoprecipitate was analyzed by two-dimensional SDS-PAGE under non-reducing conditions in the first dimension (10% acrylamide) and under reducing conditions in the second dimension (10% acrylamide). The gel was dried and autoradiographed. The phosphoproteins drop off of the diagonal from a non-reduced configuration of about 68 kD, indicating that the complex consists of disulfide-linked dimers.

Differential Phosphoprotein Association Between IgM and IgD

We next determined whether these structures were associated with both mIgM and mIgD on B cells. Cells were permeabilized, stimulated with ALF_4^- and labeled with [^{32}P]-ATP as before, and then lysed with digitonin and precipitated with monoclonal anti-μ (Bet-2, Kunz et al, 1981) or anti-δ (11-26C, a gift from Dr. J. Kearny) derivatized Sepharose. Precipitates were analyzed by one dimensional reducing gels and autoradiography. As shown in figure 4, a striking difference exists in the migration of the smallest phosphoprotein species which coprecipitates with these receptors. While the apparent molecular weight of the mIgM-associated molecule is ~32 kDa, the mIgD-associated molecule appears slightly larger, ~33 kDa in size. The pp34 and pp37 appear associated with both receptors. Interestingly when normalized to pp32/pp33, the pp34 and pp37 associated with mIgM are lower in intensity than those associated with mIgD suggesting that less pp34 and pp37 are associated with mIgM-relative to mIgD. Alternatively, these molecules may for some reason simply be less labeled when associated with mIgM. Such a difference in mIgM and mIgD associated pp32/pp33 (B34) has also been observed by Reth et al (personal communication) and is thought to reflect different forms of MB-1. Further analysis of this difference in the mIgM- and mIgD-associated protein complexes may resolve differences in signal transduction between these isotypes.

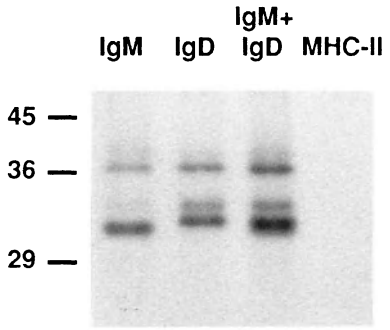

Figure 4 - **Distinct phosphoproteins are associated with mIgM and mIgD.** Permeabilized B cells were labelled in the presence of aluminum fluoride, lysed, and immunoprecipitated with monoclonal antibodies directed toward IgM (Bet-2), IgD (11-26c), or MHC class II (D3.137.5.7). Immunoprecipitates were analyzed using 10% polyacrylamide SDS-PAGE under reducing conditions and autoradiography. The lower associated phosphoprotein species differed slightly between IgM (pp32) and IgD (pp33), while the upper species (pp34 and pp37) were identical between the two receptor isotypes.

Phosphorylation of the mIg-Associated Phosphoprotein Complex

We next initiated studies to determine if mIg-associated proteins are tyrosine phosphorylated. This would be predicted for MB-1 since, based upon deduced amino acid sequence, it has 4 tyrosine residues in its intracellular domain but no identifiable consensus serine phosphorylation sites. For this experiment, B cells were stimulated with ALF_4^- and labeled as described earlier, and digitonin lysates were precipitated with various antibodies. As shown in figure 5, anti-phosphotyrosine (p-TYR) antibodies (IG2, Huhn et al, 1987) precipitated bands of equivalent molecular weight as anti-κ (187.1) antibodies suggesting that one or more of the subunits of the complex is phosphorylated on tyrosine. The anti-p-TYR precipitation is specific, since precipitation of the complex is blocked by p-TYR. To determine if the molecules which coprecipitate with mIg were the same as those which precipitate with anti-phosphotyrosine, preclearing experiments were performed. As shown in figure 5, anti-kappa antibodies cleared the anti-P-TYR precipitable pp32/pp33, pp34 and pp37, indicating that the species precipitated by the two antibodies are the same and further that all of the P-TYR-labeled complex in the cell is mIg-associated. In the inverse preclearing combination, anti-P-TYR only partially cleared the complex precipitable with anti-kappa. This could result from the fact that 1G2 is a low affinity antibody, or may indicate that some of the ^{32}P-labeled mIg-associated complex is not tyrosine phosphorylated. In results not shown, similar partial preclearing is seen with the PY20 and PY69 anti-P-TYR monoclonals, suggesting that the latter possibility is correct.

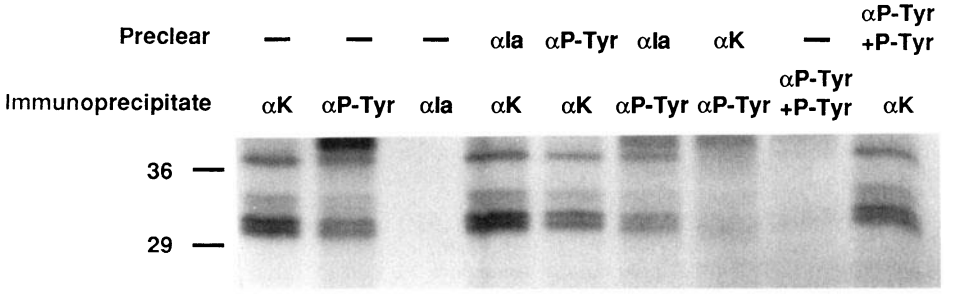

Figure 5 - The mIg-associated phosphoproteins contain phosphotyrosine. Immunoprecipitations were prepared from aluminum fluoride-stimulated [^{32}P]-phosphorylated cell lysates as in figure 1. Immunoprecipitating monoclonal antibodies were directed toward mouse kappa chain (αK, 187.1), phosphotyrosine (αP-TYR, 1G2), and MHC class II (αIa, D3.137.5.7). Precleared samples were immunoprecipitated with preclearing antibody, and subsequently lysate was removed and immunoprecipitated with another antibody. A control immunoprecipitate was prepared with anti-phosphotyrosine antibody (αP-TYR, 1G2) in the presence of 10 mM phosphotyrosine (P-TYR). Anti-phosphotyrosine antibody immunoprecipitated phosphoproteins which were identical to those coimmunoprecipitated with anti-kappa chain antibody. The ability of anti-kappa chain to preclear the tyrosine phosphorylated 33, 34 and 37kDa proteins indicates that the protein complexes immunoprecipitated by the two antibodies are the same and that essentially all of the tyrosine phosphorylated form of the phosphoprotein complex is associated with mIg.

While these studies suggest that mIg-associated complex contains phosphotyrosine, it does not distinguish whether ALF_4^- induced phosphorylation is serine/threonine or tyrosine specific. Resolution of this question requires formal phosphoamino acid analysis which is currently in progress. Preliminary findings suggest upon ALF_4^- stimulation that ^{32}P is introduced into tyrosine, serine and threonine residues in pp32/pp33, pp34 and pp37. Further physical characterization and analysis of the roles of these proteins in signal transduction and receptor regulation are in progress.

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Molecular Components of the IgM Antigen Receptor Complex

J.M. Hombach, T. Tsubata, L. Leclercq, H. Stappert, and M.G. Reth

The antigen receptors on mature B-lymphocytes are membrane-bound immunoglobulins (Ig) of the IgM and IgD class (Vitetta and Uhr 1976). Cross-linking of these receptors by polyvalent antigens results in B cell proliferation and differentiation (Cambier and Ransom 1987). How these membrane-bound Ig chains which lack a cytoplasmic tail generate a cell activation signal is presently unknown. We could show that the IgM antigen receptor is associated in the membrane of a mature B cell line with two other proteins (IgM-α and IgM-β). IgM-α is a 34-Kd glycoprotein which forms a homodimer of 68 Kd, while IgM-β is a non-glycosylated monomer of 40 Kd. IgM-α is most likely the product of mb-1, a B cell-specific gene (Sakaguchi et al.1988) which encodes a transmembrane protein with sequence homology to proteins of the T cell antigen receptor (TCR)/CD3 complex. Surface expression of IgM requires the formation of the appropriate complex between IgM and the associated proteins.

That membrane-bound IgM may be connected to other components was first indicated by a transfection experiment: introduction of an expression vector, coding for the membrane form of the μ chain (μm) into the λ chain producing myeloma line J558L did not result in surface IgM (sIgM) expression, although complete IgM molecules were assembled intracellularly (Fig. 1A) (Sitia et al. 1987; Hombach et al. 1988). From this experiment we postulated that there may exist proteins whose presence is required for sIgM expression. A sIgM positive myeloma variant (J558Lμm3) was isolated using a cell sorter. This variant differs from the sIgM negative line in that it contains transcripts of the mb-1 gene and a 34-Kd glycoprotein (B34/IgM-α) forming a homodimer, which is co-purified with membrane-bound IgM molecules (Fig. 1B) (Hombach et al. 1988). To elucidate the relationship between mb-1, IgM-α and sIgM expression, we have constructed a vector which expresses the mouse mb-1 gene under the control of the V_H promotor and enhancer. This vector was introduced into the myeloma line J558Lμm which synthesizes membrane-bound IgM molecules without bringing them onto the cell surface (Fig. 1A,C). mb-1 positive transfectants (J558Lμm/mb-1m) expressed IgM as strongly as the myeloma variant line J558Lμm3 on the cell surface (Fig. 1D and B). Analysis of biosynthetically labelled and affinity-purified IgM antigen receptors of J558Lμm/mb-1m cells showed again the IgM-α protein. This finding give strong evidence that IgM-α is encoded by the mb-1 gene. This conclusion is also supported by the sequence of the mb-1 gene (Sakaguchi et al. 1988) which encodes a glycosylated transmembrane protein with a free cystein for dimerisation and with a molecular weight similar to that of IgM-α.

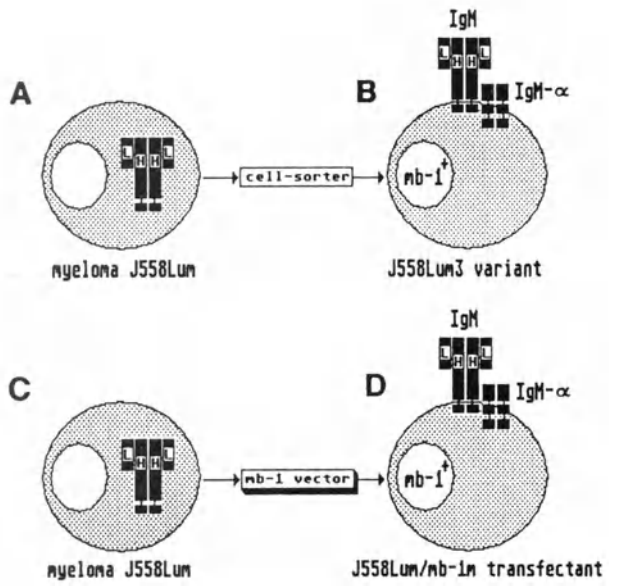
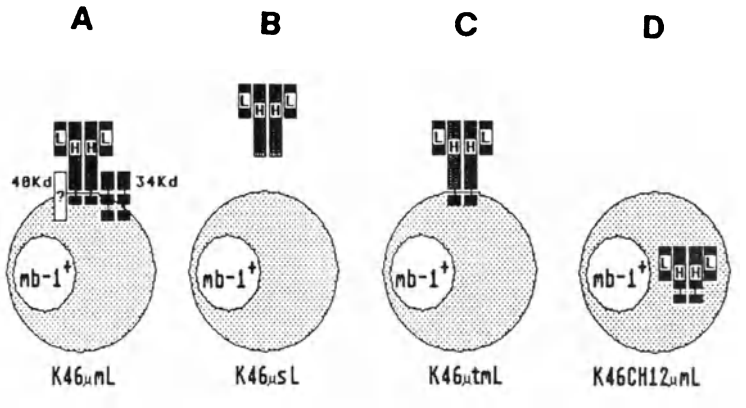


Fig. 1. Surface IgM expression in myeloma cells is dependent on the presence of IgM-α.

Association between IgM-α and membrane-bound IgM has so far been found in sIgM positive pre B and myeloma cells. To analyse a mature B cell line we transfected μm and λ chain encoding vectors into the B lymphoma line K46 (Kim et al. 1979) which expresses endogenous κ and γ_{2a} chains. Co-expression of the μm and λ chain is required for the assembly of an anti-NP IgM molecule which allows affinity purification of the IgM antigen receptor. All of the K46μmλ transfectants were $sIgM^+$. Biosynthetically labelled and affinity-purified IgM molecules of the transfectant (K46μmλ) showed the associated protein IgM-α and a novel protein of 40 Kd (IgM-β) (Fig. 2A). IgM-β is a monomeric protein and, in contrast to IgM-α, it is not glycosylated.

The specificity of the association of IgM-α and IgM-β to the μm chain was confirmed by introducing various μ-vectors into K46λ and analysing the expressed IgM antigen receptors for the presence of associated proteins. These vectors coded for the secreted form of the μ chain (μs, Fig. 2B), a μm chain in which the transmembrane exons of μm were replaced by the exons encoding the membrane and cytoplasmic part of an MHC class I ($H\text{-}2K^k$) molecule (μtm, Fig. 2C) and a truncated μm chain, lacking the CH_3 and CH_4 domain but still containing the μm transmembrane region ($CH_{12}\mu m$, Fig. 2D). After transfection into K46λ the various μ chains were correctly expressed and together with λ light chains formed NP-binding molecules. However, none of these IgM molecules were associated with the IgM-α



+	-	-	-	IgM-α/IgM-β
+	-	+	-	sIgM

Fig. 2. Surface IgM expression in K46 B lymphoma cells transfected with various μ chain vectors. Presence of the associated proteins IgM-α and IgM-β.

or IgM-β protein . This result demonstrates that IgM-α and IgM-β are specifically associated with membrane-bound IgM molecules and that their binding requires the presence of the C-terminal CH-domains as well as the appropriate μm transmembrane sequence.

In conclusion our data suggest that the IgM antigen receptor of a mature B cells is a complex of different transmembrane proteins ($\alpha_2\beta\mu_2$) and as such similar to the antigen receptor on T cells ($\alpha\beta\gamma\delta\varepsilon\xi_2$) (Clevers et al. 1988) and to the IgE Fc-receptor ($F_c\varepsilon R$) on mast cells ($\alpha\beta\gamma_2$) (Blank et al. 1989). A comparative study of these three receptors may result in better understanding of their signaling function. In particular it will be important to elucidate how the signals derived from the IgM antigen receptor control the early differentiation and activation of B cells.

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Membrane Immunoglobulin Is Associated with a Phosphoprotein Complex

M.K. Newell, A.J. Noë, and M.H. Julius

INTRODUCTION

The ligation of antigen receptors on T and B cells results in the generation of cellular activation signals (Kappler et al. 1983 and Pernis et al. 1971). Unlike many receptors involved in transducing signals (Sibley 1985), these receptors have short cytoplasmic tails (Leo et al. 1987). It is currently thought that the CD3 complex couples TCR, once ligated, with the generation of subsequent intracellular signals (Samelson et al. 1985 and Leo et al. 1987). This argument has been strengthened by the observation that antibodies against this complex mimic the effects of antigen. Antigen receptor ligation of both B and T cells results in similar signalling cascades, including polyphosphoinositide metabolism, Ca^{++} mobilization, and activation of protein kinase C (Coggeshall et al. 1985; and Imboden et al. 1985). However, the mechanism through which the B cell antigen receptor, membrane immunoglobulin, mIg, is coupled to the generation of second messengers remains unclear. The following study addresses whether mIg is coupled to a signalling complex analogous to CD3.

RESULTS AND DISCUSSION

Our experimental approach was to apply the conditions in which CD3/TCR association can be observed to the biochemical analysis of mIg and associated molecules. To establish appropriate conditions, detergent cell lysates were prepared from the $V_\beta 8$ positive T cell clone T94 (Julius et al. 1986) which were cell surface labelled with ^{125}I. The immunoprecipitates from lysates were analyzed by 9-23% gradient SDS-polyacrylamide gel electrophoresis, dried, and autoradiographed. Immunoprecipitation with the anti-CD3 epsilon antibody 145.2C11 (Leo 1987) revealed roughly equivalent amounts of the 24 kd ε chain and the 40-45 kd TCR α/β heterodimer, Fig. 1, panel A, lane 1. Given the potential differences in how these three chains can be iodinated, one cannot address questions

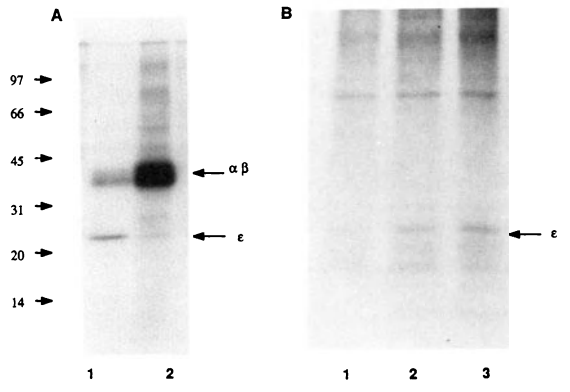

Figure 1

of stoichiometry. However, when the anti-$V_{\beta}8$ antibody F23.1 (Staerz et al. 1985) was used, the predominant species immunoprecipitated was the α/ß heterodimer of the TCR, Fig. 1, panel A, lane 2. These results demonstrate the inefficiency of indirectly precipitating CD3 components with an anti-TCR antibody. The generation of antibodies to individual CD3 components was required for biochemical and functional analysis of the complex. Until such time as this can be done for mIg associated molecules, anti-mIg antibody is the only probe available. Hence, the conditions of detergent lysis and immunoprecipitation become critical factors in isolating molecular components associated with mIg.

In studies which identified the CD3 molecules, the observation was made that the components of this complex become phosphorylated as a consequence of stimulating T cells with PMA or antigen. We reproduced this observation with T94. After loading with ^{32} P-orthophosphoric acid for 4 hours at 37°C, the cells were stimulated,or not, with PMA for 25', washed in ice cold PBS, and lysed in 0.5% Triton X-100. The lysates from unstimulated, lane 1, and PMA stimulated cells,lanes 2 and 3, were immunoprecipitated with F23.1, Fig. 1, Panel B, lanes 1 and 2, or 145.2C11, lane 3. Phosphoproteins in the 16-32 kd molecular weight range were precipitated with both 145.2C11 and F23.1 from PMA stimulated cells. The intensity of these signals derived from a clone of T cells provided a guideline for the results one might expect from immediately ex vivo B cells.

Our premise for the experiments with B cells was that if a functional analogue to CD3 is associated with mIg, it should become phosphorylated upon B cell activation and precipitable with antibodies to mIg. Toward this end, B cells were isolated by percoll density separation, loaded with ^{32}-P ortho-phosphoric acid for 4 hours at 37°, and stimulated or not, with phorbol myristate acetate (PMA) at 1 μg/ml for 25'. Cells were lysed with 0.9% Digitonin at 5 x 10^7 /ml for 1 hr. on ice, in the presence of protease and phosphatase inhibitors. Lysates were centrifuged at 12,000 x g for 30'. Lysates from unstimulated cells, (Fig. 2) lane 3 and 4, or PMA stimulated cells, lanes 1 and 2, were immunoprecipitated with 33.18.12 (Grutzman 1254), a rat anti-mouse kappa antibody, followed by Protein-G Sepharose, lanes 2 and 4, or Protein G-Sepharose, lanes 1 and 3. Immunoprecipitation from lysates of PMA stimulated cells with 33.18.12 followed by Protein G-Sepharose, revealed four phosphoproteins of approximate molecular masses: 12 kd, 27 kd, and a doublet at 19-21 kd, Fig. 2, lane 2.

A Phosphoprotein Complex Can be Immunoprecipitated With Anti-Immunoglobulin From PMA Stimulated B Cells

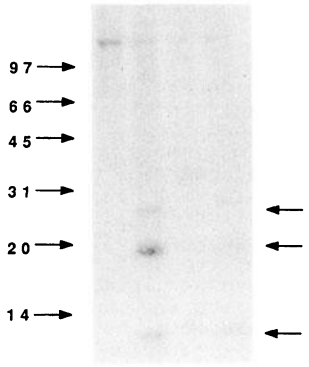

Figure 2 1 2 3 4

Specificity of mIg Associated Phophoprotein Complex

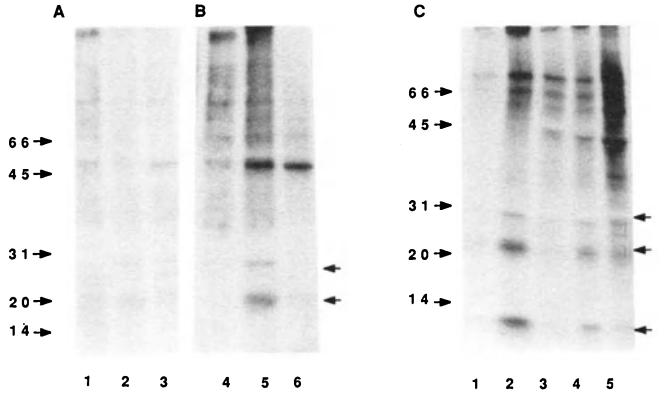

Figure 3

Using the same conditions of activation, we assessed whether this phosphoprotein complex was specifically associated with membrane immunoglobulin. Lysates from unstimulated B cells, Fig. 3, panel A or PMA stimulated cells, panel B, were precipitated with Protein G-Sepharose alone, lanes 1 and 4, or with precipitating antibodies 33.18.12, lane 2 and 5, and anti-class 1 antibody M1.42 (Bhattacharya et al. 1981) lanes 3 and 6. These data indicate that the phosphoproteins were preferentially co-precipitated with mIg. Immunoprecipitation with M1.42 from lysates of PMA stimulated B cells also revealed the 45 kilodalton α chain of class 1, Fig. 3, panel B lane 6. Furthermore, the complex was immunoprecipitated with both μ and δ isotypes of mIg. Lysates from PMA stimulated cells were immunoprecipitated with Protein-G alone, lane 1; 187.1 (Yelton et al. 1981), lane 2; anti-class II, M5.114 (Springer, T.A., 1980) lane 3; b-7-6 (Julius et al. 1984), an anti-μ, lane 4; and JA12, an anti-δ (Tonkonogy and Cambier 1982).

While most experiments have shown a preferential association of this phosphoprotein complex with mIg, in several instances the complex co-precipitated with MHC class I and MHC class II. There are precedents for the association of MHC class II molecules with mIg. In addition, recently MHC class I and class II have also been shown to be physically associated (Szollos et al. 1989). Because mIg, class II and class I are capable of delivering signals to the B cell, the phosphoprotein complex described above may be involved in receptor mediated signaling through one or a combination of these cell surface receptors.

The possibility exists that under the conditions of low stringency required to co-precipitate the complex with mIg, non-specific immunoprecipitation of these molecules may result. It was important, therefore, to determine how the conditions for lysis and precipitation necessary to visualize the phosphoprotein complex affect our ability to co-precipitate class I and class II. We surfaced labelled B cells using ^{125}I lactoperoxidase and immunoprecipitated under the same lysis and precipitation conditions described for the phosphorylation experiments, Fig. 4. Monoclonal antibodies against MHC class II, M5.114, lane 1, and MHC class I, M1.42, lane 2 revealed MHC class II and class I, respectively, as well as mIg. Negative controls for these studies included 145.2C11, lane 3, and F23.1, lane 4. As the positive

^{125}I - Surface Labelling of PMA Stimulated B Cells

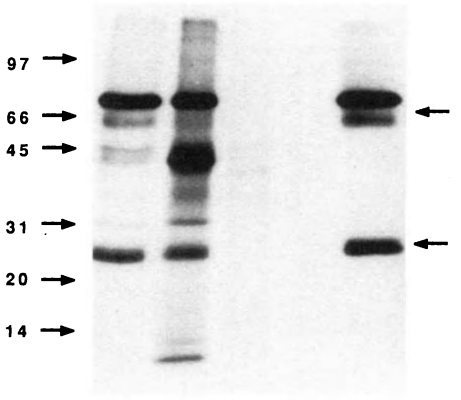

Figure 4 1 2 3 4 5

control, membrane immunoglobulin was precipitated with 187.1, lane 5. These results suggest that mIg can also be co-precipitated with class I and class II, and therefore, it is not surprising to find the phosphoproteins precipitable with these molecules under some conditions.

Acute stimulation of resting B cells with anti-μ and anti-δ antibodies has not to date resulted in the phosphorylation of molecules detectably associated with mIg. One potential problem is that the precipitating antibodies are blocked by the inducing antibodies. This seems not to be the case, since short preincubation of resting B cells with anti-μ antibodies, anti-δ antibodies, or a combination of the two did not alter the fluorescence staining profile obtained with the monoclonal anti-kappa antibodies used in precipitation experiments (data not shown). However, the amount of mIg precipitable from resting B cells pretreated with anti-Ig was greatly reduced (data not shown). It has been reported that ligation of mIg results in its rapid (seconds) association with elements of the cytoskeleton (Braun 1982). It follows that the phosphoproteins in our experiments may indeed be present in solubilized lysates of anti-Ig stimulated B cells but no longer associated with mIg, or, alternatively, they may be associated with mIg in the detergent insoluble fraction.

An mIg Associated Phosphoprotein Can Be Precipitated From Anti-Immunoglobulin Stimulated B Cells

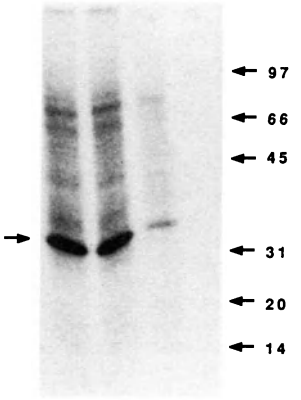

Figure 5 1 2 3 4

As an alternative method of determining whether antigen receptor mediated activation of B cells results in the phosphorylation of mIg associated molecules, a mitogenic anti-μ monoclonal antibody b-7-6 was used to stimulate resting B cells for 48 hours. Precipitation with anti-kappa and Protein G-Sepharose revealed a 34 kilodalton phosphorylated molecule, Fig. 5, lane 2. Protein G-Sepharose alone also precipitated this molecule, Fig. 5, lane 1, likely due to the persistence of stimulating antibody b-7-6. M1.42 (anti-class 1) and Protein G-Sepharose precipitated a much lower level of this molecule. This could be the consequence of either residual stimulating b-7-6 binding to the available

Protein G sites on the M1.42-coated Protein G-Sepharose or, alternatively, the co-precipitation of low levels of mIg with the class I molecules as discussed above. Note the absence of the phosphorylated MHC class I α chain after anti-Ig stimulation.

Prolonged stimulation of B cells with anti-Ig results in either the phosphorylation of the 34 kd phosphoprotein or the tighter association of the molecule with mIg. Neither acute anti-Ig nor PMA stimulation resulted in a detectable 34 kd phosphoprotein co-precipitating with mIg. It is possible that the species is not available. This 34 kd phosphoprotein may be the mIg associated molecule recently described by several laboratories (Hombach et al. 1988, and Sakaguchi et al. 1988) as being associated with mIg. Further characterization is necessary to confirm this possibility.

The molecular complex present in acutely stimulated B lymphocytes also appears in the B cell tumor 70Z3 (Paige et al. 1978). 70Z3 is induced to express surface IgM upon stimulation with LPS. For these experiments induced and non-induced 70Z3 were ^{32}P orthophosphoric acid loaded, lysed, and precipitated as described above. Lysates from both non-induced, lanes 1 and 2, and induced cells, lanes 3 and 4, were precipitated with Protein G-Sepharose alone, lanes 2 and 4, respectively, and with 33.18.12, lanes 1 and 3, respectively. The phoshoprotein complex was precipitable using 33.18.12 from the induced cells, lane 3. The expression of this phosphoprotein complex in a B cell tumor will enable their further biochemical characterization.

An mIg Associated Phosphoprotein Complex Can Be Precipitated from LPS Activated 70Z3

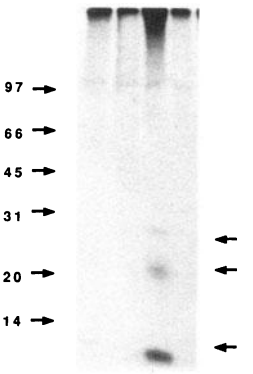

Figure 6

We conclude that mIg is associated with a phosphoprotein complex. The phosphorylation state of this complex varies with the state of B cell activation, or alternatively, cellular activation may result in the association of these molecules with mIg, their association with other cell surface molecules is not excluded. Lastly, while we have observed five mIg associated molecules, we cannot be sure that these represent the entire complement.

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Signalling via Surface Immunoglobulin, Fcγ and Interleukin-4 Receptors on B Lymphocytes

G.G.B. Klaus, M.M. Harnett, K.P. Rigley, and M. Holman

INTRODUCTION

The activation, clonal expansion and differentiation of immunocompetent B cells are controlled by a number of signals interacting with cell surface receptors. These include antigen receptors (sIg), receptors for a variety of cytokines, including interleukin-4 (IL-4), and other regulatory receptors, such as the Fcδ receptor (FcR). In recent years our understanding of the nature of the second messengers utilized by antigen receptors on both T and B cells has progressed rapidly. We have focussed on the control of signalling via sIg receptors on murine B cells, using anti-receptor antibodies as a polyclonal model for studying the biochemical and biological consequences of antigen-induced crosslinking of these receptors. We will briefly review our current understanding of the signalling cascade induced by anti-Ig antibodies, with particular emphasis on the role of guanine nucleotide-binding (G) proteins in signalling. Then we will discuss how this cascade is regulated by engaging the FcR. Finally we will present preliminary data which yield some clues as to the nature of the signalling machinery utilized by receptors for IL-4 on B cells.

SIGNALLING VIA SIG RECEPTORS

Crosslinking of sIg receptors on B cells by mitogenic forms of anti-Ig antibodies (such as $F(ab')_2$ fragments of rabbit anti-Ig) induces the rapid and prolonged hydrolysis of phosphatidylinositol 4,5 bisphosphate (PIP_2) in the plasma membrane. This results in an elevation in intracellular Ca^{2+} levels and the activation and translocation of protein kinase C (PKC) from the cytosol to the cell membrane (reviewed by Cambier et al. 1987; Klaus et al. 1987). Both sIgM and sIgD receptors are coupled to the effector enzyme in this signalling cascade (the polyphosphoinositide-specific phosphodiesterase, PPI-PDE) via an as yet uncharacterized guanine nucleotide-binding protein termed G_p (Gold et al. 1987; Harnett & Klaus, 1988b). The evidence for this is that in permeabilized normal B cells (or B lymphoma cells) the breakdown of PIP_2 can be reconstituted by co-stimulation of the PPI-PDE by anti-Ig plus a nonhydrolyzable GTP analogue (GTPδS). Little is known about the

nature of G_p in B cells, except that it is not a substrate for either pertussis or cholera toxins, which ADP-ribosylate some better-characterized G-proteins involved in signal transduction.

The involvement of G_p in this signalling cascade affords several potential points of regulation: the first of these may be at the level of ligand-receptor interaction (since it is clear that effective signalling and B cell activation via the antigen receptors requires substantial receptor crosslinking). Other possible regulatory sites could be at the level of sIg/G_p coupling, G_p/PPI-PDE interaction or finally modification of the activity of the PPI-PDE itself. It is worth emphasizing that we do not yet know if other proteins (perhaps representing the B cell equivalent of the CD3 complex) are involved in coupling sIg to G_p. One potential candidate is the recently described mb-1 gene product (Sakaguchi et al. 1988; Hombach et al. 1988).

Our recent studies have concentrated on the regulation of sIg-mediated signalling. It is well-established that activation of PKC by appropriate phorbol esters inhibits anti-Ig-induced PIP_2 hydrolysis and DNA synthesis in B cells (e.g. Mizuguchi et al. 1986). In permeabilized B cells PKC-activators such as PMA or PDB inhibit both coupled PIP_2 breakdown (induced by anti-Ig plus GTPγS) and direct activation of G_p by the GTP analogue alone. Furthermore, these agents also suppress direct activation of the PPI-PDE by high concentrations of Ca^{2+} (which bypasses the need for G_p). This therefore demonstrates that PKC activation in B cells exerts direct inhibitory effects on the PPI-PDE (perhaps by phosphorylating the enzyme), hence providing a negative feedback loop for control of signalling (Harnett & Klaus 1988a).

Isotype-specific anti-μ or anti-δ antibodies elicit indistinguishable biochemical (and biological) effects in murine B cells. We have therefore designed a series of experiments to ask whether sIgM and sIgD share a common pool of G_p and hence the same signalling pathway. These experiments demonstrated that costimulation of intact cells with anti-μ plus anti-δ did not induce additive responses, either in terms of Ca^{2+} mobilization or PIP_2 breakdown. Sequential stimulation with isotype-specific reagents revealed a complex, dynamic series of regulatory events, which has not been fully elucidated. Studies in permeabilized cells showed that stimulation via one class of receptor for 60 min. induces activation of the PPI-PDE: as a result the basal levels of both inositol phosphates and intracellular Ca^{2+} in these cells are elevated. However, the PPI-PDE in these preactivated cells is substantially refractory to restimulation via the heterologous receptors. Further analysis of this desensitization revealed that it appears to operate at the level of coupling between G_p and the PPI-PDE. After 4 - 8 hr stimulation the responsiveness of these cells had recovered. That is, the basal activity of the PPI-PDE had returned to normal, and the enzyme could again be restimulated via the heterologous receptors (Harnett et al. 1989).

These observations are best explained by postulating that sIgM and sIgD share a common G-protein. Furthermore, the results suggest that after stimulation of sIg receptors the PPI-PDE may remain activated for some time, thereby providing a feedforward mechanism

to maintain signalling, perhaps even after receptors have been shed and/or endocytosed. The (temporary) heterologous receptor desensitization observed may reflect co-modulation of G_p with the crosslinked receptors, which would deprive the other class of receptor of adequate levels of G-protein. Similar (although more long-lasting) heterologous sIg receptor desensitization has also been described by Cambier et al. (1988).

The evidence for a pivotal role for G_p in signal transduction via both classes of sIg is therefore clear. We have used two approaches to investigate the role of G-proteins in the long-term regulation of B cell activation via various polyclonal activators. The first was to study the effects of cholera or pertussis toxins on DNA synthesis induced by anti-Ig, lipopolysaccharide or PMA plus the Ca^{2+} ionophore ionomycin. Pertussis toxin does not detectably affect B cell activation by any of these stimuli. Cholera toxin has dramatic effects, which depend on the nature of the activator used (Klaus et al. 1987b). Very low concentrations of the toxin (10^{-14}M) abrogate DNA synthesis induced by $F(ab')_2$ anti-Ig, whilst 10,000-fold higher (nonspecifically toxic) concentrations are required to inhibit the response to LPS. Paradoxically, concentrations of cholera toxin which inhibit the response to anti-Ig, significantly enhance proliferation induced by PMA plus ionophore. The toxin has minimal effects on anti-Ig stimulated PIP_2 breakdown (Harnett & Klaus 1988b), and does not inhibit the early phases of B cell activation, such as the increase in Ia antigen levels (Klaus et al. 1987b). We therefore speculate that cholera toxin may affect another G-protein, distinct from G_p, involved further downstream in the sIg-induced activation cascade.

Recently, we have extended these findings by introducing nonhydrolyzable analogues of GTP and GDP (to activate or inhibit putative G-proteins, respectively) into reversibly permeabilized B cells. This was done by incubating cells with ATP in the absence of divalent cations: ATP^{4-} produces small pores in the membranes of certain cell types which can then be closed simply by adding Mg^{2+}. As shown in Fig. 1 GDPβS did not affect responses of intact cells. In permeabilized, resealed cells it specifically suppressed background [^{3}H]TdR uptake and the response to anti-Ig, but not those induced by LPS, or by PDB plus ionomycin. The effect appears to be specific for GDP, since CDP, UDP or IDP were ineffective. The fact that the response to anti-Ig was only partly suppressed presumably reflects the loss of GDPβS over the 72 hr culture period. This short exposure to GDPβS does not affect anti-Ig-stimulated increases in Ia antigen levels. GTPβS had no detectable effect in this assay, either on its own or in combination with the mitogens tested. Although these results are preliminary, they are nevertheless in line with the concept that B cell activation via sIg crosslinking may involve multiple G-proteins, acting at different sites in the activation cascade. They also further underscore the well-established fact that LPS and anti-Ig activate B cells by fundamentally different mechanisms.

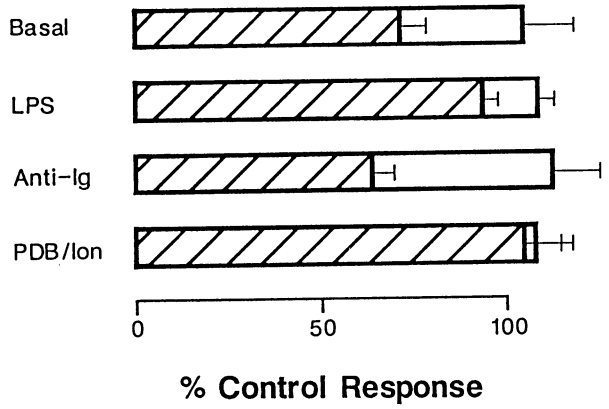

Fig. 1. Effects of introducing GDPβS into reversibly permeabilized B cells on DNA synthesis induced by various B cell mitogens. Purified B cells were incubated with 1mM GDP S, either with (hatched bars) or without (open bars) 500 uM ATP in Ca^{2+} and Mg^{2+}-free Tyrode's buffer, pH 7.8 for 25 min at 37°C: they were then resealed by adding excess Mg^{2+}, washed and plated out in 200 ul microwells which were cultured in medium alone or with 50 ug/ml $F(ab')_2$ anti-Ig, 50 ug/ml LPS, or 100 ng/ml phorbol dibutyrate plus 500 ng/ml ionomycin. $[^3H]$TdR uptakes were determined after 72 hr. Data are pooled and normalized results from 14 experiments (n = 42).

FUNCTION OF THE FcγR ON B CELLS

Murine B cells carry FcγR which bind complexed IgG antibodies. Crosslinking of FcR and sIg on B cells (e.g. by antigen-antibody complexes, or intact rabbit anti-Ig antibodies) inhibits their activation (reviewed by Sinclair and Panaskoltsis 1988). The biochemical basis of these effects has now been partially elucidated, from studies with rabbit anti-Ig. The intact (IgG) antibodies do not induce B cells to synthesize DNA, and inhibit the mitogenicity of $F(ab')_2$ anti-Ig. Biochemical studies have shown that the intact antibodies induce abortive activation of the PPI-PDE, so that release of IP_3 is abrogated after ca. 1 min. (reviewed by Klaus et al. 1987a). Our recent studies using permeabilized B cells have demonstrated that co-ligating sIg and FcR with intact anti-Ig uncouples sIg receptors from their associated G-protein (Rigley et al. 1989). The evidence for this is that stimulation of permeabilized B cells with a mixture of $F(ab')_2$ and intact antibodies inhibits the coupled release of inositol phosphates elicited by $F(ab')_2$ anti-Ig plus GTPγS, but not basal stimulation of the PPI-PDE elicited by the GTP analogue alone. This clearly indicates that the signalling cascade is interrupted at the level of sIg/G_p interaction. The mechanisms involved in uncoupling are unknown.

CONTROL OF SIGNALLING VIA IL-4 RECEPTORS

One of the first documented effects of IL-4 was its capacity to induce resting B cells to synthesize DNA in conjunction with low concentrations of anti-Ig antibodies (Howard et al. 1982). This suggests that the signalling pathway utilized by IL-4 receptors on B cells somehow synergizes with the second messengers elicited by ligation of sIg. However, several groups have shown that stimulation of B cells with IL-4 does not induce PIP_2 hydrolysis, Ca^{2+} mobilization, or PKC translocation, nor does IL-4 detectably modulate second messenger production in response to anti-Ig (reviewed by Cambier et al. 1987). An immediate question is therefore which of the known second messengers produced as a result of sIg crosslinking is involved in inducing B cell proliferation with IL-4. We have recently approached this problem by co-culturing B cells with IL-4 and the Ca^{2+} ionophore ionomycin, in the presence or absence of PKC activators, such as PDB. The results have been clearcut: co-stimulation of B cells with IL-4 and PDB induces substantial levels of DNA synthesis, whereas co-culture with the ionophore does not. Furthermore, this combination effectively stimulates B cells from CBA/N (xid) mice (Fig. 2), which respond poorly to soluble anti-Ig plus IL-4 (Nakajima et al. 1987). IL-4 causes (modest) suppression of DNA synthesis in cultures stimulated with an optimally mitogenic combination of PDB plus ionomycin.

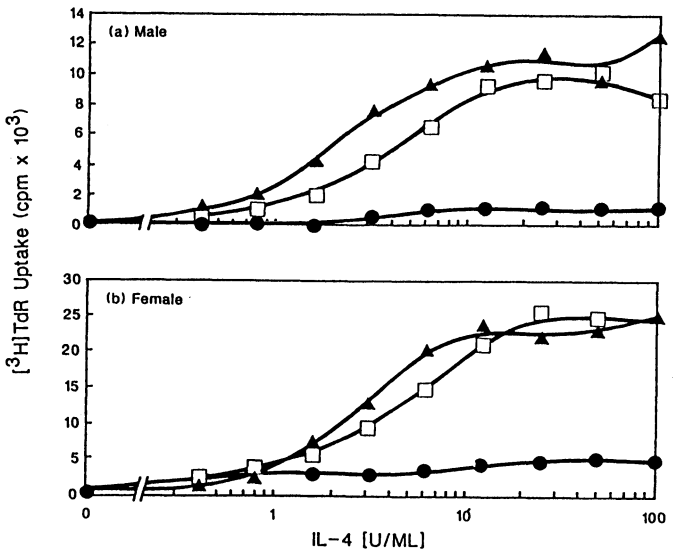

Fig. 2. Induction of DNA synthesis in B cells from (CBA/N X Balb/c/Fl male (panel A) or female (panel B) mice with IL-4 plus PDB. 2 x 105 purified B cells were cultured in 200 ul with the indicated concentrations of recombinant mouse IL-4 with 1.0 (●), 10 (▲) or 100 ng/ml PDB. [3H]TdR uptakes were determined after 72 hr. Incorporation with medium alone was 70 or 5000 cpm and with 100 U/ml IL-4 alone was 2390 or 4950 cpm for male and female B cells, respectively.

In further experiments B cells were activated by various stimuli for 24 hr, washed and subsequently restimulated in secondary cultures, which were assayed for [^{3}H]TdR uptake. These showed firstly that both PDB and IL-4 need to be present both early and late for the induction of DNA synthesis. Secondly, stimulating B cells for 24 hr with 100 ng/ml PDB inhibited their subsequent responsiveness to any stimuli which utilize PKC (e.g. PDB + IL-4, PDB + ionomycin). This is due to down-regulation of PKC, which has been demonstrated in many cell types, including B cells (Mond et al. 1987). However, priming cells with PDB + IL-4 abrogates this inhibition, and these cells respond well to restimulation with the above agents.

The biochemical basis of these effects is currently under investigation. Our working hypothesis is that IL-4 receptors interact with the sIg signalling cascade via the key regulatory enzyme PKC. It is possible that IL-4 may prevent down-regulation of PKC by phorbol esters (and by extrapolation by endogenous diacylglycerol as well). This could then result in subtle alterations of the feedback control of antigen receptor-induced phosphoinositide hydrolysis. These results in B cells are certainly consistent with the known capacity of IL-4 and PMA to co-stimulate DNA synthesis in both thymocytes and in peripheral T cells (Zlotnik et al. 1987; Hu-Li et al. 1987).

CONCLUSIONS

The evidence we have discussed clearly shows that sIg receptors are coupled to a classical second messenger cascade. The level of signalling can be regulated in various ways, by modulating the activity and/or interactions of the various component proteins which comprise the second messenger generating system. We have shown how this can also be brought about by engaging Fc receptors, for example, and think it likely that IL-4 receptors may function in a similar fashion. The concept of receptor crosstalk is well-established in other cell types and there is growing evidence for this as an important regulatory device in both T and B cells.

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Is There Specificity Involved in Fcε Receptor Aggregation Which Leads to an Effective Secretory Stimulus?

I. Pecht, R. Schweitzer-Stenner, and E. Ortega

INTRODUCTION

Immunological stimulation of mast cells and basophils to secretion of mediators of the immediate-type hypersensitivity is initiated by antigen induced aggregation of the "high-affinity" receptor for Fc_ε (FcεRI) (Ishizaka and Ishizaka 1984; Froese 1984; Metzger et al 1986). The antigen specificity of this stimulus is provided by IgE class antibodies which are bound to the FcεRI probably by their Fcε2 and Fcε3 domains (Helm et al 1988). This cell-membrane antibody - antigen binding and crosslinking process, which initiates the secretory response, is rather interesting for its own sake yet is also a useful paradigm for many other cell-stimulatory processes initiated upon receptor aggregation. Hence, extensive theoretical and experimental work has been done in order to obtain a detailed physicochemical understanding of the triggering process. Still this goal has so far been only partly attained (De Lisi and Siraganian 1979; Ishizaka and Ishizaka, 1984; Metzger et al 1986).

Two main approaches were employed in the experimental studies aiming at a better understanding of the triggering process. In one, different reagents which would cause FcεRI aggregation have been developed and employed. These included: a) the combination of chemically synthesized divalent haptens and specific IgE (Siraganian et al 1975; Schweitzer-Stenner et al 1987); b) Chemically crosslinked oligomers of monoclonal IgE class antibodies fractionated into discrete oligomers (i.e. dimers, trimers) (Segal et al 1977). c) Specific antibodies to surface exposed parts of the FcεRI (Ishizaka et al 1977; Ishizaka and Ishizaka, 1978). Results of experiments using these reagents have indeed led to the notion that FcεRI clustering is required for initiating secretory response (Ishizaka et al 1970). They produced some evidence that a minimal initial size of an effective FcεRI aggregate is a dimer (Segal et al 1977). Furthermore, possible orientational constraints on the dimers formed in terms of their signalling capacity were resolved (Ortega et al 1988).

The second experimental approach employed lipid haptens carried in liposomes as triggering agents, for specific IgE mediated, mast cells secretion (Balakrishnan et al 1982). Freely mobile lipid haptens carried in liposome bilayers were shown to cause mediator secretion effectively from mast cells of RBL-2H3 line. It did not appear that the lipid haptens can bring about receptor crosslinking by mechanisms analogous to those attained by the above described reagents. Hence the conclusion brought forth by McConnell and his associates (Balakrishanan et al 1982; Weiss et al 1982) was that, if receptor clustering is a requirement for cell-activation, it does

not necessarily involve molecular crosslinking of the above described type. Moreover, the simplest general model formulated by these authors, assumes the existence of a pre-equilibrium between monomeric and specific molecular complexes of receptors in the cell membrane. In the resting state, the concentration of such molecular complexes of FcεRI is too low to yield significant degranulation. Receptor crosslinking would then be but one pathway to displace this equilibrium, increase the concentration of such complexes and cause secretion. These authors proceeded further to suggest that if one assumes that there are limited regions of membrane-membrane contact between the mast cell and the hapten-lipid carrying liposome, then it is easy to imagine that IgE-FcεRI complexes would diffuse and concentrate into these contact regions. The enhanced FcεRI concentration would then also cause the concentration of specific molecular complexes to increase above the critical local level, resulting in degranulation (Balakrishanan et al 1982). The passive diffusion and accumulation of FcεRI into such contact regions induced by IgE-hapten binding, has been observed already in the above studies and was later corroborated further by McCloskey and M.m. Poo (1986).

We have tried to develop reagents that would enable a quantitative and rigorous analysis of the FcεRI mediated triggering process. For our studies, we used the well characterized mucosal mast cell line RBL-2H3 (Barsumian et al 1981) and monoclonal IgE of known specificity and affinity (Liu et al 1981). First, the aqueous solution equilibria of IgE oligomerization was systematically studied as induced by a series of synthetic divalent haptens which have defined length and flexibility (Schweitzer-Stenner et al 1987). However, since the secretory response of RBL-2H3 cells to these divalent haptens was found to be rather low, we could not extend the analysis done in solution to the cell membrane FcεRI crosslinking and the eventual secretion. Therefore, an alternative approach has been pursued; several monoclonal antibodies (mAbs) specific for the FcεRI have been raised and used as homogeneous and chemically defined crosslinking agents for this receptor on the RBL-2H3 line (Ortega et al 1988). These mAbs were found to be rather useful agents in the analysis of the FcεRI mediated secretory response (Ortega et al 1988). The intrinsic affinities of three such mAbs for the FcεRI on the RBL-2H3 were determined using their radiolabeled Fab fragments. In parallel, IgE binding to cells of the same batches were also carried out. As the FcεRI binds a single IgE molecule, these measurements yielded also information about the stoichiometry of Fab fragments binding to the receptors. Indeed, though the affinities of the examined three different Fabs differed by over twenty fold, the stoichiometry of all three was 1 Fab: 1 FcεRI. This result implied that these three IgG class mAbs may crosslink the receptors into dimers only. We have further measured the binding of the labeled intact mAbs to the RBL-2H3 cells on identical batches to those on which IgE and the respective Fabs binding were done. These parallel measurements yielded information about the extent of binding of the mAbs and also, the degree to which they induced FcεRI dimerization. In Figure 1 the ratio of bound mAbs to that of the bound IgE molecules on the cells is presented as function of concentration of the three mAbs. For each of the mAbs, this ratio reaches the maximal value expected for a dimer formation process, i.e. 0.5, supporting the notion that they crosslink the receptors into dimers.

Dose response of secretion induced by each of these mAbs showed that while two caused intermediate levels of mediator release at their respective optimal concentration (H10 and J17), the third mAb (F4) caused markedly higher response (Fig. 2). In order to determine

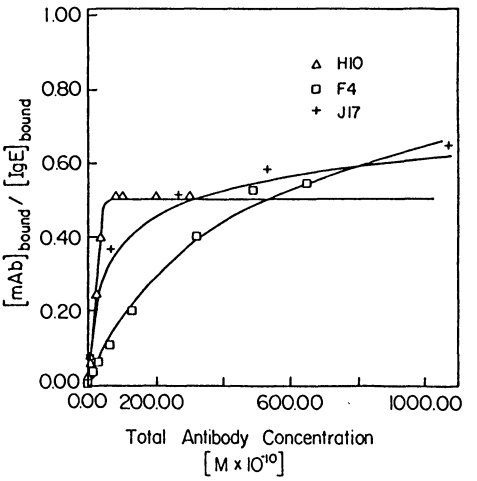

Fig. 1. Ratio of RBL-2H3 cell-bound intact monoclonal antibodies specific to the FcεRI and bound IgE molecules at saturation to cells of the same batch, as a function of total mAb concentrations. The solid lines are calculated by combining results of measuring the intrinsic affinities of the Fabs of these mAbs, titrations with IgE and binding measurements of the intact mAbs (Ortega et al., 1988). Both the marked difference in affinity between H10 and J17 or F4 as well as the divalent binding stoichiometry of the intact mAbs are illustrated by these data points and the fitted lines.

whether the reason for this difference originates in the distinct affinities of the mAbs and hence in the extent of FcεRI dimerization, we have calculated it by combining the above results, i.e.: the number of FcεRI per cell, the intrinsic affinity of each mAb and its binding as an intact divalent mAb to these cells (Ortega et al 1988). The unexpected outcome of these calculations was that rather than having the highest response produced by the mAb which causes the largest extent of dimerization, we observed the converse. Namely, mAb F4 which was found to crosslink maximally ca. 25% of the cell receptors into dimers is the one which causes the highest release (> 80% of the cell contents) while H10, which converts practically all the cell's FcεRI into dimers (already at ~ 5 x 10^{-9} M) induces maximally the secretion of 35% of the cells' mediator contents (Ortega et al 1988). These intriguing results may have two potential rationales, both based on the differing properties of the FcεRI dimers produced initially by the different mAbs. (The possibility that binding per-se e.g., by Fab fragments, could cause changes in the receptor which would lead to the observed distinct patterns was excluded by experiments described later below).

1) The first potential explanation is based on dynamic considerations (cf. for example De Lisi, 1980) and assumes that if receptors stay in close proximity for times longer than a critical period they would transduce a stimulatory signal to the cell. Therefore, FcεRI encounters which are shorter than the given period would not be productive whereas longer ones would. We have therefore studied the kinetics of association and dissociation of these three mAbs to and

from the cell's FcεRI, so as to determine the average lifetimes of dimers produced by each mAb. Results obtained to-date do not support this interpretation since they do not correlate with the amplitudes of the secretory response to the mAbs.

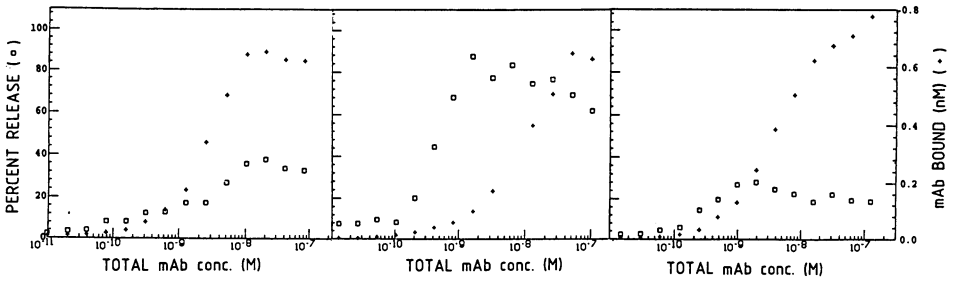

Fig. 2. A sample of experimental data illustrating the secretory-dose response of RBL-2H3 cells to FcεRI crosslinking by each of the three specific mAbs (-□-) and their binding to the cells (-+-). (Left to right H10, F4 and J17) Identical monolayers of the cells were treated with increasing concentrations of the respective [^{125}I]-labeled mAb for 30 min at 37°C. Secretion was then assayed in supernatant aliquotes by monitoring the released β-hexoseaminidase activity and is presented as fraction of total activity observed in lysed cells. The amount of bound mAbs was determined on the washed monolayers.

2) The second rationale assumes that since each of the mAbs binds its antigenic epitope in a particular way it would bring two FcεRI's together into a dimer with distinct spatial properties. In view of the complex multi-subunit structure of the receptor, one would expect that the relative apposition of the FcεRI subunits in each dimer would differ considerably and hence, this could importantly effect the signalling capacity of the dimer. According to this notion, it is not only the number of crosslinked FcεRI's which determines the amplitude of the secretory response but rather, other constraints imposed by each individual mAb are also decisive. We proceeded to suggest that these constraints are primarily of orientational nature implying an expected specificity in the interactions between the clustered receptors.

Significantly, these results carry a very interesting analogy to those obtained by McConnell et al. (1982), who showed that using lipid haptens carried on liposomes, a clear distinction can be observed between binding of the hapten-lipids to the cells and stimulating secretion. Thus, while there were concentrations of hapten-lipid (surface densities) which yielded extensive binding, no secretion was observed. Increasing the concentration above a critical value lead to an exponential rise in secretion whereas no significant increase in binding was noted.

We are currently developing protocols, using physicochemical methods to examine the notion that the distinct secretory response to the different mAbs is a result of specific orientational constraints imposed by the binding and dimerization by each of these "individual" agents. Some support for it already emerges from immunochemical experiments illustrated in Figure 3: Monovalent Fab fragments prepared from each of the mAbs were bound to the cells. No mediators were released by the cells at this stage. Polyclonal, goat

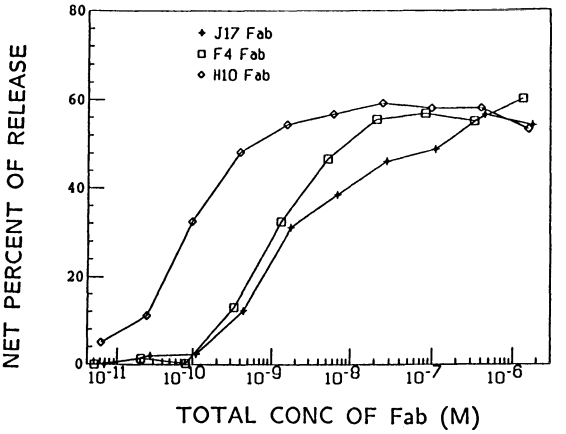

Fig. 3. Mediator release by RBL-2H3 cells as induced by crosslinking of cell-bound Fab fragments prepared from each of the three FcεRI specific mAbs, by goat anti-mouse Ig antibodies. Monolayers of [^{3}H] serotonin loaded cells (10^5 cells/100 μl in microtiter plates) were incubated with given concentrations of the different Fabs for 45 min at 25°C. The monolayers were then washed and 150 μl of goat anti-mouse Ig (15 μg/ml) were added per well. Following 30 min incubation at 37°C, supernatant samples were taken to determine the extent of secretion. Points are the means of triplicates. Nearly the same results were obtained in three independent experiments.

anti-mouse-Ig antibodies were then used to crosslink the different FcεRI bound Fabs. As can be seen in Fig. 3, the secretory dose-response pattern to the three Fabs reflects their intrinsic binding affinities and, most important, reaches practically the same maximal value. Thus, the individual features observed upon crosslinking by the intact mAbs are lost when the Fabs are being aggregated by the polyclonal anti-mouse Ig antibodies, which bring about random orientation of the crosslinked FcεRIs. This result also showed that Fab-binding by itself does not induce specific structural or configurational changes in the FcεRI which would affect the subsequent secretory response.

We have recently proceeded to study the relationship between FcεRI crosslinking, secretory response and the intermediary biochemical processes which are assumed to act as coupling elements between the stimulus and secretion. The transient rise in free cytosolic Ca^{2+} ions concentration is one important element of that kind (Beaven et al 1984; Sagi-Eisenberg et al 1985). We have monitored it using the fluorescent cell-permeable Ca^{2+} chelator Quin 2-AM (Beaven et al 1984). Results of such experiments are summarized in Figure 4. There are several noteworthy aspects to these data starting with their amplitudes and time courses as well as their temperature dependence. Here, only the rather different dose responses to the three mAbs can be briefly discussed. In marked contrast to the secretory response, the maximal rise in $[Ca^{2+}]_i$ induced by F4 and J17 is comparable, both in actual value and in its dependence on the mAbs concentration. mAb H10, however, yields a very small change in $[Ca^{2+}]_i$ at H10 concentrations (~ 10^{-8} M) similar to those required for maximal secretion by it. Thus, we observe only a qualitative correlation between the levels of this second messenger and the secretory response induced by the three mAbs. This observation

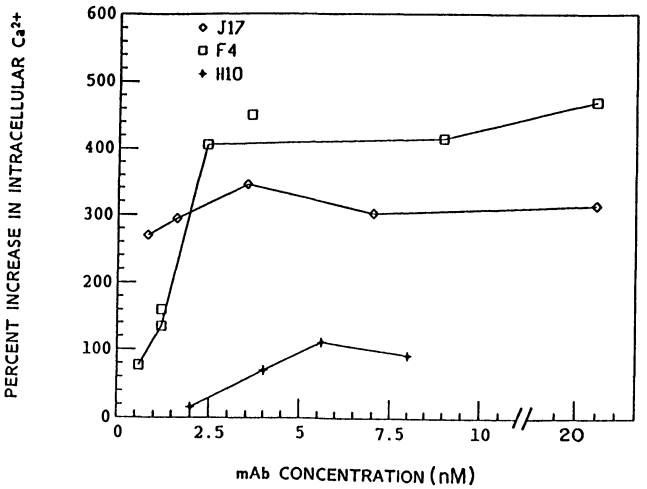

Fig. 4. Dose-response patterns of the maximal increase in free cytosolic Ca^{2+} ions concentrations induced by the FcεRI specific mAbs; F4 (-o-), J17 (-o-) and H10 (- Δ -). (Please note that the H10 concentrations employed are ten-fold higher those for the two other mAbs). $[Ca^{2+}]_i$ was determined using quin 2 signals as described elsewhere (Sagi-Eiseinberg et al 1985). Excitation wavelength was 339 nm, emission was monitored at 492 nm. Data are presented relative to the basal value (100-200 nM) observed without any stimulation. All data presented are from measurements at 25°C. Qualitatively similar results were obtained at 37°C.

illustrates the complexity of the processes coupling the initial redistribution of FcεRI's and the final degranulation. Still, it may very well be in line with the above ideas that specific interactions between the FcεRI components are induced by the distinct orientations that each mAb causes upon dimerizing the receptor. Since several different coupling elements are probably determining the eventual amplitude of secretion, the observed differences among levels of $[Ca^{2+}]_i$ induced by the three mAbs and the secretory response may be a reflection of this situation. Indeed, monitoring the secretion of LTC_4, as induced by these three mAbs (Ortega et al, submitted 1989) shows that both F4 and J17 cause secretion of this de novo produced mediator while LTC_4 secretion could not be detected as a result of H10 binding.

In summary, we have employed a system in which a quantitative examination of the secretory response to FcεRI stimulation by mast cells could be carried out; A well defined mucosal mast cell line, three different, yet homogeneous crosslinking agents - i.e. monoclonal antibodies specific for the FcεRI and, as a reference for FcεRI binding, a monoclonal IgE of known specificity. Results of these studies thus far show that: 1) The mAbs employed form, at least initially, FcεRI dimers and cause secretion by the RBL-2H3 cells in response to this dimerization, though to different extents. 2) The markedly different secretory responses observed upon crosslinking FcεRI by these reagents are probably the result of specific orientations of the receptors in the dimers. Hence, it is not only the extent of FcεRI crosslinking that determines the secretory response but also the specific properties of the crosslinked species which are formed.

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The Association of $p56^{lck}$ with the CD4/CD8 Antigens: Implications for T-Cell Function

C.E. Rudd and S.F. Schlossman

INTRODUCTION

The CD4 and CD8 antigens define discrete T-cell subsets involved in the restricted recognition of MHC class II and I antigens, repectively. The CD4 antigen also appears to serve as a receptor for the human immunodeficiency virus (HIV), the causative agent of AIDS. Both sets of antigens are members of the immunoglobulin superfamily and appear to synergize with the T-cell receptor complex (Ti/CD3) in the initiation of T-cell proliferation (Eichmann et al. 1986, Anderson et al. 1987). An important question has been to understand the underlying molecular basis of CD4 and CD8 function. In this paper, we briefly review data demonstrating a physical interaction between the CD4 or CD8 antigens and the protein-tyrosine kinase $p56^{lck}$ and outline its potential importance in the regulation of T-cell growth.

$p56^{lck}$ Is Associated With the CD4 and CD8 Antigens

The CD4 and CD8 antigens have been found to be physically associated with the catalytically active form of the protein-tyrosine kinase $p56^{lck}$ from T lymphocytes (Rudd et al,1988; Barber et al.,1989). As seen in Figure 1, immunoprecipitates of CD4 and CD8 were found capable of transfering the [γ] phosphate of [γ-ATP] to a co-precipitated substrate resulting in the labelling of a band at 55-60KD (lane 2 and 3, respectively). Further, phosphoamino acid analysis indicated labelling at a tyrosine residues, a extremely rare form of protein modification, accounting for only 0.01-0.02 percent of phosphorylation in the cell. Neither CD4 nor CD8 possess definable protein-tyrosine kinase domains. It was therefore likely that a protein tyrosine kinase was being co-purified with both CD4 and CD8. One candidate was the protein-tyrosine kinase ($p56^{lck}$) with a Mr of 56-62KD which has been reported to be expressed specifically in human T cells. To assess whether $p56^{lck}$ was associated with the CD8 receptor, an antisera to the carboxy terminus of $p56^{lck}$ was shown to specifically re-precipitate the 55-60Kd band after denaturation of the anti-CD8 precipitate in SDS (lane 10). Labelling at tyrosine was confirmed by re-precipitation with an anti-phosphotyrosine antibody (lane 11) and by phosphoamino acid analysis (Fig. 1B). Identical results were obtained in re-precipitation studies from anti-CD4 precipitates (Rudd et al. 1988). Two dimensional NEPHGE/SDS-PAGE further showed that the kinase associated with the CD8 antigen had the same Mr and charge (approximate pI of 4.8 to 5.8) as that associated with the CD4 antigen (Fig. 1C, middle panel and lower panel, respectively), and with that recognised directly by the anti-$p56^{lck}$ antiserum from detergent lysates (Fig. 1C, upper panel). Collectively, these data revealed that similar if not identical forms of catalytically

active $p56^{lck}$ are associated with the CD4 and CD8 antigens from T cells. The association is specific in that numerous other T-cell antigens have failed to co-precipitate kinase activity (Rudd et al., 1988).

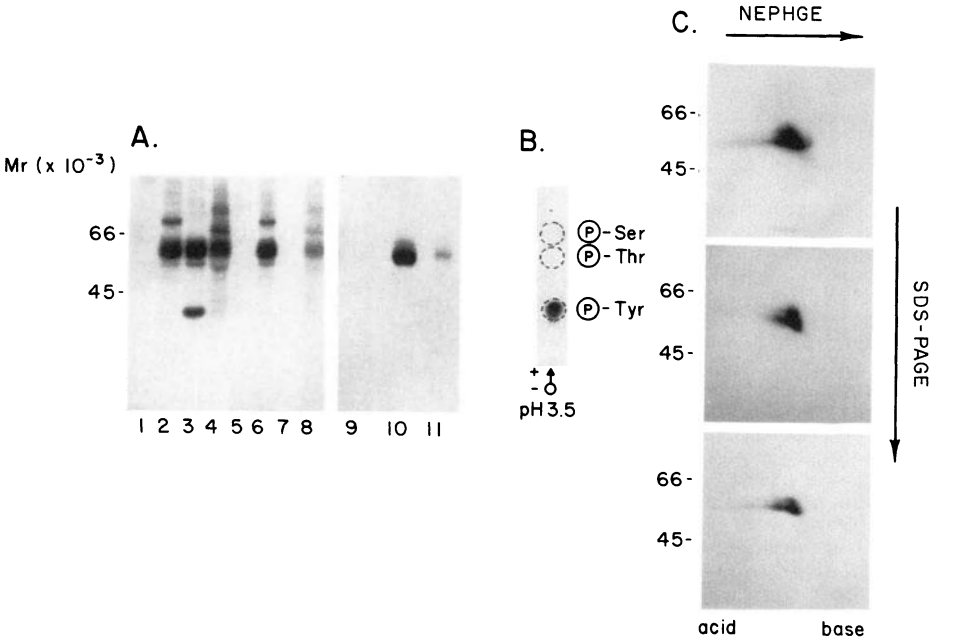

Figure 1 $p56^{lck}$ is associated with the CD4 and CD8 antigens. (A) Phosphotransferase activity of immunoprecipitates derived from REX (lanes 1-4) and MOLT 4 cells (lanes 5-8). (1,5) rabbit anti-mouse antibody (2,6) anti-CD4 antibody; (3,7) anti-CD8 antibody; (4,8) anti-$p56^{lck}$ antiserum; (9) anti-CD8 reprecipitated with the W6/32 antibody; (10) anti-CD8 reprecipitated with the anti-$p56^{lck}$ serum; (11) anti-CD8 reprecipitated with an anti-phosphotyrosine antiserum. (B) Phosphoamino acid analysis of the 55-60KDa band. (C) Two Dimensional NEPHGE/SDS-PAGE of $p56^{lck}$ Labelled in the Phosphotransferase Assay. Upper panel: anti-$p56^{lck}$ antiserum; middle panel: anti-CD8 antibody; lower panel: anti-CD4 antibody.

The CD4/CD8:$p56^{lck}$ Complex Phosphorylates the Ti(TcR)/CD3 Complex

The CD4 and CD8 antigens are known to synergise with the Ti(TcR)/Ti complex in the stimulation of T cells. Increasing the physical proximity of the CD4 and CD8 antigens with the Ti (TcR)/CD3 complex potentiates the activation of T-cells (Emmerich et al.,1986; Anderson et al.,1987). An important question is whether this interaction is mediated by CD4 and CD8 associated $p56^{lck}$. Importantly, as seen in Figure 2B, the CD4 and CD8:$p56^{lck}$ complexes readily phosphorylated members of the CD3 complex when co-purified and labelled in a phosphotransferase assay. The anti-CD3 immunoprecipitate alone did not co-precipitate kinase activity, while the anti-CD8 antibody precipitated the labelled $p56^{lck}$ at 55-60KD (lanes 1 vs 3, respectively). However, the addition of [γP^{32}]-ATP to the CD8:$p56^{lck}$ complex in the presence of CD3 resulted in the dramatic labelling of additional bands at 20 to 26KD

(lane 2). These bands co-migrated with CD3 bands precipitated from iodinated T cells (lane 5). The α/β chains of the T cell receptor at 42KD and 49KD found in the anti-CD3 precipitate from iodinated cells was generally not labelled in the phosphotranferase assay (lane 2). Re-precipitation analysis by an anti-phosphotyrosine antibody (Fig. 2C, lane 2) as well as phosphoamino acid analysis (Fig. 2C, last lane) confirmed that the CD3 band(s) were labelled at tyrosine residue(s).

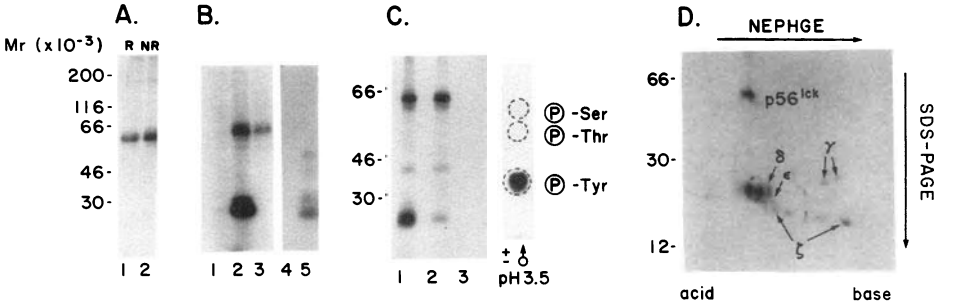

Figure 2 The CD8:p56^{lck} Complex is Non-covalently Linked and Can Phosphorylate Various Subunits of the CD3 Complex. (A) (1) anti-CD8 associated p56^{lck} (reduced); (2) anti-CD8 associated p56^{lck} (non-reduced); (B) (1) anti-CD3 precipitate; (2) combined anti-CD3 and anti-CD8 precipitates; (3) anti-CD8 precipitate; (4) rabbit anti-mouse precipitation from iodinated peripheral blood lymphocytes; (5) anti-CD3 precipitate from iodinated peripheral blood lymphocytes. (C) Re-precipitation Analysis of the [^{32}-P] ATP Labelled CD3 Subunits. (1) anti-CD4/CD3 immunoprecipitate; (2) denatured anti-CD4/CD3 re-precipitated with the anti-phosphotyrosine antisera; (3) denatured anti-CD4/CD3 re-precipitated with a W6/32 control. Last lane: phosphoamine acid analysis of the CD3 band. (D) Two-dimensional NEPHGE/SDS-PAGE analysis of the anti-CD4/CD3 immunoprecipitate labelled in the phosphotransferase assay.

Identical results were obtained with the CD4:p56^{lck} complex and separated by two dimensional NEPHGE/SDS-PAGE analysis in order to identify the nature of the phosphorylated CD3 subunits (Fig. 2D). In both cases, the 2-D patterns revealed a spectrum of spots in the positions of the γ chain at 26KD with an approximate pI of 6.5 to 7.2 as well as the δ and ε chains at 20KD with a pI of 5.0 to 6.0. In addition, the 2-D pattern also showed a series of spots stretching from 16KD to 20KD, corresponding to the ζ chain of the human CD3 complex. Intriguingly, the CD3 ζ chain labeled less well than the ε chain in this assay (unpublished results). Only the CD3 polypeptides were found to act as substrates for p56^{lck} from amongst twelve different T-cell antigens tested (CD1,CD2,CD6,CD9,CD25,CD29,CD45 etc.).

Potential Implications

Many mammalian growth receptors (including the EGF, PDGF, I-GF, CSF-1, insulin receptors) regulate cell growth via a pathway mediated by protein-tyrosine kinases. In an analogous manner, we have shown

that the CD4 and CD8 antigens are physically associated to the protein-tyrosine kinase $p56^{lck}$ which is catalytically active in its ability to phosphorylate components of the Ti(TcR)/CD3 complex (Fig. 3). Unlike conventional kinase receptors, CD4 and CD8 lack a kinase domain as part of their structure and instead interact by means of their cytoplasmic tail with $p56^{lck}$. CD4 receptors lacking the cytoplasmic region fail to associate with $p56^{lck}$ (Rudd et al., submitted). Further, a comparison of the cytoplasmic sequences of CD4 and CD8α reveals a possible binding sequence Lys/Arg Lys/Arg X Cys X Cys Pro X X X X Lys Thr/Ser with the Lys to Arg and Thr to Ser representing conservative substitutions. The kinase itself is also attached to the inner face of the membrane by a myristic group. Thus, the CD4/CD8:$p56^{lck}$ complex defines a new class of protein tyrosine kinase receptors and provides a precendent by which other members of the src family in T cells (such as fyn and hck) may eventually be found to associate with T cell surface molecules.

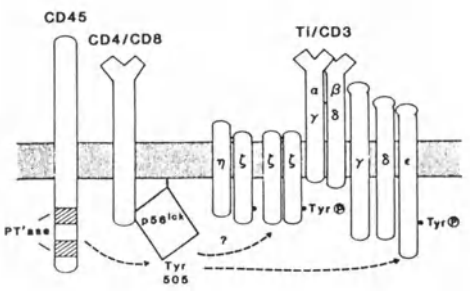

Figure 3: Postulated Role for the CD4/CD8:$p56^{lck}$ Complex and CD45 in T-cell Activation

As shown in Fig. 3, the CD4/CD8:$p56^{lck}$ complex is postulated to regulate T-cell growth by interacting with the Ti(TcR)/CD3 complex and CD45. We have shown that CD3 subunits can be phosphorylated *in vitro* at tyrosyl residue(s) in a somewhat specific manner by the complex (Fig. 2). Studies have now identified a direct physical interaction between CD4:$p56^{lck}$ and the Ti/CD3 complex (Rudd et al., submitted). Engagement of the CD4/CD8 receptor by MHC antigens, perhaps in the context of the Ti(TcR)/CD3 complex, would be expected to transiently activate $p56^{lck}$ activity. The net result of CD3 phosphorylation may be to alter the molecular associations within the Ti(TcR)/CD3 complex and/or regulate the activity of the kinase leading to a cascade of events linked to DNA replication. Other structures such as CD45 with tyrosine-phosphatase activity (Charbonneau et al.,1988) could modify the kinase acitivity of the CD4/CD8: $p56^{lck}$ complex. Therefore, the interaction of these cell surface structures is postulated to modify signals along a tyrosine phosphorylation pathway linked to T cell activation.

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Signal Transduction by the T Cell Antigen Receptor

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INTRODUCTION

As a consequence of the interaction of T cell surface molecules with antigen, associated major histocompatibility complex (MHC) molecules and other cell surface molecules on the antigen presenting cell, T cells become activated to secrete lymphokines, express a variety of cell surface molecules, proliferate and develop cytolytic activity. These events, which are manifestations of T cell activation, result from signal transduction by a variety of cell surface molecules expressed on the T cell. However, of primary importance in regulating T cell activation is the T cell antigen receptor (TCR), which in addition to its antigen recognition function also functions as a signal transduction molecule.

The structure of the TCR reflects both of its functions. It may be viewed as consisting of a two chain ligand-binding subunit responsible for antigen/MHC recognition comprised of the the α/β (or γ/δ) heterodimer (Ti) and a five chain subunit, CD3, which has been assumed to play a role in signal transduction (Allison and Lanier 1987; Clevers et al. 1988; Weiss and Imboden 1987). Such a role for CD3 is supported by the agonist effects of anti-CD3 monoclonal antibodies (mAbs) (Chang et al. 1982; Van Wauwe et al. 1980) as well as by studies with somatic cell mutants defective in TCR-mediated signal transduction (Goldsmith and Weiss 1988b; Goldsmith and Weiss 1987; Sussman et al. 1988a)(see below).

Currently, two signal transduction pathways have been shown to be regulated by the TCR: the inositol phospholipid (IP) pathway and a tyrosine kinase pathway. In the case of the IP pathway, stimulation of the TCR with antigen/MHC on antigen presenting cells or anti-TCR mAbs results in the activation of a phospholipase C enzymatic activity (PLC) which hydolyzes phosphatidylinositol 4,5-bisphosphate (Imboden et al. 1987; Imboden et al. 1985). This results in the generation of two potent intracellular second messengers, inositol 1,4,5-trisphosphate and diacylglycerol which in turn function to mobilize cytoplasmic free calcium ($[Ca^{2+}]_i$) and activate protein kinase C (pkC) (Berridge 1987). How the TCR is coupled to PLC is not known but indirect evidence has suggested the involvement of an as yet unidentified nucleotide binding protein (Imboden et al. 1986). Which if any of the several types of phospholipase C (Ryu et al. 1987) are activated in response to TCR stimulation is also not clear.

The importance of the inositol phospholipid pathway is suggested by several lines of evidence including: 1) the agonist effects of calcium ionophores and phorbol esters which activate pkC (Truneh et al. 1985; Weiss et al. 1984a); 2) the inhibitory effects of calcium chelators or pkC inhibitors (Weiss et al. 1984b; Nel et al. 1987); 3) the ability of calcium ionophore alone to induce interleukin 2 (IL-2) production from a cell expressing a constitutively active pkC enzyme (Muramatsu et al. 1989); 4) the identification of calcium and phorbol ester responsive elements in the IL-2 enhancer (Crabtree 1989); and 5) the failure of somatic cell mutants selected for their failure to

increase $[Ca^{2+}]_i$ in response to TCR agonists also to produce IL-2 (Goldsmith et al. 1987).

The second signal transduction pathway that is regulated by the TCR involves a tyrosine kinase which, unlike many of the growth factor receptors, is not intrinsic to the structure of the TCR (Samelson et al. 1986). Several tryrosine kinases have been identified in T cells but none of these have yet been directly linked to TCR stimulation. The relative importance of the tyrosine kinase pathway to cellular activation has been suggested by: 1)the ability of murine hybridoma cells defective in the inositol phospholipid pathway to produce IL-2 in response to TCR agonists (Sussman et al. 1988b); and the ability of the tyrosine kinase v-src to induce constitutive IL-2 production by a murine T cell hybridoma(O'Shea et al. 1989)

The relative importance of the inositol phospholipid pathway and tyrosine kinase pathway to later T cell responses is not clear. A more thorough understanding of the cellular components involved in the signal transduction pathways and the intermediate events involved in later cellular responses is required in order to understand how these signal transduction pathways relate to T cell activation.

SOMATIC CELL MUTANTS WITH DEFECTIVE TCR SIGNAL TRANSDUCTION

To more fully characterize the cellular elements involved in the TCR signal transduction pathways and the relative importance of these pathways in T cell activation, we have developed a methodology to derive somatic cell mutants of the human T cell line Jurkat which are defective in components necessary for a TCR-induced increase of $[Ca^{2+}]_i$ in response to TCR agonists (Goldsmith et al. 1987). The isolation of these mutants takes advantage of the inability of the wild-type cells to grow in the presence of TCR agonists and the use of the calcium-sensitive fluorescent dye indo-1 and the cell sorter to isolate cells which express high levels of TCR but fail to increase $[Ca^{2+}]_i$ in response to anti-TCR mAb. Using this methodology we have isolated three mutants which have defects in distinct components of the TCR activated IP pathway (Goldsmith et al. 1988b; Goldsmith et al. 1989; Goldsmith et al. 1987). Table 1 summarizes the $[Ca^{2+}]_i$ response phenotypes of these cells to anti-TCR mAbs.

The failure of these three mutants to increase $[Ca^{2+}]_i$ to anti-Ti and group 1 anti-CD3 mAbs correlates well with their failure to increase inositol phosphates. Although group 2 anti-CD3 mAbs do induce increases of $[Ca^{2+}]_i$ in J.CaM1 and J.CaM3, changes in inositol phosphates are substantially lower that those observed in the wild-type cell (Goldsmith and Weiss 1988a). The relative preservation of the $[Ca^{2+}]_i$ response may reflect a greater sensitivity of inositol phosphate receptors which regulate $[Ca^{2+}]_i$ mobilization for small changes in inositol phosphates.

Several features of these mutants are worth noting. All three cells express high levels of TCR on the cell surface and biochemical characterization of the component chains of the TCR on these cells has failed to reveal a deficiency or gross alteration of the Ti α or β or CD3 δ, ε, γ or ζ chains. The TCR in all three of these mutants can be internalized in response to an anti-Ti mAb (C305) despite the fact that the mAb fails to induce inositol phospholipid metabolism (Goldsmith, et al., 1987). This suggests that signal transduction and resultant activation of protein kinase C may not be necessary for

Table 1

Summary of the peak $[Ca^{2+}]_i$ responses of Jurkat and TCR signal transduction mutants

	Responding Cell			
Stimulus	Jurkat	J.CaM1	J.CaM2	J.CaM3
anti-Ti	+++	-	-	-
anti-CD3				
Group 1*	+++	-	-	-
Group 2**	+++	+++	-	+++

* Group 1 mAbs includes OKT3, UCHT1 and A32.1.
** Group 2 mAbs includes Leu4, 235, L142 and L143.

ligand-induced TCR internalization. Finally, these cells fail to produce IL-2 in response to TCR ligands, even in the presence of phorbol myristate acetate (Goldsmith et al. 1987).

The identification of the mutations responsible for these signal transduction deficits is of great interest. Initial attempts have focused on the α and β chains of Ti since these chains are derived from allelically excluded genes and, thus, are effectively haploid. However, utilizing a complementation analysis in which the response of heterokaryons to TCR stimulation is analyzed within 60 minutes of fusion has ruled out the α or β chains as the site of the mutation in each of these three signal transduction mutants (Goldsmith et al. 1988b; Goldsmith et al. 1989). These complementation assays have also established that the defect in each of the mutants resides in a distinct protein product. Although the sites of the mutations in these cells have not been identified, the finding that the Ti molecules are normal in J.CaM1 and J.CaM3 together with their abilities to respond to a degree to some anti-CD3 mAbs supports the notion that CD3 functions to couple Ti to intracellular signal transduction mechanisms.

Studies are in progress to analyze the possibility that other chains of the TCR within the CD3 complex are defective. In addition, we are assessing the integrity of other components of the IP signal transduction pathway and the status of the tyrosine kinase pathway in these mutants. Two lines of investigation have added to our understanding of TCR signal transduction and provided some insight into these mutants.

IDENTIFICATION OF TWO PROTEINS THAT ASSOCIATE WITH THE AGONIST OCCUPIED TCR

In a number of systems, agonists that bind to receptors involved with signal

transduction induce the association of such receptors either with molecules that may be involved with signal transduction directly or with molecules which serve to couple these receptors to intracellular effector molecules involved with signal transduction. We have recently identified two nondisulfide-linked cell surface glycoproteins, not previously characterized, which associate with the TCR in the agonist occupied state (Fraser, Goldsmith, & Weiss, 1989). They are glycoproteins of 34 and 38 kD (gp34 and gp38) and have been detected on three independent T cell leukemic lines. These proteins associate with the TCR in less than5 minutes following binding of agonist Ti or CD3 mAb and are visualized by cell surface iodination and coprecipitation with the TCR. Coprecipitation of these proteins with the TCR requires alklyation following the addition of the TCR ligand. Such a requirement for alkylation to detect this association is not without precedent. It has also been observed in the preservation of the association of the β2-adrenergic receptor with the stimulatory guanine nucleotide binding protein Gs (Korner, Gilon, & Schramm, 1982).

The functional significance of the association between the TCR and gp34 and gp 38 is suggested by: 1) the failure of these proteins to associate with the TCR when mAbs to other cell surface molecules are added to the cells prior to alkylation and solubilization; 2) the failure of these proteins to associate with the TCR of the signal transduction mutants J.CaM1 or J.CaM3; and 3) the ability of these proteins to associate with the TCR of J.CaM2. This latter finding suggests that this association is not linked to processes leading to the internalization of the TCR and does not occur as a consequence of signal transduction. The exact function of these proteins in TCR-mediated signal transduction and in T cell activation awaits their further characterization.

FUNCTION OF THE MUSCARINIC RECEPTOR IN JURKAT T CELLS AND THE SIGNAL TRANSDUCTION MUTANTS

One approach towards identifying the mutations in the Jurkat-derived signal transduction mutants and towards understanding the contribution of the IP pathway to later T cells responses is to express a heterologous receptor which normally couples to the IP pathway in Jurkat or the mutants. The human muscarinic subtype 1 (HM1) receptor is a seven transmembrane domain receptor which is normally expressed in neuronal, smooth muscle and cardiac tissue and activates the IP pathway in cells from these tissues. The genomic DNA encoding this receptor (Peralta, et al., 1987) was subcloned into an expression vector and transfected into Jurkat and the signal transduction mutants and the function of the HM1 receptor in these T cell hosts was assessed (Goldsmith, et al., 1989).

In Jurkat cells, no binding sites could be detected for the muscarinic antagonist quinuclidylbenzilate (QNB). Following the introduction of the HM1 receptor DNA, 8800 saturable high affinity binding sites were detected with a K_D for QNB of 32 pM on the Jurkat-derived cell J-HM1-2.2. In this T cell host, we found that the HM1 receptor can activate the IP pathway, as assessed by the increases in inositol phosphates and $[Ca^{2+}]_i$ in response to the muscarinic agonist carbachol. These responses are inhibitable with the muscarinic antagonist atropine. Moreover, stimulation of the HM1 receptor induces the expression of the α-chain of the IL-2 receptor and the production of IL-2 (Desai and Weiss, manuscript in preparation). Thus, the HM1 receptor which is structurally distinct from the TCR and is normally not expressed in

T cells can functionally activate the IP pathway in T cells. Furthermore, as a consequence of the signal transduction pathways it activates, HM1 can induce later responses specifically associated with the differentiated functions of T cells. These findings suggest that the activation of the inositol phospholipid pathway may be sufficient to induce at least some late T cell functions.

Finally, it was of interest to determine whether the HM1 receptor could functionally activate the IP pathway in J.CaM1-3 the signal transduction mutants. Following transfection with the HM1 receptor DNA, saturable high affinity QNB binding sites were detected on each of the three signal transduction mutants (Goldsmith, et al., 1989). Stimulation of the HM1 receptor with carbachol resulted in the activation of the inositol phospholipid pathway in each of these signal transduction mutants. Although it is not clear that the TCR and HM1 receptor activate the inositol phospholipid pathway through the same intracellular components, these studies suggest that the more distal components of this signal transduction pathway are similar.

CONCLUSIONS

The activation of T cells occurs as a result of receptor-initiated signal transduction events. We have only a superficial view of the immediate molecular events that occur following ligand binding. Through complementary genetic and biochemical approaches, a clearer view of the molecular events which occur following the binding of antigen to the TCR that lead to T cell activation will emerge.

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III Effector Phases

Molecules and Cells in Allergic Reactions

Molecular Biology of Mast Cell Secretory Granule Proteoglycans and Proteases

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INTRODUCTION

Mast cells and a large number of other hematopoietic cells have secretory granules which contain a unique family of proteoglycans. In the case of the mast cell, these proteoglycans are exocytosed when the IgE receptors on the cell's plasma membrane are cross-linked with antigen (Yurt et al. 1977). While a number of functions have been attributed to secretory granule proteoglycans of hematopoietic cells, it appears that a major function is to interact specifically with basically charged secretory granule proteins (Yurt and Austen 1977; Serafin et al. 1986). Mast cells have been found to contain large amounts of basically charged serine proteases that are enzymatically active at neutral pH (Woodbury et al. 1978; Schwartz et al. 1981; Dubuske et al. 1984; Shechter et al. 1986; LeTrong et al. 1987). In the rat serosal mast cell (Everitt and Neurath 1980), the mouse bone marrow-derived mast cell (BMMC) (Serafin et al. 1987), and the human skin mast cell (Goldstein et al. 1987)], the proteoglycans and serine proteases have been shown to be also packaged with basically charged carboxypeptidases. These endopeptidases and exopeptidases are stored in active form in secretory granules, and therefore it is likely that one of the functions of proteoglycans in mast cells is to package the enzymes in a configuration that minimizes their autolysis. When the composition of the protease/proteoglycan macromolecular complex in the mouse BMMC was examined during the stage of differentiation in which the cell was synthesizing both chondroitin sulfate E proteoglycan and heparin proteoglycan, it was discovered that the macromolecular complex was preferentially enriched for heparin proteoglycans (Serafin et al. 1986). In addition, when different Kirsten sarcoma virus immortalized mast cell (KiSV-MC) lines were examined for their protease activities and biosynthesis of proteoglycans, a strong positive correlation was observed in carboxypeptidase A content and biosynthesis of heparin proteoglycans relative to chondroitin sulfate E proteoglycans (Reynolds et al. 1988). Thus for proteoglycans, the type of the glycosaminoglycan that is polymerized onto the serine/glycine-rich peptide core may be related to the type of protein with which the proteoglycan is to interact inside the secretory granule.

MOLECULAR BIOLOGY OF THE HUMAN, RAT, AND MOUSE SECRETORY GRANULE PROTEOGLYCAN PEPTIDE CORE GENE

Molecular biology studies have been initiated on the cDNAs and genes that encode the mouse, rat, and human secretory granule proteoglycan peptide cores. A 3' gene-specific fragment of a rat L2 yolk sac tumor cell-derived cDNA (Bourdon et al. 1985) was constructed and used to demonstrate that this gene is expressed at relatively high levels in a variety of hematopoietic cells that possess secretory granules (Tantravahi et al. 1986). Despite the fact that the proteoglycans of large granular lymphocytes and natural killer cells have fewer and less sulfated glycosaminoglycans bound to them relative to those in mast cells, it appears that the same peptide core is used by all mast cell (Avraham et al. 1988) and lymphocyte

populations that have been examined so far. The selection of the type of glycosaminoglycan that will be synthesized onto a peptide core therefore appears to be a cell-specific event that is not exclusively dependent on the translated peptide core.

A 1.3 kb transcript was identified when a blot containing total RNA from human promyelocytic leukemia HL-60 cells was probed with the rat L2 cell proteoglycan peptide core cDNA. Thus, a HL-60 cell cDNA library was screened under conditions of low stringency with the rat probe in order to identify and isolate the human analogue of this proteoglycan peptide core (Stevens et al. 1988). Sequence analysis of resulting human cDNA clones indicated that the putative proteoglycan peptide core that is expressed in HL-60 cells is 17,600 M_r and contains an 18 amino acid glycosaminoglycan attachment region that consists primarily of alternating serines and glycines. Blot analysis of genomic DNA from a number of human/mouse and human/hamster somatic cell hybrids probed with the human proteoglycan cDNA indicated that the gene that encodes this molecule resides on chromosome 10. Subsequent *in situ* hybridization studies revealed that this gene resides on the long arm of chromosome 10 at band q22.1 (Mattei et al. 1989). A human genomic library has been probed under conditions of high stringency with a 5′ fragment of the HL-60 cell cDNA to isolate 18-kb genomic fragments that contain the entire human proteoglycan peptide core gene. A restriction map of this human gene has been constructed and the genomic fragments have been subcloned into Bluescript plasmid in order to determine the exon/intron organization of this gene and the nucleotide sequence of the 5′ flanking 621 nucleotides. Based on the deduced amino acid sequence of this gene, a 16 amino acid peptide that corresponds to a region of the peptide core preceding the putative glycosaminoglycan attachment region was synthesized, covalently coupled to hemocyanin, and injected into rabbits to elicit antibodies. Because these anti-peptide rabbit antibodies immunoprecipitated a 20,000 M_r protein from HL-60 cells that had been labeled for 2-min with [^{35}S]methionine and an approximate 150,000 M_r [^{35}S]proteoglycan from HL-60 cells labeled for a longer period of time with [^{35}S]methionine or [^{35}S]sulfate, it is now established that the isolated cDNA encodes the peptide core of a HL-60 cell secretory granule proteoglycan.

A 1.0-kb cDNA has been isolated from a mouse BMMC-derived cDNA library that encodes a 16,700 M_r proteoglycan peptide core that contains a 21 amino acid glycosaminoglycan attachment region consisting of alternating serines and glycines (Avraham et al. 1989). When the predicted amino acid sequence of the mouse proteoglycan peptide core was compared with the predicted amino acid sequences of the homologous rat and human molecules, the N-terminus was found to be a highly conserved region of the molecule. This suggests that the N-terminus of the peptide core may be important for the structure, function, and/or metabolism of this family of proteoglycans. The peptide cores of the rat, mouse, and human secretory granule proteoglycans were found to possess an Asp/Glu-Asp-Try amino acid sequence preceding the glycosaminoglycan attachment region. Since it has been proposed that a least one acidic amino acid must precede the first serine that is glycosylated in proteoglycans (Bourdon et al. 1987), it is likely that these two acidic amino acids are important for regulating glycosaminoglycan glycosylation of this hematopoietic cell proteoglycan. Areas of identity in the 3′ and 5′ untranslated regions in the human, rat, and mouse proteoglycan cDNAs were also observed. Interestingly, these 3′ and 5′ conserved regions were almost identical in the rat cDNA that encodes rat mast cell protèase II (Benfy et al. 1987), suggesting that these nucleotide sequences may be important for coordinated regulation of those genes that encode proteins destined to reside in secretory granules.

A mouse liver genomic library was probed under conditions of high stringency with a 3′ gene-specific fragment of the mouse BMMC cDNA to isolate an 18 kb genomic clone

which contains the three exons that encode the mouse secretory granule proteoglycan peptide core (Avraham et al. in press). Cotransfection of rat-1 fibroblasts with the genomic clone and the selectable marker pSV2 *neo* resulted in the establishment of fibroblast cell lines that had integrated both foreign genes into their genome. RNA blot analysis revealed that two of the fibroblast cell lines contained low, but detectable, levels of a 1.0-kb specific mRNA transcript that hybridized under conditions of high stringency to the gene-specific 3′ fragment of the BMMC-derived proteoglycan cDNA. The demonstration of mouse secretory granule proteoglycan peptide core mRNA in the two cotransfected fibroblast cell lines indicated that the mouse genomic clone contained the entire three exons of the mouse proteoglycan gene. The exon/intron organization of the mouse gene has recently been determined, as well as the 504 bp sequence that is upstream of the mouse proteoglycan gene. S1 nuclease protection and primer extension reactions were performed to determine that a site ~40 nucleotides upstream of the translation-initiation site is where transcription begins in mouse BMMC, human HL-60 cells, and rat RBL-1 cells (but not in rat L2 tumor cells). Neither a classical TATA box nor a GC-rich element was detected 30 bp upstream of the transcription-initiation site, indicating that this gene has an unusual promoter. Because no other gene that encodes a distinct proteoglycan peptide core has been isolated and characterized in its entirety, it is not yet possible to determine the significance of the deduced exon/intron organization of this gene and the uniqueness of the nucleotide sequences in its promoter region. Nevertheless, the ability of the transfected rat-1 fibroblasts to transcribe the mouse gene indicates that some of the regulatory elements in the gene's promoter are present in the isolated mouse genomic clone. When the 504 bp 5′ flanking region of this mouse gene was compared to the corresponding 5′ flanking region of the analogous human gene, a 119 bp region that immediately precedes the transcription-initiation site was found to be nearly identical. This nucleotide sequence was in fact more highly conserved across species than any similar sized region of the gene that is translated into protein, implying that this 5′ flanking region contains *cis* regulatory elements that are critical for expression of the secretory granule proteoglycan peptide core gene in mast cells and other hematopoietic cells.

MOLECULAR BIOLOGY OF RODENT MAST CELL SECRETORY GRANULE PROTEASES

The development of the transformed rat basophilic leukemia (RBL-1) cell line and the mouse KiSV-MC cell lines have now enabled investigators to identify and characterize at the molecular level those serine proteases and carboxypeptidases that are specifically expressed by rodent mast cells. Both RBL-1 cells (Seldin et al. 1985) and rat mucosal mast cells (Woodbury et al. 1978) contain rat mast cell protease-II (RMCP-II) in their secretory granules. Because the amino acid sequence of this serine protease had been determined (Woodbury et al. 1978), a RBL-1 cell-derived cDNA library was prepared and probed with synthetic [^{32}P]oligonucleotides that were constructed based on the amino acid sequence of RMCP-II to isolate a cDNA that encodes this rat MMC/RBL-1 cell secretory granule serine protease (Benfy et al. 1987). Analysis of the deduced amino acid sequence of the RMCP-II cDNA revealed that the translated rat mast cell prepro-enzyme is 247 amino acids and contains a 18 amino acid signal peptide. The pro-enzyme is predicted to differ from the stored secretory granule enzyme in that it has two additional glutamic acid residues at its N-terminus and three additional amino acids (Thr-Ser-Ser) at its C-terminus. A rat liver genomic library was probed with the RMCP-II cDNA to isolate and characterize this mast cell gene (Benfy et al. 1987; Sarid et al., 1989). Comparative nucleotide studies and gene transfection studies revealed that a 5′ flanking region of the RMCP-II gene contains an element that

enhances transcription of this gene in RBL-1 cells, but not in rat and mouse fibroblasts and in mouse B lymphoma cells.

The N-terminal amino acid sequences of a novel serine protease and a novel carboxypeptidase that are both present in the secretory granules of mouse KiSV-MC have been determined. A cDNA library, prepared from KiSV-MC, was used to isolate distinct cDNAs that encode both enzymes (Reynolds et al. 1989a & b). By probing the KiSV-MC cDNA library under conditions of low stringency with the cDNA that encodes RMCP-II, a 953-base pair cDNA was isolated that encodes a 28,000 M_r basically-charged serine protease which has been designated mouse mast cell protease-2 (MMCP-2). The deduced amino acid sequence of the MMCP-2 predicts that the prepro-enzyme is 26,700 M_r and consists of 244 amino acids. Based on the -3, -1 rule for cleavage of signal peptides (von Heijne 1984), the initially-translated enzyme contains an 18 amino acid signal peptide. Thus, it was concluded that the pro-enzyme consists of 226 amino acids. Two glutamic acids are then predicted to be removed from the N-terminus of the pro-enzyme during its subsequent post-translational processing to form a mature 24,700 M_r enzyme that contains 224 amino acids. Since a potential N-linked glycosylation site was identified in the deduced amino acid sequence, it is likely that the mature mouse mast cell serine protease has a larger M_r because it contains an oligosaccharide. Based on the deduced amino acids in the substrate binding site, the substrate preference of MMCP-2 is predicted to be chymotryptic rather than tryptic. Recently, this serine protease has been shown to be selectively expressed by those mast cells that are present in the proximal small intestines of mice infected with *Nippostrongylus brasiliensis*.

Based on the N-terminal sequence of a 36,000 M_r carboxypeptidase that was isolated from the secretory granules of mouse KiSV-MC, an oligonucleotide probe was synthesized and used to isolate a 1470-base pair cDNA that encodes this novel mouse mast cell exopeptidase. The deduced amino acid sequence revealed that the prepro-enzyme consists of 417 amino acids. This enzyme is predicted to have a 15 amino acid hydrophobic signal peptide and a 94 amino acid activation peptide. RNA and DNA blot analyses revealed that mouse peritoneal mast cells, KiSV-MC, and BMMC all express a prominent 1.5 kilobase MC-CPA mRNA which is transcribed from a single gene. Based on the amino acids in its active site, the substrate preference of this mast cell exopeptidase is predicted to be more similar to pancreatic carboxypeptidase A than carboxypeptidase B.

The isolation of the above cDNAs that encode organelle specific proteoglycans and mast cell-specific neutral protease mRNAs should be invaluable reagents for future studies in the mouse on mast cell secretory granule development, mast cell differentiation, and mast cell responses to specific stimuli.

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Differentiation of Mast Cells and Phenotypic Change Between Subpopulations

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INTRODUCTION

Mast cells are a progeny of multipotential hematopoietic stem cells. However, the differentiation processes of mast cells are unique and different from those of other progenies of stem cells. First, we will describe the unique differentiation processes of mast cells. In contrast with the unique differentiation processes, regulation mechanisms of mast-cell differentiation appear to be comparable to those of other progenies of hematopoietic stem cells. Second, we will describe regulation mechanisms of mast-cell differentiation. Mast cells are observed in various tissues of the body, and phenotypes of mast cells are different from tissue to tissue. However, the phenotypes of mast cells are not rigidly determined, and can be changed with experimental manipulations. Third, we will describe the phenotypic change between mast-cell subpopulations.

DIFFERENTIATION PROCESSES

Hematopoietic cell origin of mast cells was first shown by using giant granules of beige (C57BL/6-bg-J/bg-J, Chediak-Higashi syndrome) mice as a marker. Transplantation of bone marrow cells from C57BL/6-bg-J/bg-J mice to irradiated normal (C57BL/6-+/+) mice resulted in development of bg-J/bg-J-type mast cells with giant granules (Kitamura et al 1977). Two other mutant mice are also useful for investigations of mast-cell differentiation. Mice of either W/W-v or Sl/Sl-d genotype are deficient in mast cells (Kitamura et al 1978; Kitamura and Go 1989; Galli and Kitamura 1987). From the results of transplantation experiments, the absence of mast cells is attributed to a defect of precursor cells in W/W-v mice and to a defect of the tissue environment necessary for differentiation of mast cells in Sl/Sl-d mice (Table 1).

We demonstrated that mast cells are the progeny of multipotential hematopoietic stem cells by using W/W-v mice as recipients (Kitamura et al 1981). Most of progenies of multipotential stem cells such as erythrocytes, platelets, neutrophils, eosinophils and basophils leave the bone marrow after they differentiate. However, mast cells do not complete their differentiation in hematopoietic tissue. Morphologically unidentifiable precursors of mast cells leave the bone marrow, migrate in blood, and invade into the connective or mucosal tissues, proliferate and differentiate into morphologically identifiable mast cells (Kitamura et al 1987).

Table 1. In vivo analysis of mast cell deficiency of mutant mice of W/W-v and Sl/Sl-d genotypes

Treatment	Genotype Donor	Recipient	Development of mast cells
Bone marrow transplantation	+/+	W/W-v	Yes in all tissues examined
	+/+	Sl/Sl-d	No
	Sl/Sl-d	W/W-v	Yes in all tissues examined
Skin transplantation	W/W-v	+/+	Yes in grafted skin
	Sl/Sl-d	+/+	No
	W/W-v	Sl/Sl-d	Yes in grafted skin

Turnover of mast cells was investigated; +/+ mice were continuously supplied with water containing bromodeoxyuridine (BrdUrd), which was incorporated into the nucleus of the cells in S phase of the cell cycle. The incorporated BrdUrd was identified by using anti-BrdUrd monoclonal antibody. Sixty days after starting the experiment, more than 60 percent of mast cells in the gastric mucosa were labeled with BrdUrd, whereas less than 20 percent of mast cells in the skin were labeled (Fukuzumi et al, unpublished data). This indicates the slow turnover of mast cells in the skin. Within the stomach, proportion of labeled mast cells was significantly lower in the muscularis propria than in the mucosa. The turnover of mast cells appeared to be influenced by the tissue at which mast cells are present.

Although most progenies of multipotential hematopoietic stem cells lose the proliferation potential when they fully differentiate, some morphologically identifiable mast cells have an appreciable proliferation potential (Sonoda et al 1984).

Neutrophils, eosinophils and basophils appear to die after functioning. Recently, we investigated the fate of mast cells after degranulation. Purified peritoneal mast cells were sensitized with monoclonal anti-dinitrophenol (DNP) IgE antibodies and stimulated with DNP conjugated with human serum albumin. Mast cells were vitally stained with neutral red, and highly degranulated mast cells were identified under a phase-contrast microscope and individually picked up with the micromanipulator. When these highly degranulated mast cells were individually plated in methylcellulose, their potential to produce a colony was comparable to that of morphologically intact mast cells. Moreover, when highly degranulated mast cells were injected into the skin of mast cell-deficient W/W-v mice, the proportion of injection sites at which mast cell colonies appeared was comparable to the value observed when morphologically intact mast cells were injected, indicating that proliferative potential of mast cells is not reduced by their degranulation (Kuriu et al in press).

REGULATION OF DIFFERENTIATION

T Cell-derived Growth Factors

Striking proliferation of mast cells in the intestinal mucosa is observed during the infection of certain intestinal parasites in mice

and rats. By contrast, genetically T cell-depleted nude athymic mice lack such a response, suggesting the involvment of T cells for proliferation of mast cells (Ruitenberg and Elgersma 1976).

T cells produces many hematopoietic growth factors, among which IL-3 is the principal growth factor for differentiation of mast cells. IL-3 stimulates differentiation of mast cells from hematopoietic stem cells, and supports the proliferation of differentiated mast cells in suspension culture. The coexistence of IL-4 augments the effect of IL-3 on suspension-cultured mast cells. On the other hand, the combination of IL-3 and IL-4 was essential for clonal growth of differentiated peritoneal mast cells in methylcellulose (Hamaguchi et al 1987).

Fibroblast-dependent Proliferation

The concentration of mast cells in the skin of nude athymic mice is comparable to that of normal congenic mice, implicating the presence of regulatory mechanisms without T-cell involvement. We investigated the role of fibroblasts in the proliferation of mast cells. Without addition of IL-3 and IL-4, bone marrow-derived cultured mast cells may continue to proliferate on a monolayer of the NIH/3T3 fibroblast cell line (Fujita et al 1988b). Since NIH/3T3 fibroblasts synthesized neither IL-3 nor IL-4, and since direct contact of mast cells with NIH/3T3 fibroblasts was necessary for the proliferative response, we considered that the supportive effect of the NIH/3T3 cell line on the cultured mast cells was mediated neither by known mast-cell growth factors nor by unknown diffusible substances.

We analysed the mechanism of mast-cell deficiency in mutant mice of W/W-v and Sl/Sl-d genotypes by co-cultureing mast cells and fibroblasts. T cells of both W/W-v and Sl/Sl-d mice may produce IL-3 and IL-4. Mast cells develop when bone marrow cells of either W/W-v or Sl/Sl-d mice are cultured in the medium containing IL-3 and IL-4. Therefore, IL-3 and IL-4 do not appear to be involved in the actions of the W and Sl mutant genes. Some fibroblast cell lines derived from mouse embryos (including the NIH/3T3 cell line) supported the growth of cultured mast cells derived from normal (+/+) mice. In contrast, none of these fibroblast cell lines supported cultured mast cells derived from W/W-v mice (Table 2). By synchronizing cultured mast cells at G-1 phase of the cell cycle, the defect of W/W-v mast cells was further characterized as a failure to transit G-1 and enter S phase upon contact with fibroblasts (Fujita et al 1988a). This suggests that the W gene product expressed on the surface of mast cells is mandatory for the fibroblast-dependent proliferation.

Table 2. In vitro analysis of mast cell deficiency of mutant mice of W/W-v and Sl/Sl-d genotypes

Genotype of cells		
Mast cells	Fibroblasts	Fate of mast cells
+/+	+/+	Proliferate
W/W-v	+/+	Disappear
Sl/Sl-d	+/+	Proliferate
+/+	Sl/Sl-d	Disappear

Cultured mast cells derived from Sl/Sl-d mice were maintained as well by the NIH/3T3 cell line as were cultured mast cells derived from +/+ mice, indicating the normal response of Sl/Sl-d mast cells to fibroblasts (Table 2). The fibroblast cell lines were established from Sl/Sl-d and control +/+ embryos. The fibroblast cell lines derived from +/+ embryos induced the G-1 to S transition in synchronized +/+ mast cells upon contact, but the fibroblast cell lines derived from Sl/Sl-d embryos did not (Table 2) (Kitamura et al 1989; Onoue et al in press). This suggests that Sl gene product expressed in 3T3 fibroblasts is indispensable for the fibroblast-dependent proliferation of cultured mast cells.

Jarboe et al (1989) reported comparable experiments. They used morphologically non-granulated mast-cell precursors instead of cultured mast cells and BALB/3T3 cells instead of NIH/3T3 cells. The sauce of the mast cell precursors was mesenteric lymph nodes of mice infected with Nippostrongylus brasiliensis (Nb). They claimed that the conditioned medium prepared from the monolayer of the BALB/3T3 cell line supported the development of mast-cell colonies from mesenteric lymph node cells of Nb-infected mice.

Jarboe and Huff (1989) also analysed the mechanism of mast-cell deficiency of W/W-v and Sl/Sl-d mice by using their experimental system. Mesenteric lymph node cells harvested from Nb-infected W/W-v mice did not produce mast cell colonies in methylcellulose cultures supplemented with fibroblast-conditioned medium. In contrast, mesenteric lymph node cells from Nb-infected Sl/Sl-d mice developmed mast cell colonies in the same culture condition, suggesting that Sl/Sl-d mice but not W/W-v mice produced the mast-cell precursors that responded to fibroblast-conditioned medium. They prepared monolayers from skin connective tissues of either Sl/Sl-d or W/W-v mice. Fibroblast-conditioned medium from monolayers prepared from W/W-v but not Sl/Sl-d mice supported development of numerous mast cell colonies in methylcellulose.

The W-19H mutation has been known to be a deletion of chromosome 5 including the W locus (Lyon et al 1984). Recently, Chabot et al (1988) and Geissler et al (1988) showed that the proto-oncogene c-kit is also deleted in the W-19H mutant. Since two loci cannot be segregated by backcross analysis, the W and c-kit genes appeares to be tightly linked. Moreover, the c-kit gene is disrupted in two mutant W alleles, W-44 and W-x. These results implicate that the c-kit is identical with the W locus (Geissler et al 1988). The c-kit encodes a transmembrane tyrosine kinase receptor that is structurally similar to the receptors for colony-stimulating factor-1 (CSF-1) and platelet-derived growth factor. The CSF-1 receptor is encoded by the c-fms proto-oncogene and is expressed in macrophages, proliferation of which is stimulated by CSF-1.

Cultured mast cells strongly expressed the c-kit gene. Thus, results of ours (Kitamura et al 1989) and Jarboe and Huff (1989) can be explained if the Sl gene is assumed to be expressed in the fibroblast cell lines. We consider from our results that the ligand encoded by the Sl gene is bound on the surface of fibroblasts or on the matrix produced by the fibroblasts (Kitamura et al 1989), whereas Jarboe and Huff (1989) consider that the ligand is secreted from the fibroblasts. We are now investigating the cause of the discrepancy.

Inhibition of Differentiation

Overproduction of mast cells such as observed in patients of urticaria

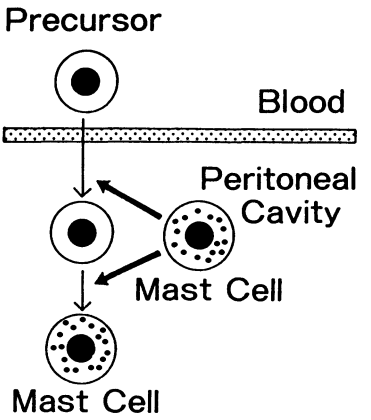

Fig. 1. Suppression of mast-cell differentiation by differentiated mast cells in the peritoneal cavity of mice. The invasion of precursor cells and their differentiation into mast cells are suppressed.

pigmentosa, a benign skin tumor of mast cells, induces unpleasant symptoms. Therefore, suppression rather than induction of mast-cell differentiation appears to be important in the ordinary life of men. Recently we investigated a mechanism for the suppression of mast-cell differentiation.

Two experiments were done. First, peritoneal mast cells of +/+ mice were eradicated by intraperitoneal injection of distilled water, and the regeneration process was analyzed by estimating the changes in numbers of precursor cells and morphologically identifiable mast cells (Kanakura et al 1988). The eradication of mast cells resulted in the invasion of precursor cells from blood into the peritoneal cavity. If purified peritoneal mast cells were injected 2 days after the water injection, the increase of precursor cells did not occur.

Second, the invasion of precursor cells into the peritoneal cavity of W/W-v mice was investigated after the bone marrow transplantation from the congenic +/+ mice. If cultured mast cells had been injected into the peritoneal cavity of W/W-v mice, the invasion of precursor cells was significantly suppressed (Waki et al, unpublished data). The results of these two experiments suggested that the presence of differentiated mast cells inhibited the invasion of precursor cells. Differntiation of the invaded precursor cells into mast cells was also inhibited by the differentiated mast cells.

PHENOTYPIC CHANGE BETWEEN SUBPOPULATIONS

Mast cells in various tissues differ in their phenotypes (Kitamura 1989). The difference is remarkable in rats and mice, and in these

species mast cells are classified into two distinct populations; connective tissue-type mast cells (CTMC) and mucosal mast cells (MMC). CTMC are present in the skin, peritoneal cavity, and muscularis propria of the stomach. CTMC contain heparin proteoglycan, store relatively large amount of histamine. In contrast, MMC are prominent in the mucosal layer of the gastrointestinal tract, contain chondroitin sulfate proteoglycan and relatively small quantities of histamine. The interrelation between subpopulations had not been systematically investigated until we demonstrated the phenotypic change from suspension-cultured mast cells to CTMC. Suspension-cultured mast cells were used since they are obtained as a homogeneous population and since many characteristics of them are common with those of MMC.

We cultured mast cells from the bone marrow of normal (+/+) mice, and transferred them into the peritoneal cavity of congenic W/W-v mice. At various weeks after the intraperitoneal transfer, mast cells were recovered from the peritoneal cavity. The density of the cultured mast cells is significantly less than that of CTMC harvested from the peritoneal cavity of +/+ mice, but the density increased and became comparable to that of the peritoneal CTMC 10 weeks after the transfer (Nakano et al 1987). The recovered mast cells acquired the histochemical and electron microscopic features of CTMC. Furthermore, the histamine content increased more than 20-fold after the transfer (Nakano et al 1985).

The phenotypic change from suspension-cultured mast cells to CTMC was confirmed by biochemical and immunological criteria. Cultured mast cells derived from +/+ mice synthesized chondroitin sulfate proteoglycan, but mast cells recovered from the peritoneal cavity of W/W-v mice after the intraperitoneal transfer synthesized heparin proteoglycan. Forssman glycolipid was weakly expressed on the surface of the suspension-cultured mast cells, but it appeared strongly after the transfer (Otsu et al 1987).

The phenotypic change occurs to the opposite direction as well (Kanakura et al 1988). A fluorescent dye, berberine sulfate, stains heparin-containing CTMC but not MMC and bone marrow-derived suspension-cultured mast cells. Mast cell colonies derived from peritoneal CTMC contained both berberine sulfate-positive and berberine sulfate-negative mast cells. When these mast cells were transferred to suspension culture, they generated populations which were 100% berberine sulfate-negative, and which synthesized predominantly chondroitin sulfate proteoglycans. When these MMC-like cultured mast cells derived from +/+ peritoneal CTMC were injected into the peritoneal cavity of W/W-v mice, the adoptively transferred mast cell population became 100% berberine sulfate-positive. In methylcellulose culture, these 'second generation peritoneal CTMC' formed clonal colonies containing both berberine sulfate-positive and berberine sulfate-negative mast cells. Thus, clonal mast cell populations initially derived from a single peritoneal CTMC exhibited multiple and bidirectional alterations between CTMC-like and MMC-like phenotypes (Kanakura et al 1988).

The fate of CTMC derived from +/+ mice was investigated in the stomach wall of W/W-v mice. After the injection of a single CTMC, mast cells may appear both in the mucosa and the muscularis propria. Mast cells which appeared in the mucosa showed histochemical and electron microscopic features of MMC, whereas the cells which appeared in the muscularis propria showed the features of CTMC (Sonoda et al 1986). The phenotypic change between mast cell subpopulations may be possible

due to the extensive proliferation potential of differentiated mast cells (Kitamura 1989). Although the phenotype is influenced by environments, the mechanism remains to be clarified.

CONCLUSION

In the recent 10 years, differentation of mast cells has been clarified from various aspects. These informations are useful for understanding the immune responses, in which mast cells are involved. Furthermore, these informations have made mast cells a unique and interesting model for the study of cell differentiation.

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Nerves, Neuropeptides and Mast Cells

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INTRODUCTION

The association of mast cells and peripheral nerves has been known and commented on for a number of years, and was first reviewed by Olsson (1968). Since that time a number of other aspects of mast cell/nerve association, including evidence for functional and physiological interactions, have been brought forward. It is the purpose of this paper to briefly review this evidence.

MORPHOLOGICAL EVIDENCE OF NERVE/MAST CELL ASSOCIATIONS

Numerous descriptive reports of close associations between nerves, nerve tumours and mast cells have appeared in the literature over the years. These have referred to both myelinated and unmyelinated nerve fibers and bundles, in tissues as varied as the mesentery, diaphragm, skin and intestine, in both healthy and disease states (see Stead et al 1989a for review). One paper even reported limited bouton formation between intestinal mucosal mast cells (IMMC) and enteric nerves in the rat (Newson et al 1983). In a careful morphometric study of normal and inflamed rat intestine we observed in the light microscope that two thirds of IMMC were associated with neurone specific enolase containing nerve bundles (Stead et al 1987a). This was confirmed in the electron microscope where membrane/membrane associations were frequently seen between IMMC and nerves which often contained dense core vesicles at the sites of contact. Immunohistochemistry showed that the apposed nerves contained substance P (SP) and calcitonin gene related peptide (CGRP) (Stead et al 1987a, 1988). We have recently confirmed these observations for the human intestine (Stead et al 1989b).

AXON REFLEXES INVOLVING MAST CELLS

A number of reports suggest that in response to noxious stimuli or antidromic nerve stimulation, mast cells and nerves (which probably contain SP) are involved in so called neurogenic inflammation and are responsible for the flare reaction (see Stead et al 1989a). Such neurogenic edema can be reduced by pretreatment with compound 48/80 or by histamine (H1) antagonists (Lembeck & Holzer 1979; Kiernan 1977; Fjellner & Hagermark 1981). Pretreatment with capsaicin, which depletes SP containing nerves, also reduces the effects of nerve stimulation (Lembeck & Holzer 1979; Kiernan 1977). More recently Kowalski and Kaliner (1988) have questioned these antidromic nerve stimulation experiments, since they themselves were only able to obtain evidence of mast cell degranulation after prolonged nerve stimulation. The reasons for this disparity are not, at present, clear.

Leff et. al (1986) showed that vagal stimulation in dogs reduced the threshold for antigen-induced bronchoconstriction. Bani-Sacchi et. al (1986) showed that field stimulation of rat ileum caused mast cell degranulation and histamine release which was reduced by atropine and tetrodotoxin (a nerve blocker). We ourselves have recently shown that nerve stimulation of the superior laryngeal nerve caused elevation of the level of the rat mucosal mast cell specific protease (RMCP II) in a perfused trachea preparation (Kakuta et al 1989). Matsuda et. al (1989) have shown that substance P injections into the skin of w/w^v (mast cell deficient) mice, produced little or no local eosinophil or neutrophil influx unless the skin was previously repopulated with bone marrow derived cultured mast cells. Most recently Yano et. al (1989) showed that in w/w^v mice, SP-induced tissue swelling, vascular permeability and granulocyte infiltration were almost entirely mast cell dependent. Similar observations have been made by Foreman in skin, with regard to somatostatin (SOM), vasoactive intestinal polypeptide (VIP), SP, and CGRP (Foreman 1987; Piotrowski & Foreman 1985).

An interesting observation was recently reported by Miura et. al (1989) in atropinized and propranolol treated cats subjected to vagal stimulation. Antigen induced bronchoconstriction and histamine release were inhibited, suggesting a non-adrenergic, non-cholinergic (NANC) pathway for inhibition of mast cell degranulation.

Neuropeptides and a number of putative neurotransmitters have been shown to have degranulating effects on mast cells and basophils from a variety of species. These putative neurotransmitters include neurotensin, SOM, VIP, CGRP and SP (see Stead et al 1989a). The concentration at which these are effective <u>in vitro</u> are questionably physiologic, although they are said to be achieved adjacent to nerve terminals and varicosities.

Mast cell degranulation is accompanied by release of mediators which include potent proteolytic enzymes. It is interesting that many of these enzymes are capable of degrading some of the polypeptide neurotransmitters. Indeed Brain and Williams (1988) reported an elegant series of experiments in which they showed that when SP was injected into rat skin at the same time as CGRP and compared to either SP or CGRP alone, the effect of CGRP on blood flow was markedly diminished due to degradation by mast cell proteases. The effects of local tissue enzymes which modulate exogenously administered neuropeptides are interesting in this regard. Perhaps the best example is the presence of enkephalinases in the respiratory tract which cause immediate degradation of aerosolized SP and effectively prevent bronchoconstriction and mucous secretion (Said 1987).

MAST CELL/NERVE INTERACTIONS & EPITHELIAL TRANSPORT

The perfusion with antigen of intestine from sensitized rats causes epithelial cell permeability and a net decrease of absorption of solutes, water and ions. In Ussing chamber experiments using such tissues, increases in short circuit current were noted on challenge with antigen (Perdue et al 1984, 1986a, 1986b). These were shown to be dependent on epithelial chloride ion secretion. Mast cell mediators such as histamine and serotonin, as well as arachidonic acid metabolites, also appear to be involved (Castro et al 1987; Russell 1986). These effects were antigen specific; and the abnormalities were inhibited by doxantrazole, which stabilizes both mucosal and

connective tissue mast cells, whereas cromoglycate which only stabilizes connective tissue mast cells, had no effect (Perdue & Gall 1986a). Furthermore tetrodotoxin reduced the ion transport abnormalities induced by antigen (Baird & Cuthbert 1987).

Rats treated neonatally with capsaicin to deplete SP and other neuropeptides, also showed decreased response to antigen in Ussing chamber experiments and similar effects were seen in a tracheal preparation from sensitized rats (Perdue et al 1989; Sestini et al 1989a). In the latter group of experiments, neonatal capsaicin treatment almost completely abrogated the inhaled antigen-induced increase in solute permeability in the lung, as measured by technetium labelled aerosolized DTPA (Sestini et al 1989b).

Taken together, these types of results strongly suggest that ion transport and water handling can be directly affected by an antigen specific, mast cell and nerve dependent unit, which presumably involves an axon reflex. It is likely that in the models tested, SP containing nerves are involved in both the trachea and intestine. Following antigen presentation, the early portion of the short circuit current response appears to depend in the rat on histamine and serotonin. The later phases of the response are probably due to products of arachidonic acid metabolism.

EFFECTS OF NERVE GROWTH FACTOR

Nerve growth factor is a polypeptide found especially in male mouse submandibular gland secretions. It is essential for the development, growth, differentiation and survival of sensory afferent and sympathetic neurons (Levi-Montalcini & Calissano 1986). There is greater than 90% homology between this murine molecule and its human counterpart. NGF causes phosphatidylserine-dependent histamine release from mast cells, and enhances antigen-dependent mast cell degranulation, without added phosphatidylserine (Pearce & Thompson 1986; Tomioka et al 1988). In vivo, injection of NGF in neonates causes significant increases in both connective tissue and mucosal mast cells (Aloe & Levi-Montalcini 1977; Tomioka et al 1989, submitted). In more recent experiments we have shown that disodium cromoglycate prevents the mastopoiesis induced in vivo by NGF (Marshall et al 1989, submitted). This and other observations suggest that the mechanism whereby NGF promotes mastopoiesis is at least in part dependent on mast cell degranulation. In this regard it is interesting that cultured mast cells appear, upon activation, to secrete and increase their messenger RNA for IL-3, -4, -5 and -6, as well as GM-CSF (Wodnar-Filipowicz 1989; Plaut et al 1989).

In hemopoietic colony growth assays involving methylcellulose cultures of human peripheral blood, NGF promoted granulocyte, macrophage and basophil, as well as eosinophil colony growth. The effect was somewhat more pronounced on histamine containing colonies, and was abrogated by the removal of T cells from the starting cell population (Matsuda et al 1988). Recently we have shown a synergy between NGF in these assays and GM-CSF. Synergistic effects were also found with human recombinant IL-5, whereas no synergy was seen with IL-3 (Tsuda et al 1989a&b, submitted).

COCULTURE EXPERIMENTS WITH MAST CELLS AND NERVES

In order to develop a system to test for functional interactions between mast cells and nerves in culture, we made nerve preparations from superior cervical ganglia from neonatal mice, and cultured them either as explants, or as dissociated neuronal cultures after enzymatic digestion. While the majority of our experiments have involved the rat basophil leukemia cell line RBL 2H3, we have confirmed many of our findings with rat peritoneal mast cells. (The RBL cell type is considered analogous to IMMC, since they have similar proteoglycan and protease contents). When these cells were cocultured, neurites formed permanent contacts with RBL over periods of 8 to 92 hours (Blennerhassett et al 1987). The neurites in contact with mast cells invariably showed dense core vesicles when examined in the electron microscope. Examination of the electrophysiology of such contacts showed that the input resistance of RBL in contact with neurites was on average 57% lower than the intrinsic control values in the same dish. Neurite contact increased the conductance of the RBL cell membranes, which can be interpreted as representing increased membrane permeability. Neither acetylcholine nor noradrenaline affected membrane resistance values in vitro (Blennerhassett & Bienenstock 1989, submitted). Indeed the changes in conductance were only reproduced by SP and this effect was shown to be dose dependent. We suspect that these in vitro findings reflect and confirm the findings that we have made and reviewed above in vivo.

One other observation that we have made in this coculture system may also be of interest: namely that the RBL in association with nerves ceased to divide. With a normal doubling time in culture of approximately 12 hours, observations over 96 hours would be expected to produce evidence for at least 7-8 divisions. However, no doublings were found in RBL associated with nerves. Furthermore, the cells associated with nerves increased their granularity significantly, suggesting that this association was in some way promoting their maturity.

PSYCHOLOGICAL (PAVLOVIAN) CONDITIONING OF MAST CELL MEDIATOR RELEASE

Because of the relationships described above between mast cells, neuropeptides and nerves, we wondered whether it would be possible to psychologically condition animals for mast cell degranulation. Russell et. al (1984) had previously shown in the guinea pig that histamine release was conditionable; and that this was due to "psychological mechanisms", since they were able to demonstrate that repetition of the psychological cue extinguished the histamine release response. However, histamine can be released from a variety of cell types, so we decided to set up a series of experiments in which rats were sensitized to egg albumin with adjuvants. Animals were then infected with Nippostrongylus brasiliensis,to promote IgE antibody synthesis and intestinal mastocytosis, and subsequently trained by association of antigen injections with an audio-visual cue. Finally, animals were exposed only to the audio-visual cue and serum samples were taken one hour later for RMCP II determination. With the appropriate controls, these experiments clearly showed that in conditioned animals, audio-visual cues alone could cause significant serum RMCP II elevations (MacQueen et al 1989a). Subsequent physiological experiments with this model, using Ussing chambers, have shown that trained animals exposed to the audio-visual cue alone have an increased intestinal epithelial cell secretion of chloride ion, supramaximal responses to antigen compared to controls, and also

oscillatory bursts of activity which are blocked by tetrodotoxin (MacQueen et al 1989b). These data strongly suggest that mucosal mast cell degranulation has occurred in the intestine and that local physiological effects have ensued in response to the psychological cue. It would seem that the central nervous system can not only affect peripheral mast cells, but can also thereby have secondary effects on local physiological function.

DISCUSSION AND CONCLUDING REMARKS

We have briefly reviewed some of the evidence which suggests that mast cells and nerves not only may have an intimate anatomic relationship, but that this may cause some functional consequences. It is clear from the morphologic and morphometric studies that this association is not an obligatory one, and the factors which regulate it are unclear. We know that in a variety of tissue injuries, nerve growth factor as well as many other cytokines are produced by locally activated cells, including fibroblasts. We also know that mast cells are often seen in situations where wound healing and repair processes are active. It is tempting at this point to implicate NGF, and possibly other cytokines, in this process. While we are not certain of the role of mast cells in tissue repair it is becoming clear that mast cells synthesize and secrete a number of molecules over and above the long list of mediators such as histamine, serotonin and products of arachidonic acid metabolism, upon which emphasis has been placed for so long. Recent evidence suggests that mast cells may synthesize cytokines such as IL-3, -4, -5, -6 and GM-CSF upon activation (Wodnar-Filipowicz 1989; Plaut et al 1989). The local release in tissues of such factors must have very potent affects on both the immune and hemopoietic systems. It could be predicted that local production of these molecules would affect both T and B cell responses, especially synthesis of IgE and IgA, as well as the immigration into tissues of both progenitor as well as mature cells of the inflammatory series. In addition, the innervation and neuropeptide levels must be important in the local homeostasis of repair processes. The well documented and diverse actions of neurotransmitter substances on immune cells would support this (Stead et al 1987b).

It is now recognized that molecules classified as interleukins also modulate nerve cells. For example, IL-1 enhances the production of NGF in sciatic nerve explants (Lindholm et al 1987) and IL-6 induces neurite outgrowth from PC12 cells (Satoh et al 1988). This data suggests the possibility for changes in the local innervation during inflammation. In recent <u>in vivo</u> experiments we have demonstrated nerve remodelling during the time course of Nippostrongylus brasiliensis infection in rats. The intestinal mucosal neuronal mass increased about four-fold (determined by morphometric analysis of nerve areas) 10 days after infection and subsequently reverted to normal (Stead et al 1989c, submitted) The nerve changes appeared to be inversely co-ordinated with changes in mast cell density. Accordingly, we have hypothesized that mast cells might be involved in peripheral neuronal plasticity, both structurally and phenotypically (Bienenstock et al 1989).

While we have focussed on the mast cell in this paper, it is important for the reader to recognize that other cell types including lymphocytes appear to have similar interactive relationships with the nervous system. Indeed the fields of neuroimmunology and psychoneuroimmunology have grown up, as a consequence of such findings. Blalock and colleagues have shown that lymphocytes can

communicate with the nervous system as a result of a variety of molecules synthesized and secreted by these cells (Carr & Blalock 1986). Morphologic evidence for the apposition of noradrenergic nerve endings with lymphocytes, particularly in T cell dependent areas in lymph nodes, has also been brought forward (Felten & Olschowka 1987). Since antigen injection peripherally can cause increased firing rates of hypothalamic cells in the brain (Besedovsky & del Rey 1986), the circle or network of multiple interactions involving the nervous and immune systems appears to be complete. The puzzle with which we are faced now, is to discern which pathways are the most important, so as to establish the principles by which these systems operate, to define their role, and determine how to use this information therapeutically.

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The High Affinity Receptor for Immunoglobulin E: New Developments

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Introduction

Over the last few years considerable progress has been made in the field of the high affinity receptor for immunoglobulin E ($Fc_\varepsilon RI$). The recent achievements were made possible by the rapid development of the techniques of molecular genetics. The cloning of complementary DNA for the rat α (Kinet et al 1987)(Shimizu et al 1988), the rat β (Kinet et al 1988), and the rat γ subunits (Blank et al 1989) of the receptor unveiled the respective DNA and amino-acid sequences of the subunits which became the basis for a new model of the receptor.

Through further molecular cloning it has been possible to confirm this model and to define which portions of the subunits are the most conserved between species and which therefore, may be functionally relevant. Interesting homologies were also detected between the subunits and other polypeptides raising the possibility of functional relatedness.

The techniques of gene transfer in receptor-negative cells have generated new ways to study the receptor binding site and the transmembrane signalling function of the receptor.

A model for $Fc_\varepsilon RI$

Six years ago Metzger et al (1983) proposed a tetrameric model for $Fc_\varepsilon RI$ with one IgE-binding α chain, one β chain and two identical disulfide-linked γ chains. Although this model is still valid, it has been considerably refined (Fig.1).

Polypeptide structure of the subunits

Potential leader peptides and transmembrane domains can be predicted from hydropathicity plots of the polypeptide sequences. Regardless of the species studied (see below), these plots give identical results increasing the likelihood that these structural interpretations are correct. Both α and γ - but not β - contain an amino terminal leader peptide which is processed post-translationally. There are seven transmembrane domains in the

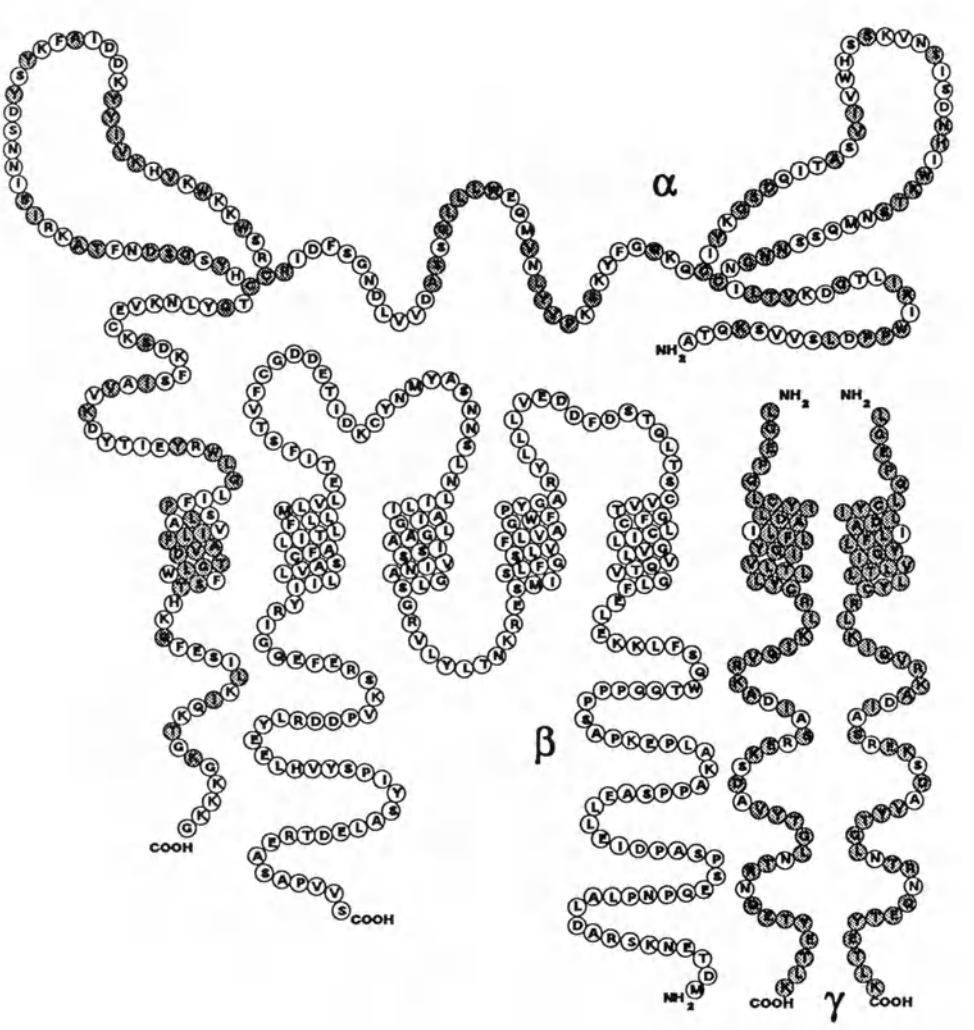

Figure 1 : Model of the high affinity IgE receptor

The amino acid sequence (single letter code) is from the rat receptor (Blank et al 1989). The residues in α and γ which are identical between rat, mouse and human are shaded. The model is based on the fully processed subunits. The extracellular portion of α is shown at the top and the remainder of the chain on the left. To the right of α is the β subunit with its four transmembrane domains and to the right of β is the dimer of γ subunits. The putative transmembrane segments are shown as consisting of 21 residues.

tetrameric receptor: one in α, four in β and one in each of the γ chains.

The α subunit contains seven potential N-linked glycosylation sites and two immunoglobulin(Ig)-related domains of 40 and 42 residues respectively. These domains are each defined by a pair of cysteines residues which most likely interact with each other. Neither β nor γ contain carbohydrates although three potential N-linked glycosylation sites are found in β. Two cysteines between the third and fourth transmembrane segments of β could form a disulfide bond but this remains to be demonstrated.

Biosynthetic labeling indicate that the plasma membrane-bound γ does not contain any histidine residue as would be predicted by the γ cDNA sequence. This apparent discrepancy could be explained by a post-translational processing which clips off the C-terminus of γ before insertion in the plasma membrane.

Topology of the subunits

The topology of the subunits was assessed by using antipeptide antibodies or monoclonal antibodies against defined epitopes (Ra et al 1988)(Kinet et al 1988). It was independently confirmed by using the algorithm of von Heijne (Metzger et al 1989) from which extracellular and intracellular segments of polypeptide sequences can be predicted. Other considerations were also taken into account. For example, the γ chains can be iodinated on inverted vesicles but not on intact cells (Holowka and Baird 1984). Therefore tyrosine residues should only be found in the cytoplasmic segment. Indeed, the model implies that this assumption is true.

Subunit interactions

The direct interaction of β with γ was first suspected when complexes of β and γ were generated upon immunoprecipitation of the receptor (Kinet et al 1983). The coordinate dissociation of β and γ from the α when the receptor is exposed to detergent micelles supports such an interaction (Kinet et al 1985). But the precipitation of dissociated βγ complexes with anti-β is even a stronger argument (Rivera et al 1988). From the same studies it seems that the interaction of β with γ is relatively stronger than the interaction of the complex βγ with α.

The direct interaction of α with γ was recently demonstrated in transfected cells: the human α associates with γ on the cell surface (Miller et al 1989)(Küster and Kinet, unpublished).

Preliminary results on the assembly of the receptor suggest an interaction of α with β: the complex αβ seems to be the first to appear (Rivera and Metzger, unpublished). The model presented in Fig. 2 tries to incorporate these data on the subunit interactions.

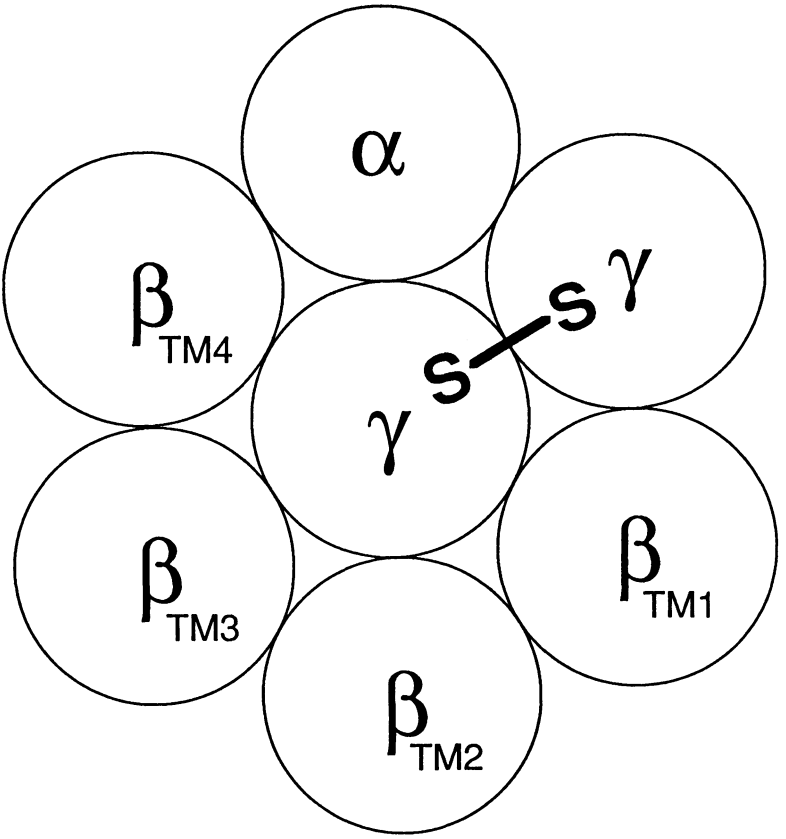

Figure 2: Model of interactions between the transmembrane domains of FcεRI subunits.

The rationale for the specific configuration is given in the text.

Comparisons between species: functional relevance

The cloning and sequencing of the three subunits of mouse $Fc_\varepsilon RI$ has now been accomplished (Ra et al 1989). The sequences of human α (Kochan et al 1988)(Shimizu et al 1988) and human γ (Küster and Kinet, unpublished) are also available.

The degree of sequence conservation in α and γ between the three species (rat-mouse-human) is strikingly different. The consensus sequence of α shows only 38% identical residues compared with 87% in the consensus sequence of γ (Fig.1). The analysis of subregions of the α subunits also gives surprising results. The cytoplasmic tails share only 16% identities between species and their lengths are variable: 20 residues in the rat, 25 in the mouse and 31 in the human α. By contrast, the transmembrane domain is highly conserved: 62% identities between the three species including nine consecutive residues. Because functionally relevant sequences are usually highly conserved through evolution, these observations suggest a functional importance for the γ subunit and the transmembrane domain of the α subunit (Kinet 1989). The unavailability of the human β sequence prevents similar comparisons.

Chromosomal localization and linkages: implications

The single genes found for α and γ are linked to the gene for $Fc_\gamma RII$ (Ly-17) at the distal end of mouse chromosome 1 (Huppi et al 1988,1989). At least one of the two forms of $Fc_\gamma RII$ has recently been shown to contain γ chains identical to those from $Fc_\varepsilon RI$ (Ra et al 1989). Therefore the close association of these unrelated genes - the γ gene on the one hand and the $Fc_\varepsilon RI$ and $Fc_\gamma RII$ genes on the other hand - suggests that selective pressure during evolution has maintained these genes as a cluster for purpose of regulation.

The β chain is encoded in a single gene, linked to the Ly-1 (Ly 12) locus in a region of mouse chromosome 19 that also contains the loci for Ly-44 (CD20) (Huppi et al 1989). The latter codes for a polypeptide with interesting similarities to the β chain (see below).

Homologies with other proteins

The <u>α subunit</u> is a member of the immunoglobulin (Ig) superfamily, homologous to the Ig-binding polypeptide of other Fc receptors but particularly to mouse $Fc_\gamma RIIa$ (Ravetch et al 1986) and human $Fc_\gamma RIII$ (Scallon et al 1988). The two Ig-related domains are homologous to the corresponding domains of these Fc receptors but the most conserved region of homology is in the transmembrane domain where eight consecutive residues are found in the consensus sequence

of human-rat-mouse α with mouse $Fc_\gamma RIIa$ and human $Fc_\gamma RIII$. This may be functionally significant although it may simply reflect the common association of these receptors with γ.

The proximity of the genes for the β subunit and Ly-44(CD20) (Tedder et al 1988) on mouse chromosome 19 helped us to identify interesting similarities between these two molecules (see above). Their predicted topology is strikingly similar, with four transmembrane domains and both amino and carboxy termini being cytoplasmic. Furthermore they share 29% identical residues over a stretch of approximately 100 residues. This includes two clusters of higher homology with approximately 50% identities respectively.

The γ subunit is homologous to the ζ chain (Weissman et al 1988) of the T cell receptor: 55% identical residues are found in their corresponding 29 residue N-terminal segment (Miller et al 1989). More recent data indicate that the extent of the homology is even greater than initially thought. They not only share homologous residues at the C-terminus but they show an analogous organization of their respective gene (Küster and Kinet, unpublished).

Expression by gene transfer

Efficient expression of receptors on the cell surface can be induced by gene transfer. Co-transfection of cDNA for the α, β and γ subunits is required to yield surface expression of the *rodent* receptor (Blank et al 1989). Surprisingly, efficient surface expression of the *human* receptor can be achieved after co-transfection of α and γ without apparent need for β (Miller et al 1989). The differential requirement for surface expression of the rodent and human receptor is under study and may increase our understanding of both the assembly and the structure of the receptor.

This success in expressing the receptor has also provided us with the tools to study IgE-receptor interaction in the human system.

The receptor binding site

Rodent IgE binds both the rodent and human receptor with high affinity. However human IgE does not bind the rodent receptor. To take advantage of this difference we generated chimeric constructs between human and rodent α to try to determine which portion of the rodent α can be substituted with human sequences to generate the binding characteristics of human α. Answering that question should permit identification of the sequences responsible for the binding specificities and hopefully for the binding itself. Preliminary results (Blank and Kinet,unpublished) suggest that the binding specificity probably requires two or more discrete portions of the α chain.

The complete understanding of IgE-receptor interactions will come only from crystallographic analysis. To reach this goal we have engineered truncated α chains which are secreted by transfected cells and which still bind IgE with high affinity. This should allow us to produce sufficient protein for crystallization.

Strategies to study receptor function: preliminary results

The ability to selectively modify by genetic means the subunits of the receptor and to transfect cells with the cDNAs for the receptor, now permits a new approach to investigating the mechanism by which the receptor initiates a variety of cellular changes. The strategy involves four steps: 1) construction of the desired modified cDNAs by site-directed mutagenesis, 2) testing for surface expression of the mutant receptor, 3) identification of suitable cells which lack the receptor but have the apparatus that will respond to "activation" (aggregation) of the receptor, and 4) transfecting such cells with the mutant receptors to determine the effect on transmembrane signalling.

So far a variety of mutant cDNAs have been constructed and their sequences verified. Several of these have been transiently transfected into COS cells and their ability to be incorporated into surface-expressed receptors assessed by the rosetting technique previously described (Blank et al 1989). The principal finding from these initial experiments was that good expression could be obtained with single-defect mutants in which one of the subunits at a time was modified - for example by truncating their presumptive cytoplasmic extensions.

The COS cells appear to lack the mechanism that generates some of the early biochemical events triggered by the aggregation of the IgE receptor. Thus neither a rise in intracellular Ca^{2+} nor hydrolysis of phosphoinositides was observed in these cells even though they expressed ample numbers of transfected wild-type receptors.However, one of the consequences of the receptor aggregation - interaction of the aggregates with cytoskeletal structures - does seem to occur. This should allow us to probe by mutational analysis which part(s) of the receptor are required for this interaction.

It appears that a mouse mast cell analogue which can be permanently transfected with receptors will be a suitable cell in which to study some of the early biochemical signals. This line of cells lacks the α and β chains of $Fc_{\varepsilon}RI$ although it contains γ chains. Early transfection experiments with wild-type subunits have given sufficiently promising results, so that we are already testing mutated receptors for their ability to initiate hydrolysis of phosphoinositide, a rise in intracellular Ca^{2+}, release of incorporated arachidonic acid and other signals.

We plan to utilize the information gained by these studies to identify other cellular components with which the receptor interacts. The failure to identify unambiguously such molecules, remains as one

of the principal impediments to the further analysis of the mechanism of transmembrane signalling.

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FcεR2/CD23: Regulation and Functional Roles in Cell Activation

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SUMMARY

Cloned human low affinity receptor for Fc portion of IgE (FcεR2/CD23) has a homology to animal lections and the extracytoplasmic portion has a property to cleave off as a IgE binding factor (IgE-BF). Although FcCR2 probed to be same to CD23, a B cell differentiation antigen, it is now known to be not unique to B lymphocytes but is expressed on variety of cell lineages including T lymphocytes, macrophages and eosinophils. IL-4 is a general inducer of FcεR2/CD23 on all of these cell types, whereas γIFN down-regulates FcεR2/CD23 on B cells and up-regulates on macrophage and eosinophil cell lines. As predicted by the expression of FcεR2/CD23 on some HTLV-I(+) T cell lines, FcεR2/CD23 probed to be induced on normal peripheral T lymphocytes by IL-4 or IL-2 in the presence of additional permissive signals.

FcεR2/CD23 and Tac/CD28 (IL-2 receptor/p55) are often co-expressed on hematopoietic cells. We found a interesting co-regulation between these two receptors. On some cells, IL-2 induced FcεR2/CD23 while triggering of FcεR2/CD23 resulted in the enhanced expression of IL-2R/p55(Tac). It is also suggested that the triggering of cell surface FcεR2/CD23 by natural ligands may buffered by soluble FcεR2/CD23 (IgE-BF).

MOLECULAR ASPECTS OF HUMAN FcεR2/CD23

Cloned human cDNA (Kikutani 1986, Ikuta 1987, Ludin 1987) for FcεR2/CD23 has a homology with animal lectins such as ASGPR (asialoglycoprotein receptor) and MBP-C (mannose binding protein C). As is the case with these proteins, FcεR2/CD23 is inserted to the plasma membranes with the N-terminus inside of the cells. Despite the homology to animal lectins, there is some evidence indicating that carbohydrates of IgE may not be necesary for the IgE binding to FcεR2/CD23 (Vercell et al).

Another unique property of this receptor is a spontaneous cleaving as a soluble receptor molecule with IgE binding capacity (IgE binding factor ; IgE-BF) (Figure 1).Comparatve studies with monoclonal antibodies (mAbs) against FcεR2 and CD23 revealed that FcεR2/CD23 is identical to CD23 B cell differentiation antigens (Yukawa 1987). However, subsequent studies have shown that FcεR2/CD23 is not a specific marker for B lymphocytes leneages but is an inducible receptor on a variety of hematopoietic cell lineages. The biological significance, molecular variations, and the regulation of the expression of FcεR2/CD23 and IgE-BFs on different cell types will be discussed in this paper.

MOLECULAR HETEROGENEITY OF IgE-BF AND FcεR2/CD23

Previous studies have shown that essentially all the cell lines bearing FcεR2/CD23 spontaneously produce soluble FcεR2/CD23 (IgE-BF) regardless of the cell types (B, T lymphocytes, macrophasges, eosinophils) (Table 1). (Kawabe 1988)

To see the mechanisms of the cleaving of soluble FcεR2/CD23 (IgE-BF), we established stable transformant by transfection of the cDNA for FcεR2/CD23 to various mammalian cell lines. These transformant not only expressed FcεR2/CD23 on their cell surfaces, but also spontaneously released the soluble FcεR2/CD23 (IgE-BF). When we characterized the property of the soluble FcεR2/CD23, the affinity-purified soluble FcεR2/CD23 from some transfected cell lines (Jurkat) had the size of c.a.

25kDa as was the case with IgE-BF derived from EBV(+) RPMI8866 cells on sodium dodesyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). By contrast, U937 (monoblast) cell line and YT (LGL cell line) transfected with FcεR2/CD23 cDNA produced heterogenous soluble FcεR2/CD23 (Figure 2).

This heterogeneity of soluble FcεR2/CD23 from YT cells was also confirmed by the analysis with ion exchange chromatography using mono-Q column in HPLC. These multiple protein peaks after affinity purification retained the antigenecity recognized by anti-FcεR2/CD23 mAB H107 (Noro 1986) on Western blot analysis. The charge heterogeneity associated with the apparent size heterogeneity on SDS-PAGE may be due to either aberant proteolysis of the FcεR2/CD23 or the alteration of glycosylation. At this moment, it is unknown whether the heterogenous components of soluble FcεR2/CD23 have different biological activitis or not.

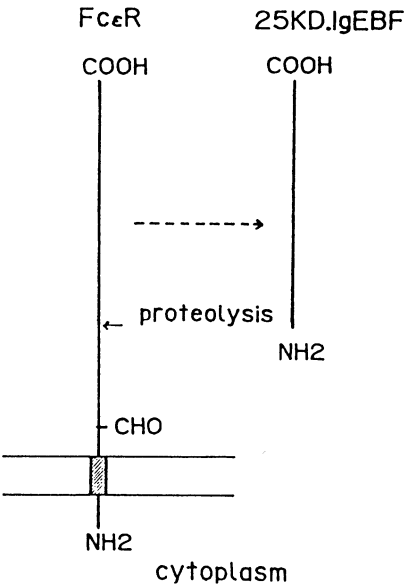

Figure 1. The relationship between FcεR2/CD23 and IgE-BF.
IgE-BF is cleaved from cell bound 43kD FcεR2/CD23 by proteolysis.

Table 1. Production of IgE-BF by FcεR2(+) cell lines.

	IgE-BF in culture supernatant (pg/ml)	$Fc\varepsilon R_2$ Ag in cell lysate (pg/ml)
JIJOYE (-)	≤8	≤8
(IL-4)	21	49
U937	51	76
ED	92	58
EOL-3	36	58
Jurkat	≤8	≤8

Recently a new heterogeneity has been shown in the products of FcεR2/CD23 gene. Yokota (1989) have shown the two types of FcεR2/CD23 molecules transcribed from different initiation sites and use different exons of the same FcεR2/CD23 gene. These molecules had the different cytoplasmic domain and the same extracytoplasmic domain giving rise to essentially the same soluble FcεR2/CD23 molecules. The tissue specificity and the difference in the biological function of these products remain to be answered.

The possible utility of the cell surface FcεR2/CD23 and the soluble FcεR2/CD23 (IgE-BF) in clinical medicine is not concluded yet. In collaboration with Mayumi et al. we have observed the enhanced expression of FcεR2/CD23 on peripheral lymphoid cells in atopic childrens. Mayumi et al. also found the possibility that the measurement of soluble FcεR2/CD23 by ELISA in cord blood may predict the future appearance of atopic symptoms in these children. The possible use of recombinant IgE-BF as the receptor blokade would be theoretically feasible at least against FcεR2/CD23 on various hematopoietic cell types. The regulatory effect of IgE-BF on IgE mediated pathological reaction IgE/FcεR2 interaction is to be critically evaluated. Indeed, such a direction is important with the emerging recognition of the importance of FcεR2/CD23 on eosinophils and macrophages in hyper-IgE patients (Spiegelberg 1979, Capron 1986).

By contrast, the competitive inhibition of IgE/high affinity IgE receptor (FcεR1) with soluble FcεR2/CD23 (IgE-BF) seems theoretically debateful, although Suemura and Kishimoto have presented a significant evidence for it (personal communication). Finally, the enhanced production of IgE-BF in HIV-infected patient (Carini 1989) may give a new patameter for monitory their immunodeficiency condition.

EXPRESSION AND REGULATION OF VARIOUS HEMATOPOIETIC CELLS

A)Variable regulation of FcεR2/CD23 on lymphoid and macrophage cell line by cytokines and ligands.

We comparatively analysed the regulation of FcεR2/CD23 and the soluble receptor of monocyte (U937), T (ED) and B (Jijoye) cell lines (Kawabe 1988, Mayumi 1989). As was the case with normal B cells (DeFrance 1987), IL-4 induced FcεR2/CD23 on Jijoye and U937 cells, although DE cells constitutively expressed the receptor without response to IL-4. By contrast, the regulatory effect of γ-interferon (γIFN) on FcεR2/CD23 expression was variable depending on the cell types. γIFN suppressed the expression of γIFN on IL-4 stimulated normal B lymphocytes (DeFrance 1987). On the other hand, γIFN enhanced the FcεR2/CD23 gene expression and the production of FcεR2 and IgE-BFs on U937, whereas no significant effect on the FcεR2/CD23 expression on ED cells. The effect of γIFN on Jijoye cells were more complicated. In Jijoye cells there was a dissociation between the surfance expression of FcεR2 and mRNA expression treated with γIFN plus IL-4. γIFN enhanced the FcεR2 mRNA expression and the IgE-BF production on IL-4 stimulated Jijoye cells in spite of the down-regulation of the surface expression of FcεR2. The results indicated the possibility that the expression of FcεR2 and IgE-BFs is resulated by γIFN at least two different levels: (1) on the transcriptional levels and (2) the levels of cleavage of the surface FcεR2 to release IgE-BF.

Ligands binding to the FcεR2 such as IgE and anti- FcεR2 mAb also enhanced the surface expression of FcεR2 on these FcεR2 positive cell lines. This was mainly due to the surface accumolation of the receptors on Jijoye and U937. However, the stimulation of ED cells by mAb H107 resulted in a significant enhancement of the mRNA for FcεR2. This indicating that FcCR2 synthesis may be up-regulated by the specific ligands in some cell types (Kawabe 1988). These results indicate that the expression and processing of FcεR2/CD23 is under complicated regulation by various cytokines and other ligands.

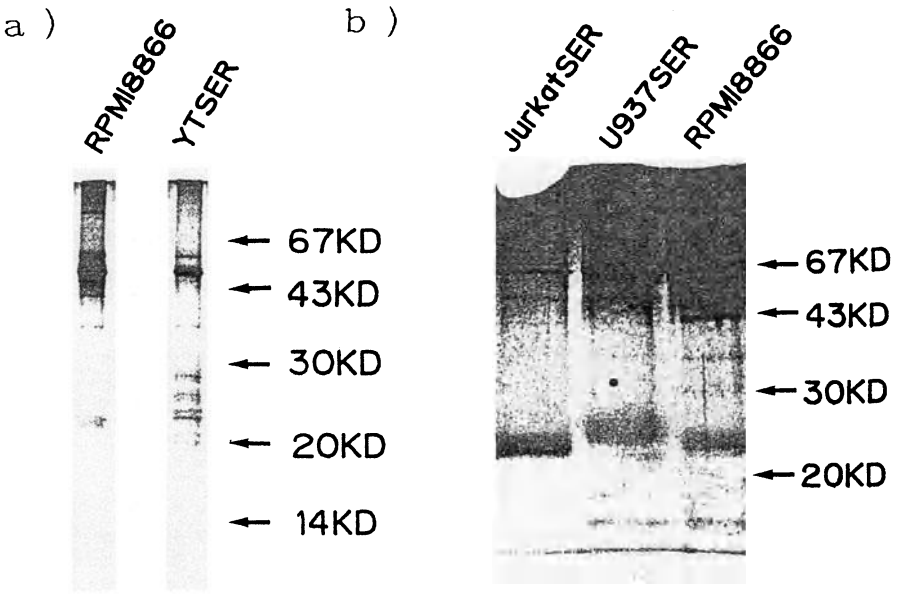

FIgure 2. a) SDS-PAGE and immunoblott analysis of soluble FcεR2/CD23 derived from TYSER and RPMI8866.
YTSER is a stable trnsfectant of YT (NK/LGL cell line) established by the transfection of a vector (pSV2neoSER) which included FcεR2/CD23 cDNA. The affinity purified soluble FcC R2/CD23 from conditioned medium of YTSER or native RPMI8866 by H107 were applyed on 15% SDS-PAGE under nonreduced condition and stained with mAb H107 and HRP-G/M.
b) SDS-PAGE analysis of soluble FcεR2/CD23 deroved from RPMI8866, JurkatSER, U937SER cells.
JurkatSER and U937SER are stable transformant of Jurkat (T cell line) and U937 (monoblast cell line) respectively. RPMI8866 is EBV(+) B cell line which produces soluble FcCR2/CD23 spontaneously. Affinity purified soluble FcεR2/CD23 using mAb H107 were applied on 15% SDS-PAGE under nonreducing condition followed by staining with silver.

B) Property of Fc εR2/CD23 on eosinophil cell lines

Recently, the nature and the roles of FcεR2/CD23 on eosinophils has been widely studied. FcεR2 expression on human and rat eosinophils and its functions on IgE dependent anti-parasitic functions had been reported by Capron et al (1986). In collaboration with H. Saito, we confirmed the expression of the FcεR2/CD23 on huamn eosinophil cell line Eol-1 and Eol-3 (Saito 1985). Ige binding to Eol cells was almost completely inhibited by mAb H107 (anti-FcεR2 mAb). The expression of FcεR2 on Eol-3 was up-regulated by IL-4 and γIFN as is the case with monoblast cell line U937. Eol cells also produced the soluble FcεR2/CD23 (IgE-BFs). The production of IgE-BF correlated with the expression of the surface FcεR2/CD23 and was regulated by cytokines (Hosoda 1989). The regulation of the FcεR2 expression on normal eosinophils is remain to be clarified.

C) Induction of expression by activated zymosan

Recently we found that the stimulation of the FcεR2/CD23 (+) U937 cells with activated zymosan particles markedly enhances the expression of FcεR2. The IL-4 dependent gene expression of FcεR2/CD23 was enhanced by these serum-activated zymosan but not by control zymosan. Similar enhancement of FcεR2/CD23 was also observed in B cell line (Jijoye). Although the involvement of complement components such as C3d has been sugested, the possible involvement of other serum components on FcεR2/CD23 induction is not excluded yet. Because of the binding of immunogloblins to the serum-treated zymosan particles, possible activation of the target cells via FcA receptors is also tobe dissected. These results suggest the network regulation between FcεR2 pathway and other receptors such as FcA rteceptors or complement component receptors, indicating the important roles of FcεR2/CD23 in inflammatory reactions involving immune complex.

FcεR2 on HUMAN T CELLS

It had been reported that the FcεR2 were expressed on the human T lymphocytes of the atopic patients and the hyper-IgE patients. Recently several laboratories reported the expression of FcεR2/CD23 on T cell lines transformed with HTLV-I. The characteristics of the FcεR2 and the IgE-BFs expressed on the HTLV-I positive T cell line is essentially the same as those of the B cells in molecular size, isoelectric point, and the size of the mRNA (Sarfati 1988).

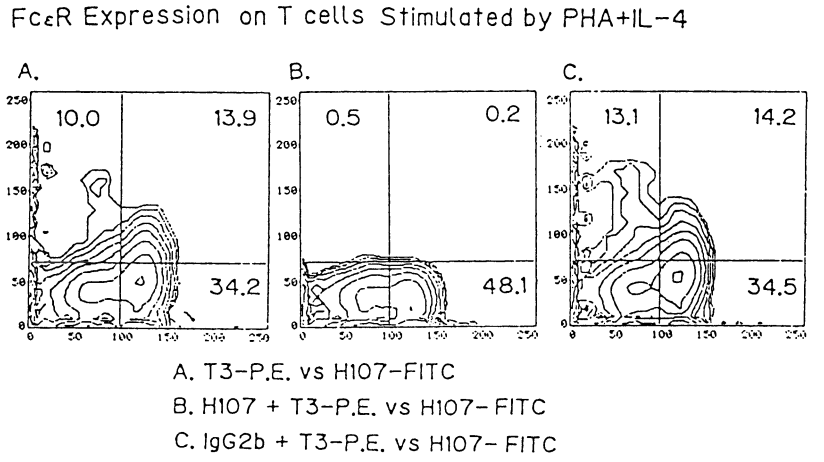

Fig. 3 FcεR$_2$/CD23 expression on T cells stimulated by PHA + IL-4

Recently we found that normal human peripheral T lymphocytes can express II/CD23 after activation with T cell mitogens such as Pytohemagulutinin (PHA). The expression was markedly enhanced by the addition of IL-2 and IL-4 (Figure 3). Interestingly, rebitt anti-IL-4 antiserum completely suppressed the expression of FcεR2/CD23 on PHA activated T lymphocytes induced not only by IL-4 but also by IL-2. This observation strongly indicated that IL-2 enhanced the expression of FcCR2/CD23 on T lymphocytes via IL-4 dependent pathway.

In other experiments PBL depleted of B lymphocytes could not induce FcεR2//CD23 on PHA activated T lymphocytes in spite of the presence of IL-4. By contrast macrophage-depletion of PBL before PHA stimulation did not effect the expression of FcεR2//CD23 on T lymphocytes in response to PHA plus IL-4. This induction of FcεR2//CD23 on human T lymphocytes accompanied the expression of mRNA, indicatind at least part of the FcεR2//CD23 expressed on T lymphocytes are the product of T lymphocytes. These dara showed that the expression of FcεR2//CD23 on PHA activated human peripheral T lymphocytes requires the B cells and IL-4, indicating the possible requirement of T-B interactionfor the FcεR2/CD23 expression on human T lymphocytes.These ecidence is indicative of the importance of the FcεR2/CD23 displayed on T lymphocytes.

CO-EXPRESSION OF FcεR2/CD23 AND IL-2 RECEPOR/p55 (Tac)

While human helper T cell lines transformed by HTLV-I ofen express FcεR2/CD23 constitutively, essentially all the HTLV-I(+) T cell lines express high number of IL-2R/p55(Tac) as already described. The eosinophilic cell line Eol-3 also proved to be positive for not only FcεR2/CD23 but also IL-2R/p55 antigen. In B cell lineage, similar co-expression appears to be physiologically more relevant. When normal peripheral B cells were stimulated with SAC (Staphylococcus aureus Cowas I), there was an enhacement of the expression of both of the inducible receptors. In case of a B-CLL (chronic B lymphocytic leukemia), the stimulation of the leukemic cells with various agents resulted in the co-expression of FcεR2/CD23 and IL-2R/p55(Tac). Furthermore, on some EBV-transformed B cell lines such as 3B6, IL-2R/p55(Tac) as well as FcεR2 were co-expressed.

To clarify whether the co-expression of IL-2R/p55(Tac) and FcεR2/CD23 is physiologically relevant or not, we examined the possible effects of the triggering of either receptors on the expression of the other. We recently found a "bilateral co-regulation network between FcεR2 and IL-2R/p55.

TRIGGERING OF FcεR2/CD23 INDUCED IL-2R/p55(Tac)

When FcεR2/CD23 was triggered by anti-FcεR2 mAb (H107) with polyclonal goat anti-mouse IgG, the expression of IL-2R/p55(Tac) was enhanced on several lymphoid cell line, including 3B6 (EBV cell line), YT transfectant (NK/LGL cell line transfected with FcεR2 cDNA) and leukemic cells of B-CLL (Figure 4). The induction of IL-2R/p55(Tac) was associated with the increase of IL-2R/p55 mRNA as determined by Northern blot analysis.This findings may be related to the previous report by Gordon et al (1986) who observed that normal B cells activated with phorbol ester progressed into active cycle by stimulation with anti-CD23 mAb MHM-6.

It is to be clarified whether natural ligands of FcεR2//CD23 such as IgE or (BCGF?) may also trigger the receptor and induce the expression of IL-2R/p55(Tac) or not. The possible inhibiting effect of soluble FcεR2/CD23 to this signaling FcεR2/CD23 is also to be clarified.

On some lymphoid cells co-expressing both IL-2R/p55(Tac) and FcεR2/CD23, stimulation of the cells with IL-2 enhanced the expression of FcεR2/CD23. In the case of 3B6 cells and a fresh B-Cll cells, IL-2 also enhanced the proliferation of the cells. In conclusion, these mutual pathway to regulate

FcεR2/CD23 and IL-2R/p55(Tac) expression indicate that the co-expresion of both FcεR2/CD23 and IL-2R/p55(Tac) might be biologically important for the collaboration of these two receptor systems.

MODULATION OF IL-4 DEPENDENT IgE FORMATION BY FcεR2//CD23

In vitro IgE production by human PBL was slightly enhanced by mAb H107 in the presense of low concentration (2U/ml) of IL-4, whereas IgE production was markedly suppressed by H107 in high concentration of IL-4 (200U/ml).

Furthermore, mAb H107 enhanced the proliferationof IL-4 preactivated PBL, indicating the possible involvement of FcεR2/CD23 for the facilitation of cytokine-geared lymphocyte activation. It remains to be clarified whether the target molecule of mAb H107 in these reaction is the cell surface FcεR2 or soluble FcεR2 (IgE-BFs).

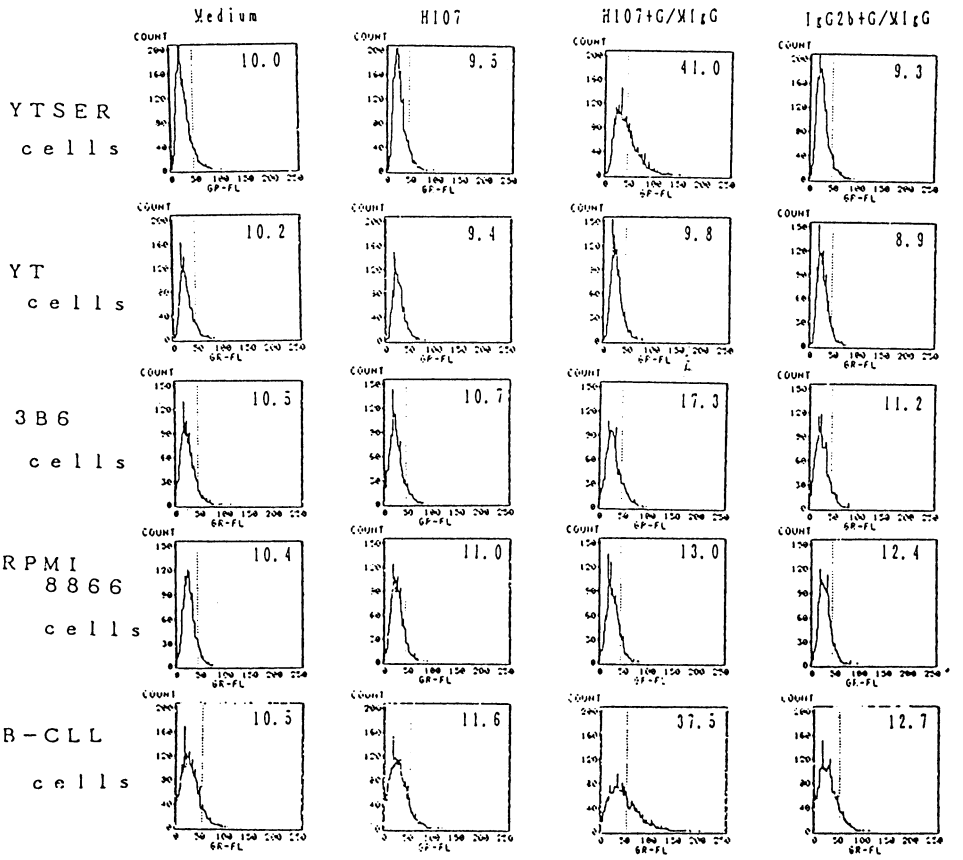

Fig. 4 Triggering of FcεR2/CD23 resulting in the induction of IL-2R/p55(Tac)
IL-2 DEPENDENT REGULATIONOF FcεR2/CD23 EXPRESSION

Acknowledgement

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Human Eosinophils: Synthesis and Expression of CD4 and Class II MHC Proteins

P.F. Weller, A. Nicholson-Weller, and D.R. Lucey

INTRODUCTION

Human eosinophilic leukocytes are prominent participants in a number of immunological responses, including those during allergic and helminthic parasitic diseases. In addition to the heightened blood and tissue eosinophilia that frequently accompanies these responses, eosinophils normally are richly distributed in tissues which interface with the external environment, such as the respiratory and gastrointestinal tracts. Within the environment of the tissues, eosinophil functioning can be affected by cytokines released by fibroblasts, endothelial cells, lymphocytes and other cells. Mechanisms for eosinophils to potentially interact cooperatively with other cells, however, have not been defined. We have now demonstrated that mature, blood-derived human eosinophils can express cell surface ligands not previously recognized on cells of this class of leukocytes. Eosinophils can express CD4 and can be induced *in vitro* to synthesize and express HLA-DR.

HUMAN EOSINOPHIL CD4 EXPRESSION

Eosinophils were isolated by methods including Percoll density gradient centrifugation from the blood of 4 normal donors and 5 donors with eosinophilia due to filarial parasitic infections (*Loa loa*), the hypereosinophilic syndrome or idiopathic etiology. Although some neutrophils contaminated these eosinophil-enriched leukocytes, both cytochemical staining and flow cytometric analyses with fluoresceinated anti-T cell and anti-monocyte/macrophage mAbs specifically demonstrated the absence of lymphocytes and monocytes. After staining with fluoresceinated mAbs anti-Leu 3a and b, eosinophils from all donors 9 donors expressed detectable CD4. The varying degrees of eosinophil expression of CD4 ranged from 5% to 83% CD4+ (Table 1), with a mean for freshly isolated eosinophils from all donors of 24% CD4+ eosinophils (Lucey et.al. 1989a).

Table 1 CD4 Expression by Human Eosinophils

	Donors		Blood Eosinophils	
	#	Diagnosis	% Eos*	% CD4+
Normal:	1	-	5	13
	2	-	2	10
	3	-	2	5
	4	-	5	14
Eosinophilic:	5	Filariasis	17	48
	6	Idiopathic	11	9
	7	Hypereos. Syndrome	46	11
	8	Filariasis	63	23
	9	Hypereos. Syndrome	74	83

* % peripheral blood eosinophilia

Eosinophils from each of these donors were cultured with 50 pM rhGM-CSF on monolayers of murine Swiss 3T3 fibroblasts. During culture contaminating neutrophils died yielding virtually pure populations of eosinophils. The absence of any lymphocytes or monocytes was again ascertained by cyto-chemical staining and by the absence on flow cytometric analyses of cells staining with lymphocyte- or monocyte-specific mAbs. As the eosinophils were maintained in culture over 7 days, the expression of CD4 on eosinophils fluctuated and, as displayed in Fig 1, generally increased over that detectable on freshly isolated blood eosinophils (Lucey et al 1989a).

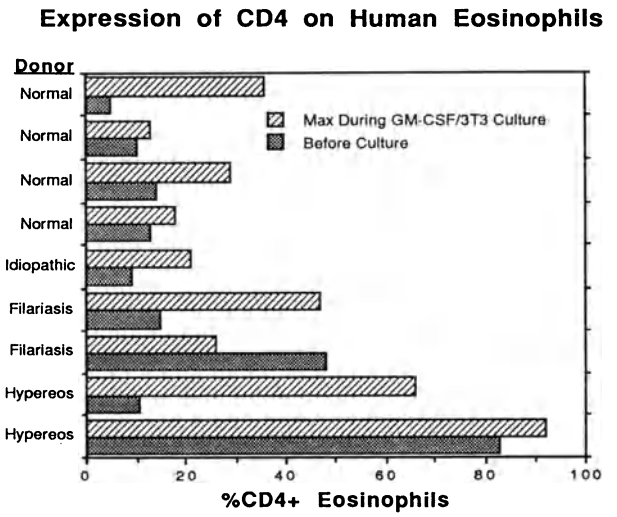

Fig. 1. The expression of CD4 on human eosinophils at the time of isolation from the blood of nine donors (before culture) and at the maximum during 7 days of *in vitro* culture with 50 pM rhGM-CSF and murine 3T3 fibroblasts.

To evaluate the identity of CD4 detectable by flow cytometry on eosinophils, 8.7 x 10^6 human eosinophils (>99% eosinophils), after 15 days of culture with rhGM-CSF/3T3 fibroblasts, were metabolically labeled with ^{35}S-methionine. Two CD4-specific mAbs, anti-OKT4 and anti-OKT4A, both immunoprecipitated a ~55 kD, ^{35}S-labeled protein from the eosinophil lysate, confirming the identity of the eosinophil protein recognized by several anti-CD4 mAbs and also establishing that mature eosinophils retain the capacity to synthesize this protein. The ability of eosinophil CD4 to bind HIV-1 gp120 was demonstrated by flow cytometry. HIV-1 gp120 competitively blocked the staining of eosinophils with mAb OKT4A, but not with mAb OKT4, which does not interact with a gp120 binding epitope on CD4 (Lucey et al 1989a).

HUMAN EOSINOPHIL HLA-DR EXPRESSION

When eosinophils purified from the 9 donors were evaluated by flow cytometry for expression of the Class II MHC antigen, HLA-DR, only 1 donor's eosinophils displayed detectable HLA-DR (23% HLA-DR+). On 9 occasions, blood eosinophils from the other 8 donors were HLA-DR-. These donors included 4 normals and 5 with hypereosinophilia (2 with filariasis, 2 hypereosinophilic syndrome, 1 idiopathic) (Lucey et al 1989b). These hypereosinophilic donors included those with high percentages of circulating "hypodense" eosinophils.

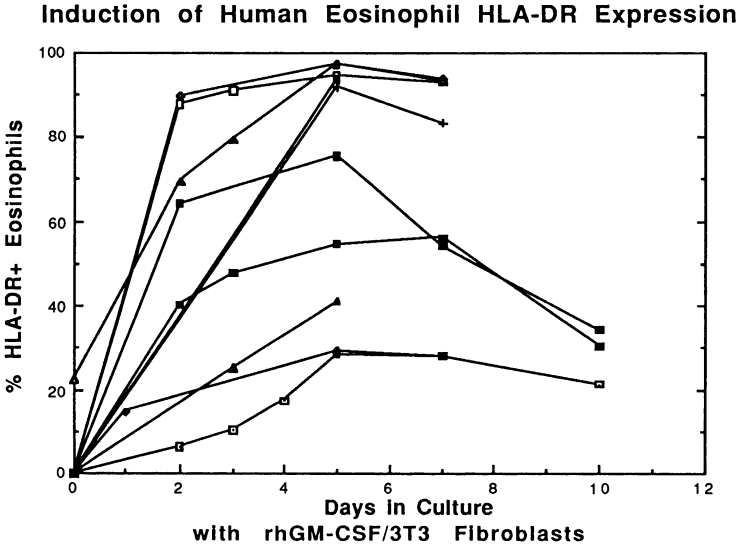

Fig. 2. Time course of HLA-DR expression by purified human eosinophils before (day 0) and during culture with 50 pM rhGM-CSF and 3T3 fibroblasts. HLA-DR expression was quantitated by flow cytometry, relative to a subclass control mAb. Data are derived from 10 sequential studies on eosinophils from 9 donors.

The purified eosinophils were ascertained at the outset and again routinely during culture to be devoid of any monocytes or T cells that might be HLA-DR+, by cytochemical staining and by flow cytometric analyses with specific mAb's (anti-Leu-1 for CD5 on T cells and anti-Leu-M3 for monocytes/macrophages). When these eosinophils were maintained in cultures with rhGM-CSF and 3T3 fibroblasts, HLA-DR expression was induced on eosinophils from all 9 donors (Fig 2) (Lucey et al 1989b).

To corroborate the expression of HLA-DR on eosinophils flow cytometrically detectable with one anti-HLA-DR mAb, eosinophils (8.7×10^6, >99% pure, 60.4% HLA-DR+) were metabolically labeled with ^{35}S-methionine. A second anti-HLA-DR mAb, LB3.1, immunoprecipitated the ^{35}S-labeled heavy and light chains of HLA-DR from lysates of these eosinophils (Lucey et al 1989b). These findings provided evidence that the augmented expression of HLA-DR detectable on eosinophils maintained *in vitro* with rhGM-CSF/3T3 fibroblasts was attributable to enhanced synthesis of HLA-DR and not due primarily to mobilization of this protein from intracellular sites.

DISCUSSION

These findings with peripheral blood eosinophils obtained from both normal and eosinophilic donors demonstrate that under appropriate conditions, such as exposure to cytokines *in vitro*, mature eosinophils retain the capacity to synthesize specific proteins. This capacity for protein synthesis has not been recognized previously when eosinophils were assumed to be only end-stage effector cells. Notably, the proteins demonstrated to be synthesized by eosinophils were not "house-keeping" proteins or the quantitatively predominant granule proteins but rather cell-surface proteins that could serve as ligands in facilitating the interactions of eosinophils with other cell types. With the currently recognized capabilities of HLA-DR to mediate binding with CD4 and to participate in the process of antigen-presentation to CD4+ lymphocytes, it is conceivable that eosinophils within tissues, especially in submucosal sites, might cooperatively interact with lymphocytes. Similarly, the recognition that eosinophils express CD4 provides a ligand by which the functional responses of eosinophils may be effected by other cells or CD4 binding cytokines. CD4 expression may also place the eosinophil at risk for infection with HIV.

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Effector Function of Eosinophils: Isotype-Dependent Activation

M. Capron, C. Grangette, M. Tomassini, J. Khalife, L. Prin, R.L. Coffman, and A. Capron

INTRODUCTION

Whereas considerable knowledge has been accumulated on the cellular and humoral mechanisms underlying immediate-type hypersensitivity reactions, one main concept has largely dominated the field of allergy. This general concept is related to the major role played by chemical mediators derived from mast cells and basophils. However, convergent observations have clearly demonstrated that this concept based on the intervention of an unique cell population is not exclusive. Allergic reactions are complex and result from a cell interaction network involving the release of pharmacological mediators by other cells than the classical mast cells or basophils. The identification of IgE receptors ($Fc_{\varepsilon}RII$) on inflammatory cells, such as mononuclear phagocytes, eosinophils and platelets, all found to infiltrate the site of allergic reactions, has certainly drawn the attention on their direct participation in the basic mechanisms of hypersensitivity reactions (Capron A. et al. 1986).

Eosinophils can be incriminated in the pathology of allergic reactions by generating various newly or preformed mediators such as LTC4, PAF-acether or granule cationic proteins (Major Basic Protein, MBP ; Eosinophil Cationic Protein, ECP ; or Eosinophil Peroxidase, EPO), directly involved in bronchoconstriction or tissue damage (reviewed in Gleich and Adolphson 1986). Eosinophils possess membrane receptors for IgG, IgE and IgA, which allow them to interact with the respective isotypes of antibodies, and to release pharmacologically active mediators, in response to the addition of antigens.

In the present review, we will briefly summarize the characteristics of eosinophil Fc receptors with a particular emphasis on the comparison of eosinophil $Fc_{\varepsilon}R$ and CD23, and on the newly described IgA receptor. The release of granule mediators in response to isotype dependent activation (specially IgE and IgA antibodies) of eosinophils from allergic patients will then be reported. Finally, a synergistic effect of IgE and IgA antibodies in eosinophil-mediated cytotoxicity will be suggested, as well as the potentiating effect of various lymphokines. Taken all together these studies point to the prominent role of eosinophils in local inflammatory reactions.

CHARACTERISTICS OF Fc RECEPTORS

Fc Receptor For IgG

In parallel to the demonstration that human IgG antibodies could be involved in eosinophil-mediated cytotoxicity (Butterworth et al. 1977), an Fc receptor for IgG was identified on the membrane of human eosinophils. Whereas previous reports have concerned heterologous systems using animal immunoglobulins, more recent studies on the binding of human IgG to human eosinophils were performed. In summary, by a rosette assay, the receptor for IgG binds to the four IgG isotypes without distinct specificity (Walsh and Kay 1986) but the affinity for the different subclasses might vary, as shown for rat IgG2a and IgG2c (Khalife et al. 1985). The MW of human eosinophil IgG receptor (Fc gamma receptor II) is different from that of the neutrophil Fc gamma receptor III (43 kDa versus 52 - 68 kDa) and their binding affinity for the various IgG subclasses seems distinct (Kulczycki 1984). Eosinophil Fc gamma R is inductible as suggested by its increased expression after incubation with various soluble factors in vitro, among which chemotactic factors such as Eosinophil Chemotactic Factor of Anaphylaxis or ECF-A (Capron M. et al. 1981), FMLP or LTB4 (Kay and Walsh 1984).

Fc Receptor For IgE

IgE receptors have been demonstrated on human eosinophils and they participate directly in the effector function of eosinophils against parasite larvae. The main characteristics of eosinophil IgE receptors have been reviewed in detail in Capron M. et al. (1989a). The production of a monoclonal antibody (BB10) raised against human Fc_ER-positive eosinophils and able to inhibit the IgE-dependent cytotoxicity of eosinophils, platelets and monocytes, has confirmed the common antigenicity of these Fc_ER, now referred to as Fc_ERII (Capron A. et al. 1986). The eosinophil receptor for IgE was recently characterized by using immunosorbent chromatography of eosinophil detergent extracts, passed over either IgE or BB10 immunosorbents (Jouault et al. 1988). Under reducing conditions, 3 polypeptide fragments were obtained with apparent molecular weights of 45-50, 23 and 15 kDa. Similar results were recently obtained by immunoprecipitation (Grangette et al. 1989). Western blotting procedure with human myeloma IgE followed by the addition of peroxidase-labelled anti-IgE, on molecules immunoprecipitated by BB10, revealed the binding of IgE to the components of 45-50, and 20-25 kDa. These findings together with the negative results obtained with heat-inactivated IgE confirm the specific binding of IgE to the major components of eosinophil IgE receptor immunoprecipitated by the mAb BB10 (in spite of the low affinity of Fc_ERII).

The apparent homology between the MW of the components of eosinophil Fc_ER and those reported for B lymphocyte Fc_ER, together with previous results using polyclonal antibodies suggesting antigenic similarities (Capron M. et al. 1984), led to further comparative studies between eosinophil and B cell Fc_ERII or CD23. Preliminary attempts performed to investigate the binding of BB10 to B cell lines and the binding of anti-CD23 mAb to eosinophils gave controversial results (Capron M et al. unpublished

observations) indicating some level of heterogeneity among the Fc_ERII present on these various cell populations, as recently reported (Yokota et al. 1988). Eosinophil Fc_ER, although recognized by polyclonal antibodies to human B lymphoblastoïd cell lines (Capron M. et al. 1984) is not detected by all anti-CD23 monoclonal antibodies, but by one monoclonal anti-CD23 antibody named mAb135 (Rector et al. 1985). Whereas BB10 immunoprecipitates 2 components at 45-50 and 25 kDa on a B lymphoblastoïd cell line (WIL-2WT), mAb135 against human lymphocyte Fc_ERII/CD23 binds to a major 45-50 kDa polypeptide both on eosinophils and on B cells.When immunoprecipitation is followed by immunoblotting, mAb135 binds to the component of 45-50 kDa immunoprecipitated by BB10 and reciprocally BB10 recognizes this component first immunoprecipitated by mAb135, both on eosinophils and on WIL-2WT cells (Grangette et al., 1989). This study confirms the existence of a common structure between the IgE receptors present on eosinophils and B lymphocytes, since the major component of 45-50 kDa recognized by the two monoclonal antibodies on cell populations can bind IgE.

A cross-reactivity between BB10 and a mAb anti-Leishmania gp63, which is a "fibronectin (Fn)-like" molecule, containing the RGD cell attachment domain indicated the presence of such a sequence in the common structure present on eosinophil and B cell Fc_ERII. These findings were confirmed by showing that anti-Fn mAb was able to immunoprecipitate the 45-50 kDa major component of surface or metabolically labelled eosinophils, suggesting that this "Fn-like" component was not a passively adsorbed structure, but a newly synthetized component (Grangette et al. 1989). Moreover, immunoprecipitation followed by immunoblotting procedures indicated that the 45-50 kDa component and slightly the 20-25 kDa Mr of the Fc_ERII immunoprecipitated either by BB10 or by anti-CD23 mAb135 are also recognized by anti-Fn mAb and by anti-RGDS polyclonal Ab, on eosinophils and on WIL-2WT cells. When the amino-acid sequence of the human B lymphocyte Fc_ER/CD23 is carefully analysed, a PDGR sequence is observed in the COOH-terminal region (aminoacid residues 308-311). This peptide is the inverse representation of RGDP where proline is subtituted to serine in RGDS sequence (Gordon et al. 1989). We could recently show that the binding of BB10 and anti-Fn mAb to eosinophils and WIL-2WT cells is inhibited both by the synthetic peptide RGD and by the inverted peptide sequence DGR. These data indicate that the common structure recognized by BB10 and by anti-Fn mAb on eosinophils from human patients and on a B cell line could be the adhesion sequence (DGR) present in the C-terminal extracellular domain of Fc_ERII. The absence of cross-inhibition between BB10 and mAb 135, together with the absence of inhibition of the binding of mAb 135 by RGD or DGR containing peptides suggest in addition the existence of one different epitope, common to eosinophil Fc_ERII and to CD23.

The functional role of such an adhesion sequence has to be envisaged on eosinophils but also on B cells. In the case of eosinophils, both anti-CD23 and anti-Fn mAb, together with anti-RGDS polyclonal antibodies or the SDGR peptide inhibited the IgE-dependent eosinophil-mediated killing of parasites, in a range similar to that obtained with the mAb BB10 (Grangette et al. 1989). In this respect, it is interesting to recall that mAb directed against the alpha chain of CR3 were also able to inhibit IgE dependent cytotoxicity by human hypodense eosinophils, indicating the role of such adhesive molecules in cell-mediated effector

function (Capron M. et al. 1987). Moreover, it is also suspected that these adhesion proteins and their receptors, part of the integrin family, may include members serving functions other than cell adhesion (Ruoslahti and Pierschbacher 1986). They can be involved in the interaction with different matrix molecules during differentiation. In this respect, it has to be pointed out that $Fc_{\varepsilon}RII$ represents an unique differentiation antigen transiently expressed on mature B cells before isotype switching (Kikutani et al. 1986). In parallel, a functional $Fc_{\varepsilon}RII$ can be considered as a marker of eosinophil heterogeneity, present on activated hypodense eosinophils (Capron M. et al. 1984). Further studies are needed to understand the role of such sequences associated to eosinophil $Fc_{\varepsilon}RII$ and to CD23. The molecular genetic analysis of eosinophil $Fc_{\varepsilon}RII$, currently in progress, would certainly improve comparative studies between $Fc_{\varepsilon}RII$ present on these different cell populations.

Fc Receptor For IgA

Whereas receptors for IgA have been detected on various leukocyte populations, their presence has been only recently reported on human eosinophils (Capron M. et al. 1988b). After the demonstration of surface IgA on intestinal eosinophils from parasite infected mice, and on blood eosinophils from patients with filariasis (Capron M. et al. 1989b), the existence of receptors for IgA (Fc alpha R) has been confirmed by using flow cytofluorometry (Capron M. et al. 1988b). The binding of monomeric IgA to purified human eosinophils (> 95 % purity) was evaluated by flow cytofluorometry analysis after staining with fluorescein-labelled antihuman IgA. Between 5 and 60 % of eosinophils were able to bind IgA (Capron M. et al. 1988b). The use of secretory IgA instead of monomeric IgA led to an increase of fluorescence intensity. Eosinophil receptor for IgA has been more recently characterized by immunosorbent chromatography and immunoprecipitation, as a major component of 55-60 kDa . An overnight incubation of purified human eosinophils with secretory IgA increased the proportion of molecules able to bind to an IgA-Sepharose column, suggesting that eosinophil IgA receptor is inducible by IgA as shown for the Fc alpha R on lymphocytes (Yodoï et al. 1982). Experiments of binding of radiolabelled IgA revealed a higher binding affinity of eosinophil Fc alpha R for secretory IgA and confirmed the induction of the receptor by IgA. Taken all together these results indicate a large variability in the expression of Fc alpha R on eosinophils from hypereosinophilic patients. Two non mutually exclusive hypotheses could be envisaged : either variations in IgA levels among the different patients, or effects of cytokines like IL-5 known to participate both to eosinophil differentiation and to IgA synthesis (at least in mice).

IMMUNOGLOBULIN-DEPENDENT ACTIVATION

Release of Mediators

IgG-dependent activation, The triggering of eosinophil IgG receptors by human IgG coated on Sepharose beads, induces the release of LTC4 and higher amounts are generated by hypodense eosinophils (Wardlaw and Kay 1987). However, increased levels of LTC4 are obtained when normodense eosinophils are activated by FMLP or Eosinophil Activating Factor (EAF) (Wardlaw and Kay 1987).

Similar results were obtained with IgG antibodies directed against *Aspergillus fumigatus* in the presence of the specific antigen. The production of H_2O_2 in response to IgG dependent stimulation has also been reported (Yazdankaksh et al. 1985). Eosinophils release twice as much H_2O_2 as do neutrophils upon interaction with IgG1-, IgG2- or IgG3-coated Sepharose beads, but this difference becomes fivefold with IgG4 coated Sepharose. This favours the view of distinct functional interactions between eosinophil IgG receptors and the IgG subclasses.

IgE-dependent activation, The demonstration of surface IgE on eosinophils from patients with increased IgE levels and particularly on lung eosinophils confirmed the biological relevance of the interaction between eosinophils and IgE antibodies (Capron M. et al. 1985). In order to investigate the role of cytophilic IgE in eosinophil function and to evaluate their specificity, further experiments measuring the release of various mediators were then performed. Eosinophil peroxidase (EPO) was detected in the supernatants of eosinophils from hypereosinophilic patients after incubation either with the specific antigen or with antiIgE antibodies. This was obtained not only in the case of patients with parasitic infections (Khalife et al. 1986) but recently confirmed in the case of allergic patients (Capron M. et al. 1989b). Only allergens related to the patient's allergy and giving positive skin tests induced extracellular release of EPO. Moreover no release of EPO was obtained when the same allergens were added to eosinophils from non allergic patients. The involvement of cytophilic IgE antibodies in allergen-mediated release of EPO was suggested by the significant correlation between EPO release induced by allergen or by anti-IgE antibodies (Tomassini et al. in preparation). These results suggest that cell-bound IgE with very restricted antibody specificity could play a role in the activation of hypodense eosinophils in inducing the release of granule proteins with potent cytolytic functions.

Selectivity Of Mediator Release, Eosinophil Peroxidase (EPO) was released after addition of anti-IgE but not of anti-IgG antibodies (Khalife et al. 1986). In contrast, ECP measured by a radioimmunoassay using monoclonal antibodies (kindly performed by Dr P.C. Tai, London) was only detected after addition of anti-IgG antibodies. These results showing no correlation between EPO and ECP release suggested a variability in the response of eosinophils to different stimuli (IgE versus IgG). Similar results have been recently reported concerning a differential release of EPO and ECP in response to activation by opsonized zymosan (Venge et al. 1989). Finally, the production of a newly formed mediator such as PAF-acether in response to IgE-dependent triggering, but not to IgG-dependent stimulation of hypodense eosinophils confirm the selectivity in the mediators produced by eosinophils according to the stimulus of activation (Capron M. et al. 1988a). These findings have to be considered in relation to human eosinophil heterogeneity. Indeed it has been recently shown that lung eosinophils produced 1000-fold more PAF-acether in response to anti-IgE antibodies than corresponding blood eosinophils from the same patients (Bruynjzeel et al. 1989). Moreover normodense eosinophils seemed to produce significant although limited amounts of PAF, in response to IgG triggering but not to IgE triggering (Kay et al. 1988).

IgA-Dependent Activation, The existence of surface IgA bound to eosinophils from allergic or parasite-infected patients led us to investigate the IgA-dependent release of mediators. EPO was evaluated after addition of anti-IgA antibodies to highly purified eosinophils. A significant release of EPO (between 2.5 and 6 times more than in controls) was obtained after addition of anti-IgA. These results suggested that similarly to IgE, surface IgA could participate to eosinophil activation, by inducing the release of mediators (Capron M. et al. 1988b). In a different assay, it was recently shown that Sepharose beads coated with secretory IgA induced the release of another granule protein, the eosinophil-derived neurotoxin or EDN (Abu-Ghazaleh et al. 1989). These findings, associated to the higher affinity of eosinophil Fc alpha R for secretory IgA indicate the functional interactions between eosinophils and secretory IgA at mucosal surfaces, specially in helminth infections and hypersensitivity diseases. Preliminary results suggest in addition a synergy between IgE- and IgA-dependent stimulation but not with IgG (Tomassini et al. in preparation).

Eosinophil-Mediated ADCC

IgG-dependent cytotoxicity, The effector aspects of IgG receptors have been first evidenced in IgG-dependent ADCC mechanisms against parasitic targets. The appropriate triggering of human eosinophils by IgG antibodies coated to parasite targets induced the lysis, involving cationic proteins such as MBP or ECP, as well as oxidative metabolism (reviewed in Gleich and Adolphson 1986). This IgG-mediated ADCC mechanism is strongly enhanced by a variety of factors such as Eosinophil Activating Factor (EAF), Gm CSF, PAF-acether, Tumor Necrosis Factor (TNF) or endothelial cell supernatants (reviewed in Capron M. et al. 1989a). Very little data exist that describe the role of each IgG antibody isotype in this event. Very recently, it was shown that the killing effect was associated with IgG1 and IgG3 antibodies. In contrast, IgG4 antibodies blocked the killing mediated by IgG effector antibodies. The function of IgG2 antibodies was dual : either an effector function in the presence of activated eosinophils or a blocking effect in the presence of normal eosinophils (Khalife et al. 1989). These results underline the differential effector function of human IgG subclasses in the presence of eosinophils (as previously demonstrated in experimental models, Khalife et al. 1985).

IgE-Mediated Cytotoxicity

The direct involvement of eosinophil $Fc_{\varepsilon}RII$ in effector function was recently confirmed by showing that not only the BB10 mAb or cross reacting anti-Fn mAb or anti-RGDS polyclonal Ab but also the anti-CD23 (135) mAb were able to significantly inhibit the IgE-dependent eosinophil mediated killing of parasites (Grangette et al. 1989). The recent description of Fc alpha R on eosinophils and its participation to eosinophil degranulation and release of mediators led us to investigate the role of Fc alpha R in eosinophil-mediated cytotoxicity. The preincubation of human eosinophils with myeloma IgE but also with IgA induced a significant decrease in ADCC mediated by human anti-*S. mansoni* immune serum. The increased inhibitory activity of heat-aggregated

or secretory IgA versus serum IgA might be related to the higher affinity of Fc alpha R for polymeric IgA. The hypothesis of a cross reactivity between IgA and IgE receptor was ruled out in binding experiments showing the absence of competition between the two isotypes.

These results, confirmed in the rat experimental model (Grezel et al. in preparation), suggest a synergistic association between IgE and IgA receptors on eosinophils. In addition, they might have a particular functional significance in inflammatory reactions involving eosinophils in given tissues such as the lungs or the gastrointestinal tract.

Enhancing Effect of Interleukins

In this context, it was particularly interesting to investigate the enhancing effects of interleukins and specially IL5, known to induce both eosinophil differentiation and increase of IgE and IgA synthesis. Using the same experimental model (antiparasite cytotoxicity mediated by human eosinophils and human immune serum), we could recently show that both IL5 and Gm CSF were able to strongly enhance eosinophil mediated cytotoxicity in the presence of IgE antibodies. IL4 and alpha TNF had more limited effects whereas gamma IFN was without effect. This effect of IL5 on eosinophils might be mediated through a specific receptor for IL5 that we have recently characterized on human eosinophils (Chihara et al. in preparation).

In conclusion, these findings have enabled the demonstration of a novel cellular pathway in hypersensitivity reactions. The existence of receptors for IgE and for IgA on eosinophils has certainly broaden current views on the cellular mechanisms of allergic reactions. The further demonstration that the activation of these receptors is associated with the selectivity and the dynamics of pharmacological mediator release might have significant implication in the understanding of the physiopathology of allergic and inflammatory reactions. More generally, these studies have clearly established that eosinophils which have been for years ambiguous cells for immunologists, are essential cellular components of protective mechanisms against parasites as well as effectors of hypersensitivity reactions.

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III Effector Phases

Inflammation

Endothelial Activation and Inflammation

R.S. Cotran and J.S. Pober

INTRODUCTION

Recent studies have shown that vascular endothelial cells respond to various stimuli, particularly to cytokines, by undergoing specific alterations in their normal constitutive properties, and by acquiring new capacities that may perform important functions in both acute and chronic inflammation. These induced alterations have been referred to as "endothelial activation" (Pober and Cotran 1989) akin to the induced changes that occur in activated macrophages and lymphocytes. In this paper we will highlight the responses of various cytokines on endothelium in culture, and in vivo, and examine the possible role of endothelial activation in inflammatory injury.

RESPONSES TO CYTOKINES IN ENDOTHELIAL CULTURES

Interleukin-1 (IL-1) and Tumor Necrosis Factor (TNF)

These pleiotropic mediators have been shown to share numerous inflammatory properties, and their effects on endothelium are also very similar. Most of these effects are also mimicked by lymphotoxin (TNF-β).

One of the best studied endothelial effects of IL-1 and TNF is the stimulation of leukocyte adhesion. Increased adhesivity has been shown for neutrophils (Bevilacqua et al. 1985, 1987; Gamble et al. 1985), lymphocytes (Cavender et al. 1986), eosinophils (Lamas et al. 1988), basophils (Bochner et al. 1988), monocytes and leukocyte cell lines (Bevilacqua et al. 1985). The effect is maximal four to six hours after cytokine treatment of endothelium and declines by 24 hours, but adhesion remains above control values at 24 hours for neutrophils and monocytes. Increased adhesion for neutrophils at four to six hours has been shown to be due, in large part, to the induction on the endothelial cell surface of an adhesive protein, named endothelial adhesion molecule 1 (ELAM-1) (Bevilacqua et al. 1987). ELAM-1 expression can be detected by monoclonal antibodies H4/18 (Pober et al. 1986) and H18/7 (Bevilacqua et al. 1987). cDNA for ELAM-1 has been recently sequenced and the predicted structure of the ELAM-1 molecule is a 610 amino acid, type I transmembrane protein, having a lectin-binding domain at the amino terminal, an EGF-like domain, six consensus repeats resembling sequences in complementary regulatory proteins, and a short cytoplasmic tail (Bevilacqua et al. 1989). The structure resembles that of two other adhesive proteins, GMP 140 found in platelet granules and endothelial cells, (Johnson et al. 1989) and Mel-14, an adhesion protein on mouse lymphocytes, which is involved in lymphocyte homing to lymph node high endothelial venules (Siegelman et al. 1989; Laskey et al. 1989). The ligand for ELAM-1 has not been identified, but it is not a component of the leukocyte adhesion molecule of the CD11/18 complex. IL-1 and TNF also induce increased expression of ICAM-1

(intercellular adhesion molecule-1), an adhesion molecule present on the surface of fibroblasts, lymphocytes, and normal endothelium (Dustin and Springer 1988; Pober et al. 1986). Increased ICAM-1 expression is maximal after 24 hours of cytokine treatment and is sustained as long as the cytokines remain in the culture medium. ICAM-1 is one of the ligands for the leukocyte adhesion molecule LFA-1 (CD11 a/CD18)(Dustin and Springer 1988), and studies with monoclonal antibodies have shown that ICAM-1 may be involved in adhesion of neutrophils (Smith et al. 1988), and B lymphocytes (Dustin and Springer 1988). Recent studies have described a second ligand for LFA-1, called ICAM-2 (Staunton et al. 1989). ICAM-1 expression can also be induced by γinterferon (IFN-γ)(Pober et al. 1986). TNF differs from IL-1 in that it also acts on neutrophils to rapidly induce a leukocyte-dependent increase in adhesivity, which can be blocked by antibodies to the CD11/18 complex (Gamble et al. 1985). In early primary cultures of endothelial cells, TGF-β can antagonize these actions of neutrophil adhesion (Gamble and Vadas, 1988).

IL-1 and TNF also can influence leukocyte interactions with endothelium by induction of endothelial cell derived cytokines. Activated endothelium secretes Il-1, Il-6, GM-CSF and other colony stimulating factors (reviewed in Pober and Cotran 1989), as well as low molecular weight neutrophil (Streiter et al. 1988) and lymphocyte chemoattractants (Larsen et al. 1989). They also secrete a factor that inhibits neutrophil adhesion for activated endothelium (Wheeler et al. 1988).

The second major effect of IL-1 and TNF is to enhance the surface thrombogenicity of endothelium. Endothelial cells possess on their surface both anticoagulant and procoagulant molecules. In its basal state, the endothelial surface activates neither the intrinsic nor extrinsic clotting pathways, and is non-thrombogenic. IL-1 and TNF treatment increase the endothelial surface expression of tissue factor (Bevilacqua 1984, 1986); decrease endothelial surface thrombomodulin, thus markedly inhibiting the anticoagulant effects of protein S and protein C (Nawroth et al. 1986); increase secretion of the inhibitor of tissue plasminogen activator and may decrease secretion of tissue plasminogen activator itself (Bevilacqua 1986; Emeis and Kooistra 1986; Nachman et al. 1986). These effects tip the scale of the coagulant-anticoagulant balance towards fibrin deposition and intravascular coagulation. Indeed, infusion of IL-1 into rabbits leads to activation of coagulation and fibrin deposition on an apparently intact endothelium (Nawroth et al. 1986). With low doses of TNF, this increased thrombogenicity TNF <u>in vivo</u> is restricted to tumor vessels (Nawroth et al. 1988). To date, however, tissue factor antigen has not been localized to endothelium <u>in vivo</u>.

IL-1 and TNF have numerous other endothelial effects (Pober and Cotran 1989; Cotran and Pober 1989). These include alterations in endothelial cell morphology and cytoskeletal organization (Stolpen et al. 1986), the release of prostacyclin, particularly enhanced in the presence of thrombin (Zavoico et al. 1989), and for TNF, stimulation of class I MHC antigens (Pober et al. 1986).

Finally, IL-1 and TNF may render endothelial cells more susceptible to injury. Varani et al. (1988) have shown that pre-treatment of rat pulmonary artery endothelial cells with TNF or IL-1 increases in time and dose-dependent manner the sensitivity to killing by neutrophil stimulated with phorbol esters or C5a.

To summarize then, interleukin-1 and TNF have profound effects on endothelium which influence leukocyte adhesion, surface thrombogenicity, leukocyte activation, and a variety of other inflammatory events. Indeed, it may very well be that the major effects of these cytokines <u>in vivo</u> rest on their

induction of endothelial responses. Cytokine production by endothelial cells themselves may also play a role in the cascade of inflammatory events.

γInterferon (IFN-γ)

IFN-γ has profound effects on endothelial cells in culture. It is the only cytokine that causes endothelial cells to express class II MHC antigens (Pober et al. 1983; Wagner et al. 1985). This effect is gradual, being first detected six to eight hours after IFN-γ treatment and reaching a plateau by four to six days. Increased class II MHC expression permits vascular endothelium to act as an antigen presenting cell to T cells *in vitro*. IFN-γ also increases the expression of class I MHC antigens, ICAM-1 (Pober et al. 1986), proteins of the alternate complement pathway (Ripoche et al. 1988), and a cytoplasmic antigen normally found only in lymph node high endothelial venules (Duijvestijn et al. 1986).

IFN-γ also induces increased adhesion of leukocytes, but its effect is restricted to lymphocytes, particularly T cells (Thornhill et al. 1989) and is more gradual than the effect of IL-1, endotoxin, and TNF. IFN-γ also causes morphological changes similar to those induced by TNF: two or three days after addition of IFN-γ, normally polygonal endothelial cells become markedly elongated, overlap extensively, and exhibit reorganization of actin cytoskeletons (Stolpen et al. 1986).

In addition to its own direct effects, IFN-γ synergizes with TNF in inducing increases in class I MHC and ICAM-1 expression (Lapierre et al. 1988; Pober and Cotran 1989), morphological changes in endothelium (Stolpen et al. 1986), and changes in membrane protein lateral mobility (Stolpen et al. 1988). IFN-γ also accelerates and prolongs ELAM-1 expression induced by TNF (Doukas and Pober; manuscript submitted).

Other Cytokines

IFN-α and -β inhibit endothelial cell growth and migration and increase class I MHC antigen expression. Transforming growth factor-β inhibits endothelial cell growth, influences endothelial matrix protein biosynthesis, and is angiogenic *in vivo*, although it is not clear that the angiogenic effect is direct or is due to the induction of monocyte influx. GM-CSF has also been reported to induce endothelial migration *in vitro* and angiogenesis *in vivo*. In contrast, IL-2 has no unequivocal direct endothelial effects. Likewise, there are no reports to date that either IL-4 or IL-6 affect known endothelial cell functions or modulate the expression of ELAM-1, ICAM-1, or MHC antigens (reviewed in Pober and Cotran, 1989).

ENDOTHELIAL EFFECTS OF CYTOKINES *IN VIVO*

Cytokines induce a multitude of effects when injected locally or systemically in experimental animals and humans. Here we shall restrict our discussion to those effects which are relevant to the endothelium in inflammation.

There is substantial evidence that the effects of cytokines on neutrophil attachment and induction of adhesion molecules described from *in vitro* work, also occurs *in vivo*. Intracutaneous injections of IL-1 or TNF in mice,

rabbits, and baboons causes neutrophil adhesion and infiltration (Cybulsky et al. 1986, 1988; Munro et al. 1989). We have recently (Munro et al. 1989) examined the effects of intradermal injection IL-1, TNF, and IFN-γ upon endothelial antigen expression and leukocyte influx in the baboon (Papio anubis) using immunocytochemical reagents developed in humans which cross react with baboon tissues. We found that TNF causes early (by two hours) adhesion of neutrophils to endothelium, associated with increased ELAM-1 expression and later (six to nine hours), increased expression of ICAM-1 associated with influx and infiltration by mononuclear cells. At 24 and 48 hours, TNF-induced lesions that are characterized by a perivascular infiltrate with the same immunophenotypic nature as the infiltrate in delayed hypersensitivity reactions. IFN-γ caused increased expression of HLA DP (class II MHC antigen), in the microvascular endothelium associated with scant mononuclear cell infiltrate. These studies indicate that the cytokine effects uncovered from culture studies can also be mimicked in vivo and will prove to have relevance in inflammation.

There is also evidence that TNF and IL-1 are involved in the Shwartzman phenomenon (Movat et al. 1987; Rothstein et al. 1988; Pober and Cotran 1989). For example, IL-1 and TNF administered at a single injection can replace endotoxin in priming for the local Shwartzman phenomenon in rabbits, and a Shwartzman-like local reaction in mice can be induced by intradermal injections of endotoxin followed at 24 hours by TNF.

Cytokine actions on endothelial cells also undoubtedly contribute to several of the features of the hypersensitivity reactions. For example, class II expression in endothelial cells occurs as a very early event in experimental delayed hypersensitivity in guinea pigs (Sobel et al. 1984). Vascular endothelial cells enhance T cell responses by markedly augmenting IL-2 concentrations (Guinan et al. 1989). Cytokines may also be involved in the recruitment of neutrophils in the early phases of delayed hypersensitivity reactions, and the mononuclear cell infiltrate, at a later time, as suggested previously in the baboon model. In addition, anti-cytokine sera inhibit the development of these infiltrates in the guinea pig (Geczy et al. 1976). Anti-IFN-γ anti-sera reduce the size of the infiltrate in rat hypersensitivity reactions (Issekutz et al. 1988). Activation of endothelial antigens can readily be induced in human delayed hypersensitivity reactions (Cotran et al. 1986) and can be found in a variety of human pathological conditions associated with cell-mediated immunity (Cotran et al. 1988). Ultrastructural changes of endothelial activation, identical with those seen in human delayed hypersensitivity reactions can also be mimicked by injections of cytokines intradermally in baboons (Munro et al. 1989).

Cytokines may also play a role in increased vascular permeability in inflammation. Local injections of IL-1 and IFN-γ cause immediate transient increased permeability that is probably mediated by the release of vasoactive amines (Martin et al. 1988). In addition, by inducing PGI2 synthesis, and causing leukocyte adhesion, cytokines promote local vasodilatation and leukocyte-dependent plasma extravasation (Rampart et al. 1986; Williams et al. 1988). Vascular leakiness has been reported as a complication of treatment with cytokines, such as with combinations of TNF and IFN-γ , or GM-CSF, but most commonly after IL-2 infusions used for cancer therapy (Rosenstein et al. 1986). We have shown that patients treated with systemic infusions of IL-2 exhibit endothelial expression of ELAM-1, and increased expression of ICAM-1 and class II MHC antigens in dermal endothelial cells, sometimes associated with a perivascular inflammation (Cotran et al. 1988). These changes resemble those seen in delayed hypersensitivity reactions, which are also associated with vascular leakiness. The studies suggest that endothelial activation may play a role in the vascular leak syndrome. Since IL-2 itself does not cause the increased expression of these antigens directly in vitro, it is likely

that the endothelial activation in this setting is induced by endogenous mediators such as IL-1, TNF, IFN-γ, and lymphotoxin, which are known to be stimulated with interleukin-2 (Cotran 1988 et al.). There is in addition, evidence of endothelial cell toxicity, induced by activated T cells in the IL-2 induced vascular leak syndrome.

Finally, there is evidence that the cytokine-induced endothelial activation antigens may play a pathogenetic role in certain types of vasculitis. In particular children with Kawasaki disease, who develop a childhood vasculitis characterized by generalized immune activation, exhibit expression of ELAM-1, and increased expression of ICAM-1, in dermal endothelium (Leung et al. unpublished). Sera from these patients have complement-dependent antibodies that lyse cultured endothelial cells which have been exposed to IL-1 or TNF for four hours, or γinterferon for 72 hours. The sera do not lyse normal endothelial cells, and disappear during convalescence (Leung et al. 1986a,b). It is hypothesized that cytokines, induced by activated lymphoid cells in this condition, induce the expression of novel endothelial cell surface antigens, which in turn stimulate the production of autoantibodies that injure endothelial cells and initiate the events leading to vascular injury.

To summarize, cytokines, and in particular IL-1, TNF, and IFN-γ have profound effects on endothelial cell function and structure in vitro. There is also now considerable evidence that many of the endothelial effects induced by cytokines in vitro also occur in vivo and may play important roles in the evolution of the inflammatory response and in vascular injury.

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Pathogenesis of Tissue Fibrosis: Growth Factor Gene Expression in Mononuclear Phagocytes

B.C. Trapnell and R.G. Crystal

"Tissue fibrosis" is a pathologic process characterized by the replacement of normal tissue architecture by mesenchymal cells and a collagenous extracellular matrix (Agelli and Wahl 1986). The processes involved are similar to those modulating scar formation in skin wounds, including inflammation followed by an accumulation of fibroblasts that deposit extracellular matrix material in the local milieu (Wahl 1981). However, while normal scar formation is localized in time and place, fibrosis of internal organs usually involves the entire organ and typically is chronic (Agelli and Wahl 1986, Wahl 1981).

Evidence from the study of pathogenesis of tissue fibrosis of various organs has lead to the general acceptance of a paradigm that first involves "injury" and hence architectural derangement of the organ (Fig. 1). If these derangements are sufficiently extensive such that the normal architecture cannot be re-established, subsequent "repair" processes utilize mechanisms common to wound healing i.e., fibroblast proliferation and the subsequent deposition of a collagenous matrix in an attempt to maintain structural integrity of the injured tissue. In this regard, tissue fibrosis can be conceptualized as a normal biologic process, but exaggerated in extent and time.

A variety of mechanisms may participate in the injury and derangement of tissue architecture that precedes the accumulation of mesenchymal cells. However, all available evidence supports the concept that the cells which "direct" mesenchymal cell accumulation are the tissue representatives of the mononuclear phagocyte system (Fig. 1). In this regard, mononuclear phagocytes have the capacity to express a variety of genes coding for polypeptide growth factors capable of stimulating resting mesenchymal cells to enter and proceed through their growth cycle and replicate. To illustrate the central role of mononuclear phagocytes in this process, we will use the clinical example of idiopathic pulmonary fibrosis (IPF), a chronic, fatal disorder characterized by diffuse fibrosis of the alveolar walls in which alveolar macrophages (the lung representatives of the mononuclear phagocyte system) play a central role. For details concerning the clinical aspects of IPF and how the alveolar macrophages are recovered and studied, several reviews are available (Crystal et al. 1984, Hunninghake et al. 1979).

Classification of Alveolar Macrophage Mesenchymal Cell Growth Factors

Depending on their source and the conditions under which they are evaluated, mononuclear phagocytes are capable of expressing the genes for a variety of polypeptides with growth factor activity for mesenchymal cells including platelet derived growth factor (PDGF), fibronectin (FN) insulin-like growth factor-I (IGF-I), interleukin-1α and β, and basic fibroblast growth factor (Agelli

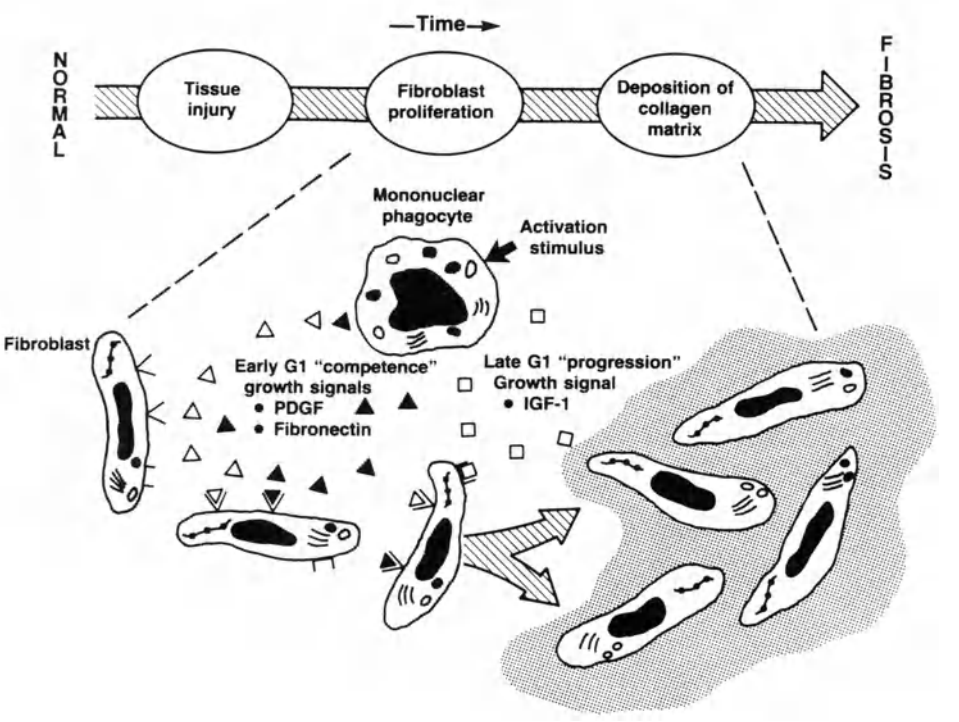

Figure 1. Central role of the mononuclear phagocyte in directing fibroblast proliferation in the development of tissue fibrosis. The current concept of the pathogenesis of tissue fibrosis holds that the tissue proceeds from "normal" to "fibrosis" by sequential processes including tissue injury followed by proliferation of fibroblasts and the deposition of a collagen matrix by the fibroblast. Since fibroblasts produce primarily type I collagen, the result is an increased mass of fibroblasts and their type I collagenous matrix replacing the normal tissue architecture. The mononuclear phagocyte (for example the alveolar macrophage in the lung) is activated and consequently releases polypeptide growth signals that signal fibroblasts to proliferate. Included among these growth signals are those that function early in the G1 phase of the growth cycle providing "competence" to the target cell. Examples of such growth signals are platelet-derived growth factor (PDGF) and fibronectin. The mononuclear phagocyte also releases growth signals that function late in the G1 phase of the cell cycle (so-called "progression" signals) that allows the cell to proceed through G1 and then proliferate. An example of this class of growth signals is insulin-like growth factor-I (IGF-I). The fibroblasts have specific receptors for each of these growth factors. Each fibroblast secretes collagen matrix material which surrounds the cell and as the number of fibroblasts increase so does the amount of extracellular collagenous matrix ultimately resulting in tissue fibrosis.

and Wahl 1986, Joseph-Silverstein et al. 1988). Activated mononuclear phagocytes also express the gene for tumor necrosis factor, a polypeptide with many functions overlapping interleukin-1, and which stimulates fibroblast proliferation (Yamauchi et al. 1987). Using sensitive methods such as the polymerase chain reaction, mRNA transcripts for transforming growth factor α have also been observed in mononuclear phagocytes (Rappolee et al. 1988). Mononuclear phagocytes express the gene for transforming growth factor β, a multifunctional protein that suppresses the growth of diploid human fibroblasts, but upregulates the expression of genes for fibroblast products such as type I collagen and fibronectin (Yamauchi et al. 1987). Despite this multifaceted potential growth factor armamentarium, current evidence suggests that the alveolar macrophage uses only PDGF, FN and IGF-I to direct the expansion of fibroblast numbers in the alveolar walls of individuals with IPF.

A convenient classification for these alveolar macrophage mesenchymal-cell polypeptide growth factors is based on the "competence/progression" model (Pledger et al. 1977), which groups the growth factors on the basis of where they act in the cell replication cycle. "Competence" factors induce resting (G_0) fibroblasts to enter the G_1 phase of the cell cycle, and "progression" factors stimulate the "competent" cells to continue through G_1, synthesize DNA, proceed through G_2 and proliferate. In this classification, PDGF and FN act as "competence" factors and IGF-I acts as a "progression" factor.

Platelet-Derived Growth Factor

Two chains, A (18 kDa) and/or B (16 kDa) make up a functional PDGF molecule of 30 kDa (Ross et al. 1986). The PDGF dimers can be AB, AA, or BB, although the AA form has been observed only in tumor cell lines (Ross et al. 1986). BB and AB dimers appear to be equipotent, and have 10-fold greater growth stimulatory activity than AA dimers (Heldin et al. 1988). The A-chain gene is comprised of 7 exons encompassing >25 kb on chromosome 7 at pter-q2 (Bonthron et al. 1988). Through alternative splicing, mRNA transcripts coded by the A-chain gene may contain sequences of all 7 exons, or transcripts missing exon IV. The B-chain gene consists of 7 exons spanning 24 kb on chromosome 22 at q12.3-q13.1 (Rao et al. 1986). All PDGF B-chain transcripts include sequences coded by all 7 exons. PDGF interacts with two types of receptors on mesenchymal cells, designated class A and B. The class A receptor binds all three dimeric forms of PDGF whereas the class B receptor binds PDGF BB with high affinity, PDGF AB with low affinity and does not bind PDGF AA (Heldin et al. 1988). The major mitogenic effect of PDGF appears to be mediated via the class B receptor (Heldin et al. 1988). Following binding of PDGF to it receptor, several genes are activated in the target cell, including the cellular protooncogenes c-fos and c-myc (Ross et al. 1986). As a consequence of these events, the mesenchymal cell enters G1 and becomes "competent" to respond to a "progression" factor.

Alveolar macrophages are capable of expressing both the PDGF A and B chain genes, but A-chain expression is very low (Mornex et al. 1986, unpublished observations). Normal resting blood monocytes, the precursors of alveolar macrophages, do not normally express either gene. When activated by PMA, monocytes rapidly transcribe both genes with a concomitant increase in cytoplasmic mRNA levels. If monocytes are placed in culture and allowed to mature to

macrophages without any activation, the PDGF A-chain gene remains turned off, but the B-chain gene is expressed, and B-chain mRNA levels progressively rise. This is consistent with the observation that normal alveolar macrophages constitutively express the B-chain gene of PDGF at both the transcription and mRNA levels. This observation is of interest in the context that the gene coding for the PDGF B-chain is the c-*sis* protooncogene, the human cellular analog of the v-*sis* oncogene, a gene that has the capacity to transform fibroblasts *in vitro* (Ross et al. 1986). Thus, while the viral oncogene v-*sis* renders infected mesenchymal cells unable to control their growth, the c-*sis* protooncogene is constitutively expressed at low levels in normal human alveolar macrophages.

Evaluation of the secreted products of normal alveolar macrophages is consistent with this observation i.e., a low level of PDGF (likely B-chain homodimers) are detected (Mornex et al. 1986). Strikingly, alveolar macrophages recovered from the lungs of individuals with IPF release 4-fold more PDGF than do normal alveolar macrophages i.e., the expression of the PDGF gene is markedly upregulated (Martinet et al. 1987). In the context of the knowledge of the fibroblast transforming ability of the v-*sis* oncogene, the observation in IPF of the upregulation of PDGF gene expression in alveolar macrophages in association with local fibroblast proliferation suggests there is a continuum, in which the same class of genes are implicated in neoplasms, pathologic tissue fibrosis and normal scar formation. In this regard, monocytes (the mononuclear phagocyte recruited to skin wounds) can be activated to express the c-*sis* gene (in a brief and tightly regulated manner while tissue macrophages associated with chronic inflammatory lung disease like IPF have an exaggerated, chronic expression of this gene. In contrast, neoplasms are driven by uncontrolled and/or greatly exaggerated expression of growth factor genes in cells that do not normally express significant levels of these genes.

Fibronectin

FN, a dimer of 220 kDa glycoproteins found in plasma and the extracellular matrix, contains several binding sites that mediate important cell-matrix interactions (Ruoslahti 1988). Through these binding domains, FN plays a central role in normal tissue turnover, wound repair, and inflammatory processes characterized by tissue remodeling. FN is coded for by a gene of at least 50 exons encompassing 50 kb (Hirano et al. 1983) and is expressed in a variety of cells including mononuclear phagocytes (Rennard et al. 1981). In addition to its ability to mediate the attachment of cells to the matrix, FN functions as a fibroblast chemoattractant and as a competence factor for fibroblast proliferation (Bitterman et al. 1983).

Like the PDGF B-chain gene, alveolar macrophages constitutively express the FN gene. In contrast, blood monocytes cannot express this gene, independent of how the monocyte is stimulated. However, as monocytes mature in culture, the FN gene begins to be expressed, consistent with its observed expression in normal alveolar macrophages. Also like the PDGF B-chain gene, the FN gene is upregulated in IPF. The levels of mRNA transcripts are markedly higher in alveolar macrophages from individuals with IPF compared to normals (Adachi et al. 1988). This is reflected at the protein level; alveolar macrophages from individuals with IPF are

spontaneously releasing 2-3-fold more FN than are normal alveolar macrophages (Rennard et al. 1981).

Insulin-Like Growth Factor-I

IGF-I is comprised of a family nonglycosylated polypeptides present in plasma and tissues. Somadomedin C, the plasma form of IGF-I, is a 70 amino acid, 7.6 kDa protein that binds to specific "IGF-I binding proteins" in plasma (Van Wyk 1984). Tissue IGF-I molecules are a heterogeneous collection of 18 to 30 kDa proteins produced by liver, fibroblasts, Sertoli cells, and macrophages (Rom et al. 1988). Both the plasma and tissue forms of IGF-I function as progression factors for mesenchymal cells i.e., fibroblasts exposed first to a competence factor like PDGF or fibronectin will, after exposure to IGF-I, proceed through G_1 synthesize DNA, proceed through G_2 and proliferate (Van Wyk 1984). Human IGF-I molecules are coded for by a single gene of at least 5 exons spanning >45 kb on chromosome 12 at q22-q24.1 (Brissenden et al. 1984, Rotwein et al. 1986). Multiple IGF-I mRNA molecules ranging in size from 7.7 kb to 1.1 kb are expressed from the single IGF-I gene through differential transcription and alternative splicing. Two forms of alternatively spliced IGF-I mRNA are found within the 1.1 kb size class. IGF-IA mRNA utilizes exons I-III and V and codes for a 153 amino acid, 17.5 kDa polypeptide while IGF-IB mRNA utilizes exons I-IV and codes for a 194 amino acid, 22 kDa polypeptide (Rotwein et al. 1986). It is assumed that these heterogeneous tissue IGF-I precursors are processed into the smaller plasma form of somadomedin C. Monocytes cannot express the IGF-I gene, nor can IGF-I gene expression be induced by *in vitro* maturation of monocytes. In contrast, normal alveolar macrophages contain IGF-I mRNA. Interestingly, normal alveolar macrophages do not secrete IGF-I unless activated. However, alveolar macrophages recovered from the lungs of individuals with IPF spontaneously secrete functional IGF-I molecules capable of acting as a progression factor in promoting fibroblast growth. Studies of the regulation of IGF-I gene expression in the macrophage-like cell line U937 indicate that surface stimulation simultaneously increases IGF-I transcription, decreases IGF-I mRNA levels and causes extracellular release of IGF-I from a preformed storage pool (Nagaoka et al. 1989). Importantly, blockage of transcription and/or protein synthesis does not affect the rapid release of preformed IGF-I thus, demonstrating protein release as the major regulatory point in IGF-I gene expression in mononuclear phagocytes (Nagaoka et al. 1989).

Implications for Therapy of Tissue Fibrosis

In disorders such as IPF, the etiology is unknown and therefore avoidance of the causative agent is impossible. However, regardless of the causative agent, the knowledge that tissue macrophages are expressing genes that include competence (e.g., fibronectin, PDGF) and progression (e.g. IGF-I)-type growth factors, provides a class of biologic targets that may prove vulnerable to therapeutic modulation, thus suppressing exaggerated fibroblast accumulation and deposition of extracellular matrix that characterizes these disorders.

PDGF gene expression in mononuclear phagocytes is regulated, at least in part, at the level of transcription and mRNA accumulation. Fibronectin gene expression also appears to be

regulated at the level of mRNA accumulation either through transcriptional control or differential mRNA stability. In contrast, IGF-I gene expression seems to be regulated primarily at the level of protein release.

Thus, for PDGF and FN, therapy might be directed towards transcriptional deactivation of the genes. Alternatively, if differential mRNA stability proved to be the major mechanism of mRNA accumulation, therapy might be directed here, although the mechanisms which regulate stability of specific mRNA molecules are poorly understood at present. Therapeutic intervention of IGF-I gene expression in macrophages should probably not be directed at transcriptional or mRNA stability-type mechanisms, but rather at the mechanism of IGF-I release from the cellular storage pool. In the context of these concepts, it is of note that standard glucocorticoid therapy is not effective at suppressing alveolar macrophage release of either fibronectin nor IGF-I in IPF patients even though these cells have glucocorticoid receptors (Lacronique et al. 1984). However, colchicine significantly blocks alveolar macrophage release of both fibronectin and IGF-I _in vitro_ (Lacronique et al. 1984). Although the toxicity associated with levels of colchicine necessary to suppress growth factor release prohibit its use in patients, this observation provides a conceptual basis for designing rational therapies for this disease.

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Inflammation and Angiogenesis

J. Folkman, W. Li, and R. Casey

Acute inflammation (i.e., caused by a superficial infection) is generally associated only with dilated vessels in the microcirculation of the skin. In chronic inflammation, dilated blood vessels are also accompanied by neovascularization. In certain tissues, such as the cornea, chronic inflammation (i.e., from infection or alkali burns) is so frequently associated with neovascularization that the two processes have not been separable either operationally or conceptually. In fact, before the discovery of specific angiogenic molecules (Folkman and Klagsbrun, 1987) early workers in the field of angiogenesis were criticized for studying "non-specific inflammation".

We now show that angiogenesis and chronic inflammation are distinct and separable processes. The evidence for this concept is based on two recent discoveries: (i) angiogenic polypeptides; and (ii) angiostatic steroids.

Because the cornea is avascular and hypocellular, it provides a good model for the study of the sequential events of inflammation and angiogenesis. Agents that are injurious to the cornea usually induce inflammation and concomitant neovascularization. For example, silver nitrate, alkali burns or injection of endotoxin, all lead to inflammatory cell infiltration and angiogenesis in the rabbit cornea (Ausprunk et al., 1978). In fact, the corneal edema resulting from inflammation was so commonly associated with neovascularization that up until about the late 1970's, the hypothesis that corneal edema was the cause, or at least a pre-requisite for neovascularization was widely accepted (Cogan, 1962).

The first clue that angiogenesis could occur in the absence of inflammation was the demonstration that tumor cells implanted in the rabbit cornea could induce intense angiogenesis in the absence of any corneal edema or inflammatory cell infiltrate (Gimbrone et al., 1974). Tumor implants in the anterior chamber also induced angiogenesis without an inflammatory cell infiltrate. (Gimbrone et al., 1972). Further confirmation was possible when pure angiogenic peptides became available (Shing et al., 1984). Angiogenic molecules such as basic fibroblast growth factor (bFGF) implanted in sustained release pellets of ethylene vinyl acetate co-polymers stimulated neovascularization in the rabbit cornea, yet there was no inflammatory infiltrate or edema by light or electron microscopy (Shing et al., 1985).

Hydrocortisone, an anti-inflammatory steroid, partially suppressed the corneal neovascularization associated with inflammation, but had

little or no effect on tumor angiogenesis. Nor could hydrocortisone, dexamethasone and related anti-inflammatory glucocorticoids inhibit angiogenesis in the chick embryo chorioallantoic membrane when they were applied alone (Folkman et al., 1983) (Crum et al., 1985). Vessel growth in the chick embryo chorioallantoic membrane is not driven by inflammation or by an inflammatory cell infiltrate.

We found that cortisone and hydrocortisone could be converted to potent angiogenesis inhibitors if they were co-administered with heparin (Folkman et al., 1983). An inactive metabolite of hydrocortisone, tetrahydrocortisol-S which has no glucocorticoid or mineralocorticoid activity inhibited angiognesis and was also greatly potentiated by heparin in the chick embryo chorioallantoic membrane.

Heparin-cortisone combinations inhibited tumor-induced angiogenesis in the cornea, whereas, cortisone alone did not. However, heparin had to be implanted in the corneal stroma, in a slow-release pellet because heparin is poorly diffusible across the cornea and could not be applied topically.

There were other problems with heparin. Its potentiation of anti-angiogenic activity varied from batch to batch and with each manufacturer. Furthermore, it was not clear why the steroid-heparin "pair" was so important or so critical for maximal inhibition of angiogenesis whereas either compound alone had little or no effect.

These problems were resolved with the recent discovery that sulfated cyclodextrins could mimic the ability of heparin to potentiate the anti-angiogenic effect of steroids. (Folkman et al., 1989) (for review see Folkman and Ingber 1989). The alpha-, beta-, and gamma-cyclodextrins are cyclic oligomers of 6, 7, and 8 glucose rings respectively. The center cavity of the beta form can easily include a large portion of a steroid molecule. Beta-cyclodextrin tetradecasulfate was the most potent of the cyclodextrins tested so far. It was able to potentiate the anti-angiogenic activity of hydrocortisone and other angiostatic steroids in the chick embryo at 100 to 1,000 times lower concentrations than heparin. Beta-cyclodextrin tetradecasulfate gave reproducible activity from batch to batch. It is a simple synthetic molecule of low molecular weight and it also diffuses across the cornea. Thus, it can be applied topically as eyedrops in experimental animals and potentially in patients with corneal neovascularization.

We employed an endotoxin model to stimulate inflammation and angiogenesis in the rabbit cornea by modifying the method of Howes et al. (1982). Endotoxin was implanted in the cornea in sustained-release polymer pellets as previously described (Grayson et al., 1988) (Li et al., 1989). The endotoxin pellets induced reliable corneal neovascularization. Liebovich et al. (1987) and Frater-Shroder et al. (1987) showed that endotoxin: (i) recruited or mobilized macrophages and (ii) activated these macrophages to release cytokines, among which, tumor necrosis factor-alpha (TNF-alpha) is a potent angiogenic molecule. Topical application of hydrocortisone alone, suppressed the inflammatory infiltrate in endotoxin-containing corneas, but had little effect on the angiogenesis, reducing vessel growth only down to 78% of the untreated control corneas.

Hydrocortisone plus beta-cyclodextrin tetradecasulfate caused regression of growing blood vessels and suppressed angiogenesis down to 16% of untreated animals.

Tetrahydrocortisol-S alone reduced angiogenesis down to 47%. Histologic sections revealed no suppression of the inflammatory cell infiltrate. Tetrahydrocortisol-S plus cyclodextrin reduced vessel growth down to 26% of untreated controls, but again, there was little or no effect on the inflammatory infiltrate.

These results show that the process of inflammation can be dissociated from the process of neovascularization. The biological and biochemical distinction among these events has immediate clinical importance, especially in ophthalmology. There are at least twenty diseases in which corneal neovascularization may lead to blindness in patients. The majority of these diseases are caused by inflammatory lesions such as infections, trauma, alkali burns, and other chronic injuries to the cornea. Conventional therapy is based mainly on hydrocortisone or related anti-inflammatory steroids and these often have to be pushed to their toxic limits. Furthermore, many of them fail to cause regression of capillary blood vessels because the ability of hydrocortisone to suppress blood vessel growth depends upon an indirect action against inflammatory cells and the release of their various cytokines. The use of hydrocortisone or its glucocorticoid relatives is associated with complications such as corneal thinning, microbial keratitis and glaucoma. When hydrocortisone is used at its maximal dose of approximately 10 mg/ml in eyedrops, most ophthalmologists will discontinue its use after 10-12 days because of the risk of corneal thinning or melting.

The availability of purely angiostatic steroids such as tetrahydrocortisol-S which have no glucocorticoid effect, may alleviate or prevent these side effects. Preliminary evidence in the rabbit endotoxin model suggests that while hydrocortisone causes significant corneal thinning by approximately 10-12 days, tetrahydrocortisol-S at the same dose, does not.

We propose that specific angiostatic steroids which are not inflammatory may be used for long-term management of corneal neovascularization. Where an inflammatory infiltrate is present it may be managed separately by short-term pulses of anti-inflammatory steroids.

This conceptual and operational distinction between inflammation and angiogenesis may also be important in re-thinking our approach to the treatment of arthritis, another disease in which prolonged anti-inflammatory steroid therapy, especially when given systemically, has many side effects. Pure angiostatic steroids may also be useful in psoriasis, where therapy with anti-inflammatory steroids could then be limited to short duration.

CONCLUSION

These studies show that inflammation and neovascularization can be distinguished from each other at the cellular and biochemical levels. If hydrocortisone represents those corticosteroids that are mainly anti-inflammatatory, then tetrahydrocortisol-S represents a group of corticosteroids which are almost solely angiostatic. We propose that angiostatic therapy may be useful in many diseases such as corneal neovascularization, where abnormal capillary growth is a prominent feature. Thus, separate and specific therapies for neovascularization as well as for inflammation may improve current therapy and may possibly avoid the side-effects of prolonged treatment with anti-inflammatory steroids.

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Novel Neutrophil-Activating Peptides and Their Role in Inflammation

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INTRODUCTION

The family of peptides illustrated in Fig. 1 has attracted much attention following the discovery of the neutrophil-activating peptide, NAF/NAP-1 (Walz et al.1987; Yoshimura et al.1987b; Gregory et al.1988). Alignment according to their four cysteine residues differentiates the sequences into two groups. The two first cysteines are separated by one amino acid in the peptides of the upper group and are adjacent in those of the lower one. NAF/NAP-1 has considerable sequence homology with peptides from human platelet alpha-granules, platelet basic protein (PBP) (Holt et al.1986), connective tissue activating peptide III (CTAP-III) (Castor et al.1983; Niewiarowski et al.1980) and platelet factor 4 (PF-4) (Deuel et al.1977). NAP-2 is a neutrophil-activating truncation product of PBP (Walz and Baggiolini 1989). NAF/NAP-1, has structural and biological similarities with macrophage inflammatory protein 2 (MIP-2) (Wolpe et al.1989) which could represent the murine homologue of NAF/NAP-1. Identical relative positions of the cysteines and sequence homology is also found in two proteins obtained from human cell lines, gamma-IP-10 (Luster et al.1985) and gro or MGSA (Anisowicz et al.1987; Richmond et al.1988). Two murine macrophage inflammatory proteins (MIP-1-α and -β) (Sherry et al.1988), and a novel peptide that is chemotactic for human monocytes (MCP-1) (Furutani et al.1989; Robinson et al.1989) belong to the species with adjacent cysteines 1 and 2. The peptides of the upper group presumably have a two-loop configuration resulting from the linkage of the first to the third and the second to the fourth cysteine (Begg et al.1978) as shown for NAF/NAP-1 in Fig. 2. The disulfide bridges are essential for NAF/NAP-1 activity (Peveri et al.1988).

The cDNA coding for NAF/NAP-1 was cloned from an mRNA expressed by stimulated blood mononuclear cells (Schmid and Weissmann 1987) only a few months before NAF/NAP-1 was identified as a neutrophil-activating peptide. The cDNA indicates that NAF/NAP-1 is generated as a 99-amino acid precursor with a leader sequence of 22 amino acids. The largest mature form of NAF/NAP-1 consists of 77 residues. Other mature forms have been identified, and the major naturally occurring one consists of 72 amino acids (Lindley et al.1988).

```
NAP-2                                          A E L R C M C . . . .
CTAP-III                    N L A K G K E E S L D S D L Y A E L R C M C . . . .
PBP          S S T K G Q T K R N L A K G K E E S L D S D L Y A E L R C M C I K T T S - G I H P K N I Q S L E
PF-4                                        E A E E D G D L Q C L C V K T T S - Q V R P R H I T S L E
NAP-1                                          S A K E L R C Q C I K T Y S K P F H P K F I K E L R
GRO                                  R R A A G A S V A T E L R C Q C L Q T L Q - G I H P K N I Q S V N
IP10                                        V P L S R T V R C T C I S I N Q - P V N P R S L E K L E
mMIP-2                                      A V V A S E L R C Q C L K T L P - R V D F K N I Q S L S

MCP-1                                    Q P D A I N A P V T C - C Y N F T N R K I S V Q R L A S Y -
mMIP-1α                                  A P Y G A D T P T A C - C F S Y - S R K I P R Q F I V D Y -
mMIP-1β                                  A P M G S D P P T S C - C F S Y T S R Q L H R S F V M D Y -

-------------------------------------------------------------------------------------------------------

PBP/NAP-2    V I G K G T H C N Q V E V I A T L K D - G R K I C L D P D A P R I K K I V Q K K L A G D E S A D
PF-4         V I K A G P H C P T A Q L I A T L K N - G R K I C L D L Q A P L Y K K I I K K L L E S
NAP-1        V I E S G P H C A N T E I I V K L S D - G R E L C L D P K E N W V Q R V V E K F L K R A E N S
GRO          V K S P G P H C A Q T E V I A T L K N - G R K A C L N P A S P I V K K I I E K M L N S D K S N
IP10         I I P A S Q F C P R V E I I A T M K K K G E K R C L N P E S K A I K N L L K A V S K E M S K R S P
mMIP-2       V T P P G . . . . . .

MCP-1        R R I T S S K C P K E A V I F K T I V - A K E I C A D P K Q K W V Q D S M D H L D K Q T Q T P K T
mMIP-1α      - F E T S S L C S Q P G V I F L T K R - N R Q I C A D S K E T W V Q E Y I T D L E L N A
mMIP-1β      - Y E T S S L C S K P A V V F L T K R - G R Q I C A N P S E P W V T E Y M S D L E L N
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Fig. 1. Amino acid sequences of NAP-1 and related peptides. Sequences are aligned according to their four cysteine residues (bolded). For NAP-2 and CTAP-III, which represent truncations of PBP, only a few amino-terminal residues are indicated (top).

BIOLOGICAL PROPERTIES OF NAF/NAP-1

NAF/NAP-1 was characterized originally as a peptide inducing chemotaxis (Yoshimura et al.1987b; Schroder et al.1987) and enzyme release (Walz et al.1987). Its biological activity was therefore compared with that of known chemotactic agonists, formylmethionyl peptides (Schiffmann et al.1975), the anaphylatoxin C5a (Fernandez et al.1978), platelet-activating factor (PAF) (Lee and Snyder, 1985) and leukotriene B_4 (LTB_4) (Ford Hutchinson et al.1980).

Following chemotactic stimulation, neutrophils marginate, adhere to the endothelial cells and migrate into the extravascular space. Single functional responses can be monitored in vitro. Our studies show that NAF/NAP-1 acts in a similar way as the classical chemotactic peptides, C5a and f-Met-Leu-Phe, and induces activation of the motile apparatus, directional migration, expression of adhesion molecules, release of enzymes and other macromolecules from different storage organelles, and production of superoxide (Peveri et al.1988; Thelen et al.1988). The transduction of the NAF/NAP-1 signal depends on a rapid, transient rise in cytosolic free calcium. All NAF/NAP-1 mediated responses, including the calcium changes, are abrogated when the cells are pretreated with *B. pertussis* toxin, indicating that signal transduction depends on a GTP-binding protein. The respiratory burst induced by NAF/NAP-1 is inhibited by staurosporine, implying a role for protein kinase C, and by wortmannin, a fungal metabolite that

prevents the respiratory burst and exocytosis by blockade of what appears to be a calcium-independent transduction event (Thelen et al.1988; Dewald et al.1988). Desensitization experiments with C5a, f-Met-Leu-Phe, PAF and LTB_4 suggest that NAF/NAP-1 acts via a selective receptor (Schroder et al.1987; Peveri et al.1988). It was reported that neutrophils have on average 20,000 NAF/NAP-1 receptors with a K_D of $8x10^{-10}$ M (Samanta et al.1989). Our own studies indicate the existence of about 5,000 high-affinity (K_D = $1-3x10^{-11}$ M) and 30-40,000 low-affinity (K_D = $6-8x10^{-10}$ M) binding sites per cell (Besemer et al., J. Biol. Chem., in press).

NAF/NAP-1 appears to be rather selective for neutrophils: it does not stimulate mononuclear phagocytes or platelets (Thelen et al.1988), and has only borderline effects on eosinophils (Schroder et al.1987) and basophils from normal and atopic individuals (Dahinden et al., Int. Archs Allergy appl. Immun., in press). NAF/NAP-1 was reported to be chemotactic for lymphocytes (Larsen et al.1989). Human blood lymphocytes, however, show no cytosolic free calcium changes upon stimulation with NAF/NAP-1 (Thelen et al.1988). Blood lymphocytes and monocytes separated by elutriation specifically bind low amounts of ^{125}I-labelled NAF/NAP-1. Although essentially free of neutrophils, these cell preparations are not completely pure, and it cannot be excluded that the binding is due to contaminants bearing the receptor (Besemer et al., J. Biol. Chem., in press).

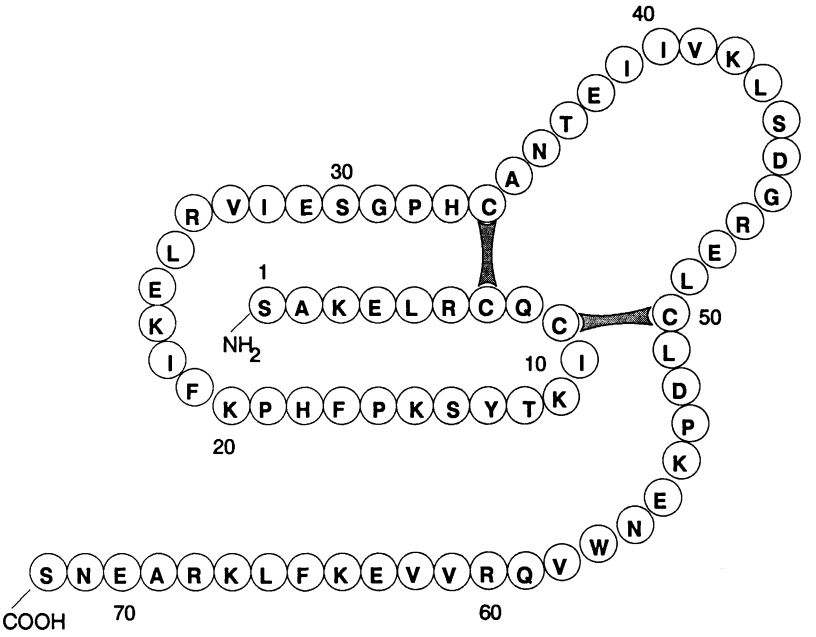

Fig. 2. The structure of NAF/NAP-1.

EFFECTS IN VIVO

Neutrophil accumulation in the tissues is observed in a wide variety of pathological conditions and is generally considered as a sign of inflammation. Neutrophils are known to collect in large numbers at sites of infection to phagocytose and kill the invading microorganisms. In other conditions, such as ischaemia, trauma and cancer, they may be recruited as scavengers of damaged tissue or unwanted extracellular materials. Migrating and phagocytosing neutrophils release oxygen radicals, bioactive lipids and granule enzymes. Several of these products induce inflammation, and in fact neutrophil invasion is typically associated with vasodilatation, plasma leakage and pain.

The in vivo effects of NAF/NAP-1 were studied in different animal species. Injection into the skin of rabbits induces plasma exudation and a massive neutrophil accumulation. Infiltration is long-lasting and apparently restricted to neutrophils (Colditz et al.1989). Less severe and more transient lesions are obtained with f-Met-Leu-Phe or E.coli lipopolysaccharide (LPS). Massive neutrophil infiltration is also observed upon intradermal injection of NAF/NAP-1 in rats, mice, guinea-pigs and dogs (Zwahlen et al., submitted for publication). The long duration of action is probably due to the peptides' remarkable resistance to enzymatic cleavage and to denaturation (Peveri et al.1988).

NAF/NAP-1 IS PRODUCED BY MANY CELLS

In the light of the classical theory of inflammation, which describes the sequential migration of neutrophils and mononuclear phagocytes, it was surprising to realize that monocytes produce large amounts of a neutrophil-attracting peptide. The subsequent finding that a wide variety of cells are able to produce NAF/NAP-1 upon stimulation was therefore critical for the understanding of the potential role of the new mediator in pathology. High levels of NAF/NAP-1 mRNA are expressed in fibroblasts, epithelial cells, alveolar macrophages, endothelial cells and hepatocytes in response to IL-1-α, IL-1-β and TNF-α (Strieter et al.1988; Kunkel et al.1989; Dixit et al.1989;). In some cells, like monocytes, macrophages and endothelial cells, expression can also be induced with LPS (Peveri et al.1988; Strieter et al.1988; Dixit et al.1989; Schroder and Christophers 1989). On the other hand, NAF/NAP-1 is not generated when interleukin-6 (Strieter et al.1988) interleukin 2 and type I or type II interferons are used as stimuli (Peveri et al., unpublished).

PATHOPHYSIOLOGY OF NAF/NAP-1

As a product of stimulated tissue cells NAF/NAP-1 may represent a ubiquitous mediator of inflammation arising upon disturbance of tissue homeostasis. In this connection it is particularly interesting that IL-1 and TNF, two major inflammatory cytokines, induce NAF/NAP-1 production in all tissue cells studied so far. There are already clear indications for a potential role of NAF/NAP-1 in disease. A neutrophil-activating peptide differing from C5a has been demonstrated in psoriatic skin lesions (Schroder and Christophers 1986) where

NAF/NAP-1 was subsequently identified (Gregory et al.1988). IL-1, which is also present in high amounts, could function as inducer, and locally-generated NAF/NAP-1 is likely to be the cause of the neutrophil-rich micro-abscesses which are characteristic for this disease. NAF/NAP-1 may also be involved in arthritis, since it has been shown that IL-1-stimulated synovial cells produce a neutrophil chemotactic peptide (Watson et al.1988). The inflamed synovium may thus be involved in recruiting neutrophils which are considered as a rich source of cartilage-degrading enzymes (Baggiolini et al.1979). Neutrophil infiltration is of major importance in lung diseases such as idiopathic pulmonary fibrosis (IPF) and asbestosis (Hunninghake et al.1981; Rola Pleszczynski et al.1984). A similar, but more dramatic inflammatory process is observed in the adult respiratory distress syndrome (ARDS), where extensive tissue damage is largely attributed to neutrophil elastase (McGuire et al.1982; Idell et al.1985; Parsons et al.1985).

NAF/NAP-1, however, could also be involved in processes that are not overtly inflammatory. It may for instance cause neutrophil infiltration in myocardial infarction areas and thus contribute to the reperfusion damage that is associated with the release of inflammatory cell products (Romson et al.1983). Furthermore, under physiological conditions, NAF/NAP-1 may function as an attractant for the steady emigration of neutrophils from the blood to the tissue compartment that drives turnover. High numbers of neutrophils are eliminated across the epithelial barrier of the gastrointestinal tract (Teir et al.1963). NAF/NAP-1 produced by the gastrointestinal epithelium or by the endothelial cells of intestinal microvessels could be the mediator of neutrophil emigration. The venular endothelium may turn out to play a key role in NAF/NAP-1 dependent neutrophil diapedesis, since IL-1, TNF and LPS, which stimulate endothelial cells to produce NAF/NAP-1, also induce the expression of adherence proteins (Bevilacqua et al.1985; Pohlman et al.1986) that promote neutrophil interaction.

DISCOVERY OF NAP-2 - A GROWING NAP FAMILY?

We have recently identified NAP-2 in cultures of unfractionated human blood mononuclear cells stimulated with LPS or lectins (Walz and Baggiolini 1989). As shown in Fig. 1, NAP-2 consists of 70 amino acids and has, therefore, about the same size as NAF/NAP-1. NAP-2 corresponds to part of the sequence of the platelet alpha-granule proteins, PBP (Walz and Baggiolini 1989) and CTAP-III (Castor et al.1983), and is partially homologous to platelet factor 4 (PF-4) (Deuel et al.1977). It is not found in platelets (or other component of the mononuclear cell cultures), and appears to be formed following release (Walz, unpublished observation). NAP-2 exhibits the typical properties of chemotactic receptor agonists and induces cytosolic free calcium changes, chemotaxis and exocytosis in the same molar range as NAF/NAP-1, while PBP, CTAP-III and PF-4 have little if any activity at 100 to 10,000 times higher concentrations (Walz et al., submitted for publication). On the basis of the biological information presently available, we expect NAP-2 to be as effective as NAF/NAP-1 and C5a in the recruitment of neutrophils. It is likely to have a role in thrombosis where it could attract neutrophils involved in the recanalization of obstructed vessels.

CONCLUSIONS

With the discovery of NAF/NAP-1, a new group of tissue-derived mediators of inflammation has been highlighted. The information on MIP-1 and MIP-2, and the recent data on NAP-2 and a new peptide which is chemotactic for human monocytes (See Fig. 1) indicate that a whole family of structurally and biologically homologous mediators may be involved in the regulation of inflammatory reactions. The emerging understanding of the potential role of these mediators constitutes a new basis for the design of antiinflammatory agents.

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Neuromediators of Immunity and Inflammation

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INTRODUCTION

The nature and extent of the alterations in immunity resulting from neural lesions, and of the neural adaptation to immune reactions and inflammation suggested initially that the two systems cooperate in host defense (Ader 1981; Goetzl & Spector 1989). The elucidation of patterns of innervation of organs of the immune system, effects of immune cytokines on neuroendocrine functions, and mechanisms of response of immune cells to neuromediators has provided models and techniques for molecular and cellular studies of neuroendocrine interactions (Goetzl and Spector 1989). The findings of similar membrane protein antigens, enzymatic pathways, mediators, and receptors in some neuroendocrine and immune cells supported further the evolving concept of a multisystem network. A conceptual integration of currently available data suggests that some distinct phases of immunity and inflammation are particularly susceptible to neural influences. The potency and specificity of neuropeptide mediators in chronic immunological reactions, involving predominantly macrophages and lymphocytes, will be described as the background for discussion of recent studies of immune variants of some neuropeptides and the corresponding lymphocyte receptors.

Immediate Hypersensitivity and Acute Inflammation

Tissue responses resembling allergic and inflammatory reactions are evoked rapidly by the introduction of endogenous neuromediators, through excitation of local nerves, or of exogenous synthetic neuromediators into skin, lungs, or joints (Levine et al. 1987). The detection of nanomolar concentrations of several neuropeptides in nasal fluids of allergic patients reacting to pollen antigen and in fluids of acute inflammatory reactions confirmed the involvement of peripheral neurons and stimulated further investigations of the nature of neuropeptide effects on mast cells, basophils, and PMN leukoctyes *in vitro*. SP and a wide variety of other neuropeptides elicit release of preformed and newly-generated mediators from mast cells of some species. The mechanism of activation of mast cells by neuropeptides is specific for a distinct domain of each peptide, IgE-independent and different in terms of transductional biochemistry than the IgE-directed stimuli (Foreman et al. 1983). Concentrations of 1-10 micromolar are required for maximal release of mast cell mediators by SP and other neuropeptides, and basophils are not susceptible to activation by most neuropeptides (Fig. 1).

The low apparent potency of neuropeptide stimuli of mast cell functions may be attributable to the peptidases and proteases released by challenged mast cells, that selectively cleave SP, VIP, CGRP, and other neuropeptide mediators of immediate hypersensitivity. Only SOM exhibits

full inhibitory activity for IgE-dependent activation of basophils at picomolar to nanomolar concentrations, and of some mast cells at 0.1-100 nanomolar (Goetzl and Payan 1984). Nanomolar concentrations of nerve growth factor (NGF) enhance the release of histamine from sensitized ratperitoneal mast cells stimulated by antigen or other secretagogues, as a result of apparent increases in expression of mast cell receptors (Tomioka et al. 1988).

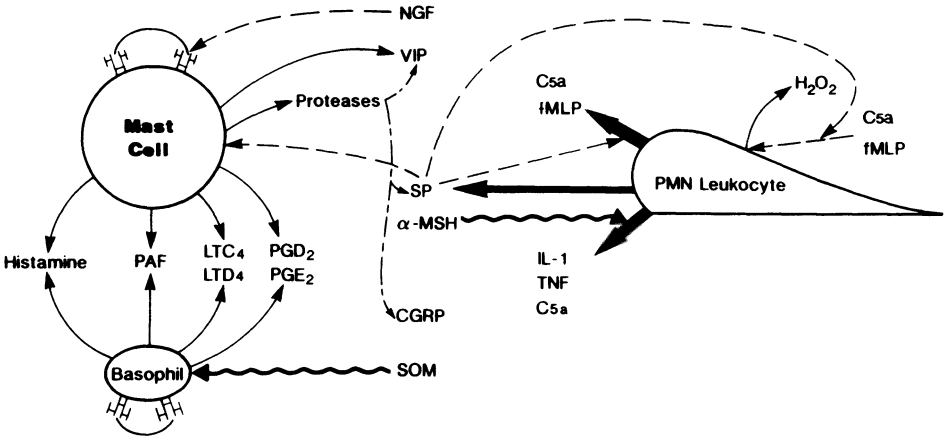

Fig. 1. Neuropeptide mediation of immediate hypersensitivity and acute inflammation. ➡ = chemotaxis; ⟶ generation or release; ⇢ = stimulation or enhancement; ⇢ = peptidolysis; ⇝ = inhibition; HH = IgE.

As for mast cells, PMN leukocytes are only chemotactically and biochemically responsive to 0.1-10 micromolar SP and some other neuropeptides (Serra et al. 1988) (Fig. 1). The principal effect of SP, as a representative neuropeptide, is to modulate the responses of PMN leukocytes to more potent stimuli, rather than to exert direct effects. At concentrations as low as 0.1 nanomolar, SP enhances both the chemotactic and H_2O_2-generating responses of neutrophils to fMLP and C5a by 50-100% (Perianin et al. 1989). The neuropeptide alpha-MSH, when administered intraperitoneally to mice in amounts as low as 0.1 nanomole, suppresses neutrophil influx into subcutaneously implanted sponges containing IL-1, TNF-alpha, or C5a (Mason 1988). Again, alpha-MSH lacks any direct inflammatory activity, but acts solely to modulate the responses to other mediators (Fig. 1).

Chronic Inflammation and Lymphocyte Functions

Neuropeptides are potent mediators of many of the activities of macrophages and numerous types of lymphocytes (Fig. 2). SP is one of the most selectively active chemotactic factors for monocytes and macrophages, as picomolar concentrations elicit maximal responses in vitro in contrast to the micromolar concentrations required to stimulate neutrophil chemotaxis and activation (Ruff et al. 1985). Other neuropeptides alter macrophage biochemical pathways, without or with the

involvement of lymphocytes. Picomolar-nanomolar concentrations of beta-endorphin suppress the production of O_2^- and H_2O_2 by human monocytes incubated with phorbol myristate acetate or opsonized zymosan, whereas growth hormone enhances porcine macrophage oxidative metabolic responses in vitro and in vivo activates the responses of macrophages from hypophysectomized rats to a level similar to that achieved by IFN-gamma (Peterson et al. 1986; Edwards et al. 1988). The generation of H_2O_2 by human macrophages and their function as antigen-presenting cells are inhibited by preincubation with picomolar concentrations of CGRP and calcitonin (Nong et al. 1988). In vivo, lymphocytes may be the primary target of some neuropeptide effects on macrophage functions. Inhibition of prolactin secretion in mice by bromocryptine, prevents helper T-cell induction of macrophage cytotoxicity and concomitantly suppresses lymphocyte proliferation and production of interferon (IFN)-gamma, with reversal of all effects by exogenous prolactin (Bernton et al. 1988). In some instances, the reduction in generation of IFN-gamma results from cellular cooperation, as when beta-endorphin evokes macrophage secretion of PGE2 and oxygen-metabolites that suppress T-cell production of IFN-gamma.

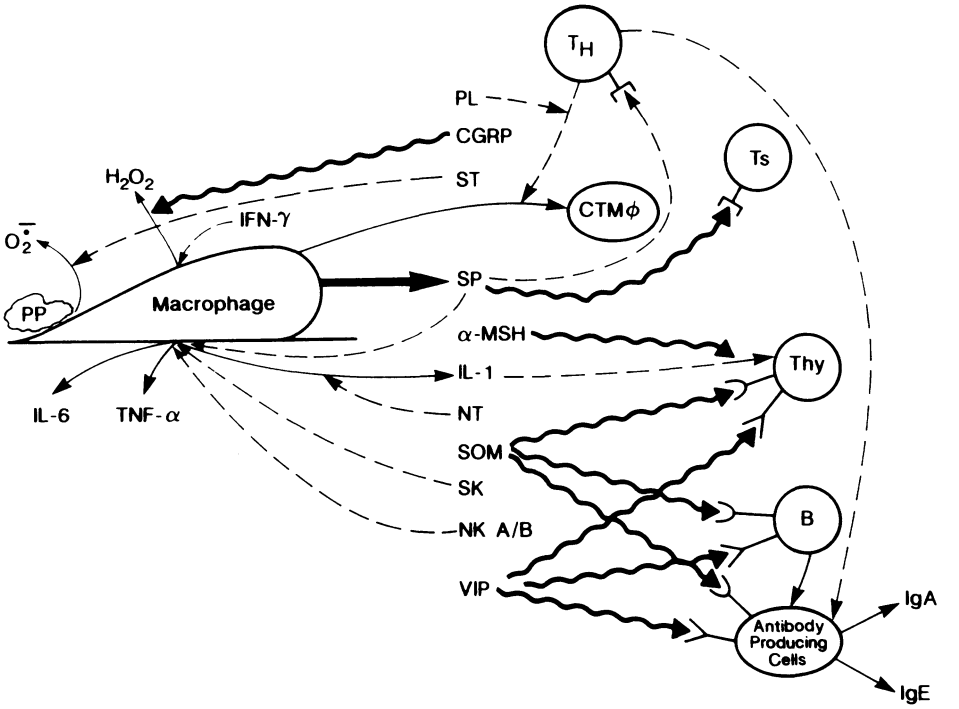

Fig. 2. Neuropeptide mediation of chronic inflammation and lymphocyte functions. pp = phagocytosable particle; CTMØ = cytotoxic macrophage; ⊣, ⟨, ⋎ , = neuropeptide receptors; all other symbols are as in Fig. 1.

IL-1 and neuropeptides interact functionally in several immunological reactions. The generation of IL-1, TNF-alpha, and IL-6 by human blood monocytes is induced by SP and SK, with maximal effects at concentrations of 10 nanomolar or greater for either neuropeptide (Lotz et al. 1988). At similar concentrations, SP and neurokinins A and B evoke the production of IL-1 by cultured mouse macrophages (Kimball et al. 1988). Neuropeptides that lack a primary effect on IL-1 synthesis

may substantially alter the generation and release processes, as for the enhancing activity of neurotensin (Lemaire 1988). At another level of regulation, 10-100 nanomolar alpha-MSH selectively blocks the stimulation of thymocytes and fibroblasts by IL-1 in vitro and the inflammatory effects of IL-1 and TNF in mice (Mason and Van Epps 1989; Cannon et al. 1986).

An array of neuropeptides have major effects on the differentiation, tissue homing and recycling, and immunological functions of lymphocytes (Goetzl and Spector 1989). The most recently demonstrated effects of neuropeptides and other neuromediators include development and functional priming of lymphocyte subsets, substitution for one type of regulatory T-cell, and unmasking of autoimmune reactivity. Low concentrations of beta-endorphin permit antigen-directed production of anti-herpes antibodies by human blood mononuclear leukocytes from some subjects, where antigen alone has no effect (Williamson et al. 1988). Beta-endorphin and Met-enkephalin facilitate the generation of cytotoxic T-cells from murine spleen cells in one-way mixed lymphocyte reactions. Arginine vasopressin (AVP) replaces helper T-cells in the generation of IFN-gamma by Lyt-2^+ mouse splenic lymphocytes (Torres and Johnson 1988). AVP is recognized through an AVP receptor on mouse lymphocytes, that exhibits a novel profile of affinities for defined antagonists. Estrogens and other potent steroid hormones mediate deletion of an intermediate developmental stage of T-cell $receptor^-$, $CD3^-$, IL-2 $receptor^-$ lymphocytes acutely, and then both earlier and later forms of regulatory T-cells after long-term treatment of mice (Screpanti et al. 1989). The production of antibodies to autologous erythrocytes by mouse $CD5^+$ B-cells is augmented by estrogens, as a result of more efficient production by each responding B-cell (Ahmed et al. 1989). The synthesis of immunoglobulins by mouse splenic and mesenteric lymph node lymphocytes increases in parallel with proliferative responses to mitogens after an intravenous infusion of SP, with the greatest effect on IgA as expected from the results of in vitro studies (Scicchitano et al. 1988). Thus, the stimulatory effects of SP and the inhibitory effects of VIP and SOM on diverse lymphocyte functions are observed in vivo, just as they were originally described in vitro. The finding that neuropeptides have their most potent influence on mononuclear leukocytic activities suggested the importance of elucidating the properties of lymphocyte-receptors for neuropeptides and of defining any variants of neuropeptides recognized more efficiently by immune receptors, than the receptors of neuroendocrine cells.

Immunological Generation of Neuroendocrine Mediators

The SP, CGRP, and SOM recovered from fluids of patients with allergic and delayed-type hypersensitivity reactions were largely the authentic neuropeptides, by chromatographic analyses of immunoreactivities. Lymphocytes generate ACTH, beta-endorphin, thyrotropin, and corticotropin-releasing hormone, and eosinophils produce VIP and SP indistinguishable from the corresponding neuroendocrine peptides (Goetzl and Spector 1989). In other instances, studies in vitro have elucidated the nature of variant neuropeptides from non-neural sources. The VIP-related peptides from mast cells and basophils (Goetzl et al. 1988), SOM-like peptides from monocytes and mast cells, and enkephalin-like peptides from lymphocytes, macrophages, and mast cells (Martin et al. 1987) differ structurally from previously defined neuropeptides.

The immunological equivalents of SOM_{28} and Met-enkephalin appear larger, but the amino acid sequences have not been completed. VIPs from RBL cells and some cultured lines of murine mast cells consist of VIP_{10-28} free acid and a mixture of amino-terminally extended "big"

VIPs (Goetzl et al. 1988), which appear to be derived from a novel preproVIP encoded by an alternatively-spliced mRNA. The polymerase chain reaction (PCR) technique has been applied to amplify the VIP message in poly(A) mRNA from RBL cells, using a set of primers derived from the previously defined sequence of neural preproVIP. Agarose gel electrophoresis of PCR-amplified DNA fragments from RBL cDNA reveals differences in size from those obtained with cDNA of control neural cell lines. This additional evidence for alternative splicing of VIP mRNA in RBL cells remains to be supported by determination of the sequences of the DNA fragments. The isolation of distinctive fragments of VIP_{1-28} from suspensions of lymphocytes, mast cells, and other leukocytes after incubation in vitro confirmed the involvement of post-translational peptidolysis (Goetzl et al. 1989a) in the structural diversity of some VIPs in immunological responses. The recent findings that genetic and proteolytic variants bind differently to lymphocyte than neural receptors suggest that each member of the VIP family may have unique immunoregulatory roles.

More detailed studies have partially delineated the genetic messages for growth hormone and ACTH in human and rodent mononuclear leukocytes. Maximal levels of mRNA for growth hormone in the cytoplasm of blood mononuclear leukocytes are observed after 4 hr of incubation, and are polyadenylated with an Mr of 1.0 kb. The bioactivity, molecular weight, and antigenicity of mononuclear leukocyte-derived growth hormone indicate identity with pituitary growth hormone. Messenger RNA specific for pro-opiomelanocortin (POMC) is observed in normal human blood mononuclear leukocytes, and leukocytes of lymphoid and myeloid malignancies. This message is translated into at least three forms of immunoreactive POMC/ACTH, exhibiting a chromatographic pattern similar to that of pituitary POMC/ACTH. Immune cells that generate a neuropeptide in vitro may be the principal non-neural source in complex tissue reactions. The SP generated in granulomas of murine Schistosomiasis is largely attributable to the eosinophils, as demonstrated by the histochemical localization of SP immunoreactivity and mRNA (Weinstock et al. 1988).

Immune Cell-Specific Receptors for Neuropeptides

The neuroendocrine modulation of immune functions necessitates the expression of specific receptors for neuroendocrine peptides on cells of the immune system. Opioid peptide receptors on human monocytes, granulocytes and lymphocytes, SP and human growth hormone receptors on cultured human IM-9 B-lymphoblasts, prolactin receptors on NK cells, nerve growth factor receptors on rat mononuclear spleen cells, and ACTH receptors on human mononuclear leukocytes have been characterized in terms of the affinity and specificity of binding of the respective neuroendocrine peptide (Goetzl and Spector 1989). Our finding that cultured human T- and B-lymphocytes both express distinct subsets of receptors for SOM and VIP, with affinities and specificities different from those of the corresponding neural receptors, supports the possibility of immunomodulation by the neuroendocrine system.

The binding of [^{125}I-Tyr^{10}] VIP and [^{125}I-Tyr^{11}] SOM to T- and B-lymphocytes of cultured lines was quantified at room temperature, in the presence of the peptidase inhibitors DL-thiorphan and phenylmethylsulfonyl fluoride, and bound peptide was separated from unbound by centrifugation of the lymphocytes through a cushion of n-butyl phthalate and dinonyl phthalate (7:2, v:v).

The PEER, Jurkat, and Molt-4 lines of cultured T-cells show high levels of specific binding of SOM, and the latter two lines also show evidence

for VIP binding. In contrast, a greater variability is observed in studies of B-cell lines. The MAK EBV-infected human B-cell line, the U266 IgE-producing human myeloma line, and the J558 IgA-producing murine myeloma line exhibit high levels of specific binding of SOM. These lines also demonstrate specific VIP binding, as did the DAKIKI IgA-producing human lymphoblasts and the U937 human monocyte line, both of which failed to bind SOM. The 2F.11.15 and S49 lines of B-cells did not bind either SOM or VIP.

Two representative human cell lines, the IL-2-secreting Jurkat leukemic T-cells and the IgE-secreting U266 myeloma B-cells, were studied by flow cytometry for binding of fluoresceinated VIP and SOM. The majority of the cells of both lines recognize SOM and VIP. The presence of an excess of unlabeled SOM or VIP, respectively, prevents more than 50% and 40% of the corresponding fluorescent labeling of the cells. The specific binding of [^{125}I-Tyr^{11}]SOM or [^{125}I-Tyr^{10}]VIP to Jurkat and U266 cells at 22^{o}C are time-dependent and can be reversed with the addition of excess unlabeled SOM and VIP, respectively. Computer-based Scatchard analyses of the competitive inhibition of binding of [^{125}I-Tyr^{11}]SOM to Jurkat and U266 cells, by different concentrations of unlabeled SOM, revealed 10^2 and 10^3 high-affinity sites with Kd values of 3 pM and 5 pM, respectively (Sreedharan et al. 1989a). A large number of low-affinity sites for SOM also were identified on both cell lines with Kd values of 66 nM and 100 nM, respectively. The results of studies of the competitive inhibition of [^{125}I-Tyr^{10}]VIP binding to Jurkat and U266 cells by different concentrations of unlabeled VIP indicate the presence of 10^4 sites with mean Kd values of 5.2 nM and 7.6 nM, respectively (Finch et al. 1989). Neither U266 nor Jurkat cells bind SP.

Analogs of SOM, including the naturally occurring twenty-eight amino acid SOM 28, mono-iodinated [Tyr^{11}] SOM and [D-Trp^{8}, D-Cys^{14}]SOM also inhibited the binding of [^{125}I-Tyr^{11}]SOM to both cell lines (Sreedharan et al. 1989a). Similarly the principal mast cell-derived variant VIP$_{10-28}$, as well as the L-8-K and the [Ac-Tyr^{1}, D-Phe^{2}]GRP$_{1-29}$ amide peptide analogs of VIP displace [^{125}I-Tyr^{10}]VIP from Jurkat and U266 cells (Finch et al. 1989). VIP$_{10-28}$ exhibits an affinity 1/10 that of VIP for lymphocyte receptors, whereas it has only 1/10,000 the affinity of VIP for neural receptors of rodent tissues. SP, CGRP, and VIP failed to competitively inhibit the binding of [^{125}I-Tyr^{11}]SOM to Jurkat and U266 cells, respectively, whereas SP and SOM have no effect on binding of [^{125}I-Tyr^{10}]VIP to either cell line. Biotinylated SOM and VIP analogs, which will be used in the purification of their respective receptors, have been prepared and preliminary studies have indicated that biotinylated SOM 28 can displace [^{125}I-Tyr^{11}]SOM from U266 cells.

The interaction of VIP with its receptor is known to elevate cAMP levels by stimulating adenylate cyclase in plasma membrane preparations of human blood mixed T-cells and Molt-4 T-lymphoblasts (O'Dorisio and Tseng 1989). In the same population of T-cells, VIP also activates a cAMP-dependent protein kinase that phosphorylates a 41 kD membrane protein. While it has been demonstrated that VIP alters significantly the synthesis of IgA and IgM, but not IgG, by mixed lymphocytes from murine spleen and Peyer's patches, there is no additional information about the functions of B-lymphocyte receptors for VIP or the effects of mast cell-derived variants of VIP on T- and B-lymphocytes. SOM and VIP receptors expressed on the same cell may exhibit functional interactions, as in the pituitary line of cultured GH_4C_1 cells. SOM consistently and rapidly inhibits the stimulation of cyclic AMP by VIP, and this inhibition is blocked in some types of cells by pertussis toxin, indicating a functional role for G_i proteins.

Conclusions

The capacity of immune cells to recognize, through specific high-affinity receptors, and respond to neuromediators provides a molecular basis for the effects of the nervous system in immunity and inflammation. The development of immune cell-specific antagonists of neuropeptides will permit further analyses of their role as mediators and modulators in animal models and human diseases.

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Cytokine Induced Changes in the Endothelial Cell Surface Membrane: Significance for Lymphocyte Traffic in Inflammation

D.O. Haskard, M. Thornhill, U. Kyan-Aung, G. Kingsley, C. Pitzalis, and G.S. Panayi

INTRODUCTION

The emerging realization that vascular endothelial cells (EC) both respond to and secrete pro-inflammatory cytokines has focused attention on the active role that EC may play in the control of inflammatory responses (Cavender et al 1987a, Pober 1988). This article describes recent observations on lymphocyte-EC interactions and discusses their significance in the context of lymphocyte traffic into chronic inflammatory lesions.

THE CONTROL OF LYMPHOCYTE-ENDOTHELIAL CELL ADHESION BY CYTOKINES

Using an *in vitro* assay that measures the adhesion of 51-chromium labelled lymphocytes to human umbilical vein EC monolayers in microtiter wells, it was demonstrated that EC adhesiveness for lymphocytes could be increased by preincubation of EC for 4-6 hr with the cytokines interferon gamma (IFN) (Yu et al 1985), interleukin 1 (IL1) (Cavender et al 1986), or tumour necrosis factor alpha (TNF) (Cavender et al 1987a). These observations suggest that the release of cytokines in inflamed tissue can result in a similar effect on endothelium *in vivo*, leading to an increased adhesion of circulating lymphocytes to the vessel wall. Lymphocytes immobilized in this way would then emigrate along chemotactic gradients into the inflammatory lesion, thereby adding to the pool of lymphocytes capable of taking part in a localized immune response. This would constitute a positive feedback leading to an increase in the magnitude and breadth of the immune mediated component of an inflammatory reaction.

The effect of phorbol esters

Stimulation of lymphocytes with phorbol esters lead to an enhanced adhesiveness of lymphocytes for EC that was detectable after 1 min and maximal after 15 min and which was additive to the enhanced adhesion due to cytokine stimulation of EC (Haskard et al 1986a). Lymphocytes therefore have the capacity, under appropriate stimulation, to alter their adhesion rapidly to EC. It is possible that this mechanism might be involved in the process of adhesion and and subsequent detachment from the EC surface during lymphocyte migration through the endothelium into the perivascular space.

The preferential adhesion to EC of "memory" T lymphocytes

Analysis of several non-lymphoid tissues including rheumatoid synovial tissue (Duke et al 1986) and synovial fluid (Pitzalis et al 1987) has shown that most lymphocytes carry the "memory" cell markers CD29 and UCHL1 and that very few are of the "naive" cell (CD45R+) phenotype. When the adhesion to EC of CD29+, UCHL1+ and CD45R+ T cells was compared, CD29+, UCHL1+ T cells were found to be more adhesive than CD45R+ T cells to both unstimulated and IL1 stimulated EC monolayers (Pitzalis et al 1988), suggesting that the preferential acccumulation of "memory" lymphocytes in inflammatory tissues might be at least partially attributable to a relatively greater adhesiveness of this subset to EC.

THE MOLECULAR BASIS OF LYMPHOCYTE ADHESION TO ENDOTHELIAL CELLS

The enhanced adhesiveness of cytokine stimulated EC for lymphocytes is likely to be due to the induction or upregulation of adhesion receptors on the EC surface membrane as (1) enhanced adhesion of lymphocytes is prevented by performing cytokine stimulation in the presence of RNA or protein synthesis inhibitors (Cavender et al 1987b), and (2) enhanced EC adhesiveness is stable to light fixation of EC with 2% paraformaldehyde-lysine-periodate (Cavender et al 1986). Experiments conducted to determine the molecules involved in lymphocyte adhesion to EC indicate that multiple receptor ligand interactions may be involved, and that the various molecular interactions may summate in determining the total strength of lymphocyte-EC adhesion.

Role of Lymphocyte Function Associated Molecule-1 (LFA-1)

The involvement of LFA-1 in lymphocyte-EC adhesion is indicated by the inhibitory effect of anti-LFA-1 monoclonal antibodies (mAb) on unstimulated and phorbol ester stimulated lymphocyte adhesion to unstimulated EC (Haskard et al 1986b).

Role of Inter-cellular adhesion molecule-1 (ICAM-1)

ICAM-1 is a single chain glycoprotein which is present on a number of cell types including EC (Dustin et al 1986), and which is known to act as a receptor for LFA-1 (Marlin and Springer 1987). The surface expression of ICAM-1 on EC can be upregulated by IL-1, TNF or IFN (Pober et al 1986), suggesting that one of the mechanisms by which these factors enhance lymphocyte-EC adhesion is by increasing the capacity of lymphocytes to adhere to EC by a LFA-1-ICAM-1 dependent mechanism. ICAM-1 is not, however, the only receptor for LFA-1 on EC, as judged by the failure of anti-ICAM-1 mAb to inhibit lymphocyte-EC adhesion as effectively as anti-LFA-1 mAb (Dustin and Springer 1988), and by the recent cloning of another receptor for LFA-1, ICAM-2. In contrast to ICAM-1, ICAM-2 mRNA does not appear to be inducible by cytokines (Staunton et al 1989).

Evidence for novel cytokine inducible adhesion receptors

Although anti-LFA-1 mAb effectively inhibited unstimulated and phorbol ester stimulated lymhocyte-EC adhesion, they did not inhibit the enhanced adhesion induced by IL1 or TNF (Haskard et al 1986b). Furthermore, lymphoblasts derived from a child with CD11/CD18 deficiency showed normal enhanced adhesion to IL1 or TNF stimulated EC, suggesting the existence of other cytokine inducible adhesion receptors on EC besides ICAM-1 (Haskard et al 1989). These receptors, whose identity is at present unknown, must be at least partially different from the cytokine inducible EC adhesion receptors for neutrophils as IFN does not enhance EC adhesiveness for neutrophils and the IL1 or TNF enhanced EC adhesiveness for lymphocytes is delayed compared to that for neutrophils (Thornhill et al 1989). Furthermore, ELAM-1, which is an IL1 or TNF inducible receptor for neutrophils, is not thought to be involved in lymphocyte adhesion (Bevilacqua et al 1987, Bevilacqua and Gimbrone 1987).

The preferential adhesion of "memory" lymphocytes

The process of activation of lymphocytes results not only in the transition of phenotype to CD29+, UCHL1+, but also in the increased surface expression of other surface adhesion molecules including LFA-1, CD2, LFA-3 (Sanders et al 1988). As alterations in LFA-1, CD2 or LFA-3 are unlikely to account for the difference in adhesion to EC between CD45R+ and CD29+/UCHL1+ T cells (Haskard et al 1986b, Pitzalis et al 1988), it is possible that the loss of CD45R+ and acquisition of CD29 and UCHL1 is associated with the induction or upregulation of other adhesion molecules that mediate attachment to EC. As IL1 and TNF do not enhance the adhesion of "memory" T cells selectively (Pitzalis et al 1988), the EC receptors involved in the selective adhesion of "memory" cells are likely to be distinct from those receptors responsible for cytokine enhanced adhesion.

CONCLUSION

Increased understanding of the action of cytokines in promoting lymphocyte adhesion to endothelium and of the adhesion molecules involved is not only of great scientific interest but may lead the way to the development of anti-inflammatory agents that act by the inhibition of lymphocyte migration into inflammatory tissues.

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III Effector Phases

Autoimmunity

Paul Ehrlich and the Foundations of Autoimmunity

R.S. Schwartz

An invitation to convene a symposium on autoimmunity in Berlin warrants a celebration of one of the principal architects of immunology, Paul Ehrlich. The occasion is appropriate, for it is now almost 100 years since Ehrlich was invited by Robert Koch to join him in the new Institute for Infectious Diseases in Berlin, where Ehrlich first applied his talents to problems of immunology.

Paul Ehrlich was born in Silesia on March 14, 1854. He received his M.D.from the University of Leipzig in 1878, at the age of 24. For his remarkable medical thesis, *Contributions to the Theory and Practice of Histological Staining*, Ehrlich applied his knowledge of organic dyes to the development of technics for the differential staining of blood cells, and thereby quickly discovered the neutrophil, the eosinophil, the basophil, the mast cell and the reticulocyte. The foundations of modern hematology rest on this work, carried out while Ehrlich was still a medical student. It contains the seeds of ideas that dominated the rest of his scientific career. After graduating from medical school, Ehrlich joined the clinical staff of the Charité Hospital in Berlin, where he continued to pursue his interest in organic dyes, this time to stain bacteria. Shortly after Koch's announcement of the discovery of the tubercle bacillus, Ehrlich invented the "acid fast" stain for demonstrating the presence of Koch's bacillus, even when present in small numbers or when contaminated by the presence of other microorganisms.

Ehrlich arrived at a time of extraordinary ferment. Emil von Behring, another of Koch's brilliant recruits, had joined the the Institute for Infectious Diseases in 1889, and within a year he and Shibasaburo Kitasato announced their historic discovery of antibodies against the toxins of diptheria and tetanus. The following year, 1890, von Behring and Kitasato showed that passive immunization with antitoxins could protect animals against both organisms. On the Christmas eve of 1891 the first patient, a child with diphtheria, was effectively treated with von Behring's "antitoxin"; in 1892 the first diphtheria antitoxin preparation was released to the medical profession. Progress in the conquest of diphtheria had been phenomenal: *1883*, Klebs finds diptheria bacillus in stained smears of diphtheritic membranes; *1884*, Löffler isolates *C. diphtheriae* from patients with diphtheria, induces

a fatal diphtheritic disorder in guinea pigs with a pure culture of the organism, and concludes that the disease is due to a soluble bacterial toxin; *1888*, Roux and Yersin, at the Institut Pasteur, identify the pathogenic exotoxin of *C. diphtheriae*; *1890*, antitoxin antibodies were discovered and passive immunization of experimental animals against diphtheria was demonstrated ; *1891*, first successful human trial of antitoxins was carried out; *1892*, horse antitoxin was marketed. In just nine years a disease responsible for thousands of deaths, mainly of children, seemed close to defeat.

However, enthusiasm over these remarkably swift advances was dampened by the lack of practical methods for the production of potent, standardized antisera. Ehrlich was assigned to the problem and he soon perfected a method for measuring the neutralizing potency of the antitoxins, thus inaugurating his career in immunology with a notable practical application to clinical medicine. So successful was Ehrlich in perfecting diphtheria antitoxin, that he was provided with his own research establishment at Steglitz, near Berlin, in 1896. His publication in the following year of "The assay of the activity of the diphtheria-curative serum" was a landmark event because it was the first demonstation of what we now refer to as a bioassay: a biological yardstick of the activity of a substance whose active ingredient could not be isolated in pure form or measured by any chemical test.

Ehrlich's experiments on the standardization of diphtheria toxin were revalatory not only for their practical results but also because they began the road that lead him to his extraordinary side chain theory. From this work arose his ideas about antibody affinity, antibodies as receptors, and antigens as haptens or haptophores (from the Greek, *apten*, to fasten). Ehrlich stipulated unmistakably that "immune cells" possess surface-bound receptors for antigens, and the role of antigen is to select cells for the production and secretion of the corresponding antibodies. This was the earliest clonal selection theory of the immune system. The revolution it should have ignited had to wait almost 50 years, for McFarlane Burnet, because the idea that immune receptors exist for all antigens -- even synthetic compounds -- was altogether too implausible to be be taken seriously. The eminent immunochemist Felix Haurowitz has recollected how the side chain theory was received in Ehrlich's own time:

> Ehrlich's diagrams of complementarily fitting antigen and antibody were considered by chemists and immunologists as too speculative and fantastic. When the receptor theory was advanced, it was not yet clear that the determinant groups of bacterial and cellular antigens were chemical groups of definitive structure; nothing was known at that time about a definite shape and a definite conformation of macromolecules. Uncertainty concerning the reality of Ehrlich's diagrams ... may have discouraged biologists for more than 50 years from reviving Ehrlich's view

on a selective action of the antigen. (Haurowiz, F., *Immunochemistry and the Biosynthesis of Antibodies*. Interscience Publishers, New York, 1968, p. 98).

No less a figure than Landsteiner, Ehrlich's contemporary, rejected the possibility that the immune system already knew how to make anti-nitrophenyl antibodies before any contact with the hapten:

> Ehrlich tried to account for the specificity of antibodies by the assumption that antibodies are normal constituents of the body which in the cell protoplasm act as receptors and are responsible for the toxic action and the fixation of the antigen; as a result of this specific union ... the antibodies are regenerated in excess and enter the blood stream. Apart from the minor points that antibodies for proteins are usually not demonstrable in normal serum and that differences exist between normal and immune antibodies, this hypothesis is untenable on account of the unlimited number of physiological substances which it would presuppose. (Lansteiner, K. *The Specificity of Serological Reactions*. Dover Publications, Inc., New York, 1962, p. 148.

The mistaken idea that antigens served as templates against which primordial antibodies molded their final shapes was used to explain Landsteiner's demonstrations of the exquisite specificity of anti-hapten antibodies. It is a sobering lesson that this Lamarckian instructionist (template) theory and its variants reigned immunology for a period of 30 years, during which most other biological sciences succumbed to the impact of Darwinian selectionist ideas.

Thoughts of receptors must have been on Ehrlich's mind when he formulated his dictum of *horror autotoxicus* -- the idea that the immune system responds to foreign antigens but not to autoantigens. Ehrlich enumerated several reasons for the body's lack of self-reactivity, among which he explicitly stated that cells of the immune system lacked receptors for self antigens. *Horror autotoxicus* has been much maligned and wrongly interpreted to mean that autoimmunization is impossible. Perhaps Ehrlich's own remark that an immune response to one's own red blood cells would be "hard to believe" had a role in this. In any event, what Ehrlich actually had in mind by the term is a mechanism that *prevents* autoimmunization. This mechanism would include not only elimination of cells with anti-self receptors but also what we refer to today as *immunoregulation* and even anti-idiotypes! In fact, Ehrlich had actually carried out some of the earliest experiments on idiotypes. Although technically flawed by today's standards, Ehrlich's experiments came close to the first demonstration that an anti-idiotype could block antigen binding by an antibody-1.

It is not widely appreciated that Ehrlich had carried out clinical investigations of the first autoimmune disease, paroxysmal cold hemoglobinuria, the rapid development of intravascular hemolysis after exposure to cold. He showed that local intravascular hemolysis and erythrophagocytosis occurred when a finger of a patient with the disease was ligated with an elastic band and immersed in ice water. The disease was also studied by Karl Landsteiner. One of the great ironies of the history of autoimmunity is that shortly after Ehrlich had written "it would be difficult to believe" that autoantibodies could be formed against one's own red blood cells, Donath and Landsteiner discovered the immunological basis of paroxysmal cold hemoglobinuria: a complement-fixing autoantibody. Ehrlich had ignored that possibility, which he attributed to toxins produced by blood vessels as a result of cold.

A few years after the turn of the century, Ehrlich resumed his work on organic dyes, with the hope of developing new forms suitable for chemotherapy. He was stimulated by the work of Laveran and Mesnil (the latter a student of Metchnikoff), who showed that certain trypanosomes responsible for natural infections in man could be made to infect rats and mice, in which they could be passaged indefinitely. Ehrlich and Shiga produced trypan red, which they showed had a curative effect on trypanosome infections in mice; this compound served as the starting point for a campaign of research on *chemotherapy* (a word coined by Ehrlich) which culminated in the discovery of salvarsan -- compound 606 -- the first effective remedy for syphilis. This *magic bullet* (another of Ehrlich's neologisms) was the crowning achievement in Ehrlich's scientific career, and the one for which he generally most noted.

Almost a century after Ehrlich we continue to grapple with the very questions that occupied his mind. Of the major problems in immunology, the mechanism of the distinction between self and not-self and its parallel, the origins of autoimmunization, still withstand a comprehensive solution. The applications of advanced molecular technics to the problem have uncovered fascinating phenomena but their significance is unclear and they have not brought us too much closer to the end. The reductionist approach to autoimmunization, exemplified by the transgenic mouse, is hardly likely to explain the complex elements that culminate in an autoimmune disease in humans.

The following listing of some of the general features of autoimmunization and of autoimmune diseases may provide a perspective on the problem. It is now clear that *multiple genes determine susceptibility to both autoimmunization and to autoimmune diseases.* Not so evident is the fact that *multiple genes also determine the pathological manifestations that can result from autoimmunization.* For example, the autoimmune disease of the NZB mouse is autoimmune hemolytic anemia, but (NZBxNZW)F_1 hybrids

develop lupus nephritis and no evidence of autoimmune hemolytic anemia. However, despite these important genetic influences, *stochastic elements contribute to autoimmunization.* In identical twins, the concordance rate for type I diabetes is only about 50%. Environmental factors (infectious agents?) have been implicated as the cause of this discrepancy, but the discordance holds even for identical twins raised in the same household. It is unlikely that the immune systems of identical twins are *identical*: random rearrangements of V genes and somatic mutations of immunoglobulin V genes surely must contribute to somatically acquired differences immunological between germline identical twins. Such stochastically generated differences could be of greater importance than hypothetical environmental factors.

Another generality to be dealt with is that *the instigating stimulus to autoimmunization is rarely evident.* It is striking that, in most caes, the disease appears "out of the blue", and the most painstaking investigation of what may have triggered the pathogenic events is fruitless. In short, if there is an infectious or toxic agent to be implicated, then it invades in silence and leaves no distinctive mark. For that reason, the Epstein Barr and other herpesviruses and some retroviruses, which have those characteristics, under intense scrutiny as the cause of diseases like rheumatoid arthritis and multiple sclerosis. Molecular mimicry of a microbial antigen has been championed as a mechanism of autoimmunization, but it is equally likely that an entirely endogenous autoimmune response could result in authentic autoantibodies that cross react, through molecular mimicry, with microbial antigens that have nothing to do with the disease.

A related problem is that *autoantigens are not necesarily autoimmunogens.* In other words, the immunogen (if any) that incites the disease and the antigen that stimulates the patient's T cells *in vitro* or that binds to the autoantibodies of the disease are not necessarily equivalent. Indeed, identification of the immunogens that incite autoimmune diseases is one of the major unsolved problems in autoimmunity.

Clear distinctions are generally made between organ-specific and systemic autoimmune diseases. However, detailed analysis has shown that *organ-specific and systemic autoimmunization often overlap.* Furthermore, the tendency to develop one kind of autoimmune disease may be accompanied by susceptibility to a second type. The BB rat, for example, develops not only type I diabetes but also autoimmune thyroiditis.

Finally, the fact that *women are more susceptible than men* to autoimmune diseases must be mentioned. This is a neglected area of research, and little is known about it. The facts are striking: autoimmune thyroiditis is 40 times more frequent in women than in men, and systemic lupus is 10 times more frequent in young women (20-40 years) than in men of the same age. Many phenomena relevant to this sex difference have been observed, the actual mechanism is an enigma.

These seven issues, it seems to me, are the at the core of the problem of autoimmunization and autoimmune diseases. Perhaps the power of molecular biology joined with Ehrlich's intuitive and holistic style could provide the answers to them.

The Physiology of Autoimmune Reactivities

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THE LAST TEN YEARS OF AUTOIMMUNITY : EMERGENCE OF FUNCTIONAL AUTOREACTIVITIES IN NORMAL INDIVIDUALS

The three years frequency of the International Immunology Congresses make them appropriate to consider the place of progress in a given area. This decade was certainly marked by the structural solution of the T cell receptor problem, first announced in Kyoto (1983). However, if less dramatically, the common sense in other central areas of Immunology has also significantly evolved. This is particularly evident in what concerns autoimmunity. While in 1980 (Paris), there was hardly any mention of autoimmune reactivities in normal individuals, we are starting the 1989 Congress with Symposium lectures on the physiology of autoimmunity. The previous decade had seen the isolation and description of very promising animal models of spontaneous and induced autoimmune diseases, an increasingly powerful technology, and the demonstrations that "important" diseases (e.g., diabetes) were actually autoimmune. Many immunologists were attracted to this area, and the greatest expectations could be entertained. The present consensus is less optimistic and, in spite of much work, it seems that we have advanced very little towards the goal of treating and/or preventing autoimmune disease. We do feel, however, that the increasing attention given to autoreactivities in normal individuals, and the shift in perspective that this implies, have opened new possibilities: we can now compare physiology with pathology of autoreactivity, while

not longer than 10 years ago, we were trying to understand autoimmune disease with no information, not even consideration, of what the physiology of those processes would be.

Undoubtly, the breakthrough has been the study of normal individuals, prior to immunization or any other manipulation. Interestingly, the various groups contributing in this area were often pursuing quite distinct interests, such as natural antibody specificities (Avrameas et al. 1983), models for autoimmune disease (Klinman et al. 1988), B cell subspopulations (Herzenberg et al. 1986), lymphocyte repertoire selection (Martinez-A. et al. 1988; Marcos et al. 1986), or the basis for the internal activities in the normal immune system (Coutinho et al. 1984). The first critical observations concerned the presence of circulating autoantibodies in healthy donors (Guilbert et al. 1982), and the vulgarization of the hybridoma technology rapidly lead to the accumulation of reports describing autoreactive monoclonal antibodies isolated from normal individuals (see e.g., Prabhakar et al. 1984; Dighiero et al. 1985). Since the production of hybridomas requires fusion with cycling cells, both of those observations indicated that autoreactive B cells were not only functionally competent, but actually activated under normal conditions. Other technologies for lymphocyte cloning (Andersson et al. 1977), combined with lymphocyte separation by cell activation criteria (Pereira et al. 1985), contributed quantitative evidence for the notion that autoreactive B lymphocytes in normal individuals are indeed activated (Portnoï et al. 1986) and "naturally" secrete autoantibodies (Klinman et al. 1988) that circulate in the serum (Avrameas et al. 1983). Actually, experiments on immunoabsorption of normal serum Ig on autoantigen affinity columns, together with the fact that natural autoantibodies reacting with over 150 structurally different "self" proteins have already been identified in normal serum, lead to the notion that the majority (all?) of the circulating Ig is autoreactive (Avrameas et al. to be published). These notions were supported by the findings of normal levels of circulating IgM and of IgM-secreting plasma cells in the spleen of mice that are secluded of all environmental antigens (Hooijkaas et al. 1984; Pereira et al. 1986). Under these conditions, lymphocytes are exclusively exposed to stimulation by "internal" antigens, and their activities can be appropriately classified as autoreactive. T lymphocytes, although less well analysed, did not escape

this change in approaches and perspective. In spite of the recent interest in "thymic deletions", the notion of autoreactive T cells has made its way. Adding to the abundant observations on "autologous MLR", the technology of cloning and T cell hybridoma production, lead to the frequent identification of autoreactive clones in normal donors. Moreover, the separation of lymphocytes according to activation state together with the development of new assays for effector activity (Pobor et al. 1982; Pereira et al. 1985), has again revealed that autoreactive T cells are depleted from the resting lymphocyte pool and accumulate among activated cells of normal individuals (Coutinho et al. 1989). Interestingly, the activation of some autoreactive B cells was shown to require the help of T lymphocytes that are, themselves, activated in normal donors (Huetz et al. 1988).

CURRENT MODELS

These observations imposed a reevaluation of the prevalent concepts, which equated all autoimmune activities with disease. Thus, if, by the end of the 70's, only few contested the existence of autoreactive B cells in normal individuals (Cohn et al. 1980; Nossal and Pike 1980), the predominant conviction was that such cells remained either resting, suppressed, or perhaps anergic, in any case inactive under physiological conditions. Indeed, the development of autoimmunity required the injection of B cell mitogens (Izui et al. 1977) or anti-host T lymphocytes (Gleichamn et al. 1976), in order to activate antibody secretion by such cells. Furthermore, normal individuals score as negative in the tests for autoantibodies, used since long in diagnostics of autoimmune diseases. What has changed in this decade? Technological progress has certainly played a major role, allowing for better quantitation, sensitivity and analytic power of our measurements. On the other hand, the theoretical contributions should not be ignored, particularly the impact of the "network theory" (Jerne 1974), essentially based on notions of autoreactivity. Some of the early demonstrations of lymphocyte autoreactivity have indeed concerned variable regions of antibodies (Holmberg et al. 1984) or other lymphocytes (Martinez-A. et al. 1984). What is then the difference between pathology and physiology? . Aside

from somewhat "ideologic" positions, that range from the defense of autoreactivity as the essence of a normal immune system, to the refusal of the available evidence discarded as artifactual, two basic types of general models can be found today. One keeps to the traditional view that autoimmune pathology can be correlated with the activity of isolated lymphocyte clones, while the other argues that physiology and pathology are systemic properties that cannot be understood by the analysis of single clones. We shall refer to these alternatives as the "clonal" and the "systemic" views, respectively.

Clonal models maintain that normal autoantibodies are qualitatively different from "pathogenic" autoantibodies, usually displaying very low affinity to the "target" self antigens, and multireactivity. These reactivities are not functionally meaningful, at the level of deletion, induction and effector functions. Autoantibodies associated with disease, in contrast, show properties conventionally ascribed to "immune" antibodies, that is, high affinity and specificity, as well as structural characteristics which demonstrate (auto)antigen-driven clonal selection and affinity maturation by somatic mutation (Schlomchik et al. 1987). Furthermore, in most instances, pathogenic autoantibodies are of the IgG class, while those of normal individuals are, invariably, germ-line encoded IgM molecules (Naparstek et al. 1986) . Besides, pathology and physiology also differ on a quantitative basis, the titers of antoantibodies in patients being usually much higher than those in normal subjects. These views, shared by a majority of the specialists, consider normal autoantibody production as the result of (1) leakage in the mechanisms ensuring deletion or inactivation of autoreactive lymphocytes, followed by (2) antigen-nonspecific (polyclonal) activation of B cells driven by unrelated challenges, or mitogenic activation by microbial products. The recent demonstration that B cells carrying high affinity antibody receptors to autologous antigens are either deleted (Nemazee and Burki 1989) or anergized (Goodnow et al. 1988) in transgenic mice is amply used as evidence for those arguments. Within these perspectives, however, there are no explicit models proposing a global explanation for the origin of autoimmune diseases. Several alternatives exist in the literature, the most frequent being: (1) Polyclonal activation of B cells, associated with intrinsic "hyperreactivity", depression in "suppressor cell function", or "general

disregulation", all of unknown origin; in this case, however, it has to be assumed that normal autoantibodies can indeed be "pathogenic", what has actually been shown in lupus nephritis and in Berger nephropathy. Although genetic experiments segregate polyclonal activation from disease (Datta et al., 1982), the frequent association of these two conditions remains an interesting aspect of autoimmunity. (2) Immune responses stimulated by microbial antigens which crossreact with self structures; here, again, it has to be accepted that potentially autoaggressive cells are not normally purged by deletion. (3) Induced exagerated or aberrant expression of normal tissue antigens, leading to stimulation of low affinity autoreactive cells or, by the bias of MHC-presentation, to activation of organ-specific T lymphocytes available in the normal repertoire. Recent experiments with organ-specific promoters controling the expression of MHC transgenes, however, have not produced the autoimmune syndromes expected by this model (Lo et al., 1988). (4) Stimulation of high affinity autoreactive clones, following putative deficiencies in the normal processes of deletion or regulation. Here again, no example of this nature has been identified.

In contrast, the systemic view argues that isolated clonal activities are not by themselves the cause of autoimmunity. First of all, only in few cases, has it been possible to transfer disease with antibodies or with single clones of lymphocytes, under conditions where the participation of the recipients immune system can be excluded. Furthermore, the temporal correlation between the titers of autoantibodies and disease is poor, sometimes completely absent, just like in mouse strains that are susceptible or resistant to the induction of several autoimmune diseases and all produce the same titers of autoantibodies. It is argued that, whenever analysed, the avidities of normal autoantibodies are in the conventional range for effective function, and that the Ig-transgenic mouse models developed so far cannot be compared with physiology, given the very selected nature of the antibodies utilized (encoded by hypermutated genes, which would not be expressed in normal individuals). Moreover, the mechanisms supposed to ensure tolerance by thymic "clonal deletion" operate as well in autoimmune individuals (Kotzin et al., 1989), while disease-provoking autoreactive T cells can readily be induced in normal individuals, both observations dissociating tolerance from clonal elimination and disease from the presence of

autoreactive clones. For these views, autoreactive cells are activated in perfectly normal, (self)antigen-specific, manners; the predominance of autoantibodies in normal serum is simply the consequence of the overwhelming exposure to autologous antigens; the multireactivity of normal autoantibodies should actually be expected since they are encoded by nonmutated germ-line genes (Baccala et al., 1989; Naparstek et al., 1986) unselected for increased affinity by clonal expansion, and selected for multireactivity by the multiplicity of different antigens interacting with the responding lymphocyte in its immediate environment. Also here, models explaining disease are unsatisfactory. Thus far, physiological behaviours are said to lack autoaggresion for they are "at equilibrium" with self, but the models are not explicit on the origins of such putative desequilibria. It has been suggested that all autoreactive cells are recruited by activation into a compartment of idiotypically "connected" clones, that are thereby forced into dynamic behaviours incompatible with the development of immune responses with large clonal amplifications and antigen-driven affinity maturation (Huetz et al. 1988; Stewart et al. 1989 ; Coutinho 1989). This model, therefore, ascribes to V-region "connectivity" of autoreactive clones the difference between normality and disease, but it falls short of explaining why all autoreactive cells are "connected", and how this rule would brake down in pathological conditions.

These two general types of models include quite different propositions for therapeutic intervention in autoimmune diseases. While the clonal view proposes specific suppression of the clones responsible for the pathology (or nonspecific immunosuppression if that is not possible), the systemic view considers that pathology is most likely accompanied by deficient activation of the autoreactive clones, or of those which connect them. For the latter, therefore, autoimmune patients should be "stimulated", rather than suppressed, as suggested by the well known association of autoimmune pathology with primary imunodeficiencies (Marcos et al., 1986).

SUMMARIZING SOME *A PRIORI* CONVICTIONS

1. Autoimmunity is not synonimous with autoimmune disease, and the understanding of the pathology will have to develop from a solid basis of knowledge in the physiology of autoreactivities. We might need novel approaches and techniques to progress in the description and quantitation of these physiological processes.

2. Autoimmune diseases constitute an heterogeneous group of conditions, and we find it unlikely that a single, global scheme, based on any given kind of mechanisms, can interprete them all. We should, however, not get lost in phenomenology, and search instead for the most common grounds of autoimmune disfunction.

3. Pathological autoimmunity always corresponds to multiple deviations from normality, and is never reducible to the malfunction of an isolated clone. Even if triggered as a clonal process, disease will only be manifested when large areas of the whole immune system have been implicated even if as reactive compensations attempting to recover a new equilibrium. This is incompatible with simple "clonal solutions" for specific therapy.

4. Lymphocyte function is controled by a variety of structural and regulatory genes, and lymphocyte repertoires are selected under the influence of many loci encoding or regulating "somatic" components. It follows that autoimmune diseases will always show multiple and complex genetic controls, severely limiting interventionist strategies aiming at "gene correction".

5. The selection of lymphocyte repertoires is recursive and, therefore, early developmental influences are likely to leave profound imprints in the future evolution of the system. It is thus certain that maternal influences and other perinatal experiences will markedly contribute to disease susceptibility and evolution.

6. The physiology of autoreactivities also pertains to the participation of immune components, in other biological systems of the body's economy. Pathological behaviours of any sort reflect alterations of the normal equilibria, which include, therefore, immune activities. It follows that it might be possible to correct non immunological diseases by immune manipulations. Conversely, primary immune disfunctions may possibly be corrected by manipulating autoantigens.

7. We take it as a good sign of our little progress the fact that the immunological treatment of a lupus or a diabetes is essentially the same as that of an allogeneic kidney recipient. Specific therapy is the obvious goal, but significant progress will require the utilization of the reagents that the system uses in its own operation, most likely natural antibodies.

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Reverse Immunogenetics: Investigations of HLA-Associated Disease Based on the Structural and Genetic Identification of Candidate Susceptibility Genes

G.T. Nepom

INTRODUCTION

"Reverse genetics" is a term used to describe the analysis of inherited traits in which the genetic defect, rather than the trait itself is the primary target of analysis, so that the nature of the defect is reduced by working backwards from basic information about the gene (Orkin 1986). In an analogous sense, we are entering an era of "reverse immunogenetics", in which basic structural and genetic information about HLA genes contributing to susceptibility can be used to predict models of disease. In this article, we will briefly illustrate this concept for two HLA class II-associated diseases: Type I diabetes and rheumatoid arthritis.

HAPLOTYPES WITH WELL-CHARACTERIZED HLA CLASS II ASSOCIATIONS WITH DISEASE

Over the last 15 years, a large number of diseases have been studied which are associated with a variety of HLA specificities (Tiwari and Terasaki 1985). Recent application of molecular-based techniques such as restriction fragment length polymorphism detection, oligonucleotide hybridization, gene amplification, and DNA sequencing has refined the analysis considerably. Since most of the HLA specificities are broad public serologic or cellular recognition elements shared by more than one gene and more than one haplotype, haplotype analysis using molecular genetic markers is a more precise and informative method of analysis. Table I lists some of the diseases and HLA haplotypes which account for a large share of the genetic contribution to the diseases listed.

Table I. Haplotypes with well-characterized HLA class II associations with disease.

	DQB1 (DQβ)	DQA1 (DQα)	DRB1 (DRβ)	DRβ3/4	HLA-DR serotype
Type I Diabetes	DQ3.2	DQ4.1	Dw4	DRw53	DR4
	DQ3.2	DQ4.1	Dw14	DRw53	DR4
	DQ3.2	DQ4.1	Dw10	DRw53	DR4
	DQ2	DQ2	Dw3	DRw52	DR3
Rheumatoid Arthritis	DQ3.1	DQ4.1	Dw4	DRw53	DR4
	DQ3.2	DQ4.1	Dw4	DRw53	DR4
	DQ3.2	DQ4.1	Dw14	DRw53	DR4
Celiac Disease	DQ2	DQ2	Dw3	DRw52	DR3
Pemphigus Vulgaris	DQ3.2	DQ4.1	Dw10	DRw53	DR4
	DQ1.9	DQ1	Dw9	DRw52	DR6

The identification of disease-associated haplotypes, such as those listed in this table, has itself contributed to both practical and theoretical improvements in the field of HLA and disease. On the practical side, once a specific haplotype is identified as associated with a particular disease, then any genomic marker characteristic of that haplotype serves as a marker in the population for susceptible individuals. In practice, this means that any linked polymorphism, such as an RFLP for any linked site on chromosome 6, can be used. On the theoretical aspect, the analysis of linked polymorphisms within a set of disease-associated haplotypes can now be used to direct the next step in the process of "reverse immunogenetics"; namely, the identification of specific candidate HLA class II susceptibility genes.

BEYOND HAPLOTYPES: CANDIDATE SUSCEPTIBILITY GENES

Among the HLA class II haplotypes associated with type I diabetes (IDDM), as listed in Table I, all the HLA-DR4-positive haplotypes share identical DQ genes. This is in marked contrast to other DR4-positive haplotypes, which carry different DQ genes, that are not associated with IDDM (Kim 1985). Careful analysis of these haplotypes indicated that a specific DQß gene, the DQ3.2 allele, was the most discriminating marker distinguishing susceptible from non-susceptible haplotypes within the DR4 family, and was the basis for the hypothesis that the DQß3.2 gene accounts for the DR4-associated susceptibility to IDDM (Nepom 1986a,b; B Nepom 1986). Genomic studies demonstrated that genes telomeric of DQß were frequently identical in disease-associated and non-associated haplotypes (Holbeck and Nepom 1986) and also demonstrated that genes centromeric of DQß were variable even within IDDM-associated haplotypes (B Nepom 1986); these observations effectively "map" susceptibility on DR4-positive haplotypes to the DQ3.2 gene itself.

Similar analysis of haplotypes associated with rheumatoid arthritis (RA) implicates a different set of linked loci. Different DR4-positive haplotypes associated with RA encode different DQ alleles (B Nepom 1986; Nepom 1989) and different DP alleles (Begovich 1989), effectively ruling out a contribution of genes centromeric to HLA-DR. Since the DRw53 allele is present on haplotypes which are not associated with RA, this appears to rule out the contribution of genes telomeric of HLA-DR, and focuses attention on the DRB1 locus itself (Nepom 1987). Additional support for this conclusion derives from comparisons of sequence polymorphisms within DR4-associated DRB1 alleles. One of these alleles, the Dw10 gene, differs from Dw4 and Dw14 by just four amino acid substitutions, clustered within a small immunogenic region of the DRB1 molecule. Since DR4 haplotypes that carry this Dw10 gene are not associated with RA, this difference also suggests that the DRB1 gene product itself on susceptible haplotypes accounts for the association with RA (Gregersen 1987; Winchester 1986).

Similar arguments have recently been made by other investigators which appear to implicate the DQ2α and ß genes as accounting for the HLA-DR3 association with celiac disease (Sollid 1989; Bugawan 1989); in the case of pemphigus vulgaris, similar analysis suggests that a DRB1 gene may account for the contribution of DR4-positive haplotypes, but that the DQB1 gene may account for the association corresponding to DR6 haplotypes (Scharf 1989).

BEYOND CANDIDATE GENES: EPITOPES AND CONFORMATION

"Reverse immunogenetics" differs from "reverse genetics" in one important regard: Expression of the candidate disease gene does not in itself identify the function of this gene in disease. Rather, because of the critical function of HLA class II molecules in establishing and permitting immune recognition properties within an individual, structural clues which identify the disease-associated properties of class II molecules are likely to be contained within just a few key polymorphic residues within each susceptibility gene product.

The effect of such discrete limited substitutions upon function hinges upon at least three variables: first, the specific residue itself; second, the impact of that residue's substitution upon the conformation of its polypeptide; and thirdly, the impact of that substitution and conformation upon the bimolecular interaction between HLA class II α and ß chains. We have begun, through a systematic process of site-directed mutagenesis and gene expression, to analyze polymorphic residues within candidate class II susceptibility genes for each of these three parameters. Some of these studies have been reported elsewhere (Kwok 1989a,b,c) and have been reviewed in some detail (Kwok and Nepom 1989); they will be summarized here in the context of a structural model useful for predicting the role of specific disease-associated epitopes in immune recognition and disease.

The Dw14 Epitope Associated with Rheumatoid Arthritis

The primary candidate susceptibility genes for rheumatoid arthritis are encoded within the DRB1 locus. Products of these genes are expressed on the cell surface in heterodimers associated with the DRα polypeptide. Since DRα genes are non-polymorphic, the contribution of specific DRB1 alleles to disease susceptibility is likely to be a function of the ß chain itself. Polymorphisms within DRB1 alleles which distinguish disease-associated from non-associated alleles predominately cluster within the portion of the molecule corresponding to amino acids 57 through 86 (Gregersen 1986). Structural models for the class II molecule, based on the x-ray crystallographic analysis of homologous HLA class I molecules (Bjorkman 1987; Brown 1988) predict that residues 57 through 86 lie on a long α helical loop, which is the dominant structural element of the class II ß chain exposed to its environment, i.e., immunogenic peptides and T cell receptors. A schematic view based on this model is shown in Figure 1, in which the α-helical loop of the ß chain, lying on a structural platform composed of ß-pleated sheet structures, is responsible for exposure of the molecule to both the interior groove [the putative peptide recognition site], and to the upper and outer sides of the molecule.

There is a remarkable example of sequence identity within this α-helical stretch of the DRB1 gene which suggests that residues within this helix are highly associated with RA. The Dw14 DRB1 gene, one of the major RA-associated susceptibility alleles, carries a characteristic sequence of Leu-67, Arg-71, Ala-74 that distinguishes it from other alleles within the DR4 family of genes. Due to the spacing of these residues along the turns of an α helix, each of these sites is a primary candidate for "lining up" along a single face of the α helix, as illustrated in Figure 1. Depending on the exact orientation of the helix, this face could either point "up" into the putative T cell receptor, or could point into the

groove, the site of probable peptide interactions. Analysis using an alloreactive T cell clone which uniquely recognizes this sequence (Seyfried 1988) suggests that this Leu-67, Arg-71, Ala-74 sequence may be predominantly a T cell receptor interaction site, although interactions with peptides in allorecognition are a possibility.

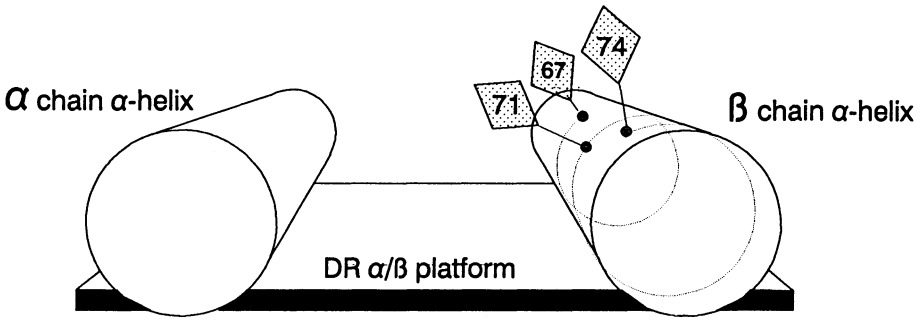

Figure 1. Schematic view of predicted structure of HLA-DR α/ß polymorphic domains. Likely orientation of critical residues along the ß-chain α-helical loop is highlighted to illustrate their potential for T cell interaction, consistent with experimental observations (see text for details).

In experiments using an oligonucleotide probe which uniquely hybridizes to the DNA sequence corresponding to residue 67 through 74, an interesting observation was made: two other DRB1 alleles hybridize to this probe, indicating an identical sequence in this region; neither were HLA-DR4. In one case, DR1-associated DRB1 genes were identified and, remarkably, accounted for the majority of the non-DR4 positive patients with rheumatoid arthritis in a well-characterized study (Nepom 1989). The other example is the Dw16 gene, a rare DR6-associated allele found in Native American populations. Interestingly, these populations are known for a high prevalence of non-DR4-associated arthritis (Willkens, 1982) raising the possibility that this sequence corresponds to almost all of the non-HLA-associated genetic contributions to RA outside the DR4 family of genes. Site-directed mutagenesis binding experiments are in progress to fully characterize the contributions of specific residues to this interesting disease-associated sequence.

Polymorphic Residues within the DQ3.2 IDDM Susceptibility Gene.

There are three closely related members of the DQw3 family of DQB1 alleles, termed DQ3.1, 3.2, and 3.3 (Hiraiwa 1989). Polymorphisms which distinguish among these alleles occur at four sites in the amino terminal domain of the molecule, at residues 13, 26, 45, and 57. DQ3.2, the most likely candidate for a diabetes susceptibility gene in Caucasian populations, differs from DQ3.1, which is not associated with disease, at all four of these residues. DQ3.3, the

most highly associated HLA gene in Oriental IDDM, differs from the DQ3.1 gene at only three of these positions, codons 13, 26 and 45. These comparisons thus pinpoint a restricted set of polymorphic residues as likely contributors to the structural basis for DQ-associated susceptibility to IDDM. Based on the modeling predictions for class II structure, the proposed orientation of these polymorphic residues is illustrated in Figure 2.

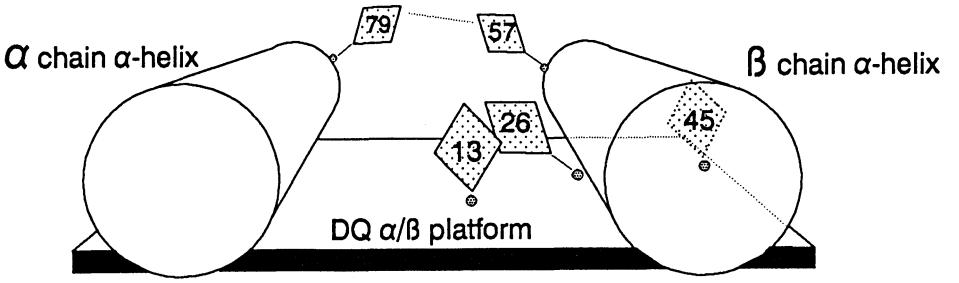

Figure 2. Schematic view of HLA-DQ α/β domains, illustrating the predicted structural contributions of key polymorphic sites in the molecule characteristic of susceptibility to IDDM. The presence of Glu-45 determines the major serologic allospecificity, termed TA10, distinguishing DQ3.1 from DQ3.2 molecules; also shown is the potential "salt bridge" between the two helices at one end of the antigen-binding groove postulated to be dependent on Asp-57. Polymorphic residues at codons 26 and 13 also contribute to functional parameters of DQβ recognition (see text for details).

There are major differences in the structural and functional predictions of this model compared to the model illustrated in Figure 1. Whereas critical residues in the rheumatoid arthritis-associated Dw14 epitope cluster along an α helical loop, and are predicted by structural and functional analysis to interact with T cell receptor structures (Fig. 1), polymorphic sites corresponding to IDDM susceptibility alleles are located at sites which appear to be critical for conformation and antigen interactions (Fig. 2). We have analyzed the contribution of each of these polymorphic sites within the DQ3 family of alleles using site-directed mutagenesis and retroviral mediated expression (Kwok 1989a,b). Residues at codon 45 are immunodominant with respect to the serologic epitopes which distinguish DQ3.1 from DQ3.2 and DQ3.3. The presence of a glutamic acid at codon 45 determines the TA10 epitope, the major allospecificity associated with this gene. When tested for allostimulation against a panel of DQw3 reactive T cell clones, substitutions at codon 45 altered most, but not all, recognition events. In contrast, none of the substitutions at codons 13, 26, or 57 affected the serologic recognition of the DQβ chains. This is consistent with the model in which these latter residues

interact with the "inside" of the class II structure, very likely interacting with putative bound antigenic peptide or with the heterologous α polypeptide chain.

Other experimental observations are also consistent with this model: Depending on the specific alloreactive T cell clone used, single amino acid substitutions at either codons 13, 26, or 57 were sufficient to ablate T cell recognition (Kwok 1989b). However, these experiments also identified a number of subtle interactions occurring within the DQß structure. For example, while the substitution of an aspartic acid at codon 57 was sufficient to interfere with T cell recognition for 2 out of 3 alloreactive clones tested when the mutated DQß chain was expressed in the context of a DQ2 α chain, only one of these clones was similarly affected when codon 57 DQß mutants were paired with a DQ3 α chain. Even more intriguing is that substitution of an aspartic acid at codon 57 actually enhanced some T cell reactivity patterns when the ß chain was paired with the DQ7 α chain (Kwok 1989b). These data strongly suggest that the role of substitutions at codon 57 is involved in interchain interactions dependent on both α and ß polypeptides. These data are consistent with the hypothetical model proposed by Brown (1988) in which the side chain of residue 57 may potentially form a "salt bridge" interacting with a side chain of residue 79 on the DQα chain (Fig. 2).

Our findings with respect to the function of residue 26 are very similar to those for residue 57. Namely, substitution of a tyrosine, as found in DQ3.1, for a leucine, as found in DQ3.2 and DQ3.3, markedly alters T cell recognition, dependent on α chain interactions. In the proposed structural model for class II polypeptides, residue 26 lies on the platform of the antigen binding groove, near the α helix of the DQß polypeptide. If this model is correct, then the interaction between residue 26 and the DQα chain is presumably an indirect interaction, perhaps mediated by structural constraints on peptide binding (Kwok and Nepom 1989).

In addition to these major structural effects of codons 26, 45, and 57, substitutions at codon 13 ablated the reactivity of 1 out of 4 DQ reactive T cell clones. Substitutions at this position (Ala [DQ3.1] - Gly [3.2,3.3]), therefore, are also potential contributors to functional parameters of the molecule; as shown in Fig. 2, the structural model predicts that the side chain of residue 13, on the platform structure, also potentially interacts with bound antigenic peptide.

STRUCTURAL DETERMINANTS OF FUNCTION

There are several conclusions which can be drawn from these experiments, which have implications for the "reverse immunogenetics" approach to understanding the HLA contribution to immune-mediated events in type I diabetes. First is that the functional topography of the molecule is a result of interactions between polymorphic sites on the ß chain and their interactions with α chain polymorphisms. Because of these interactions, there are multiple structural elements within the molecule acting in concert to create a unique conformation. This indicates that the specific antigenic peptide interactions and specific T cell receptor interactions involved in recognition of the DQ3.2 polypeptide are not easily attributed to a single residue in isolation. Rather, the function of the molecule appears to be a

result of a combined set of key polymorphic residues. One way of demonstrating this conclusion functionally was summarized above, in which substitutions of different α chains markedly affect the recognition of discrete ß chain substitutions. Another demonstration comes from our experiments in which two different ß chain substitutions interact with each other. For example, T cell clonal reactivity against the dimer formed by products of the DQ7α and DQ3.2ß alleles is diminished by 50% with ß chain substitutions at either codons 45 or 57. Remarkably, a "double mutant" with the same substitutions at both codon 45 and 57 actually reconstitutes the activity three-fold compared to either mutant alone (Kwok 1989b).

A second conclusion from these studies is that the structural contributions to disease susceptibility appear to be very different for different diseases. While the primary contribution of the Dw14 epitope in rheumatoid arthritis appears to be a discrete helical epitope interacting with T cell receptors, the contribution of residues within DQß3.2 to IDDM, as noted above, is considerably more complex, and appears to primarily involve interactions with the peptide binding site of the class II α/ß dimer. These distinct structure/function models are potentially important clues for deducing the function of class II susceptibility genes in specific diseases. If the inference drawn from these studies are correct, then it may be more fruitful to focus on T cell selection and T cell receptor specificities in Dw14-associated rheumatoid arthritis, and, in contrast, to focus on specific antigen binding requirements and determinant selection events associated with the DQ3.2 molecule in type I diabetes. Future work along these lines will determine whether the clues deduced from the basic genetics and structure of class II susceptibility genes will illuminate critical steps in the pathogenesis of disease.

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Autoimmunity in the Nervous System: Functional Properties of an Encephalitogenic Protein

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INTRODUCTION

There are many ways to analyse immunological self tolerance. For example, studies on the generation of T cell clonal diversity and of the regulation or elimination of self reactive T cell clones in the thymus have been invaluable for better understanding self-nonself discrimination. We have chosen an alternative approach studying autoimmunity and self tolerance by means of experimentally induced, autoimmune diseases with the aim to define the factors that have led to this aberrant situation. Identification of the defective factors may not only help us to understand the pathogenic mechanisms of clinical autoimmunity, but, by extrapolation, the same information can be expected to help us understand, why, under normal conditions, the immune system tolerates the own organism.

EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

Experimental Autoimmune Encephalomyelitis (EAE) of rodents is a model for organ specific autoimmune diseases, which has been remarkably useful for better understanding cellular mechanisms involved in autoimmune reactions, and, in addition, for elucidating immune reactivity within the central nervous system, in a more general sense (Wekerle et al. 1986).

The model has several interesting features. First, EAE is an organ specific autoimmune disease with exquisite tissue specificity. Second, the target autoantigen has been identified and characterized in molecular terms. Third, it has been established that the disease is mediated by T lymphocytes and that this immune response is under the strict control by Ir genes localized within the major histocompatibility complex (MHC).

EAE is induced in susceptible experimental animals by injection of CNS homogenates or CNS myelin suspended in appropriate immune adjuvant. This treatment unfailingly leads to paralytic disease, which after an incubation period of 10-14 days, starts at the tail and takes an ascending course. The disease is mediated by T lymphocytes, which invade the CNS via the endothelial blood brain barrier, and which cause a mononuclear infiltration concentrated mainly around small postcapillary vernules.

It is of interest that in all species studied so far, the pathogenic T cells activated by myelin autoimmunization are specific for one

protein component of the myelin membrane: Myelin Basic Protein (MBP). MBP, a phylogenetically conservative protein of about 170 amino acids, is localized at the cytoplasmic inner surface of the myelin membrane. The gene for MBP is organized in 7 exons, which, by alternative splicing, can give rise to up to 5 isoforms (Campagnoni and Macklin 1988).

Why is MBP such a dominant autoantigen? What distinguishes this protein from all other peptide sequences to render it encephalitogenic? The molecular properties listed above are certainly not sufficient to confer encephalitogenicity on MBP. As a minimal requirement for a protein to be encephalitogenic, we will propose three criteria: 1) the protein must elicit a strong T cellular immune response in the host, due to favorable Ir gene control; 2) the protein must be presented in an appropriate way within the target organ, CNS; 3) the protein must select/activate T cells with cytotoxic potential.

AN ENCEPHALITOGEN MUST ELICIT A STRONG, IR GENE CONTROLLED T CELL IMMUNE RESPONSE.

The first requirement for an effective encephalitogen is, quite obviously, its capacity of triggering a vigorous T cell immune response within the host´s immune system. As in any T cell reaction, this response must be controlled by Ir genes that are expressed via the trimolecular complex formed by MHC class II determinants (the actual gene products of Ir genes) binding critical (auto-) antigenic protein fragments, on the one hand, and the variable regions of complementary T cell receptor chains, on the other.

The rodent T cell response to MBP is indeed under very strict Ir gene control. It is known for about 15 years that the Lewis rat is a genetically determined high responder for MBP and that this high reactivity is associated with its particular MHC haplotype (Gasser et al. 1973; Günther et al. 1978). The dominant T cell epitope of Lewis rats has been identified as sequence p68-88 (Kibler et al. 1977). Only Lewis T cells recognize this epitope, which is ignored by encephalitogenic T cells from other strains of rat. In the mouse, different T cell epitopes have been mapped. PL/J mice recognize preferentially MBP sequence p1-10, as recently demonstrated by two groups (Acha-Orbea 1989; Kumar et al. 1989). In contrast SJL/J ($H\text{-}2^s$) have T cells that are focussed on epitopes located within the peptide sequence p89-109 (Acha-Orbea 1989; Kumar et al. 1989). Further epitopes may reside localized within regions 68-88 (B6.bm12; Wekerle, unpublished) and 31-50 (PL/J and B10.PL; Kumar et al. 1989). In general, both in rat and in mouse, the T cell responses against MBP are focussed on singulary epitopes, or on epitopes clustered within a short peptide sequence of MBP.

In man, T cell reactivity against MBP seems to be much more complex. A panel of T cell lines reactive against human MBP was selected both from normal donors and from patients with multiple sclerosis. All donors had the HLA DR2 haplotype, which in Caucasian populations is associated with some mildly enhanced disease susceptibility (Svejgaard et al. 1983). All T lines analyzed were able to recognize human MBP presented by L cells transfected either with DR2a or DR2b, the ß chain genes of the DR2/Dw2 haplotype (Wilkinson et al. 1988). Most lines recognized their antigen in the context of DR2a, whereas only a few of MS patient derived lines responded to MBP/DR2b. Screening a panel of partly overlapping proteolytic fragments of

human MBP showed that both control and MS patient derived DR2a restricted anti-MBP T lines recognized epitopes situated at least at 3 different parts of the MBP molecule (Pette et al., submitted).

Considering the diversity of HLA D region products, which principally could serve as MBP presenting elements, and considering that among one defined element, DR2a, can present an undetermined, but considerable number of epitopes to T cells, it seems safe to predict that the human anti-MBP T cell response is directed against an enormous number of distinct peptide epitopes that may be distributed all along the MBP sequence.

Ir genes influences the immune reactivity not only in the mature immune response by controlling antigen recognition by T cells. Together with germ line genes for the T cell receptor, they certainly have a profound impact on the formation and composition of the clonal T cell repertoire. Both gene classes should affect the proportion of potentially autoaggressive T cell clones within the immune system.

The existence of potentially encephalitogenic T cells within the normal immune repertoire has been most directly demonstrated by Schluesener and Wekerle (1985), who isolated MBP specific T lines from completely naive, nonimmunized Lewis rat lymph node populations. Transfer experiments established that these lymphocytes were indeed lethally encephalitogenic when transferred in doses higher than 0.5×10^6/rat. Given the absence of somatic mutations in T cell receptor V region genes, the clonal precursors of the encephalitogenic T lines must be considered "normal" components of a "normal" immune system.

Like the number of encephalitogenic epitopes of MBP, the number of V region genes used for the antigen receptor of MBP specific T cells is remarkably low in rodents. This was first shown in the PL/J mouse, where almost all encephalitogenic T lines use the Vß8.2 element. In addition, most of these clones express either the V-alpha-2 or V-alpha-4 gene product (Acha-Orbea et al. 1989; Kumar et al. 1989). It was especially noteworthy that also the encephalitogenic T cells of Lewis rats use only one TcR Vß element, and that this element was highly homologous to the mouse Vß8.2 (Chluba et al. 1989; Burns et al. 1989). It should be stressed that Lewis T cells recognize a different MBP epitope (p68-88) than PL/J T cells (p1-10).

In mouse and rat the simplified usage of Vß elements in encephalitogenic T lines has been successfully used for new strategies of immunotherapy. Monoclonal antibodies against Vß8.2 determinants have been impressively efficient in preventing and treating actively induced or passively transferred EAE in PL/J mice (Acha-Orbea et al. 1988) and Lewis rats (Owhashi and Heber-Katz 1988). Whether similar regimens will be of promise also in the treatment of human myelin specific autoimmune disease, will depend on the results of T cell receptor V region analyses which are currently being carried out in several laboratories including our own. Considering the stunning complexity of epitope presentation/recognition in humans, the hopes for a readily designable anti-T cell receptor therapy should not be too high at present.

AN ENCEPHALITOGEN MUST BE PRESENTED WITHIN THE TARGET ORGAN IN AN OPTIMALLY RECOGNIZABLE CONTEXT

Clearly, high reactivity of the immune system is necessary, but by no means sufficient for a peptide to be a strong encephalitogen. To be an effective encephalitogen, a candidate protein must be presented within the target organ to be readily recognizable for the pathogenic T cells. That this requirement is nothing less than trivial may become clear from an examination of Experimental Autoimmune Neuritis (EAN), an autoimmune disease inducible in rodents by injection of myelin from peripheral nerves. EAN shares most of its principles with its companion model, EAE. EAN is actively induced in susceptible rats by immunization with peripheral nerve myelin or with the dominant neuritogenic protein, P2. It is mediated by CD4 positive T lymphocytes recognizing defined peptide epitopes of the P2 protein, like the Lewis rat sequence 59-72 (Uyemura et al., to be published). Stable neuritogenic, P2 specific T cell lines have been established (Linington et al. 1984).

Like EAE, EAN induced actively, or by transfers of T lines is highly tissue specific. P2-specific T lymphocytes cause violent inflammation with demyelination in the peripheral nerve, but leave adjacent CNS tissue completely untouched (Izumo et al. 1985). This may not be unexpected, as in the rat, P2 is expressed exclusively in peripheral nerve, but not in CNS myelin. It is much more wonderous that, as mentioned, MBP specific T lines attack exclusively the CNS, but not the peripheral nerves, although MBP is contained within peripheral nerve myelin in rather high quantities (Brostoff, 1984). Why is P2 neuritogenic, and why isn't MBP?

This apparant paradox may be solved by a new in vitro model of EAN which is based on coculturing a permanent Schwann cell line SC1.17 (Porter et al. 1987) with Ia compatible P2 specific T lymphocytes (Nomura et al., submitted). SC1.17 is a permanent, autocrine Schwann cell line, which conserves most normal SC markers including galactocerebroside, Ran-1, myelin proteins and inducibility of MHC class II determinants (Porter et al. 1987; Nomura et al., submitted). When Ia induced SC1.17 cells are coincubated in vitro with Ia compatible neuritogenic T line cells, the lymphocytes respond by strong proliferation irrespective, which is not further increased by addition of exogenous, soluble P2 protein. This response represents "classical" Ia restricted presentation/recognition of the neuritogenic P2 epitope 59-72. SC1.17 cells are thus able to efficiently and autoimmunogenically present their own, autochthonous P2 protein to specific T line cells. The situation is completely different with MBP, another main protein component of peripheral myelin. MBP-specific, Ia compatible T line cells are only marginally stimulated by Ia induced SC1.17 cells in the absence of soluble MBP. Addition of MBP to these cultures, however, leads to maximal T cell proliferation. These observations make several points relevant for understanding the pathogenesis of EAN in particular and tissue specific autoimmunity in general. First, the data demonstrate that membrane bound Ia determinants are "constitutively" complexed with protein epitopes produced by the same cell. Similar findings have been reported by Lorenz et al. in the case of hemoglobin (Lorenz and Allen 1988). Second, not all cellular proteins seem to be bound and presented by Ia determinants equally well. Some proteins - like P2 in Schwann cells - are presented more efficiently than others (e.g. MBP). The basis for this autoantigenic hierarchy is unknown. It remains to establish whether metabolic differences between potential autoantigens have an essential role, or whether competition of peptide fragments for binding sites on Ia molecules is a relevant factor.

AN ENCEPHALITOGEN MUST ACTIVATE OR SELECT CYTOTOXIC T LYMPHOCYTE

Mere recognition of a tissue specific autoantigen by a T lymphocytes may not necessarily cause pathogenic tissue damage. Additional factors, including auxiliary inflammatory cells and their mediators, may be required for a full autoimmune attack. In myelin specific autoimmune models, however, the autoaggressive T lymphocytes seem to be autonomous in mediating disease. A key observation was made by Sedgwick et al. (1987), and was fully confirmed in our systems. MBP specific T lymphocytes transfer EAE with equal efficiency to normal and to lethally irradiated recipients. Injection of MBP specific T line cells into irradiated rats causes peracute EAE which follows the same kinetics as in normal and recipients. Clinical disease develops after a lag phase of 2-3 days, lasts for about one week, and in the case of sublethal cell doses spontaneously remits. The assumption that the lymphohemopoietic system of the irradiated recipient rats is unable to contribute inflammatory or immune cells to the development of transfer EAE is borne out by histological analyses of the EAE lesions. The lesions in irradiated rats show scarcely any round cell infiltrates, which are so typical for normal EAE. There are just a few scattered activated T cells infiltrating the parenchyme around CNS blood vessels. In contrast, hemorrhagia documents a violent attack against the endothelial blood brain barrier (R. Meyermann, unpublished).

These observations are most plausibly explained by a direct cytotoxic attack of autoaggressive T lymphocytes against local autoantigen presenting cells in the nervous system. Indeed, encephalitogenic, MBP specific T line cells are able to completely destroy syngeneic astrocytes in vitro, provided the target cells are Ia induced and the cultures contain MBP. Electron microscopy shows that under these conditions, T line cells make contacts with the MBP presenting astrocytes within the first few hours of coincubation, and that within the subsequent four hours, they completely lyse the monolayers (R. Meyermann, unpubl.). This lytic interaction has been quantified using a conventional isotope release assay (Sun and Wekerle 1986). This work confirmed that the lytic interaction between encephalitogenic T line cells and antigen presenting astrocytes critically depends on the concentration of soluble MBP, and that the reaction is restricted by the appropriate MHC class II determinants.

It is essential to stress that the lytic capacity of MBP specific T cells is by no means limited to astrocytes as target cells, but that any competent antigen presenting cell, e.g. peritoneal macrophages, will be attacked. Even more important, not all Lewis rat T lines recognizing "their" specific protein antigen on an appropriate presenter cell are competent for cytotoxic killing. The cytotoxic potential of rat $CD4^+$, Ia restricted T lymphocytes is unseparably associated with their specificity for pathogenic epitopes on myelin proteins, and with their autoaggressive potential in vivo. Lewis T lines recognizing the encephalitogenic epitope p68-88 and mediating EAE in adoptive transfers, are maximally active in lysing MBP presenting target cells in vitro. In striking contrast, T lines specific for a nonencephalitogenic epitope (e.g. p43-67) are non-lytic. They behave exactly like T lines specific for control antigens, like ovalbumin and tuberculin (Sun and Wekerle 1986).

These findings have been fully reproduced and extended using neuritogenic, P2 specific T lymphocytes as effectors and SC1.17 as targets. Neuritogenic T line cells recognizing the neuritogenic P2 epitope 59-72 most efficiently lyse line SC1.17. This interaction

is, like the induction of proliferation described above, independent of exogenous P2, and is restricted by MHC class II. Non-neuritogenic T lines recognizing distinct epitopes are nonlytic. Interestingly, MBP specific encphalitogenic T lines (e.g. line Q4) are inert against SC1.17 in the absence of MBP, but completely lyse these targets after addition of MBP (Table 1). These data corroborate our previous conclusion that SC1.17 autoimmunogenically present autochthonous P2, but not MBP to potentially autoaggressive T lymphocytes.

Table 1. Association of target epitope, cytolysis and neuritogenicity of P2 specific T lymphocyte lines

Line	Epitope	Cytotoxicity*	Neuritogenicity
N15	P2, p59-72	++	++
N29	P2, p59-72	++	++
N14	P2, not p59-71	$\pm$	0
Q4	MBP, p68-88	$\pm$ *$	0&
L.OA	Ovalbumin	0	0

* tested against ^{51}Cr labelled SC1.17, in absence of soluble P2;
$ lyses SC1.17 in presence of soluble MBP;
& strongly encephalitogenic;

The molecular basis of the association between target cell killing by $CD4^+$ autoaggressive T cells and epitope specificity is not understood at present. To date, it is not even established, whether the lytic interaction represents "classical" lytic pathways, or whether they are the consequence of lymphotoxin release. It is furthermore open, whether the differential cytoloytic potential exhibited by rat T lines recognizing different protein epitopes is related to the selection of Th1 or Th2 subsets, as proposed by Mosman and Coffman (1989) in the mouse. In any event, the strong linkage of in vitro killing and in vivo autoimmune potentials documented in two independent models of experimentally inducible organ specific autoimmune diseases makes it tempting to speculate that these lytic events have a critical role in the pathogenesis of the autoimmune lesion in situ. In fact, cytotoxic attacks to relevant glia cells in the central and peripheral nervous system could quite plausibly explain the key pathological changes seen in EAE and EAN respectively. As discussed previously (Wekerle et al. 1986), astrocytes have vital functions in the CNS, which besides presentation of antigens include spatial septation, maintenance of the ionic equilibria, mediator balances and trophic state. Astrocytes are, however, not involved in formation of myelin. Indeed in T line mediated EAE, nerve function is impaired in the absence of gross myelin destruction. In contrast, in EAN, Schwann cells are potential target of pathogenic T cells. These peripheral glia cells which share many of the functional properties with astrocytes form the myelin sheaths around nerval axons. Consequently, it is remarkable that in T line mediated EAN, myelin destruction is a conspicuous pathological change (Izumo 1984).

CONCLUSION

We feel that in the future, one major line of autoimmune research must be focussed on the identification and molecular characterization of potential autoantigens. This work should help to understand why one self protein sequence, but not another one can trigger a crippling autoimmune disease. Answering this question is by no means of purely academic interest, but pave the way to design specific therapies. These therapeutic strategies may include manipulation of the autoimmune T cell response by synthetic peptides competing for the binding site of relevant MHC class II (or I) products. Other possible strategies could make use of monoclonal antibodies directed against MHC product- bound autoimmunogenic epitopes or against the T cell receptor V region. Some of these strategies have been successfully applied in experimental models. Furthermore, more detailed knowledge of the molecular properties of autoantigens could be an essential step in designing new "vaccination" strategies (see Cohen, this volume) using attenuated autoimmune T cells, or their T cell receptors structures as triggers for immune specific counterregulation.

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Inflammatory Destruction of Pancreatic Islets in Interferon Gamma Transgenic Mice

N. Sarvetnick, D. Liggitt, and T. A. Stewart

INTRODUCTION

The histological finding of discrete accumulations of lymphocytes within and surrounding the islets of Langerhans in the pancreas of newly diagnosed diabetic patients, termed "insulitis," implicated immune effector cells as mediators of the pathology in this disease (Gepts, 1965). Similar lesions have also been observed in the non-obese diabetic (NOD) mouse and the Biobreeding (BB) rat, two animal models of type I diabetes mellitus (Like and Weringer, 1988; Tarui *et al.*, 1986). These accumulations of lymphocytes within the islets implies an ongoing immunological reactivity. In our studies on novel animal models of diabetes, we have been interested in tracing the pathway of such a response.

It has been reported that there is increased expression of major histocompatibility complex (MHC) class I and II antigens on the islet cells of diabetic patients (Bottazzo *et al.*, 1985). This has lead to much speculation as to whether or not this is a cause or a consequence of the immune response. It has been hypothesized (Bottazzo *et al.*, 1983) that the MHC antigens overexpressed on non-immune cells might allow these cells to act as antigen presenting cells. These cells might then present their endogenous antigens to the immune system. If the host is not tolerant to such antigens, it is possible that the host would be able to respond to the presented antigens in the tissue. An alternative hypothesis is the hyperexpressed MHC antigens have nothing to do with the disease, and are hyperexpressed as a result of the inflammatory processes nearby. We and others have begun to investigate the consequences of overexpression of MHC antigens in the periphery *in vivo* (Sarvetnick *et al.*, 1988; Lo *et al.*, 1988; Allison *et al.*, 1988). While no research group has completely disproved the "overexpression of MHC leads to autoimmunity" concept, there appears to be a general trend in this direction from the cumulative data. Most conclusively was the study by Markman *et al.* (1989) who demonstrated that when islet cells present antigen, they induce non-responsiveness in the antigen-specific T cells. This presumably reflects the lack of a secondary "co-stimulatory signal". The experimental approaches involving ectopic expression of MHC antigens all separated MHC expression from other aspects of immune reactions, including the production of immunologic mediators, lymphokines. *In vivo*, this would not occur.

Lymphokines produced in response to infection have the ability to upregulate MHC antigens on a wide variety of cell types. The most noteworthy of these is IFN-γ which has been demonstrated to induce class II MHC expression on a wide variety of cell types in tissue culture (Wong *et al.*, 1985). IFN-γ is a highly pleiotropic molecule which is capable of cooperating in many poorly understood cellular activities. Among other activities, it is thought to have a role in bone remodeling and to be involved in endothelial cell function. IFN-γ also has a protective effect to cells in tissue culture when they are infected with virus after pretreatment..

The fact that IFN-γ is known to induce MHC antigens and is made in response to viral infections has lead to its being indicated as a mediator of autoimmune disease. For this reason we decided to express this molecule locally in transgenic mice. In part, we were interested in recapitulating the effects of a viral infection locally in the pancreas. Transgenic mice were thus created that express IFN-γ in the pancreatic beta cells (Sarvetnick *et al.*, 1988). This was accomplished by creating fusion genes in which the human insulin promotor was fused to the murine interferon gamma gene. This chimeric gene (INS-IFN-γ) was then microinjected into fertilized zygotes to create transgenic mice.

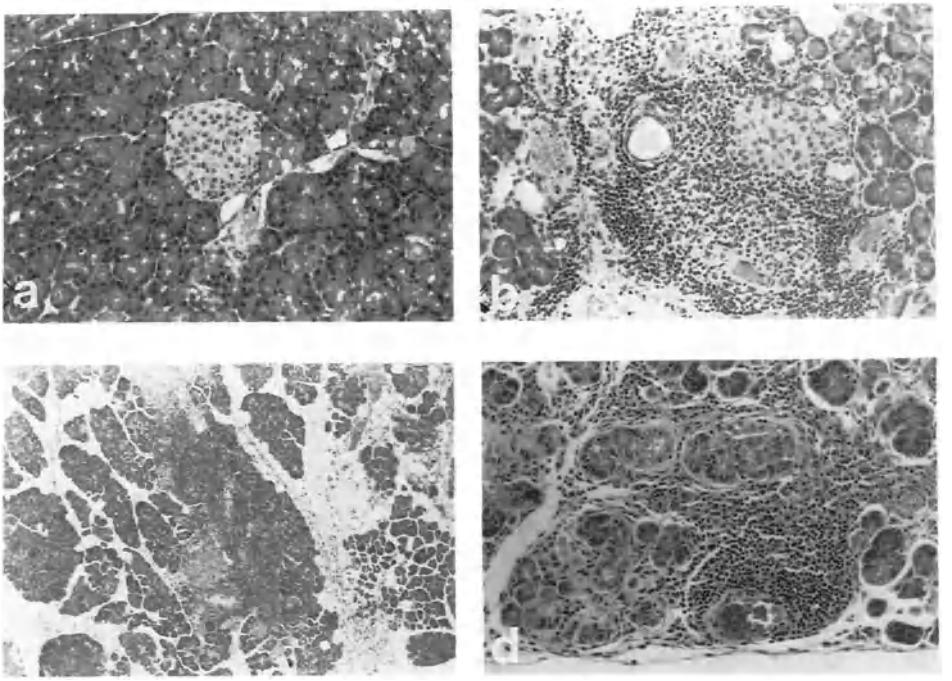
EFFECTS OF IFN-γ EXPRESSION BY PANCREATIC BETA CELLS

Three founder lines of INS-IFN-γ transgenics were created. The first line (461-2) showed signs of insulin-dependent diabetes mellitus between 10-20 weeks of age. The 462-4 line becomes diabetic with a variable latency starting at about 20 weeks of age. A third line (454-4) has never shown any signs of disease. Both diabetic lines can be maintained and bred on exogenous insulin.

Histological Analysis

We were curious to determine the histopathology of this disease. Tissues were fixed in 10% neutral buffered formalin embedded in paraffin, and sections stained with haematoxylin and eosin for morphogenic evaluation . We then examined the pancreas histologically to determine the cause of the beta cell malfunction. At the age of 20 weeks the 461-2 pancreas looks quite disorganized. Its structure has been disrupted by the influx of numerous inflammatory cells. These cells consist of lymphocytes and histiocytes. There is also a significant fibroblastic component to the disease which affects the older animals. The pancreas in these animals consists of exocrine tissue, widely dilated ducts, scattered endocrine cells, and inflammatory cells.

We next performed a sequential histological study of the progression of the lesion in these mice. At three weeks of age, histological examination of the pancreas in these



Histopathology of the INS-IFN-γ Transgenic Mice

Sections of the formalin fixed paraffin embedded tissue were stained with haemotoxylin and eosin. a. Normal BALBc pancreas showing an islet of Langerhans. b. Section of a three week old INS-IFN-g pancreas. Note lymphocytic infiltrate. c. Eight week old animal showing more widespread inflammation. d. Four month old animal showing marked duct cell proliferation and chronic inflammation.

animals revealed that at this time there exists an insulitis or perinsulitis. This infiltration was primarily lymphocytic and involved the islets and the area immediately surrounding them. In older animals the lesion became more widespread and involves the adjacent exocrine tissue.

Leukocyte Subsets within the IFN-γ Pancreas

The leukocyte subsets present in this infiltrate was then characterized. Cyrostat sections were reacted with an antibodies to leukocyte subsets and visualized by the immunoperoxidase technique using DAB as a chromogen. These studies determined that the lymphocytic infiltrate consisted largely of T lymphocytes. The majority of these infiltrating cells are helper T cells (70%) accompanied by cytotoxic T lymphocytes (25%). The remaining cells are macrophages. The cell types that produce the actual damage remain to be determined.

DISCUSSION

We have produced transgenic mice that express IFN-γ in the pancreatic islets. Two lines of transgenic mice developed of diabetes. The mechanism of the islet cell destruction in these transgenic mice is probably distinct from the INS-MHC transgenic mice which also became diabetic. In transgenic mice expressing MHC class II antigens, no inflammatory component is seen (Sarvetnick *et al.*, 1988).

The INS-IFN-γ transgenic mice suffer an inflammatory destruction of the pancreas leading to diabetes. This is undoubtedly a complex process. One component could certainly be an indirect chemotactic effect which allows the efficient infiltration of the pancreas with leukocytes. This effect is mediated through specialized vessels, high endothelial venules, which are found in the inflamed pancreas. High endothelial venules are normally found in organized lymphoid tissue. They are also reported to be found at sites of inflammation. The destruction of the tissue seems largely confined to and directed at the pancreatic islets, implying that a specific immune-mediated destruction of the islets may have been initiated. Late in the disease there is also a exocrine atrophy, fibrosis, and duct cell proliferation. Some of the damage might be initiated by the IFN-γ itself. This could be either separately or in combination with other lymphokines which are produced by the infiltrate. Other lymphokines have been demonstrated to have cytotoxic activity for beta cells *in vitro*. Studies are currently underway investigating the mechanism of islet cell destruction in these mice, which should illuminate the contributions of immune system to the development of this disease.

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Tolerance in Transgenic Mice to a Non-MHC Self-Protein is not a Result of Clonal Deletion

P.J. Whiteley and J.A. Kapp

INTRODUCTION

The immune system is designed to provide the host with mechanisms of self-defense against invasions of viruses, fungi, bacteria, and parasites. Under normal conditions, individuals do not develop overt immunity to autologous proteins. Thus, a cardinal feature of the immune system is its unique ability to distinguish between self proteins and foreign invaders ie "nonself". However, in some cases the immue system works in a deleterious manner and results in autoimmune disorders. Tolerance normally prevents lymphocytes that recognize self-proteins from causing autoimmune diseases; although the mechanisms by which tolerance to self-proteins is initiated and maintained are not well understood.

Three potential mechanisms have been proposed to explain how T cells maintain self-tolerance. In the first model, clonal deletion, T cells that bear the appropriate receptor are physically eliminated during ontogeny and maturation in the thymus (Schwartz 1978). In the second model, active inhibition, self-reactive T cells are present in the periphery but are functionally inhibited. The inhibition could occur via antigen-specific suppressor T cells (Tada 1984). In the third model, clonal anergy, the T cells are in the periphery but they are functionally inactivated, presumably through a failure in the recognition of a costimulator signal (Jenkins 1987). These mechanisms need not be mutually exclusive and evidence for all three have been found.

Tolerance to certain cell surface self-proteins such as MHC Class II molecules (Kappler 1987) the minor lymphocyte stimulatory (Mls) antigen (Kappler 1988), and the male antigen, H-Y (Kisielow 1988) have been studied. In these cases tolerance is maintained by deletion of the relevant clones in the thymus. Elimination of the T cell clones is feasible since all of these antigens are present on cells that are either in the thymus or traverse through the thymus during development. However, the mechanisms underlying tolerance to non-MHC self proteins have been difficult to examine under physiological conditions. Tolerance to autologous proteins that are not associated with the thymus could also occur via clonal deletion, but this seems unlikely as it would require

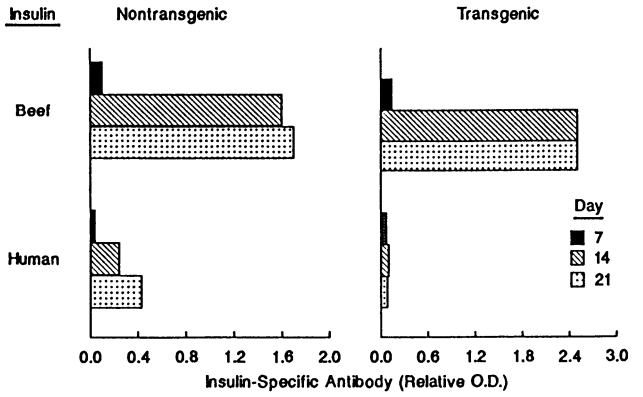
special mechanisms to insure that sufficient levels of all self-antigens reached the thymus at the appropriate time. It seems more likely that extra thymic (peripheral) mechanisms of tolerance could have developed. In fact, it has recently been shown that mature peripheral T cells rendered tolerant by intravenous injection of antigen are not deleted but are anergic (Rammensee 1989).

Many experimental models to study the development of tolerance involve injection of neonatal mice with a bolus of exogenous proteins (Siskind 1984). Although the neonatally tolerized mice remain nonresponsive to the antigen as adults, these models cannot directly address the mechanisms of tolerance induced by physiological levels of circulating protein antigens. We have used transgenic mice to directly examine mechanisms of tolerance to self-proteins that are not cell-surface molecules and are not synthesized by cells in the thymus. Our studies investigate the development of tolerance in transgenic mice that express the human preproinsulin gene. These mice have previously been shown to express the mRNA for human proinsulin, the precursor form of insulin, only in pancreatic islets (Selden 1986). In the islets the human proinsulin is enzymatically cleaved into insulin and C-peptide which are subsequently secreted in response to normal physiological signals. The transgenic mice secrete physiological amounts of insulin and C-peptide containing a mixture of both mouse and human proteins (Selden 1986).

INSULIN-SPECIFIC TOLERANCE IN TRANSGENIC MICE

Primary in vivo responses to human insulin have been characterized in normal (B10.BR x B10)F1 ($H\text{-}2^{k/b}$) and H-2 matched transgenic mice. Neither normal nor transgenic mice produce insulin-specific antibody in preimmune sera or seven days post intraperitoneal (ip) injection of human insulin in CFA (Whiteley 1989) (fig. 1). However, 14 days post ip injection of human insulin in CFA, normal F1 mice produced insulin-specific antibodies, whereas, transgenic mice did not. The lack of an antibody response in the transgenic mice is not due to a generalized defect in the immune system since they responded normally to beef insulin (fig. 1). Furthermore, tolerance to human insulin is complete and long-lived in the transgenic mice (Whiteley 1989). These observations provide direct evidence to support the hypothesis that under physiological conditions tolerance to self-proteins is acquired.

Fig 1. Primary immune responses In Vivo



Transgenic H-$2^{k/b}$ and syngeneic nontransgenic littermates were injected with 50 ug human or beef insulin in CFA. Sera was collected at 7, 14, and 21 days post injection and analyzed for insulin-specific antibody by ELISA. Data is expressed as the O.D of a 1:100 dilution of the sera relative to a known standard.

CELLULAR SITE OF TOLERANCE

The immune antibody response is composed of a complex set of cellular interactions involving B cells, T cells and antigen presenting cells. The next set of experiments were designed to determine whether B and/or T cells were nonresponsive to human insulin in the transgenic mice. B cell responsivneness to human insulin was assessed by comparing the avidity of insulin-specific antibodies for different species of insulins. Transgenic and nontransgenic mice were injected with beef insulin and their sera were analyzed for insulin-specific antibodies that bound to ELISA plates previously coated with beef insulin. A competition ELISA was performed to determine how well other species of insulin compete for binding of insulin-specific antibodies to beef insulin (Table 1). We found that all the species of insulin tested had comparable 50 percent inhibitory concentrations (IC_{50}). Thus, anti-beef insulin antibodies cross-react extensively with beef, sheep, pork, and human insulin. Furthermore, there were no differences in the IC_{50} of the various species of insulins between transgenic and nontransgenic immune sera. Thus, the B cells recognize all species of insulin, including human insulin, and they were not deleted in the transgenic mice.

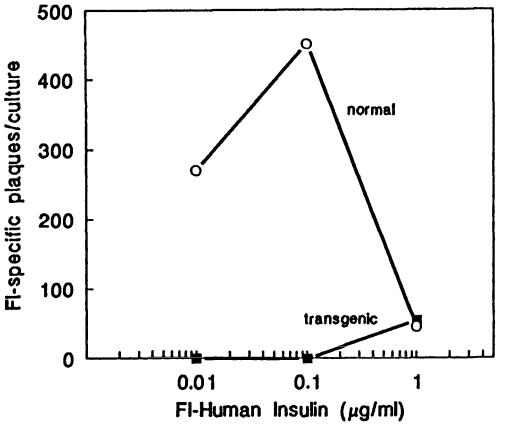
Table 1. Crossreactivity of insulin antibodies

	IC_{50} (ug/ml) Competitor			
	Beef	Sheep	Human	Pork
Transgenic	50±4	49±6	40±10	62±5
Nontransgenic	32±6	46±8	74±12	40±4

Transgenic and nontransgenic mice were immunized with 50 ug beef insulin in CFA. Sera was analyzed for insulin-specific antibodies by ELISA using beef insulin coated plates. Competition of binding was assessed by preincubation of the sera with various species of insulin prior to incubation on beef insulin coated plates. The data is expressed as the concentration of insulin necessary to inhibit 50 percent of the binding of the sera to a beef insulin-coated ELISA plate.

In contrast to the B cells, a striking difference was found in the helper T cell activity of lymph node T cells from human insulin-primed transgenic and nontransgenic mice. LN T cells derived from human insulin primed transgenic mice did not provide helper T (Th) cell activity for antibody responses to haptenated human insulin whereas, nontransgenic derived Th cells did (fig. 2).

Fig 2. Secondary immune response in vitro.



Lymph node (LN) T cells from human insulin-primed normal (B10.BR x B10)F1 and H-$2^{k/b}$ transgenic mice were cultured under Mishell Dutton conditions with B cells from normal syngeneic mice primed with fluorescein (Fl) ovalbumin and various concentrations of Fl-human insulin (Jensen 1984). Carrier specific helper T cell activity was assessed by enumeration of Fl-specific plaque forming cells (PFC).

Although the transgenic T cells did not provide help in a Mishell-Dutton assay, they developed a significant proliferative response to human insulin. Human insulin-primed LN T cells from transgenic mice have one-third the proliferative activity of primed nontransgenic LN T cells (Table 2). In addition, human insulin primed LN T from both transgenic and nontransgenic mice also responded to beef insulin.

Table 2. Proliferative response of insulin-primed LN cells

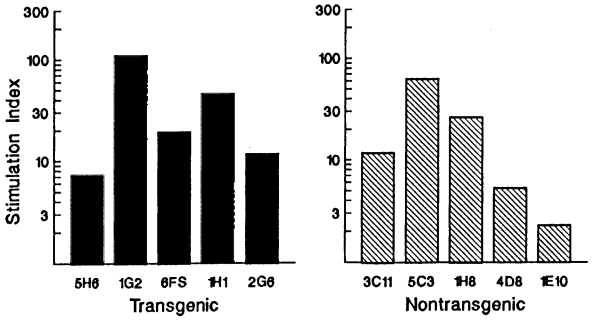
	Mean [3-H] thymidine incorporation in the presence of		
Primed LN T cells	No antigen	Human insulin	Beef insulin
Normal	1,787±531	34,101±2999	25,991±4452
Transgenic	3,307±760	15,467±2074	11,428±2199

Normal (B10.BR x B10)F1 and syngeneic transgenic H-$2^{k/b}$ mice were injected in the footpads with 50 ug human insulin in CFA. After 7 days the lymph node cells were harvested and enriched for T cells by panning (Jensen 1984). The LN T cells were incubated at 4 x 10^5 cells per well with or without insulin. After 4 days the cells were pulsed for 4 hours with [3-H] thymidine and harvested (Whiteley 1988). The data is the mean [3-H] thymidine incorporation from triplicate wells.

MECHANISM OF TOLERANCE

Results from the proliferative responses suggest that human insulin-specific T cells are present in transgenic mice. In order to further characterize the T cells that recognized self insulin we developed human insulin-specific T cell hybridomas by fusing human insulin-primed spleen cells to BW5147. Figure 3 shows the IL-2/4 production from representative hybridomas. Human insulin-specific T cell hybridomas were readily produced from both transgenic and nontransgenic mice. Furthermore, no differences in the responsiveness of the hybridomas from nontransgenic and transgenic mice have been detected.

Fig. 3. Il-2/4 production by hybridomas.



Splenic cells from transgenic and nontransgenic mice primed ip with human insulin in CFA were stimulated in vitro with human insulin; after four days the viable blasting cells were hybridized with the HAT sensitive fusion partner, BW5147 (Allen 1987). The resulting hybridomas were assayed, after stimulation with syngeneic antigen presenting cells in the absence or presence of human insulin, for lymphokine production using the IL-2/4 sensitive cell line HT-2. The stimulation index is equal to (mean cpm in the presence of insulin)/(mean cpm in the absence of insulin).

CONCLUSIONS

Our work demonstrates that transgenic mice which have circulating physiological concentrations of human insulin are tolerant to human insulin; whereas H-2 identical inbred mice are not. The tolerance is not a result of inactivation or clonal deletion of insulin-specific B cells. However, there were marked differences in the T cells between transgenic and nontransgenic mice. Human insulin-specific T cells are functionally inhibited in the transgenic mice. The nonresponsiveness of the Th cells is not a result of clonal deletion as we were able to produce insulin-specific Th hybridomas from both transgenic and nontransgenic mice. Thus, the tolerance of the human insulin-specific T cells, in the transgenic mice, must be due to functional inhibition of the helper activity. However, the mechanism by which these Th cells are inhibited is unknown. Furthermore, we have yet to determine if the nonresponsiveness of the Th cells is reversible. The lack of human insulin-specific Th activity, in transgenic mice, could be a result of an active antigen-specific suppression mediated by a human insulin-specific suppressor T (Ts) cell. If this were the case then removal of the antigen-specific Ts cells should unmask human insulin-specific Th cells. The possibility also exists that antigen-specific suppression is an irreversible event and that the affected Th cells are rendered anergic. Thus, clonal anergy could result from peripheral antigen-specific suppressor mechanism. Alternatively, clonal anergy may be induced by an unrelated mechanism not yet defined.

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Autoabzyme Catalyzed Cleavage of Vasoactive Intestinal Peptide

S. Paul and R.J. Massey

CATALYSIS BY ANTIBODIES

Antibodies, like other proteins, are versatile biological mediators. Traditionally, antigen binding has been thought to be the sole function of the antibody combining site. The forces underlying enzyme-substrate and antibody-antigen interactions are identical. It is now becoming evident that antigen binding by antibody can be followed by chemical transformation of the antigen. Elegant approaches have been employed to design antibodies that, like enzymes, can catalyze chemical reactions. These include: (a) generation of antibodies to the presumed transition states of chemical reactions (Tramontano, 1986), (b) introduction of a catalytic functional groups in the antibody combining site (e.g., a thiol) (Pollack *et al.*, 1988), (c) induction of antibodies complementary in charge to antigen (Shokat *et al.*, 1989), and (d) provision of metal cofactors to assist antibody-mediated catalysis (Iverson and Lerner, 1989). The immune system encounters many peptide and protein antigens. Efficient cleavage of peptide bonds by designer antibodies has been difficult to achieve. We have identified autoantibodies capable of catalytic cleavage of peptide bonds in vasoactive intestinal peptide (VIP).

HUMAN AUTOANTIBODIES TO VIP

Airway hyperresponsiveness in asthma may arise from an imbalance in autonomic mechanisms regulating muscle contraction and relaxation. VIP is the likely neural mediator of non-adrenergic, non-cholinergic relaxation of the airway smooth muscle (Said, 1987). A recent study suggests that nerves supplying the asthmatic airways are deficient in VIP (Ollerenshaw *et al.*, 1989). We found about 18 percent of adult asthma patients and 16 percent healthy subjects to be positive for plasma autoantibodies to VIP, mainly of the IgG class (Paul *et al.*, 1989a). The healthy individuals carrying VIP autoantibodies usually have a history of habitual muscular exercise (Paul and Said, 1988). Specificity of these autoantibodies for VIP is indicated by their low reaction with secretin, growth hormone releasing factor and peptide histidine isoleucine, peptides homologous to VIP. The mean VIP binding affinity of antibodies in asthmatics is about 60-fold higher than that of antibodies in non-asthmatic individuals. An antibody preparation from an asthmatic is shown to inhibit binding of VIP by receptors in the lung (Paul *et al.*, 1989a).

Candidate antigenic stimuli responsible for VIP autoantibody formation include: (a) viral epitopes similar in sequence to VIP (e.g., peptide-T, an epitope of the AIDS virus) (Ruff, *et al.*, 1987), (b) dietary ingestion of avian or fish VIP known to be structurally different from human VIP (Nilsson, 1975; Dimaline and Thorndyke, 1986), and (c) degradation fragments of VIP or larger precursor forms of VIP that may be more antigenic than VIP itself.

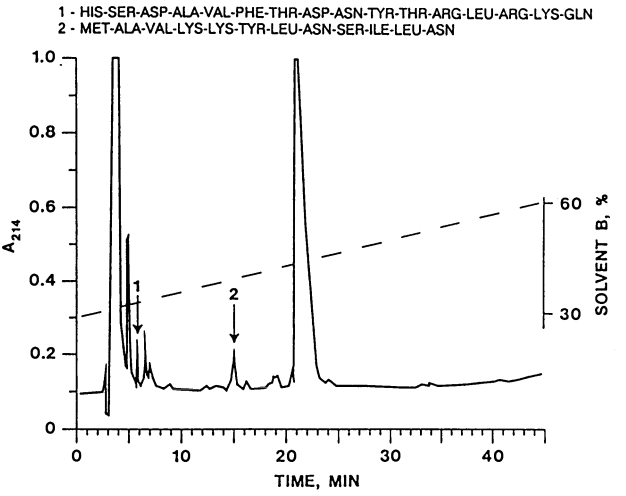

Fig. 1. Identification of VIP fragments produced by autoantibody mediated hydrolysis. The peptides labeled 1 and 2 were absent in a nonimmune IgG treated VIP preparation. Following purification by a second round of HPLC, 1 and 2 were identified as VIP(1-16) and VIP(17-28) by amino acid sequencing.

Table 1. Binding and catalytic characteristics of the anti-VIP autoantibody

Anti-VIP[a] Concentration (fmol/mg IgG)	K_d[a] (nM)	K_m[b] (nM)	k_{cat}[b] (min^{-1})	k_{cat}/K_m $min^{-1}M^{-1}$)
73.4	0.4±0.1	37.9±5.5	15.6±0.6	$4.1x10^8$

[a]Determined by Scatchard analysis of (Tyr^{10}-^{125}I)-VIP binding under conditions that did not lead to hydrolysis.
[b]Obtained from Lineweaver-Burke plots of (Tyr^{10}-^{125}I)-VIP hydrolysis by anti-VIP in the presence of increasing concentration of unlabeled VIP (7.5-480 nM).

Table 2. Induction of VIP hydrolytic activity in IgG

IgG Treatment[a]	(Tyr^{10}-^{125}I)-VIP (CPM hydrolyzed ± S.E.)
None[a]	-117 ± 156
Dialysis[a,b]	2461 ± 37
Ultrafiltration[a,c]	2346 ± 272
Washing on Protein G-Sepharose[a,d]	2662 ± 77
Purification on VIP-Sepharose[e]	1660 ± 289

[a]75 μg IgG per assay
[b]96 h with 3 buffer changes
[c]Two cycles (10 kD cut-off)
[d]1 mg IgG, bound to protein G-Sepharose, washed at neutral pH and eluted at pH 2.7
[e]Obtained by acid-elution; 100 ng specific antibody per assay

CATALYTIC HYDROLYSIS OF VIP BY AN AUTOANTIBODY

(Tyr^{10}-^{125}I)-VIP was used to study hydrolysis of the peptide by autoantibodies. IgG was prepared by chromatography on DEAE-cellulose and protein G-Sepharose columns. The (Tyr^{10}-^{125}I)-VIP was treated with immune IgG or IgG from a non-immune individual. Reverse phase HPLC showed that the treatment with immune IgG caused the appearance of an early eluting peak of the radioactivity (retention time - 10 min) distinct from intact (Tyr^{10}-^{125}I)-VIP (retention time - 25 min). When unlabeled VIP was used as the substrate, treatment with immune IgG produced two peptides (labeled 1 and 2 in Fig. 1) absent from a VIP preparations treated with non-immune IgG. Following purification by a second round of HPLC, these peptides were subjected to amino acid sequencing and fast atom bombardment-mass spectroscopy (Paul, *et al.*, 1989b). These studies indicated that the immune IgG cleaved a single peptide bond in VIP, viz., Gln^{16}-Met^{17}.

In order to characterize the hydrolysis of VIP further, a rapid assay method was developed. Precipitation with 10% trichloroacetic acid was used to separate intact (Tyr^{10}-^{125}I)-VIP from the radioactive fragment, VIP(1-16). The amount of radioactivity rendered TCA soluble by the IgG correlated well with the amount of radioactivity in the early eluting fraction (retention time - 10 min) seen in reverse phase HPLC of (Tyr^{10}-^{125}I)-VIP after treatment with the immune IgG. The Fab fragment of the IgG, prepared by papain treatment and purified by protein A-agarose chromatography, hydrolyzed (Tyr^{10}-^{125}I-)VIP in a dose dependent fashion. Specific autoantibodies were purified by affinity chromatography on VIP-Sepharose, and these antibodies were able to hydrolyze VIP (unpublished). Precipitation with anti-human IgG resulted in loss of most of the hydrolytic activity of the IgG (Paul, *et al.*, 1989b). VIP hydrolysis by the immune IgG was saturable, as seen in experiments in which (Tyr^{10}-^{125}I)-VIP mixed with increasing concentrations of unlabeled VIP was used as substrate. The reaction appeared to conform to Michaelis-Menten kinetics. Scatchard analysis of VIP binding by the IgG under conditions that did not result in hydrolysis of the peptide suggested the presence of a single type of antibody with a high binding affinity. Likewise the K_m value obtained from kinetic analysis of VIP hydrolysis was relatively low, suggesting tight binding of VIP by the antibody. For an antibody catalyzed, reaction the observed k_{cat} and k_{cat}/K_m values are impressive (Table 1). It is likely that the hydrolysis of VIP by the antibody is not greatly susceptible to product inhibition. This, coupled with the high binding affinity of the antibody, is probably responsible for the catalytic efficiency of this antibody.

The nucleic acid sequences coding for the CDR regions of IgG mutate with high frequency during maturation of the antibody response. In principle, substitution of reactive amino acid residues (e.g., Asp, His and Ser) for relatively non-reactive residues could confer catalytic activity to an antibody, provided antigen recognition is still maintained (these and other reactive amino acids are known to be involved in catalysis by enzymes). Naturally occurring catalytic antibodies could arise by such means.

Peptide bonds are relatively stable. Traditional approaches to design antibodies that can efficiently catalyze peptide bond hydrolysis have met with limited success. The study of the mechanism of VIP hydrolysis by autoantibodies is likely to be instructive in defining the rules governing efficient antibody-catalyzed peptide bond hydrolysis.

PATHOPHYSIOLOGY OF CATALYTIC VIP AUTOANTIBODIES

Systematic studies of the relationship between catalytic VIP autoantibodies and airway pathophysiology remain to be performed. The autoantibody described in this study was obtained from a healthy subject. Little or no VIP hydrolytic activity was present in the IgG fraction of this subject when tested directly after its purification on immobilized protein G (Table 2). The VIP hydrolytic activity was observed when the IgG was subjected to the following treatments: (a) two cycles of ultrafiltration, (b) extensive dialysis, (c) prolonged washing of the IgG at neutral pH when bound on protein G-Sepharose, and (d) chromatography on a VIP-Sepharose column. These data are consistent with the presence of a tightly bound, relatively small-sized inhibitor, the removal of which confers catalytic activity on the antibody. Although, the chemical nature of this putative inhibitor is not known, the induction of hydrolytic activity in autoantibodies purified on immobilized VIP suggests that it is probably active-site directed. We have recently identified a bronchitis patient positive for circulating VIP hydrolytic autoantibodies. IgG prepared from this patient was able to hydrolyze VIP without "activating" treatments listed in Table 2. It is not inconceivable that airway dysfunction may be related to the absence or presence of the putative inhibitor, rather than the catalytic antibody itself.

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Evidence Suggesting a Retroviral Etiology for Human Autoimmune Disease

N. Talal, M.J. Dauphinee, H. Dang, S. Alexander, and R. Garry

INTRODUCTION

Through recent scientific developments in our laboratory, two previously independent areas of research have come together leading to important new observations about retroviruses, germline immunoglobulin (Ig) genes and autoimmune diseases.

These new developments began with immunoblotting studies against HIV-1 proteins utilizing serum from primary Sjogren's syndrome (SS) patients (Talal et al 1989 and Talal et al, manuscript submitted [a]). Our interest in SS began 25 years ago with the report of lymphoma accompanied either by hypogammaglobulinemia or monoclonal immunoglobulins developing in this usually benign autoimmune rheumatic disease (Talal N, Bunim JJ 1964). A possible retroviral etiology for SS was first suspected when AIDS was linked to HIV-1. This interest grew stronger with the realization that many immunologic abnormalities are shared in common between patients with primary SS and patients with AIDS (Talal 1985).

For example, increased $CD5^+$ B cells occur in autoimmune mice (Herzenberg LA et al 1986) and also in SS (Dauphinee et al 1988) and in AIDS patients (Moody et al manuscript submitted). Abnormalities of transmembrane lymphocyte signalling revealed by exposure to PMA and ionomycin occur spontaneously in SS (Dauphinee et al 1989) and can be induced in normal peripheral blood lymphocytes (PBL) by exposure in vitro to HIV-1 (Linette et al 1988). There are now many examples of arthritic and dermatologic symptoms occurring in subjects infected with HIV-1 (Kopelman and Zolla-Pazner 1988). This includes approximately forty cases of an SS-like disease (including dry mouth and dry eyes, arthralgias, salivary gland and pulmonary lymphoid infiltrates, positive rheumatoid factor and anti-nuclear factor) in HIV-positive individuals (Schiodt et al 1989). These subjects can range from asymptomatic carriers through ARC to severe AIDS. In contrast to patients with primary SS, these HIV-infected patients lack anti-Ro(SS-A) and anti-La(SS-B).

RESULTS

We have recently found that 14 of 43 patients with primary SS are reacting immunologically to the p24 gag protein of HIV-1 (Talal et al 1989 and Talal et al, manuscript submitted [a]). Three patients also react to p17 gag. Four react with p24 gag of HIV-2. Specificity was confirmed by reactivity with recombinant p24. There was no reactivity with envelope proteins of HIV-1 or with any protein of HTLV-I. Clinically, these 14 patients showed a broad spectrum of disease (Table I) but, like HIV-infected subjects presenting with an SS-like illness, they lack anti-Ro-(SS-A) and anti-La(SS-B). This is important because anti-Ro(SS-A) and anti-La(SS-B) are expected in 50-75% of primary SS patients.

Table 1. Sjogren's syndrome patients positive for reactivity to p24

Patient	p24	Ro(SS-A)	La(SS-B)	Clinical Features
F.C.	2+	-	-	SS/SLE
M.C.	2+	-	-	Peripheral nueropathy
S.E.	2+	-	-	CNS
G.F.	2+	-	-	CNS
B.P.	2+	-	-	SS/SLE
B.R.	2+	-	-	Chronic renal failure (? cause)
M.Y.	2+	+	-	SS/SLE
K.B.	3+	-	-	Lymphoma
M.B.	3+	-	-	Bell's palsy
J.S.	3+	-	-	Pseudolymphoma
A.C.	4+	-	-	Polymyositis
B.F.	4+	-	-	Recurrent episodes of "mononucleosis"
D.T.	4+	-	-	Chronic fatigue
N.C.	5+	-	-	SS/SLE

A search for other diseases in which patients are reacting to p24 gag has been conducted. Twenty-one out of 60 SLE patients (35%), 10 out of 50 scleroderma patients (20%) and 4 out of 16 juvenile rheumatoid arthritis patients (25%) produce antibodies to p24 gag compared to 1 out of 115 normal subjects (< 1%). About 10% of patients producing anti-p24 gag also react to p17 gag. Thus, there is evidence of an antibody response to conserved gag proteins of HIV-1 in four different autoimmune rheumatic diseases.

In SLE (Table II), 19 of the 21 patients (91%) making antibodies to p24 gag (Talal et al, manuscript submitted [b]) also express an Ig idiotype (Id 4B4) previously identified in our laboratory on a human/human monoclonal antibody (Mab) prepared from PBL of an SLE patient (Takei M et al 1988). Both the serum of this patient and the Mab bind the small nuclear ribonucleoprotein (snRNP) called Sm. Id 4B4 localizes to the CDR2 region of the Ig H chain and is conserved between species since it is also present on a murine Mab prepared from the spleen of an autoimmune MRL/lpr mouse. We cloned the gene for this Id and find it to be VH_{III} and entirely germline (Sanz et al 1989).

Table 2. Systemic lupus erythematosus patients positive for antibodies to retroviral gag protein p24

Patient	p24	ID 4B4	Sm antibody
T.T	2+	+	-
J.K.	2+	+	+
A.C.	2+	+	+
I.T.	2+	+	-
C.C.	2+	+	-
F.C.	2+	+	-
B.P.	2+	+	+
M.Y.	2+	+	-
G.A.	2+	+	+
S.P.	2+	-	+
R.M.	2+	-	-
M.S.	2+	+	-
G.T.	2+	+	-
M.Z.	2+	+	-
J.E.	2+	+	-
C.O.	2+	+	-
I.V.	3+	+	-
F.G.	3+	+	-
G.W.	4+	+	+
N.C.	5+	+	+
S.C.	5+	+	-

Seven of the 21 SLE patients reacting to p24 gag also bind Sm, suggesting cross-reactivity between the retroviral gag protein and the snRNP. Evidence in support of cross-reactivity came from competitive inhibition experiments demonstrating that Sm inhibited the binding of p24 in SLE serum. Anti-Id 4B4 antibodies were also able to inhibit p24 binding. These results suggest that autoantibodies to Sm arise through a mechanism involving "molecular mimicry" and that the immunogen for this response may be viral rather than host in origin.

DISCUSSION

We interpret these findings to suggest that approximately 20-30% of patients with primary SS are reacting immunologically to retroviral gag proteins. We have recently succeeded in growing such a retrovirus from salivary gland biopsy material removed from two patients. The virus buds intracisternally and can be identified as an A-type retrovirus by electron microscopy. It is more dense (1.22 gm/cm^3) than is the AIDS virus (1.16 gm/cm^3). Characterization of the reverse transcriptase (RT) produced by this virus shows it to be different from the RT of HIV-1 by virtue of having a Mn^{++} rather than a Mg^{++} dependency.

The following points emerge from the SLE studies: 1) SLE patients, like SS patients, react to p24 gag of HIV-1; 2) Sm may be the internal image for p24 gag, 3) "autoantibodies" may arise by immunization to viral rather than to self antigens, and 4) there is a relationship between immunity to p24 gag and germline

genes as revealed by Id analysis. The latter point raises the possibility that certain germline genes may predispose to immunization against retroviruses.

These results suggest that autoimmune disease in some patients has an infectious etiology. Our results to date indicate that this may be true for approximately 20-30% of patients with several different autoimmune diseases. The most important next step is to better characterize the virus or viruses that cause these infections. Preliminary results suggest an A-type retrovirus in two patients with primary SS. Evidence for a retrovirus has also recently been found by Southern hybridization analysis in Graves' Disease (Ciampolillo et al 1989). If these results continue to find support in other laboratories, treatment of autoimmune disease patients with AZT and other forms of anti-retroviral therapy becomes a strong possibility.

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III Effector Phases

Suppression

Is Genes in the Mouse

N.A. Mitchison

Progress in understanding suppressor T cells since the last International Congress can be summarised in the following terms. The phenomenon of suppression of this type has not been seriously challenged, and instances of suppression continue to accrue. Some are in areas of clinical relevance, such as organ transplantation (Gassel 1987). Others are in branches of immunology opened up by molecular genetics such as the new array of mycobacterial antigens (Ivanyi 1989). There have also been setbacks. For instance one of the best pointers to suppression has been dominant inheritance of unresponsiveness. MHC-controlled dominant unresponsiveness to a particular antigen never provided more than a clue that suppression might be operating, but nevertheless it served as a source of encouragement. Now it has become clear that a second major cause of such unresponsiveness operates through deletion of large segments of the T-cell-repertoire by negative selection in the thymus (McDuffie 1988). MHC molecules are powerful inducers of tolerance, and as such can delete the T-cell response not only to themselves but also to other antigens. This discovery significantly weakens the value of dominant unresponsiveness as a clue for suppression. Another setback has been in the area of suppressor epitopes. Sercarz and his colleagues have pioneered study of these structures; that group has now introduced the concept of a 'processing epitope', which can influence the ability of antigen-presenting cells to present other epitopes on the same protein (Sercarz 1986 and pers. comm.). A processing epitope that inhibits processing behaves much like a suppressor epitope, and detailed analysis will be needed to sort out one from the other. In summary, much of what was taken as evidence of suppression will need to be reevaluated, and the number of instances of suppression may shrink in consequence.

In terms of molecular mechanism, nothing has yet been discovered that is unique to suppressor T cells. It remains entirely possible that these cells express their function exclusively as a result of the context within which they operate, and thus that the term 'suppressor' has a semiotic rather than a mechanistic meaning. Indeed as time passes the likelihood of a unique mechanism being discovered diminishes, although vigorous attempts to discover new molecules continue.

This summary is expanded in Tables 1-5, which present the main features of antigen-specific suppresion mediated by T cells as they are at present understood. Discoveries made since the last Congress, or substantially expanded since then, are marked with an asterisk, and this discussion will focus on them. References to earlier work may be found in reviews (Germain 1981; Oliveira 1989; Mitchison 1989).

Table 1. Main features of suppression (a) at the level of the overall response

(1) Suppression occurs fairly frequently
(2) Occurs particularly with
- large doses of antigen, especially when administered intravenously as 'clean' protein
- chronic exposure to antigen ("sprinkling"). Also in prolonged anti-transplant reactions
- oral administration of antigen
- cell-bound antigen
* - 'idiosyncratic' responses

* (3) Is genes often determine suppression
(4) Certain antigens, e.g. F liver protein, tend to induce suppression
(5) Major feature of certain diseases, e.g. lepromatous leprosy
(6) Not important in tolerance-of-self, but can counteract autoimmunity

* new information since last Congress

In Table 1, we note that progress has been made in delineating Is genes. So far all of these genes, whether well established or only hinted at from immunogenetical studies, map to MHC Class II; there,presumably, they either mediate activation of suppressor-inducer cells, or of suppressor cells that operate more directly through the pathways shown below in Table 6. The first move in bringing order to a previously haphazard collection of genes was made by J. Klein and his collaborators, who proposed that suppression is preferentially mediated by H-2E genes in the mouse (Baxevanis et. al. 1982). T. Sasazuki has similarly developed the hypothesis that HLA-DQ preferentially mediates suppression in man, a subject of his accompanying paper at this meeting. As H-2E and HLA-DQ are not homologous, these hypotheses imply that suppressor function has flipped between loci during the course of mammalian evolution. For this reason and others Oliveira and I have proposed that specialization of function among Class II loci has evolved largely in response to hypersensitivity generated by chronic infection (Mitchison 1987); and we have recently reviewed the numerous instances of putative H-2E-restricted suppression (Oliveira 1989). The main evidence for this view, the apparent specialization of the H-2E locus, is however now seriously flawed by the possibility mentioned above that tolerance rather than suppression may mediate some of its effects. The present situation is outlined in Fig. 1. Just how much true H-2E-restricted suppression will eventually be left is unclear; the final answer will presumably emerge from the kind of experimentation mentioned at the foot of the figure.

Fig.1 PRESENT STATUS OF THE CLASS II SPECIALIZATION HYPOTHESIS

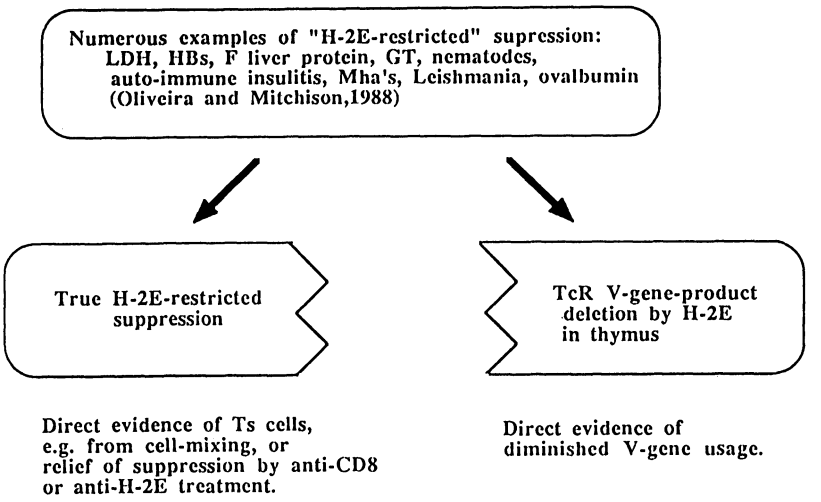


Table 2. Main features of suppression (b) at the cellular level

(1) Inducer cells ("Ts1") usually CD4, effector cells ("Ts2") usually CD8
(2) Targets of suppressor effector cells are usually helper T cells
* (3) Markers (e.g. CD45R, Leu8) and lymphokine profile (e.g. IFNγ secretion) characterise Ts in some systems
(4) A third effector population ("Ts3") mediates suppression of DTH
(5) Antigen-presenting cells: unclear. Depletion of APC claimed to facilitate suppressor induction, but APC specialised for suppression also claimed. Strong UV effect
* (6) Ts clones and hybridomas increasingly available

* new information since last Congress

Two new features at the cellular level are mentioned in Table 2. The subject of markers has been evolving rapidly. Leu 8 is in some ways a particularly valuable marker, but it has features that make it hard to handle and in practice its use has hardly extended out of California (Damle 1987). CD45R on the other hand has been the subject of extensive research in man (Merkenschlager 1988), rat (Powrie 1989), and mouse (Marvel 1988). At one time it was hoped that the CD45R isoforms would provide a lineage marker of a T-cell subset that would include all suppressor-inducer cells. That view is no longer tenable, and CD45R now emerges as a marker of 'virgin' T cells that is lost upon activation (Merkenschlager 1988; Powrie 1989). That of course does not prevent CD45R from associating with functional activities (such as suppression), or lineage markers (such as H-2E or HLA-DQ) (Mitchison 1988). This is only

speculative, for little progress has been made in broadening the analysis of suppresion mediated by CD45R cells, and none at all in determining whether this marker is expressed preferentially on T-cells restricted by particular MHC genes. As for the lymphokine secretion profile of suppressor cells, a clearer picture is beginning to emerge from the excitement and confusion over the Th1 and Th2 subsets that were first described at the last Congress. That aspect of suppression will be dealt with by Dr M Berton in this session of the Congress.

The main advance in understanding the role of antigen presenting cells has come from studies on ablation of the 'second signal' given by these cells (Jenkins 1988). While not yet known to lead to the induction of suppressor cells, it seems likely that some such mechanism may be involved.

More and more hybridomas and clones of suppressor T cells have successfully been made, although clear-cut recipes for how to do so are still needed (Kuchroo 1988). One is always impressed by results from one's own laboratory; successive colleagues have had little difficulty in generating Ts cells specific for minor transplantation antigen (Mha's) by injecting CBA mice intravenously with 10^8 B10.BR spleen cells and now N. Nanda and E. Thompson have been able repeatedly to grow these Ts cells as lines and clones (pers. comm.). An outstanding puzzle is how a clone can mediate a function associated with both CD4 and CD8, which would normally be expected to involve two cells.

Table 3. Main features of suppression (c) biochemistry

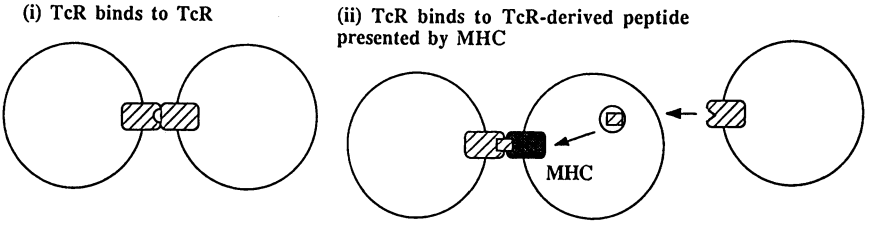
*(1) Ts clones, hybridomas are mainly (?all) $CD3^+$, have normal TcR rearrangements
(2) Several examples of normal MHC restriction known
(3) IJ still highly controversial. Molecular genetics unclear; may be an epitope of H-2E or $H\text{-}2E^-$ receptor; or, alternatively, may be an auxilliary molecule like CD4 but with polymorphism under MHC epistatic control ("epirestriction")
(4) Influence of IgV genes ("epirestriction") and of Ig-idiotypes
(5) Direct binding to antigen (usually but not always amino-acid copolymers or haptens)
(6) Soluble antigen-specific factors ("TsF's")

Progress in the biochemistry of suppression (Table 3) has been slower, as also has been the study of suppressor factors or 'TsF's' (Table 4). Several lines of suppressor cells that have been growing in vitro have been investigated for TcR gene structure, and most of them turn out to have conventional α and β rearrangements (Kuchroo 1988; N Nanda and I Mills pers. comm.). Unrearranged or deleted genes do not turn up as often as the first reports suggested, and it is becoming clear that the $\gamma\delta$ receptor is not preferentially expressed by suppressor cells.

Restriction by I-J and by IgV genes remains an area where striking observations have been made but not matched by unequivocal interpretations. At the last Congress I proposed that both these phenomena should be interpreted as network effects, and indeed published a picture of how that part of the network might operate (Mitchison 1986). The term 'epirestriction' was offered there to describe phenomena in which the restricting element is encoded by

one gene-complex (e.g. the TcR genes), but is controlled in its expression by another gene-complex (e.g. IgV genes). My ideas at that time were guided by T. Tada, who since then has come to regard the IJ product more as an auxillary molecule along the lines mentioned in Table 3 (Nakayama 1989). Immunologists interested in this question eagerly await resolution of the claim made for an impact of Ig-transgenes on the network (Weaver 1986).

Fig. 2 POSSIBLE MECHANISMS OF RECOGNITION BY T CELLS OF T-CELL-IDIOTYPES



As the importance of this aspect of the network grows, a resolution of the question posed in Figure 2 becomes more urgent. My figure at the last Congress showed one TcR binding to another, as inalternative (i) in Figure 2. That reflected ideas generally current at the time, and is certainly easier to reconcile with the repeated finding that TcR's on suppressor T cells, or the TsF's which they release, can bind to anti-IgV-idiotypic antibodies (O'Hara 1988). But as the implications of the Bjorkman-Wylie model sink in, alternative (ii) in the figure becomes more attractive. This is a matter that I. Cohen's work (presented in this session) bears on, and that has been discussed before (Batchelor 1989) and by A. Livingstone (pers. comm.). Perhaps their research will resolve this deep issue. In the meanwhile, the network continues mysteriously to haunt suppression, and vice-versa.

Table 4. Main features of suppression (d) TsF's

(1) Bind antigen, id^+
(2) IJ^+
(3) ~65KD, can be dissociated into 2 chains, one antigen-binding and the other IJ^+
(4) Little progress. Possibility that TsF's are cleaved TcR's not excluded. Very unlikely that TcR transcripts could be processed to encode a non-membrane-anchored form

Table 5. Suppressor epitopes

1. Of great potential practical value. Amputation of such epitopes might improve vaccines against infectious disease, and conjugation might improve vaccines against immunological disease.

2. Several such epitopes have been described, but none have yet passed all the tests, such as suppressing by conjugation what would otherwise be an immunogen.

3. At present the search for such epitopes remains largely empirical, but rules may emerge, such as that these epitopes resemble those recognised by B cells.

*4. For most of the presently known such epitopes, it is unclear whether they act by engaging appropriate TcR's (i.e. at level of repertoire), or by affecting antigen processing (i.e. at level of APC's).

5. Molecular genetics is likely to facilitate studies on amputation and conjugation. However use of recombinant antigens will bring problems, for instance in preparing proteins clean enough for intravenous administration in large doses.

Table 5 deals with suppressor epitopes. S. Char, J. Colston, K. Simon and I have tried over the last two years to contribute to this subject using the p65 antigen of Mycobacterium leprae, a protein that has the desirable features of being a strong antigen froman infectious agent known to generate specific immunosuppression, of having well-worked molecular genetics, and of having its structural gene available in a production plasmid. We found it difficult to isolate recombinant p65 in a form clean enough to use for induction of suppression. Yes, molecular genetics has much to offer for epitope analysis, but suppression may alas be particularly unamenable to this approach because of the need for non-denatured protein.

Fig. 3 POSTULATED SUPPRESSOR MECHANISMS

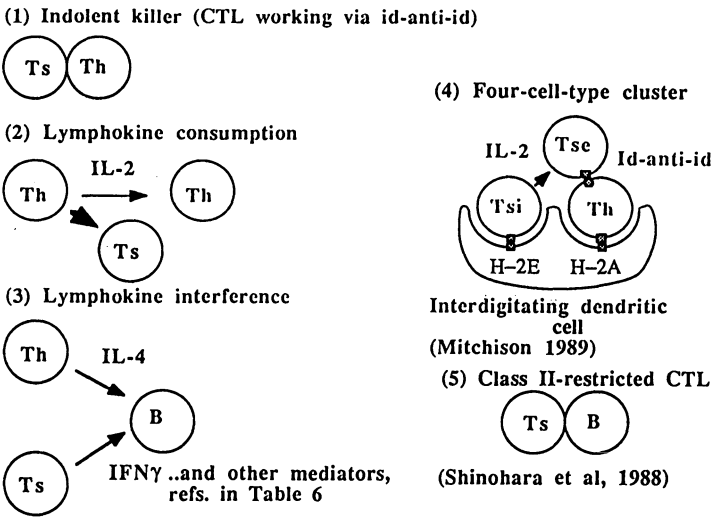


Table 6. Down-regulatory antigen-non-specific mediators and adhesion molecules

TGF-β (Ranges 1987)
IFN-γ (Pene 1988)
Soluble FcR's (Ishizaka 1988; Unkeless 1988)...

CD29 (Groux 0989)
CD45 (Alexander 1989; Fisher 1989)...

My last topic is the mechanism of suppression. Fig. 3 sets forth five of the possibilities that have been most widely discussed and Table 6 expands the list of mediators and adhesion molecules that can mediate suppression within various experimental systems in vitro. Although their activities are not antigen-specific, these mediators could implement epitope-linkage (i.e. produce suppression only when suppressor and helper epitopes are presented on the same structure) provided that they function within a 3-cell-type cluster of mediator-secreting cell, target cell, and dendritic cell (Mitchison 1987). Thus even though non-specific mediators may play a key role, the contacts that a cell makes would determine which target gets suppressed. Adhesion molecules provide another example of how a non-specific mechanism can be given direction by antigen-mediated cell contact. Of special interest is CD45, where an internal balance between phosphatase and phosphokinase activites seems to control activation of T cells. In a more general sense, negative signals from mediators and adhesion molecules may act at every conceivable level, from receptor down-regulation through second messengers to nuclear events. This is an active area where we can expect progress to be rapid.

It is something of a criticism of our subject that so little progress has been made in resolving these very different mechanisms shown in Fig. 3. Furthermore the figure underates the variety, for TsF's have not been included. This omission stems from deep uncertainty about the in vivo significance of these factors, and also from the difficulty in drawing any schematic picture of them until the question posed in the previous figure (Fig. 2)has been answered. Another mechanism not shown is the veto cell. At the height of the Nagy-Klein theory of suppression it looked as though vetoing might fit nicely into the overall framework of suppression, for the theory postulated that a helper cell recognised antigen (LDH) plus MHC Class II on the surface of a mouse suppressor T cell and died in consequence (Baxevanis et. al. 1982). In the continuing absence of evidence for Class II expression on mouse T cells that theory has faded. The views on this matter of R. Miller, the original proponent of the veto cell, can be found in his accompanying paper.

Most of the possibilities here set forth rest on experiments performed in vitro. Our main task now is to determine which, if any, of these mechanisms can account for the phenomena of suppression as they are encountered in human disease and in model experiments in mice. Progress in this area will be much needed before our next Congress.

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Differential Roles of HLA-DR and DQ in Immune Regulation

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INTRODUCTION

Whether or not there are suppressor T cells which regulate the immune response, in experimental animals and in humans has remained controversial. One of the major problems in studying immune suppression has been difficulty in obtaining lines or clones of a suppressor T cell or a helper T cell for suppression which would facilitate acquisition of information essential to understanding the immune suppression at cellular and molecular levels. We have postulated the existence of HLA-linked immune suppression genes (Is-genes) in humans which control the low or nonresponsiveness, through antigen specific suppressor T cells. We summarize here evidence for these HLA-linked Is-genes, and related cellular mechanisms were elucidated using T cell lines of a suppressor T cell or a helper T cell for suppression.

HLA-DR AS THE IR-GENE PRODUCTS

The H-2 linked immune response genes (Ir-genes) were first described by McDevitt et al.(1972), and Benacerraf and McDevitt (1972). It has long been assumed that these Ir-genes are present in humans. Indeed there is little doubt that HLA-DR molecules act as products of the HLA-linked Ir-genes in high responders, because (1) HLA-DR molecules are restriction elements in interactions between CD4+ helper T cells and antigen presenting cells (APC) to respond to various antigens such as streptococcal cell wall antigen (SCW) (Nishimura and Sasazuki 1983; Sone et al. 1985; Hirayama et al. 1986) schistosomal antigen (Sj) (Hirayama et al. 1987) *Mycobacterium Leprae* antigen (ML) (Kikuchi et al. 1986) and so on, and (2) anti-HLA-DR monoclonal antibodies abolish the immune response to the antigens (Nishimura and Sasazuki 1983; Sone et al. 1985; Hirayama et al. 1986; Kikuchi et al. 1986; Hirayama et al. 1987).

EVIDENCE FOR THE PRESENCE OF NONRESPONDERS TO SEVERAL ANTIGENS IN HUMAN POPULATIONS

Fig.1 summarizes the immune response to natural antigens in human populations. There is a small area in Japan where schistosomiasis is endemic. Individuals infected with Schistosoma Japonicum were divided according to their response to Sj into two groups, high and low responders (Sasazuki et al. 1980 b). A small proportion of the high responders developed postschistosomal liver cirrhosis whereas the low responders did not (Ohta et al. 1982). Healthy individuals

showed either a high or a low T cell response in vitro to SCW (Sasazuki et al. 1980a). In case of leprosy there are at least two well defined clinical types, tuberculoid and lepromatous leprosy. Peripheral blood lymphocytes (PBL) from patients with tuberculoid leprosy showed a vigorous T cell response to ML in vitro, whereas the T cell response was not observed in patients with a lepromatous leprosy (Kikuchi et al. 1986). This nonresponse of patients with lepromatous leprosy to ML was antigen specific, because the patients show a strong T cell response to other antigens such as SCW and cedar pollen antigen (CP). In the early spring about 10 to 15% of Japanese individuals suffer from type I allergy, cedar pollinosis, following extensive exposure to pollens from Cryptomeria Japonicum. In most of the unaffected individuals there was no appreciable IgE response provoked in the presence of purified antigen from cedar pollens whereas the affected individuals showed evidence of IgE antibody production to the antigen (Matsushita et al. 1987). All these observations clearly indicate that there are low or nonresponders in human populations to several antigens, after natural exposure to these antigens.

Since we measured the immune response after a natural exposure, we could not control the dose of antigen and the period of exposure. To overcome these problems we investigated the immune response after planned immunization(Fig. 1). Two weeks after the injection of tetanus toxoid (TT) 84.8% (78/92), medical students showed a strong T cell response to TT in vitro yet 15.2% showed no response (Sasazuki et al. 1978). In the case of hepatitis B vaccine, we measured the IgG response to hepatitis B virus surface antigen (HBs), both in vivo and in vitro. Even after the 3rd immunization 22.4% (19/85), medical students showed no evidince of antibody production (Watanabe et al. 1988). Thus, we conclude that there are nonresponders to several antigens in the human population, even after planned immunization.

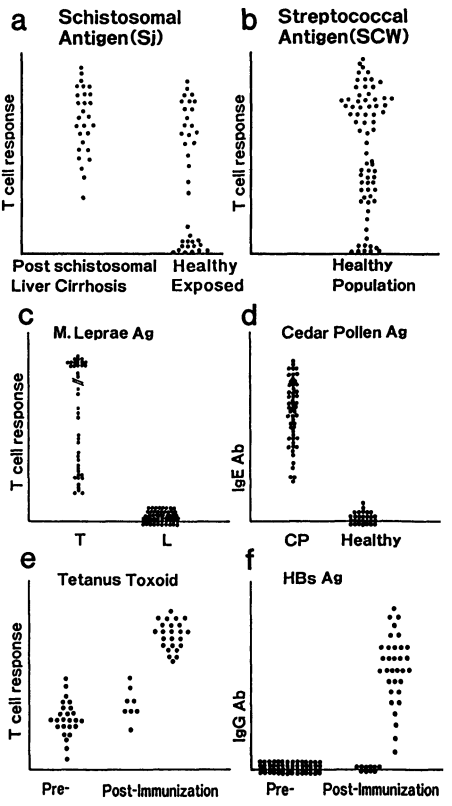

Figure 1. Polymorphism of immune responsiveness to natural antigens or vaccines in humans. Proliferative response of peripheral T cells specific to Schistosoma Japonicum antigen (a), streptococcal cell wall antigen (b), *Mycobacterium Leprae* antigen in patients with

tubercloid leprosy (T) or lepromatous leprosy (L) (c) or tetanus toxoid in healthy individuals pre and post immunization (e). IgE levels specific to cedar pollen antigen among patients with cedar pollinosis, or healthy controls (d). IgG levels specific to hepatitis B virus surface antigen in healthy vaccinees, pre and post immunization(f).

EVIDENCE FOR THE HLA-LINKED DOMINANT TRAIT GENETIC CONTROL OF NONRESPONSIVENESS

T cell response to SCW was measured in vitro and the correlation coefficiency in the immune response to SCW between monozygotic twins was 0.79, whereas that between dizygotic twins was less than 0.40, thesеby indicating that the immune response to SCW was controlled genetically. Genetic analysis of the immune response to SCW in 23 families revealed that nonresponsiveness is a dominant genetic trait, closely linked to HLA (Sasazuki et al. 1980a; Sasazuki et al. 1983). Genetic analysis of the response to HBs using the formula of Thomson & Bodmer also suggested that the nonresponse to HBs was a dominant genetic trait closely linked to HLA (Watanabe et al. 1988). As shown in Table 1, the statistical association between nonresponsiveness to various antigens and HLA-class II alleles also confirmed genetic control of the immune response and suggested that genes controlling low responsiveness to those antigens may be mapped within the HLA class II region.(Sasazuki et al. 1978; Sasazuki et al. 1980b; Sasazuki et al. 1983; Kikuchi et al. 1986; Watanabe et al. 1988).

Table 1. HLA-linked immune suppression genes

Antigen	Immune response observed as	Mode of inheritance of low responsiveness	Marker of suppressor T cell	Linkage with	Linkage disequilibrium with
SCW	T cell proliferation	Dominant[a]	CD8	HLA[a]	DR2,DR5,DQw1
Sj	T cell proliferation	(Dominant)	CD8	HLA	DR2-DQw6-Dw12
ML	T cell proliferation	Dominant	CD8	HLA	DR2-DQw6-Dw2
CP	IgE response	Dominant[a]	CD8	HLA[a]	DQw3(negative)
HBs	IgG response	Dominant	CD8	HLA	DR4-DRw53-DQw4-Dw15

a The HLA-linked dominant inheritance of low responsiveness was elucidated in a family study.

POSSIBLE MECHANISMS OF NONRESPONSIVENESS CONTROLLED BY HLA-CLASS II GENES.

The nonresponsiveness controlled by HLA-class II genes can be explained by at least four mechanisms, as follows. (1) Lack of Ir-genes: HLA-class II molecules of nonresponders cannot bind processed antigen (Buus et al. 1986; Ceppellini et al. 1989). (2) Cross tolerance: HLA class II molecules of nonresponders share epitopes with the antigen in question. (3) Clonal deletion of T cell repertoire: HLA class II molecules of nonresponders eliminate certain T cell clones during intrathymic differentiation resulting in a "hole" in the T cell repertoire (Kisielow et al. 1988; Marrack et al. 1988). (4) Suppression: HLA classII molecules of nonresponders generate an active suppression (Araneo and Kapp 1980).

If nonresponsiveness was due to the lack of HLA-linked Ir-genes, nonresponsiveness should be recessive and responsiveness should be dominant. Genetic analysis revealed that nonresponsiveness is dominant, therefore, the first suggested mechanism can be ruled out. If nonresponsiveness is due to cross tolerance or to clonal deletion, restoration of the immune response in nonresponders cannot be expected. Only when nonresponsiveness is due to active suppression, restoration of the immune can response be expected, by a blockage of active suppression. Therefore, we focused our investigations on the restoration of immune response in nonresponders.

EVIDENCE FOR THE ACTIVE SUPPRESSION.

After removal of CD8+ T cells from PBL, even nonresponders showed a strong immune response, an observation which suggested that even nonresponders have CD4+ T cell clones to respond to antigens in question (Nishimura and Sasazuki 1983; Ohta et al. 1983; Kikuchi et al, 1986: Matsushita et al. 1987). When we put the CD8+ T cells back into the culture system, the response was abolished.

EPISTATIC INTERACTION BETWEEN HLA-DR AND DQ TO CONTROL THE IMMUNE RESPONSE.

It is interesting to note that monoclonal antibody directed against HLA-DQ also restored the immune response to Sj, in vitro and in nonresponders (Hirayama et al. 1987). This observation provides a good contrast with the finding that monoclonal antibody to HLA-DR completely block the immune response. Furthermore, the restored immune response of the nonresponders after depletion of CD8+ T cells was also blocked by the anti-HLA-DR monoclonal antibody. Thus, HLA-DR2-DQw6 (Dw12) controls the nonresponse to Sj, but even the nonresponders with HLA-DQw6 (Dw12) showed a potent immune response to Sj after the removal of CD8+ T cells, this response was abolished by anti-HLA-DR monoclonal antibody, thereby suggesting that HLA-DR2 on the HLA-Dw12 haplotype acts as the Ir-gene for Sj in the nonresponders. The nonresponse to Sj is therefore not due to lack of the Ir-gene but to the presence of an active suppression mediated by CD8+ T cells most likely generated by HLA-DQw6, as the immune suppression gene.

DIFFERENCE IN STRUCTURE, EXPRESSION AND CONTRIBUTION TO MLR BETWEEN HLA-DR AND DQ

HLA-DR and DQ belong to the HLA-D multigene family, together with DP, DO and DN. The α chains of DR and DQ exhibited a 64% homology in amino acid sequence and the β chains of DR and DQ exhibited a 68% homology. The majority of DQ β chains are eight amino acids shorter in the cytoplasmic tail due to base substitution from G to A right before exon5, in comparison to DRβ or murine β chains of I-A and I-E, even though DQ β chains from HLA-Dw12, Dw9 and DB7 utilize exon5 because of no base substitution (Tsukamoto et al. 1987). Expression of the DQ molecules on B cells, macrophages and activated T cells is much less than that of DR molecules, a finding which may be explained by the base substitution in the promoter region of DQ α gene; one base substitution from C to T in the X box and from G to A in the Y box within the 5' flanking region which controls expressivity of the DR and DQ genes (unpublished observations).

In the allogeneic mixed lymphocyte reaction (MLR) DR is the major element related to stimulation, with DQ contributing little, if at all. On the other hand, when the frequencies of precursor T cell reactive to class II molecules are estimated in the presence of recombinant IL-2, utilizing murine L cells transfected with HLA class II genes and a limiting dilution analysis, the frequencies of precursor T cells reactive to autologous or allogeneic DQ molecules were unexpectedly as high as that of precursor T cells reactive to allogeneic DR molecules (Fujisawa et al. submitted). Because the expressivity of HLA-DQ on B cells and monocytes is smaller than that of HLA-DR, the contribution of HLA-DQ molecules in allogeneic MLR becomes smaller than that of HLA-DR molecules, despite the high frequency of DR reactive precursor T cells. Thus, HLA-DR and DQ possess significant differences in structure, expression and function.

DISTINCT ROLE OF HLA-DR AND DQ IN IMMUNE REGULATION

To investigate the role of HLA-DQ in the low responsiveness to SCW, antigen specific T cell lines were generated from high and low responders, as described (Hirayama et al. 1986, Sone et al. 1985). The restriction molecules of these T cell lines were identified using murine L cells transfected with HLA-class II genes. In high responders, DR molecules were mainly recognized in the context of SCW by T cell lines. On the other hand, DQ molecules as well as DR molecules were used as restriction molecules in low responders. Therefore, the SCW specific T cell lines established from low responders were a mixture of T cell lines restricted by DR or DQ molecules. A small proportion of $CD8^+$ T cells was also found.

These T cell lines from low responders were co-cultivated with SCW, IL-2 and irradiated allogeneic PBL which shared HLA-DR or DQ with the donor of the T cell lines, to establish T cell lines restricted by either HLA-DR or DQ. After this selection, T cell lines were co-cultivated with irradiated autologous PBL as the source of APC. $CD3^+$ $CD4^-$ $CD8^-$ and $CD3^+$ $CD4^-$ $CD8^+$ T cells were propagated in the HLA-DQ restricted T cell lines and the T cell line consisted of $CD3^+$ $CD8^+$ T cell receptor(TCR)αβ, $CD3^+$ $CD8^+$ TCRγδ,$CD3^+$ $CD4^-$ $CD8^-$ TCRγδ and $CD3^+$

$CD4^+$ TCRαβ T cell subpopulations, in almost the same proportion. The irradiated DQ restricted $CD4^+$ T cell subpopulation stimulated the $CD4^-$ T cell subpopulation to proliferate in the presence of SCW and autologous monocytes. The irradiated $CD4^-$ T cell subpopulation suppressed the proliferative response to SCW of freshly separated autologous $CD4^+$ T cell in the presence of autologous monocytes, whereas the irradiated $CD4^+$ T cell subpopulation exhibited no suppressive activity. On the other hand, $CD4^+$ T cells dominated and $CD8^+$ T cells disappeared in the HLA-DR restricted SCW specific T cell lines.

From these observations we propose a tentative model for the activation of $CD4^-$ suppressor T cells, as follows (Fig.2): The classical $CD4^+$ helper T cell (THB) which activates B cells to produce immunoglobulin recognizes antigen in the context of the DR molecule. On the other hand, a new category of $CD4^+$ helper T cell (THS) which activates $CD4^-$ suppressor T cells (Ts) to suppress the proliferative response of $CD4^+$ T cells recognizes antigen in the context of DQ molocules. Because $CD4^-$ suppressor T cells contain at least three T cell subpopulations, $CD8^+$ TCrαβ, $CD8^+$ TCRγδ and $CD4^-$ 8^- TCRγδ, it is important to identify the true suppressor T cell among these three subpopulations. $CD8^+$ T cells, in general, recognize class I major histocompatibility antigens (Ratnofsky et al. 1987) and TCRγδ T cells may also recognize class I antigens, including Qa or TL-like antigens (Strominger 1989). Therefore, $CD4^-$ suppressor T cells may recognize self or non-self antigens in the context of class I antigen expressed on $CD4^+$ THB and possibly APC to suppress the proliferative response of THB.

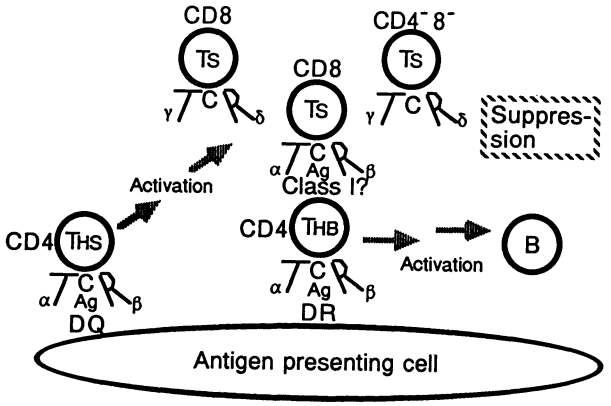

Figure 2. A tentative model for the interaction between antigen presenting cell, $CD4^+$ helper T cell for B cell (THB) or suppressor T cell (THS) and $CD4^-$ suppressor T cell (Ts) through the recognition of a self or non-self antigen (Ag) in the context of distinct HLA antigens.

Taken together we suggest that HLA-DR upregulates the immune response and HLA-DQ downregulates it through induction of $CD4^-$ suppressor T cells activated by DQ restricted $CD4^+$ helper T cell for suppression. The immune suppression gene may be a particular allele(s) of the HLA-DQ locus and HLA-DQ might be epistatic to HLA-DR in the regulation of immune response in humans.

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Role of the Veto Phenomenon In Vivo

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Under normal circumstances, T cells do not mount destructive responses against self antigens. Recently, much progress has been made in understanding how this state of self tolerance is established. We will first briefly review the different mechanisms which have been proposed and the evidence supporting them. The veto phenomenon is one such mechanism. Conclusions from older work describing the veto phenomenon in vitro will be summarized and newer evidence supporting the existence of veto activity in vivo will be described. We conclude with a brief description of how the veto phenomenon might form the basis of the blood transfusion effect.

More than 95% of mature T cells recirculating through lymphoid tissue in the periphery bear the TcR (T cell receptor) dimer α/β. These cells recognize processed antigen in association with MHC molecules. They can be split into two classes, depending upon the expression of the cell surface markers CD4 and CD8.
(i) $CD4^+$ $CD8^-$. These recognize peptides in association with class II MHC molecules and are primarily T helper cells.
(ii) $CD4^-$ $CD8^+$. These recognize peptides in association with class I MHC molecules and are primarily CTL precursors.
We here ignore the remaining TcR-α/δ T cells, whose function and specificity are unclear at the present time.

Self products in the form of processed self proteins (self peptides) can associate with self MHC molecules on APC (antigen presenting cells) and activate allogeneic T lymphocytes to undergo an immune response (Lorenz and Allen 1989; Lin and Stockinger 1980). However, syngeneic T lymphocytes are not activated, i.e. they are tolerant. Yet it seems very likely that self reactive T lymphocytes are produced during ontogeny. What happens to these cells? Two main possibilities have been considered.
(i) Tolerance is established during ontogeny through processes which act on immature cells and/or
(ii) Tolerance is established in the periphery through processes which act on mature cells.

In the last year, very substantial evidence supporting (i) has appeared. Immature thymocytes that have successfully expressed an α/β-TcR carry both CD4 and CD8 surface markers. If they are stimulated through their TcR at this point, either non specifically by anti CD3 antibody (Smith et al. 1989) or specifically by APC carrying the processed antigen they recognize

(Matzinger and Guerder 1989), the cells are induced to die. APC which activate mature T cells will stimulate this death process in immature double positive thymocytes (Matzinger and Guerder 1989).

This process should be effective for establishing tolerance for all self proteins which can be processed and presented by APC in the thymus. However, it seems unlikely that all possible self antigens can be presented in the thymus and that there must be additional mechanism(s) acting in the periphery. There is, in fact, direct evidence that such additional peripheral mechanisms must exist (e.g. Zamoyska et al. 1989). It might well be that there are different peripheral mechanisms for tolerizing $CD8^+$, class I-restricted and $CD4^+$, class II-restricted T cells. Here, we will focus on class I-restricted cells.

One possible mechanism for establishing peripheral tolerance is that there are two different kinds of APC.
(i) Stimulatory APC. Mature T cells become activated on recognizing this type of APC. Examples of such APC are dendritic cells and macrophages.
(ii) Deletional APC or Veto Cells. Mature T cells become inactivated on recognizing this type of APC. Examples of such APC, capable of inactivating CTL precursors are mature CTL (Claesson and Miller 1984; Fink et al. 1984).

The existence of deletional APC or veto cells has been most clearly demonstrated in in vitro studies of the MLR (mixed lymphocyte reaction) (Miller 1980, 1986; Muraoka and Miller 1980; Fink et al. 1988). Letting A, B and C stand for three MHC-different inbred mouse strains, cell subpopulations from strain B can be found which, when added to an A anti-B MLR, reduce production of anti-B CTL. The same subpopulation of B strain cells has no effect on CTL production in a B anti-A or A anti-C MLR. In limiting dilution studies, it was found that the response reduction was due to an apparent deletion of CTL precursors and that only CTL precursors capable of recognizing the added B subpopulation cells are deleted. Note that from the point of view of the added cells, the specificity of the response reduction is against an anti-self response.

The above studies have established the following properties of the veto phenomenon.
(i) Veto activity is possessed by cells of varying phenotype, possibly all in the T lineage.
(ii) Veto cells must carry the antigenic determinant (including the class I MHC restricting element) against which the response is being suppressed.
(iii) Veto cells act early in an immune response and produce functional deletion of CTL precursors capable of recognizing them. They are ineffective against mature CTL.
(iv) Veto activity can occur in the presence of appropriate stimulatory APC and lymphokines such as IL-2.

All the above conclusions concerning the veto phenomenon were reached in in vitro studies. Can similar results be obtained in vivo? It has been established that when parental mice (strain A) are injected with semallogeneic F_1 (strain F_1(AxB)) viable lymphoid cells, the ability of lymphoid cells from the recipient to produce anti-B CTL in a subsequent in vitro MLR is greatly

reduced whereas the ability to respond against unrelated third party stimulator cells (strain C) is unaffected (Miller and Phillips 1976). This result was unexpected in that exposure *in vivo* to foreign (B) antigen produced response reduction rather than priming in the subsequent *in vitro* test systems. Response reduction occured throughout the recirculating lymphocyte pool and was rapid, being detectable within 20 min of injection of F_1 cells and reaching maximum within 2 days. Rammensee and colleagues (1984, 1987) demonstrated that this response reduction appeared to be due to functional deletion of CTL precursors and could be interpreted as a veto phenomenon, i.e. A anti-B CTL precursors in the recipient became inactivated rather than stimulated on encountering B antigen on the injected lymphoid cells.

We have recently reinvestigated this system (Martin and Miller 1989). Before injection, we labelled the F_1 cells with fluorescein isothiocyanate (FITC) so that we could follow their fate *in vivo* and, if desired, remove them from cells suspensions by cell sorting before *in vitro* testing. The FITC-conjugated F_1 cells could be easily identified in cell suspensions prepared from i.v. injected recipients (10^7 injected per recipient). They could be detected in lymph nodes within minutes of injection, reached a plateau value of about 2% of all lymphoid cells in both lymph node and spleen within 24h, and could be easily distinguished from host cells for at least 8 days following injection, despite a loss in fluorescence intensity of 5-10% per day. In a 2 dimensional flow cytometric analysis of forward angle light scatter intensity (related to cell size) vs fluorescence intensity, injected FITC-labelled parental or F_1 cells produced identical patterns. Thus, the injected F_1 cells appear to become part of the normal recirculating lymphocyte pool. FITC conjugation of the F_1 cells had no effect on their ability to induce response reduction.

As stated above, when cells from the F_1-injected animal are tested *in vitro*, their ability to mount an anti B response is reduced. Does this response reduction require the F_1 cells to be present in the *in vitro* culture, i.e. has the reduction already occurred *in vivo* or is there some continuing process going on in the *in vitro* MLR? Lymph node cell suspensions from A mice injected 2-8 days previously with FITC-F_1(AxB) were freed of F_1 cells by cell sorting. The response reduction was unaffected.

Thinking generally, there are three possible explanations for the above result: (i) Suppressor cells of recipient origin had become activated *in vivo* and mediated suppression *in vitro*;
(ii) Suppressor cells of donor (F_1) origin became activated *in vivo*, in the process losing their fluorescent label and mediated suppression *in vitro*;
(iii) Recipient CTL precursors became inactivated *in vivo* before the cells were placed in the MLR.

To distinguish among these possibilities, we used limiting dilution analysis to measure the frequency of CTL precursors reactive against B. The precursor frequency was shown to be reduced by an amount roughly equivalent to the reduction in cytotoxic reactivity. This suggests the response reduction is entirely the result of functional deletion of CTL precursors *in vivo* and not due to the induction of suppressor cells of either recipient or donor origin. As a further test for the presence of

suppressor cells acting in vitro, we measured the frequency of CTL precursors in an equal mixture of cells from control and F_1-injected responder cells on the assumption that any suppressor cells would have an equal effect on the CTL precursors of both populations. The measured CTL precursor frequency was equivalent to the predicted mean of the CTL precursor frequencies determined for each population measured separately, suggesting very directly that no suppressor cells were present.

What has happened to the functionally deleted CTL precursors? Are they still there but anergic or have they been removed from the recirculating pool? This question would be most easily addressed if one had a cell surface marker specific for the functionally deleted CTL precursor population. This is, in fact available. We used cells from C57BL/6 (B6) female mice expressing α and β transgenic TcR genes specific for the male H-Y antigen in association with D^b (Kisielow et al. 1988). Most T cells in the periphery of these mice carry the transgene receptor and, due to positive selection in the thymus (Teh et al. 1988), are predominantly $CD8^+$. Monoclonal antibodies specific for the α transgene (T3.70) and β transgene (F23.1) are available (Teh et al. 1988). In normal mice, T3.70 reacts with <1% of T cells whereas F23.1 reacts with about 20%, reflecting the relative use of the V_α and V_β genes of the transgenic receptor in the normal T cell repertoire.

Our experimental design was to FITC-label lymphoid cells from a female transgenic mouse and inject them into normal B6 female mice (Martin et al. 1989). After waiting 2 days to allow the injected cells to equilibrate with the recirculating pool, one group of recipient mice was injected with B6 male lymphoid cells (experimental group), and a second (control) group with either female lymphoid cells or nothing. After a further 2-6 days, lymph node and spleen cell suspensions were prepared and analyzed by flow cytometry. Injected transgenic cells could be distinguished from host or injected male cells by virtue of their FITC (green) label. Cell suspensions were also labelled with phycoerythrin (red)-conjugated monoclonal antibodies against CD4, CD8, F23.1 and T3.70. In the experimental group, transgenic cells (green label) fell in number relative to control, the decline being detectable on day 2 and plateauing by day 5 to 6. The cells which disappeared appeared to be confined to those simultaneously expressing CD8 and T3.70, i.e. those cells capable of recognizing H-Y in association with class I MHC. This is precisely the population of cells that one would expect to be vetoed by the male cells. Numbers of $CD4^+$ cells and of $CD8^+$, $T3.70^-$ cells were unchanged relative to control. This provides direct evidence that the $CD8^+$, $T3.70^+$ cells have been physically removed from the recirculating lymphocyte pool and not merely rendered anergic.

We conclude this brief review with an experiment of potential relevance to what has been called the "transfusion effect" in tissue transplantation. Although first described in man (Opelz et al. 1981), it has been best characterized in animal model studies (e.g. Faustman et al. 1982). Briefly, when an A strain animal is injected with F_1(AxB) viable T cells, at later times one observes enhanced survival of a B graft (e.g. skin or heart) but no effect on the survival of a C graft. The graft-survival-enhancing ability can be transferred to a naive animal by injecting it with

T cells from the primed animal. Since the effect is antigen specific and depends upon T cells, this has been taken as evidence supporting the existence of antigen specific T suppressor cells (reviewed in Roser 1989). Alternatively, it may be that the injected F_1 T cells are acting as deletional APC, or veto cells. Since they persist in the first recipient, it may well be that they continue to act in the same way when transferred into a second recipient.

To distinguish between the two possibilities outlined above, we injected A mice with FITC-conjugated F_1(AxB) lymphoid cells. As described earlier, the ability of these mice to respond against B in an MLR was greatly reduced. Here, we took lymphoid cells from these primary recipients, sorted them into fluorescent and non fluorescent cells, and injected them separately into secondary recipients. Secondary recipients injected with fluorescent (F_1) cells were greatly reduced in their ability to respond against B in an MLR; those injected with non fluorescent cells were unaffected (Martin and Miller 1989). This provides direct evidence against the induction of antigen specific suppressor cells in the cells of the first recipient. Nor is it likely that antigen specific suppressor cells have been induced in the F_1 cells: primary recipient cells cultured with or without F_1 cells were equally reduced in responsiveness (see above). The simplest explanation of the above results is that the injected F_1 cells are acting as deletional APC or veto cells. One caveat should be added. We have ruled out the existence of specific suppressor cells only for up to 8 days following injection of F_1 cells. It may well be that the deletion of CTL precursors produced by the injected F_1 cells sets up conditions favourable to the generation of specific suppressor cells at later times.

We feel the experiments described above provide strong evidence that veto cells (or deletional APC) play an important role in deleting self-reactive class I-restricted CTL precursors that either escape censure during development in the thymus or develop via an extrathymic pathway. Whether they also play a role in the control of self-reactive, class II-restricted T helper cells remains to be determined, although there is evidence this may not be the case: class II-restricted self-Mls reactive T cells are anergized in the periphery rather than deleted (Rammensee et al. 1989). Whether deletional APC play a role in normal immunoregulation is an intriguing possibility which is currently being investigated.

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T Cell Vaccination and Suppression of Autoimmune Disease

I.R. Cohen

INTRODUCTION

Immunological suppression may be defined as inhibition of an immune response in the face of an otherwise adequate stimulus. Based largely on investigations of the induction of "infectious tolerance" to foreign antigens (Gershon and Kondo,1970), it was once widely accepted that immunological suppression was the task of a special class of CD8 suppressor T cells that specifically recognized the tolerogen (Dorf and Benacerraf,1984). However, the inability of many to clone antigen-specific suppressor T cells has given rise to skepticism about the very existence of such cells.

For some years my colleagues and I have been investigating the suppression of experimental autoimmune diseases induced by a process termed T cell vaccination (Ben-Nun et al.,1981; Ben-Nun and Cohen,1982; Cohen et al.,1983; Cohen,1986,1988,1989). T cell vaccination may be defined as the administration of specifically autoimmune T cells, avirulent or attenuated, to prevent or to treat autoimmune disease. T cell vaccination has been shown to induce suppression mediated by T cells, but in this case the suppressor cells are anti-idiotypic rather than antigen-specific (Lider et al.,1988,1989).

The aims of this article are to review information, recently published or about to be published, illustrating the effectiveness of T cell vaccination and exploring how T cell vaccination affects antigen-specific suppression.

METHODS

Adjuvant arthritis (AA)

This progressive polyarthritis is induced in Lewis rats by immunization with killed *Mycobacterium tuberculosis* (MT) organisms (Holoshitz et al.,1983). The key MT antigen appears to be a 65KD heat shock protein (hsp65) that has about 50% sequence homology with mamalian hsp65 (van Eden et al.,1988) and cross-reacts immunologically with joint tissue (van Eden, et al.1985).

NOD mouse diabetes

In contrast to AA and the other experimentally induced autoimmune diseases to which we have applied T cell vaccination, the autoimmune diabetes of NOD mice develops spontaneously (Rossini et al.,1985). The diabetes is caused by T cells that destroy the insulin producing beta cells of the pancreatic islets (Bendelac et al,1987). Mononuclear cell infiltration of islets usually begins about 4-6 weeks of age and progresses until overt insulin dependent diabetes mellitus (IDDM) surfaces at about 4-5 months of age. The incidence of IDDM in the female mice in our colony is about 90% while the incidence of IDDM in male mice is about 50%.

T cell vaccination

The preparation of vaccines using specific T lines or clones, or lymphocyte populations from antigen-primed or sick animals has been described elsewhere.(Lider et al.,1987) Three elements are important for the effectiveness of the vaccine: the cell population has to contain T cells specific for the target antigen, the T cells have to have been activated in vitro by culture with the specific antigen or with a T cell mitogen (Naparstek et al.,1983) and often but not always, the activated T cells have to be treated with a chemical cross-linker such as glutaraldehyde (Lider et al.,1987). Some virulent lines or clones of T cells can induce resistance without causing the autoimmune disease if irradiated (Ben-Nun et al.,1981) or if administered alive at doses below the threshold for pathogenicity (Beraud et al.,1989; Lider et al.,1988,1989. Three T cells were used in the investigations described here: A2b,M1 and B9. Clone A2b recognizes an epitope of hsp65 defined by a peptide comprising amino acids 180-188(van Eden, et al.1988). Line M1 recognizes a partially defined portion of the hsp65 molecule distal to residue 277. M1 does not respond to the 180-188 peptide. A2b and M1 can vaccinate against AA. Clone D9 recognizes the major encephalitogenic peptide of guinea pig myelin basic protein (BP) and can cause experimental autoimmune encephalomyelitis or vaccinate against the disease (Vandenbark et al.,1986).

Anti-idiotypic T cells

T cells responding specifically by proliferation to irradiated T cell lines or clones are termed anti-idiotypic T cells (Lider et al.,1988,1989).

Suppressor cells

The activity of suppressor cells in the spleens of rats with developing AA was measured by irradiating the spleen cells and adding them to cultures of various T cell lines or clones responding to their antigens. Control spleen cells were taken from naive rats. If suppression of the T cell response by the AA spleen cells required the presence of a specific AA antigen, then the suppression was termed antigen-specific. If the T cells were suppressed by the AA spleen cells in the absence of specific AA antigens, the suppression was termed non-specific.

RESULTS

Therapeutic applications of T cell vaccination

T cell vaccination was used initially as a preventive measure (Ben-Nun et al.,1981). The first indication of therapeutic effectiveness was seen in studies in which the anti-hsp65 clone A2b was used to treat established AA (Lider et al.,1987). Treatment of AA was also obtained using mitogen activated T cells from the lymph nodes of MT primed rats (Lider et al.,1987). In addition, mitogen-activated lymph node cells of rats suffering from AA were used to induce lasting remission of AA in other rats. (Cohen,1988). The most telling experimental trial of T cell vaccination has been carried out by Dana Elias and her colleagues in the NOD mouse model of spontaneous IDDM (in preparation). To prepare a vaccine, spleen cells were taken from 3 month old mice, about 1 1/2 months before the outbreak of clinical diabetes. Since T cells from mice of that age

were able to transfer diabetes to very young pre-diabetic NOD mice (Bendelac et al.,1987), we assumed that T cells specific for the beta cell antigen would be present in such spleens. In the absence of a specific antigen, we used the T cell mitogen concanavalin A to activate the spleen cells. Felix Mor has shown (in preparation) that the T cells involved in an ongoing autoimmune process are more responsive than naive T cells to stimulation by mitogen. Indeed, the frequency of antigen specific T cells from AA rats increased from 1:1000 to 1:25 upon activation with concanavalin A.

NOD mice of 5-6 weeks of age were treated by 10^7 of the mitogen-activated T cells. In the absence of treatment with glutaraldehyde, these cells actually produced hyperglycemia and insulitis. However, after cross-linking with glutaraldehyde, the cells became avirulent. More important, all of the recipient female mice were free of IDDM at 6 months of age. Control NOD mice receiving non-activated spleen cells developed IDDM with same incidence as control non-vaccinated mice (about 80%). By 10 months of age over 90% of the control mice were diabetic or dead of IDDM, while only about 20% of the vaccinated mice developed diabetes. Thus mitogen-activated and cross-linked spleen cells of pre-diabetic NOD mice were effective in vaccinating other pre-diabetic mice to control the process of beta cell destruction. These findings regarding a spontaneous autoimmune disease suggest the possible utility of T cell vaccination in clinical disease (Cohen and Weiner,1988).

Suppressor mechanisms accompanying induction of autoimmune disease

T cell vaccination appears to induce or activate at least two types of T cell responses in treated animals: anti-idiotypic T cells that respond to the vaccinating T cells specifically (Lider et al.,1988,1989), and anti-ergotypic T cells that respond to activated T cells irrespective of their specificity (Lohse et al.,1989). The anti-ergotypic response seems to be transient and less effective than the specific anti-idiotypic response. Both responses appear to involve CD4 and CD8 T cells. The laboratory of Wekerle has shown that CD8 anti-idiotypic T cells can protect rats against encephalomyelitis produced by the anti-BP idiotypic T cells (Sun et al.,1988). The anti-idiotypic T cells are cytotoxic in vitro and destruction of idiotypic T cells may be a factor in resistance to disease. Nevertheless, why does T cell vaccination work so well? How can vaccination of rats with a single clone such as A2b affect the course of AA induced by whole MT?

To approach such questions it would be helpful to know more about the immunological environment within which T cell vaccination works. By defining the regulatory mechanisms set in motion by the autoimmune disease itself, we can begin to investigate how T cell vaccination interacts with the immune system to affect the natural course of events. Such studies were undertaken by Nathan Karin (in preparation) and I shall briefly review the salient findings.

The strategy was to induce AA by immunization to MT and then to study T cell reactivity and suppression at various times before the appearance of arthritis, during its acute peak, and as it declined into the chronic phase. Responses were measured to the hsp65 antigen, to its 180-188 peptide and to undefined antigens of MT. In addition, T cell responses were measured to irradiated anti-hsp65 A2b and M1, or to anti-BP D9 T cells as stimulators. Suppression was detected by studying the effects of irradiated spleen cells on the responses of A2b, M1 or D9 to their specific antigens with or without added MT or other antigens.

As early as two days after immunization with MT, the rats demonstrated antigen-specific suppressor cells in their spleens. The response of D9 to BP was suppressed by about 50% when irradiated spleen cells were added together with hsp65. AA spleen cells without added hsp65, or naive spleen cells with hsp65, did not suppress the anti-BP response of D9. The specific suppressor epitopes of hsp65 are now being characterized using fragments of hsp65 genetically engineered by Jan van Embden of the Netherlands (van Eden,et al.,1988).

The other response detected early before the appearance of arthritis was a specific T cell proliferative reaction to the M1 anti-hsp65 T line. There was no appreciable response to A2b or to D9. The spontaneous response to M1 progressively increased in magnitude as arthritis developed. Thus, the earliest detectable manifestations of immune reactivity preceding arthritis were antigen-specific suppression and anti-idiotypic T cell reactivity to the M1 anti-hsp65 T cell line.

The T cell responses to MT, to hsp65 and to its 180-188 peptide emerged only later, a few days before the appearance of clinical arthritis on days 13-15. As arthritis progressed to its peak, the magnitude of the anti-MT response increased and then waned.

At the peak of arthritis, around day 25, the spleen cells of the affected rats manifested for a few days non-specific suppression; they inhibited the responses of all the T cells tested irrespective of their antigens.We are now investigating the possibility that the non-specific suppression was caused by anti-ergotypic T cells (Lohse et al.,1989).

In addition to the kinetics of these responses, we measured their magnitude as a function of the severity of the arthritis. Intuitively one would guess that the magnitude of the immune response to the hsp65 antigen or to MT would be positively associated with the severity of arthritis; after all, the immune response caused the arthritis. Conversely, one would expect that the magnitude of the antigen-specific and the non-specific suppression would have a negative correlation with severity of arthritis; suppression should negate the cause of the arthritis. In fact, the opposite was observed. Severe arthritis was associated with the strongest suppression, both antigen-specific and non-specific, and with relatively low T cell reactivity to hsp65 or MT. Mild arthritis was associated with higher T cell responses to antigen and lower suppression.

T cell vaccination modulates both suppression and antigen reactivity

The above experiments provided a baseline for analyzing the effect of T cell vaccination on the immunology of AA. Rats were vaccinated with activated, glutaraldehyde treated A2b clone cells and a month later they and non-vaccinated control rats were immunized with MT to induce AA. The vaccinated rats were completely protected against AA while the controls developed severe disease. How did this successful T cell vaccination influence the immune response?

Two effects were observed. First, the vaccinated rats showed an earlier but much abbreviated response to hsp65 compared to the non-vaccinated rats who developed AA. The vaccinated rats' anti-hsp65 T cell response was significantly elevated on day 4, peaked on day 10, and thereafter declined rapidly. The non-vaccinated response to hsp65 was quite low on day 10 and peaked only on day 14. Thereafter,

the response decayed at a slower rate than that of the vaccinated rats.

The second effect of T cell vaccination was that both antigen-specific and non-specific suppressor cells were undetectable. T cell vaccination thus accelerated the kinetics of the anti-hsp65 response and abolished conventional suppressor cells while activating anti-idiotypic T cells and inhibiting arthritis.

DISCUSSION

The therapeutic effects of T cell vaccination on AA and NOD IDDM are clear, although many details remain to be clarified, such as the identity of the effector and suppressor cells, the signals that activate them, their changes in number and life span, and their flow through the lymphoid and tissue compartments. It is unlikely, however, that this information alone will resolve more fundamental paradoxes. Common sense would suggest that the severity of the disease results from the magnitude of the immune response and strong suppression should lead to mild or no disease. Nevertheless we found that severe disease was associated with a relatively low response and strong suppression. Granted that the suppression ought to lower the response, but should not a reduced response reduce the disease? It's as if the disease itself triggered antigen-specific and non-specific suppression. Compatible with this conclusion were the observations that mild disease was accompanied by mild antigen-specific suppression and that T cell vaccination, which prevented disease, abolished the antigen-specific suppression. However, if the disease triggers suppression, why should antigen-specific suppression appear so early, even before the response to the hsp65 antigen is detectable?

The findings that the hsp65 antigen induces a T cell response to an anti-hsp65 T cell (M1) and that vaccination with an anti-hsp65 T cell accelerates the anti-hsp T cell response, indicate the existence of a functional network relating hsp65 with anti-anti-hsp65 at the T cell level. Such a network may be called an idiotypic network (Jerne,1974).

Moreover, the anti-idiotypic (anti-M1) response and hsp65 suppressor cells appeared so rapidly after immunization to MT that it is conceivable that these control elements pre-existed the immunization. Thus, the response to hsp65 may be anticipated by an idiotypic network that is also connected to antigen-specific suppressor cells. H. Atlan and I have developed a formal analysis of such a network ,. that may be read for more details (Cohen and Atlan,1989).

A pre-existing network of regulatory T cells could serve at least two important functions in autoimmunity. The first and obvious function would be to prevent autoimmune disease by controlling the nature of the autoimmune response. This implies that disease could result from a malfunctioning network. Hence, the basic fault may not be in the act of self-recognition itself, but in the failure of the network to regulate the consequences of self-recognition.

The second function of a pre-existing network could be to direct the autoimmune response to a particular antigen; to ensure that if an autoimmune process happens to be triggered, it will be expressed against a chosen antigen and not to any antigen at random. In other words, a pre-existing network could ensure the immunological

dominance of the antigen around which the network is centered. As we saw, T cell vaccination with an anti-hsp65 T cell actually accelerated the anti-hsp65 response,albeit this response was short-lived and arthritis did not develop. In this way a regulatory network may focus the immune system towards a particular antigen.

The idea of immunological focus on a self-antigen and its resulting dominance is obviously the antithesis of self-tolerance by clonal deletion, but this is no reason to reject the idea. Indeed, the common self-antigens are quite dominant. For example, BP is the dominant nervous system antigen for almost all species, from mouse tc human. Even healthy rats harbor T cells specific for BP (Steinman et al.,1980). Mice and humans produce lupus antibodies to the same antigens (Mendlovic et al.,1988) and such antibodies are also found in normal, healthy individuals (Avrameas et al.,1983). The antigen hsp65 is the dominant antigen in non-autoimmune mycobacterial diseases such as tuberculosis and leprosy (Young et al.,1988), although it is 50% self and arthritogenic in rats (van Eden et al.,1988) and possibly in humans (Res et al.,1988).

As the dominance of self-antigens tends to be uniform for genetically diverse individuals and species, it cannot be attributed to MHC alleles. The MHC clearly determines which peptides of BP will be seen by the T cells, but the cross-species dominance of BP as an antigen is beyond the MHC. Our work with T cell vaccination and networks in autoimmunity suggests that it is the pre-formed idiotypic networks which determine self-antigen dominance.

The advantage of a limited set of dominant self antigens is that autoimmune responses will tend to be expressed against predictable target molecules. Dominance establishes law and order. The autoimmune responses are thus pre-empted into defined channels. Most important, these dominant self-antigens are precisely the ones for which strong regulatory mechanisms already exist. Thus, in most situations, pre-emptive autoimmunity will not produce disease. If, however, the network malfunctions, an autoimmune disease will be expressed against the self-antigen, the dominance of which is encoded in the network. Therefore, it would be reasonable to expect that strengthening the regulatory elements of the network might control the disease. If these speculations have some truth, they could explain why T cell vaccination is effective; it both uses and strengthens the idiotypic network that nature may have provided herself.

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Antigen Specific Suppressor Cells, Receptors, and Factors

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Following the original description of suppressor T cell (Ts) activity, extensive efforts were devoted to describing the various subpopulations of Ts. Although some investigators noted differences among the populations of Ts, the data generally can be consolidated into a limited number of operational Ts subsets. The two most frequently described Ts are the $CD4^+$ inducer subset which we refer to as Ts_1 and the $CD8^+$ effector suppressor cells which we term Ts_3 (Dorf and Benacerraf, 1984). These Ts populations display antigen specificity and representative cells have been immortalized by cell fusion techniques. The biological activities of the Ts hybridoma cells are mediated by antigen specific suppressor factors (TsF) which are released into the culture media.

We use this occasion to review recent developments in the field of antigen specific Ts. Since it's inception this field has been based largely on phenomenology and has been criticized for its failure to provide a molecular basis for suppressor phenomena. A more detailed description of Ts receptor genes would help allay some of these shortcomings. We will first inspect the T cell receptor (TcR) genes focusing on data gathered in a murine model system of hapten specific suppression directed to the antigen 4-hydroxy-3-nitrophenyl acetyl (NP). Initial reports (Hedrick et al, 1985) demonstrated that such antigen specific Ts hybridomas frequently deleted donor TcR-β genes, suggesting that the conventional $\alpha\beta$-TcR may not be responsible for antigen specific binding, an interpretation which now appears flawed.

Hybridomas representing the Ts_3 subset have recently been reinspected for TcR expression. To enrich for TcR expression selected populations of $CD3^+$ Ts_3 hybridomas were prepared by panning parental Ts hybridomas over anti-CD3 or antigen coated plates followed by cloning at limiting dilution. The Ts activity of the CD3-selected population is enhanced by up to 100-fold by such procedures (Kuchroo et al, 1988a). The CD3-selected populations of Ts_3 hybridomas can rosette NP-coupled sheep erythrocytes (NP-SRBC) indicating that Ts selected in this fashion directly bind antigen which has not been processed and is not presented in the context of H-2 molecules. This antigen binding is specifically inhibitable with free antigen or can be down modulated by preincubation with anti-CD3 antibodies (Kuchroo et al 1988a). The selected hybridoma cells express conventional TcR-CD3 complexes which can be visualized by flow cytometry and immunoprecipitated with anti-CD3 or anti-TcR-α chain specific antibodies, yielding typical immunoprecipitates of TcR-CD3 complexes on SDS-PAGE gels.

The TcR genes from the CD3 selected NP-specific Ts_3 hybridomas have been analyzed. Southern analyses clearly demonstrate that TcR-δ genes are deleted from all three lines. In addition, Northern analyses demonstrate that expression of TcR-γ genes is variable. The combined data indicate that the $\gamma\delta$-TcR is not responsible for specific antigen binding among this group of Ts hybridomas. In contrast, all 3 lines express full- length in-frame TcR-α RNA of Ts donor origin. These TcR-α genes each contained distinct Vα and Jα segments (Table 1). In addition, 2 of 3 lines simultaneously express the BW5147 derived TcR-α. All 3 Ts_3 hybridomas express functional TcR-β genes only derived from the BW5147 fusion partner. Attempts at identifying additional or alternatively spliced forms of the TcR-α or TcR-β chain genes have been unsuccessful.

Table 1. TcR genes expressed in Ts hybridomas

Hybridoma Clone	TcR α Chain	TcR β Chain
BW5147	Vα1.1, JαTT11	Vβ1, Dβ2, Jβ2.5
B6-Ts_3-8	Vα3, novel Jα #1	Vβ1, Dβ2, Jβ2.5
CKB-Ts_3-3	Vα1.1, novel Jα #2 Vα1.1, JαTT11	Vβ1, Dβ2, Jβ2.5
CKB-Ts_3-9	Vα2, JαTA28 Vα1.1, JαTT11	Vβ1, Dβ2, Jβ2.5
CKB-Ts_1-38	Vα2,	Vβ6,
CKB-Ts_1-53	Vα2,	donor β present
BW1100	aberrant	none

Table 2. TcR β chain mRNA expression is required for antigen binding but not for TsF production

B6-Ts_3-8 Variants	TcR Genes		Percent Rosettes	Dilution of TsF_3 for 40% Suppression	
	Donor α	BW β		PFC	DTH
H3	+	-	21 $\pm$ 3	200	10
G4	+	+	71 $\pm$ 6	20	NT
G7	+	+	69 $\pm$ 7	200	10
G9	+	$\pm$	29 $\pm$ 1	200	NT
BW5147	-	+	15 $\pm$ 1	-	-

Since antigen or anti-CD3 selection enriches for both TcR surface expression and specific antigen binding, we conclude that the antigen binding receptors on the Ts_3 hybridoma clones consist of conventional TcR-$\alpha\beta$ chains. Although the selection caused increases in TcR expression and TsF release only minor differences have been detected in the levels of TcR-$\alpha\beta$ RNA. This suggests that the panning procedure selects for translation factors which control the level of both TcR surface expression

and TsF release. Alternatively, the selection may affect expression of one of the CD3 chains which indirectly controls these parameters. Recent preliminary data from the analysis of 2 CKB-derived Ts_1 hybridomas prepared by fusion with the BW1100 line which express an aberrant Vα which cannot be translated and a deleted TcR β gene (Born et al, 1988) suggests that both Ts_1 hybridomas use Vα2 genes. Combined with the previous data on Ts_3 Vα utilization, it appears that 3 of 4 CKB derived NP specific Ts use the same Vα gene (Table 1), demonstrating a restricted pattern of gene utilization and implying that TcR-Vα genes play a critical role in NP-specific Ts activity.

To study the relationship of TcR genes with TsF production, a series of B6-Ts_3-8 hybridoma subclones were compared. Of 4 subclones examined in detail, 2 showed significant binding of NP-SRBC whereas two others showed little or no rosetting above background levels. The ability of these 4 subclones to form rosettes correlated with the expression of TcR-αβ chains (Table 2). In contrast, supernatants derived from each of these four subclones mediated antigen specific suppression. Titrations indicated that all four lines made roughly comparable amounts of TsF (Table 2). Analysis of the subclone termed H3 indicated its inability to form a functional TcR was due to a 1000 fold decrease in the level of TcR-β mRNA, resulting in an average of 1 TcR-β RNA molecule per 10 cells. We conclude that expression of functional TcR-β genes is not necessary for production of TsF and that TsF is not a shed form of the cell surface TcR-CD3 complex. These data are consistent with the findings of Zheng et al (1989) who demonstrated that antisense oligonucleotides corresponding to V-α but not V-β blocked production of another antigen specific regulatory T cell factor.

In contrast to the above findings, the data of Fairchild et al. (1988) demonstrate that serological epitopes associated with the presence of TcR-β chain are also expressed on TsF. At present we cannot account for this apparent paradox without suggesting that the TsF described by the latter group has different characteristics. It lacks some of the antigenic epitopes associated with NP-specific TsF and displays distinct genetic restrictions (Fairchild et al, 1987).

For years data from numerous groups suggested that effector TsF_3 molecules consist of disulfide linked heterodimers (Taniguchi et al, 1984). The present data can be interpreted to support a model in which the TcR-α chain is associated with a second molecule, i.e. the TcR-α + X model. We speculate that this hypothetical X molecule would also be a member of the immunoglobulin supergene family, thereby providing the architectural framework to permit TcR-α chain to bind antigen. This putative second chain has often been associated with several serological determinants, including the mysterious IJ epitopes and as will be detailed later, lipomodulin (Steele et al, 1988). Alternatively, the TsF molecule may be totally unique, i.e. the old ?? model. If so, the selection procedures used to identify Ts hybridomas with enriched biological activity, may select for transcription or translation factors which cause increases in both cell surface TcR expression and TsF production.

To investigate the functional interactions of Ts hybridomas with antigen we noted that cell growth as monitored by ^3H thymidine incorporation is blocked following antigen specific activation of

Ts hybridomas. This technique allows additional analysis of antigen specific MHC restrictions and lymphokine release from antigen binding Ts. Specific antigen (NP coupled to various protein carriers or closely related compounds such as an iodinated form of NP (NIP)), induce growth arrest of Ts_3 hybridomas, but uncoupled carrier or carrier coupled with irrelevant haptens fails to reduce ^3H thymidine incorporation. Furthermore, these hybridomas show no apparent requirement for H-2 restriction since changing the origin of the antigen presenting cells (APC) used in these assays has no influence on the pattern of growth inhibition (Table 3). Finally, monoclonal anti-CD3 and anti-TcR-$\alpha\beta$ antibodies show antigen-like effects in that they inhibit growth of $CD3^+$ Ts hybridomas (Table 3).

Table 3. Antigen dependent growth inhibition of Ts hybridomas

			Percent Growth Inhibition	
Antigen	APC	Antibody	CKB-Ts_3-9	CKB-Ts_1-53
none	CKB	none	0	0
NP-KLH	CKB	none	67	85
NIP-BSA	CKB	none	70	75
ABA-BSA	CKB	none	0	2
KLH	CKB	none	-18	14
NP-KLH	none	none	24	14
NP-KLH	CKB ($H\text{-}2^k$)	none	63	94
NP-KLH	B6 ($H\text{-}2^b$)	none	61	24
NP-KLH	3R (IA^b IJ^b)	none	62	21
NP-KLH	5R (IA^b IJ^k)	none	59	28
NP-KLH	4R (IA^k IE^b)	none	NT	16
NP-KLH	CKB	anti-IE^k	NT	14
NP-KLH	CKB	anti-IA^k	NT	83
none	CKB	control Ig	0	6
none	CKB	anti-CD3	80	91
none	CKB	anti-TcRα/β	39	81
none	CKB	anti-Thy1	5	13
none	CKB	anti-LFA1	10	6

Use of this growth inhibition assay permitted initial screening for a new series of NP-specific inducer CD4 bearing Ts_1 hybridomas. Of over 800 clones screened for growth inhibition, 14 hybridomas specifically recognized NP-protein-conjugates. Of these only 3 constitutively release antigen specific TsF as detected by biological assays and serological screening with anti-TsF_1 specific reagents (Steele et al, 1989). This ability to release antigen specific TsF into the culture media is a property of relatively few NP reactive $CD4^+$ hybridomas. With the exception of TsF production we have been unable to distinguish between these groups of $CD4^+$ cells. Thus, in contrast with Ts_3 hybridomas the growth arrest of Ts_1 hybridomas was dependent on the H-2 haplotype of the APC (Table 3). The two CKB ($H\text{-}2^k$) derived Ts_1 hybridoma clones examined in detail expressed IE^k restrictions as evidenced by the failure of 4R APC to permit growth inhibition and the specific ability of anti-IE^k antibody to block antigen mediated growth arrest (Table 3). However, the antigen binding TsF_1 derived from these and other Ts_1 cells lack H-2 restriction (Dorf and Benacerraf, 1984).

Interestingly, $CD4^+$ Ts_1 hybridomas release detectable levels of IL-2/IL-4 following antigen triggering, but the $CD4^-$ Ts_3 hybridomas fail to secrete IL-2 or IL-4 under these conditions. This suggests that Ts_3 hybridoma cells, have a different physiological response compared with $CD4^+$ inducer Ts or Th hybridomas. It is interesting to draw parallels between the failure of the Ts_3 hybridomas to release IL-2 and the observations of clonal anergy in which there is also selective inhibition of IL-2 release following engagement of the TcR-CD3 complex (Jenkins and Schwartz, 1987).

Although the biological activity of TsF has been described by many groups, little has been reported on the cellular targets of these factors. Evidence from our laboratory indicates that macrophages are the targets of TsF (Ishikura et al, 1988). This was shown by adoptive transfer experiments in which one can demonstrate the target of H-2 restricted TsF using TsF pulsed parental macrophages which are transferred to F_1 recipients. Under these conditions the restriction specificity of the TsF is controlled by the phenotype of the macrophage. The genetic restrictions of the $CD8^+$ Ts_2 and Ts_3 derived TsF molecules map to the putative "IJ region" which is defined as a genetic polymorphism between the B10.A(3R) and B10.A(5R) mouse strains (Ishikura et al, 1988).

The mode of action of TsF remains a matter of speculation. However, the finding that TsF can be specifically bound by anti-lipomodulin antibodies indicates that TsF shares a common antigenic determinant with phospholipase inhibitory proteins such as lipomodulin (lipocortin) and glycosylation inhibitory factor (GIF) (Jardieu et al, 1986). In addition, the demonstration that these molecules possess glycosylation regulatory activity suggests a possible mode for their bioactivity. Thus, GIF activity exhibited by TsF may modify the carbohydrate expression of MHC or accessory molecules involved in the cognate interactions between cells in the immune system. Alternatively, the lipomodulin determinants on TsF may provide phospholipase inhibitory activity which may result in modifications of membrane fluidity or cause alterations in antigen-membrane association either of which could disrupt helper activity or stimulate suppressive signals. Since macrophages have been shown to be the target of TsF, macrophages could be the direct target of the GIF and/or lipomodulin activity. Alternatively, macrophages may focus this bioactivity onto lymphocytes.

To identify and characterize the APC responsible for Ts induction, we used a protocol in which Ts donor animals were immunized with hapten coupled cells, and lymphocytes from these donors were then used to adoptively transfer suppressor activity to naive recipients. With this protocol we showed that the accessory cells involved in Th and Ts induction are at least functionally distinct (Ishikura et al, 1988). Most notably sensitivity to ultraviolet irradiation has been used to operationally distinguish between these APC populations. However, this parameter does not determine whether the cells are derived from distinct lineages or represent different macrophage activation or differentiation states.

The APC capable of inducing Ts can be obtained from either spleen, peritoneal exudate, skin, or liver granulomas using

techniques which are known to enrich for macrophages. The cells express Ia and Mac-1 determinants but lack conventional lymphocyte markers including Thy-1, CD5 and immunoglobulin. Furthermore, the cells are contained within the 24 hr adherent, phagocytic, Fc receptor bearing cell fraction. In contrast, other Ia bearing cells such as dendritic or B cells are unable to induce Ts activity (Table 4). To date, the only non macrophage-like cell lines capable of inducing antigen specific Ts activity are thymic stromal cells, which suggests that the process of Ts induction may play a role in self tolerance (Table 4).

Table 4. The cells responsible for inducing Ts activity

NP Coupled Cells for Immunization	Treatment	Percent Suppression
splenic macrophages	none	52
dendritic cells	none	19
resting B cells	none	11
thymic stromal clone #2	none	81
macrophage #63	none	52
macrophage #63	anti-IJ-IM	7
macrophage #63	anti-Ia	59
macrophage #59	none	10
macrophage #59	100 U/ml rIFN-γ	63
macrophage #59	rIFN-γ + anti-IJ-IM	3
macrophage #59	rIFN-γ + 10^{-5}M H7	7
macrophage #59	rIFN-γ + 10^{-5}M HA1004	72

To further characterize the cells involved in Ts induction, we prepared a series of macrophage hybridoma lines from which we identified one clone that constitutively induced Ts activity after antigen coupling. This clone, termed macrophage #63, is able to induce Ts activity in an antigen specific, H-2 restricted fashion. In contrast, a second clone (#59) which expresses comparable levels of class II molecules was unable to induce Ts activity under identical experimental conditions (Table 4). The major advantage of identifying a cloned cell line that induces Ts activity was to examine if and how such cells induce IJ-restricted Ts.

As predicted from studies with heterogeneous APC populations, NP coupled macrophage #63 cells (NP-63) functionally induce Ts activity in an IJ-restricted manner. These observations of genetic restriction imply that Ts express receptors which recognize ligands (cell interaction molecules) on the APC. To account for the IJ restrictions associated with Ts cells we refer to the T cell structures as Ts-IJ and the cell interaction molecules expressed on macrophages as IJ-IM (Ishikura et al, 1988). Antibodies against these putative IJ-IM molecules block the Ts inducing activity of this macrophage cell line (Table 4). Neither anti-IA nor anti-IE reagents were able to influence the generation of Ts cells, suggesting that IJ-IM expression is functionally distinct from class II (Table 4).

To evaluate whether the cloned macrophage hybridoma cells that induce Ts can also induce immunity, antigen coupled macrophage hybridomas were used to induce primary DTH responses. NP-59 cells consistently induced DTH immunity. In contrast, NP-63 cells

induced little or no primary immune response when injected into normal H-2 compatible hosts. Thus, although macrophage 63 expresses comparable levels of Ia molecules, it is unable to induce primary immune responses in vivo. To determine if NP-63 cells were potentially capable of inducing Th activity we used 3 strategies aimed at eliminating the Ts activity generated by these cells. First, following immunization with NP-coupled macrophages, mice were treated with cyclophosphamide, which eliminates Ts activity. Second, macrophage 63 was treated with anti-IJ-IM antibody prior to NP coupling. Finally, the haptenated macrophages were injected into IA compatible but IJ incompatible recipients. All 3 protocols resulted in augmented DTH responses, indicating that clone 63 has the ability to generate strong primary immune responses but that under physiological conditions the generation of Ts activity predominates (Kuchroo et al, 1988b).

As indicated above, clone 59 macrophage hybridoma cells normally induce immunity but not suppression. However, recombinant derived interferon-γ (IFN-γ) is able to endow macrophage 59 cells with the capacity to induce suppressive activity in a time dependent manner. The Ts activity is genetically restricted by the putative IJ region. The mechanism by which IFN-γ acts to induce clone 59 cells to induce suppression may not involve class II expression since H-7, an inhibitor of phosphorylation, selectively inhibits the ability of clone 59 to induce Ts although there is no detectable effect on Ia expression (Table 4). These observations do not exclude the possibility that the IJ-IM molecules represent a complex of Ia antigen with either a phosphorylated peptide or a peptide that requires phosphorylation for proper degradation.

In summary, Ts hybridoma cells can be selected for TcR expression by passage over antigen or anti-CD3 plates. The selected cells are enriched for TcR expression and TsF production. The cells express conventional TcR-CD3 complexes through which they bind antigen in the absence of H-2 molecules. The TcR-$\alpha\beta$ genes have been cloned from several Ts hybridomas. Each contains a donor derived TcR-α chain and 3 of 5 hybridomas use a related Vα2 gene. TcR-β expression is not correlated with TsF production. The TsF molecule appears to be a heterodimer containing an antigen binding chain plus a second chain which expresses lipomodulin determinants. Macrophages are the targets of TsF and also serve to induce Ts responses. The ability of macrophages to induce Ts vs. Th activity can be modulated, implying that the same macrophage clones can induce either Ts or Th cells depending on experimental conditions. Although we still do not have a complete molecular understanding of Ts activity, the principle features of receptor structure and accessory cell requirements which apply to other T cells generally apply to the Ts populations.

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Extrathymic Acquisition of Tolerance by T Lymphocytes: Is Suppression Involved?

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INTRODUCTION

The immune response does not unfold following a simple interaction of antigen and immunocompetent cells: it is the result of the activation of an elaborate network of interacting cells (Jerne 1974). In addition, each response is a highly amplified reaction and, as is the case with other amplification systems, each step must be subjected to appropriate control mechanisms. Cells involved in this control must be identified if we are to learn how to manipulate the immune response to our advantage.

The finding that cells from tolerant animals, when mixed with cells from normal animals and transferred to appropriate hosts, specifically suppressed the ability of the normal cells to respond (McCullagh 1970; Gershon and Kondo 1971) raised hopes that a regulatory cell may be identified and used to down-regulate undesired immune responses as for example in graft rejection or autoimmune diseases. Indeed, the suppressor element was confined to the T cell compartment and the concept of a suppressor T cell circuit was formulated in which both various T cell types and factors derived from them were involved (reviewed by Dorf and Benacerraf 1984). Unfortunately all attempts to clone suppressor factors and to create suppressor T cell lines have so far either failed or have not been confirmed.

Our interest in suppression was reawakened as a result of experiments designed to study tolerance to self components synthesized outside the thymus. Other investigators had clearly shown that differentiating T cells bearing high affinity receptors directed to antigens on thymic stromal cells were clonally eliminated within the thymus (Kappler et al. 1987, 1988; MacDonald et al. 1988; Kisielow et al. 1988). The question however arose as to whether T cells could acquire tolerance once outside the thymus when they encountered antigens synthesized only in extrathymic tissues. To determine this we used transgenic mice in which a particular major histocompatibility complex (MHC) class I gene, H-2K^b, was expressed in the pancreatic islet β cells (Allison et al. 1988), or in the hepatocytes, exocrine pancreas and kidney tubules (Morahan et al. 1989b). In all these cases the tissues were not subjected to autoimmune attack and the animals were specifically tolerant of H-2K^b-bearing cells in vivo, although some could generate cytotoxic T lymphocytes (CTL) in vitro. Hence, although the intrathymic environment may be the dominant site for negative selection, it is clear that ancillary mechanisms exist in the periphery to ensure that tolerance is achieved to antigens not synthesized in the thymus.

TRANSGENIC MICE EXPRESSING H-2K^b IN PANCREATIC β CELLS

The MHC H-2K^b class I gene was linked to the rat insulin promoter (RIP) and the DNA construct micro-injected into fertilized mouse eggs of different haplotypes to create transgenic mice. These showed abundant expression of H-2K^b heavy chain protein in the islet β cells which normally express only very low levels of class I molecules. Flow cytometric analysis revealed low level of surface transgene expression, presumably because the heavy chain requires β-2 microglobulin for transport to the cell surface. Initially, we anticipated a T cell reaction against the β cells, and hence insulin-dependent diabetes mellitus (IDDM), but only in mice in which the transgene product was allogeneic. Since transgene expression could not be detected in the thymus by immunoperoxidase staining, differentiating thymus cells should not encounter such a product and the clone of T cells able to respond to it should not therefore have been eliminated. On the other hand, no reaction was expected in syngeneic mice expressing a transgene H-2K^b product identical with their own endogenous MHC molecules. IDDM occurred at a very early age, regardless of whether the mice were syngeneic or allogeneic with respect to the transgene product and in the absence of T cell involvement. Histology of the pancreas from diabetic mice revealed markedly abnormal islets, disorganized in structure and depleted of β cells. The α and δ cells persisted as the disease progressed until all the β cells were lost by about 50 days after the onset of IDDM (Allison et al. 1988).

No lymphocytic infiltration was observed at any stage. This suggested that the mice were immunologically tolerant of the transgene product. Indeed, deliberate immunization with H-2K^b-bearing spleen cells failed to provoke any lymphocytic infiltration in the islets. Likewise, no infiltration was observed in transgenic recipients of normal spleen cells from syngeneic nontransgenic donors. As a formal test for tolerance to H-2K^b, spleen cells from 12 day-old RIP-K^b mice of the 31-2 line (of SJL background and developing severe IDDM early in life) were cultured with irradiated B10.A(5R) (K^{b}D^d) stimulator cells and tested for their ability to lyse ^{51}Cr-labelled EL4 (H-2^b) and P815 (H-2^d) targets (results are summarized in Table 1). Cells from 12 day-old transgenic mice killed the H-2^d targets but not those bearing H-2K^b. When supernatants from concanavalin A stimulated (CAS) spleen cells were added to cultures of spleen cells from 14 day old RIP-K^b transgenic mice, the splenocytes were as responsive to H-2K^b as cells from nontransgenic littermates. Recombinant interleukin (IL)-2, but not rIL-1α, could substitute for CAS in inducing responsiveness to H-2K^b-bearing targets. Spleen cells from 17-wk old transgenic mice could kill both targets in the absence of added IL-2. At this stage, severe IDDM had developed and the islet β cells were markedly depleted. Tolerance was thus dependent on persisting critical amounts of the H-2K^b antigen. Thymocytes from 16 day old transgenic mice could, however, kill both H-2K^b and H-2D^d targets implying that, at this stage, developing anti-H-2K^b T cells had not been clonally eliminated within the thymus (Morahan et al. 1989a).

In vivo tests of tolerance were performed by skin grafting. Mice of the 50-1 line of endogenous H-2^k haplotype and CBA background develop, after about 3-5 weeks, a less severe form of IDDM which does not initially require insulin treatment. They were crossed with bm1 mice and the progeny grafted with B10 skin. At 6 weeks of age, the transgenic mice did not reject skin grafts whereas mice grafted at 10 weeks did do so (Table 2) (Miller et al. 1989). This again suggested waning of tolerance with time and with increasing severity of IDDM.

TRANSGENIC MICE EXPRESSING H-2K^b IN MANY NONLYMPHOID TISSUES

To investigate the effects of increased expression of MHC class I molecules in cells other than pancreatic β cells, transgenic mice were produced which expressed the H-2K^b gene under the control of the sheep metallothionein (sMTp-K^b) promoter (Morahan et al. 1989b). Zinc administration enhanced trangene expression, this being greatest in liver, kidney and exocrine pancreas. In contrast to the situation with the RIP-K^b mice, however, the sMTp-K^b transgenic mice showed no evidence of dysfunction in organs expressing the transgene. Pancreatic β cells may thus be unusually sensitive to the effects of overexpression of MHC molecules.

Table 1. Responsiveness of lymphocytes from transgenic mice to H-2K^b

Age of donor	Cells	Transgene status	rIL-2	specific lysis[a]	
				EL4	P815
12 days	spleen	transgenic RIP-K^b	none	-	+
		nontransgenic	none	+	+
14 days	spleen	transgenic RIP-K^b	added	+	+
		nontransgenic	added	+	+
17 weeks	spleen	transgenic RIP-K^b	none	+	+
		nontransgenic	none	+	+
16 days	thymus	transgenic RIP-K^b	none	+	+
		nontransgenic	none	+	+
12 weeks	spleen	transgenic sMTp-K^b	none	+	+
		nontransgenic	none	+	+

[a]Cells from transgenic RIP-K^b or sMTp-K^b mice and their nontransgenic littermates were cultured at $2x10^6$ ml^{-1} with an equal number of B10.A(5R) (H-2K^{b}D^d) irradiated (2000 rads) stimulator cells. After 4 days, the cultures were harvested and the cells tested in triplicate for their ability to lyse ^{51}Cr-labelled target cells, P815 (H-2^d) or EL4 (H-2^b) at an effector target ratio of 50:1. Percent specific lysis (+ = lysis; - = no lysis; for detailed data see Morahan et al. 1989a,b) was calculated from the formula [(test release-background release)/(maximum release-background release)] X 100.

Table 2. Survival of C57BL/10 skin grafts on transgenic mice[a]

Mice	Number	Graft survival (days)			
		<20	20-30	31-50	>60
transgenic RIP-K^b 6 wk old	3				3
transgenic RIP-K^b 10-12 wk old	6	3	2	1	0
transgenic sMTp-K^b 10 wk old	9				9
nontransgenic littermates	14	14	0	0	0

[a]Transgenic RIP-K^b mice from the 50-1 line and transgenic sMTp-K^b mice were crossed to bm1 mice and the progeny grafted with C57BL/10 skin. All the mice rejected BALB/c skin grafts.

No lymphocyte infiltration indicative of an autoimmune response was found in transgene-producing tissues of sMTp-K^b transgenic mice allogeneic to H-$2K^b$. Splenic T cells from these mice, however, could be stimulated in vitro to lyse H-$2K^b$ bearing target cells (Morahan et al. 1989b) (Table 1). To determine whether the sMTp-K^b mice were tolerant of H-$2K^b$ bearing cells in vivo, they were crossed to bm1 mice and their progeny were grafted with skin from B10 donors. While the nontransgenic progeny showed a first-set rejection of their grafts, all the transgenic mice maintained the H-$2K^b$-bearing skin graft for a period in excess of 5 months (Table 2) (Miller et al. 1989). Tolerance was thus fully evident in vivo, though not in vitro.

To investigate the basis for this in vivo specific unresponsiveness, normal syngeneic spleen cells or T-cell depleted bone marrow cells were used to reconstitute sublethally irradiated sMTp-K^b transgenic recipients. Whether they were Zn-induced or not, there was no evidence of graft-versus-host disease (GVHD) in bone marrow recipients: differentiation of T cells within the transgenic environment resulted in their inability to respond to the transgene product. In contrast, transgenic recipients of mature T cells developed GVHD lesions in those organs expressing the transgene product, while other organs including the thymus and spleen soon became histologically normal. Three weeks after reconstitution, signs of acute hepatitis were apparent: apoptotic hepatocytes, intra-lobular infiltrating lymphocytes, focal necrosis and mitotic activity suggesting some degree of liver regeneration. In the portal tracts were dense collections of activated lymphocytes which mediated piecemeal necrosis of periportal hepatocytes. After a further 3 weeks, there was less lobular infiltrates and less hepatocellular death. The pathology progressively diminished with time and by 12 weeks many of the portal tracts were free of infiltration while the others showed no necrosis (Morahan et al. 1989b). All these results point to the existence of peripheral mechanisms, not involving clonal deletion or permanent clonal anergy, preventing immune responses to self molecules, including MHC antigens.

TARGET CELLS OF TOLERANCE INDUCTION TO EXTRATHYMIC ANTIGENS

In both the RIP-K^b and sMTp-K^b transgenic mice, transgene expression was not detectable at the protein level in the thymus. H-$2K^b$ molecules might, however, be shed from the transgene expressing tissues into the circulation and presented in the thymus by cells responsible for imposing tolerance in the precursors of anti-H-$2K^b$ cytotoxic $CD8^+$ T cells. In the RIP-K^b model, three observations argue against this: (1) tolerance in spleen cells was reversed by exogenous rIL-2; (2) the spleen cells of older mice did not show tolerance in vitro, and (3) thymus cells of young or old mice were not tolerant in vitro. In the sMTp-K^b model, spleen cells from transgenic mice were fully capable of killing targets bearing H-$2K^b$ in vitro.

Since different antigen processing pathways operate for the two classes of MHC molecules (Braciale et al. 1987), shed extra-thymic antigens may be processed and presented in association with class II molecules by cells which impose negative selection in the thymus. In the INS-I-E transgenic mice expressing the I-E molecule in the pancreatic islet β cells, the thymus lymphocytes were unresponsive to I-E in a classical mixed lymphocyte reaction (Lo et al. 1988) and yet $V\beta17^+$ T cells (normally deleted intrathymically in $I\text{-}E^+$ mice) were found circulating, although unable to be stimulated in vitro by cross-linking their receptors (Burkly et al. 1989). Inactivation, rather than clonal elimination, may thus be imposed upon class II reactive

thymocytes by shed antigens. In our transgenic models, therefore, class-II restricted CD4 helper T cells encountering shed extra-thymic antigens presented intrathymically may have been tolerized. By contrast, class I-restricted CTL reactive to extra-thymic antigens would not be affected. Indeed, there may be no need to impose tolerance on these cells per se: witholding "help" would ensure an effective state of tolerance in vivo. Since, however, thymocytes from the RIP-K^b mice and spleen cells from sMTp-K^b mice did not behave as tolerant cells, it is unlikely that $CD4^+$ helper T cells would have been inactivated.

Alternatively, the target cell for tolerance could be the $CD8^+$ T cell able to help class I alloresponses (Singer et al. 1987). Tolerance would not be imposed upon these cells within the thymus since $CD8^+$ class I molecules do not present exogenous antigens and $CD8^+$ cells do not "perceive" shed antigens. Hence these cells could help the alloresponse of thymus lymphocytes. By contrast, tolerance could be imposed on these cells in the periphery.

In both our transgenic models tolerance might be imposed only upon those T cells having receptors with the highest affinity for H-$2K^b$, lower affinity T cells being spared. In the RIP-K^b model, the low affinity cells would be able to lyse H-$2K^b$ targets in vitro provided they received exogenous help (IL-2). In vivo, however, exogenous help may not be sufficient to allow the cells to reject skin grafts. In the sMTp-K^b model, responsiveness was observed in vitro possibly because only cells with the highest affinity for H-$2K^b$ had been inactivated leaving cells with sufficient affinity to respond in vitro in the absence of exogenous help but still insufficiently well equipped to reject skin grafts in vivo.

MECHANISMS OF TOLERANCE INDUCTION TO EXTRATHYMIC ANTIGENS

Several mechanisms could induce tolerance to extrathymic cellular antigens. For example, the veto effect could account for peripheral tolerance: this postulates that precursors of CTL are inactivated when they recognize some antigen on the surface of $CD8^+$ T cells (Fink et al. 1988). This, however, is unlikely to be operative in our transgenic models because the transgene was not expressed in lymphoid organs. Furthermore, irradiated transgenic sMTp-K^b mice reconstituted with nontransgenic bone marrow cells displayed no signs of GVHD.

Cells not equipped to deliver a co-stimulatory signal might inactivate T cells that recognize some cell surface antigen as suggested by Lafferty some years ago (Lafferty et al. 1980). Indeed, antigen presentation in the absence of such a signal has been shown to induce unresponsiveness in $CD4^+$ T cell lines (Essery et al. 1988; Mueller et al. 1989; Markmann et al. 1988), although $CD8^+$ cell lines have not yet been tested. In vivo, tolerance has also been demonstrated in adult mice given either transfusions preoperatively (Dallman et al. 1987) or chemically modified splenocytes (Jenkins and Schwartz 1987) and in adult mls-1^b mice made unresponsive to mls-1^a (Rammensee et al. 1989). In all these systems the T cells may not be physically deleted but rendered anergic in much the same way as has been postulated for B cells (Nossal 1983).

Another possible peripheral T cell tolerance-inducing mechanism may involve the activation of some type of suppressor cell. Indeed, in the sMTp-K^b model, the inability of normal spleen cells to produce a GVHD-type reaction unless the transgenic recipient has been irradiated hints at the existence of some radiosensitive suppressor cell network.

To date, however, neither we nor others (Lo et al. 1989), have been able to demonstrate suppression in any transgenic model. Our attempts to show suppression are summarized in Table 3 and Fig. 1.

Table 3. Attempts to demonstrate suppression in sMTp-K^b mice

Procedure	Result
Cyclophosphamide given to sMTp-K^b mice	No lymphocytic infiltration in transgene-expressing tissues
Adult thymectomy of sMTp-K^b mice followed by anti-I-J serum[a]	No lymphocytic infiltration in transgene-expressing tissues
Anti-I-J serum treatment of pregnant mother and of RIP-K^b babies[a]	CTL assay shows tolerance to H-$2K^b$ targets (see Fig. 1)
bm1 spleen cells + bm1 sMTp-K^b spleen cells injected into newborn C57BL/6	GVHD not prevented

[a]The anti-I-J serum was a generous gift of Professor Basten and shown to be active in his systems (Gibson et al. 1985)

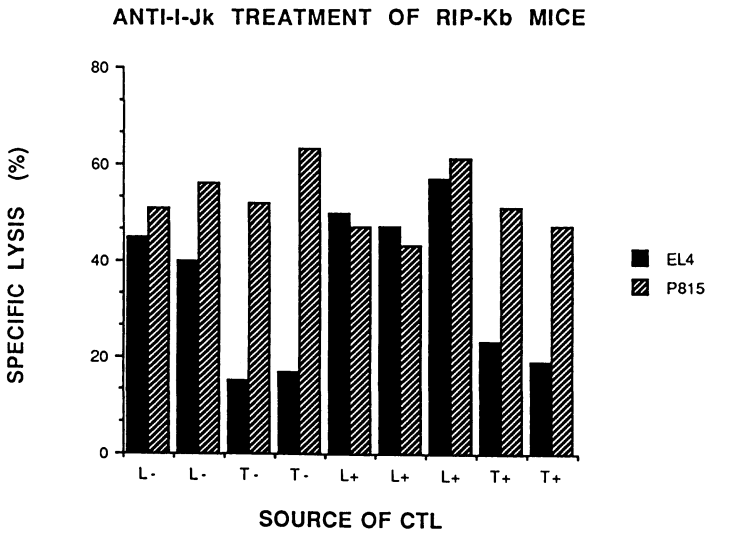

Fig. 1. Effect of anti-I-J^k serum on tolerance to H-$2K^b$ in RIP-K^b mice. Pregnant mice of the 50-1 line were injected intravenously with anti-I-J^k serum and their offspring given further injections. Spleen cells collected when these were 12 days old were stimulated to generate CTL as in Table 1 and tested on EL4 (black bars) and P815 (striped bars) targets. L = nontransgenic littermates; T = transgenic mice; + = recipient of anti-I-J^k serum; - = recipient of normal B10.A(3R) serum. The mean of triplicate samples tested at an E:T of 25:1 are shown.

CONCLUSIONS

Induction and maintenance of tolerance to self components is so essential a recipe for normal health that it is not surprising that several mechanisms should be involved in establishing the tolerant state. While there is clear evidence for intrathymic clonal deletion of self-reactive T cells, the studies presented here, as well as recent work by others (e.g. Lo et al., 1988, 1989), prove the existence of extrathymic mechanisms inducing tolerance to what may be considered as authentic self antigens, i.e. components synthesized by tissues rather than introduced from outside, and present in unmanipulated mice, not in chimeric animals. Exactly how extrathymic tolerogenesis operates is not known, although clonal anergy resulting from inappropriate antigen presentation and the activation of suppressor cells are likely candidates. Possible reasons why we have so far failed to demonstrate suppression in our transgenic models are many: e.g. the cells which initiate a suppressor cascade may be present in very low numbers and in sites which may not be easily accessible to experimental manipulation; they may not possess markers which distinguish them from other T cell subsets; they may not survive in vitro; they may act by producing short lived lymphokines which antagonize the effects of other lymphokines involved in cell activation; alternatively, they may act via an idiotype network on effector cells that have been generated only in the transgenic environment and hence are lacking in normal spleen. These difficulties should not deter efforts to identify and characterize suppressor cells as they may play a crucial role in immunoregulation and self tolerance and their activation may help in controlling graft rejection and autoimmunity.

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Antigen Specific T Cell Nonresponsiveness in Man: Lepromatous Leprosy, a Model Disease

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The leprosy spectrum: responders and nonresponders

One of the most intriguing aspects of leprosy is the close correlation between its clinical and immunological spectrum. There are two polar forms of the disease, tuberculoid and lepromatous leprosy, with a number of intermediate stages. Tuberculoid (TT) or high responder patients usually have only a few, localized lesions with rarely detectable leprosy bacteria. They usually display a strong cell mediated immune response to Mycobacterium leprae. On the other pole of the spectrum are lepromatous (LL) leprosy patients that have numerous diffuse skin lesions with vast numbers of M.leprae organisms residing in unactivated macrophages. Lepromatous leprosy patients typically show a selective T cell nonresponsiveness to antigens of M.leprae, in vivo and in vitro, which is selective because T cell responses to closely related organisms like M.tuberculosis are usually intact. The extraordinary specificity of the defect in T cell reactivity to M.leprae antigens makes lepromatous leprosy a model disease for the study of antigen specific T cell unresponsiveness in man (Bloom and Godal 1983).

The lepromatous lesion: an excess of the CD8+CD28- suppressor T cell phenotype

What is the histopathology of the lepromatous lesion? Besides the large number of leprosy bacilli found in these lesions, there is a lack of granuloma formation accompanied by a reversed CD4/CD8 ratio, in sharp contrast to tuberculoid lesions, as reviewed by Modlin and Rea (1988). In addition, the lepromatous CD8+ T cells lack the CD28 marker and thus seem to belong to the suppressor rather than to the cytotoxic subset (Modlin and Rea). This fact raises the following question:

Do M.leprae specific suppressor T cells suppress M.leprae reactive helper T cells in lepromatous leprosy?

If this is the case, it should be possible:
(1) to detect latent helper T cell responses to M.leprae by interfering with suppressor T cell activity, and
(2) to isolate a pure population of suppressor T cells.

1. To test the first hypothesis, we used various approaches.

First, we postulated that, based on the work of Hirayama et al. (1987) in the schistosomal and streptococcal system, M.leprae specific suppressor T cells might be (epi)-restricted by HLA-DQ.

Because M.leprae reactive helper T cells are almost always restricted by HLA-DR and not DQ (Ottenhoff et al. 1986a), one would expect that antibodies to DQ would selectively interfere with suppressor T cell activity and thus lead to latent helper T cell responsiveness to M.leprae. Recent results show that this can be indeed the case in a variable fraction of the lepromatous patients tested (approx. 10%) (Ottenhoff and Sazasuki, unpublished; Li and De Vries, in press).

A second way to restore T cell proliferation to M.leprae in lepromatous nonresponders was the stimulation of T cells with fractions of M.leprae rather than with complete M.leprae. To that end, M.leprae sonicates were run on SDS PAGE and electroblotted onto nitrocellulose paper that was then cut into small horizontal strips which subsequently were brought in a suitable form to stimulate peripheral blood mononuclear cells directly. Figure 1 shows an example of a lepromatous patient who was a non-responder to M.leprae but became a strong responder when such individual fractions were used as stimulating antigens (Ottenhoff et al. 1989). Using this method one third of the lepromatous patients tested became responders to M.leprae fractions. Antigenic fractions to which lepromatous patients responded were often in the higher molecular weight range (i.e. > 70 kD), whereas tuberculoid patients responded typically to the lower m.w. fractions (i.e. 10-70 kD).

A third way to trigger T cell responses to M.leprae antigens in LL patients was the use of affinity purified or recombinant antigens of M.leprae in the presence of low doses of rIL2 (Ottenhoff et al., unpublished).

These in vitro studies showed that M.leprae responsive T cells are indeed detectable in nonresponders. They are also complemented by in vivo studies in which either repeated vaccinations with BCG + M.leprae (Convit et al. 1986) or the intradermal injection of rIL2, rIFNgamma or PPD (Kaplan et al. 1989) resulted in a shift from the lepromatous to a more tuberculoid phenotype of the local lesion (Kaplan et al. 1989) or of the disease itself (Convit et al. 1986).

Thus we conclude, that it is possible to restore T cell responses to M.leprae in a significant number of M.leprae nonresponder lepromatous patients.

2. Is it possible to obtain more direct evidence for suppression by isolating T suppressor cells? Kikuchi et al. (1986) tested this by using purified CD8+ peripheral blood T cells from a lepromatous patient and observed that the addition of these cells to HLA class II compatible T cells of a M.leprae responder strongly suppressed proliferation of the latter cells to M.leprae.
Are the above experiments however sufficient to convince critics who argue that a separate subset of suppressor T cells simply does not exist (Möller 1988)? They are most probably not. They do not really address one of the major objections, namely that ".. so far no one has had a pure population of suppressor T cells in a test tube .." (Möller 1988).

Cloned antigen specific suppressor T cells

This urged us and others to clone suppressor T cells (Ottenhoff et al. 1986; Modlin et al. 1986). These clones were able to suppress polyclonal - as well as monoclonal - helper T cell responses when added to the latter in increasing concentrations (figure 2). Sup-

pression was specific since responses to unrelated antigen (e.g. HSV (Herpes Simplex Virus or DHA; see figure 2) or to bystander antigens were not suppressed. The suppressor clones recognize M.leprae together with an HLA-DR coded restriction determinant. The functionally determined polymorphism of the latter however seems to be more complex than that of restriction elements seen by the autologous helper T cell clones. Although we believe that those T suppressor cells are expanded through the recognition of M.leprae plus class II, its suppressive effector function is not necessarily mediated through the same receptor as will be briefly discussed below.

The suppressor T cell clones do not suppress helper T cells by simply killing them. Figure 3 summarizes several experiments in which helper T cells were used as 51 Chromium labelled target cells, (in the absence or presence of M.leprae pulsed autologous macrophages) and suppressor T cells were used as effector cells. Helper T cells were not lysed whereas K562 tumor cells, coated with the WT31 monoclonal antibody that specifically stains TCRαβ T cells, were efficiently lysed. These results demonstrate that our suppressor T cells do possess lytic capability but do not use that for suppression.

The suppressor clones are all CD3+ TCRαβ+γδ- CD25+ DR+ and either CD4-CD8+ or CD4+CD8- or CD4+CD8+. However, none of the suppressor clones expressed the CD28 marker, a molecule involved in TCR independent signal transduction. In contrast all T cells that could be suppressed (i.e. M.leprae responsive helper T cells) were CD28+, whereas T cells that could not be suppressed, e.g. those that responded to HSV, were CD28-. These results suggest the interesting possibility that the CD28 molecule may be involved in the reception and/or transduction of suppressive signals (Haanen et al. 1989) and this is currently under investigation. Another series of interesting experiments was recently reported by Salgame, Modlin and Bloom (1989). They noted that M.leprae specific suppressor T cell clones can induce a longterm functional impairment of M.leprae specific helper T cell function since the latter became anergic to restimulation by M.leprae plus class II. It would be interesting to correlate their findings to our observation on the absence of CD28 on suppressor T cells and its presence on M.leprae reactive helper T cells.

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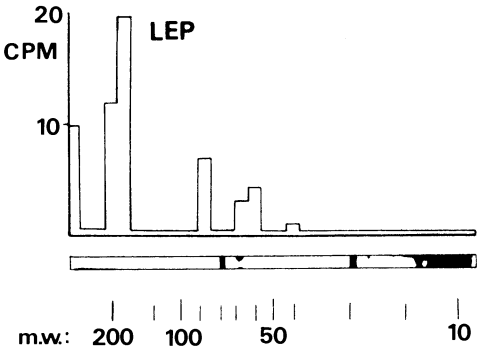

Figure 1

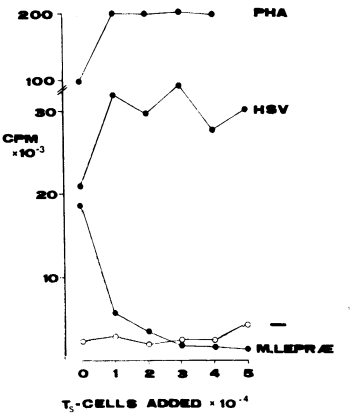

Figure 2

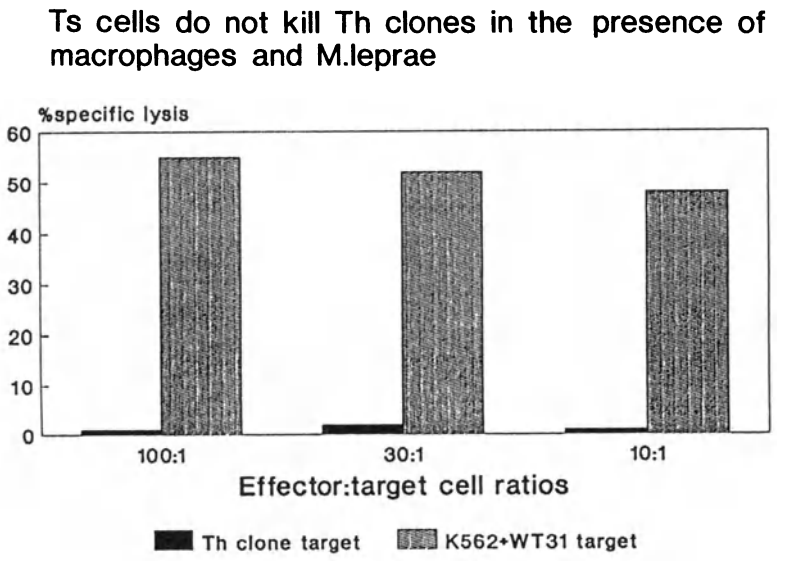

Figure 3

IV Defense

Virus-host Relationships

CD4+ and CD8+ T Cells in Murine Virus Infections: Experiments with Lymphocytic Choriomeningitis and Influenza

P.C. Doherty, Z. Tabi, A. Cleary, W. Allan, and S. Carding

INTRODUCTION

Much of the analysis of T cell function in virus-specific cell-mediated immunity [CMI] in the mouse has concentrated on the CD8+, class I major histocompatibility complex [MHC] restricted effector population. Part of the reason for this is that the early cytotoxic T lymphocyte [CTL] assays were done only with class I MHC+, virus-infected taget cells [Zinkernagel and Doherty, 1979]. Also, it was apparent from the initial adoptive transfer experiments with the lymphocytic choriomeningitis [LCM] model that both the induction of inflammation in the central nervous system [CNS] and the promotion of virus clearance from extraneural sites were properties of the class I MHC-restricted T cell subset. Compatibility between donor and recipient for class II MHC phenotype alone was without effect [Doherty et al, 1976]. The paradigm that developed was that the class I MHC restricted effectors were particularly concerned with viruses, or with any agent that could directly modify the surface of the cell, while the class II MHC restricted population would be targeted principally to macrophages or to other cells having the capacity to phagocytose exogenous material. It is now apparent that antigen processing is essential in both cases, though the net result is still partitioning of the response as a consequence of the intracellular sites where protein degradation occurs [Yewdell and Bennink, 1989] The following discusses our current understanding of the respective roles of CD8+ class I MHC-restricted and CD4+ class II MHC restricted T cells in the mouse influenza and LCM models, and describes some recent phenotypic and functional studies of influenza pneumonia.

The T cell Response in LCM

The acute immunopathological disease that occurs in the CNS of previously unexposed adult mice that are injected intracerebrally [i.c.] with LCM virus is the classical model for T cell-mediated inflammatory process in a virus infection. The case that this massive extravasation of T lymphocytes and monocytes into cerebrospinal fluid [CSF] is mediated by CD8+, and not by CD4+, effector T cells is summarized in Table 1.

Table 1. Role of T cells in the induction of LCM immunopathology

Model	Reference
1. in vitro depletion of Thy 1+ cells prevents the development of immunopathology.[a]	Cole et al, 1972
2. nu/nu and ATxBM mice are protected	Doherty &Zinkernagel, 1974
3. class I, but not class II, MHC-restricted T clls induce meningitis.[a]	Doherty et al, 1976, 1988
4. in vivo depletion of CD8+, but not CD4+, T cells is protective.	Moskophidis et al, 1987, Leist et at, 1987
5. cloned CD8+ effectors induce fatal LCM in irradiated mice.[b]	Baenziger et al, 1986.
6. CD4- immune spleen cells are enriched for effectors while CD8 depletion is inhibitory.[a]	Dixon et al, 1987 ; Doherty et al, 1988a
7. the inflammatory exudate in the CSF is dominated by CD8+ T cells.	Ceredig et al, 1987.

a. adoptive transfer into immunosuppressed and unsuppressed, virus-infected recipients.
b. i.c. injection of T cells into LCMV-infected mice.

The CD4+ lymphocytes are required to promote the B cell response in LCM [Ahmed et al 1988] but, if help provided by the CD4+ population is important for the generation of the CD8+ effectors, this is not particularly obvious from the pattern of events in either the regional lymph node [Lynch et al, 1989] or in the CSF [Table 1]. However the minimal role attributed to the CD4+ subset in LCM could, at least in part, reflect that the virus may grow in a proportion of CD4+ T lymphocytes. [Ahmed et al 1987; Tishon et al, 1988]. Also, the relative lack of class II MHC+ cells in the blood-brain barrier might tend to minimize the involvement of CD4+ T cells at the site of pathology.

The general picture in the induction of fatal LCM is thus that the inflammatory process is triggered by a relatively small number of activated CD8+ Tcells, which recognize class I MHC glycoprotein+LCMV peptide [Oldstone et al, 1988] on the surface of virus infected cells located in the blood-brain barrier . The likely target is endothelium [Doherty and Allan, 1986], though this is by no means proven. These lymphocytes, many of which have the morphological characteristics of cytotoxic effectors [Young et al, 1989], then recruit large numbers of "resting" CD8+ [but not CD4+] T cells and monocytes in a non-MHC- restricted way [Doherty et al, 1988b]. The net result is breakdown of the barrier between blood and CSF and massive cellular extravasation.

Effector T cells in Influenza

The generalization made for the LCM model that the CD8+ T cell is responsible for eliminating virus, while the CD4+ lymphocytes provide help for B cells, would seem in many respects to be also valid for influenza . However there is an important difference between the two experimental systems in that the influenza-immune CD4+, class II MHC- restricted population can apparently [on

adoptive transfer into virus infected recipients] induce more severe inflammation than the CD8+ effectors that mediate virus clearance [Leung and Ada, 1982: Ada and Jones, 1986]. One possible reason for this is that there are greater numbers of class II MHC+ antigen presenting cells in the lung than in the blood-brain barrier. Another factor is that, unlike LCMV [Ahmed et al, 1987; Tishon et al, 1988], influenza virus is not known to grow in CD4+ T cells.

The influenza system also has the great experimental advantage that, while in vitro grown T cell clones do not recirculate normally following intravenous [i.v.] injection into mice, they do home to the lung. The consequence of this is that when, for instance, potent, LCMV-immune CD8+ CTL clones were given i.v. to LCMV carrier mice, the result was fatal pneumonitis rather than neurological disease [Byrne and Oldstone,1986]. The adoptive transfer of T cell clones in influenza more closely approximates the naturally occuring situation. Cloned CD8+ T ells have been shown to clear influenza virus and, at least in some experiments, this is correlated with a capacity to produce gamma interferon [Morris et al,1982; Lukacher et al, 1984; Taylor and Askonas, 1986].

The unexpected result is, however, that class II MHC restricted, CD4+ T cell clones can also eliminate influenza virus from the lung [Lukacher et al, 1986]. This is at variance with the earlier findings from the adoptive transfer of immune spleen populations where only the CD8+ subset was effective in this regard [Jones and Ada, 1986]. What is even more surprising is that these CD4+ effectors were only capable of clearing an influenza A virus for which they were specific, and did not reduce the lung titres for another, non cross-reactive influenza A virus. The implication is thus that these CD4+ effectors are interacting directly with the cells that are supporting virus growth. This could reflect that the targets are class II MHC+, a situation that can be induced in endothelium by gamma interferon [Collins et al, 1984]. An alternative is that class II MHC+ antigen presenting cells, such as macrophages, are closely associated with class II MHC- virus infected targets and the CD4+ T cells secrete factors that operate at short range to limit virus growth.

A possible explanation for this divergence in findings for adoptively transferred immune spleen populations and T cell clones that were developed in vitro is that, though the CD4+ lymphocytes can operate effectively to clear virus, these CD4+ effectors constitute only a minority component in the CMI response induced following infection with an influenza A virus. Dominance of non-lysosomal pathways for the generation of class I MHC-influenza peptide complexes in virus infected cells could ensure that the normal host response is skewed toward the CD8+ compartment . However, this does not explain the observation that CD8-immune spleen cells from influenza-primed mice induce severe inflammatory pathology in the lungs of infected mice but do not eliminate the virus [Ada and Jones,1986].

CD4+ and CD8+ T Cells in Lymph Node and Lung of Mice With Influenza

The difference in roles attributed to the influenza-immune CD4+ T cell component from adoptive transfer experiments utilizing immune spleen cells and T cell clones illustrates the difficulty in

seperating what can happen from what normally does happen during the course of a virus specific host response. Our current analysis is exploiting a combination of flow microfluorimetry [FMF] and functional techniques to define the changes occuring in the regional lymph node and lung of mice with influenza, an approach that proved extremely informative with the LCM model [Ceredig et al, 1987; Doherty et al 1988b; Lynch et al, 1989]. The part played by CD4+ and CD8+ influenza-specific T cells in the development of both the induction and effector phases of immunity is being assessed by in vivo depletion with monoclonal antibodies [Cobbold et al 1987]. The overall intention is to develop a quantitative model of the cellular events occuring during the host response to a pathogen.

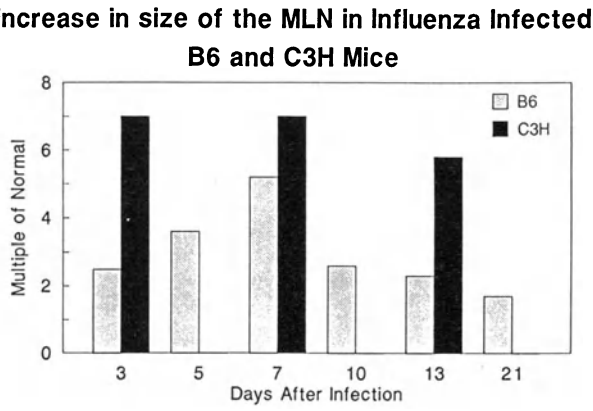

Fig. 1. The increase in cellularity of the MLN of C3H and B6 mice following i.n. infection with 600 HAU of HKx31 influenza virus, expressed as a multiple of the values for uninfected controls.

A very obvious change in mice infected intranasally [i.n.] with a large dose of influenza virus is that there is a massive accumulation of mononuclear cells in the mediastinal lymph nodes [MLN]. This peaks at about day 7, but the MLN are still enlarged at 21 days after infection [Fig1]. All categories of lymphocytes are represented, and the relative proportions of the different subsets are substantially similar to those found in a normal lymph node [MLN, Table 2]. Also, cell-cycle analysis

Table 2. Ratio of lymphocyte subsets in the lymph node and lungs

Day of experiment	T:Bb		CD4:CD8	
	MLN	LIE	MLN	LIE
5	0.9	>71.0	1.0	0.2
7	1.0	>81.0	1.0	0.3
10	1.4	43.0	0.9	0.3

a The cells were typed by FMF after, in the case of the LIE, adherence to plastic .
b = Thy1+:B220+

indicates that the overall frequency of proliferating lymphocytes is close to normal. However, selective multiplication of a small set of cells could be masked if the large component of recently-recruited lymphocytes is turning over at a slower rate than the population that is normally found in lymphoid tissue. Even so, the increase in size of the MLN in mice with influenza seems largely to reflect the non-specific recruitment/retention of circulating T cells and B cells.

Evidence of much greater selectivity is seen for the recruitment/retention of the cell population that can be washed out from the lungs of mice with influenza. This lung inflammatory exudate [LIE] is almost entirely devoid of B lymphocytes, while the T cell component is dominated by the CD8+ subset [LIE, Table 2]. The Thy-1+ cells in the LIE are larger than those in the MLN [Fig 2], and the level of CTL activity is higher. The analysis of size for the CD8+ lymphocytes in these two sites can be superimposed on the profiles shown for all T cells in Fig 2, while the CD4+ cells in the lung are no bigger than those in the node. It thus appears likely that the virus-specific component of the 100 to 200-fold increase in the number of cells that can be washed out of the lung at 7 days after i.n. infection with an influenza A virus is normally dominated by activated, CD8+ effectors.

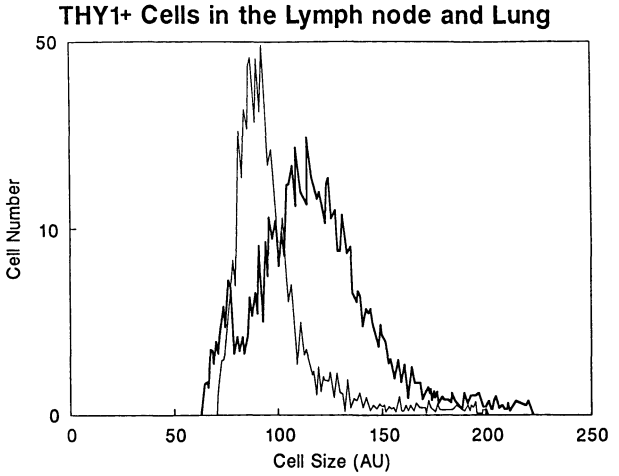

Fig. 2. Relative sizes of Thy1.2+ cells in the MLN and LIE of influenza infected B6 mice on day 7.

Removal of the CD4+ T cells by in vivo administration of the GK1.5 monoclonal antibody [mAb] always decreases the cellularity of the MLN [Table 3]. This is usually the case following depletion of the CD8+ population with the 2.43.1 mAb, though the MLN are sometimes larger following this procedure. In the lung, substantial inflammation still occurs in the absence of one, or both, of the lymphocyte subsets, but the level is never higher than that found in normal, virus-infected mice [Table 3]. Another consistent finding is that the percentage of Thy1+CD4-8- cells is greatly increased in the LIE of CD8- mice [Table 4]. This population is also present in the lungs of intact mice and in those depleted of both CD4+ and CD8+ T cells, at a level of 9% in both cases for the one experiment done to date [Table 4].

Table 3. Consequences of T cell subset depletion for the cellularity of the MLN and LIE in B6 mice at 7 days after infection

in vivo depletion	% reduction in cell content[b]		% recovery after plastic aherence of LIE
	MLN	LIE	
CD4-	57	38	56
CD8-	23	10	37
CD4-8-	63	30	30
Normal mouse	73	NT	NT

[a] Treated with the GK1.5 or 2.43.1 mAbs, either separately or together.
[b] The cell counts per mouse for the untreated group were $18.2x10^6$ for the MLN and $1.5x10^6$ for the LIE.

Table 4. T cell phenotypes of the LIE from B6 mice on day 7

in vivo depletion	% cells staining for:[a]		
	Thy1	CD4	CD8
Nil	94	25	65
CD4-	93	0	88
CD8-	85	55	0
CD4-8-	9	0	0

[a] The cells for FMF were stained with the AT83, H.129.9 and 31M mAbs as described by Lynch et al, 1989.

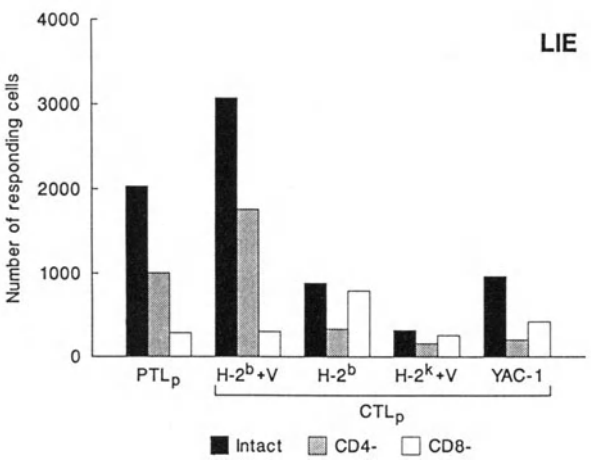

Fig 3. The numbers of responding lymphocytes were calculated from the frequency determined under LDA conditions [Owen et al, 1982]. The proliferating cells were assayed on day 5, and the cytotoxic effectors on day 7 after dividing the contents of each well into 2 aliquots.

Limiting dilution analysis of the LIE shows that in vivo CD8 depletion removes most of the proliferating cells and all the virus- immune, class-I MHC-restricted CTL activity above the background for non- specific effectors [Fig 3]. For the experiment shown in Fig 3 elimination of the CD4+ subset dropped the numbers of most categories of responding cells by about half, with the diminution being greater for the NK population . However, the effect of CD4 depletion on the CD8+ virus- immune CTL is variable with, in some experiments, the count being greater than that found in untreated mice.

Infectious influenza virus is eliminated from the lungs of mice treated with either GK1.5 or 2.43.1, though clearance may be delayed for 2 or 3 days in those lacking the CD8+ effectors. The question is whether, as suggested by the analysis of Braciale and colleagues [Lukacher et al, 1986], virus-immune CD4+ T cells are the operative population, or if some other lymphocyte is involved. An obvious candidate is the γ-δ T cell which, from preliminary studies, is present in the lungs of mice with influenza [Table 5]. However the very limited amount of data that we have to date indicates that the frequency of γ-δ positive cells is decreased in the LIE of mice depleted of CD8+ T cells.

Table 5. T cell receptor expression in the LIE of influenza infected B6 mice

Probe	Days after infection	% cells positive[a]
V-J-C γ	7	12.6
C γ	7	12.2
C β	7	16.8
CD3	7	31.4
V-J-C γ	10	37.6
C γ	10	39.2
C β	10	8.9
CD3	10	59.7

[a] The cell frequency was determined by in situ hybridization for mRNA as described by Carding et al, 1989. The cytospin preparations were from cell populations that had first been separated by plastic adherence.

Effector T cells and "T-T help"

The concept that the CD8+, class I MHC restricted T cells require "help" provided by the CD4+, class II MHC restricted set is not strongly supported by experiments with LCMV [Table I], influenza [see above], vaccinia or ectromelia [Bennink and Doherty, 1978; Buller et al, 1987]. The findings for other viruses vary. Experiments with two different models of murine cytomegalovirus infection indicate that elimination of the CD4+ T cells has either a minor [Jonjic et al, 1989] or a major effect on virus clearance [Erlich et al, 1989]. In the latter system, it is not clear whether the key function of the CD4+ cells is to act as effectors, or to provide help for T cells or B cells. Lack of the CD4+ population delays clearance of Herpes simplex virus from some sites, but does not in any way inhibit the CD8+ T cell response [Nash et al, 1987]. There is evidence from a virus-tumor model that the CD4+ T cells are needed to generate the CD8+ effectors [Weyand et al, 1989]

An indication from the influenza experiments is that "T-T help" effect could operate at the level of lymphocyte recruitment to the regional node, which is reduced by about 50% for influenza-infected B6 mice depleted of CD4+ T cells [Table 3, and unpublished data].

However there is still a substantial increase in the cellularity of the MLN following influenza infection in the absence of CD4+ T cells. In contrast, the lack of CD4+ lymphocytes in comparable mice given an inert antigen [allantoic fluid] i.n. prevented any increase in the size of the node [unpublished data].

One possibility is that the need for virus-specific CD4+ T cells to promote generalized, lymphokine dependent recruitment/retention of B cells and other T cells to lymphoid tissue is partially circumvented in some infections by the virus-induced production of cytokines [such as alpha/beta interferon] which are known to induce lymph node enlargement [Korngold and Doherty, 1985].There is no doubt that the CD4+ T cells provide direct help for B cells [Lightman et al, 1987; Ahmed et al, 1988; Jonjic et al, 1989]. However it seems likely that the interaction between the CD4+ and CD8+ populations is much more indirect, and may depend rather on the contribution that the CD4+ cells make to creating an appropriate microenvironment in lymphoid tissue for the stimulation of the CD8+ effectors.

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Immune-Protection Versus Immunopathology by Antiviral T Cell Responses

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INTRODUCTION

Virus-specific cytotoxic T cells dominate early cellular immune responses against many viruses and seem to be potently protective (Zinkernagel and Doherty, 1979; Blanden, 1974). One may question the physiological role of cytolytic T cells: why should T cells lyse infected cells? There is some evidence that cytotoxic T cells destroy virus-infected cells before viral progeny are assembled (Zinkernagel and Doherty, 1979; Blanden, 1974), thus eliminating virus during the eclipse phase of virus replication. Virus elimination via immunological host cell destruction is, in the case of cytopathic viruses, an efficient way to prevent virus spread and the resulting more extensive virus-mediated cell and tissue damage. In the case of non-cytopathic viruses, this immunological defense mechanism becomes less attractive, because host cells are not destroyed by virus but only by the T cell immune response. Because T cells apparently cannot distinguish cytopathic from non-cytopathic viruses, immune mediated cell and tissue damage results, in the latter infections, often in immunopathology (Zinkernagel and Doherty, 1979).
Examples of infections with non-cytopathic viruses are lymphocytic choriomeningitis (LCM) in mice (Hotchin, 1962; Lehmann-Grube, 1971) and Hepatitis B in humans (Bianchi, 1981; Mondelli and Eddleston, 1984). Lymphocytic choriomeningitis in mice develops only in immunocompetent animals after intracerebral injection of LCM virus (LCMV). Mice infected as embryos vertically by infected mothers, or mice lacking T cells or those immunosuppressed by irridiation or cytostatic drugs do not develop inflammatory reactions or LCM disease, but they fail to eliminate the virus and as a result become LCMV-carriers (Traub, 1936; Zinkernagel and Doherty, 1979; Lehmann-Grube, 1971); they are tolerant to LCMV at least at the level of cytotoxic T cells (Doherty and Zinkernagel, 1974; Oldstone and Dixon, 1967). LCM disease as well as elimination of LCMV after acute infection has been carefully analyzed and has been clearly shown to be cytotoxic T cell-mediated (Cole et al. 1972; Baenziger et al. 1988; Byrne and Oldstone, 1984). Thus, LCMV-induced disease in mice is an immunopathologically mediated disease caused by T-cell mediated destruction of infected host cells.

Demonstration of perforin and granzyme A in an antiviral cytotoxic T cell activity in vivo.

The above reasoning implies that anti-viral CD8 expressing T cells act cytolytically. Two cytolysis mechanisms are being discussed: Either cytotoxic T cells trigger via class I MHC somehow a self destructive mechanism in target cells or T cells release lytic molecules such as perforin or possibly granzymes which lyse target cells (Dennert and Podack, 1983; Henkart, 1985; Möller, 1988).
The analysis of gene expression in cytotoxic T cells by in situ hybridization with antisense RNA of liver and brain sections from

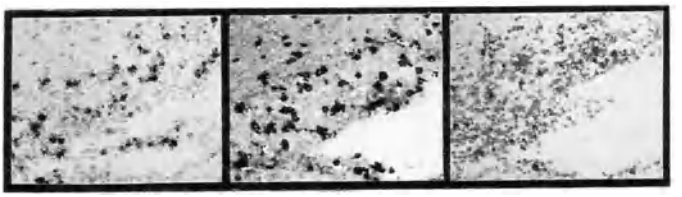

Fig 1: Expression of granzyme A and perforin mRNA. Brain sections showing part of a ventricle of a C57Bl/6 mouse infected with LCMV 6 days previously. In situ hybridisation with an antisense RNA probe specific for granzyme A (left panel), specific for perforin (right panel) or stained with an anti-CD8 antibody (middle panel); the three sections were cut in sequence from left to right.

mice infected with LCMV (Fig.1) and immunostaining with virus-specific antibodies revealed a close histological association of infiltrating lymphocytes expressing the perforin- and granzyme A-frequency of perforin mRNA containing cells on liver sections preceded by about two days maximal LCMV-specific cytotoxicity of the lymphoid cells infiltrating the liver. A key question is obviously, whether only $CD8^+$ T-cells produce and release perforin. This question was adressed by a combination of immunostaining against T-cell surface markers and subsequent in situ hybridization. Unfortunately, cytoplasmic mRNA could not be hybridized any more on tissue sections which were first treated to detect T-cell subclasses by immunohistochemistry. Based on the staining of serial sections with antibodies against T-cell markers and in situ hybridizations, it appears most likely that the perforin- and the granzyme A-genes are expressed in the $CD8^+$ T cell subset during an LCMV infection of the liver and brain. This conclusion is supported by the finding, that about 80 to 90% of the infiltrating T-cells were $CD8^+$ during LCMV-infection of the liver and the brain and the highest frequency of perforin- and granzyme A mRNA positive cells was found in both organs at the time of maximal frequency of LCMV infected cells. We cannot formally exclude however the possibility, that a minor fraction of $CD4^+$ cells or even non T-cells can express the perforin- and/or granzyme A gene during a viral infection in vivo. These results are compatible with the view that perforin is involved in cell-mediated cytotoxicity in vivo. These data confirm the recent experiments (Kramer et al. 1989) using antibodies to granzyme A and of others (Young et al. 1989) who used antibodies against perforin and double staining procedures to show that $CD8^+$ T cells express perforin.

Virus-triggered acquired immune suppression

It had been known for some time that LCMV causes immunosuppression in mice (Bloom and Rager-Zisman, 1975; Lehmann-Grube, 1984). When re-evaluating this in various mouse strains using different LCMV-isolates (Table 1), we found that an LCMV infection of mice suppressed their capacity to mount an IgM or IgG response to vesicular stomatitis virus (VSV) (Roost et al. 1988; Leist et al. 1988b). Mice preinfected with LCMV were considerably more susceptible to disease caused by VSV which usually is non-pathogenic for mice if injected subcutaneously or intravenously. The extent of immune suppression by LCMV depended upon the following parameters: Different virus isolates influenced immune reactivity differently. LCMV-WE and some other LCMV isolates such as LCMV-AGG or LCMV-DOC caused immunosuppression whereas LCMV-ARM only rarely did so. Mouse strains differed considerably with

Table 1: Immunesuppression by LCMV

Mice	Pretreatment with	Antibody response to VSV IgM d4	IgG d8-12
normal	LCMV 10^5-10^6 pfu d-8	-	-
	none	+++	++++
nude	LCMV 10^5-10^6 pfu d-8	+++	-
	none	+++	-
normal	LCMV neonatal carriers	+++	++++
normal	LCMV 10^5-10^6 pfu d-8 plus anti-CD8 d-6,-4	+++	++++

(Summarised from Leist et al. 1988b

respect to susceptibility to this immunosuppression; the MHC played some yet poorly defined role, but non-MHC genes also had a major influence. The kinetics of induction of the described impairment of mice to respond with T cell-independent IgM and/or a strictly T cell dependent IgG response to a subsequent virus infection paralleled that usually characteristic for the induction of an anti-LCMV T cell response starting on day 6 after LCMV infection and reaching maximum levels around day 8-10. The impairment to mount an IgM and/or IgG anti-VSV response after LCMV infection of mice was transient or of rather long duration (up to 4-5 months, the longest period measured to date) again dependent upon the LCMV isolate and doses used and on the mouse strain which was infected.

The following experimental results (Leist et al. 1988b) suggested that, similar to LCMV-hepatitis (Zinkernagel et al. 1986), the antiviral T cell response was responsible for immunosuppression. When neonatally infected LCMV-carrier mice were evaluated with respect to their immune responsiveness they were found to mount anti-VSV, IgM and IgG responses comparable to normal control mice. responses. This indicated that LCMV alone is not immunosuppressive and that the observed immune suppression is not caused by the action of interferons on VSV. In contrast, LCMV infected nude mice that received LCMV immune cytotoxic T cells exhibited suppressed antibody responses. Also, while LCMV infected mice failed to mount an antibody response, similarly infected mice that were treated with anti-CD8 antisera (anti-Lyt 2) some days before they received the VSV infection mounted normal IgM and IgG responses.

These results are compatible with the view that anti-viral cytotoxic T cells are responsible for immune suppression in this model infection. Accordingly, LCMV may infect lymphocytes and/or antigen presenting cells, which are involved in antibody responses; these infected cells are then in turn destroyed by anti-LCMV specific cytotoxic T cells.

This immune suppression has been analysed further by monitoring lymphocyte subset composition in spleens of mice acutely infected with LCMV by using FACS analysis. We did not find any reduction in the absolute numbers of T helper cells or B cells but an increase in the number of cytotoxic T cells.

Studies of the lymph follicles and germinal centers in spleens of T cell competent ICR mice showed the following characteristic

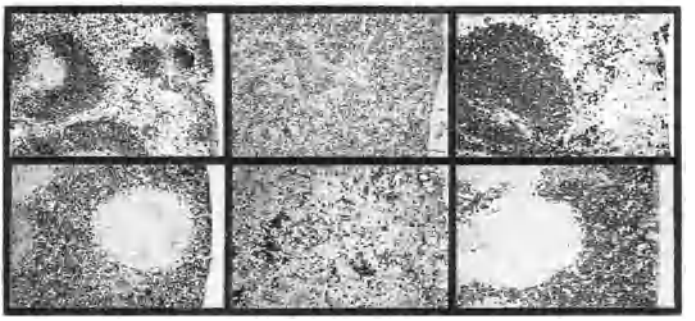

Fig 2: Morphology of lymph follicles in LCMV infected mice. Spleen sections of T cell competent ICR mice uninfected (left upper and lower panel) infected with 10^5 pfu of LCMV on day -8 (middle two panels) or of nude ICR infected identically (right two panels). Frozen sections were stained with an anti-IgM antibody (upper panels) or an anti macrophage antibody (F4/80, lower panel).

changes during an acute LCMV infection (Fig.2): By day 3-4 most of the typical distribution of cells expressing CD4,CD8, IgM or macrophage markers were comparable to that in controls. However in immunocompetent mice the follicular structure was destroyed massively between day 6 and 14 after LCMV infection. The usual architecture of germinal centers in spleens reappeared slowly after day 12. Thus the orderly structure of germinal centers is drastically altered and this may be responsible for the failure of LCMV infected mice to mount an IgM and IgG response.
These findings may be relevant to our understanding of acquired immunodeficiency which is mainly caused by the human immunodeficiency viruses (HIV) (Fauci, 1988). As in LCMV infections, recent evidence suggests that cytotoxic T cells may actually be instrumental in controlling HIV replication, but may also be responsible for destroying lymphocytes and macrophages and thus causing immune suppression (Walker et al. 1986). The example of HIV-infections in humans may therefore in a way represent the ultimate perversity in the balance between host immune system and infectious agent: non-cytopathic viruses infect lymphocytes and macrophages, the essential partners of an immune response, which are then destroyed by the immune response; i.e. the virus infection forces the immune system to destroy itself partially.

Role of IFN-gamma in protecting lympho-hemopoietic cells against virus infection?

If virus infected lympho-hemopoietic cells can be destroyed by virus specific cytotoxic T cells, or alternatively by the cytopathic effects of other viruses, their protection against infection should be of utmost importance. The role of gamma interferon (IFN-γ) induced during a viral infection in the ability of the host to acquire antiviral immunity was studied in mice (Leist et al. 1988a) (Table 2). Animals were injected subcutaneously daily with a sheep anti-IFN-γ antibody preparation able to neutralize 10^4 U of IFN-γ. Specificity of the anti-IFN-γ antiserum was demonstrated by the absence of detectable activity against natural IFN-α and -β. Controls were treated with a similarly prepared normal sheep serum. Treatment with the IFN-γ-specific antibody preparation had no influence on the ability of mice to generate anti-vaccinia virus-or anti-vesicular stomatitis virus (VSV)-specific cytotoxic T-cell responses or T helper-

Table 2: Effects of sheep anti-IFN$_\gamma$ serum on LCMV replication and antiviral T cell responses

	Treatment with normal sheep serum	Treatment with sheep anti-IFN$_\gamma$
Relative LCMV titers on d6	100	10^4 - 10^5
Susceptibility to LCM disease (mortality)	100%	0 - 20%
Relative cytotoxic T cell respones to LCMV	100	1 - 10
Relative cytotoxic T cell responses to vaccinia virus	100	100

(Summarised from Leist et al. 1988a)

dependent immunoglobulin G responses to VSV. In contrast, in mice T cell responses against LCMV were impaired. In addition, under the experimental conditions used, it prevented lethal LCM. Cytotoxic T-cell activity measured in the spleens of anti-INF-γ-treated mice was comparable to that found in mice initially infected with a 100-fold larger dose of LCMV. Evaluation of the effects of treatment on the kinetics of virus replication revealed that in both euthymic and athymic nude C57BL/6 mice, anti-INF-γ treatment led to an increase of virus titers up to 100-fold compared with control mice. Therefore, IFN-γ may play an essential role in controlling viruses with a tropism for lymphocytes and monocytes/macrophages, such as LCMV. Compatible results have been ontained by Lehmann-Grube et al (Wille et al. 1989); these authors interpret their results to indicate a role of IFN-gamma in the generation and amplification of cytotoxic T cells.

Analysis of transgenic mice expressing a T cell receptor specific for LCMV

To study tolerance to LCMV in vivo and to obtain cloned effector T cell populations that are not influenced by in vitro culture induced alterations which limit their recirculation in vivo, we have generated mice expressing LCMV-specific T cell receptors. Transgenic mice were generated with T cell receptor (TCR) αß genes originally isolated from a cytotoxic T cell clone P14 (Pircher et al. 1989a). This $CD8^+$ clone T cell recognizes LCMV glycoprotein in the context of $H\text{-}2D^b$. The α ($V_{\alpha 2}J_{\alpha\,TA31}$) and the ß ($V_{ß8.1}D_ßJ_{ß2.4}$) TCR cDNA were cloned separately into an expression vector driven by a MHC class I promoter (Pircher et al. 1989b). Both α and ß constructs were coinjected into fertilized eggs of (C57Bl/6 x DBA/2) F2 mice. Transgenic offsprings were mated with C57Bl/6 ($H\text{-}2^b$) and BALB/c ($H\text{-}2^d$) mice. Heterozygote transgenic and transgene negative offsprings were analysed in parallel. Because of the Vß8.1 gene segment used these transgenic mice express TCR specific not only for LCMV+D^b but also for Mls^a (Kappler et al. 1988). Thymocytes exhibited the following CD4/CD8 phenotypes (Table 3)

Table 3: Cellsurface markers of T cells in TCR (LCMV+D^b, Mls^a) transgenic mice

Mice	H-2	Mls	Thymocytes: % positive 8^+	4^+8^+	4^-8^-	% Total Thymocytes	Lymphnode T cells: % positive 4^+ ($V\beta8^+$)	8^+
normal	b	b	3	82	2	100	36 (12)	23 (14)
transgenic	b	b	20	65	13	100	6 (69)	46 (94)
transgenic	d	b	4	80		100		
transgenic	b	a	2	80	12	100	7 (31)	9(-20) (37)
transgenic	b LCMV carrier	b	<9	<30	<44	1-5	5 (23)	6 (33)

(Summarised from Pircher et al. 1989a)

(Pircher et al. 1989a). Whereas control mice lacking the trangenes had 2% double negative (DN) $CD4^-8^-$, 83% double positive $CD4^+8^+$, 12% single $CD4^+$ and 3% single $CD8^+$ positive thymocytes, transgenic $H\text{-}2^b$ mice possessed around 19% single $CD8^+$ versus 2% single $CD4^+$ positive T cells. The relative dominance of $CD8^+$ single positive cells was also seen in lymphnode cells. Transgenic mice had 46% $CD8^+$ T cells (vs 23% in controls) of which 94% expressed the transgenic $V_{\beta8}$chain and only 6% were $CD4^+$ of which 70% were transgene $V_{\beta8}$ positive. The skewing of T cells towards the $CD8^+$ subsets in TCR αß transgenic mice was only observed in $H\text{-}2^b$ but not in $H\text{-}2^d$ mice. The dominance of $CD8^+$ single positive T cells reflects the origin of the transgenic T cell receptor from a $CD8^+$ and $H\text{-}2^b$ restriced cytotoxic T cell clone. These findings can be explained by mechanisms of positive selection of T cells recognizing self MHC molecules in the thymus. Similar results have been obtained with αß TCR transgenic mice by two other groups using H-Y specific and alloreactive TCR (Teh et al. 1988; Sha et al. 1988a).

T cell tolerance to LCMV was studied in P14 αß transgenic mice carrying the virus after neonatal infection. The following changes were seem in these LCMV-tolerant transgenic mice: Total thymocyte numbers were decreased to between 1 - 10% of control values. Transgenic LCMV carrier mice had fewer single $CD8^+$ and considerably more single $CD4^+$ thymocytes whereas $CD4^+8^+$ double positive cells were reduced by half. The $CD8^+$ peripheral T cell subset was drastically reduced from 46% to 7% in transgenic carrier mice; of those only 33% expressed Vß8. Five percent of the carrier transgenic animals.$^+$Double staining with monoclonal antibodies specific for CD3, CD4, CD8 and TCR $V_{\beta8}$ further revealed a larger number of double negative $CD4^-8^-$ $CD3^+$ T cells (10-20%) which expressed the transgenic receptor. This result suggests that besides absence of the transgenic receptor expressing $CD8^+$ T cells, failure to express CD8 may be an alternative pathway to

achieve nonreactivity. This results complements data obtained in the H-Y and allo-MHC TCR transgenic models showing that T cells carrying a potentially autoreactive TCR are present but have down modulated CD8 (Kisielow et al. 1988; Sha et al. 1988b). The double negative ($CD4^-8^-$) T cells in the periphery are apparently different from those found in the thymus. The 30-40% double negative $CD4^-8^-$thymocytes in the transgenic LCMV carrier mouse are high CD3+ but are $V_{\beta 8}^-$ and TCR $\alpha\beta^-$ and probably reflect the $\gamma\delta$ thymocytes which were not affected by tolerance induction. These results strongly suggest that immunological unresponsivenes to LCMV in the LCMV carrier mouse is due to clonal deletion of LCMV specific T cells in the thymus at an early $CD4^+8^+$ differentiation stage.
These findings in LCMV tolerant transgenic mice are simular to those in the H-y model (Kisielow et al. 1988) and contrast with those in Mls^a transgenic mice (Pircher et al. 1989b); the latter exhibited about normal high levels of $CD4^+$ and low levels of CD4-8- thymocytes. Thus in the same transgenic mice expressing a TCR with the double specificity for LCMV+D^b and for Mls^a the thymocyte differentiation stage at which tolerance to the two antigens is induced differs drastically. This probably reflects distinct affinities or different patterns of "self"-antigen expression in the thymus with respect to localisation and possibly also with respect to kinetics of presentation during ontogeny.
The effector function of T cells from the transgenic mice has been analysed in vitro and in vivo. Upon stimulation in vitro with LCMV infected macrophages spleen cells or lymph node cells from transgenic mice but not from transgene negative littermates virus specific cytotoxic activity can be induced in vitro within 2-3 days without previous priming in vivo. Similarly, transgenic mice but not mormal controls generated high cytotoxic LCMV-specific T cell activity within 4 days after infection in vivo. Preliminary studies revealed that the primary swelling reaction of footpads after local injection of LCMV was generated very rapidly in transgenic mice by day 3-4, peaking on day 4-5 and disappearing by day 7; whereas control mice showed swelling first on day 6-7 that peaked on day 8-9 and disappeared by day 13-15. These observations fit the notion, that virus replication locally in the foot recruits effector T cells rapidly in transgenic mice, similar to a mouse primed to LCMV some weeks previously. Since virus is eliminated relatively rapidly the swelling reaction disappears much more quickly when compared with the controls.

The transgenic mice expressing a TCR α and ß-chain for LCMV+D^b and for Mls^a will offer excellent opportunities to study mechanism and ontogeny of tolerance to viral antigens and the minor histocompatibility antigen Mls^a, to evaluate homeing and effector function of antiviral effector T cells and to analyse what kind of signals (interleukins, cellular contacts and triggering via cell surface determinants) may activate transgenic $CD8^+$ effector cell precursors in vitro and in vivo.

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Recognition of Viral Antigens by Cytotoxic T Lymphocytes

A.J. McMichael, F.M. Gotch, P.A. Robbins, H. Bodmer, S. Huet, and J. Rothbard

Introduction:

Cytotoxic T lymphocytes (CTL) form a major part of the immune response to virus infection. Their role in recovery from acute virus infection has been well documented for influenza in mice, (reviewed by Askonas et al. 1982). A similar role is probable in human infection where levels of inducible specific CTL were correlated with clearance of virus,(McMichael et al 1983). CTL also control but do not clear virus in persistent infections such as Epstein-Barr virus or cytomegalovirus.(Rickinson et al 1981,Boryseiwicz et al 1983). However they may also contribute to pathology where the persistent virus infection itself causes minimal cell dysfunction, for example lymphocytic chorio-meningitis virus (LCMV) (Buchmeier et al 1980). The role of CTL in human immunodeficiency virus infection is particularly intriguing; it is not clear whether they are beneficial or harmful or whether they are capable of clearing the virus,(see Plata 1989). In view of their importance in these and other infections, a complete understanding of their specificity is desirable and may be essential for design of efficient vaccines that induce or boost CTL immunity.

Specificity of virus specific CTL:

Zinkernagel and Doherty showed in 1974 that CTL specific for LCMV recognised virus antigen plus self major histocompatibility (MHC) class I molecules. This finding was generalised (Zinkernagel and Doherty 1979) and recognition of self MHC was found to be exquisitely specific for self, sensitive to alterations of only a very few amino acids in the sequence of the MHC molecule (e.g. Krangel et al 1984). In the influenza system which has been the most extensively studied, CTL were shown to recognise internal virus proteins, made in the cytoplasm of infected cells.(Bennink et al 1982, Townsend and Skehel 1982, Townsend et al 1984, Gotch et al 1986). These were shown to be presented on the cell surface, not as intact proteins but as peptide fragments,.(Townsend et al 1985,1986). A number of peptide epitopes derived from the sequence of influenza virus proteins nucleoprotein (NP),matrix-1 (M1) and haemagglutinin (HA) have been identified, (Townsend et al 1986, Bastin et al 1987, Gotch et al 1987, Braciale et al 1987).In each case the peptide is restricted by a single H-2 or HLA allelic product. It is

clear that HLA or H-2 type determines epitope specificity, directly or indirectly.(McMichael et al 1986,Taylor et al 1987).
In 1987 Bjorkman et al. described the crystal structure of HLA A2. Of particular interest was the groove in the top of the molecule that is bounded by the alpha helices of the alpha-1 and alpha-2 domains and beta pleated sheet in the floor (Figure 1). Unidentified electron density was found in this groove which was almost certainly peptide.

HLA A2-peptide association:

We have made a detailed analysis of the association between the influenza A matrix peptide 56-68 which is presented to CTL by HLA A2.(Gotch et al 1987,1988, McMichael et al 1988). All HLA A2 positive donors who carry the common HLA A2 variant (A2.1) recognise this peptide with HLA A2. This may be because the CTL response is polyclonal; analysis of clonal fine specificities, using variant and mutant HLA molecules and analogue peptides, has revealed at least five patterns of reactivity, each unique to one clone. These are shown in Table I.

Clone	Specifity						
	Aw69	Aw69+MP	A2-Y9	A2-70-80	60-68	A59	Y62
01	+	-	-	-	nt	-	nt
J2	+	-	+	-	+	nt	nt
D10	+	-	+	+	-	+	+
K6	-	-	+	-	-	+	-
K2	-	+	-	-	+	+	+

Table I: Recognition patterns of five CTL clones specific for HLA A2 plus peptide M56-68, from one donor JM. Recognition is shown as "+"(>30% specific lysis at killer:target ratio of 2:1) or "-"(<5% lysis at the same ratio). Targets were: cells that were A2- but Aw69+ (Aw69), these cells + peptide 56-68 (Aw69+MP), cells transfected with A2 with a substitution of Y for F at position 9 plus peptide 56-68 (A2-Y9),cells transfected with A2 with mutations at positions 70-74-76-77-80 plus peptide 56-68 (A2-70-80),truncated matrix peptide 60-68 on A2+ cells (60-68), peptide with A sustituted for I at position 59 on A2+ cells (A59),peptide with Y substituted for F at position 62 (Y62).

The relationship between these matrix specific HLA A2 restricted CTL clones and HLA Aw69 is intriguing. One clone sees this as an alloantigen, possibly with a self or EBV derived peptide. Another clone can see Aw69 only in the presence of the matrix peptide (but cannot see virus infected cells (Bodmer et al 1989). The entire HLA Aw69 molecule is derived from HLA A2 except for the alpha-1 domain which is from HLA A68,(Holmes andParham 1985). It differs from HLA A2 in six amino acids at residues 9,62,63,6670 and 74.(Figure 1).

	HLA A2	Aw69
9	F	Y
62	G	R
63	E	N
66	K	N
70	H	Q
74	H	D

Figure 1. HLA A69. The residues at which it differs from HLA A2 are shown in their positions on the alpha-carbon backbone trace of the alpha-1 and alpha-2 domains,(from Bjorkman et al 1987 a,b).

HLA mutant molecules with alterations at each of these six positions have also been tested.(McMichael et al 1988). Again different results were seen with each clone. Although lysine to isoleucine at position 66 in HLA A2 affected all clones, the asparagine at this position in HLA A69 had no effect for clone K2 at least in the context of the other changes. Mutation at position 9 in HLA A2 also affected most but not all clones. Because this mutation is in the floor of the groove this is very unlikely to directly affect interaction with the T cell receptor (TCR), it is more likely to affect conformation of the peptide thus interfering with recognition by only some T cell receptors.

Two mutations in the alpha-2 helix also affected recognition, at residues 152 and 156,(Hogan et al 1988,McMichael et al 1988)., whereas a mutation at 149 had no effect.(Hogan et al 1988) In the floor of the groove, mutation at position 99 had no effect, but 114 and\or 116 affected all clones, (Gotch and McMichael unpublished)

We have also tested these mutant A2 molecules for their ability to present an influenza B virus NP peptide to CTL(Robbins et al submitted). Mutations in HLA A2 had different effects on recognition of the B peptide compared to the A matrix peptide. The mutation at 66 had no effect, but 9, 74 and 99 abolished recognition. . These data imply that the two peptides do not fit into the same position in the groove in the HLA A2 molecule.

Table II

HLA-A2 Mutation			A matrix56-68 CTL	B Nucleoprotein CTL
9	F	Y	+/-	-
43	Q	R	+	+
62\63	G\E	R\D	-	-
66	K	I	-	+
70	H	Q	+/-	-
74	H	D	+	-
99	Y	C	+	-
107	W	G	+	+

Table II: Point mutations in HLA A2 affect recognition by CTL of two influenza virus peptides differently. +and - indicate recognition and non-recognition by specific CTL; +/- indicates that some but not all clones recognise the peptide.

More recently we have tested mutations in HLA B27 on presentation of two peptides, from HIV gag (Nixon et al 1988) and Influenza A virus NP (Huet et al unpublished), to CTL; mutations at the right hand end of the alpha helix (as in Fig 1.) at positions 77,80 and 81 affected recognition.(unpublished in collaboration with J.Lopez de Castro and E.Weiss). Changes in these positions did not interfere with HLA A2 restricted presentation of influenza A matrix peptide 57-68.(McMichael et al 1988) Thus it is highly likely that the position for peptide binding will be different in different class I molecules. Given the variation in these molecules in the position of their side chains around the groove and of the differing pockets in it's floor, there may be no simple universal rule to describe peptide conformation.

Mutations in the peptide:

In order to explore the interaction between the matrix peptide and HLA A2 in more detail, a series of peptide 'mutants' each differing in one amino acid from the 'index' peptide M56-68 was tested with HLA A2 restricted CTL (Gotch et al 1988). Those that were not recognised were tested for their ability to inhibit CTL recognition of the index peptide,competing at the level of the target cell. In addition we have recently used a binding assay, as described by Bouillot et al (1989) with modifications (Gotch et al submitted 1989), to measure direct binding of the analogue peptides to HLA A2.
The results are summarised in Table III. It can be seen that there is a good correlation between functional data and binding data. No peptide was found that failed to bind in the direct assay but was recognised by CTL. Further, the data allow some points to be made about the peptide recognition. Peptide 57-68 is the optimum peptide but within this peptide residues 57,58,59,66,67 and 68

contribute nothing to specificity of recognition. However, as their deletion does affect recognition, they probably serve a structural purpose. Residues 60-65 confer binding and CTL recognition specifity. The glycine at position 61 is crucial for binding. A small residue at this point is essential,possibly allowing bending of the chain or allowing the adjacent residue to make contact into a pocket within the groove. The phenylalanine at position 62 is also very important. A hydrophobic substitution at this position allowed weak binding but changing to a charged residue abrogated binding. Position 63 may also be involved in binding; substitution of charged or large side chains for that of valine resulted in peptides that bound but did not compete, perhaps due to low affinity interaction. Given the evidence from the HLA A2 mutants that all three sides of the groove are involved in peptide binding, we have suggested (Gotch et al 1988) that F62 points down and interacts at the floor of the groove; G61 needs to be small for stearic reasons possibly because it faces a large protruding side chain from one of the alpha helices; V63 would also interact with one of the alpha helices and would have an effect on the positioning of the peptide. These orientations could be achieved with a one-turn alpha helix, which would be disrupted by the substitution of proline at position 64 (see Table III). Residues 60,64 and 65 would primarily interact with the T cell receptor.Their exact positions might be influenced by binding of this receptor.

If this hypothetical folding of the peptide is correct,there are some clues as to it's position in the groove. The most critical region of the floor of HLA A2 is around residues 9,114 and 116; the F62 may be accomodated here. G61 might face the protruding histidine at position 70 and V63 might face valine 152. The side chains at 60,64 and 65 would then be free to make contact with residues such as lysine 66, histidine 74 and leucine 156 as well as the Tcell receptor.

The eventual solution of this peptide-HLA A2 interaction will probably require co-crystallization. This has until now proved to be an insuperable problem as peptide-Class I MHC binding has been very difficult to achieve and would be a prerequisite to purifying the complex. However recent developements offer more hope that it can be done. Townsend et al(1989). have found a mutant cell line that fails to express surface class I molecules; incubation of these cells in appropriate peptide induces expression of the class I molecules. It is probable that these molecules contain the peptide in the groove. Thus high levels of specific peptide-class I may be obtained. Transfection of HLA A2 into this cell-line may make it possible to obtain A2-matrix peptide complexes in high yield at the cell surface. Also it is now possible to see 10-30% binding of HLA A2 to peptide in cell free systems(Bouillot et al. 1989). Finally Bodmer et al(submitted) have found an anti A2 monoclonal antibody that probably enhances peptide association with HLA A2. The application of these findings to the problem may well facilitate purification and crystallisation of HLA A2-peptide complex.

In conclusion, we are now at the stage of delineating the fine details of virus-peptide-MHC Class I associations and of the recognition of these complexes by cytotoxic T lymphocytes. This information should permit a more rational design of vaccines or other modulators of these important contributors to anti virus immunity.

Table III: CTL and Binding data on Influenza A matrix peptide 57- 68 analogues with HLA A2

			57		59		61		63		65		67	
Bind	**CTL**	**Inhib CTL**	**K**	**G**	**I**	**L**	**G**	**F**	**Y**	**F**	**T**	**L**	**T**	**Y**
+	+		E	D	A	D				S		E	D	K
+	+		F	Q	E	T				N		R	A	
+	+		R	S	K					Y		F		
+	+		Q	K	F							S		
+	+		H	F	Y									
+	+		E	L										
+	+			E										
+	-	+				S				Q	K			
+	-	+				N								
+	+/-	-						Y		I	G			
+	+/-	-						I						
+	+/-	-						V						
+	-	-							R					
+	-	-							Y					
+	-	-							E					
-	-	-					V	K		P				
-	-	-					L	D						
-	-	-					D							
-	-	-					E							
-	-	-					T							
			K	**G**	**I**	**L**	**G**	**F**	**Y**	**F**	**T**	**L**	**T**	**Y**

Results are shown as peptide binding to HLA A2 using a direct binding assay (Bind); as recognition by CTL specific for matrix peptide and HLA A2 (CTL); and inhibition of CTL recognition of this peptide in a competition assay (Inhib CTL).

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Blockade of MHC Class I Molecule Transport from the Endoplasmic Reticulum Inhibits Presentation of Protein Antigens to Cytotoxic T Lymphocytes

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INTRODUCTION

The only well defined function of MHC class I molecules is to bind foreign antigens and present them to cytotoxic T lymphocytes (CTLs). Class I molecules consist of a β_2-microglobulin molecule non-covalently complexed to an α subunit, a 44Kd type I integral membrane glycoprotein. Regions of proteins recognized by CTLs are believed to bind to a groove located at the tip of the α subunit (Bjorkman *et al.*, 1987). Like other integral membrane glycoproteins and secretory proteins, α and β_2-microglobulin-subunits are believed to be co-translationally inserted into the endoplasmic reticulum (ER) during their synthesis on ribosomes. Folding of the subunits and association into class I molecules occurs within minutes following their synthesis. Assembly is believed to occur in the endoplasmic reticulum (ER). Class I molecules are then transported to the cell surface via the *cis*-, *medial*-, and *trans*-cisternae of the Golgi complex (GC).

In the past few years, we have attempted to determine where and when following their biosynthesis, class I molecules associate with foreign antigens. In this paper we summarize four independent lines of evidence that lead to the following conclusion: exogenously added peptide determinants associate with class I molecules at the cell surface, while determinants processed from the cytosol associate with class I molecules in an intracellular compartment, most likely, the (ER).

I. Depletion of intracellular class I MHC molecules.

We previously demonstrated that proteins derived from non-infectious influenza virions are processed for CTL recognition via a

cytosolic route provided that the viral neuraminidase is inactivated (Yewdell *et al.*, 1988). In these experiments we noted that antigen presentation was not affected by incubation of cells with protein synthesis inhibitors. More recently, we treated L929 cells ($H2^k$) for various times with a cocktail of protein synthesis inhibitors prior to the addition of non-infectious influenza virus. We found that treatment of cells for up to 3 hours prior to the addition of virus had no discernable effect on antigen presentation. Since class I molecules fold and assemble rapidly, this finding strongly suggests that antigens can associate with fully folded and assembled class I molecules.

Longer treatment of cells with protein synthesis inhibitors prior to the addition of non-infectious virus resulted in decreased presentation of two influenza virus proteins, hemagglutinin (HA) and nucleoprotein (NP). Preincubation of cells for 12 to 16 hours with protein synthesis inhibitors was sufficient to reduce CTL recognition of HA and NP to non-detectable levels. This cannot be attributed simply to the resistance of target cells to CTL lysis since a synthetic peptide containing the NP epitope (NP 50-63) (Basten *et al.*, 1986) was able to sensitize target cells for CTL lysis. Furthermore, CTL recognition of one of the influenza virus polymerases (PB1) was not greatly affected by treating cells for as long as 32 hours.

The differential effect of protein synthesis inhibitors on NP and HA versus PB1 presentation likely reflects a difference in the time required to deplete the intracellular pool of nascent class I molecules. This conclusion is based on two previous observations. First, in prior work we found that H-2^k restricted CTL recognize HA and NP exclusively in conjunction with K^k, while PB1 is restricted primarily by D^k-restricted CTLs (Bennink and Yewdell, 1988). Second, Williams and his colleagues (1985) have reported that the half time for egress of K^k and D^k from the ER of a B lymphoma cell line differ by approximately 10 fold (30 minutes versus 5 hours). We have confirmed that K^k and D^k behave similarly in L929 cells. Thus, our findings are consistent with the idea that antigens processed from the cytoplasm associate with newly synthesized class I molecules, while the NP 50-63 peptide associates with cell surface class I molecules.

II. Effect of Brefeldin A on CTL Recognition.

Brefeldin A (BFA) is a fungal metabolite that blocks the exocytosis of newly synthesized integral membrane and secretory proteins (Takatsuki and Tamura, 1985). Our recent studies (Doms *et al.*, 1989), and the findings of Lippincot-Schwartz *et al.* (1989) indicate that integral

membrane proteins synthesized in the presence of BFA are translocated into the ER, glycosylated, folded, and assembled with normal kinetics. BFA prevents proteins from reaching the GC, and remarkably, induces the redistribution of itinerant and resident proteins from the *cis*- and *medial*-GC to the ER. BFA also arrests transport of proteins that have reached the *trans*-GC, but fails to prevent delivery to the cell surface of proteins that have reached post-GC vesicles (G. Russ and J. Yewdell, unpublished observations).

We recently described the effects of BFA on antigen presentation (Yewdell and Bennink, 1989). In brief, we found that BFA completely inhibited the presentation of antigens by L929 cells sensitized with either infectious or non-infectious influenza virus to H-2^k restricted CTLs specific for NP, HA, NS1 and PB1. BFA also inhibited the presentation of vaccinia virus (Vac) antigens to Vac-specific H-2^k-restricted CTL. Recognition of virus infected P815 cells (H-2^d) by H-2^d restricted anti-influenza and anti-vaccinia CTLs was also abrogated by BFA. Thus BFA is a general inhibitor of antigen presentation to CTLs.

The effect of BFA cannot be attributed to disruption of previously existing class I-antigen complexes since (i) BFA did not effect the presentation of HA by a L929 cell line that constitutively expresses the HA gene; and (ii) inhibition of cells sensitized with non-infectious influenza virus or infectious influenza or Vac viruses was only observed if BFA was added within 90 minutes following the addition of virus preparations to target cells.

The effect of BFA on the presentation of influenza virus antigens was rapidly and completely reversible. Removal of BFA for 10-15 minutes at 37°C from virus sensitized-cells sufficed to recover antigen presentation. The reversibility of the BFA block in antigen presentation demonstrated a striking temperature dependence. When BFA was removed for 90 minutes from virus-sensitized cells, antigen presentation was recovered only when the incubation was performed at temperatures greater than 20°C. Importantly, the reversal of the BFA blockade of cell surface expression of membrane proteins (including class I molecules) demonstrated an identical temperature dependence.

Preincubation of cells for as long as 5 hours with BFA failed to diminish the ability of the NP 50-63 peptide to sensitize target cells for recognition by H-2^k restricted, NP-specific CTLs. The failure of BFA to affect the presentation of exogenous peptides to CTLs indicates that

peptides associate with class I molecules after they have exited the GC. Transport of H-2^k class I molecules between the Golgi complex and the cell surface occurs with a half time on the order of 5 to 10 minutes. The fact that pre-incubation of cells for 5 hours with BFA did not effect the presentation of peptides strongly suggests that exogenous peptides associate with class I molecules present at the cell surface. In contrast, the inhibitory effect of BFA on the presentation of viral proteins suggests that their association with class I molecules, occurs during the intracellular transport of newly synthesized class I molecules to the cell surface.

III. Ability of fixed cells to present synthetic peptide determinants.

To further examine the possibility that peptides associate with class I molecules at the cell surface we examined the ability of paraformaldehyde fixed cells to present the NP 50-63 peptide to CTLs (Yewdell and Bennink, 1989). Since fixation destroys the ability of cells to serve as target cells in ^{51}Cr release assays, we compared the ability of peptide pulsed- fixed and non-fixed cells to inhibit lysis of ^{51}Cr labelled, virus infected cells. L929 cells fixed by 15 minute incubation with 0.5% paraformaldehyde at 20°C maintained the ability to serve as specific inhibitors of NP-specific CTL when pulsed with the 50-63 NP peptide. This experiment demonstrates that peptides can associate with class I molecules on metabolically inert cells, presumably at the cell surface.

IV. Effect of Adenovirus E19K Protein on Antigen Presentation.

Our interpretation of experiments in which inhibitors were used to alter the transport of class I molecules was limited by the possibility that the observed inhibition of antigen presentation was due to alterations in the cellular metabolism of non-class I molecules. As an alternative, more specific approach to inhibiting the transport of class I molecules, we have used an adenovirus glycoprotein, termed E19. E19 is coded by a region of the adenovirus genome that is not required for *in vitro* replication. E19 has previously been shown to have two remarkable characteristics. First, it is retained in the ER following its biosynthesis. Second, it specifically binds class I molecules in the ER and blocks their intracellular transport (Signas *et al.*, 1982; Paabo *et al.*, 1983; Wold *et al.*, 1985; Anderson *et al.*, 1985; Burgert and Kvist, 1985, Paabo *et al.*, 1986). The effect of E19 on antigen presentation has been examined only to the extent of documenting that recognition of adenovirus infected cells by CTLs is diminished in parallel with a decrease in cell surface class I expression (Anderson *et al.*, 1987; Burgert *et al.*, 1987; Tanaka and Tevethia, 1988.).

We initially attempted to use adenovirus itself to express E19 in antigen presenting cells. This approach was hindered by difficulties in achieving high levels of expression of E19 in mouse cells suitable for ^{51}Cr-release assays. This problem was circumvented by the construction of a recombinant vaccinia virus (Vac) containing the gene encoding E19 under the control of the Vac 7.5K promoter (the recombinant is termed E19-Vac). The 7.5K promoter enables expression of inserted genes throughout the infectious cycle. We established that E19 was produced at high levels in a variety of mouse antigen presenting cells within 2 hours of infection with E19-Vac. E19 produced during E19-Vac infection was retained in the ER as determined by the sensitivity of its two N-linked oligosaccharides to endoglycosidase H (endo H) (oligosaccharides acquire endo H resistance during their passage through the cis- or medial GC).

The co-expression of E19 with Vac or influenza virus proteins introduced by co-infection with other Vac recombinant viruses has a profound effect of the presentation of Vac or influenza virus antigens to CTL. The magnitude of this effect is governed by the identity of the restricting class I molecule. Recognition by L^d and K^d restricted specific CTLs is greatly reduced, while the effect on recognition by D^d, D^k, and K^k restricted CTL ranges from slight to nondetectable.

E19-mediated inhibition of K^d-restricted recognition of protein antigens cannot be attributed to diminished levels of K^d expression at the cell surface. This conclusion follows from two findings. First, equivalent amounts of K^d were detected by cytofluorography on the surface of E19-Vac and control-Vac infected cells. Second, the ability of cells to present a K^d-restricted peptide to CTL was not diminished by infection with E19-Vac.

^{35}S-methionine pulse-chase radiolabelling experiments revealed that the effect of E19 on K^d-restricted recognition is attributable to the blockade of K^d transport from the ER. Immunoprecipitation of K^d from E19-Vac infected cell lysates results in the co-precipitation of roughly equimolar amounts of K^d and E19 as detected by SDS-PAGE. Unlike cells infected with a control Vac, in which newly synthesized K^d became endo H resistant with a half time of roughly 30 min, K^d in E19-Vac infected cells remained completely endo H resistant over a 6 hour chase period. By contrast, D^d, D^k, and K^k synthesized in E19-Vac infected cells failed to co-precipiate E19 and demonstrated only a slight to no retardation in temporal

acquisition of endo H sensitivity as compared to control Vac infected cells. Experiments in which cells were co-infected with E19-Vac and a Vac recombinant containing the K^d gene (Coupar *et al.*, 1986) provided additional confirmation that E19 prevents the surface expression of newly synthesized K^d.

Conclusions

Our findings indicate that exogenously added peptide antigens can associate with class I molecules located at the plasma membrane. This conclusion is supported by the findings of Hosken *et al.* (1989) who found that cells lightly fixed with glutaraldehyde maintained the ability to present peptides to ovalbumin specific CTLs. Indeed, the presence of class I in the cell membrane is not required for peptide binding as demonstrated by Chen and Parham (1989) who found that isolated class I molecules could bind oligopeptides, and the elegant work of Kane *et al.* (1989) who found that purified class I molecules immobilized on plastic could present peptides to CTL clones. These findings are in keeping with the far more extensive results concerning the interaction of exogenously added antigenic peptides with class II MHC molecules. It remains to be determined whether (i) the association of determinants with class I at the cell surface is of physiological significance during the course of a normal immune response; and (ii) exogenously added peptides can also associate with class I molecules in endosomal or extra-endosomal intracellular compartments, a possibility suggested by observations that plasma membrane class I molecules are internalized under certain conditions in some cell types (Machy *et al.*, 1982; Tse and Pernis, 1984; Machy *et al.*, 1987).

In contrast to peptide determinants, the presentation of proteins processed from the cytosol was blocked by either emptying the ER of class I molecules by inhibiting protein synthesis, or by preventing the exit of class I molecules from the ER by the use of BFA or the E19 glycoprotein. These findings demonstrate that transport of class I molecules from the ER is required for antigen presentation. Additional studies are required to ascertain whether antigens associate with class I molecules in the ER, or in a post-ER exocytic compartment that is rapidly cleared of class I molecules following the blockade of class I molecule transport from the ER.

The idea that antigen association with class I molecules depends on the continued transport of class I molecules from the ER has important implications for the regulation of antigen presentation. Since many cells in

an organism are not actively dividing, they require only enough class I biosynthesis to replace degraded or shed molecules. Should this rate be low, these cells might depend on external stimuli to boost synthesis of class I molecules to a level associated with effective antigen presentation. This could serve to enhance presentation of foreign determinants, and prevent autoimmune destruction of cells expressing tissue specific proteins excluded from the universe of tolerogenic self proteins.

There is ample evidence for transcriptional and translational control of class I biosynthesis. Klar and Hammerling (1989) have recently demonstrated that the expression of class I molecules can also be under post-translational control. These authors identified several mouse cell lines in which α chains and β_2-microglobulin failed to associate and were not transported out of the ER. Assembly and transport were induced by γ interferon. Might this represent a mechanism for rapidly switching on class I transport and antigen presentation *in vivo*?

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Measlesvirus-Induced Cellular Autoimmune Reactions

U. G. Liebert and V. ter Meulen

SUMMARY

Subacute encephalomyelitis (SAME) in Lewis rats following infection with a neurotropic measles virus (MV) is associated with a cell-mediated autoimmune response (CMAI) to myelin basic protein (MBP) and proteolipid apoprotein (PLP). Grown *in vitro* the autoreactive T cell lines are $CD4^+$, class II MHC-restricted, and the MBP-specific T cell lines are encephalitogenic when adoptively transferred into naive recipients. MBP-selected $CD4^+$ T cell lines both from measles infected animals as well as from rats with experimental allergic encephalomyelitis (EAE), as a consequence of challenge with MBP, shared the same antigenic specificity. Our data indicate that MV infection of the central nervous system (CNS) enhances the susceptibility of the CNS to autoimmune T cell aggression.

INTRODUCTION

Autoimmunity has been observed in acute and chronic viral infections both with RNA and DNA viruses in animal and man (Notkins and Oldstone 1984). Autoantibodies only occur temporarily in the course of such viral infections, are usually of low titer and probably not involved in the development of a disease process since these antibodies disappear when the causative viral agent is cleared by the host defence mechanisms. In contrast to this, little is known about cell-mediated immune responses against autoantigens (CMAI). CMAI which may persist longer has been detected in meningo-encephalitides which occasionally follow acute viral infections or vaccination with measles virus (MV), varicella zoster, rubella, influenza, mumps and rabies viruses (Lisak et al. 1974; Johnson et al. 1984; Hemachudha et al. 1987). These diseases present neuropathologically as disseminated encephalomyelitides with perivascular demyelination. The observed demyelinating process is, however, not due to direct injury, for example, lytic infection of oligodendrocytes, but rather, is mediated by some form of hypersensitivity (Johnson 1982). Although there is little direct evidence to suggest this view and the pathologic role of a CMAI is largely unknown in these conditions, it is nevertheless clear that clinical disease and neuropathological changes may be mediated by autoantigen-specific immune reactions such as is seen following the adoptive transfer of MBP-specific $CD4^+$ T cells and subsequent induction of EAE in rats and mice (Zamvil et al. 1985; Sedgwick et al. 1987).

The acute postinfectious measlesencephalitis (APME) is still of great medical importance as it affects one in 3000 patients with acute measles and is fatal in about 20 % of cases (Johnson 1982). MV

cannot usually be recovered from infected brain material and attempts to demonstrate viral antigens in the brain lesions have failed (Gendelman et al. 1984). In 47 % of patients the peripheral lymphocytes were shown to proliferate in vitro in the presence of MBP (Johnson et al. 1984). These findings provide circumstantial evidence for CMAI pathologically linked to APME. Since positive proof for an existing immune-mediated process in this disease and in other chronic inflammatory CNS diseases such as multiple sclerosis is missing (Hallpike et al. 1983), it is important to study the aspect of MV-induced autoimmunity in an experimental animal model. Recently, we have observed that the intracerebral MV infection of Lewis rats leads to acute and subacute disease processes of the CNS (Liebert and ter Meulen 1987). Moreover, animals developing a subacute measles encephalomyelitis (SAME) contain T cells primed for MBP or PLP. MBP-specific $CD4^+$ T cell lines may be isolated capable of adoptively transferring EAE into naive syngeneic recipients (Liebert et al. 1988). These cell lines exhibit the same specificity and have an identical function as the encephalitogenic MBP-specific T cell lines derived from rats with EAE. Our results indicate that MV infection of the CNS may lead to the generation and/or expansion of encephalitogenic MBP-specific $CD4^+$ lymphocytes. This process may contribute to a virus-induced immunopathological process within the CNS.

RESULTS

Subacute Measles Encephalomyelitis

The intracerebral inoculation of weanling Lewis rats (Zentralinstitut für Versuchstiere, Hannover, West-Germany) with the neuroadapted CAM/RBH strain of MV (Liebert and ter Meulen 1987) lead to the occurrence of a fatal acute encephalitis in more than 75 % of infected animals. About 50 % of those rats which survived the infection developed SAME after incubation periods ranging from 3.5 to 12 weeks following the intracerebral infection. The disease was characterized by weight loss, unsteadiness, ataxia with abnormal posturing of the limps and paresis. Approximately 20 % of the diseased rats died during the course of this monophasic disease and no relapses have been observed in survivors. The neuropathological changes of the SAME animals consisted of extensive perivascular infiltrations of mononuclear cells of the gray and white matter of the brain hemispheres, midbrain and upper spinal cord. Infectious virus could not be isolated from brain material, and the immunohistologic investigation revealed decreasing numbers of infected cells as the incubation period prolonged beyond 4-5 weeks. Furthermore, a progressive reduction of infected cells expressing the viral envelope proteins, M, F, and H, was seen (Tab. 1). The molecular biological characterisation of the MV replication in the SAME brain revealed, as the basis for these findings, transcriptional and translational restriction of the MV envelope gene expression (Schneider-Schaulies et al. 1989). Animals which were killed at various intervals after recovery from clinical disease had neuropathologically either persisting inflammatory lesions or residual changes typical of a previous encephalitic attack in the absence of immunohistologically demonstrable viral antigen. As controls, rats were mockinfected with rat brain homogenate containing no MV antigens. No clinical disease or histological lesions were observed in the mockinfected animals.

Tab. 1. Expression of MV structural proteins in brain cells of MV infected Lewis rats[a].

disease type	days p.i.	number analyzed	number of infected brain cells	relative expression of MV structural proteins N	P	M	F	H
AE	6-14	6	550	100	96	29	16	26
-	16-20	4	250	100	98	24	12	12
SAME	26-38	5	106	100	93	2	3	2
SAME	58-86	4	51	100	85	0	1	1
SAME	56,75, 113,158	4	0	0	0	0	0	0

a. Double-lable immunofluorescence with monoclonal antibodies was done as decribed (Liebert and ter Meulen 1987).

Determination of Cellular Immune Reaction

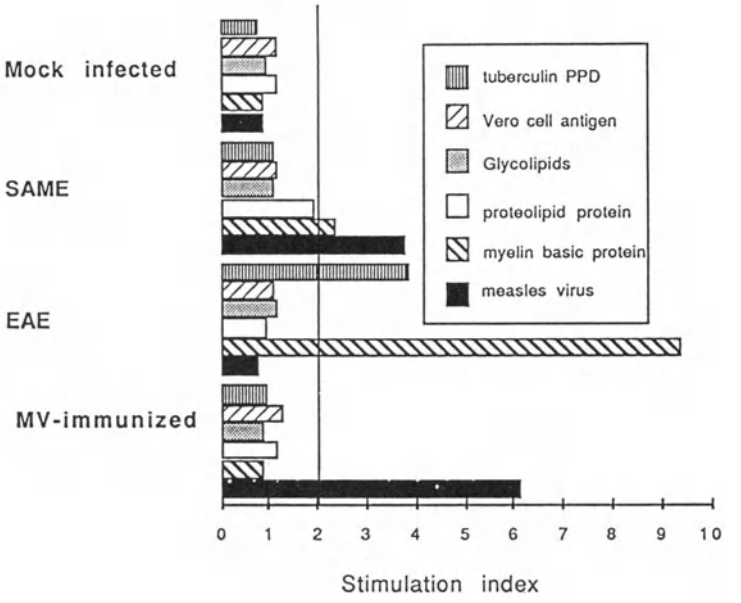
The discrepancy of persisting mononuclear cell infiltration and absence of viral antigen and RNA within the sensitivity of the tests employed, directed us to investigate the cellular immune reaction of rats with SAME (Liebert et al. 1988). Single spleen cell suspensions were cultured in the presence of a panel of antigens or mitogen and the lymphoproliferation was measured by the uptake of [^{3}H]-thymidine. As summarized in Tab. 2 and Fig. 1 the *in vitro* proliferative response of spleen cells to MBP and PLP was significantly elevated in ill SAME rats and in animals which had recovered from clinical disease but still had histologically active encephalitis. Although the observed stimulation indices (SI) for MBP are low in SAME when compared to those animals with EAE the mean SI is still higher than that of the controls not exposed to MBP or PLP. However, in the rats which had recovered from SAME and histologically had no signs of an active encephalitis, no significant in vitro proliferation to MBP was observed. Furthermore, positive proliferation to MBP in the various control groups was only seen in rats in which EAE was actively induced. Moreover, all measles-infected or immunized rats exhibited a significant proliferative response to MV independent of clincial and neuropathological status. In the control groups which had not been exposed to the virus no proliferative response to MV could be detected. In no rat of the different control groups was a significant proliferation to the various glycolipids, including galactocerebroside, gangliosides, sphingo- or phospholipids, detected.

Tab. 2. Proliferative response of spleen lymphocytes from Lewis rats

disease type	number analyzed	[^{3}H] thymidine incorporation* medium	ConA	MV	MBP	PLP	VC	PPD
SAME[a]	25	952	73.9	3.8	2.4	1.9	1.0	0.9
ex-SAME[b]	26	991	73.1	3.8	0.9	1.1	1.0	1.1
EAE[c]	6	1378	44.8	0.6	9.4	1.4	1.6	3.9
mock[d]	12	1o94	59.7	0.9	1.0	1.1	1.2	0.9
MV/CFA[e]	6	1328	44.3	6.6	1.0	0.8	1.5	1.0

*[^{3}H]-uptake in c.p.m. is shown as baseline when no antigen is added to the cultures; in the other colums the lymphoproliferative response is expressed as a stimulation index representing the ratio of the [^{3}H]-uptake in stimulated to unstimulated cells;
a: rats with histologically active encephalitis during or after recovery from clinical disease; b: 3 or more weeks after clinical disease, but no active encephalitis; c: EAE was induced by s.c. immunization with 100 µg of MBP and 100 µg mycobycterium tuberculosis in 100 µl of complete Freund´s adjuvant (CFA) 11-14 days prior to removal of draining lymph nodes; d: mockinfection was done by intracerebral injection of rat brain homogenate containing no MV; e: rats were immunized with 100 µg purified MV in 100 µl CFA.

Fig. 1.
Proliferative response of polyclonal splenic lymphocyte cultures



In vitro and in vivo Studies with T Cell Lines

T cell lines with specificity for MBP or MV could be established from rats with SAME (Liebert et al. 1988). All cell lines were antigen-specific and exhibited no cross-reactivity with inappropriate antigens (Fig. 2). The phenotype of the lines was $CD4^+$, $CD8^-$ as determined by FACS analysis. In blocking experiments it could be shown that all T cell lines investigated regardless of their antigen-specificity were predominantly of the class II MHC-restricted subset (Fig. 3).

To investigate the possible pathological effects of the T cell lines *in vivo*, freshly activated T cell lines of MV or MBP-specificity were adoptively transferred into naive recipients via the tail vein. Each of the MBP-specific T cell lines was effective and induced clinical disease and histological lesions typical for EAE within 4 to 5 days post-cell transfer (Tab.3). In contrast to this, the MV-specific T cell lines induced neither clinical nor histological abnormalities in naive recipient rats (data not shown). Animals having received T cell lines were killed and the CNS was examined for neuropathological changes and for evidence of MV replication. No viral antigen or infectious virus could be detected in the brain tissue of recipients of T cell lines. The neuropathological lesions induced by transfer of MBP-specific T cell lines were located in the lower spinal cord and thus clearly different than in the measles encephalitis.

Tab. 3. Adoptive transfer of MBP-specific T cell lines SAME3

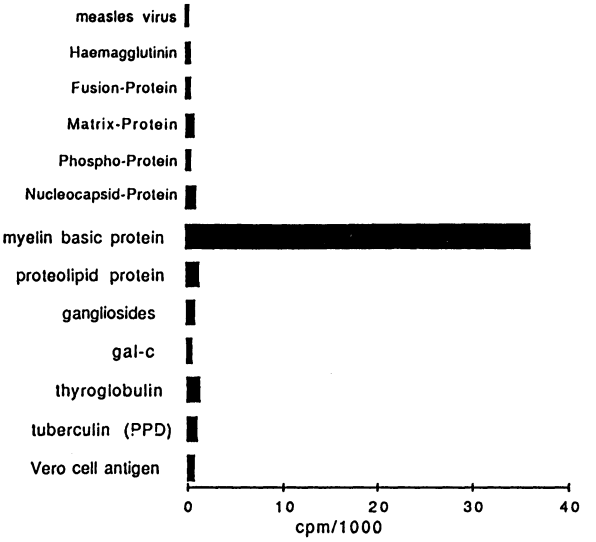
dose	mean	incidence of EAE	
$x10^6$	clinical score	clinical	histological
1	0	0/4	1/4
2	1.0	2/4	3/4
5	2.0	4/4	4/4
10	2.7	3/3	3/3
20	3.0	3/3	3/3

Freshly activated T cell lines were injected via the tail vein into 8 week-old naive syngeneic recipients. Clinical disease started at day 3 post transfer, histology was performed 1-4 days later.

DISCUSSION

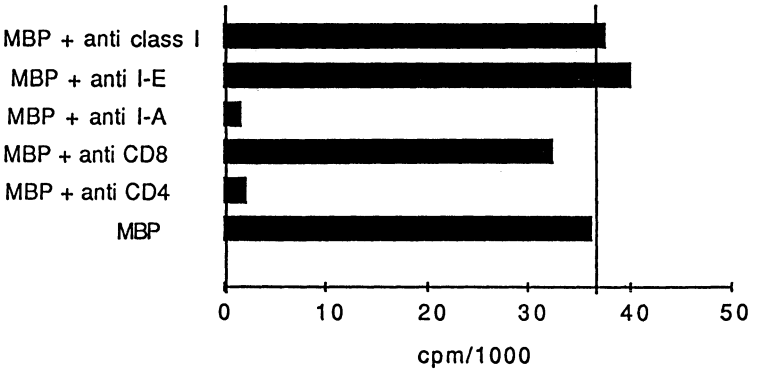
MV-infection of Lewis rats leads to the development of SAME, that occurs on the basis of a persistent MV infection of the CNS. Even after the virus has disappeared from the brain, histological changes of an acute encephalitis may persist, and immunological features of SAME resemble those observed in EAE. From rats with SAME, MBP and PLP-specific T- lymphocytes can be isolated. MBP-specific T cell lines proved in adoptive transfer experiments to be encephalitogenic in naive syngeneic recipients. The clinical disease and the neuro-

Fig. 2. Specificity of MBP-specific T cell lines



A typical pattern of the LPR of the MBP-specific T cell line from a rat with SAME is shown.

Fig. 3. Characterisation of MBP-specific T cell lines



The MHC-restriction and the surface phenotype of antigen-specific T cell lines was tested in blocking experiments in which monoclonal antibodies specific for the MHC-gene products or for CD4/CD8 molecules were added to antigen presenting cells 15-30 min prior to addition of antigen and T cell lines. As example the results of the MBP-specific T cell line as in Fig. 2. are shown. The reduction of the MBP-specific uptake of [^{3}H] thymidine was recorded and expressed in relation to the maximal uptake without monoclonal antibodies.

pathological changes observed after the transfer of MBP-specific T cell lines are not mediated by transfer of contaminating MV for two reasons. First, MV could not be reisolated from or viral antigen detected in T cell lines and brain tissue of recipient rats. Second, clinical disease or neuropathological lesions were not detected in normal syngeneic recipients following transfer of MV-specific T cell lines or polyclonal lymphocytes either incubated with MV *in vitro*, or directly isolated from the brain of infected rats without *in vitro* restimulation.

The antigenic specificity of MBP-specific T cell lines is supported by the failure of all these lines to proliferate in the presence of MV particles, MV antigens and other control antigens or peptide sequences. Vice versa, MV-specific T cell lines only respond to MV proteins but not when MBP or other brain antigens were added to the cultures.

The antigenic specificity of T cell lines for MBP together with the lack of responses to viral antigens suggests that MV particles do not apparently directly contribute antigenic sequences for the development of MBP-responses. While sequence homologies have been found between the N and C proteins of MV and human MBP (Jahnke et al. 1985), direct immunization of Lewis rats with inactivated MV fails to induce EAE-like activity or the expansion of autoreactive MBP-specific T cells probably because pathologically relevant homologous sequences between MBP and MV do not exist (data not shown).

MBP-specific T-cells have also been detected in Lewis rats infected with the murine coronavirus of JHM strain that develop a demyelinating encephalomyelitis (Watanabe et al. 1983).

The observation that viral infections may enhance the susceptibility to EAE has been made in other studies showing that a preceding MV infection potentiates the development and severity of EAE in guinea pigs (Massanari et al. 1979) or that a Semliki Forest virus infection helps to induce EAE in resistent B6 mice (Mokhtarian and Swoveland 1987). To explain this phenomenon several possibilities exist: It is conceivable that virus-induced damage to CNS tissue facilitates the subsequent priming or clonal expansion of pre-existing myelin-reactive T cells for example, via the induction of MHC class II on astrocytes (Massa et al. 1987) and efficient presentation of neural antigen by these cells (Fontana et al. 1984; Sun and Wekerle 1986). Additionally, in measles viral infection, alterations in the surface of infected cells occur potentially resulting in the exposure of cellular components together with the viral envelope proteins. Such exposure taking place in infected brain cells may lead to the development of CMAI against MBP (and PLP) that usually are not detectable in non-infected rats. Moreover, changes in the integrity of the blood-brain-barrier as a consequence of a viral infection of the brain may facilitate the entry of antigen-specific $CD4^+$ T cells to the CNS. Obviously, the development of virus-induced autoimmune reactions against CNS tissue depends on several factors which have to be analyzed before one may fully understand how a virus infection leads to immune pathological reactions.

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Requirements for Recognition of Epstein-Barr Virus-Infected Target Cells by Human Cytotoxic T Lymphocytes

R.J. Murray, C.D. Gregory, D.J. Moss, and A.B. Rickinson

INTRODUCTION

Epstein-Barr virus (EBV) is a widespread herpesvirus which infects the vast majority of individuals in all human populations worldwide. In most such individuals, infection occurs asymptomatically in childhood and leads to the establishment of a life-long virus carrier state. The virus is known to persist in at least two habitats in vivo: (1) in epithelial cells of the oropharynx from which infectious virus can be readily isolated, and (2) in peripheral blood B cells which when placed in culture give rise to continuously proliferating lymphoblastoid cell lines (LCLs). Such LCLs can also be easily produced by exposure of resting B cells to an exogenous source of EBV in vitro. The persistent infection in healthy EBV carriers is believed to be controlled by cytotoxic T lymphocytes (CTLs), since all such individuals possess CTL precursors in the circulating T cell pool which can be reactivated in vitro to lyse EBV-infected target B cells in an EBV-specific and HLA class I antigen-restricted manner (Rickinson, 1986). It is now clear that efficient lysis of EBV+ target B cells by CTLs requires two phases of recognition, the first dependent upon cell adhesion molecules mediating an antigen-independent CTL-target cell conjugation, and the second involving specific recognition of viral target antigens by the T cell receptor (Gregory et al., 1988). These two aspects of the CTL recognition of EBV-infected target B cells will be the subject of this brief review.

EBV infection alters the B cell phenotype

EBV infects resting B cells by virtue of its ability to bind to the CR2 complement receptor molecule (CD21) on the cell surface. Once internalised, the virus induces a series of cellular changes coincident with the expression of a number of so-called 'latent' viral proteins. The sequence of these changes is summarised in Fig. 1. Fig. 1A shows that the first viral proteins expressed by the infected cell (within 24 hrs) are the EB nuclear antigens, EBNA 2 and EBNA-leader protein (LP). Subsequently, EBNA 1 and the EBNA 3 family appear, whilst the EBV latent membrane protein LMP is not demonstrable until day 4 post-infection. The full spectrum of EBV latent proteins is thereafter constitutively expressed by all continuously proliferating LCLs. Expression of the EBV latent proteins by the infected B cell is accompanied by alterations in cellular gene expression and thus in cellular differentiation

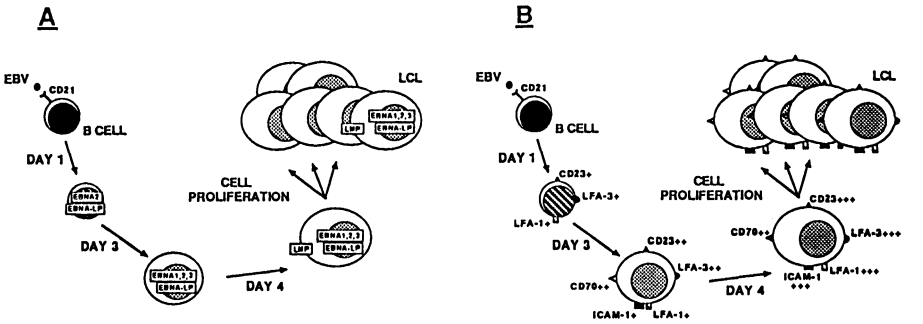

Fig. 1. Transformation of resting B lymphocytes following infection with EBV in vitro. A. Time course of EBV latent protein expression summarised from (our unpublished) Western blotting and immunofluorescence data. B. Time course of EBV-induced cell surface antigen expression. Relative levels of expression are summarised from FACS data and are represented as +, ++ and +++. Note high expression of adhesion molecules is not demonstrable until day 4 post-infection.

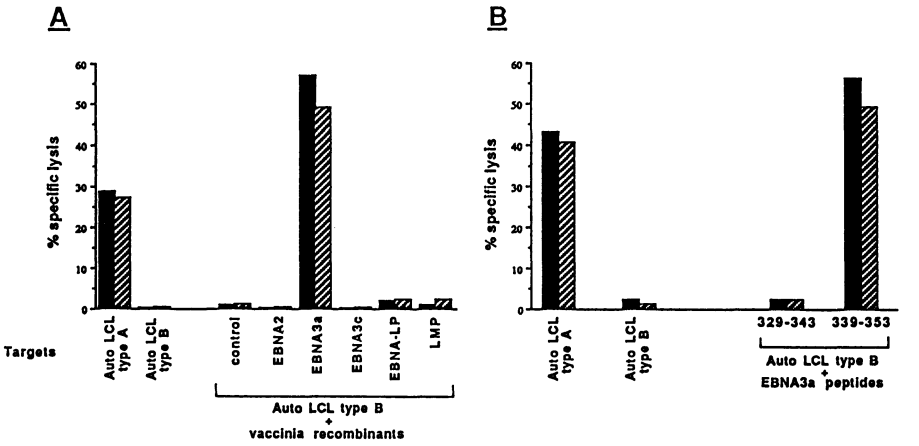

Fig. 2. Lysis of EBV type B strain LCL by autologous type A strain-specific CTLs after expression of type A strain EBNA 3a by infection with recombinant vaccinia virus. A: The type B strain LCL was infected at a multiplicity of infection of 10:1 8 hours prior to inclusion in a 5 hour chromium release assay. Results are shown as % specific lysis. The two columns denote effector:target ratios of 20:1 and 10:1. B: Lysis of EBV type B strain LCL by an autologous type A EBNA 3a-specific CTL clone after treatment with a type A EBNA 3a-derived peptide. EBNA 3a-derived peptides were included in a 5 hour chromium release assay at a final concentration of 10 µg/ml. Results are shown as % specific lysis. The two columns denote effector:target ratios of 10:1 and 5:1.

markers. A summary of some of the known surface markers expressed by EBV-infected B cells is shown in Fig. 1B. Thus, infection is followed by the rapid appearance of low levels of CD23, a surface molecule known to be involved in B cell growth signalling, and of the intercellular adhesion molecules LFA-1 (CD11a/18), LFA-3 (CD58) and subsequently ICAM-1. These various cellular markers ("activation antigens" such as CD23 and CD70, and the adhesion molecules) are induced from low to high level expression by day 4 post-infection, and thereafter remain strongly expressed by all LCLs. The interaction of ICAM-1 with LFA-1 between adjacent cells provides the major pathway of homotypic cellular adhesion leading to the characteristic growth phenotype typical of all LCLs established by in vitro transformation.

The binding of CTLs to target cells has been shown to be mediated through two major antigen-independent pathways (Shaw et al., 1986). The first of these involves the interaction of LFA-1 on the T cell with ICAM-1 on the target, whilst the second employs CD2 on the T cell and its ligand LFA-3 on the target. Our previous studies have demonstrated the importance of these antigen independent pathways in the recognition of EBV-infected B cells by CTLs (Gregory et al., 1988). The inference is that the increase in adhesion molecule expression observed in B cells after EBV infection, which takes several days to reach high levels, is a necessary pre-requisite for the efficient recognition of these target cells by EBV-specific CTLs. This will hold irrespective of the precise viral target antigen against which the response is being directed.

B cell targets expressing LMP display high levels of adhesion molecules and increased CTL binding capacity

The above change in cellular adhesion molecule profile is clearly driven by virus gene expression since, by comparison, UV-inactivated virus preparations elicit only a transient low level response. In order to investigate the relative significance of individual EBV latent proteins in influencing antigen-independent CTL-target cell binding, EBV-negative B cell lines were transfected with single EBV latent genes as previously described (Wang et al., 1988) and subjected to phenotypic analysis. Table 1 summarises some of our observations using transfectants based on two EBV genome-negative BL backgrounds, Louckes BL and BL41. As shown in the table, of all the transfectants tested, only those expressing LMP showed increased amounts of intercellular adhesion molecules ICAM-1 and LFA-3. Moreover, high LMP-expressing clones achieved levels of these molecules which were at least comparable to those found on LCLs. LMP-expressing clones also displayed an altered growth pattern from one of single cells to one of multicellular aggregates. In addition the increased levels of adhesion molecules shown by LMP transfectants were reflected in their increased ability to form conjugates with CTLs (Table 1).

It is significant that in the natural infection of resting B cells (see Figure 1), high adhesion molecule expression is achieved coincident with the first appearance of LMP in the infected cells. The above transfection experiments may therefore be revealing an important consequence of LMP expression in normal EBV-infected B cells.

Table 1. Phenotypic changes in EBV genome-negative BL transfectants expressing individual EBV latent proteins

Transfectant clone	EBV latent protein expressed	Adhesion molecules (FACS MFI)[a]		Conjugate-forming capacity[b]	
		ICAM-1	LFA-3	Medium	MAb Block
Louckes BL					
Lgpt 1	None	29	4	5	0
Lo1.16 C10	EBNA 1	31	4	Nt	Nt
Lo2.4 C6.5	EBNA 2	53	6	5	0
Lo3.3	EBNA 3	33	1	5	0
LoT65C9	EBNA-LP	44	6	Nt	Nt
LgLM5	LMP	100	32	26	0
LgLM6	LMP	164	70	25	0
LgLM8	LMP	94	58	26	0
BL41					
BL41 gpt1	None	23	6	8	0
BL41 gpt2	None	42	4	4	0
BL41 MTLM2	LMP	139	79	21	1
BL41 MTLM5	LMP	143	33	20	0
BL41 MTLM11	LMP	151	97	23	2
LCL	ALL	91	50	31	3

[a] FACS data summarised as Mean Fluorescence Intensity.
[b] Data expressed as % effectors forming conjugates following analysis by FACS as described (Gregory et al., 1988). Conjugates were formed either in culture medium alone or in the presence of a mixture of monoclonal antibodies against LFA-1 and LFA-3 to block the antigen independent adhesion pathways. Effector T cells were from a polyclonal EBV-specific CTL line (class I HLA-mismatched).

Viral antigen recognition by EBV-specific CTLs

It is believed that antigen-independent conjugation of activated CTLs to their targets both precedes and facilitates the recognition of specific antigen by the T cell receptor. LMP clearly has a role with regard to efficient conjugate formation, but the identity of the EBV-coded antigens involved in the antigen-specific phase of immune recognition is a separate issue. It is now known that CTLs recognise short peptide sequences derived from intracellular processing of viral proteins which are presented on the cell surface in conjunction with HLA class I antigens (Townsend et al., 1986). Thus any one of the EBV latent proteins has the potential to provide target epitopes for EBV specific CTLs regardless of its natural cellular location. Moreover, the identity of the target epitope will be determined by the identity of the HLA class I antigen through which recognition is mediated.

In order to determine which of the EBV latent proteins could be recognised by virus-specific CTLs we first tested EBV-negative B cell lines expressing individual EBV latent proteins for susceptibility to lysis by appropriately HLA class I-matched EBV-specific CTLs. The results indicated that LMP was recognised in the context of two different HLA class I backgrounds; HLA-A11 and presumptively HLA-B44 were thought to be the relevant restricting determinants. No evidence was obtained for recognition of any of the EB nuclear antigens in these particular experiments (Murray et al., 1988). However, these studies were limited to effector preparations restricted through only a small range of HLA class I molecules.

More recent studies have indicated that nuclear antigens can indeed provide target epitopes for EBV-specific CTLs. This work is a collaborative effort between our own laboratories and Drs. M. Kurilla and E. Kieff (Brigham and Women's Hospital, Harvard Medical School, Boston, U.S.A.) and Dr. M. Mackett (Paterson Institute for Cancer Research, Manchester, U.K.). The experiments are based on the observation that, whilst the majority of EBV-specific CTL responses are cross-reactive between different strains of the virus (types A and B), rare individuals exhibit only type A strain-specific CTL responses (Moss et al., 1988). LCLs transformed with either type A or type B strains of EBV differ in the antigenicity of their EBNA 2, EBNA 3a, 3b and 3c proteins (Rowe et al., 1989). Thus it is highly probable that one or more of these antigens provides target epitopes for the EBV type A strain-specific CTL response. The target antigen specificity of type A strain-specific CTLs was analysed using recombinant vaccinia viruses to express individual EBV latent proteins from type A strain virus in autologous type B strain LCLs. Using this system we have demonstrated CTL responses specific in one case for EBNA 2 and in another for EBNA 3a. For example, Fig. 2A shows a representative experiment in which a type A strain-specific CTL clone from one individual clearly lyses the autologous type B strain LCL only after its infection with a vaccinia recombinant expressing the type A EBNA 3a protein.

To further dissect the above type A EBNA 3a-specific CTL response, a series of overlapping synthetic peptides corresponding to EBNA 3a protein sequence fragments were screened for their ability to sensitise the autologous type B strain LCL to lysis by the EBNA 3a specific CTL clone. As shown in Fig. 2B, only one 15-mer peptide, corresponding to amino acids 339-353 of EBNA 3a was recognised by the EBNA 3a-specific clone. The very low levels of lysis obtained using peptides covering other regions of the EBNA 3a protein

sequence are represented in the figure by peptide 329-343. Further studies (D.J. Moss et al., to be published) indicate that presentation of the EBNA 3a peptide 339-353 is through the HLA-B8 class I molecule.

CONCLUSIONS

The EBV-specific CTL response can therefore be directed against epitopes derived either from nuclear or from membrane-located viral proteins depending upon the HLA class I molecule involved. However, it is probable that a newly-infected B cell cannot be efficiently recognised prior to the advent of LMP expression since only LMP-positive B cells are readily accessible to CTL conjugation through the antigen-independent binding pathways. Thus EBNA 2- and EBNA 3-specific CTL recognition might not be demonstrable before day 4 post-infection when LMP is expressed (Fig. 1A). Indeed, EB nuclear antigen-specific CTL lysis has, to date, only been demonstrated using LCLs, i.e. target cells with high levels of adhesion molecules.

In a wider context, it is thought that EBV-specific CTLs are important in controlling the growth of malignant EBV+ B cells. Thus malignant EBV+ B cell lymphomas arising in immunosuppressed individuals such as allograft recipients display a characteristic LCL-like phenotype, expressing the full spectrum of both EBV latent proteins and adhesion molecules. These lymphomas therefore should remain susceptible to the virus-specific CTL response and indeed restoration of T cell function in such patients (achieved by relaxing the immunosuppressive therapy) is accompanied by tumour regression. Conversely, EBV genome-positive Burkitt's lymphoma (BL) cells arise in the face of efficient EBV specific T cell immunity. Our studies on the phenotype of BL cells (Gregory et al., 1988; Rowe et al., 1987) indicate that this B cell malignancy is capable of evading CTL-mediated control on two counts. Firstly the tumour cells show an unusually restricted expression of EBV latent proteins (limited to EBNA 1) so that many potential target epitopes for immune recognition are not expressed; secondly the cells do not express detectable levels of cellular adhesion molecules, in particular those molecules (ICAM-1 and LFA-3) which mediate conjugate formation with immune T cells.

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Molecular Analysis of the Protective Immune Response to Murine Cytomegalovirus

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INTRODUCTION

Cytomegaloviruses (CMV), which constitute the β subfamily of herpesviruses, cause protean manifestations and diseases in their respective hosts. In general, the host can be infected throughout life. Whereas embryonal infection can lead to organ malformation and to abortion, perinatal infection is associated with chronic productive infection and disease. The infection of the immunocompetent host is usually asymptomatic. At stages of iatrogenic or acquired immunodeficiency, however, severe disease and death are common sequelae. Similar to other herpesviruses, CMV persists after infection either in the form of low level chronic productive infection, or in the state of latency from which reactivation can occur. Various cells of the hemopoietic system and the salivary glands frequently harbor the virus, but the cellular site of latency is still an issue of debate. The capacity to control the infection is closely related to the status of the host's immune system. Thus, to fully understand the mechanisms underlying the control of the CMV-host balance becomes an imperative issue.

In human beings the control of CMV infection has been correlated with the functional activity of T cells. The detailed study of human CMV (HCMV) disease is hampered by the fact that HCMV is strictly species specific. The study of CMV infections in general, however, has been greatly facilitated by the availability of CMV of other species. Our laboratory as well as others have used the infection of the mouse with murine cytomegalovirus (MCMV) as a model for human infection.

In this manuscript we review selected data from studies performed in our laboratory, which were intended to acquire new insight concerning the function of antigen-specific $CD8^+$ T lymphocytes during recovery from acute CMV infection and during establishment of latency.

PROPERTIES OF CMV

Members of the herpesvirus family share the main morphological characteristics. The core of double-stranded linear DNA wrapped on a fibrillar spool is located in a 100-110 nm icosahedric capsid that is embedded in

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an asymmetrically formed tegument. A lipid envelope surrounds the tegument, and the complete virion measures 120-200 nm in diameter. MCMV can give rise to multicapsid virions sharing a common lipid bilayer membrane. Herpesviruses are grouped either according to the structural organization of their genomes or on the basis of their biological properties. The MCMV genome of 235 kbp in length has been cloned and, unlike the genome of HCMV, it lacks large terminal or internal repeat regions (Ebeling et al 1983). Thus, the herpes-viruses of the β-herpesvirus subfamily mainly share the biological proper-ties. Typical is the relatively long reproductive cycle, the enlargement of infected cells (cytomegalia) and the inclusions formed in permissive fibroblast cells in both nucleus and cytoplasm (Weiland et al 1986). The MCMV reproductive cycle can be subdivided into a short (about 2 h) phase of immediate-early (IE) gene expression followed by an early (E) phase of about 14 h and a late (L) phase (about 8 h), which starts with the synthesis of viral DNA (Keil et al 1984). Whereas during the L-phase mainly structural proteins are synthesized, the IE-phase gives rise to a limited number of nonstructural regulatory proteins (Keil et al 1984; Koszinowski et al 1986; Münch et al 1988; Schickedanz et al 1988), and the E-phase is characterized by the synthesis of structural and nonstructural proteins.

THE IMMUNOCOMPROMISED HOST AND PROTECTION BY $CD8^+$ T LYMPHOCYTES

Clinical studies on HCMV infection show that lethal manifestations of CMV disease occur mainly during immunodeficiency. Specifically, interstitial CMV pneumonia is a risk after immunodepletion by whole body irradiation in leukemia patients receiving bone marrow transplantation. Similar conditions apply to the murine model established in our laboratory. BALB/c mice are resistant to MCMV infection over a wide dose range, and the only sites from which virus can be isolated for several weeks post infection (p.i.) are the salivary glands. In mice immunodepleted by 6 Gy irradiation the virus disseminates from the local site of infection to all organs. Animals die between day 10-18 p.i. and the terminal stage of disease is characterized by wasting, bone marrow aplasia, hepatitis, adrenalitis and interstitial pneumonia (Mutter et al 1988; Koszinowski et al 1989). Thus, with respect to the asymptomatic infection in the immunocompetent host and the severe disease with the characteristic organ manifestations after immunodepletion, the murine model reflects the main biological features of HCMV infection.

Transfer of lymphocyte populations into irradiated and infected recipients provided the experimental protocol to study the contribution of lymphocyte subsets to the control of MCMV infection. Upon prophylactic cell transfer at the day of infection, as few as 10^4 sensitized T lymphocytes limited viral replication in tissues, and transfer of 10^7 T lymphocytes established the situation observed in the immunocompetent mouse: the infection remained largely confined to the acinar glandular epithelial cells of the salivary glands (Reddehase et al 1985). Protection in vivo against spread of MCMV was not only achieved by cell transfer prior to infection. The therapeutic cell transfer, carried out about 1 week p.i., at times when virus had already colonized the tissues, was also efficient, but needed higher cell numbers. The antiviral efficacy of lymphocytes could be improved by in vivo application of interleukin 2 (Reddehase et al 1987 a). The antiviral effector function was associated with the $CD4^-CD8^+$ subset of T lymphocytes, whereas cells of the $CD4^+CD8^-$ subset had no detectable antiviral effect on their own and were also not required as helper cells. For antiviral function after transfer, $CD8^+$ memory T lymphocytes were as active as $CD8^+$ T lymphocytes taken from the peak of the immune response. Probably, memory $CD8^+$ T lymphocytes must encounter antigen in the recipient to function. This reactivation

did not require the presence of $CD4^+$ T lymphocytes in the transferred cell population nor in the recipient (Reddehase et al 1988).

In human beings, the conditions for CMV disease during HIV infection differ from those in bone marrow transplant recipients in that the immunosuppression selectively affects the $CD4^+$ T lymphocytes while sparing $CD8^+$ T lymphocytes. It was therefore of interest to explore the control of CMV infection by $CD8^+$ T lymphocytes in absence of $CD4^+$ T lymphocytes. Two questions were addressed: first, can antiviral $CD8^+$ T lymphocytes be generated in absence of $CD4^+$ helper T lymphocytes and second, if so, is this response capable of exerting a long term control of infection and to establish latency? The depletion of the $CD4^+$ T lymphocyte subset *in vivo* was achieved by weekly intravenous infusion of antibodies to the CD4 molecule. The absence of $CD4^+$ helper T lymphocytes was monitored by cytofluorography and was certified by the failure to generate antibodies to MCMV. The presence of antiviral $CD8^+$ effector cells was tested by lymphocyte transfer into lethally infected recipient mice. The results allowed two conclusions: first, an antiviral immune response by the $CD8^+$ subset can be initiated and maintained without help provided by the $CD4^+$ subset. Second, during long term depletion of the $CD4^+$ subset, $CD4^-CD8^-$ lymphocytes are generated. This subset, most probably T lymphocytes that express a TCR γ/δ, did not provide help to B cells and did not have antiviral function after cell transfer. Fully immunocompetent mice require about 6 weeks to establish latency. Infected mice depleted of the $CD4^+$ subset demonstrated a prolonged phase of asymptomatic chronic infection of organs. All organs, with the exception of the salivary glands, were, however, cleared 10 weeks p.i. Remarkably, the salivary glands remained productively infected even after a three month period. Histological examination revealed that, unlike irradiated and MCMV infected mice in which virus replicates in various cell types of the salivary gland, $CD4^+$ subset depleted mice confine the infection to the acinar glandular epithelial cells, similar to the situation in fully immunocompetent mice (Jonjić et al 1988).

Thus, apparently, the salivary gland tissue is accessible to antiviral control by $CD8^+$ effector cells and the persistent infection is based on as yet undefined but selective immune escape mechanisms characteristic of acinar glandular epithelial cells. In this context it may be of interest to determine the expression of MHC class I genes in these cells.

Altogether, the data convinced us that the $CD8^+$ T lymphocyte subset can, with the exception of establishment of latency, significantly contribute to the control of CMV infection in the normal and in the immunodeficient host.

AN IE ANTIGEN OF MCMV THAT ELICITS PROTECTIVE IMMUNITY

Cytolytic activity against infected cells presenting the relevant antigen(s) is an *in vitro* testable function of $CD8^+$ T lymphocytes and defines the subset of cytolytic T lymphocytes (CTL) in the $CD8^+$ T lymphocyte population. Although it is not proven that the antiviral activity of $CD8^+$ T lymphocytes results from cytolysis *in situ*, the cytolytic function *in vitro* usually correlates with the antiviral activity *in vivo*.

It was considered that, if cytolysis were the mechanism of virus clearance in vivo, recognition and lysis of infected cells by CTL should occur before the release of infectious progeny. It was therefore of interest to identify at which phase of viral gene expression, namely IE, E or L, the MCMV-specific CTL could interrupt the replication cascade. By using metabolic inhibitors, the replication cascade can be arrested between the IE, E and L phases resul-

ting in target cells that selectively express increasing amounts of viral proteins synthesized in the respective replication phase. This analysis revealed that already products of IE transcripts are detected by polyclonal CTL (Reddehase et al 1984 a, b). For comparing the relative importance of CTL with specificity for the viral antigens expressed at any of the three replication phases, a limiting dilution analysis was carried out to estimate the relative frequencies of antigen specific CTL. In order not to distort frequencies generated *in vivo* by *in vitro* restimulation conditions, activated precursors to CTL were expanded solely by interleukin 2. With this approach, it was found that about half of all MCMV-specific CTL detect antigens on infected cells arrested for selective MCMV IE gene expression (Reddehase and Koszinowski 1984). Evidence for an *in vivo* protective antiviral function of IE-antigen-specific $CD8^+$ T lymphocytes came from panning studies demonstrating that depletion of the IE-antigen-specific CTL activity from polyclonal CTL abolishes the capacity to control infection (Reddehase et al 1987 b). This indicated that IE antigens dominate the CTL response in BALB/c mice in a quantitative sense and are also important for antiviral function.

To understand the molecular basis of IE antigen recognition, the IE genes were mapped and characterized, and the encoded proteins were defined. Abundant IE gene transcription is derived from a 12 kbp region of the 235 kbp MCMV genome (Keil et al 1984). An enhancer is located centrally in this region (Dorsch-Häsler et al 1985) which probably represents the target sequence for cellular factors necessary for the activation of the viral transcription machinery. This enhancer sequence is flanked by two promoters. The left hand promoter (according to the prototype orientation of the genome) (Ebeling et al 1983) controls a number of transcripts which undergo splicing. There are at least five exons. Exons 2-4 contain an open reading frame for a protein of 595 amino acids. The gene encoding this protein was termed gene *ieI* and the nonstructural nuclear phosphoprotein, which represents the most abundant IE protein, was termed pp89 according to its relative mobility in gels. Other transcripts use the first three exons but are spliced into the fifth exon and constitute the ie3 transcription unit (Keil et al 1985; Keil et al 1987 a; Keil et al 1987 b). Cooperation of ie1 and ie3 encoded proteins is necessary and sufficient for the induction of MCMV early transcription in trans. The right hand promoter controls the expression of a 43 kDa protein of low abundance derived from three exons of gene *ieII*.

The comparison of the corresponding IE regions of HCMV and MCMV reveals homologies with respect to the presence and structural organization of the enhancer (Boshart et al 1985) and with respect to the structural organization and the function of products of genes under control of the ie1 promoter (Stenberg et al 1984). There is remarkably little homology in the amino acid sequence of the expressed proteins.

T lymphocyte receptors recognize peptide fragments of antigenic polypeptides, bound to MHC glycoproteins. During infection with MCMV, the immune response comprises polyclonal T lymphocytes that recognize peptides derived from more than one viral protein and presented by different MHC molecules. Given these variables, it was necessary to isolate representative CTL clones with specificity for an IE antigen to determine the restricting MHC molecule and to identify the viral protein containing the antigenic epitope. A CTL clone, termed IE1, was isolated that defines an IE antigenic determinant presented by the L^d glycoprotein (Reddehase et al 1986 a). As the next step, cells transfected with L^d were supertransfected with MCMV DNA fragments of various length. This procedure identified gene *ieI* encoding pp89 to be essential for recognition by clone IE1 (Koszinowski et al 1987 a, b). Formal proof for the recognition of pp89 was achieved when the continuous open reading frame of pp89 was expressed by a vaccinia recombinant (Volkmer et al 1987). The fact that the recombinant MCMV-*ieI*-VAC was both antigenic and immunogenic led to

the testing of this recombinant as an experimental vaccine. It was found that the IE protein pp89 was sufficient for protection against a lethal challenge with MCMV. This protection was mediated entirely by the $CD8^+$ subset of T lymphocytes since infusion of antibodies to the CD8 molecule abolished the protection *in vivo* (Jonjić et al 1988).

PROPERTIES OF THE ANTIGENIC EPITOPE OF PP89

The restriction of CTL clone IE1 for the L^d molecule is representative for all BALB/c pp89-specific CTL (Del Val et al 1989; in press). To define the epitope(s) of pp89, several in frame deletion mutants of the *ieI* open reading frame were expressed by vaccinia recombinants. Two deletion mutants, ΔF and ΔJ, located the epitope at between amino acid positions 154-249. Positive epitope definition was achieved by using synthetic peptides. Peptide P(161-179) sensitized L^d positive cells for the attack by specific CTL (Del Val et al 1988). The crystallographic structure determined for an MHC class I molecule has revealed a potential combining site that can accommodate an extended peptide of about eight residues or a helical peptide of about 20 residues (Bjorkman et al. 1987). With the sequence pattern 170-HFMPT-174, peptide P(161-179) contains a linear Rothbard motif. By screening of a series of related peptides which were reduced in length from both termini, the nonapeptide 168-YPHFMPTNL-176 was identified as the optimal antigenic peptide for clone IE1 in case of external loading of L^d (Table 1). In addition, the analysis precisely identified the predicted pentapeptide motif HFMPT as the minimal antigenic peptide, which still comprised the epitope for recognition by the TCR of clone IE1 and an agretope sufficient for specifying contact with L^d. Titration of the nonapeptide 168-YPHFMPTNL-176, the heptapeptide 169-PHFMPTN-175 and the pentapeptide 170-HFMPT-174 revealed 10^3 fold differences in the antigenic potencies (Reddehase et al 1989).

We next addressed the question of whether the identified peptide represents the only epitope or merely a strongly immunogenic sequence in a hierarchy of epitopes of different antigenic potency. The nucleotide sequence for the pentapeptide sequence HFMPT was deleted from the pp89 open reading frame, and the deletion mutant was expressed by a vaccinia recombinant. This deletion caused a complete loss of antigenicity and immunogenicity for $CD8^+$ T lymphocytes of pp89, which proved that recognition of a single epitope was responsible for the protective effects in BALB/c mice.

Although the antigenicity of an epitope is determined by its sequence, it is not yet clear to what extent sequences flanking the motif affect antigen processing. Flanking sequences do not necessarily exert major constraints on the capacity of a chimeric protein synthesized in cells to be processed and to be presented. So far, only a chimeric protein containing peptide sequences of 13 residues had been tested (Chimini et al 1989). The definition of the minimal antigenic determinant for MHC class I restricted T lymphocytes allowed us to test chimeric proteins containing pp89 epitope sequences of different length that are all antigenic as peptides. For this purpose, the 18mer sequence 162-179, the nonamer YPHFMPTNL, the heptamer PHFMPTN or the pentamer HFMPT sequences were inserted at the position 552 of pp89 using the ΔF deletion mutant that lacks the epitope. Vaccinia recombinants expressing these chimeric proteins led to different results (Table 1). While the 18mer and the 9mer insertion endowed the chimeric protein with antigenic and immunogenic properties comparable to authentic pp89, the chimeric proteins expressing the heptamer and the pentamer had lost these properties. One explanation for this result is that the pentamer sequence has a low antigenic potency and perhaps there are not enough recombinant molecules

synthesized to achieve the required molar concentration of peptides. This argument, however, is not likely to apply to the heptamer, which has a detection limit of 10^{-8} M when added to cells as a peptide. An alternative interpretation is that processing is not completely random and that peptides longer than a heptamer are generated *in vivo*. Given this is the case, the flanking sequences gain importance. Along with this interpretation is our finding that peptides which contain the pentamer or the heptamer core may be devoid of any antigenicity, which suggests that residues flanking an antigenic core motif affect antigenic potency (Reddehase et al 1989).

Table 1. Immunological properties of epitope sequences of pp89 added from without or synthesized within cells

Sequence	Peptides: Detection limit [a] (log M)	Recombinant Viruses: Antigenicity [b] (% lysis)	Recombinant Viruses: Immunogenicity [c] (% lysis)
pp89 (1-595)		20	21
pp89ΔF(Δ136-249)		0	0
18mer (162-179)	n.d. [d]	22	n.d.
11mer MYPHFMPTNLG	- 7 to - 6	n.d.	n.d.
9mer YPHFMPTNL	-12 to -10	24	27
7mers PHFMPTN	- 8 to - 7	0	0
YPHFMPT	–	n.d.	n.d.
HFMPTNL	- 5 to - 4	n.d.	n.d.
5mer HFMPT	- 3	0	2
4mers HFMP	–	n.d.	n.d.
FMPT	–	n.d.	n.d.

[a] Recognition by clone IE1 of target cells incubated with peptides at different concentrations.

[b] Recognition by BALB/c pp89-specific CTL of target cells infected with the recombinant viruses containing the indicated sequences in position 552 of pp89ΔF.

[c] BALB/c MCMV-specific memory CTL were restimulated in vitro with the recombinant viruses. CTL generated were tested on MCMV-infected cells expressing pp89.

[d] not done.

TEMPORAL CONTROL OF PP89 ANTIGEN PRESENTATION

The experimental data had shown that cells arrested for selective IE gene expression are optimal targets for pp89-specific CTL. When the specific CTL were used to monitor antigen presentation during the physiological cascade of IE, E and L gene expression in cells in absence of inhibitors, it was observed that pp89 antigen recognition was only effective at the stage of late gene expression, due to the reinitiation of expression of that gene during the L phase (Reddehase et al 1986 b). Since the expression of IE proteins is pivotal for initiation of E gene transcription, L-phase reexpression of the gene could explain the antigenic properties of L-phase

targets, but did not explain the lack of antigen presentation during the IE and E phases. Either, the physiological concentration of IE proteins required for transcriptional activation of E genes was below the threshold for detection by CTL, or there was an active interference with pp89 antigen presentation. Evidence for the latter was provided by the observation that the selective and enhanced synthesis of the IE proteins after addition of the translation inhibitor cycloheximide (CH) and its replacement by actinomycin D (act D) for preventing early gene expression provided excellent targets, whereas the omission of act D allowed E gene expression without affecting enhanced pp89 synthesis, but completely abolished pp89 antigen presentation. Thus, the expression of E genes interfered throughout the E phase with pp89 antigen presentation. This interference with the posttranslational steps of processing and presentation did not involve modifications in the expression of MHC molecules nor in their capacity to present antigen epitopes after external loading of peptides. There was also no general inhibition of the processing machinery since other MCMV proteins could be processed for L^d presentation at times when pp89 could not.

CONCLUSIONS

By using the immunodeficient mouse as a model, evidence for the protective role of $CD8^+$ T lymphocytes during acute and chronic CMV infection was provided. Although the virus infection could be controlled by the $CD8^+$ subset in absence of any other adoptive immune function, the results do not rule out a physiological contribution of antibodies and T helper functions. The failure of $CD4^+$ subset depleted mice to establish latency does in fact suggest a contribution of additional effector mechanisms.

$CD8^+$ T lymphocytes recognize fragments of processed polypeptides presented by MHC class I molecules. This way of antigen presentation does not select for or against structural components of the virion, which is illustrated by the protective immunity generated in response to a nonstructural nuclear protein. Considering the very high coding capacity of MCMV, it appears surprising that the few proteins of the IE phase apparently represent dominant immunogens in the BALB/c strain. Perhaps this merely reflects the failure of the $CD8^+$ subset to recognize many other MCMV proteins. There is ample evidence, supported also by our own observations, that not each viral protein is recognized by $CD8^+$ T lymphocytes.

One explanation for the limited number of epitopes detected by $CD8^+$ T lymphocytes is the acquisition of immunologic self-tolerance, which largely precludes that self-molecules or sequences homologous to self are recognized. However, the probability of homologous sequences is quite low. Another mechanism that could limit recognition is the unknown process operative during the E phase of MCMV replication, which prevents pp89 antigen recognition under conditions regarded as essential and sufficient for antigen presentation, namely, the presence of a sufficient amount of an antigenic protein, the integrity of processing mechanisms, and the capacity of MHC molecules to present this antigen. The pp89 is presented because of the release from control during the L phase. If such a mechanism is of general importance, it could be envisioned that some polypeptides remain completely excluded from presentation.

If we accept that only a few polypeptide fragments of a pathogen give rise to a $CD8^+$ T lymphocyte response, it is important to map such epitopes. For replacing the live pathogen, it is mandatory to provide simultaneous expression of several epitopes in a vector that should ideally have little immuno-

genicity on its own to make repeated immunizations feasible. The elucidation of principles that govern the processing and presentation of chimeric proteins is an important step in that direction.

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IV Defense

Bacterial and Parasitic Infections

Insights into Immunoregulation and Pathogenesis from a Third World Disease

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INTRODUCTION

To understand the pathogenesis of any disease that afflicts people primarily in developing countries, one must have some appreciation of that context. It is difficult to comprehend the quality of life found in the 40 poorest countries in 1988 where per capita income is $310/yr, 21% of women are allowed to become literate, 17% of the rural population has access to clean water, and there is 1 physician for 6,050 people (World Development Report , 1988). Life expectancy is 46 years, infant mortality is 136/1000 births and 31% of children suffer from malnutrition. Despite those grim statistics, the number of children in developing countries immunized with the basic childhood vaccines of diphtheria, pertussis, tetanus, measles, polio and tuberculosis has risen from 5% to over 60% in the past 15 years, largely due to the efforts of WHO and UNICEF. Clearly vaccines represent the most cost-effective measure, aside from handwashing, for the improvement of health in the Third World. Immunologists have a unique contribution to make - and special responsibility - in this area. The challenge is not merely to develop better strategies for treating and preventing diseases of the Third World, but doing so by means that can be afforded and implemented in the developing countries. This paper will argue that study of certain, often neglected diseases occurring primarily in the Third World offers much insight into fundamental and general problems of immunoregulation and immunity to infection.

IMMUNOLOGY OF LEPROSY

From the point of view of an immunologist, leprosy is fascinating because it is not a single clinical entity, but represents a spectrum that presents a diversity of clinical manifestations which correlate with immunological parameters. At one pole of the spectrum, tuberculoid leprosy, patients develop high levels of specific cell-mediated immunity that ultimately kills and clears the bacilli in the tissues. At the lepromatous pole, patients exhibit a selective unresponsiveness to antigens of *Mycobacterium leprae* and the organisms ineluctably multiply in the skin, often in enormous numbers. It is unclear why the vast majority of people exposed to infection with *M. leprae* develop immunity, and why only a minority of those who do develop clinical disease become lepromatous and remain immunologically unresponsive to antigens of the organism. Since the pathogenic agent, *M. leprae*, has never been cultivated *in vitro*, leprosy presents three challenges: 1). To understand how this intracellular pathogen that lives primarily inside of macrophages can elude immunological surveillance and cause disease; 2). How to make vaccines for a pathogen that cannot be cultivated; and 3) How to attack a universal historical stigma through modern science. Since there is no known animal reservoir for transmission of leprosy infection to man, if effective and affordable vaccines could be developed, the goal is to eradicate leprosy from the face of the earth.

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IMMUNOLOGICAL MECHANISMS OF NERVE DAMAGE

M. leprae grows essentially only in macrophages and Schwann cells. A puzzling problem is presented by patients with the tuberculoid form of leprosy, who develop high levels of cell-mediated immunity, virtually eliminate acid-fast bacilli from the tissues, but suffer major nerve damage. We developed a model for nerve damage caused by cell-mediated immunity. Guinea pigs were sensitized to killed mycobacteria and challenged with purified protein derivative [PPD] in the area of the sciatic nerve or in the ventricle (Wisniewski and Bloom, 1975). The results demonstrated primary focal demyelination of these fibers as innocent bystanders to a specific cellular immune reaction to the foreign antigen. In analyzing the mechanism of this "bystander" demyelination, we found that activated macrophages secreted neutral proteinases that could degrade purified myelin proteins *in vitro* (Cammer et. al. 1980). One of these proteinases, plasminogen activator, had the ability to activate the large reservoir of plasminogen in serum and extravascular fluids to release high levels of plasmin, a neutral protease, locally that would degrade myelin proteins. We confirmed the involvement of this mechanism in allergic encephalomyelitis, by showing that specific inhibitors of plasminogen activator blocked paralysis and demyelination *in vivo* (Brosnan et al 1980). Recent evidence by S. Kaufmann and colleagues suggest a second possible mechanism for nerve damage, namely that cytotoxic T lymphocytes may be developed capable of lysing Schwann cells stimulated by IFN-gamma to express MHC Class I molecules in the presence of mycobacterial antigens (Steinfoff and Kaufmann 1988). The pathology of nerve damage in multiple sclerosis appears similar to that in the leprosy models, and we hope that studies of nerve damage in leprosy will provide insight into general mechanisms underlying tissue damage in autoimmune diseases as well.

MECHANISMS OF MYCOBACTERIAL SELF-DEFENSE:

One of the paradoxes of *M. leprae* is that it lives all or most of its life in the human host primarily within mononuclear phagocytes, which have evolved potent and sophisticated mechanisms to kill intracellular pathogens. The most effective and best understood cytocidal mechanism is that dependant on the production of reactive oxygen intermediates, resulting from single electron reduction of molecular oxygen and the production of the superoxide anion, hydrogen peroxide, and hydroxyl radical (Babior 1987). One further curiosity about *M. leprae* is the fact that this feeble organism produces prodigious amounts of two lipid moieties --- a unique phenolic glycolipid-1 (PGL-I) and a lipoarabinomannan (LAM). We have tested the hypothesis that the ability of *M. leprae* to survive within professional phagocytic cells might result in part from the production of molecules that scavenged oxygen radicals, the best candidates for which being PGL-I and LAM. Using a system of electron spin resonance and spin trapping to measure adducts with O_2-, H_2O_2 and $^{\cdot}0H$, derivatives containing the terminal sugars of the *M. leprae* PGL-1 were found to be excellent scavengers of both $OH^{\cdot}$ and $O2^{\cdot}$ (Chan et al 1987). The other *M. leprae* glycolipid, LAM, also is an oxygen radical scavenger. Since the inability of *M. leprae* to grow precludes direct testing of the protection to killing afforded by these glycolipids, we studied a related molecule, lipophosphoglycan (LPG), produced by the protozoal parasite, *Leishmania donovani*, which grows exclusively in macrophages. This compound not only scavenges oxygen radicals, but was able to protect *L. donovani* parasites against killing by these radicals *in vitro*. These studies suggest that the long overlooked molecular species of complex glycolipids and polysaccharides of many bacterial and protozoal parasites, may have an evolutionary role in protecting against cytocidal activity of macrophages.

MECHANISM OF SPECIFIC IMMUNOLOGICAL UNRESPONSIVENESS:

Studies of patients with early lepromatous leprosy indicate that, although they are totally unresponsive to antigens of *M. leprae*. the vast majority exhibit normal cell-mediated immunity to a variety of recall antigens including PPD, This is particularly paradoxical since most antibodies prepared against the protein antigens of *M. tuberculosis* are cross-reactive with antigens of *M. leprae*. These results raise the question of how it is possible to respond to the same or cross-reactive antigens when they are associated with *M. tuberculosis*, and yet be totally unresponsive to the same or related antigens associated with *M. leprae*. We suggested many years ago that there may be one or a small number of unique epitopes associated with *M. leprae* capable of inducing T suppressor cells, that had the ability to suppress responses of potentially cross-reactive helper clones centrally or in the periphery (Bloom and Mehra 1984). In studies of more than 250 leprosy patients, we found that >80% of lepromatous patients, and many borderline patients showed lepromin-induced suppression of T cell proliferation to Con A or antigen *in vitro*. In contrast, very few patients with tuberculoid leprosy or strongly skin-test positive healthy

contacts of leprosy patients showed *in vitro* suppression. The phenotype of human Ts cells is CD8+, CD3+, FcR+, HLA-DR+, TAC+ and CD28-. The induction of suppression is specific for antigens in *M. leprae* and is not induced *in vitro* by other mycobacteria (Modlin et al 1987).

The suppressor cell hypothesis has now been tested at several levels. Removal of the CD8+ cells restored lepromin responsiveness to lymphocytes a third of lepromatous patients (Mehra et al 1982) Our interpretation for the majority is that removal of CD8+ cells could not correct "afferrent suppression", which, in a chronic disease like leprosy is likely, over time to prevent the expansion of lepromin responsive T_H cell clones to a detectable level in blood. Cells of the suppressor phenotype predominate in lepromatous and not in tuberculoid lesions (Modlin et al 1983). Methods were developed by R. Modlin and T. Rea to isolate T lymphocytes directly from diagnostic biopsies of the lesions, and after brief expansion with IL- 2 *in vitro*, we could demonstrate that over half of the lines and clones so isolated from lepromatous lesions had suppressor activity *in vitro* when stimulated with *M. leprae* antigen (Modlin et al 1986); 3). In patients given an immunotherapeutic vaccine developed by J. Convit and colleagues (Convit et al 1982), consisting of killed *M. leprae* and live BCG, under conditions where they converted to lepromin skin test positivity and showed clinical improvement or cure, in blinded *in vitro* studies we found that their *in vitro* suppressor activity disappeared.

By establishing Ts cell clones it has been possible to examine two critical characteristics of human Ts cells that have been controversial in the mouse. When CD8 T suppressor clones were added to CD4+ antigen specific T helper clones in the presence of specific antigen, they effectively suppressed proliferation of the Th cells *in vitro*. By mixing clones of different major histocompatibility haplotypes we were able to show that there is a clear genetic restriction on T cell suppression associated with MHC Class II antigens (Modlin et al 1986). Second, we established that 9/9 of our Ts clones expressed T cell receptor α,β heterdimers on their surface (Modlin et al 1987). Antibodies prepared against T cell receptor τ-chain peptides failed to disclose the expression of TCR $\tau\delta$ receptors.

MECHANISMS OF T CELL SUPPRESSION

The mechanisms by which Ts regulate T_H activity and antibody production have not been elucidated in any system. The existence of both antigen-specific T_H and Ts clones has permitted us to explore a number of possible mechanisms of suppression (Salgame, Modlin and Bloom 1989). The data indicate clearly that the CD8 Ts clones do not effect their suppression by: i) killing antigen presenting cells in the presence or absence of antigen; or ii) killing the T_H helper clones by an idiotypic or antigen- specific mechanism. Although not excluding the possibility definitively, we have thus far been unable to find evidence for a suppressive factor. It was shown that after stimulation with specific *M. leprae* antigens, Ts cells will suppress T_H cells specific for non mycobacterial antigens, e.g. influenza peptides. This result and others suggest that the mechanism of suppression is unlikely to involve "veto" or presentation of antigen/MHC to the T_H cell receptor by CD8 suppressor cells lacking appropriate co-stimulatory signals. The data are most consistent with the view that the mechanism of suppression is likely to be unique, perhaps negating positive signals at the level of the T_H cell. The end result, however, appears to be indistinguishable from clonal anergy of the CD4 T_H cells that can be produced by antigen/MHC in the absence of co-stimulatory signal (Mueller, Jenkins and Schwartz 1989).

T CELL RECEPTOR γ,δ CELLS IN LEPROSY:

One of the intriguing problems in immunology remains the function and specificity of TCR γ,δ cells. Although a limited number of germ line genes encode the TCR γ and δ subunits, extensive junctional variation particularly in the gene results in unprecedented diversity for this receptor. Yet until recently, there was little evidence for immunological specificity and the biological function of these cells remains unknown. TCR γ,δ cells are distributed as approx. 5% of the CD3+ cells in all organized lymphoid organs and skin and gut-associated tissues. In collaborative studies with R. Modlin, T. Rea and M. Brenner, surveying lesions of patients with leprosy we found a marked increase in TCR γ,δ cells in two circumstances. One was the Mitsuda test, a standard skin test with killed *M. leprae* that is read as a granuloma at 3 wks after test. The second was a reactional state seen in patients with borderline leprosy, termed reversal reactions, characterized by major increase in T cells, particularly CD4+ cells, into the lesions, sometimes associated with nerve damage, but usually accompanied by histopathological improvement in the lesions that frequently persists. The frequency of TCR γ,δ cells in such lesions was

25-35% of CD3+ cells. TCR γ,δ cells were isolated from the lesions, and cell lines were produced which showed 28-100-fold proliferation of *M. leprae* antigens *in vitro* (Modlin et al 1989). This result is similar to other recent evidence that TCR γ,δ cells can respond to mycobacterial antigens. The proliferative responses of the cells from leprosy lesions appear to be genetically restricted, but apparently not by classical MHC Class I or II antigens. In contrast to other forms of leprosy lesions, the two lesions in which TCR γ,δ cells are found to be elevated have in common active granuloma formation. Consequently, the possibility that the TCR γ,δ cells might be involved in granuloma formation was examined. Supernates of activated TCR γ,δ cells induced adhesion and aggregation of human bone marrow monocytes in vitro. The nature of the lymphokine involved is not known although both IL-4 and TNF have been implicated by other investigators as being relevant to granuloma formation. Since granuloma formation appears to be essential to resistance to mycobacterial infection (Kinder et al 1989), the results suggest that at least some TCR γ,δ cells may recognize mycobacterial antigens in lesions and contribute to granuloma formation.

DEVELOPMENT OF A NEW RECOMBINANT MULTIVACCINE VECTOR:

BCG (Bacille Calmette Guerin) vaccine against tuberculosis has been the most widely utilized vaccine in the world, having been given in over 2.5 billion people since 1948 (The State of the World's Children 1987). In four large trials, BCG has had some degree of protection against leprosy, although it varied between 20-80% in different parts of the world. Convit has developed a combined vaccine of purified killed *M. leprae* plus live BCG that has had therapeutic efficacy in engendering cell mediated immunity in immunologically unresponsive patients with lepromatous leprosy (Convit et al, 1982). Further, he has shown the generality of the effectiveness of combined vaccines with BCG with a combination vaccine of *L. mexicana* to treat patients with American cutaneous leishmaniasis (Convit et al 1987). These results suggested that BCG offers a number of extraordinary advantages as a multivaccine vehicle: i) The frequency of serious complications of BCG vaccination is significantly lower than that of the smallpox vaccine, and it appears to be very safe; ii) BCG is the only vaccine, other than oral polio, recommended by WHO to be given at birth or any time thereafter; iii) BCG requires only a single immunization that engenders cell-mediated immunity to tuberculoproteins for a periods of 5-50 years; iv) BCG is the most effective known adjuvant for induction of cell-mediated immunity in animals and man; and v) BCG costs $0.055 per dose. Because of these unique attributes, it has long been our view that if antigens from a wide variety of pathogens could be introduced and expressed into BCG, one could develop an important new polyvalent vaccine for protecting people, particularly in the Third World, against multiple infectious diseases possibly with a single immunization.

The initial problem was that there was essentially nothing known about the molecular biology and genetics of the mycobacteria. In the past three years, we have succeeded in develop a genetic system for the Mycobacteria that should enable the development of BCG into a multivaccine vehicle. While a colony of approximately 10^8 organisms of *E. coli* can be seen on a Petri dish in 8 hours, it takes 3 days to see a colony of rapidly growing *Mycobacterium*, such as *M. smegmatis*, and 24 days to get a colony from BCG. As a consequence, Bill Jacobs devised a shuttle vector strategy using cosmid cloning that permits genetic manipulation of mycobacterial DNA in *E. coli*, and transfer or exchange of that DNA into mycobacteria. Initially a "shuttle phasmid" system was developed, in which foreign genes could be cloned into a "phasmid" vector, i.e., a hybrid between a mycobacteriophage and a an *E. coli* cosmid (Jacobs et al 1987) This could be easily genetically manipulated in *E. coli* as a cosmid, and yet grows in mycobacteria as a phage. Using a temperate (non-lytic) phage capable of inducing lysogeny, it was possible stably to express the first foreign gene in *M. smegmatis* and in BCG. This was an *E. coli* gene encoding kanamycin resistance, which gave us our first selectable genetic marker for the Mycobacteria (Snapper et al 1988). Because plasmids offer the possibility of regulating copy number and are easily manipulated, and taking advantage of our selectable kan^r marker, in collaboration with T. Kieser and colleagues in Norwich, we developed a hybrid plasmid, containing a complete *E. coli* cosmid ligated to and a complete mycobacterial plasmid. This shuttle plasmid (pYUB12) contains selectable markers and origins of replication for both *E. coli* and mycobacteria, and cloning sites into which to introduce foreign genes. With this vector it has been possible by means of electroporation to transform BCG vaccine sub-strains at high efficiency to express foreign genes (Lugosi, Jacobs and Bloom).

PERFECTING BCG

There remains much to be done before one can critically assess the potential usefulness of recombinant BCG as a vaccine vehicle. At a molecular level, it will be necessary to stabilize introduced foreign genes in BCG. This will require developing auxotrophic mutants, such that the complementing gene required for survival of the auxotrophic mutant can be inserted in the plasmid containing the foreign antigen gene. In that circumstance, the only BCG that will survive are those containing the plasmid with both the gene for converting the cells to free living prototrophs and the gene encoding the foreign antigen. Alternatively, the foreign antigen gene can be integrated into the mycobacterial chromosome, either by precise gene replacement or by recombination into the att site at which temperate phages integrate into the chromosome to create lysogens. In addition, it may be necessary to develop means to stabilize the recombinant foreign proteins produced. One method would be to produce protease-deficient mutants. In addition, both to enhance antigen stability and to increase the probability that the recombinant antigens will be immunogenic, it will be useful to target recombinant antigens produced in BCG for secretion. Since natural secreted proteins in BCG have been described, it should be possible to use secretory signal sequences to allow recombinant proteins to be secreted. In this regard, it is not clear whether foreign genes expressed in BCG will be capable of inducing CTL, and it may necessary to target BCG or its secreted recombinant antigens to the cytoplasmic compartment of host cells. Finally, the spectra of AIDS represents a shadow that darkens prospects for all live vaccine. Fortunately, we and others have shown that the major T cell reactive antigens of *M. leprae* and *M. tuberculosis* reside in the cell wall, and that purified cell walls are immunogenic (Melancon-Kaplan 1988). Thus we hope to learn how to target recombinant antigens to the cell wall of BCG, so that a recombinant cell wall vaccine could be used if necessary. If these genetic modifications of BCG can be made, and if recombinant BCG can be shown to induce antibody production, T helper and CTL in model systems, it is our long-term goal to introduce and express genes for protective antigens from a variety of pathogens to create a recombinant multivaccine vehicle that is effective and affordable to improve the quality of life not only in developed countries, but in the poorest countries on earth.

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Effector T Cells in Bacterial Infections

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INTRODUCTION

Intracellular bacteria include the etiologic agents of tuberculosis, leprosy, listeriosis, legionnaire's disease and typhoid fever, *Mycobacterium tuberculosis*, *M.leprae*, *Listeria monocytogenes*, *Legionella pneumophila*, and *Salmonella typhi*, respectively. These pathogens are capable of living inside host cells. Mononuclear phagocytes (MP) represent the preferred target, although other host cells can be invaded, as well. At the same time, MP are important effector cells for the combat of bacterial infection. The original concept of protection against intracellular pathogens can be summarized as follows: Intracellular bacteria possess evasion mechanisms which allow them to evade killing by resting macrophages and hence can persist in tissue MP. Once, however, T lymphocytes have been activated they produce lymphokines which, in turn, activate infected MP to eliminate their intracellular parasites. With our increasing knowledge about the virulence factors of intracellular pathogens on the one hand and about the complexity of the T-cell system on the other, it became clear that the relationship between intracellular pathogens and their host is far more complex. At least three evasion mechanisms are known by now: (a) inhibition of phagosome-lysosome fusion; (b) interference with reactive oxygen metabolites or lysosomal enzymes; (c) transition into the cytoplasm. Furthermore it is becoming increasingly clear that many intracellular pathogens possess invasion molecules which allow them to actively enter nonprofessional phagocytes. Schwann cells infected with *M.leprae* represent an important example. It is unlikely that the host can effectively deal with such a complex and heterogeneous group of infectious agents by an inflexible response. Rather, it is likely that the host has to account for the different ways of microbial evasion by adjusting the defence armourment appropriately. Indeed, the host can choose from a plethora of immune mechanisms to accomodate the most appropriate response. However, such a complex and fine tuned system may suffer from inborn errors and every now and then turn against the host itself. Then, damage to the host by its own mechanisms may occur. It is the purpose of this review to describe T-cell functions and antigens which may contribute to host protection against intracellular bacteria and to outline harmful events which may arise.

PART I: FUNCTION

The peripheral T-cell system can be grouped into different subsets according to phenotype, antigen recognition pattern, and function. All T cells bear the CD3 molecule on their surface. The majority

of T cells recognize an antigenic peptide in the context of a major histocompatibility complex (MHC) product via a conventional T-cell receptor (TCR) which is composed of an alpha and a beta chain. These so-called alpha/beta T lymphocytes segregate into two non-overlapping sets of reciprocal phenotype. CD4 T lymphocytes see antigenic peptides in the context of MHC class II molecules; CD8 T lymphocytes recognize peptides plus MHC class I molecules. CD4 T cells are preferentially helper T cells while CD8 T lymphocytes are primarily cytolytic. More recently, a second T-cell set has been identified which in most cases is $CD4^-CD8^-$, although some CD8 T cells have been found in this group. These T cells use another TCR for antigen recognition which is composed of a gamma and a delta chain. Although in most cases the ligand of the gamma/delta TCR is unknown, it is thought that these T cells, too, can see foreign antigen in the context of a cell surface molecule.

Macrophage Activation by Interleukins

The activation of antibacterial activities in macrophages by interleukins from activated T lymphocytes is well appreciated. Using bone marrow-derived macrophages (BMM) cultured for 9 days in serum-free medium we have assessed the relative role of various interleukins in the activation of tuberculostatic activities. BMM were activated with Interferon-γ (IFN-γ), tumor necrosis factor (TNF), interleukin-4 (IL-4), or interleukin-6 (IL-6) and after 24 h were infected with viable mycobacteria (Flesch and Kaufmann 1987, Kaufmann and Flesch 1988). Mycobacterial replication was assessed by measuring ^{3}H-uracil uptake or by counting colony-forming units. Under these circumstances IFN-γ was the only interleukin capable of activating tuberculostasis. Although TNF by itself failed to activate mycobacterial growth inhibition, it showed marked synergy with low concentrations of IFN-γ. A different picture emerged when BMM were first infected with *M.bovis* and afterwards stimulated with IFN-γ, IL-4, or IL-6. Under these conditions, IFN-γ was far less active while IL-4 and IL-6 became potent activators of antimycobacterial macrophage activities. Interestingly, low concentrations of IFN-γ antagonized with IL-4 in the activation of tuberculostasis. It appears that activation of tuberculostatic macrophage functions by B-cell stimulatory factors depends on a second signal which can be provided by natural infection with mycobacteria. In a granulomatous lesion B-cell stimulatory factors may preferentially act on macrophages which already harbour microbial organisms whereas IFN-γ seems to have its preferential target in freshly immigrant monocytes. Hence, both synergistic and antagonistic interactions between different interleukins could occur in a granuloma.

Cytolytic T Lymphocytes (CTL)

Until recently, CTL have been considered important only for protection against viral infections and not against bacteria and protozoa. However, since our observation in 1979 that CD8 T lymphocytes are involved in adoptive protection against *Listeria monocytogenes* several reports have ascribed a role for CD8 cells in immunity to a variety of bacteria and parasites (Kaufmann 1988a). In the virus system, the characteristic feature of CD8 cells is their capacity to lyse infected target cells. We therefore analyzed the cytolytic potential of T lymphocytes with specificity to the intracellular bacteria *M.tuberculosis*, *M.leprae*, and *L.monocytogenes* (DeLibero et al 1988; Chiplunkar et al 1986; Kaufmann et al 1986). T lymphocytes from mice immunized with these pathogens were restimulated in vitro

with accessory cells, bacteria and T-cell growth factors. After in vitro propagation for various periods of time cells were cloned under limiting dilution conditions. Many CD8 T-cell clones could be established in this way. Using BMM primed with bacterial antigens as targets the cytolytic activity of these T cells was assessed. They lysed BMM primed with the homologous bacterial antigen to a significant degree while unprimed targets remained virtually unaffected. For many T-cell clones class I restriction was demonstrable. Several CTL clones were, however, identified which lacked apparant MHC restriction (DeLibero et al 1988; Kaufmann et al 1988). The majority of CTL clones produced significant concentrations of IFN-γ after stimulation with accessory cells, homologous bacterial antigen, and exogenous r-IL-2. As expected, supernatants containing high IFN-γ concentrations were also capable of inducing tuberculostatic functions in BMM. Independent from this IFN-γ-mediated activity CTL caused tuberculostasis by lysing mycobacteria-infected macrophages. This pathway is probably cell-contact dependent and related to destruction of infected host cells. Besides macrophages also Schwann cells presenting *M.leprae* antigens were lysed by CD8 CTL (Steinhoff and Kaufmann 1988). Among our CD4 T-cell clones with specificity to *L.monocytogenes* and mycobacteria, several clones were identified which also possessed lytic potential (Kaufmann et al 1986, 1987). Lysis by CD4 T cells of antigen presenting BMM, however, required prestimulation of BMM with IFN-γ. This is due to the fact that BMM are class II-negative and become class II positive after IFN-γ stimulation. Hence both CD4 and CD8 T cells possess cytolytic activity.

Gamma/Delta T Cells

In recent studies evidence has been accumulated to indicate that many gamma/delta T cells recognize ligand(s) expressed by mycobacteria (Janis et al 1989; O'Brien et al 1989; Modlin et al 1989; Holoshitz et al 1989). Such gamma/delta T lymphocytes have been isolated from the synovial fluids of rheumatoid arthritis patients and from early granulomatous reactions of leprosy and leishmaniasis patients. Furthermore, the percentage of gamma/delta T cells in draining lymph nodes of mice immunized with killed mycobacteria in Freund's adjuvant was markedly increased. In all these cases isolated gamma/delta T cells responded profoundly to mycobacterial preparations. Recently, we found that in-vitro stimulation of peripheral blood mononuclear cells from normal healthy individuals for 7 to 10 days results in preferential expansion of gamma/delta T cells as assessed by flow cytometry of stained cells. In the presence of mycobacterial components the percentage of gamma/delta T cells increased three to tenfold (2-9% at d 0 to 12-30% at d 7). Peripheral blood mononuclear cells, after in-vitro stimulation with mycobacterial preparations, were positively selected on a fluorescence activated cell sorter. Selected gamma/delta T cells proliferated vigorously in response to mycobacterial preparations. Furthermore, selected gamma/delta T cells lysed autologous adherent cells primed with mycobacterial preparations leaving unprimed targets unaffected. Taken together, these findings strongly argue for the participation of gamma/delta T cells in the immune response to mycobacteria and warrant for a more extensive evaluation of their functional role in protection and/or pathogenesis.

Discussion

The data summarized in part I indicate a role for interleukins and

lysis in the acquired host response to intracellular pathogens. The original concept only considered macrophage activation as an essential step in antibacterial protection and IFN-γ produced by CD4 T cells was considered the major mediator of activation. As discussed here, also the B cell stimulatory factors, IL-4 and IL-6, products of so-called T_{H2} cells, as well as TNF, contribute to macrophage activation. Others have indicated an important role for TNF in tuberculous granuloma formation and a role for IL-4 in the differentiation of multinucleated giant cells - a prominent cell type in these lesions (Kindler et al 1989; McInnes and Rennick 1988). Thus, a multitude of interleukins may be required for optimum protection to occur (Figure 1A). Besides, interleukins may be detrimental for the host either directly (like TNF which, in combination with mycobacterial products, induces necrotic reactions) or indirectly by activating secretion of harmful molecules in macrophages (Figure 1B). As shown here, IFN-γ is also produced by CD8 T cells. Class I MHC molecules (the restricting elements for CD8 T cells) are expressed by virtually all host cells, while class II molecules (used by CD4 T cells) are limited to selected cells. Therefore, the recognition spectrum of CD8 T cells is far broader. The activation of CD8 T cells by bacterial antigens was long considered incompatible with the dogma that phagocytosis allows for class II processing only. However, many intracellular pathogens are transmitted from the endosomal to the cytoplasmic compartment where their antigens can gain access to "cytoplasmic" class I processing. Thus, certain bacterial proteins can be presented in the context of class I MHC molecules. Furthermore, many gamma/delta T cells recognize mycobacterial components and evidence has been presented that such gamma/delta T cells secrete interleukins of relevance to granuloma formation (Modlin et al 1989). The genetic restriction of these T cells may be even less stringent. In addition to helper functions cytolytic functions may also participate in antibacterial immunity. CD4 and CD8 alpha/beta T cells as well as gamma/delta T cells with reactivity to mycobacteria have been shown to lyse target cells presenting mycobacterial antigens.

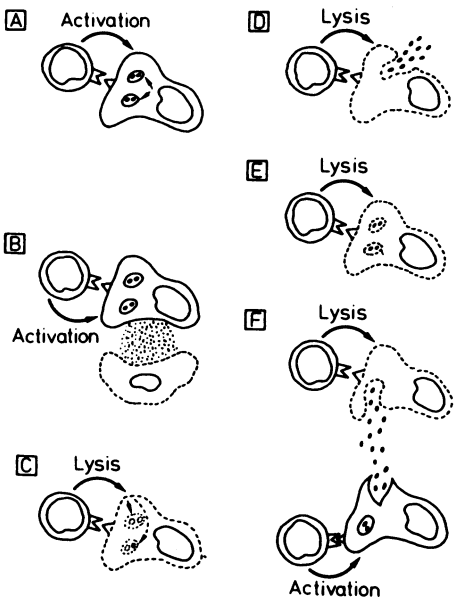

Fig. 1: Possible sequelae of different T cell functions in antibacterial immunity.

Target cell lysis may be of benefit for the host because it could directly affect growth of microbes which essentially depend on intracellular metabolites (Fig. 1C). Target cell lysis may also have detrimental consequences: first by allowing for microbial dissemination (Fig. 1D) and second, by damaging host cells which are hard to reconstitute (Fig. 1E). Damage of Schwann cells, for example, is the major pathomechanism in leprosy. Lysis of host cells which by themselves cannot limit their intracellular pathogens, however, may be unavoidable for sterile elimination since it facilitates microbial uptake by more appropriate effector cells (Fig. 1F).

PART II: SPECIFICITY

Following the cloning and expression of *M.leprae* and *M.tuberculosis* genes it was soon revealed that many of the recombinant proteins belong to the hsp family (Young et al 1988; Kaufmann et al, in press). This holds true for the 71 kD, 65 kD, 18 kD, and 12 kD proteins. Hsp are a family of highly conserved proteins which are produced under stress conditions. Not only heat, but also many other insults like depriviation of essential nutrients, attack by reactive oxygen metabolites, and anaerobiosis will induce a hsp response. It could be argued that inside MP bacteria are exposed to these and similar insults and hence produce abundant hsp and that this event leads to preferred presentation of hsp to the T-cells system (Kaufmann et al, in press). A homolog of hsp 65 has been identified in a multitude of other species. Even in man a homolog which shares more than 65% homology with the mycobacterial hsp has been identified (Jindal et al 1989).

Crossreactive CTL against hsp 65

Hsp 65 of mycobacteria and man share a marked degree of homology. Four regions consisting of more than 10 amino acid residues exist which are almost or fully identical in mycobacteria and man. To assess whether CTL against the mycobacterial hsp 65 recognize stressed host cells we took advantage of a recently described system for in-vitro activation of CTL against soluble ovalbumin by using tryptic peptide fragments (Moore et al 1988). In this system it was shown that CTL against tryptic fragments fail to recognize epitopes generated through the "natural" cytoplasmic pathway. Spleen cells were cultured with a tryptic digest of hsp 65 or ovalbumin, respectively. After 6 days, CTL had developed with the capacity to specifically lyse target cells labelled with the homologous tryptic digest. Ovalbumin or hsp 65 were introduced into the cytoplasm of EL-4 cells by osmotic shock and afterwards used as target cells for CTL against tryptic fragments. In agreement with published data (Moore et al 1988) CTL against ovalbumin peptides failed to lyse target cells presenting "naturally" processed ovalbumin. In contrast, CTL against tryptic hsp 65 lysed targets presenting epitopes of hsp 65 after cytoplasmic degradation. Thus, in contrast to ovalbumin, cleavage sites for trypsin on hsp 65 appear to be similar to those for proteases active in cytoplasmic processing. We therefore could use CTL against tryptic hsp 65 to evaluate whether T cells against cross-reactive epitopes recognize stressed MP. As a source of target cells, BMM were used. CTL raised against mycobacterial hsp 65 were able to lyse unstimulated BMM in the presence of tryptic digest, whereas unstimulated BMM in the absence of peptides showed only marginal lysis. Importantly,

BMM which had been stressed by IFN-γ activation, by cytomegalovirus infection or by *M.bovis* infection were killed (Koga et al, in press). CTL against tryptic ovalbumin fragments lysed BMM labeled with ovalbumin peptides but not BMM primed with hsp 65 peptides nor BMM which had been stressed by IFN-γ stimulation. Lysis by CTL against hsp 65 was blocked by monoclonal antibodies against the CD8 molecule and left unaffected by antibodies against the CD4 molecule. Furthermore, these CTL only lysed targets which shared the H-2D MHC locus. Thus, the relevant T cells were $CD8^+$, class I-restricted. In a similar type of experiment, using Schwann cells instead of BMM as targets, it could be shown by antibody blocking that antigen recognition involved the alpha/beta T-cell receptor. Furthermore, using antibodies against mycobacterial hsp 65 we could identify a molecule of similar size in unstimulated BMM indicating that hsp 65 is constitutively present in BMM. The most plausible explanation for these findings would be that under stress endogenous hsp are processed and presented in the context of MHC class I molecules and subsequently are recognized by CD8 CTL activated against a mycobacterial hsp.

CTL from Normal Donors with Specificity to Shared Epitopes of Hsp 65.

In another set of experiments the question was addressed as to whether the cellular response against mycobacterial hsp 65 comprises T cells with specificity for epitopes shared by mycobacteria and man (Munk et al, in press). For this purpose, synthetic peptides corresponding to sequences of the human hsp 65 which are fully or almost identical in the mycobacterial homolog were employed. T-cell lines were established by stimulating peripheral blood monocytes from healthy donors with killed *M.tuberculosis*. These T cells were capable of lysing autologous adherent cells which had been pulsed with killed *M.tuberculosis* or with tryptic digest of mycobacterial hsp 65 but not targets pulsed with intact hsp 65. Importantly, lysis was also observed using targets which had been labeled with peptides representing shared epitopes (residues 109-120; residues 269-289; residues 298-307; residues 430-441 of the human hsp 65). Using HLA-DR transfected L-cells, target cell recognition was found to be class II dependent. These data suggest that T cells against self epitopes exist in healthy individuals and that they are activated by in-vitro stimulation with killed *M.tuberculosis* organisms. Furthermore, it appears that these T-cell epitopes do not arise through "natural" endosomal class II processing. Recently, evidence has been presented that some gamma/delta T cells with reactivity to mycobacteria recognize hsp 65 (Holoshitz et al 1989; O'Brien et al 1989). In a limited number of experiments we found that gamma/delta T cells from a few normal donors, after activation with mycobacterial preparations in vitro (see above), proliferate in response to hsp 65. It is, therefore, tempting to speculate that gamma/delta T cells might also see regions of hsp 65 shared by bacteria and host cells.

Discussion

T cells with reactivity to the mycobacterial hsp 65 have been identified frequently (Kaufmann 1988b). This protein probably exists in all bacteria man can encounter and hence may provide a common antigenic stimulus for the T-cell system. By recognizing a cross-reactive bacterial epitope T cells against hsp 65 of one bacterium may contribute to immunity against another one (Fig. 2-1). Whether such a cross-reactive response provides a common and basic level of protection to various microbial infections remains to be estab-

lished. The experiments discussed here indicate that T cells to epitopes shared by hsp 65 of mycobacteria and man exist even in healthy individuals. These T cells, therefore, must have evaded tolerance deletion during thymic differentiation. The crossreactive T cells were found to recognize synthetic peptides but not "naturally" processed hsp 65 suggesting that (i) the mycobacterial preparations used for primary stimulation comprised cross-reactive epitopes and (ii) that the cross-reactive epitopes were not generated through the "natural endosomal" class II processing pathway in sufficient densities. We, therefore, have to assume that such epitopes only arise in pathological situations; e.g. they could be produced by autolytic degradation of pathogens in necrotic lesions or abscesses (Fig. 2-2). Once such T cells have been activated, they can recognize shared epitopes expressed by host cells which had been stressed by a variety of shock situations. Indeed, the data in the murine system indicate that endogenous hsp can be processed through the "natural" cytoplasmic class I pathway and that self epitopes can arise in this way. T cells against hsp may provide the host with the means to identify and eliminate transformed, infected or otherwise damaged cells and hence contribute to immune surveillance. During inflammatory processes host cells suffer under stress. Provided that T lymphocytes to hsp had been activated by previous infection they may now have the chance to recognize a shared epitope of hsp on stressed cells, and to initiate an autoimmune response. Such an event, for example, could be envisaged to occur around peripheral nerves of leprosy patients during reactional stages and also in joint cavities of rheumatoid arthritis patients. Indeed, a relationship between cellular immunity to hsp 65 and rheumatoid arthritis has been suggested (Van Eden et al 1988; Res et al 1988; Holoshitz et al 1989).

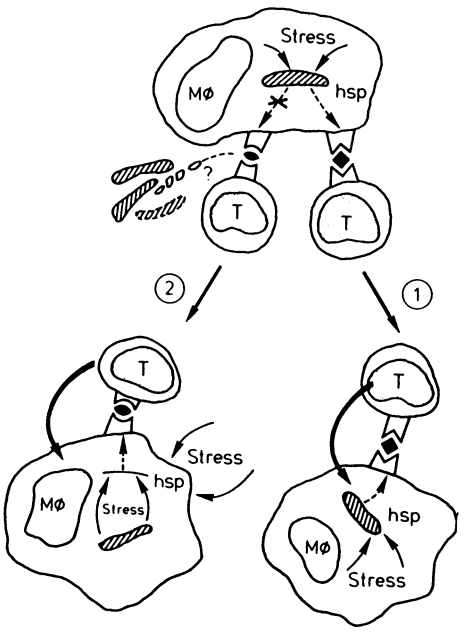

Fig. 2: Possible sequelae caused by hsp specific T cells.

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Experimental Infection of Mice with *Leishmania Major:* Analysis of the Role of T Cells in Resistance and Susceptibility

J. A. Louis and I. Müller

INTRODUCTION

The leishmaniases, a complex of infectious diseases of protozoan origin, represent an important public health and medical problem in approximately 80 countries throughout the world. Trypanosomatid protozoans of the genus *Leishmania*--the organisms responsible for these diseases--are transmitted to their mammalian hosts by the bite of female phlebotomine sandflies. During the bloodmeal the extracellular flagellated promastigotes, originating from the digestive tract of the insect vector, enter the vertebrate host's circulation and penetrate the cells of the mononuclear phagocyte lineage, where they transform into aflagellated amastigotes.

The spectrum of clinical manifestations resulting from *Leishmania* infection ranges between the localized cutaneous form (Oriental sore) and the malignant visceral form (Kala-azar). Furthermore, the characteristics of the disease appear to depend not only on the *Leishmania* species involved, but on the immune responses of the host (Turk and Bryceson 1971). Interestingly, different clinical profiles seen in humans can also be observed in mice with appropriate genetic backgrounds following experimental infection with *Leishmania major* (*L. major*). The existence of inbred strains of either susceptible (BALB/c, DBA/2) or resistant (CBA, C57BL) mice has permitted numerous studies aimed at analyzing the immune mechanisms involved in either resistance or susceptibility to infection with *L. major*. In susceptible mice severe cutaneous lesions develop at the site of infection and visceralization ensues leading to death of animals. In contrast, resistant mice develop small lesions that heal readily within a few weeks.

In the murine experimental model of infection, no clear evidence exists to indicate that specific anti-*Leishmania* antibodies have an important

role in the healing of lesions (Liew 1986). However, recent evidence suggests that specific antibodies could have some protective properties. When present at the beginning of infection, these antibodies specific for molecules at the promastigote surface could interfere with their binding to membrane receptors of macrophages. Since macrophages are the parasite's obligate host cells, the bound antibodies could inhibit the promastigotes from invading macrophages, and subsequently, prevent their survival (Handman and Goding 1985; Anderson *et al* 1983; Champsi and McMahon-Pratt 1988).

But which processes determine resistance to an established infection with *L. major*? Which processes determine susceptibility? And what specific cells and molecules mediate this protection or vulnerability?

There is now a consensus of opinion that T-cell immunity plays a major role in the acquisition of resistance to leishmanial infection. In addition, strong evidence exists to indicate that the activity of specific T cells can promote the growth of parasites *in vivo*. In this brief review, the results, obtained by several research groups including our own, pertaining to the analysis of the role of specific T cells in resistance and susceptibility will be summarized.

ROLE OF CD4$^+$ T CELLS IN RESISTANCE AND SUSCEPTIBILITY OF MICE TO INFECTION WITH *L. MAJOR*

Resistance

The pioneering work by G. Mitchell and his colleagues, which showed that reconstitution of exquisitely susceptible nu/nu mice with T cells from normal mice conferred some level of resistance, has clearly demonstrated the crucial role of T cells in resistance to infection with *L.major* (Mitchell 1984). Additionally, in adoptive cell transfer experiments, protective immunity in resistant mice was ascribed to the activity of Lyt 2$^-$ (CD8$^-$) T cells (Liew 1986). Subsequently, the important role of CD4$^+$ T cells in the resolution of lesions induced by *L. major in vivo* was demonstrated when elimination (>95%) of CD4$^+$ T cells by treating resistant mice with anti-CD4 monoclonal antibodies (mAb), rendered these mice extremely susceptible to infection with *L. major* (Louis *et al* 1986).

At present, the activation of macrophages parasitized with *Leishmania* represents the main effector mechanism by which these microorganisms are destroyed in their infected hosts. Consequently, it has generally been assumed that lymphokines, which are released by activated $CD4^+$ T cells and which are capable of activating macrophages must be critical in the healing process.

In this vein, interferon-gamma (IFN-γ) has been demonstrated to be capable of activating macrophages *in vitro* leading to the destruction of intracellular *Leishmania* amastigotes (Nathan *et al* 1983; Ralph *et al* 1983; Titus *et al* 1984). The role of IFN-γ in the resolution of *Leishmania*-induced lesions *in vivo* has been demonstrated by numerous observations. In summary these observations have shown that: a) the capacity of genetically resistant mice to spontaneously resolve their cutaneous lesions correlates with the ability of their draining lymph node T cells to release IFN-γ after stimulation with parasite antigens *in vitro* (Sadick *et al* 1986); b) compared to susceptible mice, the lymph node and spleen cells of resistant mice contain greater amounts of IFN-γ-mRNA a few weeks after infection (Locksley *et al* 1987; Heinzel *et al* 1989) and, c) the administration of neutralizing anti-IFN-γ-mAb during the course of infection in resistant mice interferes with the spontaneous resolution of lesions normally seen in these mice (Müller *et al* 1989; Scott and Sher, personal communication).

The $CD4^+$ T cell subpopulation has been shown to be comprised, at least in mice, of two functionally distinct subsets designated TH_1 and TH_2 (Mosmann and Coffman 1987). Since following stimulation, TH_1 cells produce IFN-γ and IL-2, whereas TH_2 cells produce IL-4 and IL-5, it appears that the specific T cells involved in the resolution of lesions belong to the TH_1 subset and are preferentially induced in resistant mice.

Studies with *Leishmania*-specific $CD4^+$ T cells lines and clones provide additional support that T cells involved in protective immunity against *L. major* express the TH_1 functional phenotype. Scott *et al* (1988) recently derived a T cell line recognizing some antigen present in a fraction separated from a soluble extract of *L. major* which was able to vaccinate mice against challenge with viable *L. major* (Scott et al 1987). The adoptive transfer of this $CD4^+$ T cell line to either 200 rad

irradiated or normal mice was shown to protect them against a normally fatal infection with *L. major*. After antigenic or mitogenic stimulation *in vitro*, this protective T cell line was found to produce a pattern of lymphokines (i.e. IFN-γ and IL-2) corresponding to the TH_1 subset (Scott *et al* 1988).

Recently, we have derived two *L. major*-specific cloned $CD4^+$ T cell lines which were capable of significantly protecting normal recipient BALB/c mice against challenge with virulent *L. major* promastigotes (Müller and Louis 1989). This protective effect was reflected by a large reduction in the size of cutaneous lesions and by a dramatic reduction in the number of parasites found in lesions of mice adoptively transferred with the cloned T cells. This effect was abolished by treatment of recipient mice with anti-IFN-γ-mAb. Additionally, the cloned T cells were shown to produce IFN-γ and IL-2 following specific stimulation *in vitro*. These results indicate that these cloned T cells also belong to the recently described TH_1 subset and that IFN-γ is involved in their ability to mediate protection. An important feature of the two protective $CD4^+$ T cell clones, derived in our laboratory, is that they appear to recognize antigens associated only with live *L. major* parasites (Müller and Louis 1989).

Susceptibility

Early experiments by G. Mitchell and his group (1980) suggested that T cells also participated in susceptibility to *L. major*. They showed that the adoptive transfer of high numbers of normal T cells to nu/nu mice failed to confer resistance and even abrogated the resistance resulting from the transfer of small numbers of normal T cells. Several subsequent observations strongly indicated that $CD4^+$ T cells, triggered as a result of infection could play a role in determining susceptibility. These observations include: a) treatment of susceptible mice, given a regimen which did not completely eliminate their $CD4^+$ T cells, with anti-CD4 mAb rendered these mice resistant to infection (Louis *et al* 1986); b) an increased number (10-50 times) of $CD4^+$ T cells capable of mediating specific DTH reactions was found in lymphoid tissues of infected susceptible mice in contrast to resistant mice (Louis *et al* 1986), and c) susceptible mice are rendered resistant to infection following administration of cyclosporin A (Solbach *et al* 1986). Although these experiments added significantly to our understanding of the processes involved, they did not permit one to elucidate whether

promotion of resistance or susceptibility resulted from the activity of distinct CD4 T cells.

Elegant studies conducted by Heinzel *et al* (1989) illustrated that, compared to resistant mice, the mRNA isolated from lymphoid tissues of infected susceptible mice contained less IFN-γ-mRNA, but 50 fold greater quantity of IL-4 message. This observation strongly suggested that IL-4 secreting TH_2 cells were preferentially being expanded in susceptible mice. However, the demonstration of higher frequencies of $CD4^+$ T cells, which were capable of transferring DTH reactions in normal recipients, in lymph nodes of susceptible mice (Milon *et al*, 1986) indicated that, although TH_2 cells comprised the exacerbating cells, some TH_1 $CD4^+$ T cells could also play a role in susceptibility to infection.

Studies with T cell lines and clones indicate that although parasite-specific TH_2 T cells exacerbate disease progression, some $CD4^+$ T cells with the functional characteristics of TH_1 cells can also promote disease progression. Scott *et al* (1988) have derived T cell lines recognizing antigens present in a fraction of soluble extract of *L. major* which were unable to vaccinate mice. Additionally, these T cells exacerbated the course of disease after adoptive transfer. These T cells clearly displayed *in vitro* functional characteristics of TH_2 cells.

We have derived parasite-specific $CD4^+$ T cell lines from immune lymph nodes responding *in vitro* to a crude lysate of *L. major* promastigotes and found that these cells exacerbated the course of disease after adoptive transfer to syngeneic recipients (Titus *et al* 1984). These specific T cell lines were capable of not only transferring strong DTH reactions to syngeneic recipients, but following specific activation *in vitro*, releasing substantial amounts of MAF as tested in a tumoricidal assay, granulocyte-macrophage colony stimulating factors (GM-CSF) and interleukin-3 (IL-3) (Feng *et al* 1988). Cloned T cell lines derived from these homogenous populations also exacerbated the cutaneous lesions after adoptive transfer to normal BALB/c mice . These T cell clones expressed the $CD4^+$ T cell surface phenotype and released IFN-γ and IL-2, but no IL-4 after specific stimulation *in vitro* (Titus *et al*, in preparation). Furthermore we would like to stress that these TH_1 cells, which were capable of promoting disease progression, responded *in vitro* to a lysate of *L. major*, as opposed to the protective

TH_1 cells described above, which recognized antigen(s) only associated with live parasites. Therefore, it could be hypothesized that the capacity of TH_1 parasite-specific T cells to protect against *L. major* infection depends upon their specificity. It is tempting to hypothesize that the antigens recognized by protective $CD4^+$ T cells are expressed at the surface of infected macrophages harbouring multiplying microorganisms. One possibility currently being investigated is whether the antigen(s), present in a lysate of *L. major* promastigotes, which are recognized by the TH_1 cells promoting disease progression are not presented by macrophages containing multiplying *L. major* amastigotes. Accordingly, these T cells would not be parasiticidal since they might not recognize antigens at the surface of infected macrophages, and thus, would be unable to focus the IFN-γ, that they release after stimulation, at the surface of parasitized macrophages.

Attempts have been made to relate the disease promoting activity of $CD4^+$ T cells to the specific lymphokines released by these cells. The results from recent studies show that administration of anti-IL-4 mAb to BALB/c mice reduced the progression of lesions. This observation indicates that IL-4 produced and secreted by specific TH_2 cells plays a role in disease progression (Heinzel *et al* 1989). It is noteworthy that treatment of BALB/c mice by anti-IL-4 mAb does not appear to allow the expansion of IFN-γ producing TH_1 cells in these mice (Locksley, personal communication). Furthermore, although it has been suggested that IL-4 and IL-3 could inhibit the activation of macrophages by IFN-γ (Liew 1988), previous studies have indicated that resistance of macrophages to infection with *L. major* amastigotes required IFN–γ in combination with IL-4 (Belosevic *et al* 1988). In contrast to the demonstration of a disease promoting property of IL-4, recent results have illustrated that IL-4 administered s.c. around the lesions inhibited the development of lesions, and rendered mice resistant to reinfection (Carter *et al* 1989).

IL-3 and GM-CSF have both been implicated as mediators of the disease promoting activity of T cells. This promotion of disease has further been related to the capacity of these lymphokines to increase the pool of circulating mononuclear phagocytes--the very cells in which *Leishmania* multiply (Feng *et al* 1988; Solbach *et al* 1987).

ROLE OF CD8+ T CELLS IN RESISTANCE OF MICE TO INFECTION WITH *L. MAJOR*

Although the role of $CD8^+$ T cells in the immunological control of infection with *L. major* remains controversial, results are accumulating which indicate that $CD8^+$ T cells, in addition to the $CD4^+$ T cells, participate in the healing of *L. major*-induced lesions (Titus *et al* 1987). Evidence recently obtained in our laboratory further suggests that $CD8^+$ T cells could play a more important role in controlling lesions which develop in immunized than in naive mice (Farrell *et al* 1989; Müller and Louis, in preparation).

CONCLUDING REMARKS

At present there is a general consensus of opinion that resolution and exacerbation of lesions induced by *L. major* in mice are the consequence of the activity of distinct parasite-specific $CD4^+$ T cells. It appears that cells of the TH_1 subset are the protective cells and those that exhibit the functional characteristics of the TH_2 subset play a causal role in the severity of the disease, as observed in susceptible mice. In addition, evidence exists that some TH_1 cells which can exacerbate the disease process could have a different specificity than the TH_1 protective T cells. Finally, it is noteworthy to mention that an increased understanding of the complex interplay of lymphokines and their effect on the outcome of infection with *L. major* will require further investigation.

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Progress Towards Vaccination Against Schistosomiasis

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INTRODUCTION

Schistosomiasis is a chronic, debilitating disease that affects over 200 million people worldwide, and of which around 800 000 die annually according to WHO estimates. Three species of schistosome infect man (*Schistosoma mansoni*, *S. haematobium* and *S.japonicum*) while a fourth species (*S. bovis*) infects cattle, causing extensive economic loss, particularly in East Africa. Despite the existence of effective chemotherapeutic agents, and numerous programs aimed at controlling the fresh water snail intermediate host, progress towards circumscribing the parasite has been slow. This may in part due to inadequate planning of chemotherapy campaigns (Prescott, 1987). However, the problem of drug resistance (Coles et al. 1987) and the overall economic cost mean that other approaches are necessary. Since, unlike protozoan parasites, schistosomes do not replicate in the definitive vertebrate hosts, a partial non-sterilizing immunity would greatly diminish both transmission levels in endemic areas, and the incidence of human pathology caused by the deposition of parasite eggs in host tissues (Phillips and Colley, 1978).

An effective vaccine would reduce individual worm burdens (but not completely prevent infection) and possibly affect the fecundity of female worms. In addition, a direct effect on eggs and on egg-induced granuloma formation may be possible. All three of these effects would lead to reduced levels of pathology and diminish transmission.

The detailed knowledge that has accumulated concerning immunity to schistosomiasis, and of the target molecules of acquired immunity has meant that a number of the latter have been identified and cloned (Capron A. et al. 1987). This paper will outline the approaches we have undertaken towards the characterization and cloning of protective antigens, and the prospects for a viable vaccine in the light of our increasing knowledge of the immune response to the recombinant antigens.

PROTECTIVE IMMUNITY AGAINST SCHISTOSOMES

Differences in mechanisms of protective immunity are evident between experimental models and the mechanisms operating in man are as yet unknown although common characteristics exist between rat and human responses.

Mice develop immunity to reinfection after vaccination with irradiated cercariae and this resistance is T-cell dependent

in vivo (Sher et al. 1982). Vaccinated mice produce activated, larvicidal macrophages at the site of challenge infection. In vitro studies indicate that T lymphocytes activated by schistosome antigens produce lymphokines including gamma interferon (IFNγ) capable of activating macrophages to kill newly transformed larvae or 2 1/2 week old schistosomes (Pearce and James, 1986). However, antibodies and other cell types may be involved in immunity. Murine monoclonal antibodies with specificity for schistosomulum surface antigens can mediate protection (Harn et al. 1985). Furthermore, a mechanism whereby eosinophils are responsible for very early attrition of the infection in the skin of CBA/Ca mice has been proposed (Ward and McLaren, 1988). Most data indicate that 'late' immunity acting when schistosomula migrate from the lungs to the hepatic portal system is dominant in the mouse model (Bickle and Ford, 1982).

The rat is a semi-permissive host developing a strong antibody-dependent immunity that is characterized by a marked anaphylactic antibody response, and the presence of antibody-dependent cellular cytotoxicity mechanisms (ADCC). The latter involve anaphylactic antibody isotypes and eosinophils, macrophages or platelets (Capron M and Capron A, 1986). These mechanisms can also operate in vivo as is suggested by the fact that the passive transfer of any of the three cell types from an infected rat will protect a naive animal against infection (Capron M et al. 1984 ; Joseph et al. 1983). Equally, monoclonal antibodies of both rat anaphylactic antibody classes (IgG2a and IgE) have been produced that protect rats by passive transfer (Grzych et al. 1982 ; Verwaerde et al. 1987).

The same in vitro effector mechanisms can be demonstrated using human or primate infection sera and cells (Capron M and Capron A, 1986) and the human immune response is also characterized by the production of high levels of anaphylactic antibodies. A further correlate to the rat model is the production of blocking antibodies recognizing schistosomulum surface carbohydrate epitopes (Khalife et al. 1986), the presence of which is associated with a state of susceptibility to reinfection in human populations (Butterworth et al. 1987).

In practical terms, both investigations of the target antigens of immunity in the mouse model in which cell-mediated responses were studied, and the characterization of antigens responsible for humoral responses, and that were targets of ADCC mechanisms, have led to the cloning of proteins that protect against infection. The main thrust of current work in this area is directed at elucidating the types of immune responses that these molecules elicit in primates and humans (Capron A. et al. 1987).

TARGET ANTIGENS OF IMMUNITY

Schistosomulum surface antigens

The search for target antigens of the immune response that would be good candidate vaccine molecules first focused on those determinants expressed on the surface of the schistosomulum. Newly transformed schistosomula have a restricted repertoire of molecules at their surface (Dissous et al. 1981) of which the immunodominant

species is a 38 kDa glycoprotein (GP38). This molecule was first identified (Dissous et al. 1982) using a rat monoclonal IgG2a antibody (IPLSm1) that mediated both eosinophil-dependent cytotoxicity towards schistosomula and protection of rats by passive transfer (Grzych et al. 1982). Subsequent studies have shown that the epitope involved in the protective response was glycannic in nature (Dissous et al. 1985). This, taken together with the fact that the antibody response to GP38 produced both protective (IgG2a) and blocking (IgG2c) subclasses in rats (Grzych et al. 1984) and the existence of such blocking antibodies (of the IgM class) in human sera (Khalife et al. 1986) meant that the molecular cloning of the protein portion of GP38 would be both difficult and of limited usefulness.

For this reason, an anti-idiotype strategy was developed, based on the IPLSm1 antibody (Grzych et al. 1985). Rats immunized with a monoclonal antiidiotype antibody (AB2) directed against the antigen-binding site of IPLSm1 produced specific AB3 antibodies that were highly cytotoxic for schistosomula in vitro in the presence of eosinophils, and protected rats against infection by passive transfer. Equally, immunization of rats with AB2 antibodies protected rats against infection.

At this stage, the characterization of the glycan epitope was facilitated by a chance observation when using keyhole limpet hemocyanin (KLH) as a carrier for the immunization of rats with the AB2 monoclonal antibody, that control animals immunized with KLH alone developed antibodies to GP38 (Grzych et al. 1987). This result tallied with parallel work showing that the glycan epitope of GP38 was shared not only with the intermediate host of S. mansoni, *Biomphalaria glabrata*, but with other fresh water snails including schistosome hosts such as *Bulinus truncatus* and non-hosts such as *Limnaea limosa* (Dissous et al. 1986).

The availability of KLH has meant that structural analysis of the glycans reaching with IPLSm1 has been possible and a consensus sequence has been obtained. Interestingly, an unusual structural motif of this N-glycan structure, has also been characterized in *L. stagnalis* (Van Kuik et al. 1987).

Although the chemical synthesis of this glycan epitope remains a possibility, it is far from certain that its use in a vaccine is a viable possibility given the problem of blocking antibody production. This is particularly the case since the presence of antibodies to *S. mansoni* carbohydrate epitopes is strongly correlated to a state of non-resistance to reinfection in human populations (Butterworth et al. 1987). Further efforts at characterizing and producing protective molecules have therefore concentrated on protein antigens and in particular molecules secreted or excreted by schistosomula but nevertheless present in adult worms. The reason for the latter is that adult worms constitute the major source of antigenic stimulus during infection and maintain the immune response that prevents reinfection, a phenomenon known as concomitant immunity (Smithers and Terry, 1967).

Naturally, there is no reason why such molecules should not be present on the surface of schistosomula and several groups have set out to clone the protein parts of surface glycoproteins. One such approach is based on the observation that in the mouse model

protective antibodies are in fact directed against the protein moieties of surface antigens (Omer-Ali et al. 1986). Up till now, the only published example of a cloned schistosomulum surface membrane antigen is that of a 18 kDa protein (Dalton et al. 1987). However, no data exists as to its protective value.

Excretory-Secretory Antigens In Protection

Two separate approaches led us to the molecular cloning of non-surface protective protein antigens. The first was the demonstration of the primordial importance of antigens excreted and secreted by schistosomula (schistosomula released products : SRP-A) in the induction of a protective IgE response in the rat and in primates. The second element was a pragmatic approach based on the search for protein antigens common to infective larvae and adult worms. SRP-A induces the production of IgE antibodies that are cytotoxic for schistosomula in the presence of macrophages, eosinophils or platelets and that transfer immunity to naive rats against a challenge infection (Damonneville et al. 1986). Direct immunization with SRP-A also protects rats to a large extent against infection. The IgE present in anti-SRP-A sera recognized two major antigens at 22 and 26 kDa along with other minor bands. Interestingly, an IgE monoclonal antibody that reproduced the cytotoxic and protective properties of anti-SRP-A IgE also recognized a 26 kDa antigen (Verwaerde et al. 1987). In contrast, anti-SRP-A IgG antibodies recognized the same range of schistosomulum surface antigens as infection serum, as well as a protein of about 29 kDa present in the in vitro translation products of adult worm mRNA (Pierce et al. 1985).

Molecular Cloning Of The P28-I Antigen

The definition of a 28 kDa molecule (P28-I) as a major protective antigen derived from the second element of the approach that aimed at developing polyclonal, monospecific antibody probes for screening cDNA libraries (Balloul et al. 1985). Sera were raised in rats against a series of fractions of adult worm proteins separated on SDS-polyacrylamide gels. Of the fractions tested only one, against a 28 kDa band, produced antibodies that recognized both an in vitro translation product of adult worm mRNA, and a ^{125}I-labelled schistosomulum surface protein, albeit weakly. The anti-P28 serum was highly cytotoxic for schistosomula in the presence of eosinophils and this was demonstrably due to the IgG2a antibody subclass. Both passive transfer of the anti-P28 sera, and direct immunization with the electroeluted P28 fraction was extremely protective against a challenge infection in rats (65-70 %) and mice (43 %) (Balloul et al. 1987a). In addition, helper T cell lines were developed against the P28 fraction (Auriault et al. 1987) and their passive transfer protected rats against infection (85 %). This protection was related to early production of anti-P28 antibodies.

The major protective element of the P28 fraction, P28-I was cloned from an adult worm cDNA expression library in the vector lambda gt11 (Balloul et al. 1987b). Three independent clones were actually cloned and sequenced. The full length sequence was obtained by rescreening the library with an oligonucleotide probe derived from the 5' end of the longest insert. Two more candidates were obtained

and all five clones contained overlapping sequences corresponding to a 28 kDa protein of 211 aminoacids and was termed P28-I. The sequence was confirmed by the sequences of two tryptic peptides obtained from the native protein.

The recombinant protein was initially expressed in *E. coli* as a fusion protein with the first aminoacids of the lambda phage CII protein. The protein was highly immunogenic and induced the production of antibodies specific for the native protein and cytotoxic for schistosomula in the presence of eosinophils. Initial studies showed that direct immunization of rats and hamsters induced levels of protection of 65 and 50 % respectively.

Preliminary experiments using baboons immunized with *E. coli* produced P28-I also indicated that the recombinant antigen protected against a challenge infection although wide individual variations in response were noted (Balloul et al. 1987c). It was notable, however, that the mean granuloma size in immunized animals was reduced, indicating a possible reduction in egg-induced pathology.

A subsequent experiment confirmed both aspects of protection in baboons. Of three groups of six animals immunized with different dose regimes of P28-I in the presence of aluminium hydroxide one group receiving three doses of 67 µg of recombinant P28-I were protected at a mean level of 38 %, with individual variations from 25-80 % (Boulanger et al., in preparation). A second group of animals having received two doses of 100 µg, paradoxically showed no overall reduction in worm burden. However, these animals had fewer granulomatous lesions and the latter were smaller in size than in controls. In this case, both egg production and the granuloma reaction were reduced. These effects have subsequently been reproduced in the mouse model (I. Wolowczuk, unpublished observations).

More recent studies using rats and mice have shown that multiple doses of P28-I are not necessary to induce protection. On the contrary, single doses of P28-I with either Aluminium hydroxide or BCG as adjuvant protect rats and mice significantly against infection (Grezel et al., in preparation). This is an encouraging result in the context of human vaccination where repeated doses may be difficult or impossible to administer effectively. These results are highly promising in that P28-I seems to fulfill the requirements for a candidate vaccine. However the major remaining problems concern the variability in the immune response to P28-I and its possible MHC restriction, and the fact that overall protection levels in primates are not adequate to justify human trials.

Epitopic Characteristics Of P28-I

In order to study the major epitopes of P28-I for both T and B cell responses the primary sequence was analyzed for exposed sequences and for mobility and accessibility. Peptide fragments corresponding to these criteria were synthesized and tested for their capacity to restimulate T cells from infected or immunized animals, and their recognition by antibodies. Three peptides were thus tested and two (aminoacids 24-43 and 115-131) were found to contain major epitopes for IgG antibodies in the rat, but not for IgE

(Auriault et al. 1988). The antibodies raised against the 24-43 peptide were of the IgG2a subclass, were cytotoxic in vitro for schistosomula and recognized the native P28-I antigen. The same 24-43 peptide, as well as the 140-153 peptide also contained major T cell epitopes. T cell lines specific for the 24-43 peptide when passively transferred to rats immunized with P28-I led to a significant increase in specific IgE.

The MHC restriction of the response to P28-I was tested using both recombinant antigen and synthetic peptides in the mouse model (Wolowczuk et al. 1989). A preliminary survey using H-2 congenic mice on a Balb background showed that the P28-I response was indeed under MHC control and that the $H\text{-}2^b$ haplotype determined a low response to P28-I and its peptides, whilst $H\text{-}2^d$ and k haplotypes determined high responders.

A further feature of the mouse immune response to P28-I was that resistance to infection could be transferred by T helper cell lines from Balb/c mice immunized with the recombinant antigen, but not by the corresponding immune sera. This result confirms observation made in the mouse model using other antigen preparations (James and Sher, 1986).

The humoral responses toward P28-I and its peptides of human subjects after oxamniquine treatment, that have been defined as either susceptible or resistant to reinfection has also been examined (Auriault et al. submitted). The main feature of the response toward the whole recombinant antigen was a significant increase in the IgG4 response after treatment in the susceptible population. Two of the peptides (115-131 and 140-153) contained epitopes recognized by IgE antibodies, and this response increased after treatment in both susceptible and resistant individuals. Use of the peptides also showed that specific IgG1 antibodies were produced by the immune population after treatment, whereas the susceptible population was again characterized by a strong production of IgG4 antibodies. While the implications for a vaccination programme using recombinant P28-I are difficult to assess, the results do indicate that manipulation of the immune response may be possible in order to favour a protective response. Taken together the results in experimental animals and in humans also suggest that a synthetic vaccine based on peptides 115-131 and 140-153 may be a viable prospect.

P28-I Is A Glutathione-S-Transferase

The aminoacid sequence of P28-I over much of its length displays a low level of homology to rat glutathione-S-transferase (GSH transferases) of class α and class μ, but not of class π (Taylor et al. 1988). However, high levels of homology to class α or μ subunits occur over short regions of the aminoacid sequence. Recombinant P28-I produced in either yeast or *E. coli* possesses GSH-transferase activity and when native GSH-transferase was purified by affinity chromatography from adult worms, and subjected to sequence analysis, the latter was found identical to that of the cloned molecule. No significant level of homology was found to the 26 kDa GSH-transferase previously cloned and sequenced from *S. japonicum* (Smith et al. 1986).

One consequence of this finding was that it was now possible to purify recombinant P28-I to homogeneity by a single step purification involving passage over a glutathione-agarose affinity column. This largely obviates problems of contamination due to *E. coli* components and particularly LPS and endotoxin. A second possible consequence was an eventual cross-reactivity with the mammalian enzyme. This has never been detected whatever the technique used, however.

We had previously characterized P28-I as a schistosomulum surface protein, however electron microscopy using the immunogold technique demonstrated that although the antigen is present in the adult worm tegument, protonephridial cells and subtegumental parenchymal cells, it is not exposed on the surface. The antigen is also present in the tegument of schistosomula, and also in the head gland from which tegumental components may be derived. The latter location may explain a transient surface exposure. Neither the aminoacid sequence, nor the function of the molecule are consistent with its being an integral membrane protein.

The function of GSH transferases may be consistent with a role in the defence of the parasite against the immune defences in the host. The fatty acid hydroperoxide-GSH peroxidase and GSH transferase activities expressed by P28-I indicate a possible involvement in inhibiting lipid peroxidation and scavenging hydroxyalkenals produced by the release of highly reactive oxygen species by effector cells. It is thus tempting to speculate that an effective immune response directed against the enzyme may act in part by neutralizing this defensive enzyme.

Other Cloned Antigens : Prospects For A Composite Vaccine

The problem of MHC restriction of the immune response may indicate that a single antigen vaccine against schistosomiasis will not be adequate. This is not necessarily the case since the use of different adjuvants and dosing schedules, and the selection of epitopes using synthetic peptides could well obviate the necessity for antigen cocktails.

At the present time, only two other antigens known to protect against schistosome infections have been cloned. One is the *S.japonicum* 26 kDa GSH-transferase (Smith et al. 1986) previously mentioned. It is indeed interesting that two of the protective antigens should possess the same enzyme activity although they belong to different gene families. This underlines the crucial role played by GSH-transferases in the survival of the parasite. Cross-reactivity exists between P28-I and 28 kDa molecules in other species of schistosome, including *S. japonicum*, but it remains to be seen whether the interspecific antigenic community is sufficient to permit cross-species protection.

A major target of the cell-mediated immunity developed in the mouse model after immunisation with *S. mansoni* adult-worm antigens in the presence of BCG is a 97 kDa molecule (Pearce et al. 1986). This turned out to be paramyosin, an invertebrate muscle protein involved in the 'catch' mechanism of muscle contraction (Lanar et al. 1986). Purified native paramyosin or the ß-galactosidase fusion protein both protected mice against infection by intradermal vaccination in the presence of BCG. Paramyosin is not uniquely a

target of cell-mediated immunity since it is a major allergen in rat infections, and is also recognized by IgE in some human infection sera (Boutin et al. 1989).

A large variety of schistosome antigens have now been cloned, some of which may be candidate vaccines, including a 50 kDa schistosomulum surface antigen (Havercroft et al. 1988) and a cercarial esterase (Newport et al. 1988). However, their potential remains to be demonstrated in animal models.

The main prospect for a composite vaccine at the moment could be the combination of P28-I with paramyosin and work along these lines is in progress.

CONCLUSIONS

The prospects for an effective vaccine against *S. mansoni* are very real. The recombinant P28-I antigen is a candidate molecule for such a vaccine. Although high levels of protection can be induced by single doses of P28-I, it remains likely that MHC restriction of the immune response in outbred populations will necessitate either a manipulation of the immune response by selective epitopic presentation, or the incorporation of other protective antigens in the definitive vaccine. Several candidates for a vaccine cocktail have been cloned, one of which is paramyosin. However, further work remains to be accomplished, notably on the effects of vaccinating children previously exposed to schistosome antigens in utero, and on the possibility of vaccinating individuals after chemotherapy to eliminate an ongoing infection. Indeed, such a combination of chemotherapy with a vaccine strategy could well be the main hope for control of schistosomiasis.

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Antigenic Diversity: Implications for Vaccine Design

N. Agabian

INTRODUCTION

In the last decade, molecular research in human disease has made great conceptual advances. We have discovered the genetic basis of several diseases and recombinant synthesis has provided pharmacological amounts of regulatory peptides; the potential for gene replacement *in vivo* has raised our expectations for a type of molecular surgery which may one day prevent disease by in essence "fixing" the genetic defect *in situ*. In the case of parasitic and infectious disease, where there has been a long evolutionary association between the host and parasite, understanding the genetic basis of disease has a very different meaning. In the context of parasitic disease and infection in general, we are far from identifying a single factor or set of factors which, if "fixed" in the host, will prevent disease. Rather, the challenge of molecular biology in parasite systems is complex, and requires a multidisciplinary attack on the cell biology and genetics of both the parasite and the host and the unique conditions which are manifest when these two complex organisms interact in infection. In studying the interplay between the host defense system and the regulation of parasite antigen expression, many new molecular mechanisms for immune evasion have been revealed. Consequently the cell biology of parasites becomes important, not only in understanding the nature of parasitism, but also in discovering new targets and strategies for vaccination against parasites.

The Generation of Antigenic Variation and Diversity

One of the earliest documented and best-studied examples of immune evasion in parasites is displayed by subspecies of *Trypanosoma brucei*, the parasitic protozoan which causes African sleeping sickness (Vickerman 1969; Turner 1982). The relapsing fevers and recurrent parasitemias of patients with African sleeping sickness were suggested to arise from a changing antigenic phenotype of the infecting trypanosomes which were described as early as 1910 by Levaditi and McIntosh (Levaditi 1910). Today, we know that the surface of these organisms consists of one of as many as several hundred variable surface glycoproteins (VSGs) which may be expressed from the genome of a single infecting parasite. This intrinsic capacity for an amazing repertoire of antigenically diverse, but

functionally identical polypeptides, allows parasite populations to escape the host immune system by alternative expression of one of these VSGs. It has been possible to learn how this antigenic variability and diversity is regulated as a result of molecular genetic analysis. (Borst 1982) Schematically presented in Fig. 1, are three potential modes by which surface antigen expression is regulated. A necessary, but not sufficient condition for the transcriptional activation of VSG gene expression is the chromosomal position of the VSG gene; only those genes which are located in a telomeric position can be transcribed and only one of the many telomeric VSG genes is transcriptionally active at any one time.

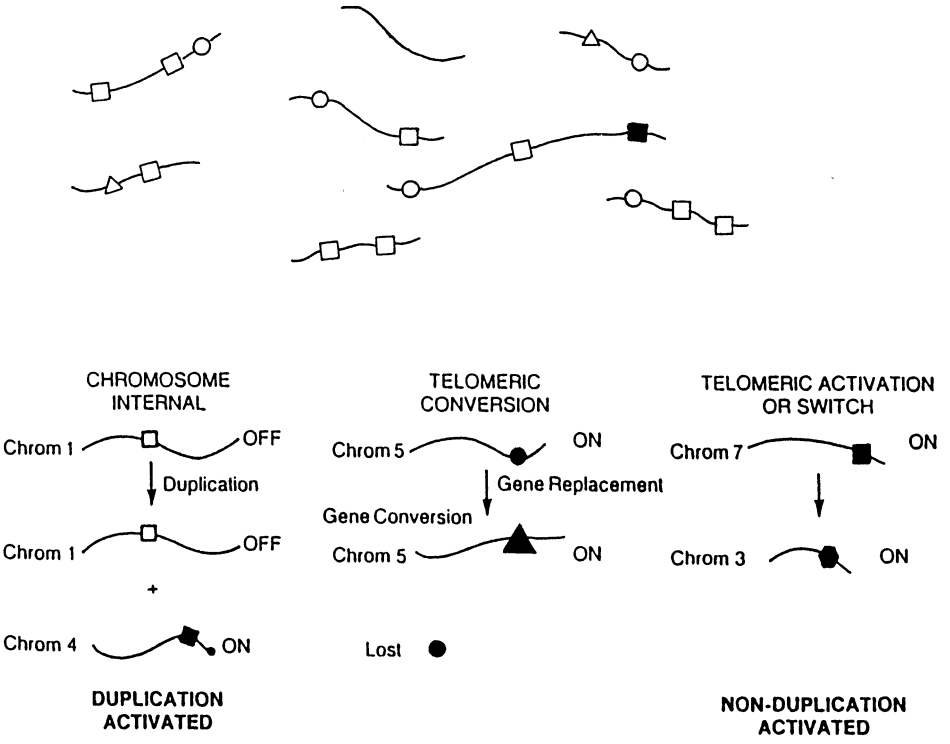

Fig 1 Schematic diagram of trypanosome genome illustrating multiplicity of chromosome size classes (top). Most chromosomes contain one or more variant antigen genes encoding distinct antigenic types (□ , ○ , △) organized in VSG gene families. A filled symbol indicates a transcriptionally active VSG gene. Normally only a single, telomeric VSG gene is expressed at any time; expression is regulated at the level of trancriptional activation; the remaining telomeric and chromosome-internal VSG genes are transcriptionally silent.

The study of gene conversion, duplication and transposition associated with the regulation of VSG gene expression occurred at a time in cell biology when the role of chromosomal rearrangement of genetic sequences was becoming recognized as a generalized regulatory strategy in gene expression. The molecular biology of VSG gene expression in trypanosomes contributed significantly to our knowledge of the role of genetic rearrangements and position effects in gene expression and thus played an important role in characterizing the molecular biology of mobile genetic elements. The molecular genetics of antigenic variation demonstrated the vast repertoire of surface antigens in African trypanosomes; their random order of appearance and their high rates of switching had clear implications for vaccinology: it was necessary to discover and define alternative targets for the immune system if these parasites were to be subject to immune control.

Through the last several years molecular analysis of surface antigens of several other infectious organisms have provided examples of similar and permuted mechanisms which likewise contribute to antigenic variation and diversity in parasite systems. *Chlamydia trachomatis* is an intracellular microbial parasite which causes not only blinding trachoma, but sexually transmitted disease and infertility in more than 400 million people in developed and developing countries. Isolates from around the world have been shown to fall into approximately 15 serotypes, each characterized by antigenically distinct epitopes expressed on the major outer mebrane protein (MOMP) of the organism (Stephens 1985). Through the work of Richard Stephens and colleagues at UCSF, the molecular biology of antigenic diversity in the MOMP gene family have been elucidated. Distinct from the case of antigenic variation in trypanosomes, each chlamydia genome possesses only a single MOMP antigen gene which itself is a mosaic of both variable and constant sequence domains. Each of the 15 serotypes can be organized into MOMP families with overlapping antigenic epitopes (Stephens 1985). Variation is expressed when a population of chlamydia expressing a different MOMP allele is introduced into the population. It is not known if antigenic diversity between the MOMP alleles can be generated by recombination between serotypes or by other mechanisms. However, as with antigenic variation in trypanosomes, this variability constitutes an example of antigenic diversity amongst a family of genes whose members are functionally equivalent but antigenically different. In the case of chlamydia, a major challenge of molecular biology and immunology is to identify and synthesize antigenic mosaics which have overlapping and broad specificity for vaccination against multiple serotypes.

Before the introduction of molecular genetic techniques, the immune system's pattern of reaction against the infecting agent provided the primary access to parasite molecular biology; the antibodies of the host defined the parasite molecules of interest. Because of this long-standing relationship between serological analysis and infectivity, when molecular technologies allowed access to parasite antigens, investigations began with the screening of recombinant expression libraries of parasite genomes with variously characterized patient sera. Through these studies, novel targets of the immune system have unexpectedly revealed themselves. One of the antigens thus characterized represents a particularly intriguing example. George Newport and colleagues, while screening sera from patients infected with *Schistosoma mansoni,* observed an immunodominant antigen of ~70KD which was uniformly detected; similarly *S. japonicum* infected patient sera recognized a protein of the same molecular weight (Hedstrom 1987, 1988). Curiously, while in both cases these were immunodominant antigens, sera from *S. mansoni* and *S. japonicum* were not reciprocally active in recognizing the protein from the alternative species (Hedstrom 1988). The genes encoding each of these antigens were cloned and shown to encode homologous sequences which, when evaluated by DNA sequence analysis, were shown to encode the schistosome homologues of the heat shock protein (HSP) 70. Recombinant peptides of the HSP70 antigen were used for epitope mapping, and it was shown that the C-terminal domain of the *S. mansoni* and *S. japonicum* HSP70 contained the immunodominant epitopes; no other antibody activity was detected in patient sera against the rest of the HSP70 molecule (Hedstrom 1988). Furthermore, the C-terminal regions of the HSP70 peptides showed no immunological cross-reactivity between *S. mansoni* and *S. japonicum*. The phenomenon of HSP70 immunodominance in a variety of bacterial, protozoan and helminth infections have since been documented (Newport 1988). In contrast to the common antigen domains of the microbial HSP65 proteins which may have a role in autoimmunity, in the case of the HSP70, the immunodominant epitopes are found in the C-terminal variable regions of the HSP70 polypeptide.

HSPs are amongst the most highly conserved and ubiquitous proteins in nature. One of the proposed roles for HSPs in cell biology suggests that they are important in the synthesis and assembly of macromolecular arrays. A current challenge of molecular biology and immunology will be to understand the specific role of HSPs in infection and the cell biology of these proteins in relation to antigen presentation and defense against microorganisms.

Strategies for Disease Control

Antigenic variation and diversity is a mirror which reflects the range of problems the parasite encounters in developing strategies for long-term survival in the host. Antigenic analysis thus presents a view on the vast new cell biology of immune evasion and modulation exercised by these organisms in establishing infection.

One of the lessons learned from these antigenic analyses is the repertoire and complexity of mechanisms for achieving immune evasion and persistence exhibited by organisms capable of establishing chronic infection. These underscore the need for alternative targets for vaccinology which may only be revealed by studying the cell biology of the parasite itself and developing a comprehension of the requirements of the parasite for survival in the host.

Understanding the cell biology of parasitic organisms provides strategies for extending the natural repertoire of the immune system to cope with disease. Through such knowledge it will be possible to define alternatives to the antigen-dependent killing/neutralization of infecting organisms which has heretofore been the primary rationale for choosing an immunogen. Understanding the nature of the molecules and enzymatic activities required for the parasite to penetrate the external defenses of the host, for entry into specific cell types, or which mediate receptor-ligand interactions necessary for parasite sequestration, provide potential targets for immuno-blockade at the host-parasite interface. Immunological interference with parasite penetration is an approach which is currently being developed in relation to the penetration of schistosome larvae through mammalian skin (Newport 1988). In this case, an elastase activity with broad substrate specificity is secreted by the cercaria and presumably facilitates skin penetration by proteolysis at the site of parasite entry (Newport 1987). In principle, anti-elastase antibodies in the host might prevent parasite penetration by interferring with enzyme secretion or activity. A second strategy involves the recruitment of the immune system as a pharmaceutical agent which interfers with enzymatic activities essential for parasite viability. Many of the parasite antigens identified through molecular genetics have turned out to be metabolic enzymes, especially those of carbohydrate metabolism (unpublished). Some of these enzymes have also been shown to elicit antibody activity in the host which partially protects against reinfection, glutathione-metabolizing enzymes of *S. mansoni* being a notable example. It has not been shown if these neutralizing antibodies diminish infectivity through inhibition of enzymatic activity, however, these studies suggest the potential for producing antibodies which may function as therapeutic reagents.

The interplay between host and parasite is a result of the intrinsic genetic repertoire of both the individual organisms and the dynamics of each as they interact with one another during infection. Modulation of the host immune response or attenuation of parasite virulence can dramatically alter the course of disease. The identification and molecular cloning of the many hormones of the immune system raise the potential for immunotherapy in parasite infections which function through the potentiation of the host response to parasite infection and employ a strategy which modulates the range of immunological activity which the host may recruit to combat the parasite. An alternative target for modulation is the parasite itself. The current move toward molecular genetics in parasite systems will enable us to discover the molecular basis of pathogenesis and the regulation of antigen expression. DNA transformation of parasite systems remains problematical and elusive, however the potential for genetic manipulation of the parasite holds much promise for the development of new strategies for immune intervention by providing access to the cell biology of these interesting and little studied classes of organisms.

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Genetic Aspects of Mycobacterial Infections in Mouse and Man

E. Schurr, E. Buschman, P. Gros, and E. Skamene

INTRODUCTION: THE *Bcg* GENE

Inbred strains of mice differ dramatically in their susceptibility to infection with *Mycobacterium bovis* (BCG). So far, all strains tested segregated in two non-overlapping groups: strains which are resistant to the growth of the bacilli and susceptible strains which permit a rapid bacterial growth in their reticuloendothelial organs (Gros et al 1981). Mendelian analysis revealed control of the resistance/susceptibility phenotype by the two alleles of a single, dominant autosomal gene which was named *Bcg*. Subsequently, the gene was mapped in a panel of BXD and BXH recombinant inbred strains (RIS) to the proximal part of mouse chromosome 1 (Skamene et al 1982). A detailed analysis of multiple infected backcross mice indicated that the *Bcg* locus was identical with the previously described *Lsh* and *Ity* loci which mediate innate resistance to infection with *Leishmania donovani* and *Salmonella typhimurium*, respectively. The *r* and *s* alleles of the *Bcg/Lsh/Ity* locus also determine resistance/susceptibility of mice to infection with *Mycobacterium lepraemurium* (Skamene et al 1984) and with a number of atypical mycobacteria, including *Mycobacterium intracellulare* (Goto et al 1989). Although vastly different in life cycle and metabolic requirements, all these pathogens are intracellular parasites of macrophages in their murine hosts. It is therefore not surprising that the host resistance locus is expressed as a T-cell independent, immunologically non-specific, enhanced state of macrophage priming for bactericidal activity (Stach et al 1984; Denis et al 1988a). Moreover, it was demonstrated that macrophages from resistant mice also induce superior acquired immune responses to both specific and non-specific stimuli, possibly due to increased Ia expression (Denis et al 1988b). The combined evidence from these experiments suggests that the *Bcg* gene can be considered as a key genetic regulatory element of mycobacterial infections in mice.

MOLECULAR GENETICS OF THE *Bcg* GENE

The investigation of macrophage biochemistry and of immunological processes, generally associated with bactericidal activity in mouse strains congenic for *Bcg* have revealed numerous phenotypic manifestations of the *Bcg* gene (Buschman et al 1988). Despite these efforts, the molecular

identity of the protein encoded by *Bcg* has so far proven to be elusive. In the absence of any known or suspected protein product, we have chosen the strategy to identify genetic markers closely linked to *Bcg* and then to use these markers as starting points for a molecular cloning of genomic sequences overlapping *Bcg*. For this purpose, we identified genes which had been co-transduced with *Bcg* during the construction of mouse strains congenic for *Bcg*. Co-transduced genes are expected to be closely linked to *Bcg* and pre-screening on congenic mouse strains thus offers a convenient method to identify genes located in the vicinity of the host resistance locus. The alleles of genes investigated were identified as restriction fragment length polymorphisms (RFLP) on Southern blots by hybridizing endonuclease restricted genomic DNA with radioactive gene-specific cDNA probes. Among numerous markers tested, five loci were found to co-segregate with the Bcg^r allele in congenic mice after 20 generations of introgressive backcrossing: *Len-2, Fn, Vil, Akp-3* (*Alpi*) and *Achrg*. The precise map location and gene order of these loci with respect to *Bcg* were then established by analyzing strain distribution patterns (SDP) in 43 AXB/BXA RIS and recombinant classes in 186 backcross mice segregating for the Bcg^r and Bcg^s alleles. The combined result of these analyses indicate the following gene order and distances: centromere - *Len*-2 - 2.6cM - *Fn* - 4.6cM - *Bcg*/*Vil* - 5.2cM - *Akp*-3 - 0.4cM - *Achrg*. The most striking observation of these experiments is that no recombination was observed between *Vil* and *Bcg* making *Vil* the closest marker of the host resistance locus known to date (Schurr et al 1989). In the meantime, we have also determined the SDP of *Vil* and *Bcg* in 26 BXD and 10 BXH RIS (Table 1).

Table 1: Strain distribution pattern of *Vil* and *Bcg* among BXH and BXD recombinant inbred strains (RIS)

Bcg	*Vil*	Progenitor	RIS
H	H	C3H/J	BXH 4, 6, 7, 8, 9, 14, 19
D	D	DBA/2J	BXD 1, 6, 8, 13, 15, 23, 25, 27, 28, BXD 30, 32
B	B	C57BL/6J	BXH 3, 10, 11; BXD 2, 5, 9, 11, 12, BXD 14, 16, 18, 19, 20, 21, 22, 24, BXD 29, 31

Again, in both mapping panels, no recombination was observed between both genes. Because the equivalent of 4 meioses occurs during the construction of 1 RIS, we can conclude that no recombination occurred between *Vil* and *Bcg* in almost 500 meioses investigated, corresponding to

an estimated maximal distance of 0.2 cM. Using the rule of thumb that 1 cM corresponds approximately to 1 million base pairs (bp), both genes are expected to be located within 200 kbp of each other, a distance amenable to long-range eukaryotic cloning techniques. Using a *Vil* cDNA clone as an entry probe in genomic libraries, we have now initiated chromosome walking experiments with the aim of cloning the closest recombinational breakpoints distal and proximal of *Bcg/Vil*, which we expect within an estimated average distance of 100 kbp from the start point. During our walk, repeat-free probes will be tested for differential expression in resistant versus susceptible macrophages and thus help to identify candidate gene sequences of the *Bcg* gene.

THE CONCEPT OF COMPARATIVE GENE MAPPING

Recent advances in gene mapping technology have led to a significant increase in the number of homologous genes which are mapped in more than one species (Nadeau 1989; O'Brien et al 1989). The majority of homologous genes have been mapped in the mouse and human genomes. In this case, the approximately 250 pairs of homologous genes make up more than one third of the murine autosomal genome. An important application of comparative gene maps is to predict the location of homologous genes in other species. For example, many mouse models for medically important mutations or disease phenotypes have been developed over the years, including models for resistance to infection as exemplified by the *Bcg* gene, or models for numerous immunological mutations (e.g. scid, gld, mo, nu). Most of the mouse genes causing the observed phenotypes have been mapped in the murine genome and in many instances comparative gene maps can be used to focus the search for corresponding human disease genes. Instead of scanning the entire 3000 cM of the human genome for possible linkage associations with the phenotype the search can be concentrated on chromosome fragments extending no more than 50 cM. The reason for linkage conservation among species is presently unknown. It is possible that linkage disruption, i.e., chromosomal rearrangements during the evolution of species, are random events which can occur at many regions within the genome with equal probability. On the other hand, it is conceivable that genome evolution selected for the physical conservation of a set of genes along the chromosome e.g., for the purpose of concerted regulation of gene expression.

CONSERVED SYNTENY BETWEEN MOUSE AND MAN OVERLAPPING *Bcg*

Close scrutiny of the mouse and human gene maps shows that human homologues of the five *Bcg* congenic loci are precisely conserved onto the

telomeric end of human chromosome 2, region q32-qter (Schurr et al 1989). Although the gene order of the five human homologues on chromosome 2q is not known, the preservation of synteny for a linkage group of five genes closely linked to *Bcg* identifies the telomeric region of chromosome 2q as the primary target region for the search of a human *Bcg* homologue. In humans, this chromosomal region is dominated by a large cluster of structural protein genes encoding nebulin, elastin, crystallins, myosin, fibronectin, villin and at least three collagens. This observation has stimulated speculation that the host resistance locus possibly encodes a structural protein of either extracellular matrix or cytoskeletal nature. Members of both groups of proteins have well characterized functions in immune and non-immune defense mechanisms. Interestingly, all pathogens which are under *Bcg* gene control are phagocytosed by macrophages via integrin receptors which usually mediate transmembrane binding of extracellular and intracellulare structural proteins. It is therefore a reasonable hypothesis that integrins on macrophages interact differently with distinct allelic forms of cytoskeletal proteins resulting in a differential T-cell independent activation of resistant and susceptible macrophages.

LINKAGE ANALYSIS IN HUMAN FAMILIES

Following the identification of a conserved linkage group between mouse chromosome 1 and the 2q chromosomal region in man we have embarked on a linkage analysis of 2q specific DNA polymorphisms with the trait of resistance/susceptibility to tuberculosis and leprosy. The investigation of linkage is done in families from endemic areas with a familial history of the disease and multiple affected offspring. Genotyping of the parents in these families indicates that the frequency of alleles detected with polymorphic DNA markers is dependent upon the ethnic background. Therefore, to define probes with high polymorphism information content (PIC), i.e. a high degree of heterozygosity, allele frequencies must be determined for each probe in matched control populations. For example, the lambda allele system of the locus D2S3 is reasonably informative among Caucasians (PIC: 0.24), whereas the degree of heterozygosity for the same allele system is 0 among a group of 22 unrelated Chinese people. We have also started to evaluate the segregation of parental alleles among affected and non-affected siblings by means of a LOD score analysis. For this analysis, the likelihood of the observed segregation of marker and susceptibility alleles is compared to the likelihood under the assumption that marker and phenotype are not linked. The $\log_{10}$ likelihood ratio is called the LOD score. Generally, a LOD score of +3 is taken as confirmation of linkage. In our study, we have not yet reached this significance limit between any marker and the disease phenotype.

INNATE RESISTANCE VERSUS ACQUIRED IMMUNITY

In mice, innate resistance and susceptibility to BCG infection has been shown to be under single *Bcg* gene control. However, the quantitative variation in acquired immuntiy to BCG infection which exists among inbred mouse strains is clearly under polygenic control. The *H-2* linked genes have been shown to modify resistance to *M. lepraemurium* infection as well as the production of gamma interferon during BCG infection (Curtis et al 1984; Huygen et al 1988). The role of non-*H-2* genes in regulating immunity to BCG infection has also been documented (Buschman et al 1988). The influence of *Bcg*-controlled macrophage activation is one example of a non-*H-2* gene which influences the development of immune responses (Buschman et al 1988). The *Bcg* gene affects immune responsiveness in two ways. First, the extent of granuloma formation, splenomegaly response and antigen-specific lymphocyte proliferation in inbred strains were found to be dependent on the magnitude of the bacterial load, this being the consequence of the multiplication of BCG *in vivo*, which is controlled by the *Bcg* gene (Skamene, 1989). Secondly, under conditions where congenic Bcg^s and Bcg^r strains accumulated an equivalent BCG burden, genetically resistant mice developed significantly more antigen-specific T cell proliferation and production of interleukin-2 compared to susceptible mice (Buschman and Skamene 1988). However, at 6 weeks following infection, T cell responses did develop in Bcg^s mice. In contrast, during infection with virulent *M. intracellulare* , T cell responses in Bcg^s, but not Bcg^r, mice remained suppressed, suggesting that bacterial virulence and genetic susceptibility are important factors in maintenance of the immunosuppressed state (Nakamura et al 1989).

As a mechanism to explain the greater development of T cell immunity in Bcg^r mice, several lines of evidence have shown that accessory cell functions were enhanced in congenic Bcg^r compared to Bcg^s macrophages. These functions included the ability of splenic macrophages to present antigen to T cells (Denis et al 1988a) and the increased expression of Class II Ia antigens by Bcg^r macrophages (Johnson and Zwilling 1985; Denis et al 1988b). As shown in Table 2, the frequency of splenic macrophages bearing Ia antigens was higher in Bcg^r compared with Bcg^s macrophages, both before and after infection with BCG. These results indicate that elevated Ia expression by Bcg^r macrophages was associated with increased antigen-presenting activity. Moreover, it was observed that macrophage populations isolated from spleens of Bcg^s mice infected with 10^6 BCG suppressed T cell proliferation. The active suppressor function of Bcg^s macrophages was revealed by co-culturing with Bcg^r macrophages, as shown in Fig. 1. Although this suppressor mechanism remains to be characterized, the data suggest that the *Bcg* gene may regulate T cell responsiveness/suppression via the macrophage.

Table 2: Ia^+ macrophages in spleens of *Bcg* congenic mice

Number of BCG injected	Percent Ia^+ Splenic Macrophages	
	Bcg^s	*Bcg*^{r}
none	38 ± 7	49 ± 9
10^4	35 ± 7	69 ± 10
10^6	29 ± 6	70 ± 7

Macrophages were isolated from normal mice or from mice infected for 3 weeks with BCG. The percentage of Ia^+ cells was determined by FACS analysis.

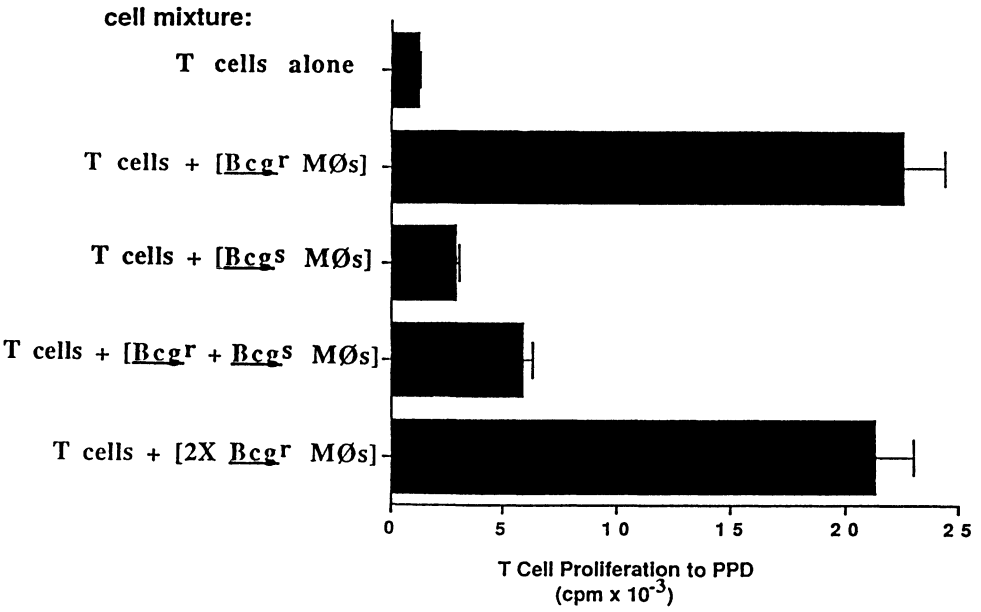

Fig. 1 Macrophages from BCG-infected *Bcg*^{s} mice suppress T cell proliferation. Purified splenic macrophages were obtained from congenic *Bcg*^{r} and *Bcg*^{s} animals 3 weeks following injection of 10^6 BCG. T cells were obtained from congenic *Bcg*^{r} mice 3 weeks following injection of 10^6 BCG.

CONCLUSION

Murine models of mycobacterial diseases are an important experimental tool for the identification of genes controlling innate resistance and acquired immune responses to mycobacterial infections. In these models, innate resistance is under single *Bcg* gene control. The *Bcg* gene has also been identified as one of several non-*H-2* genes which modulate the action of *H-2* restricted events. The *Bcg* gene can therefore be considered as an example of the interaction between *H-2* and non-*H-2* linked genes in the pathogenesis of mycobacterial infections and further our understanding of the spectral, clinical manifestations characteristic of these diseases. Most interestingly, however, the identification of a man-mouse conserved linkage group overlapping *Bcg* makes it possible to search for a homologous resistance gene in the human genome. The identification of a human *Bcg* homologue would have a profound impact on future efforts towards the control of mycobacterial diseases in man.

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IV Defense

Acquired Immunodeficiency Syndrome

An Introduction into the Immunology of AIDS

H. Wigzell

Introduction

AIDS represents an end stage following infection by HIV-1 for an average period of 5-10 years. The immune deficiency breakdown induced represents the sum of damaging events caused either directly by the virus or induced indirectly via the various forms of immune reactions initiated against the viral infection.

In the following session various features of HIV and AIDS will be presented with particular focus on immunological aspects of this disease I will here try to summarize some personal views on the present knowledge or lack of knowledge of the immunology of AIDS.

The virus

HIV carries genes allowing the production of at least 9 proteins, their functions and features will be discussed in detail by Flossie Wong-Staal. Here it is sufficient to state that these proteins when used as immunogens would seem to be comparatively trivial inducing conventional humoral and cell-mediated immuune responses.

What may be less conventional about the virus in relation to the immune system is the ability of HIV to go into hiding as a latent provirus where it could possibly reside in a dormant state in long-lived T lymphocytes for unknown periods of time without being recognized by the immune system. The latent virus can be activated by several molecules, many of which represent molecules produced by the immune system in response to foreign structures (Matsuyama et al, 1989). Anthony Fauci will address the capacity of certain cytokines to regulate HIV replication and discuss how this may direct our thoughts to immunomodulatory therapy of AIDS.

HIV has also a conspicious capacity to allow sizeable genomic variability without loosing function. This is assumed to be a primary cause for the loosing battle of the immune system against an established HIV infection. In support for such a concept is the fact that dominant sites for neutralizing antibodies and CTL:s against HIV are directerd against regions known to express sizeable diversity. Isolated specific neutralizing antibodies and

CTL:s are thus a frequent finding and failures to produce efficient group-specific immunity constitute a major problem when attempting to design future HIV vaccines. These specific questions will be dealt with in detail by Dani Bolognesi.

The target cells

HIV may kill the cells it can infect by several means, directly or indirectly by inducing an immune attack against the infected cell. HIV has a restricted cellular tropisms both in vitro and in vivo (Levy, 1989). A major restriction element in relation to infection is CD4, the cellular receptor for HIV via its high affinity for the outer envelope protein of HIV, gp 120 (Maddon et al, 1986). Robin Weiss will discuss various aspects of CD4 in relation to HIV infection and possible therapy. Cells may be infected via non-CD4 dependent routes but the latter behave like being much less efficient than entry via CD4.

In vivo dominating target groups for HIV infection are represented by the CD4+ T cells and the monocyte family with its subgroups. It may thus not come as a surprise to the immunologist that the dominating immunosuppression noted early on in an HIV-infected individual relates to functions requiring intact CD4+T lymphocytes recognizing soluble antigens on antigen-presenting cells within the monocyte family (Levy, 1989, Donnenberg et al, 1989, Krowka et al, 1989). Loss of delayed type hypersensitivity is also a sometimes comparatively early feature of HIV infection.

In the infected individual the most dramatic consequences of HIV infection in relation to cell numbers are observed in relation to CD4+T cells, where in AIDS a dramatic reduction in such cells represents the final stage of gradual decay in numbers of such cells over many years. A significant reduction of CD4+T cells is early feature in HIV-infected individuals. Whether this reduction in the CD4+T cells merely represents a result of direct cell deaith via HIV infection or involves other parameters as well will be briefly discussed below.

The primary infection

HIV confronts the immune system in a diversified manner. Free virus particles are known from the hemophiliac cases to be infectious for man. It is tacitly assumed that the same holds true for HIV-infected cells although no formal proof for this exists and is difficult to obtain. Attempts to analyse the relative viral load of cells versus free virus particles in various body fluids have yielded data suggesting a somewhat higher viral recovery from the cellular compartment (Levy, 1989). Venereal diseases associated with high amounts of mononuclear cells are also associated with higher capacity to transfer HIV, in particular if ulcers are present at the same time (Simonsen et al, 1988).

Whereas free virus and actively virus-producing cells with great likelihood can transfer infection between individuals it is less clear to what extent cells with inactive HIV provirus can be transferred and allowed to survive in a foreign host for a sufficient time for induction of infectious virus to take place. As HIV is a budding virus from T lymphocytes with no intracellular reservoirs killing of virus-producing T cells could be considered to only be negative for the virus. It is less clear whether lysis of an HIV-producing macrophage with a resulting release of infections virus particles (Wigzell, 1988) always is of benefit for the host during HIV infection.

No evidence exists that a generally strong immune system of the individual before primary infection is of relevance in relation to susceptibility to infection. On the contrary, one may argue that an individual with low numbers of CD4+T cells and monocytes for mere reasons of presenting fewer targets for HIV infection could be less susceptible than a normal individual.

HIV is a selective immunoregulator

Infection by HIV is known to have profound immunosuppressive consequences for the human immune system. Early in infection significant reduction in T cell performance against soluble antigens is found (Krowka et al, 1989). A reduction in the number of precursor cells amongst PBL:s responsive to I1-2 is also a frequent finding (Donnenberg et al, 1989). This reduction is not easily explained by direct viral infection of the participating cells in such reactions as the actual numbers of HIV infected cells amongst the PBL:s at that stage is considered to be very low. Rather is may imply that soluble factors such as cytokines and other molecules may be produced early in the infection with significant consequences, here negative, for the functions of other cells within the immune system. However, HIV may also in fact have paradoxical stimulatory consequences for certain immune functions. It is well known that HIV infections frequently is linked to increased Ig levels and activated B cells are a frequent finding in HIV infected individuals (Pahwaa et al, 1986). Normaliaztion of antibody formation after HIV infection in individuals suffering from acquired hypogammaglobulinemia exemplify the ability of HIV to enhance immune function (Wright et al, 1987). With time there is, however, a decrease in the capacity to produce specific humoral antibodies upon immunization, in particular if the antigens are previously not encountered by the immune system. Whereas NK cell activity by most researchers is found to become reduced upon HIV infection, there exist a presence of significant CTL activity against HIV in asssociation with MHC even until the individual reaches AIDS.

Is the immune reponse against HIV beneficial and suppressing viral replication?

The "classical" picture of a primary HIV infection in relation to immune reactions describes an initial viremia followed within weeks or months by an immune response which is causing a depression in viral replication. With time over a few years there is then a gradual comeback of viral replication largely parallelled by a decay in CD4+T cells in the circulation resulting in the majority of cases in the final ending in AIDS. Most of the data providing information in relation to viral production in this picture stem from blood sample analysis whereas viral replication most likely is predominantly occurring in the tissues. Antibodies against HIV envelope proteins would be expected via immune complex formation to enhance the rate of removal of virus from the circulation even in situations where the antibodies as judged by in vitro studies may not be classified as neutralizing. This may provide a picture overemphasizing the actual suppression by the immune system of the production of HIV. The admittedly scarce data on viral replication in lymph nodes during various stages of HIV infection have in fact so far failed to indicate any significant differences in the rate of production of virus/viral proteins at the different clinical stages of HIV infection (Schuurman et al, 1989).

A more detailed analysis of the immune reactions initiated against HIV as summarized in table I gives a mixed feeling as to the potential positive and negative consequences of the anti-HIV immune response. On the positive side, in vitro several reactions capable of eliminating free virus or virus-producing cells can be defined including neutralizing antibodies, ADCC and CTL:s. That they may be positive and relevant also in vivo is supported by several pieces of evidence of mostly indirect nature. Early antibodies in an individual will thus be more prone to neutralize early viral isolates from the same individual whereas later isolates are more prone to escape such inhibition. The dominant neutralizing antibodies contain strong elements of isolate-specificity and are directed against a region on the envelope protein with high tendency to variability (Rusche et al, 1988). The presence of isolate-specific CTL:s would argue in the same direction.

Table I. Good and bad reactions of the immune system in relation to HIV and AIDS

Humoral Immunity	Humoral+cellular immunity	Cellular immunity
+	+	+
Neutralizing ab:s	ADCC eliminating virus-infected cells	CTL:s killing virus infected cells
-	-	-
Enhancing ab:s	ADCC eliminating Gp120-coated non-infected CD4+ cells	CTL:s killing cells taking up HIV antigens
Autoimmune ab:s		

The interesting findings that early isolates normally act as replication deficient viruses in vitro to later in diseased individuals be replaced by more cytopathic and efficient replicators (Åsjö et al, 1986) are also in line with the concept that an intact immune system can suppress the latter forms. Support for such an argument can also be drawn from the fact that infectivity does increase at the later symptomatic stages of HIV infection, that is at the time when the in vitro agressive variants do tend to become prominent. A two-way Darwinian selection process may thus be a common feature during HIV infection with an initial in vitro aggressive form being responsible for the primary infection but at the same time being more sensitive to immune attack allowing for the later selection of the in vitro growth-deficient variants. When the immune system after some years is becoming worn down there is a resurgence of the in vitro aggressive forms of HIV which then may speed up the progression towards AIDS (Tersmette et al, 1989).

The fact that antibodies against HIV envelope proteins in vitro may allow an enhancement of HIV replication by a factor of 2-10 (Robinson et al, 1988, Wigzell et al, 1988) represents a potential dilemma for interventions using passive administration of antibodies against HIV or when attempting to vaccinate against HIV. Here it remains to establish under which, if any, in vivo conditions antibodies against HIV may be detrimental via enhancement. Titrations of antibodies necessary for such studies are only possible in systems where comparatively large numbers of experimental animals can be used, for instance in SCID mice repopulated by human hematopoietic cells and infected by HIV-1 as will be discussed by Michael McCune in this session. An alternative system would be HIV-2 or SIV in macaques.

Factors associated with prognosis of HIV infection

Definition of prognostic factors in HIV infection may give important leads in relation to immune parameters of interest in relation to prophylaxis and/or therapy. The singular most important factor defined so far is the age at which HIV infection does take place (Goedert et al, in press). Here the rate of progression towards AIDS in individuals contracting infection between the age of 1 to 20 years is approximately half compared to individuals infected after age 20. As the relative size of the lymphatic tissues is much higher in the younger age group one may argue that the better prognosis in this group is linked to a better capacity to maintain and/or repair normal T cell numbers and functions. However, more recent data of Goedert et al show that the rate of decay of CD4 T cells is comparable in the two groups leaving the question at present unanswered whether the striking difference in prognosis between the above two groups has an immunological basis.

HIV-infected mothers have a great risk to transfer their disease to their foetuses. The situtation represents a unique system where a virus may be transferred into a recipient who has already received antibodies produced against the very same variant(s) of HIV. It is here of interest to note that there exist a reactivity pattern of antibodies against certain select gp120 peptides in HIV-infected mothers significantly correlated to prognosis of transfer/non-transfer of HIV infection to the child (Rossi et al, in press). Presence of antibodies directed against some defined regions of gp120 with conserved cysteins in HIV-infected mothers will thus correlate with failure to transfer infection. Whether this in fact represents the presence of unusually active neutralizing antibodies in such mothers against HIV or merely reflects in an indirect manner the clinical stage that the mothers happened to be in now remains a most important question to solve. At present, however, such a defined peptide ELISA assay as used in these studies may give useful advice in relation to prognosis in this mother-child situation.

The tumors arising in AIDS patients

AIDS patients have been found to run a very high risk to develop certain normally relatively rare tumors. This is of interest for the present audience for one reason. The types of tumors appearing in AIDS patients are thus well known tumors to the clinical immunologist working with immunodefieiencies, be they genetic, acquired or induced. Table II gives in a summary form a comparison between tumor types and frequencies in organ recipients immunosuppressed at will and AIDS patients. Two tumor types dominate so far in the AIDS patients, namely Kaposi's sarcoma and extranodular B cell lymphomas. The very same tumor types are also grossly overrepresented in the grafted patients.

It is here of interest to note that Kaposi's sarcoma has the shortest latency period after organ grafting with B lymphomas having the second shortest latency. Immunodeficiency as such would thus seem to function as a gate opener for these tumors. HIV itself does not play any direct role for the tumors to appear. As the life span of AIDS patients is now being extended it would be of interest to observe whether skin tumors such as squamos cell carcinomas with a long latency period now would start to appear in similar frequences as in organ recipients. The relative proportion of the respective tumor types may thus depend on several factors such as the degree of immunosuppression, duration of the deficiency etc. whereas the actual immunosuppressive cause probably only plays a secondary role.

Table II. Tumors in immunodeficient individuals

Immunodificiency	Kaposi*	Extramodular lymphoma*	Ca.perineum*
Organ recipient:	4500 (23)	3900 (36)	10000 (90)
AIDS	>1000000	>4000	Increased

*First figures o/o in relation to control = 100 %;
Figures within brackets = log phase in months after organ grafting = immunosuppression

How are normal CD4 T lymphocytes maintained in an adult individual?

There is no doubt that HIV infection may kill CD4 T cells by syncytia formation or induction of single cell death by yet unknown molecular events. It is also possible that innocent bystanders may be killed by recruitment into syncytia formation via infected cells, or via attack by CTL:s or NK-ADCC mechanisms after binding Gp120 to the cellular surface. The average time period requested for the HIV infected individual to acquire AIDS is, however, in the order of 5-10 years. It is therefore highly relevant to consider the possible impact of HIV infection in relation to the normal ways of maintaining T cell numbers in adult individuals.

A summary of this would read something like follows:
Thymectomy early in life but after mature T cells have left the thymus has only minor consequences in relation to total T cell numbers late in life = there exist a thymus-independent system allowing mature T cells to expand and maintain normal T lymphocyte levels. This is further emphasized by several studies in rodents where small numbers of mature T cells inoculated in thymus-lacking animals can expand and reach close to normal cell numbers.

The profound histopathological changes in the lymph nodes of a large proportion of HIV-infected individuals with drastic changes in germinal centers with follicular expansion followed by fragmentation and a final destruction of lymph node architechture (Racza et al, 1986) may have a variety of consequences of interest in relation to HIV and AIDS. A pivotal role of germinal centers is to allow clonal expansion of B lymphocytes upon antigen stimulation, a feature being lost with time after HIV infection. Furthermore, the lymph node follicles are considered major foci for the removal of immune complexes and here the destruction may seriously impede the capacity to eliminate circulatory immune complexes which may include antibody-coated HIV particles.

Finally, any normal function of the lymph nodes in maintaining T cell function and numbers is likely to become influenced in a negative manner by the destruction. HIV infection is in addition known to result in premature involution of the thymus.

Data from studies in mice do strongly argue in favour of a dependence of mature T cells on accessibility to relevant stimulatory MHC complexes in the peripheral lymphoid tissue to maintain normal cell numbers (Marusi-Galesic et al, 1988). Prolonged infusion of antibodies against class II MHC in mice will lead to a select reduction of CD4 T cells in such animals with time, whereas anti-class I immunoglobulins will result in a corresponding reduction of CD8 T mature lymphocytes. As the lymphatic system is an area of the body with normally a high proportion of class II MHC expressing cells in close proximity to mature T lymphocytes damage in such areas may be expected to have a more profound negative effect on CD4 T cells than on CD8 T lymphocytes. In addition to this, gp41 of HIV contains a sequence of 11 amino acids which shows a significant high homology with a region of the external part of the constant beta chain regions of HLA-D molecules. This is most likely the reason why more than 1/3 of HIV infected individuals do produce antibodies which react with gp41 and HLA-D positive cells and which in vitro inhibit normal CD4 T cells from proliferating against soluble antigens (Golding et al, 1989). It is not far-reaching to assume that the production of such antibodies may add to the failure to maintain CD4 T cells in HIV-infected individuals.

One should here consider that a comparatively minor shift in the repair and maintenance profile of the CD4 T cells may result in a profound change in the slope of decay in CD4 T cells with time in HIV infected individuals and corresponding changes with regard to prognosis and clinical picture. This is thus a possibility worthwhile exploring.

Minimal requirements for a vaccine against HIV

Many viral diseases result from a high efficiency of transfer upon contact, that is infection will result with high frequency. This is not the case of HIV infection. With the exception of infection via blood transfusion even a pregnant HIV-infected mother may only have a possibility of 50 % or less to transfer a productive infection to the foetus. The likelihood of transfer of infection via sharing of infected needles and syringes is probably in the order of 1 % of contacts. Efficiency of sexual transmission is quite variable with the highest figures being in order of 5 % in the case of simultaneuous venereal disease with ulcers (Golding et al, 1989). In the absence of venereal disorders, however, the likelihood of HIV transfer when the infected individual is still asymptomatic is probably in the order of 1:1000 or less. It is thus relevant to assume that even a quite inefficient vaccine resulting in the elimination of every second infectious unit of HIV (be that a virus particle or infected cell) will have a significant reducing impact on the spread of HIV, that is on the AIDS epidemic.

Acknowledgements

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Genetic Regulation of HIV

F. Wong-Staal

Not all retrovirus infections require active viral replication to accelerate the disease course. A good case in point is Adult T-cell Leukemia associated with the human Leukemia Virus, HTLV-1. Once the patient is diagnosed with ATL, there is no sign of virus expression of any kind in the tumor cells. In contrast, clinical progression in AIDS does correlate with elevated level of virus expression and continuous recruitment of new infected cells. There are several important implications from this observation: (1) Regulation of HIV expression by viral and cellular factors play a key role in AIDS pathogenesis. (2) Exogenous factors may contribute to clinical progression. (3) Agents that inhibit the virus replication cycle would be appropriate therapeutic agents. In this paper, I would focus on two regulatory genes of HIV relating to their function and their potential use as targets in antiviral therapy.

The HIV Genome is highly complex, and contains multiple overlapping open reading frames that may be brought together by various splicing events. There are already nine well characterized genes defined on the 10 Kb genome, as compared to three (gag, pol, and env) for most retroviruses (Figure 1). The function of several of these accessory genes remain mysterious (Table 1). They are completely dispensable for virus infection and its biological effects, and at best display subtle effects (less than ten fold) on the rate of virus replication. The vif gene is necessary for infectivity of cell-free virus, but not cell-cell transmission. However, two genes stand out as being essential for virus replication. These genes, tat and rev, both encode regulatory proteins that are predominantly found in the nucleolus. Tat is a master switch that turns on all viral gene expression, while rev is a differential regulatory that post-transcriptionally activates the expression of virion associated proteins as distinct from regulatory proteins.

MECHANISM OF TAT ACTIVATION

The mechanism of tat activation is not well understood. Evidence has been presented that implicate tat in both transcriptional and post-transcriptional events, including transcriptional initiation, anti-termination, mRNA stabilization, transport, or translation (for review, see Cullen, Franza, and Wong-Staal, 1988). The location of the cis-acting tat responsive element (TAR) at +1 to +44 is consistent with tat interaction with either DNA, RNA, or both. We have examined the capacity of recombinant tat protein to bind either TAR DNA or RNA and found that partially purified tat protein expressed in either E. coli or Baculovirus Vectors showed detectable binding in a gel shift assay to TAR RNA, but not DNA (J. Rappaport, et al., unpublished). The sequence in TAR RNA forms a stable stem-loop structure which has been shown to be necessary in structure and in sequence of the loop to be important in the transactivation response. A non-functional TAR mutant which was deleted in four bases, and thus predicted to have an altered stem-loop no longer binds tat, confirming some level of specificity for the binding. However, there was no a one-to one

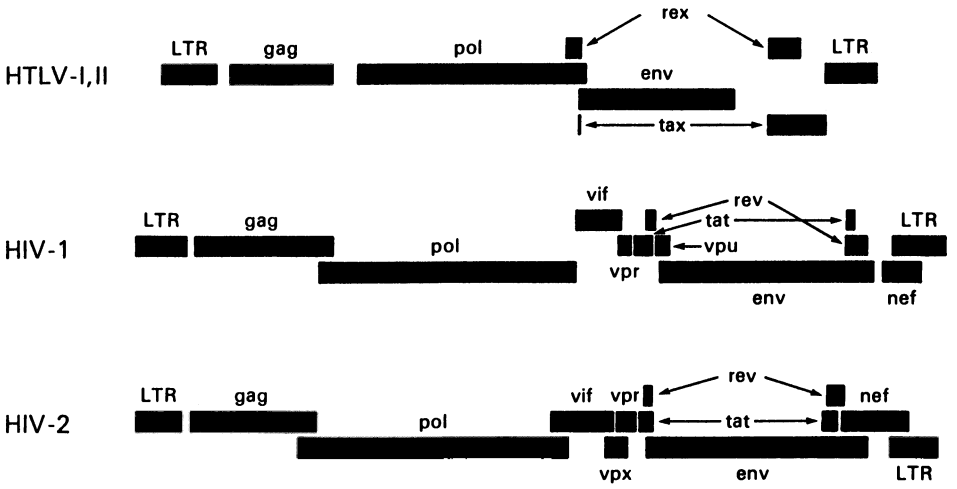


FIG. 1. GENOMIC STRUCTURE OF HUMAN RETROVIRUSES

TABLE 1. ROLE OF THE HIV ACCESSORY GENES FOR VIRUS REPLICATION

	Immuno-Genicity	Size	Cellular Localization	Function	Replication Competence of (-) Mutants
vif	+	p23	Cytoplasm/ inner membrane	Infectivity	±
tat	+	p14	Nucleus/ nucleolus	Transcriptional & post-transcriptional Activation	-
rev	+	p19	Nucleus/ nucleolus	Expression of structural proteins Modulation of transcription	-
nef	++	p27	Cytoplasm	Negative regulator	++
vpr	+	p18	?	?	+
vpu (HIV-1)	+	p15	Cytoplasm/ membrane	Assembly and Release (?)	+
vpx (HIV-2)	+	p15	Cytoplasm	?	+

correspondence of binding and activation. A TAR mutant with a substituted loop sequence was not functional but nonetheless still binds tat. We speculated that the tat-TAR interaction may recognize a stem-loop structure with certain constraints (e.g., size of the loop, some local secondary features), but additional cellular factors may be involved to further confer specificity and activity.

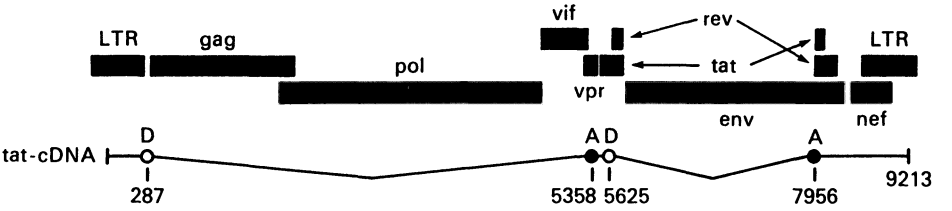
We and others have also generated mutants of the tat protein by site-directed mutagenesis. There appears to be three major functional domains (Figure 2): 1) a basic stretch that is important for nuclear localization of the protein; 2) a cysteine-rich stretch that may be involved in dimerization of the proteins, and an amino terminal region which resembles the acidic domain of transactivator proteins (Figure 3). Rappaport, et al. (in press), found that not only did alteration of any of the three acidic residues in this domain drastically reduced the activity of the protein, but also substitution of a random sequence with the same amphipathic, helical structure conferred significant transactivation activity. This provided direct evidence that this region may constitute a trans-acting domain of tat. None of the tat mutants in this or other regions are trans-dominant. A recent report suggested that mutations in an interior domain (between the cysteine stretch and basic stretch) were trans-dominant and the domain supposedly constitutes the trans-acting acidic domain (Lowenstein and Green, personal communication). We made the same mutations and observed, at most, a 2-3 fold reduction in activity. Therefore, our results are at variance with the other report.

REGULATION BY REV

Similar approaches have been taken for the rev gene. Regulation by rev involves a novel mechanism not documented in others systems. The phenotype of rev defective mutant is the aberrant distribution of viral mRNA in the cytoplasm: only the lower molecular weight, multiply spliced forms are present. Recent studies indicate that rev recognizes a specific sequence (RRE) in the intron of an unspliced viral RNA and directs it for nuclear export instead of the splicing or degradative pathways. Site-directed mutagenesis of rev revealed two areas of importance, one for nuclear localization and binding to RNA, another represents the trans-acting domain since mutations in this domain inhibit wild-type activity (Malim, et al, in press). This result raises some hope that such mutants may provide the basis for antiviral therapy, but reduction of this to practice will take time, and requires improvement of gene therapy approaches for expression and delivery.

INHIBITION OF VIRUS EXPRESSION BY ANTISENSE TO TAT/REV

We also examined the possibility of targeting the tat and rev genes by antisense oligonucleotides. In collaboration with Sam Broder and colleagues, we used a phosphorothioate derived 28'mer which is complementary to a sequence overlapping both tat and rev, and including the initiation codon of rev. Culture of chronically infected H9 cells in the presence of this oligomer completely suppressed expression of virus as measured by p24 immunofluorescence and extracellular reverse transcriptase activity (Matsukura, et al., 1989). A control with the sense-oligomer or oligomers derived from unmodified deoxynucleotide or methylphosphonate derivatives which are expected to form less

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ATG GAG CCA GTA GAT CCT AGA CTA GAG CCC TGG AAG CAT CCA GGA AGT CAG CCT AAA ACT
MET GLU PRO VAL ASP PRO ARG LEU GLU PRO TRP LYS HIS PRO GLY SER GLN PRO LYS THR

GCT TGT ACC AAT TGC TAT TGT AAA AAG TGT TGC TTT CAT TGC CAA GIT TGT TTC ATA ACA
ALA CYS THR ASN CYS TYR CYS LYS LYS CYS CYS PHE HIS CYS GLN VAL CYS PHE ILE THR

AAA GCC TTA GGC ATC TCC TAT GGC AGG AAG AAG CGG AGA CAG CGA CGA AGA CCT CCT CAA
LYS ALA LEU GLY ILE SER TYR GLY ARG LYS LYS ARG ARG GLN ARG ARG ARG PRO PRO GLN

GGC AGT CAG ACT CAT CAA GIT TCT CTA TCA AAG CAA CCC ACC TCC CAA TCC CGA GGG GAC
GLY SER GLN THR HIS GLN VAL SER LEU SER LYS GLN PRO THR SER GLN SER ARG GLY ASP

CCG ACA GGC CCG AAG GAA TAG
PRO THR GLY PRO LYS GLU END
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FIG. 2. tat GENE cDNA ORGANIZATION AND CODING SEQUENCE

stable hybrids had no effect. It is interesting that the treated cells did not display a phenotype that is either tat (-) or rev (-), but rather is consistent with partial reduction in both activities. This is consistent with the general inefficiency of the anti-sense approach. However, expression of the unspliced gag/pol mRNA appears to be acutely sensitive to rev depletion, a result also observed with low rev expressions in previous experiments (R. Sadaie and F. Wong-Staal, unpublished data). Therefore, even though tat and rev are only partially repressed, the final outcome in blocking virus is achieved. These results are again encouraging for using rev as a target for therapy.

HIV-2 INFECTION OF RHESUS MONKEYS AS AN ANIMAL MODEL FOR AIDS

A major problem is studying the pathogenesis of HIV infection, and for evaluating drugs and candidate vaccines is the lack of a suitable animal model. Other than man, HIV-1 is only known to productively infect great apes, and furthermore, without pathology. We wanted to determine if HIV-2, a related human AIDS virus which also infects and induce disease in the lower primate genus Maraca, would be an appropriate model for such considerations. We have generated infectious molecular clones of HIV-2 from patients with AIDS and ARC (Franchini, et al., 1989) and inoculated them into Rhesus monkeys. Two of two monkeys inoculated with one of the cloned viruses ($HIV\text{-}2_{isy}$) seroconverted within one month, and exhibited a significant and continuous depletion of CD4+ cells, similar to that seen with uncloned virus (Franchini, et al., unpublished). Mock infected monkeys had stable CD4+ cell count. Virus was also recovered from the infected monkeys and found to be nearly identical to the parental clone with one or two mutations detectable in the envelope gene. Thus, we have all the elements of a relatively accessible animal model to study pathogenesis, therapy, and vaccine development.

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The Role of CD4 Antigen in HIV Infection

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INTRODUCTION

Ever since the initial description of acquired immune deficiency syndrome (AIDS), it has been known that a specific depletion of $CD4^+$ T-lymphocytes in the peripheral blood accompanies the onset of immunodeficiency (Gottlieb et al 1981). Following the discovery of HIV-1, Klatzmann et al (1984a) showed that the virus had a distinct tropism for and cytopathic effect in $CD4^+$ cells. The evidence that CD4 antigen itself acts as a component of the cell surface receptor came from experiments in which HIV-1 binding, syncytium induction and infection were specifically blocked by pretreating cells with monoclonal antibodies to CD4 (Dalgleish et al 1984; Klatzmann et al 1984b; McDougal et al 1985). The cloning of the CD4 gene and cDNA allowed its expression in heterologous human cells, which then become susceptible to HIV-1 infection (Maddon et al 1986). More recently, other cell surface glycoproteins that are members of the immunoglobulin superfamily have been shown to act as viral receptors, namely ICAM-1 for the major group of rhinoviruses, and a hitherto unknown antigen as the polio receptor (White & Littman 1989).

The identification of CD4 antigen as the receptor for HIV-1, and also for HIV-2 and related simian immunodeficiency viruses (SIV) (Sattentau et al 1988), opened up several avenues of research to exploit this knowledge for potential therapeutic and preventive means (Sattentau and Weiss 1988). It has also raised questions as to whether alternative receptors for HIV exist on different cell types, and whether CD4 antigen is sufficient for virus binding and entry or is one component of a more complex receptor structure. Our laboratory's contribution to this field of AIDS research is briefly reviewed here.

COMPLEXITY AND TOPOGRAPHY OF THE CD4 RECEPTOR

Our observation (Maddon et al 1986) that CD4 gene transfer into human cells such as HeLa allowed HIV infection and syncytium induction to occur indicated that the CD4 antigen alone may serve as the CD4 receptor. Following binding, HIV enters cells in a pH-independent manner (Stein et al 1987; McClure et al 1988). However, we found that the same CD4 gene constructs as well as chimaeric mouse/human CD4 genes encoding murine C-terminal cytoplasmic domains, did not render mouse cells permissive to infection or to membrane fusion, although HIV virus particles efficiently bound to CD4 on the murine cell surface (Maddon et al 1986, 1988). These findings suggested that human cells may additionally express another receptor component,

necessary for virus internalization and for syncytium induction, that is species specific but not cell lineage specific. Attempts to identify a genetic locus for a second receptor component by somatic cell hybridization between human and mouse T-cells, however, have been unrewarding (Tersmette et al 1989). We have also transferred human CD4 to cells of a number of other non-primate species besides the mouse but have not observed HIV infection. On the other hand, natural CD4 antigen expressed on lymphocytes of a variety of old world and new world primates acts as an efficient receptor to initiate HIV infection (McClure et al 1987). The reason why only human and primate cells expressing CD4 are permissive for HIV internalization currently remains unanswered.

The affinity of gp120 to CD4 affects signalling across the membrane and MHC Class II restricted helper or cytotoxic reactions, even with gp120 bound to non-infected cells. CD4/gp120 interactions may also affect later stages of the HIV replication cycle than virion binding and entry. Gp120 expression masks and down-regulates CD4 on the cell surface of HIV infected cells (Dalgleish et al 1984; Hoxie et al 1987; Stevenson et al 1988; Yuille et al 1988). The level of CD4 expression in lymphocytes and monocytes correlates with the cytopathic effect of rapidly-growing strains of HIV in these cells (Åsjö et al 1987; Clapham et al 1987). This indicates that the complexing of newly synthesized gp120 and CD4 may play a role in cell damage, both in the internal membranes of infected cells and in cell-cell fusion between infected virus-producing cells and $CD4^+$ 'bystander' cells.

Mapping of the epitope on CD4 recognized by the envelope glycoprotein, gp120, of HIV, has been aided by the use of anti-CD4 monoclonal antibodies and site-specific mutagenesis of the CD4 gene (Sattentau et al 1986; Landau et al 1988; Peterson and Seed 1988). We have found that single amino-acid changes, for example at residues 43 and 55 in the N-terminal V1 domain, substituting 'murine' residues in the human CD4 sequences, destroy the binding capacity for HIV, as well as for Leu3a antibody (Arthos et al 1989). Although antibodies that recognize sites principally on the V2 domain (e.g. MT151) can also compete with HIV for binding (Sattentau et al 1986), soluble recombinant V1 of CD4 is sufficient to bind to gp120, and to neutralize HIV (Arthos et al 1989).

EXPLOITING HIV/CD4 INTERACTIONS FOR THERAPEUTIC AND VACCINE STUDIES

Strategies for blocking HIV binding to the CD4 receptor, these may help to prevent infection de novo, or to arrest the insidious spread of HIV in the infected individual. Figure 1 illustrates schematically some of the approaches that are being explored to take advantage of our knowledge of HIV/CD4 interactions.

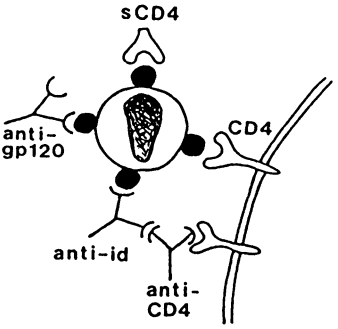

Fig. 1. Inhibition of HIV-receptor interaction. Attachment of HIV to CD4 receptors on the cell surface can be blocked by (a) soluble CD4 molecules (sCD4), (b) anti-CD4 antibodies occupying the receptor sited (c) anti-idiotypic antibodies to anti-CD4 and (d) anti-gp120 blocking the epitope recognizing CD4.

Several pharmaceutical research groups have prepared soluble, recombinant forms of CD4 (sCD4) representing the whole of the extracellular portion of the antigen, or just the N-terminal domains containing the HIV gp120 binding site. These molecules act as potent neutralizing agents for the infectivity in vitro of all strains of HIV-1, HIV-2 and SIV isolates tested, although it is less potent for inhibiting HIV-2 (Clapham et al 1989). Chimaeric constructs of sCD4 fused with the Fc fragment of immunoglobulin, or albumin domains, increase the half-life of the protein in plasma (Capon et al 1989; Traunecker et al 1989). Hybrid proteins of sCD4-toxin selectively kill cells expressing gp120 antigen (Chaudhary et al 1988).

Soluble CD4 is not obviously toxic in monkeys (Watanabe et al 1989), and fears that sCD4 itself might be immunosuppressive have not materialised thus far. It does not block CD4/MHC Class II dependent T-cell activation in vitro. Indeed, sCD4 abrogates the blocking effect of gp120 and may thus be of therapeutic value not only in neutralizing HIV infectivity but also in chelating any free gp120 that might otherwise contribute to the immunosuppressive state. Phase I/II clinical trials of sCD4 have begun and it will be of great interest to determine whether these soluble receptor molecules have therapeutic value.

CD4/gp120 interactions are also being explored with a view to developing vaccines. The gp120 antigen of HIV-1 itself has an immunodominant epitope for eliciting neutralizing antibodies (D. Bolognesi, this Symposium). The antibodies do not interfere with binding to CD4 antigen but prevent subsequent membrane fusion and infection. This epitope is variable between different strains of HIV-1, so that the antibodies reacting with it exhibit strain-specific neutralization. By contrast, the relatively well conserved CD4 binding site may provide a basis for vaccine development as a suitable target for eliciting broadly cross-protective antibodies.

The CD4 putative binding site on gp120 has been delineated (Lasky et al 1987). We have found that the putative CD4 binding site as a synthetic linear peptide (acc 409-424 of gp120) neither binds to CD4 nor abrogates the ability of sCD4 to block HIV infection, suggesting a complex, discontinuous conformation of the receptor-binding domain. The receptor binding site appears to be "immunosilent" in intact gp120, but three approaches to elicit neutralizing antibodies to it

are under investigation with a view to eventual vaccine development. The first is to determine whether internal image anti-idiotypic antibodies to anti-CD4 might neutralize HIV if they are raised against anti-CD4 monoclonal antibodies that bind to the same epitope as gp120. To date Leu3a antibody has been tested as an immunogen to generate anti-idiotypes (Chanh et al 1987; Dalgleish et al 1987). Leu3a elicits weakly neutralizing anti-idiotypic sera in mice that cross-neutralize many HIV strains. However, the failure thus far to isolate monoclonal anti-idiotypic antibodies with neutralizing properties (Beverley et al 1989) raises the question whether the neutralizing activity in the polyclonal sera might result from anti-anti-idiotypes blocking CD4, rather than anti-idiotypes binding to gp120. Further work on anti-idiotypes to anti-receptor antibodies is required using different monoclonals recognizing overlapping epitopes.

The second approach to the CD4 binding site on gp120 has been to screen panels of monoclonal antibodies specific to native gp120 for their recognition of peptides covering the predicted linear CD4 binding site. Monoclonal antibodies specific for the CD4-binding site peptides exhibit weak neutralizing activity to several HIV-1 strains (Sun et al 1989). If the epitope on the natural gp120 lies within a crevice or canyon, the relatively weak neutralizing activity of these monoclonals, as well as of the anti-idiotypic sera, may result from a lack of access of the immunoglobulin molecules or their antigen combining sites for this antigenic epitope. However, we have found that rabbit antisera to the same peptide have equivalent affinities both to peptide and to native gp120, and in the presence or absence of detergent. Thus this domain appears to be accessible on the native molecule.

The third approach which we have recently taken is to exploit polio virus as a live, recombinant vector and potential vaccine for eliciting anti-HIV humoral immune responses. HIV sequences can be substituted in the VP1 coat protein of Sabin type 1 polio vaccine strain in a region that is prominent on the surface of the polio virion. We successfully introduced a neutralization epitope of HIV gp41 and recovered viable polio/HIV chimaeric particles that elicited broadly cross-reactive neutralizing antibodies (Evans et al 1989). In our current experiments, the CD4 recognition epitope of HIV-1 gp120 has been expressed on the surface of polio virions, and we have obtained sera that cross-neutralize most HIV-1 strains and some strains of HIV-2 (J.A. McKeating et al submitted). Since live polio vaccines give rise to mucosal immunity, this approach may one day be useful in protecting against initial infection by HIV during sexual transmission.

ALTERNATIVE RECEPTORS TO CD4

It is now known that HIV can infect a much broader spectrum of cell types in vitro and in vivo than T-helper lymphocytes. Other cells of the immune system such as monocytes, macrophages and dendritic cells may act as reservoirs of HIV in chronic infection and possibly as front-line targets in early stages of infection. There is also evidence of HIV infection of non-lymphoid cells in the brain, gut and other tissues.

We addressed the question whether CD4 serves as a receptor for HIV infection of non-lymphoid cells by infecting clonal tumour cell lines in vitro. Whereas HIV infection of T-lymphocytic and monocytic leukaemic cell lines could be efficiently blocked by anti-CD4 or soluble CD4, we found that the relatively low susceptibility to infection of brain glioma cells and rhabdomyosarcoma cells was not further reduced by anti-CD4 or sCD4 treatment (Clapham et al 1989). Thus infection of these cell types appears to be CD4-independent. The nature of the alternative receptors, if specific receptors exist, remains unknown.

Certain antibodies to HIV envelope antigens can enhance the infectious titre of HIV for cells bearing Fc or complement receptors. As discussed by H. Wigzell (this Symposium) the enhancement is not as marked as seen with flaviviruses, and it is not known whether, or to what extent, enhancing antibodies may increase the efficiency of HIV infection in vivo, or exacerbate the onset of disease. There has been some doubt whether antibody enhancement of HIV infection involves CD4, though a recent report by Homsy et al (1989) indicates that CD4-independent infection can take place.

We have sought to investigate the role of Fc receptors in HIV infection by inducing FcR in human embryonic fibroblasts that do not express CD4 antigen. FcR expression can be induced in these cells by infection with cytomegalovirus (CMV). We found that HIV-1-antibody complexes, but not native HIV-1, could selectively superinfect the CMV-infected fibroblasts. Infection of these cells was not inhibited by soluble CD4, but was completely abrogated by 5% human serum containing sufficient immunoglobulin to block the CMV-induced Fc receptors (J.A. McKeating, submitted). Thus CD4-negative cells can be infected by opsonized HIV via FcR. Since CMV and HIV exhibit trans-activating effects on viral replication, the expansion of cell tropism in seropositive individuals to CMV-infected cells may have clinical significance.

In conclusion, it is clear that CD4/gp120 interactions play an important role in HIV infection and pathogenesis, and provide means of developing potential therapeutic agents and vaccines. There are, however, alternative ways by which HIV can gain entry into target cells in vitro which may also prove to be important for in vivo infection.

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Immunopathogenic Mechanisms of HIV Infection

A.S. Fauci and Z.F. Rosenberg

INTRODUCTION

The outcome of infection of CD4-bearing target cells with HIV is dependent on complex interactions between viral and cellular factors (Haseltine 1988). In addition to genes which code for the structural proteins of the virion and the enzymes which facilitate the transcription of viral RNA into DNA and the integration of the viral DNA into the host cell genome (Varmus 1988), the HIV genome contains at least six genes which act in distinct ways to regulate HIV replication. For several of these regulatory genes, the mechanism of action involves the long terminal repeat (LTR) sequences of the HIV genome. For example, the target for tat-mediated enhancement of viral replication is the trans-acting responsive (TAR) region of the LTR (Rosen et al 1985). The nef gene product has also been shown to regulate viral transcription by interacting with the negative regulatory element (NRE) of the LTR (Ahmad and Venkatesan 1988). In addition to TAR and NRE, the HIV LTR contains other regulatory sequences such as the TATAA promotor sequence, the NFkB core enhancer elements, and Sp-1 binding sites (Starcich et al 1985).

Depending on the state of activation of specific viral genes and of the target cell itself, infection with HIV may result in an acute cytopathic effect, restricted chronic virus replication, or a complete absence of virus expression (Fauci 1988). Since the activation of latent or chronic virus expression to a full-blown cytopathic infection in vitro may mimic events that occur during the progression of HIV disease in vivo, we have pursued a research strategy designed to delineate the mechanisms of HIV activation in vitro.

ACTIVATION OF HIV EXPRESSION

It has been demonstrated that cellular activation signals such as phytohemagglutinin (PHA) and interleukin (IL)-2 are required for efficient infection of T4 cells by cocultivation with peripheral blood cells from HIV-infected individuals (Gallo et al 1984; Levy et al 1984; Barre-Sinoussi et al 1985). In addition, cell-free HIV infection of normal human lymphocytes can be enhanced by mitogens (McDougal et al 1985; Folks et al 1986b). Mitogens have also been shown to activate HIV from long-term, latently infected T cell cultures from AIDS patients and to augment HIV expression in chronically infected T cell lines (Zagury et al 1986; Folks et al 1986a; Harada et al 1986).

Several investigators have found that the mechanism of mitogen-induced activation of HIV expression involves the induction of cellular proteins that bind to the core transcriptional enhancer sequences in the HIV LTR (Nabel and Baltimore, 1987; Kaufman et al 1987; Tong-Starksen et al 1987; Siekevitz et al 1987; Dinter et al 1987). The binding of one such protein, the NFkB T cell activation factor, to the NFkB region on the LTR, has been shown to activate in vitro transcription of the HIV promotor (Kawakami et al 1988).

Another, more physiologically relevant, set of factors that can activate T cells are antigens. We have shown that tetanus toxoid or keyhole limpet hemocyanin stimulation of peripheral blood mononuclear cells from HIV-negative individuals prior to HIV infection results in 10 to 100-fold higher levels of virus replication as compared with unstimulated cells (Margolick et al 1987). Similarly, others have found that stimulation of peripheral blood leukocytes with non-infectious HTLV-1 either before, during, or after HIV infection resulted in enhanced HIV replication (Zack et al 1988).

Agents which are known to induce stress responses in cells have also been implicated in the induction of HIV expression. It has recently been shown that either ultraviolet (UV) light, mitomycin C, or sunlight can activate the HIV promotor (Valerie et al 1988). In addition, upregulation of HIV expression in infected T cell lines occurred following pretreatment of the cells with UV light. We have obtained similar results in latently HIV-infected promonocytic cells exposed to UV light (Stanley et al in press).

We have recently found that exposing chronically infected cells to elevated temperatures resulted in induction of virus expression (S. Stanley and A.S. Fauci, unpublished data). Other investigators have found that heat-shock can induce expression of the chloramphenicol acetyl transferase (CAT) gene when it is under the control of the HIV promotor (Geelen et al 1988). It has been postulated that the mechanism of heat-shock induction of HIV expression involves the NFkB binding sites in the HIV LTR since a portion of these sites resemble the cellular heat-shock core sequences (Geelen et al 1988).

Activation of HIV by Heterologous Viruses

HIV-infected individuals are often coinfected with a variety of viral pathogens, some of which are known to infect the same target cells as HIV (Nahmias et al 1964; Nelson et al 1988). Therefore, it was of interest to explore whether coinfection with heterologous viruses could enhance HIV expression. A number of investigators have found that in vitro cotransfection of the immediate early genes of several herpesviruses, herpes simplex virus type 1 (HSV-1), Epstein-Barr virus, and cytomegalovirus, with the HIV LTR can result in the upregulation of expression of CAT in an HIV LTR-CAT construct (Gendelman et al 1986; Rando et al 1987; Davis et al 1987; Kenney et al 1988). Similar results have been obtained under the following conditions: cells containing HIV-LTR-CAT constructs were infected with intact HSV-1 (Mosca et al 1987a); cells were cotransfected with an infectious clone of HIV and the immediate early genes of HSV-1 (Ostrove et al 1987); and HIV infected cells were superinfected with HSV-1 (Albrecht et al 1989). More recently, the genes of hepatitis B and human herpesvirus 6 have been shown to transactivate the HIV promotor (Seto et al 1988; Twu and Robinson, 1989; Lusso et al 1989; Horvat et al 1989).

As was observed during mitogen activation of HIV expression, the mechanism of action of HIV induction by heterologous viral genes has been shown to involve the production of DNA-binding proteins that bind to the HIV LTR. However, unlike the situation with mitogen activation, investigators have found that the DNA-binding proteins interact with several regions on the HIV LTR, depending on the heterologous virus being tested. These sites may include the NFkB region, the Sp1 transcription factor binding sites, the TATAA sequences, or a combination of regions (Mosca et al 1987b; Ostrove et al 1987; Gimble et al 1988; Nabel et al 1988; Seto et al 1988)

Cytokine Induction of HIV Expression

As physiologic inductive signals, cytokines are similar to antigens, mitogens, and heterologous viruses in that they activate cells and induce the production of cellular factors that may be important in HIV expression. To further delineate the mechanisms of induction of HIV expression in latently or chronically infected cells, we investigated the role that cytokines may play in HIV expression. In this regard, we created an experimental system to study the regulation of persistent HIV infection in chronically HIV-infected cloned T and promonocytic cell lines (Folks et al 1985; Folks et al 1988). ACH-2, a T cell clone, was derived from A3.01 cells that were chronically infected with HIV. U-1, a promonocytic clone, was derived from chronically infected U937 cells. Both ACH-2 and U1 were exposed to either cytokine-containing cellular supernatants or purified, recombinant cytokines. Activation of HIV expression was evaluated by measuring reverse transcriptase (RT) activity in the cell supernatant.

In early experiments with the U1 cells, we demonstrated that cytokine-rich, PHA-induced mononuclear cell supernatants significantly enhanced HIV expression (Folks et al 1987). Exposure of U1 cells to recombinant granulocyte-macrophage colony stimulating factor (GMCSF) also resulted in an increase in RT activity. Similarly, we have shown that HIV expression can be increased following exposure of ACH-2 cells to lipopolysaccharide (LPS)-induced monocyte supernatants (Clouse et al 1989). In addition to increased RT activity, exposure of these cells to cytokines resulted in the induction of new viral protein as well as the concomitant expression of new HIV messenger RNA (Clouse et al 1989; Duh et al in press). These data suggest that the elevation in RT activity following cytokine exposure is due to an increase in new virus production and does not solely result from the release of preformed virions from the cells. It has also been demonstrated that acute infection of primary peripheral blood monocytes can be enhanced following exposure of the cells to GMCSF (Koyanagi et al 1988).

Since virus can induce the production of monokines (Aderka et al 1986), it was of interest to determine whether one of the mechanisms of heterologous virus activation of HIV expression may involve monokine production. To test this hypothesis, we exposed highly purified peripheral blood monocyte/macrophages to a panel of inactivated viral antigens from CMV, HSV-1, HSV-2, EBV, HHV-6, varicella zoster virus, HBV, vaccinia virus, and HIV. The monokine-enriched supernatants from the viral antigen exposed monocytes were then added to the culture media of U1 and ACH-2 cells. In these experiments, CMV, EBV, and HIV proteins were able to induce monocyte/macrophages to secrete factors capable of upregulating the chronically infected cell lines (Clouse et al 1989b) (Fig. 1).

Of particular note is the fact that most of the HIV-inducing activity in LPS-induced monocyte supernatants could be removed by passing the supernatants through an anti-tumor necrosis factor (TNF) immunoaffinity column (Clouse et al 1989a). Using a panel of recombinant cytokines, we have subsequently demonstrated that TNF-alpha was able to induce expression in ACH-2 cells under conditions in which IL-1, IL-2, IL-3, IL-4, IL-6, GMCSF, interferon-gamma, and transforming growth factor-beta all failed to enhance HIV expression (Folks et al 1989). It has recently been reported that TNF can enhance HIV expression and cytopathicity during de novo infection of some T4 cell lines (Ito et al 1989). In the U1 model system, TNF-alpha and IL-6, in addition to GMCSF, were able to induce HIV expression. Of particular interest was

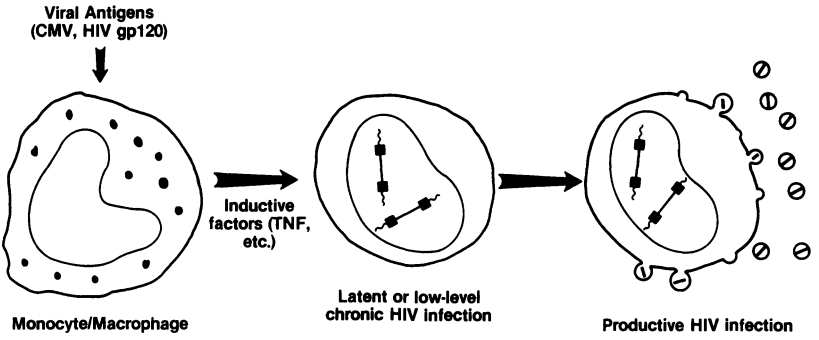

Fig. 1. Induction of HIV expression by supernatants of monocytes exposed to various viral antigens.

the fact that TNF-alpha synergized with IL-6 and GMCSF in the induction of HIV expression (G. Poli and A.S. Fauci, unpublished data).

We investigated the molecular mechanisms whereby the cytokine-induced upregulation of HIV expression occurred. By analogy to the mechanisms of induction of HIV by mitogens and heterologous viruses, we sought to determine if cytokine induction of HIV involved a transactivating mechanism whereby DNA binding proteins bound to specific sites on the LTR of HIV. Using transient transfection experiments with LTR-CAT constructs in A3.01 cells, we were able to demonstrate that TNF-alpha clearly upregulated CAT activity in the transfected cells, indicating that the mechanism of action involved the LTR of HIV (Folks et al 1989).
To more precisely define which sequences of the HIV LTR were involved in TNF-alpha induction of HIV, gel mobility shift assays were performed. Using oligonucleotide probes spanning either the NFkB binding sites or the Spl binding sites, we were able to demonstrate that TNF-alpha induced in A3.01 cells DNA binding factors which bound specifically to the NFkB region of the HIV LTR and not to the Spl region. When oligonucleotide probes were used in which deletions at specific sites of the NFkB region were performed, binding did not occur. In addition, when the NFkB portion of the LTR was deleted from the LTR-CAT constructs, TNF-alpha was unable to enhance HIV expression (Duh et al in press). Similar results have recently been reported by Osborn et al. (1989).

Using slot blot analyses and nuclear run-on assays, we determined that treatment of ACH-2 with TNF-alpha resulted in a 4 to 5 fold increase in HIV RNA levels and a 3-fold increase in HIV transcriptional activity as compared with untreated ACH-2 (Duh et al in press). Taken together these studies demonstrate that TNF-alpha was able to induce a cellular gene to produce a DNA binding factor which bound specifically to the NFkB region of the HIV LTR and induced new transcription of viral mRNA and ultimately new virion formation (Fig. 2). It is of interest to note that TNF-alpha has recently been shown to regulate IL-2 receptor-alpha gene expression through the induction of proteins that specifically bind to NFkB-like regions of the IL-2 receptor-alpha gene (Lowenthal et al 1989a; Lowenthal et al 1989b).

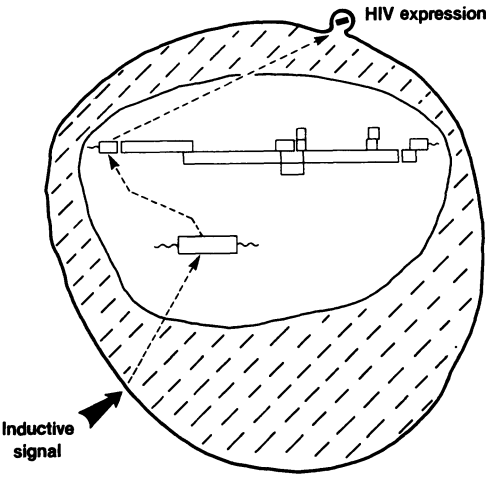

Fig. 2. Effects of inductive signals on HIV expression. Cytokine induction of HIV expression occurs as a result of the induction of specific DNA-binding proteins that bind to the promotor region of HIV. (Reprinted with permission from Fauci AS (1988) Immunopathogenic mechanisms of HIV infection. In: Aiuti F, Bonomo L, Danieli G (eds) Topics in Immunology. Proceedings of the Ninth European Immunology Meeting. Il Pensiero Scientifico Editore, Roma, Italy, p 217.

We have also demonstrated that phorbol myristate acetate (PMA) was able to induce the secretion of TNF-alpha in both ACH-2 and U1 cells as well as the expression of membrane-bound TNF-alpha on the surface of ACH-2 cells. Furthermore, there was a synergy between PMA and exogenous TNF-alpha in the induction of endogenous TNF-alpha secretion from U1 cells. In this regard, we have found that anti-TNF-alpha antibody not only suppressed TNF induction of HIV in U1 cells but also suppressed, at least in part, the PMA induction of HIV in U1 cells. In addition, anti-TNF-alpha antibody blocked the cytopathic effects of TNF-alpha as well as PMA at the same time that it blocked the induction of expression of HIV in U1 cells. Finally, transforming growth factor (TGF)-beta blocked the induction of HIV expression in U-1 cells by PMA, but did not block the induction of HIV expression in U-1 cells by TNF-alpha, suggesting at least a partial dichotomy of mechanisms of induction of HIV by PMA versus TNF-alpha (G. Poli and A.S. Fauci, unpublished data).

It had previously been reported that PMA induces a downregulation of TNF receptors on a variety of cell lines (Aggarwal and Eessalu, 1987). In support of these findings, we found that PMA stimulation of uninfected T cells (A3.01 and 12D7) and promonocytes (U937) resulted in either a decrease or no change in the level of TNF receptors. In contrast, we noted that PMA stimulation of chronically infected ACH-2 and U1 cells resulted in an upregulation of TNF receptors (G. Poli and A.S. Fauci, unpublished data). Taken together, these data indicate that TNF can function in an autocrine and paracrine manner in the induction of HIV expression in chronically infected cell lines.

The identification of TNF-alpha as an inducer of HIV expression in

vitro has potentially important physiological relevance since it has been clearly demonstrated that TNF-alpha is present in elevated concentrations in the plasma of HIV-infected individuals, particularly in the advanced stages of their disease (Lahdevirta et al 1988). In vitro, concentrations of TNF-alpha as low as 50 pg/ml have veen shown to induce HIV expression in ACH-2 cells (Folks et al 1989) as compared with TNF-alpha levels as high as 150 pg/ml that have been measured in AIDS patients (Lahdevirta et al 1988). In addition, it has recently been shown that peripheral blood monocytes from symptomatic HIV-infected individuals produce high levels of TNF-alpha as compared to controls (Roux-Lombard et al 1989).

Since TNF-alpha is produced in humans in reaction to naturally occurring infections (Cerami and Beutler, 1988), the presence of opportunistic infections that afflict individuals with AIDS may result in high levels of TNF-alpha. TNF-alpha could subsequently accelerate the replication of HIV and the course of disease in symptomatic individuals in an autocrine/paracrine manner. In this regard, the progression of neurological manifestations of HIV infection may be the result of elevated TNF-alpha levels that have recently been found in the cerebrospinal fluid of patients with bacterial meningitis (Leist et al 1988). The fact that TNF-alpha is an important component of the regulation of normal immune responses (Kehrl et al 1987) suggests that TNF-alpha may also play a role in the gradual progression of HIV infection prior to full-blown AIDS.

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Immunobiology of the HIV Envelope and Its Relationship to Vaccine Strategies

D.P. Bolognesi

INTRODUCTION

One of the many bottlenecks standing before a rational approach to vaccine design against HIV is the lack of epidemiological evidence that immune responses to the virus are able to influence the course of infection or the disease process.

This raises the important issue of whether an immunological clearance mechanism exists against this virus; or more to the point whether it is possible to induce one through vaccination or post-exposure prophylaxis. Studies of the natural immune response to HIV do indeed reveal anti-viral immune responses which would be expected to be able to suppress virus replication; but at the same time, one can also document immunological responses which are immunosuppressive or even potentiate virus infection (for review see 1). One could speculate on this basis that the situation one faces during natural infection is one of counteractive forces which initially may favor control of virus (i.e. during the protracted asymptomatic phase) but eventually the balance is shifted in favor of virus replication with concomitant adverse consequences to the host. On this basis vaccine strategies for HIV should ensure that the elements which give rise to protective responses are present, while those eliciting undesirable effects be excluded. To achieve this goal, it is necessary to understand the features of the virus which are responsible for each category. By way of example, the properties of one of the most intensely studied gene products of the virus will be reviewed in this context, namely its envelope glycoproteins.

Functions of the HIV envelope during infection

The aspects of viral infection which are the most pertinent to vaccine strategies are represented by the initial interaction of the virus with its target cells. When studied in T lymphocytes these involve the processes of 1) binding to the CD4 receptor 2) anchorage of the viral membrane to that of the target cell 3) fusion of the respective membranes and 4) entry of the viral contents within the cell.

Of these, the binding of the virus to its receptor is the best studied. It occurs between the exterior envelope glycoprotein (gp120) and a portion of the CD4 molecule. The regions of the ligand (2) and the receptor (3) involved in

binding have been identified. Monoclonal antibodies to the receptor are quite effective in blocking virus infection (4), as are soluble forms of the receptor (5) or gp120 itself (6).

An essential process for virus infectivity and pathogenicity is the phenomenon of fusion. With HIV, this occurs subsequent to virus binding to CD4 through a complex process involving both gp120 and the transmembrane envelope glycoprotein, gp41, (7). The fusogenic domain is thought to reside within the N-terminal portion of gp41 (8) but other sites on both gp120 and gp41 are likely to take part in the overall process (see below). Fusion can also occur between virus infected cells exhibiting gp120 and gp41 on their surface and uninfected cells bearing CD4. This results in multinucleated giant cell formation and represents a form of virus cytopathicity. This process also allows HIV to pass directly from cell to cell. There is no clear evidence for a cellular receptor which is specifically required for fusion to occur subsequent to the binding phase (gp120 to CD4). However, recent evidence does point to other cell surface molecules which may be involved in the process, notably LFA 1 (9).

Regions of the envelope which elicit antibodies that neutralize virus infectivity

Based on experience with other viruses, it is not surprising that the HIV envelope represents a major target for antibodies that can interfere with infection. A number of studies have been done to define the epitopes which generate such antibodies.

Reports from several laboratories (for review see 1) identify the portion of gp120 lying between residues 307 and 330 as dominant for development of high-titered, type-specific neutralizing antibodies against HIV-1. It is situated within the third hypervariable region (V3) of the virus envelope and thought to exist as a loop formed by two disulfide-linked cysteine residues. The two cysteines spanning this region are themselves highly conserved. Thus, although the amino acid sequence within this loop is variable, the V3 loop itself is present in gp120 from most if not all HIV-1 isolates.

Numerous attempts have also been made to raise neutralizing antibodies against conserved regions of gp120. To date such studies have not uncovered a dominant neutralizing epitope suggesting that if such sites exist, they are somehow inaccessible to antibodies due to extensive glycosylation or to conformation. A logical target for neutralizing antibodies to a conserved domain would be the binding site of gp120 to CD4. Antibodies directed to synthetic peptides within the putative binding domain to this region are quite effective in preventing binding of gp120 to CD4 (2). However, in contrast to antibodies to the V3 domain, these are relatively ineffective in blocking virus infection perhaps because they are unable to overcome the cooperative affinity of multiple gp120/CD4 binding interactions which occur when the virus meets the cell. Yet, one does find antibodies in HIV infected humans or chimpanzees that exhibit

high titered antibodies which block gp120 and HIV binding to CD4 and also exhibit broad virus neutralizing capacity (10). The identity of the site(s) responsible for such reactivities, if they are indeed one and the same, are clearly of major interest to establish.

Virus infection can also be prevented by interfering with the process of fusion. One class of antibodies that is very effective in blocking fusion represents those directed at the hypervariable neutralizing site on gp120. Antibodies to this region interfere with a post-binding step in virus infection which is probably linked to the fusion process (10). A plausible mechanism for this might be that the binding of gp120 to CD4 serves as a trigger for releasing the fusogenic domain within gp41 which anchors the respective membranes. The hypervariable loop may itself be associated with a critical contact region of gp120 to gp41 and antibodies to the loop may prevent the process of dissociation. Parenthetically such antibodies need not have to compete with the high affinity binding that occurs between gp120 and CD4 in order to block virus infectivity and cell fusion.

A more logical candidate for anti-fusion antibodies would be the transmembrane component of the envelope, gp41. Recent studies point to a conserved site on gp41 (amino acids 735-752) as a region which can induce antibodies which neutralize HIV and block cell/cell fusion in a broad fashion (11). Curiously, most models of how gp41 is situated in the virus outer membrane indicate that this region is probably internal. Yet monoclonals to this epitope actually stain the surface of virus infected cells (11); a paradox that remains unexplained. Finally, recent studies by Jeffrey Almond and colleagues (12) demonstrate that when this region is inserted as a surface component of poliovirus, it is not only able to induce similar antibody reactivities but is also capable of removing a good portion of the group common neutralizing and fusion inhibiting antibodies from some human sera. At the present time, this region would appear to represent one of the dominant targets for group common reactivity.

Epitopes Associated with Cellular Immunity

To date, various regions of gp120, are known to be recognized by T cells. Some of the best studied are in (a) a conserved region toward the N'terminus of gp120 (13), (b) within the hypervariable neutralization loop (14), and (c) within the conserved binding region of gp120 to CD4 (13) as well as an adjacent region towards the N'terminus (15). There are also T-cell epitopes on gp41 (16,17,18). Many more epitopes are certain to be discovered and particular emphasis is being placed on those that are targets for cytotoxic lymphocytes.

One of the epitopes that serves as a target for CTL is present within the hypervariable neutralization loop in gp120 (14). Variable T-cell epitopes may be part of the strategy of HIV to escape immune destruction, much like the targets for neutralizing antibodies. This could occur through mutations that

reflect recognition by the T-cell receptor; but also at sites which are critical for association with class I or class II MHC. Without the latter, a virus would become invisible to the immune system.

A distinct but related issue relates to regions that are targets for antibodies that mediate cellular cytotoxicity (ADCC). Although, fine mapping studies have not been done, it appears that conserved regions of the envelope, situated mainly on gp120, may be primary (19). More recently, evidence that variable regions are also targets has been obtained (D. Tyler, in preparation). Finally, it is now evident through use of human monoclonal antibodies directed toward gp41, that various sites within the transmembrane glycoprotein are also targets for ADCC (20, D. Tyler, in preparation).

Epitopes Which Mimic Products of Normal Cellular Genes

Molecular mimicry is increasingly being recognized as an important process in pathogenesis and immune suppression accompanying virus infections (21). Viruses bearing structures analogous to those present on the surface of normal cells can present such regions to the immune system in a manner that they are recognized as foreign antigens and thereby elicit immune responses which attack normal cells. Alternatively these regions may represent growth factor-like elements which could influence a variety of normal cellular functions. HIV displays examples of each of these as well as other mechanisms by which it can cause the destruction or impairment of normal cells.

The first report of molecular mimicry in HIV was a homology to IL-2 in the C'terminus of gp41 (22) (Table 1). Subsequently, homology to HLA-DR, both within gp41 (23) and gp120 (24), has been documented. Anti-DR activities are present in HIV infected individuals which can impair normal immune function (23). Finally, homology to neuroleukin, a nerve-cell growth factor has been reported to involve a region of gp120 coinciding with a putative neutralizing epitope (25). However, the homology to neuroleukin could not be substantiated and the nature of the related normal cellular sequence appears to be more related to the gene coding for 6-glucophosphoisomerase.

Immunosuppressive Effects of the HIV Envelope

Based primarily on in vitro studies, HIV has evolved some other unique ways to impair the immune system. By releasing its exterior envelope glycoprotein, a molecule is generated which actively binds to CD4 bearing normal lymphocytes. This event now targets these cells for immune attack by both antibodies with mediate ADCC (19) and cytotoxic T cells which recognize processed forms of gp120 after its internalization by CD4 positive lymphocytes (15). Both events can result in destruction of

normal CD4 positive T-cells without the necessity for HIV infection (non-infectious lympholysis). To what extent this occurs in vivo depends on the level of gp120 synthesis, secretion and shedding. To date, free gp120 has not been measured in the circulation but this is not surprising given its powerful affinity for CD4. Adding another twist, it has been suggested that gp120/CD4 complexes might give rise to new epitopes on CD4 which elicit anti-CD4 antibodies in a fraction of HIV infected patients (26).

The gp120 molecule can also suppress immune function by virtue of its ability to bind to CD4 (27). Recent studies demonstrate that this might occur as a result of its ability to occlude the binding of CD4 to class II MHC, and event which is necessary in antigen recognition (28). Indeed the evidence suggests that the binding site of CD4 to gp120 is contained within the binding domain of CD4 to MHC II (28).

Finally, immunosuppressive sequences may exist in the envelope itself. Regions homologous to those present in the transmembrane glycoprotein of animal and human type C retroviruses known to be highly suppressive for macrophage function are also present in HIV gp41 (29). In addition it has been reported that disappearance of antibodies to this region correlated with disease progression (30).

Discussion

This discussion serves to emphasize the importance of defining functional and immunogenic regions of the HIV envelope. A summary of those studied and documented to date is presented in Table 1. More are certain to be added, particularly as information becomes available on the process of infection, as well as other phases of the infectious cycle.

However, it is also possible that HIV may be able to infect cells by other mechanisms. These include antibody mediated pathways of virus entry which may apply to target cells with Fc receptors (31,32) or receptors for complement (33). To date these mechanisms have only been shown to be active in vitro using special conditions; and even so the enhancement of infectivity has not been overwhelming. Nevertheless, such mechanisms of virus entry are worth noting particularly if they result in infection of secondary target cells and knowledge of responsible epitopes would be of value.

Based on the information which has been reviewed above it should be possible to construct immunogens containing the different categories of epitopes that induce protective immunity against the virus. One can also exclude the regions of the virus which elicit undesirable responses. Thus, domains representing targets for neutralizing antibodies, ADCC and cytotoxic lymphocytes should be ranked in the order of potency. Similarly, regions responsible for molecular mimicry with host components, immunosuppressive sequences, and targets for enhancing antibodies need to be identified for exclusion. With such information in

HIV Envelope Domains

gp120

gp41

Selected Functional Domains

gp120		gp41	
105 -117	gp41 complementarity (?)	518 - 527	fusogenic sequence
269	post-binding, viral entry	579 - 601	gp120 complementarity (?)
303 -337	post-binding, fusion	691 - 712	membrane span
420 - 463	CD4-binding	840 - 862	viral infectivity, cytopathicity

Virus Neutralization Epitopes

254 - 274		582	neutralization resistant mutation
303 - 337	variable	584 - 609	
458 - 484		616 - 633	
491 - 523*		735 - 752	conserved

*Antibodies to this region are also thought to enhance infectivity in cells bearing receptors for complement

T-Cell Epitopes

112 - 124		584 - 609	
315 - 329	CTL variable		
410 - 429	CD4+ CTL		
428 - 443			

ADCC Targets

315 - 329	variable
474 - 518	conserved
651 - 670	(not precisely mapped)

Regions of Homology with Known Molecular Structures

254 - 274	6-glucophosphoisomerase, HLA-DR	518 - 527	fusogenic domain
		583 - 599	immunosuppressive sequence
		831 - 837	HLA-DR
		840 - 862	IL-2

Immunodominant Epitopes

504 - 518		579 - 601	

Table 1

Selected sites of the HIV envelope grouped into various categories.

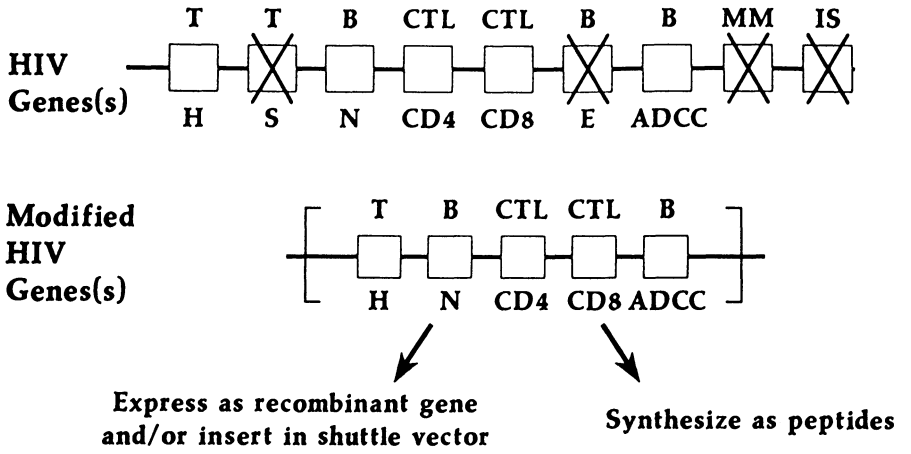

Figure 1

Selection of epitopes which induce protective responses and exclusion of undesired regions. As domains of HIV gene products that induce defined immune responses become identified, some rational selection can be made to devise an improved immunogen through recombinant DNA technology or by direct chemical synthesis. Epitopes that are conformational in nature or require post-translational modifications would probably require recombinant approaches in mammalian expression systems (some B-cell epitopes).

T/H - T-cell epitopes required for help.
T/$ - T-cell epitopes which give rise to suppressor cells.
B/N - B-cell epitopes responsible for neutralizing Ab.
B/ADCC - B-cell epitopes for ADCC.
B/E - B-cell epitopes which elicit enhancing Ab.
CTL/CD4 - Epitopes for CD4+ CTL (class II MHC dependent).
MM - Regions which mimic normal cell surface antigens.
IS - Regions which are directly immunosuppressive.

hand, viral genes can be essentially reconstructed by deletion of unwanted sequences and ligation of the portions to be included. They can then be inserted into various expression systems or chemically synthesized to produce the desired products. One can thus envision combinations of T and B cell epitopes in linear arrays which would produce a safe and effective immunogen (Figure 1).

It should be emphasized that while the envelope of HIV may well be a primary target of immune attack, other viral components are likely to be equally important. The phenomenon of antigen processing and presentation on the cell surface in association with MHC makes other HIV gene products (structural or regulatory) potential targets for immune attack. Indeed T-cell epitopes also are present on internal antigens such as p24 (34), and products of the pol (35) and nef genes (36). Depending on the level of expression on the infected target cell, it may also be that certain epitopes are more dominant than others and better suited as targets for immune attack. Thus cocktails of epitopes from products of envelope, core and regulatory genes may be needed to induce a protective response against the virus.

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The SCID-hu Mouse: A Small Animal Model for HIV Infection and Antiviral Testing

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INTRODUCTION

The human immunodeficiency virus (HIV) appears to play a primary role in the etiology of the acquired immunodeficiency syndrome (AIDS). Five years of intensive investigation have illuminated many aspects of the structure, molecular genetics, and replication patterns of this virus *in vitro*. Yet little is known about the course of HIV-induced pathology *in vivo*. Immunodeficiency is clearly an end result. Often, however, it is precisely the signs of immunodeficiency which bring the HIV-infected patient to notice in the first place. Thus, most attempts to explore and to modify the course of disease have been limited to observations made at times when the disease process itself is far advanced. More difficult to observe are the pathways leading from acute infection to diverse multi-organ involvement and to the various clinical presentations of opportunistic infections, dementia, cachexia, and malignancy.

An animal model for HIV infection would permit systematic analysis of the events surrounding infection, the course of chronic infection, and the effect of prophylactic and/or therapeutic modalities which might predictably prevent or suppress disease. Such a model might be most informative if (1) the animal harbored the human hematolymphoid target organs of HIV and (2) the etiologic agent for disease was HIV itself, and not a surrogate lentivirus. Preferably, the model would also be one in which many animals could be studied simultaneously and safely, so that multiple determinations (e.g., of antiviral effects) could be obtained in a statistically meaningful way. In practical terms, this condition suggests the use of a small animal, e.g. a mouse.

THE SCID-hu MOUSE

The SCID-hu mouse (McCune 1988) was developed in an attempt to meet these criteria. Human hematolymphoid organs were implanted into the immunodeficient C.B17 scid/scid mouse (hereafter designated as "SCID") in such a fashion that human progenitor cell differentiation can be observed. Taking advantage of the fact that fetal progenitor cells to the lymphoid lineage have not yet learned to discriminate self from non-self (Billingham 1953), human fetal liver (containing both hematopoietic progenitors and the stroma which supports the self-renewal and differentiation of these cells) may be implanted into the SCID mouse along with human fetal thymus (which contains the stromal micro-environment requisite for T cell differentiation). Thereafter, human T cell progenitors may be observed to enter and to move through the human thymus graft. Mature, single positive CD4 and CD8 human T cells are then found in the peripheral circulation of the mouse; human myelomonocytic cell differentiation is also evident within the thymic parenchyma. The maintenance of human T cells in the periphery has been documented for periods of time in excess of 18 months; in such cases, elements indistinguishable from human bone marrow are detectable within the SCID-hu mouse (Namikawa 1989). No overt or subclinical signs of graft versus host disease are found. This may be related to the migration of mouse class

II (I-A)-expressing thymic interdigitating cells into the human thymus implants, creating a microchimeric thymus which could tolerize/restrict human progenitors to mouse class II (I-A) as well as to the HLA alleles of the thymus donor.

When human lymph node is additionally implanted within the SCID-hu mouse, the subanatomic functional areas of the node are found to develop. Primary follicles containing CD20 human B cells are present along with plasma cells bearing intracellular IgM or IgG. T cell areas with $CD4^+$ and $CD8^+$ T cells are also detectable. High levels of human IgG and IgM are thereafter found to circulate in the SCID-hu mouse.

The SCID-hu mouse has offered novel experimental opportunities: first, into the process of normal human hematopoiesis; secondly, into the area of normal human T and B cell function; and also, into an evaluation of HIV infection *in vivo*, within the setting of human organs and human cells, within an easily maintained small animal.

HIV INFECTION OF THE SCID-hu MOUSE

Initially, to demonstrate that the SCID-hu mouse was permissive for infection with HIV, graded doses of the isolate HIV (JR-CSF) (a molecularly cloned HIV obtained directly from the cerebrospinal fluid of a patient with AIDS encephalopathy) (Koyanagi 1986) were introduced into the human organ implants by intra-thymic or intra-nodal injection. With time, progressively more cells were detected by *in situ* hybridization (ISH), using ^{35}S-labeled RNA probes for the 3' end of the viral genomic transcript. Given a viral inoculum of 400-4000 infectious units, 100% of SCID-hu mice showed evidence of infection by ISH after 2 weeks (Namikawa 1988). The infected cells were dispersed across the organ, with eventual and preferential localization into the thymic medulla. When counterstained with antibodies against HIV gag and env determinants, some (about 50%) of the cells positive for viral RNA showed evidence of viral structural proteins as well.

It is reasonable to expect that many of the cells infected in such circumstances are lymphocytes, most likely $CD4^+$ T cells. However, amongst these cells are varying subpopulations segregated along different stages of $CD4^+$ thymocyte differentiation. Further, there appear to be many cells in the HIV-infected SCID-hu thymus or lymph node which are not $CD4^+$ T cells at all. These cells might conceivably represent $CD4^+$ monocytes, macrophages, or dendritic cells, all of which are integral to the functioning of the lymphoid organ during an immune response. Alternatively, they may represent other unidentified subpopulations of cells which support HIV replication *in vivo*.

Given such results, it is of interest to ask: are all such populations equally permissive for infection? After infection, is virus replicated by some and not other subpopulations? If so, what are the viral or cellular parameters which denote differential regulation of replication? Such questions can be asked by analyzing discrete subpopulations of cells in the organ (e.g., after separation by FACS) with relation to viral replicative pathways. Since most of the relevant subpopulations cannot be isolated and grown *in vitro*, these questions are only approachable in the setting of an *in vivo* organ system, such as is provided in the SCID-hu mouse.

ANTIVIRAL TESTING IN THE SCID-hu MOUSE

The demonstration that 100% of SCID-hu mice show evidence of HIV infection after introduction of free virus intrathymically permits the evaluation of antiviral effect. The mouse is a living system; the infected thymus is vascularized and permits entry of both cells and macromolecules. Thus, one might ask the questions: Can a given drug be orally absorbed by the animal? Does it reach "therapeutic levels" in the bloodstream? Does the drug also penetrate the intercellular spaces of the human organ? (e.g. Is its volume of distribution inclusive of that set of spaces most likely to be involved during HIV infection?) Once within the organ, does it react equally with (or is it metabolized equally by) all of the infected subpopulations of cells? Once there and interactive with the infected cell, does it alter the course of infection? And finally, does the drug manifest overt toxicity to the uninfected human hematopoietic precursors or their progeny within the SCID-hu mouse? Such questions cannot be answered in an *in vitro* culture dish, nor can they be fully addressed when selected subpopulations of human cells are introduced intra-peritoneally into an animal in the absence of human hematolyphoid structures. Indeed, to date, these questions have only in part been asked within limited clinical studies in man.

If the SCID-hu mouse is to serve as a preclinical testing model for antivirals against HIV, many levels of relevance between it and man must be documented. First and perhaps foremost, antivirals which have known efficacy and pharmacokinetic profiles in man must be shown to behave similarly in the SCID-hu. Studies have therefore focused on the most widely-used antiviral, 3'-azido-3'-deoxythymidine (AZT). This thymidine analog is orally absorbed and thereafter tri-phosphorylated to an active intermediate within human cells; it acts to prevent reverse transcription of the HIV viral genome (Mitsuya 1985) (Furman 1986). As such, it is effective in preventing viral spread to uninfected cells.

Using an experimental protocol similar to that used previously to assess the effect of AZT on Rauscher leukemia virus (Ruprecht 1986), AZT (1 mg/ml in drinking water) was administered to SCID-hu mice 24 hours prior to intra-thymic infection of the SCID-hu mice with HIV (McCune 1989). Mice were infected with one of two viral inocula: HIV (JR-CSF), a T-cell tropic virus *in vitro*, and HIV (JR-FL), an isolate which demonstrates monocytotropism *in vitro* as well (Koyanagi 1986). Both were used at doses which had previously been titered *in vivo* to result in 100% infection after 2 weeks, as assayed by ISH. The mice were maintained on AZT for two weeks and then kept on drug-free water for an additional four weeks. Detection of viral replication in the infected thymus was carried out with two assays: (1) DNA polymerase chain reaction (PCR) for integrated viral genomes (using 60 cycles and ethidium bromide staining of PCR products on agarose gels), and (2) *in situ* hybridization (ISH) for viral RNA transcripts. Both techniques were applied to cells or tissue sections from biopsied, infected SCID-hu mice. All (40/40) mice infected with HIV in the absence of AZT prophylaxis showed viral replication at 2 weeks in the human thymus by DNA PCR and by ISH. In the latter case, more than 30 cells/x100-power field were infected. In contrast, none (0/15) of the SCID-hu mice infected in the presence of AZT showed signs of infection as assayed by DNA PCR at 2 weeks. The thymic biopsy specimens of these treated mice did, however, demonstrate rare cells (less than 3 cells/x100-power field) harboring viral RNA transcripts and detectable by ISH. When followed for an additional 4 weeks in the absence of AZT, all of these mice subsequently showed signs of HIV by DNA PCR as well. Such evidence for incomplete protection by AZT was observed with HIV (JR-CSF) as well as with HIV (JR-FL).

Two conclusions may be drawn from this study. First, AZT prophylaxis does induce a qualitative effect (100% vs. 0%) on HIV infection of the SCID-hu mouse, at least as assayed by DNA PCR. Experiments are now underway to explore the various parameters of this effect, e.g., with relation to viral inoculum, drug dose, and timing of drug

administration. Secondly, the qualitative effect demonstrated by DNA PCR becomes quantitative by the more sensitive assay of ISH. Thus, most but not all cells are "protected" from infection at 2 weeks; given 4 more weeks of viral replication in the absence of AZT, these cells produce sufficient progeny virus to be detected by DNA PCR.

The first conclusion suggests that the SCID-hu mouse might be useful in testing novel antivirals, perhaps using AZT as a standard for comparison. To facilitate this process, recent efforts have focused on techniques which might obviate the need for intra-thymic injection (by 30 gauge needle) and analysis of biopsied tissue (by razor blade). In recent experiments (Kaneshima 1989), it is now clear that 100% of SCID-hu mice with human lymph node can be infected by intravenous administration of HIV and, in a time- and dose-dependent manner, show detectable levels of viremia in the peripheral blood later. Further, and in accordance with the above experiments, prophylaxis with oral AZT suppresses viremia after infection.

The second conclusion emphasizes the need for sensitive, reproducible, and quantitative measures of infection. Such assays, under development now, might permit a more exact determination of drug effect. On the one hand, a given antiviral may only function to partially suppress viremia; such, indeed, is the case when AZT is administered to target cells *in vitro* prior to infection with HIV (Smith 1987). Alternatively, some subpopulations of cells *in vivo* may be more resistant to drug effect than others; for instance, a cell less able to triphosphorylate AZT would be correspondingly more susceptible to infection with HIV. In both settings, a quantitative analysis of virus load in the HIV-infected SCID-hu mouse would be useful in efforts to modify candidate antivirals so that they might be even more efficacious.

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IV Defense

Tumor Immunology

Structural Basis for Tumor Cell Recognition by the Immune System

L.J. Old

Opinion has varied widely as to the importance of the immune response in the development of cancer. Recurrent cycles of enthusiasm and disillusionment have characterized cancer immunology since its inception. Part of this volatility is inherent in the major objective of the field - the development of cancer therapies based on immunological principles - and the history of cancer immunology records innumerable instances of unfulfilled claims for successes in clinical trials of various forms of cancer immunotherapy. Another unsettling factor has to do with questions about the general validity of the major intellectual underpinning of the field - the immunosurveillance theory of cancer. The prediction that cancer development would be more frequent in animals or humans with suppressed immune systems, although true for certain virus-induced tumors and tumors involving the lymphoid system, has not been borne out in a variety of experimental and clinical settings. Another problem with the immunosurveillance theory is that it presupposes a target for immune recognition on cancer cells. Despite vast efforts to identify cancer-specific traits, evidence that the generality of cancers has such distinguishing features is still lacking. Nevertheless, there are cancer antigens that after many years of study retain the right to be called tumor-specific and other antigens that are not tumor-specific but nonetheless are recognized by the host of origin. The major point I want to make is that the field of tumor immunology, which has relied heavily on phenomenology, now needs a strong structural basis for its continued development, and that a precise biochemical and molecular definition of the known immunogenic cancer antigens is an essential next step.

The overview presented here will cover four areas: a summary of the origins of the field; general principles derived from experimental studies; progress in the structural analysis of cancer antigens that are targets for immune recognition; and the status of immunological approaches to human cancer therapy.

ORIGINS OF CANCER IMMUNOLOGY

The most persistent line of inquiry in cancer immunology has been the serological analysis of cancer, begun in the late 19th Century with faith that antibodies would prove invaluable in understanding and controlling cancer, a faith that appears to have survived intact as judged by current interest in monoclonal antibodies (Fig. 1). Although the search for cancer-specific antigens was the major motivation, it soon became clear that claim after claim for existence of these antigens could not be substantiated and even the most restricted cancer antigen could be found on some normal cell type. Nevertheless, much has been learned about the rich diversity of antigens expressed by normal and cancer cells, particularly cell surface antigens that distinguish cells belonging to distinct lineages or distinct phases in the same lineage, antigens that we now call differentiation antigens.

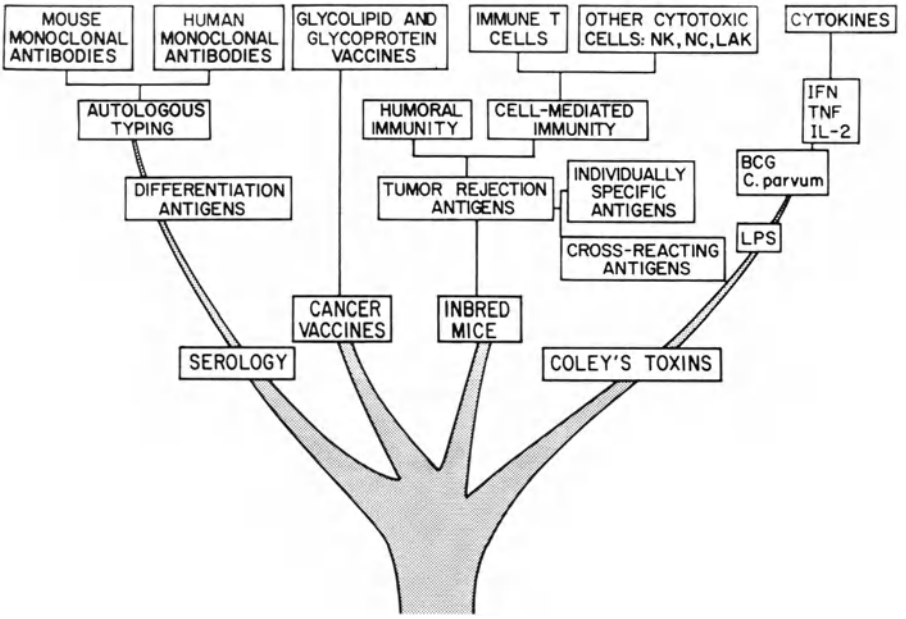

Figure 1. ORIGINS OF CANCER IMMUNOLOGY

The development of inbred strains of mice provided another way to look for tumor-specific antigens. If tumor cells gain new antigens, it should be possible to immunize mice against transplants of tumors arising in other mice of the same inbred strain. Gross, Foley, and Prehn and Main demonstrated that sarcomas induced by methylcholanthrene possessed strong transplantation rejection antigens, showing that prior growth of a tumor (followed by its removal) rendered mice resistant to challenge of the same tumor. These findings placed the field of tumor immunology on a firm experimental basis for the first time and lifted the sense of "unrelieved gloom" that had come to be associated with immunological approaches to cancer over the preceding decades, a feeling best expressed by William Woglom's statement in 1929 that "it would be as difficult to reject the right ear and leave the left ear intact as to immunize against cancer." In addition to the contributions of serology and transplantation immunology to the origins of cancer immunology, another important tributary came from observations made by physicians from antiquity to the present that human cancers can undergo spontaneous regression, and that this rare but repeatedly observed event was associated with concomitant bacterial infection in a number of cases. Attempts to mimic this by deliberately infecting cancer patients with viable bacteria, later by injecting killed bacterial vaccines, became a lifelong effort by William B. Coley, a New York surgeon. Coley's approach led to interest in the effect of microbial products on animal cancers and the discovery that a large number of microorganisms, ranging from bacteria to yeast had antitumor activity. Subsequent research showed that this effect of microbes was not a direct one but mediated through the host by a variety of mechanisms, particularly through the release of cytokines, some of which have direct antitumor activity. Attempts to exploit the Coley's phenomenon in the clinic have led to numerous trials with organisms such as BCG and *C. parvum* over the past 20 years, and present day cytokine therapy with interferons, TNF, and IL-2, with or without LAK cells can be considered modern versions of Coley's toxins.

PRINCIPLES OF EXPERIMENTAL CANCER IMMUNOLOGY

Despite much interest and effort, the cannon of tumor immunology is small, much of old vintage, and principles are hard to come by. Nevertheless, certain observations have been repeatedly made and therefore assume the status of precious fact. They can be incompletely summarized as follows: 1) Tumors vary greatly in the strength of their transplantation rejection antigens, with UV-induced tumors and tumors induced by the oncogenic DNA viruses, such as polyoma, SV40, and adenoviruses having the strongest immunogenicity, and spontaneous tumors usually showing only weak or no immunogenicity. 2) The characteristic transplantation rejection antigens of tumors induced by chemicals or physical carcinogens are individually distinct (and not cross-reacting), whereas virus-induced tumors have cross-reactive antigens, each virus inducing different cross-reactive antigens. 3) Immunity to tumor transplants is mediated by T cells, both of helper/inducer and cytotoxic phenotypes, as Bob North will be describing. Antibody, although detectable in some tumor systems, does not confer protection against transplants, except in the case of highly antigenic virus-induced leukemias. 4) Immunosuppression increases the development of tumors induced by the strongly antigenic oncogenic DNA and RNA viruses. Comparable studies with chemically induced tumors have not given consistent results - some studies showing more rapid tumor development, but others showing no effect. 5) Tumors can avoid the consequence of their immunogenicity by a number of escape mechanisms; these range from rapid growth that outpaces the immune response, tumor growth in immunologically privileged sites, immunoselection of antigen-low or -negative variants, antigenic modulation with phenotypic down-regulation of antigen expression, decrease in expression of MHC restriction elements, as Günter Hämmerling will describe, and tumor-induced general or specific immunological suppression. 6) A variety of different cell types can kill tumor cells *in vitro*; these include T cells, macrophages, NK, NC and LAK cells, mast cells and polymorphonuclear leukocytes. Because of this, there has been considerable uncertainty as to the specificity and significance of the cell-mediated immune reactions demonstrated against experimental tumor cells *in vitro;* cell cloning and identification of cytotoxic cytokines are clarifying these issues. 7) Analysis of cell surface antigens of experimental tumors has revealed several categories of antigens, in addition to MHC antigens. In the mouse, surface antigens related to endogenous leukemia viruses are commonly found on tumors of diverse origin. In the case of tumors induced by DNA viruses, the T antigens, which have a predominately nuclear localization, can also be expressed on the cell surface where they are the target of cellular and humoral immunity. Tumor cells, like normal cells, display an extensive array of differentiation antigens, some highly restricted and therefore of great interest to the tumor immunologist. One of the most exciting developments in tumor immunology points to the possibility that any mutated cellular product, including intracellular antigens, can become targets for cytotoxic immunity. In other words, protective immunosurveillance does not stop at the cell surface, but extends to all constituents of the cell. This idea has its origin in the growing understanding of antigen processing and presentation, and in the work of Thierry Boon and his colleagues on the antigens recognized by cytotoxic T lymphocytes on mutagenized tumor cells, work that he will be discussing later. These findings open up a whole new universe of potential targets for the tumor immunologist, including oncogenes such as mutated *ras* products and other intracellular antigens that have been altered during tumor initiation and progression.

STRUCTURAL ANALYSIS OF CANCER ANTIGENS

Although a large number of cell surface molecules have been defined on tumors, particularly since the advent of monoclonal antibodies, only a small number of these have been shown to elicit transplantation rejection in syngeneic animals, or specific antibody or cell-mediated immunity in syngeneic or autologous animals or humans (Table 1). Such immunogenic antigens are, of course, the most interesting to the tumor immunologist, because they can be targets for the full force of active immunity. This list of tumor antigens represents the treasure chest of tumor immunology, the combined work of many investigators over a half century. In experimental systems, the tumor antigens that have been most extensively studied are TL antigens of mouse leukemia, antigens encoded by DNA and RNA oncogenic viruses, transplantation rejection antigens of methylcholanthrene-induced tumors, and antigens recognized by cytotoxic T cells on mutagenized tumor cells. In humans, melanoma has been the focus of much study, and a series of glycoprotein and glycolipid antigens has been defined by conventional serology with human serum and human monoclonal antibodies. The list of defined surface components recognized by human monoclonal antibodies on melanoma and other types of human cancer can be expected to grow rapidly. For instance, Posner and his group (1989) have isolated several human monoclonal antibodies from a patient with chronic myelogenous leukemia in remission that show specificity for leukemia cells, and Vollmers and his group (1989) have generated a human monoclonal antibody from a patient with stomach cancer that detects an antigen with specificity for stomach signet-ring carcinoma. Although each of the antigenic systems listed in Table 1 deserve extensive discussion, I will focus on two of them. One has to do with the oldest unsolved mystery of tumor immunology, the nature of the tumor rejection antigens of chemically induced sarcomas in mice, and the second with the growing recognition of gangliosides as important tumor antigens.

Table 1. STRUCTURAL BASIS FOR IMMUNE RECOGNITION OF CANCER BY THE HOST OF ORIGIN

	TUMOR TYPE	DEMONSTRATED BY	CELL SURFACE STRUCTURE
MOUSE	LEUKEMIAS	MOUSE Ab	TL, MuLV env/gag
	MAMMARY TUMORS	MOUSE Ab	MTV env/gag
	DNA VIRUS-INDUCED TUMORS	TRANSPLANTATION REJECTION	T DETERMINANTS
	METHYLCHOLANTHRENE-INDUCED SARCOMAS	TRANSPLANTATION REJECTION	p84/86, gp96 FAMILY OF STRESS PROTEINS
	TUM^- VARIANT	CYTOTOXIC T CELL	p60 CONTAINING A SINGLE AMINO ACID SUBSTITUTION
HUMAN	MELANOMA	AUTOLOGOUS Ab	FD (gp90)
		HUMAN mAb	GM3, GM2, GD3, GD2 GANGLIOSIDES
	EPITHELIAL CANCERS	HUMAN Ab	T, Tn, GALACTOSYLGLOBOSIDE, FORSSM/
	ADULT T CELL LEUKEMIA	HUMAN Ab	HTLV-I env/gag
	BURKITT'S LYMPHOMA	HUMAN Ab	EBV-CODED
		CYTOTOXIC T CELLS	

The tumor rejection antigens of chemically induced sarcomas are classically demonstrated by their ability to induce transplantation immunity in preimmunized syngeneic mice. An extraordinary feature of these antigens is their polymorphism - each tumor appears to have its own unique antigens or set of antigens. The diversity of antigens on chemically induced tumors has commonly been ascribed to mutational changes induced by the carcinogen, and a variety of genetic systems, including genes coding for MHC, Ig, MuLV and fetal antigens, have been implicated in the generation of these antigens. MacFarlane Burnett, on the other hand, suggested that these antigens were not the consequence of carcinogen action, but existed prior to transformation as a family of polymorphic cell surface molecules on normal cells; transformation results in the clonal expansion of cells expressing one set of these antigens and because of their restricted representation in the normal animal, tolerance is not established and the immune system responds when confronted with the tumor. Although attempts have been made to distinguish pre-transformational vs. post-transformational origin of these antigens, the matter is not resolved. Recent progress has been made by Ullrich *et al.* (1986) and by Srivastava *et al.* (1985; 1987) in identifying the molecules responsible for the antigenicity of these tumors. Srivastava *et al.* have found that the transplantation rejection activity of three BALB/c sarcomas resides in a family of glycoproteins with a Mr of 96,000 (gp96). Immunization with purified gp96 elicits immunity to tumor challenge, and the specificity of the immunity is the same as immunization with tumor cells, i.e., individually specific. Despite this distinct antigenicity in transplantation tests, gp96 molecules isolated from different tumors are indistinguishable by a number of criteria, including lectin affinity, identical N-terminal sequencing, and reactions with rabbit anti-gp96 antibodies. The rabbit antibodies also detect a gp96 component that is widely expressed in normal cells, and message for gp96 is also found in both normal and tumor cells. Thus, the transplantation rejection antigens of chemically induced tumors appear to be distinct epitopes present on widely expressed molecules belonging to the same family rather than on unique molecules belonging to different families. The other possibility is that gp96 is not the tumor antigen per se but serves as a carrier for other immunogenic components, such as the mutated elements De Plaen *et al.* (1988) have described in their system. This issue will not be resolved until the structural basis for the polymorphism of chemically induced tumor antigens is defined. The transplantation rejection antigens that Ullrich *et al.* (1986) isolated from chemically induced sarcomas co-purify with proteins of Mr 84,000-86,000 and these antigens show strong homology with heat shock proteins of the hsp90 family. gp96 is also related to heat shock proteins, in this case, hsp108 (Maki, 1989). This relation of tumor antigens to heat shock or stress proteins is provocative considering the increasing evidence that heat shock proteins may be the target of immune reactions in infectious diseases and autoimmunity.

Another class of tumor cell surface molecules that are of interest to many investigators are the gangliosides (Oettgen, 1989). Gangliosides are glycosphingolipids with one or more sialic acid residues. Glycosphingolipids are amphipathic molecules consisting of a hydrophilic chain linked to a hydrophobic ceramide portion. Gangliosides are found in all normal tissues, but are particularly prominent components of the brain and other tissues of neuroectodermal origin. Melanomas, astrocytomas, neuroblastomas and other tumors of neuroectodermal origin, in contrast to epithelial cancers, display high levels of gangliosides on their cell surface. A battery of mouse monoclonal antibodies have been generated to major and minor gangliosides

expressed by melanoma and neuroblastoma, several of which are being used in clinical trials. Analysis of the human humoral immune response at the clonal level using EBV transformation and hybridoma technology shows that a surprising number of human antibodies have specificity for gangliosides (Lloyd and Old, 1989), and human monoclonal IgM antibodies have been isolated by several laboratories, including Reiko Irie's and our own, that identify GM3, GM2, GD3 and GD2. Many of these human monoclonal antibodies have complex hierarchical cross-reacting patterns with other gangliosides, in most instances predicted on the basis of shared structures. Although these human antibodies were isolated from patients with melanoma, it is not clear what role the melanoma had in eliciting their production. For this reason, the anti-ganglioside B cell repertoire of normal individuals and individuals with other types of cancers needs to be determined.

HUMAN CANCER IMMUNOTHERAPY

Before discussing how gangliosides and antibodies to gangliosides are being used in therapy, I want to say a few words about current attempts to use immunological principles in the therapy of cancer. Despite profound shifts in opinion about the prospects of immunological approaches to cancer therapy, the themes of interest have remained remarkably consistent over the past 50 years. Current explorations of monoclonal antibodies represent a direct extension of earlier studies with horse and rabbit antisera, and vaccines containing purified cancer antigens are modern day versions of earlier vaccines containing crude tumor homogenates. And as I said before, even the trials of recombinant products such as interferon, TNF and IL-2 have their precedent in the use of bacterial vaccines, such as Coley's toxins in cancer therapy, since microbial products are potent inducers of these cytokines. Reshaped by the new technologies, the old hopes of cancer immunology for cancer therapies have been rejuvenated once again, and this time around the potential of these approaches can be assessed far more critically than ever before.

Antibodies can be used for therapy in several ways, mainly as carriers of toxic agents to the tumor site and as mediators of inflammation and immune lysis of tumor cells. The carrier approach has been emphasized most and a number of novel and efficient conjugation methods have been devised. Experience with mouse antibodies conjugated with radioactive compounds, ricin and other poisons, and chemotherapeutic agents is accumulating from animal and clinical studies. The major problem with this approach in humans is the small amount of antibody that selectively localizes to the tumor - usually in the range of 0.001 - 0.01% of the injected dose per gram and far smaller in the case of antibody fragments. Without improvement in the selective delivery of antibody conjugates to the tumor, it will be difficult to achieve a meaningful antitumor response without prohibitive toxicity. The other application involves antibodies that have the capacity to kill cancer cells through complement-dependent and complement-independent immune mechanisms. Mouse antibodies with these characteristics generally belong to the IgG2a or IgG3 subclass. One such antibody, designated R24, which can serve as a prototype, was isolated by Dippold *et al.* (1980). It belongs to the IgG3 subclass and detects the disialoganglioside GD3, a prominent cell surface antigen of human melanoma (Pukel *et al.*,1982). R24 is highly active in directing melanoma cell lysis by human complement and by human effector cells in antibody-dependent cell-mediated cytotoxicity (ADCC)(Welt *et al.*, 1987a). In fact, R24 and other IgG3 antibodies to GD3 are the most potent cytotoxic antibodies that have been generated against melanoma cells. As several groups have found (Cheresh *et al.*, 1985; Hellstrom

et al., 1985; Welt *et al.*, 1987a), target cell susceptibility to GD3-targeted lysis is related to antigen density. Below a critical number of GD3 sites, immune lysis does not occur, accounting for the resistance of cultured melanocytes to R24-mediated lysis. Thus, a tumor-specific effector mechanism can be directed against an antigen that is not tumor-specific because of a threshold requirement for immune lysis by complement and immune cells. Melanoma patients receiving R24 experience erythema and urticaria at the tumor sites, and biopsies show complement deposits, mast cell degranulation and infiltration with T cells and other inflammatory cells (Houghton *et al.*, 1985; Dippold *et al.*, 1985). Tumor regressions have been seen in R24-treated patients, but they occur some weeks after antibody injection, suggesting antibody-mediated cell lysis may not be the main reason for its antitumor action. Rather, the inflammatory reaction elicited at the tumor site may augment (through a local adjuvant effect) an underlying specific immunity, and the proliferative action of R24 on a subset of GD3 expressing T cells may contribute to this more effective immune response (Hersey *et al.*, 1986; Welte *et al.*, 1987).

Cytotoxic IgG3 antibody against another ganglioside, GD2, has also shown inflammatogenic and inhibitory activity against melanoma and neuroblastoma in the clinical studies of Cheung *et al.* (1987). Hale *et al.* (1988) have recently reported clearing of tumor cells from the blood and resolution of splenomegaly and lymphadenopathy in two patients with non-Hodgkins lymphoma treated with a chimeric rat/human IgG1 antibody with complement-fixing activity. Surface antigens on tumor cells are not the only antigens that can serve as *in vivo* targets for monoclonal antibodies. Intracellular antigens released as a consequence of necrosis and cell death at the tumor site provide additional targets (Welt *et al.*, 1987b). Other targets include surface antigens on tumor fibroblasts and endothelial cells that have been induced in response to environmental stimuli in the tumor, such as cytokines released by tumor cells. F19 is one such antigen that is not expressed by normal tissue fibroblasts but is found on stromal fibroblasts in a wide range of tumors (Rettig *et al.*, 1988). As stroma can be a major component of the tumor mass, F19 represents a novel target for antibody imaging as well as therapy.

The other approach to cancer immunotherapy I want to mention involves vaccination with tumor antigens. The idea of a cancer vaccine is as old as immunological thought, and innumerable attempts have been made in the past century to alter the course of cancer by immunizing patients with various types of tumor extracts. Unfortunately, little can be learned from this massive effort, certainly not whether the vaccine approach has any validity. For one thing, endpoints based on clinical observations, such as survival, are unreliable when dealing with small and heterogeneous groups of patients. The major problem, however, was that there was no way to know whether the vaccine elicited anything in the way of a specific immune response to the tumor. New strategies for developing cancer vaccines start with a growing list of cancer antigens that are recognized by human antibodies, a list that can be expected to enlarge significantly now that the humoral immune response to tumor antigens can be dissected in such fine detail at the clonal level. Another area that has grown rapidly since the advent of IL-2 is the clonal analysis of T cell reactivity to human tumors. Studies by a number of groups (Knuth *et al.*, 1984; Knuth *et al.*, 1989; Hérin *et al.*, 1987; Anichini *et al.*, 1985; and Mukherji *et al.*, 1983; Mukherji *et al.*, 1989) have shown that cytotoxic T cell clones with specificity for autologous melanoma can be repeatedly isolated from certain melanoma patients. With the

techniques that Boon and his colleagues (De Plaen *et al.*, 1988) have developed for cloning and characterizing antigens recognized by cytotoxic T cells, it will now be possible to characterize the T cell targets on human melanoma cells, and this will represent a highly significant step for human tumor immunology. In addition, the structural definition of T cell targets on tumor cells should accelerate progress in the use of specifically sensitized T cells in adoptive immunotherapy of cancer.

Because of the frequency with which human monoclonal antibodies against gangliosides can be isolated from patients with melanoma, and the fact that gangliosides are major cell surface constituents of melanoma cells, there is considerable interest in developing ganglioside vaccines that result in high levels of serum antibodies. GM2 gangliosides have been found to be particularly immunogenic in humans (Tai *et al.*, 1985; Livingston *et al.*,1987), and vaccines containing purified GM2 and a microbial adjuvant (BCG) elicit both IgM and IgG anti-GM2 antibodies in melanoma patients (Livingston *et al.*, 1987; Livingston *et al.*, in press). Now that immunogenic GM2 vaccines have been developed and can be monitored in individual patients, the influence of high GM2 antibodies on melanoma recurrence or spread can be evaluated. However, the generation of cytotoxic antibodies mediated by human complement to a cell surface antigen expressed on the surface of melanoma cells after immunization with a pure antigen is an important step in realizing the goals of a melanoma vaccine. This approach to the development of a cancer vaccine does not differ from the steps taken in developing vaccines against infectious diseases. Only after antigen identification and development of maximally immunogenic forms of the antigen is it possible to determine the effect of vaccination on disease. Virus-induced tumors in humans represent a special case, and continued effort is needed to construct vaccines that can prevent infection or kill transformed cells associated with EBV, hepatitis virus, HTLV and HPV.

In many fields of science, the major questions don't change, only our ability to answer them, and this is certainly true of cancer immunology. Identifying cancer antigens that are recognized or have the potential of being recognized by the host of origin remains the central challenge of the field. In experimental systems, this means biochemical and molecular characterization of immunogenic tumor antigens, then dissecting the immunologic response to them, increasing their immunogenicity, and developing ways to eliminate established tumors by active immunization with these antigens. In humans, we have powerful methods to prospect for immunogenic tumor antigens recognized by humoral or cellular immunity and progress in the analysis of the human antitumor response by human monoclonal antibodies and cloned cytotoxic T cells has been extremely encouraging. Because natural tumor growth may not lead to optimal immunization, these surveys of the human immune response to tumors need to include patients undergoing active immunization with glycoprotein, glycolipid and anti-idiotype vaccines, and as knowledge grows, with synthetic carbohydrate determinants or peptides. Patients in these vaccine trials need to be carefully monitored for untoward effects of the vaccines, e.g., autoimmunity or more rapid tumor growth (tumor enhancement), and for this reason, each group should be kept small and be restricted to patients at high risk for tumor recurrence or having no other effective therapy for their cancer. It is likely that we have not begun to exploit the flexibility and adaptability of the immune system for therapeutic benefit against cancer. The induction of even low levels of active immunity may profoundly alter the biological course of cancer, and if this must be purchased at the

cost of some tolerable level of autoimmunity, it will be an acceptable price. Of course, cancer cells are also remarkably flexible, and the generation of tumor variants having low antigen or MHC expression represents a formidable problem. However, if immunity is directed at several different antigenic targets on the cancer cell, this escape route by the tumor might be effectively blocked. Precise structural definition of the tumor antigens that serve as targets for immune recognition is the next step in seeking solutions to this and the many other challenges that confront tumor immunology.

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Genes Coding for TUM$^-$ Transplantation Antigens. A Model for TSTA?

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INTRODUCTION

It has been known for a long time that some experimental tumors express antigens that constitute targets for immune rejection by the syngeneic host. The existence of these tumor-specific transplantation antigens (TSTA) was first demonstrated with chemically induced mouse sarcomas : each independent tumor was found to express a different antigen (Prehn 1957). Later, these findings were extended to ultraviolet-induced tumors (Kripke 1981). The generality of the existence of transplantation antigens specific for each tumor was however put under serious question when spontaneous mouse tumors were found to be completely incapable of eliciting an immune rejection response (Hewitt 1976). But further, experiments demonstrated that even these tumors express weak TSTA that are potential targets for immune rejection (Van Pel 1983).

What is the molecular nature of TSTA ? And what is the relation between their appearance and the tumoral transformation process ? These questions are still unanswered because the TSTA, which elicit strong T-cell mediated immune responses, do not stimulate B cells to produce antibodies. It has therefore been impossible to isolate the antigenic molecules by immunoprecipitation. This predicament is not restricted to TSTA : the minor histocompatibility antigens and the male-specific antigen H-Y remain uncharacterized for the same reasons.

We have developped a gene transfection approach aimed at identifying directly the genes that code for this type of antigen. It was applied to transplantation antigens that arise on mouse tumor cells when they are treated with mutagenic agents.

TUM$^-$ ANTIGENS

In vitro mutagen treatment of mouse tumor cells generates at high frequency stable immunogenic variants that are rejected by syngeneic mice (Boon 1977; Boon 1983). Because of their failure to form tumors, these variants were named "tum-" as opposed to the original "tum+" cell, which produces progressive tumors. With a dose of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine resulting in survival of about 0.1% of the treated tumor cells, the frequency of tum- variants usually ranges between 1 and 20% of the survivors. This phenomenon has been observed on a large number of mouse tumor cell lines of various types and also with a guinea pig fibrosarcoma (Contessa 1981; Frost 1983; Zbar 1984). Most tum- variants express new transplantation antigens not found on the original tum+ cell. The existence of these "tum-" antigens was first demonstrated *in vivo* by cross-immunization experiments (Boon 1978).

We have studied a series of tum- variants derived from mastocytoma P815, a tumor induced in a DBA/2 mouse with methylcholanthrene (Uyttenhove 1980). From clonal tum+ line P1, we obtained more than 30 different tum- variants, which rarely produce progressive tumors even when they were injected at doses exceeding thousand times a tumorigenic dose of P1. When we restimulated in vitro spleen cells of syngeneic mice that had rejected these variants, we obtained cytolytic T cells (CTL) that lysed preferentially the immunizing tum- variant (Boon 1980). From these lymphocytes, we were able to isolate stable CTL clones (Maryanski 1982). Some of these appeared to be directed against a TSTA of P815 : they lysed P1 and all P815-derived cells but not syngeneic control tumors. Others recognized the immunizing tum- variant, but neither the original tum+ cell nor the other

tum- variants derived from P815. They therefore defined new "tum-" antigens specific for each variant (Fig.1). A systematic study of the tum- antigens found on P815 variants revealed their considerable diversity : no antigen was found twice among the 15 tum- variants that were analyzed. By in vitro immunoselection with anti-tum- CTL clones it was possible to demonstrate that some tum- variants carry several tum- antigens (Maryanski 1982). These experiments also demonstrated that the tum- antigens defined by CTL are relevant to the rejection of the variants, as shown by the correlation between the loss of these antigens and the reversal of the tum- phenotype (Maryanski 1982, 1983)

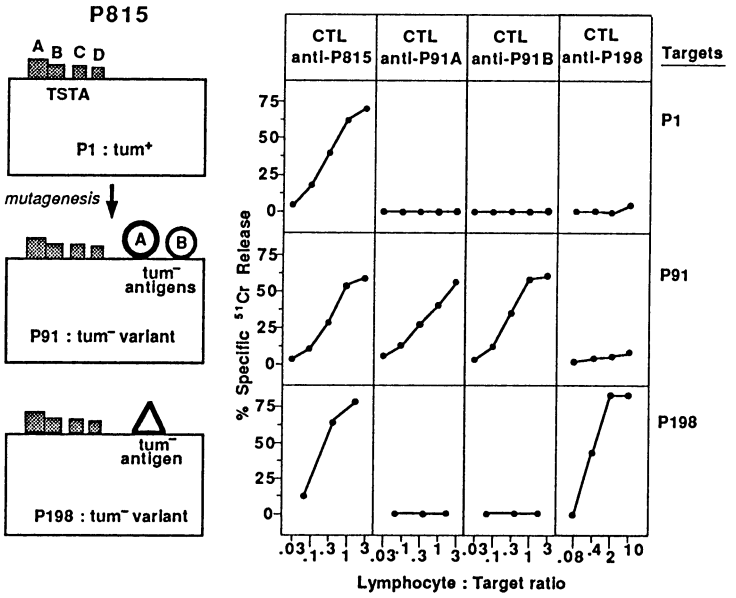

Fig. 1 TSTA and tum- antigens present on the original P815 line P1 and on tum- variants P91 and P198. The CTL clones were derived from spleen lymphocytes obtained from mice that had rejected the tum- variants. They were incubated with their target for 4 hours.

To find an explanation that could reconcile the remarkably high frequency of tum- variants with their stability and also to understand the source of their diversity, it appeared essential to identify the antigenic molecules : were they produced by a family of related genes or did they represent an array of completely unrelated molecules? We failed in our attempts to obtain antibodies directed against tum- antigens. Therefore, we undertook to clone directly the relevant genes on the basis of their ability to produce the antigens recognized by the anti-tum- CTL.

TRANSFECTION OF TUM- GENES

Whereas the transfection of genes coding for surface antigens recognized by antibodies had been demonstrated earlier (Kavathas 1983), we faced the obligation to detect our transfectants with CTL. This implied that the recipient cell must be a good target for

CTL and must express the same H-2 haplotype as P815 (Zinkernagel 1974). We were able to select from P1, the P815 tum+ cell, a highly transfectable variant, P1.HTR, by applying repeated cycles of transfections with a selectable marker (Van Pel 1985). P1.HTR, which has an efficiency of transfection of approximately 10^{-4} (compared with 10^{-6} for P1), endocytoses calcium-precipitated DNA at least tenfold better than P1 (Goethals 1987).

Considering the size of the mammalian genome (6.10^6 kb) and the observation that recipients of calcium phosphate-precipitated DNA integrate on the average approximately 1000 kb (Perucho 1980), one should expect to screen a minimum of 6,000 transfectants, to find one expressing a given single-copy gene. Testing all these transfectants individually for CTL lysis appeared impractical. We therefore explored the possibility of detecting transfectants by their ability to stimulate the proliferation of CTL clones directed against the relevant antigen. Some anti-tum- CTL clones show a considerable increase in proliferation when the proper target cell was present. Moreover, mixtures of cells expressing tum- antigens (e.g. P91) and cells that do not (e.g. P1.HTR) at a 1:30 ratio provide a clearly recognizable stimulation of these CTL clones. On the basis of this test, we set out to cotransfect P1.HTR cells with plasmid pSVtk-neoß carrying the selectable neo gene and with DNA of variant P91. Transfectants expressing tum- antigen P91A were obtained at a frequency of 1/13,000 (Wölfel 1987). Similar results were obtained later with tum- antigens P35B and P198 and with a TSTA of P815 (Table 1).

Table 1. Frequencies of transfection and cosmid rescue obtained with various antigens.

Antigen	Transfection with genomic DNA°	Transfection with cosmid library	Success rate for cosmid recovery by direct DNA packaging of the DNA of transfectants *
	No of transfectants expressing the antigen	No of independent cosmids transferring the antigen	No of independent transfectants from which a cosmid expression of the transferring antigen was recovered
	no. of drug-resistant transfectants	no. of independent cosmids in library	total no. of independent cosmid transfectants
tum⁻			
P91A	7/90,000	2/700,000	1/3
P91B	1/26,000	0/1,200,000	
P35B	3/35,000	5/700,000	1/9
P35A	0/35,000	0/700,000	
P198	2/ 9,000	3/400,000	1/8
TSTA			
P1.A	3/30,000	1/2,100,000	2/2

° Genomic DNA was cotransfected with a plasmid conferring resistance to geneticin or hygromycin as shown in fig.2.

* The DNA of all the independent cosmid transfectants expressing the tum- antigen was packaged in lambda phage components. The number of these transfectants was often higher than that of the positive cosmids because several independent transfectants were obtained with the same group of cosmids. The cosmids resulting from direct packaging were tested by transfection.

ISOLATION OF COSMIDS EXPRESSING TUM$^-$ ANTIGENS

As we failed in our attempts to retrieve gene P91A from the transfectants on the basis of its linkage with the cotransfected neo gene, we prepared a library of 700,000 cosmids with the DNA of a cell that expressed antigen P91A. It was divided into 20 subgroups that were amplified 10^8 times to produce the amount of DNA required for transfection. At the end of this process, we found that about a third of the cosmids had lost their insert and that diversity had been maintained among the others. This relatively favorable situation may have been due to the use of cosmid vector c2RB, which multiplies very well. The twenty cosmid subgroups were transfected and two subgroups produced transfectants expressing antigen P91A (De Plaen 1988).

For the isolation of the sequences expressing antigen P91A, we benefited from an observation of Lau and Kan (1983), who demonstrated that a gene transfected in a cosmid can often be retrieved by simply packaging the DNA of the transfectant into lambda phage components. This may be due to tandem integration of cosmids resulting in the positioning of the gene between two cos sites with proper orientation and distance. When we applied this to the DNA of our first cosmid transfectant, we obtained two different cosmid species. One of them proved capable of transferring at high frequency the expression of the antigen (De Plaen 1988). Various restriction fragments of this cosmid were subcloned and transfected. We identified a 3.9 kb fragment that transferred the expression of the antigen. Surprisingly, when this fragment was subdivided further, we found that a 800 bp fragment was still capable of transferring expression at high efficiency even though it was suspected and later confirmed to contain only a small part of the gene. With this 800 bp fragment, which was devoid of repetitive sequences, it was possible to identify cosmids containing both alleles of the entire P91A gene as well as cDNA clones of the homologous messenger RNA.

The procedure that led to the isolation of tum- gene P91A was applied with success to the cloning of tum- genes P35B and P198, which encode antigens expressed by other tum- variants derived from P815 (Szikora, Sibille, Chomez, unpublished observations). Recently, we have been able to obtain cosmid transfectants for TSTA P1A present on P815. This was achieved by using as transfection recipient a P1A- antigen-loss variant selected from the P1.HTR line with an anti-P1A CTL clone (Van den Eynde, Lethé, Van Pel, unpublished observations). Table 1 provides a survey of the frequencies of transfection and cosmid rescue obtained for the various antigens. As expected, the retrieval of the tum- genes by direct packaging did not work with every cosmid transfectant.

The ability of small promoterless gene fragments to transfect the expression of tum- antigens, without being cloned in expression vectors, has been observed for the three tum- genes. This observation is not yet completely understood. But it proved extremely useful for the analysis of the tum- genes, since it led us quickly to the crucial regions of these genes.

TUM$^-$ GENES AND TUM$^-$ MUTATIONS

Northern blots probed with the 800 bp fragment of gene P91A revealed a single messenger RNA species of 2.2 kb. The band was of equal intensity for tum- variant P91 and for P1, which does not express the antigen. The expression of antigen P91A is therefore not due to the activation of a silent gene.

The structure of gene P91A is shown in fig.2. It comprises 12 exons spread over 14 kb (Lurquin 1989). It does not show any similarity with Ig, T cell receptor or MHC genes. The complete sequence was obtained. It is unrelated to any sequence presently recorded in the main data banks.

Additional evidence for the role of this gene was provided by the study of three "escaping" tumors obtained with variant P91. These variants which had lost the expression of gene P91A all showed deletions in one allele of the gene (Lurquin 1989).

A sequence comparison of the normal and tum- alleles of gene P91A indicated that they differ by a point mutation in the exon, which is present in the transfecting 800 bp fragment (fig.2). This "tum-" mutation is a G to A transition that changes an arginine into a histidine in the main open reading frame of the gene (De Plaen 1988).

Southern blots of P1 and P91 DNA were hybridized with probes corresponding to various regions of the genes. They always gave identical bands for both cells. It appears therefore that no gene rearrangement occurred in the transition between the normal and the tum- allele (Lurquin 1989).

The study of the tum- alleles of genes P35B and P198 also revealed that they differ from the normal alleles by a point mutation in an exon (fig.2). The general structures and the sequences of the three tum- genes isolated so far are completely unrelated.

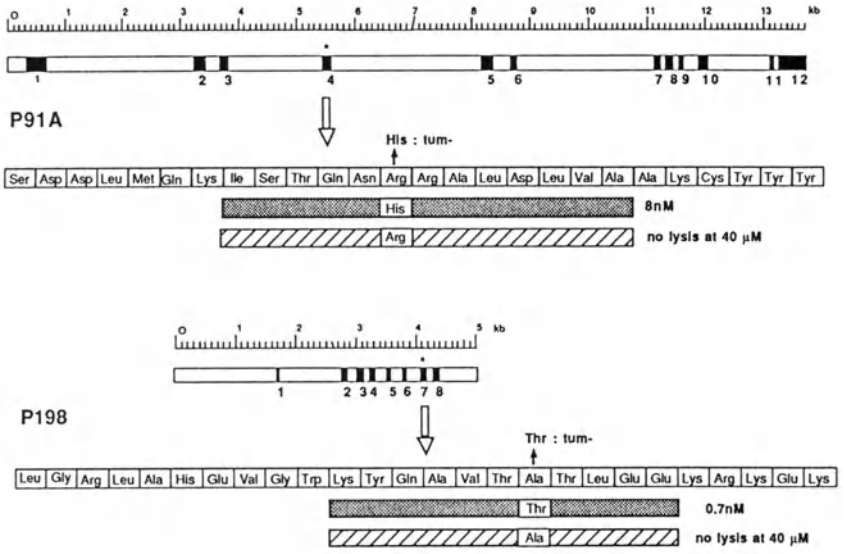

Fig. 2 General structure of genes P91A and P198. Dark regions represent exons. The exon containing the tum- mutation is marked by an asterisk. Sections of the proteins located around the mutated amino-acid are indicated. Synthetic peptides corresponding to the mutant and normal sequence of the genes are represented by boxes. They were tested for their ability to render P1.HTR cells susceptible to lysis by anti-tum- CTL. The concentration indicated to the right of each peptide provided 50% of the lysis obtained at saturating concentration of peptide.

ANTIGENIC PEPTIDES

The main open reading frame of gene P91A encodes a protein of 60 kDa, which does not have a typical N-terminal signal sequence (Lurquin 1989). In vitro translation was performed by incubating with a rabbit reticulocyte extract a RNA produced by transcribing a quasi-full length cDNA cloned in pSP65. A product of the expected size was produced. The addition of dog pancreas microsomes did not change the molecular weight even though the sequence of P91A contains two potential N-glycosylation sites (Godelaine, Amar-Costesec, De Plaen, Beaufay, unpublished results). Antigen P91A is therefore very

unlikely to be borne by a membrane protein. This is however hardly surprising, considering the recent demonstration that CTL can recognize influenza antigens corresponding to endogenous proteins remaining inside the cell, because CTL recognize small peptides that bind to surface class I MHC molecules (Townsend, 1985, 1986a,1986b). On the basis of this evidence, we examined whether we could also identify a small peptide that would trigger the lysis of P815 cells by anti-P91A CTL. In our search for this peptide we were guided by the location of the tum- mutation. A short peptide (fig.2) corresponding to the mutant sequence induced the lysis of P1 by anti-P91A CTL. Transfection and peptides studies with H-2k fibroblasts, which expressed also either Kd, Dd or Ld, demonstrated that antigen P91A is associative with Ld. Antigenic peptides corresponding to the sequence surrounding the tum- mutation were also obtained for genes P35B and P198. They associate with Dd and Kd respectively.

Studies with P91A peptides enabled us to precise the role of the tum- mutation. A priori, the mutation could influence either the production of the antigenic peptide or its ability to associate with the Ld molecule (i.e. the aggretope) or also the epitope presented to T cells by the peptide-MHC complex. Having the antigenic P91A peptide, we prepared the homologous peptide corresponding to the normal allele of the gene. This peptide did not induce lysis by anti-P91A CTL, nor did it compete with the tum- peptide. Moreover, we found that the tum- peptide competed effectively to prevent a cytomegalovirus-derived peptide from inducing lysis by CTL directed against a Ld-associative cytomegalovirus antigen. The tum+ P91A peptide did not compete (Lurquin 1989). This indicates that the P91A tum- mutation generates the aggretope of the antigen, but does not exclude that it also influences the epitope.

HUMAN TUMOR ASSOCIATED TRANSPLANTATION ANTIGENS

The demonstration that TSTA exist on animal tumors, including spontaneous tumors, inclines to believe that TSTA are also present on human tumors. But the demonstration of their existence is made very difficult by the impossibility of performing transplantation experiments. In vitro studies of immune responses are therefore the only means of studying this issue. We will refer here only to a serie of studies of anti-melanoma autologous CTL responses, even though valuable studies of responses against other types of human tumors have been reported (Anichini 1987).

Several studies of melanoma patients have shown that by cultivating autologous PBL with the melanoma lines, it is possible to obtain cytolytic T cells that lyse the autologous tumor and do not lyse autologous EBV-transformed lymphoblastoïd cells, autologous fibroblasts, or NK target K562. These CTL show therefore specificity for the tumor cell line, even though it is difficult to decide how strict this specificity is (Mukherji 1983; Knuth 1984; Anichini 1985; Hérin 1987; Degiovanni 1988; Knuth 1989). CTL clones of similar specificity have been derived from tumor-infiltrating lymphocytes (TIL) collected from melanoma tumors (Itoh 1986; Muul 1987).

To decide whether or not these autologous CTL recognize bona fide human TSTA, one must demonstrate that these antigens are present on the tumor cells in the patient, as opposed to being a culture or transplantation artefact. This is difficult to demonstrate by lysis, because fresh tumor samples usually contain too much necrotic material and foreign tissue to serve as target for chromium release tests. However, TIL derived from a melanoma were recently shown to lyse freshly thawed tumor cells (Topalian 1988). In an alternative approach, evidence was recently obtained that freshly collected melanoma cells were capable of stimulating the proliferation of a CTL clone showing specificity for the tumor (Degiovanni, Lahaye, Hainaut, Weynants and Boon, in preparation).

Another requirement of TSTA is that these antigens must be completely or almost completely absent from the other cells of the organism, so that tumor cell rejection does not degenerate into autoimmune disease. This specificity is very difficult to prove because it is clearly impossible to test the autologous anti-tumoral CTL on every cell of the organism. But, in principle, this issue should be solved completely by genetic analysis : if genes coding for the CTL-recognized antigens could be cloned and were found to carry mutations that are absent on a number of normal cells of the patient, this would demonstrate beyond reasonable doubt that these antigens are absolutely specific for the

tumor. However, this is presently far from being achieved. A first step in this direction is the analysis of TSTA by immunoselection with autologous CTL clones. One melanoma was found to express at least three different antigens recognized by autologous CTL (Knuth 1989). For another melanoma, we demonstrated the presence of six different antigens, four of which are stable (Fig. 3) (Van den Eynde in press). Thirteen independent CTL clones were obtained against three of the stable antigens. One was obtained against the fourth. It appears therefore that the diversity of transplantation antigens recognized on this melanoma by autologous CTL is limited. This is compatible with the notion that these antigens are tumor-specific. We intend to use the immunoselected cells as transfection recipients in an attempt to clone the genes that code for these human tumor-associated antigens.

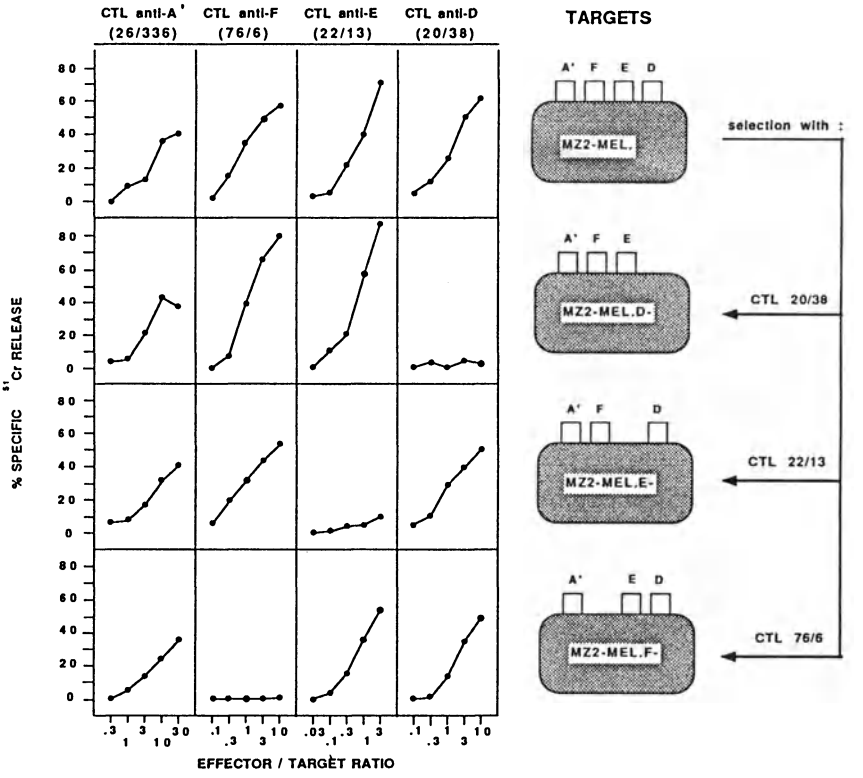

Fig. 3 Antigens recognized on a human melanoma by autologous CTL clones. Only the four stable antigens present on melanoma MZ2 are shown.

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Regulation and Functional Role of MHC Expression on Tumors

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INTRODUCTION

During the last years it has been firmly established that T lymphocytes can recognize foreign antigen only in association with major histocompatibility complex (MHC) class I or class II antigens. It has been suggested that the MHC molecules possess a groove into which peptides can be bound (Bjorkman et al 1988). In the case of MHC class I these peptides are derived from the degradation of intracellular proteins. In general, only some of the peptides have the ability to bind to a particular MHC allele. It is assumed that the MHC class I molecules transport these peptides to the cell surface where the complex consisting of MHC and peptide can be recognized by the T cell receptor of a cytolytic T lymphocyte (CTL). In the case of MHC class II, the foreign antigens are endocytosed and degraded in the lysosomal compartment. It is believed that there they are captured by class II molecules (but not class I) and recycled to the cell surface where they can be recognized by T helper cells.

This mechanism of MHC restricted antigen recognition by T cells has two important implications for the recognition and rejection of tumors by the immune system. First, tumor cells have to express not only a tumor-specific antigen which is foreign to the host, but also MHC class I molecules for the presentation of the tumor antigen to CTL. Moreover, since the antigen recognized by CTL can be an intracellular peptide complexed with MHC, it follows that tumor antigens do not have to be cell surface antigens but can be derived from intracellular proteins. This would explain why tumor antigens can be identified with relative ease with CTL, but so far only in rare cases with antibodies.

Another effector mechanism with which the immune system can eliminate tumor cells are the natural killer (NK) cells. In this brief review, evidence from gene transfection studies will be presented that class I molecules on tumor cells are indeed required for elimination by CTL. However, it will also be shown that class I molecules on tumors can drastically decrease their sensitivity for NK cells. In addition, the regulation of class I expression on various tumors is discussed. Finally, the role of MHC on human tumors will be assessed.

1.) H-2 CLASS I EXPRESSION INCREASES THE IMMUNOGENICITY OF TUMORS

A large number of tumors in both rodents and humans are deficient with regard to expression of MHC class I molecules, ranging from complete absence of all class I molecules to low expression or loss of only some class I alleles. For some of these tumors, it could be shown that restoration of class I expression by H-2 gene transfection led to an increased immunogenicity resulting in the efficient rejection of primary tumors or metastasis by tumor-specific CTL. A notable example are the (C3H x C57 Bl/6) F1 derived

and closely related fibrosarcomas IC9 and IE7. IC9 expresses of the 4 possible MHC class I antigens only D^b, but not K^b, K^k or D^k, whereas the metastastic tumor IE7 expresses D^b and D^k, but not K^b or K^k (De Baetselier et al 1980). In previous studies in collaboration with M. Feldman and S. Segal from Israel, the expression of the "missing" H-2 antigens was restored by H-2 gene transfection (Wallich et al 1985). It can be seen from Table 1 that expression of K^b on the IC9 cells prevented the growth in syngeneic (C3H x C57 Bl/6) F1 mice whereas expression of D^d, D^k or K^k had no influence. In the case of the metastatic IE7 tumor cells, the expression of K^b or K^k abrogated metastasis in syngeneic F1 mice, although the growth of the primary tumor was not affected. Subsequent studies showed that upon inoculation of K^b or K^k transfected tumor cells into the mice K^b or K^k restricted tumor-specific CTL were generated which could reject the transfected but not the parental tumor. In contrast, the parental tumor cells were not able to induce CTL (Wallich et al 1985).

In the case of the C57 Bl/6 derived lung carcinoma 3LL, it was also observed that restoration of K^b expression by K^b gene transfection led to abrogation of metastasis without influencing the growth of the primary tumor (Hämmerling et al 1987, Plaksin et al 1988). The 3LL tumor was also employed for the formal demonstration that not only murine H-2 but also the human HLA antigens can present tumor antigens to T cells. For this purpose, the 3LL cells were transfected with the HLA-Cw3 gene and injected in the syngeneic HLA-Cw3 transgenic C57 Bl/6 mice. The preliminary studies suggest that only the Cw3 transfected but not control transfected 3LL cells were rejected (Table 2). This finding suggests that the HLA-Cw3 molecule can present the tumor antigen as efficiently as the K^b molecule.

Table 1. Abrogation of growth and metastasis of IC9 and IE7 tumor cells following transfection with H-2 genes

Cell line	Transfected gene	H-2 phenotype	Tumor growth	Metastasis nodules/lung	CTL Induction
IC9	---	-, D^b	Fast	0	No
IC9-neo	neo^r	-, D^b	Fast	0	NT
IC9.K^k -1	K^k	K^k, D^b	Fast	0	NT
IC9.K^b -1	K^b	K^b, D^b	None/slow	0	Yes
IE7	---	-, D^b, D^k	Fast	>100	No
IE7.neo	neo^r	-, D^b, D^k	Fast	>100	No
IE7.K^k -1	K^k	K^k, D^b, D^k	Fast	0	Yes
Ie7.K^b -1	K^b	K^b, D^b, D^k	Medium	0	Yes

IC9 and IE7 (C3H.C57Bl/6)F_1-derived sarcomas which express only the D^b or the D^k molecule. Restoration of H-2 K^k, D^k or K^b expression was achieved by transfection with H-2 genes. NT = not tested. Growth, metastasis and tumor specific CTL induction was tested by injection into syngeneic (C3H.C57Bl/6)F_1 mice as described (Wallich et al 1985).

Similar transfection studies with class I deficient tumors have been performed by several investigators, e.g., Hui et al (1984) with the K^k negative AKR thymoma K36, Tanaka et al (1986) with the adenovirus 12 transformed line C57 AT1, Tanaka et al (1988) with the B16 melanoma, and Plaksin et al (1988) with the K^b deficient 3LL lung carcinoma. It is evident from all these studies that class I antigens are mandatory for the elimination of tumors by CTL and

that there exists a hierarchy among class I alleles. Only some, but not all class I alleles can mediate syngeneic tumor rejection, probably because in a particular tumor system only some class I alleles can present an antigenic peptide derived from the putative tumor antigens. In most cases the molecular nature of these tumor antigens is not known because, as explained above, it is extremely difficult to raise antibodies against tumor antigens.

Table 2. The murine H-2K b and the human HLA-Cw3 class I molecules can serve as restriction elements for the syngeneic recognition of the 3LL tumor

Cell line	Transfected	MHC phenotype	Growth of	Metastasis	Metastasis
3LL	---	-, D^b	fast	high	high
3LL.neo	neo^r	-, D^b	fast	high	high
3LL.K^b-1	K^b, neo^r	K^b, D^b	fast	no	high
3LL.Cw3-1	Cw3,neo^r	Cw3, neo^r	fast	no	high
3LL.Cw3-3	Cw3,neo^r	Cw3, D^b	fast	no	high

The C57Bl/6 derived and K^b deficient metastatic tumor was cotransfected with the K^b or the Cw3 gene. Lung metastasis was assessed after inoculation into syngeneic transgenic C57Bl/6.Cw3 mice. The transgenic Cw3 mice are described by Dill et al 1988. The K^b transfectants 3LL.K^b-1 and-2 also failed to develop metastasis in C57Bl/6 mice (not shown).

2.) H-2 GENE TRANSFECTION RENDERS TUMOR CELLS MORE RESISTANT TO LYSIS BY NATURAL KILLER CELLS

Another cellular mechanism by which tumor cells can be destroyed are the NK cells. Inspite of numerous studies, no precise information is available about the receptor of NK cells or about the molecular nature of the NK target structure. However, there are many reports showing that there exists an inverse correlation between the amount of MHC class I expression on the NK target cells and their susceptibility to NK mediated lysis (e.g. Kärre et al 1986, Kawano et al 1986, Harel-Bellan et al 1986). This effect was frequently observed when tumor cells were treated in vitro with interferon. The resulting increase of MHC expression was accompanied by a decrease in the susceptibility to NK lysis. In some systems, class I negative variants were shown to be more susceptible to NK lysis. Whereas all these studies suggested that MHC class I had a negative influence on NK lysis they did not provide direct evidence for a participation of the class I molecules. In addition, it has to be mentioned that in several other studies no influence of class I expression on NK lysis could be observed.

In order to address this question on a molecular level, from the H-2b positive thymoma EL4- an H-2 negative subline was selected by repeated treatment with anti-H-2 antibodies and complement and subsequent selection of negative cells with a cell sorter. An H-2 negative variant was obtained which was found to be deficient for β2m microglobulin. Upon transfection with the β2m gene restoration of class I expression could be achieved. The data summarized in Table 3 show that the parental and H-2 positive cell line is fairly resistant to NK mediated lysis (15-20% lysis) whereas the H-2 negative variant is much more susceptible (35-45%).

Table 3. MHC class I expression decreases NK lysis

Cell line	Transfection	Surface MHC			NK lysis (in %)
EL4	---	D^b	K^b	---	20
EL4.0	---	---	---	---	40
EL4.0β_2	β_2m, neo	D^b	K^b	---	19
EL4.0.IA	IA^b, neo	---	---	IA^b	44

From the EL4 thymoma an H-2 negative EL4.0 line was selected which had a β_2m defect. EL4.0 was transfected with β_2m neo or with IA^b a and β genes. NK cells were obtained from poly I:C (polyinosinic:polycytidylic acid) treated B6 mice (effector: target ratio= 200:1) (K. Sturmhöfel and G.J. Hämmerling, in preparation)

In contrast, the β2m transfected variant became resistant again. Transfection with class II genes had no influence on the NK lysis. These data provide strong evidence that the class I molecules themselves are directly responsible for the decreased susceptibility to NK lysis. The mechanism of this effect is not clear. A possible explanation is that MHC class I molecules associate with the unknown target structures for NK cells and either mask them or induce conformational changes. It will be of interest to determine if the antigen binding groove of the MHC molecules is involved. The direct role of MHC antigens was verified in another experiment in which the H-2 antigens on the EL4 cells were blocked with an antigen H-2b antibody (F(ab)2 fragment). EL4 cells treated this way became highly susceptible to NK lysis, whereas anti-Thy-1.2 antibodies had no effect. While this work was in progress, similar transfection studies with class I genes into NK targets have been described also by other investigators (Storkus et al 1989, Shimizu and DeMars 1989).

In conclusion, it is obvious from all these studies that MHC class I antigens can mediate two opposing effects on the rejection of tumor cells by the immune system. For CTL mediated rejection class I expression is required. In contrast, for NK mediated rejection low class I expression renders the tumor cells more susceptible. The model proposed above would also explain why not in all systems an inverse correlation between class I expression and NK lysis has been observed. Thus, it is possible that MHC modifies not all NK target structures, depending on their amount and molecular nature.

3.) REGULATION OF MHC CLASS I EXPRESSION ON TUMORS

The critical role of class I expression for tumor rejection and the high frequency of class I negative tumors prompted us to investigate the molecular nature of the class I defects. It is not surprising to observe that regulation of class I expression can be found on many different levels, ranging from pretranscriptional to posttranslational control. Some of the class I defects analysed in our laboratory are summarized in Table 4.
The IC9 cells mentioned above are a typical example for pretranscriptional control. The silent class I genes (K^b, K^k, D^k) could not be induced with IFN-gamma , 5-azacytidine, in somatic hybrids with H-2 positive cells, etc.. U. Maschek succeeded in cloning the "silent" K^b gene from IC9 cells which was found to be

functionally intact. After transfection it could not only be expressed in L cells, but also in the IC9 cell line from which it was isolated (Maschek et al 1989a). These data suggested a cis-regulatory effect for the silent K^b gene. Probably a dense chromatin structure around the silent K^b, K^k and D^k genes prevented their expression. This was investigated by digesting the DNA with DNase-I and specific restriction enzymes. For active H-2 genes, two DNase-I hypersensitive sites could be identified in the promotor region adjacent to the enhancer sequence. These DH sites were absent from the promotor of the silent H-2 genes in IC9 (Maschek et al 1989b). These data suggest that in the IC9 cells the chromatin structure is not open, thus preventing a digestion by DNase-I. However, an open chromatin structure is required for transcription because regulatory factors have to be able to bind to the promotor regions.

In many tumor lines, the silent H-2 genes could be induced with IFN-gamma or in somatic hybrids suggesting that transactivating factors required for transcription were missing (Hämmerling et al 1987).
Another class I defect was caused by the absence of β2m. For example, this was found in the EL4 variant described above and, interestingly, in most of the HLA-A,B,C negative human colon carcinomas investigated by us (Momburg and Koch 1989).

Table 4. Molecular mechanisms of MHC defects in tumors

1.) Chromatin structure not open (see text). Transcription of silent class I genes not inducible by IFN-gamma or in somatic hybrids. Examples: IC9 and IE7 fibrosarcomas; Maschek et al 1989a, 1989b

2.) Chromatin structure open. Transcription inducible by IFN-gamma and in somatic hybrids. Probably transactivating factors missing. Examples: Bc2 fibrosarcoma, CMT lung carcinoma, B16 melanoma; Hämmerling et al 1988; Klar et al 1989

3.) β_2m absent, H-2/HLA heavy chain present. Examples: Dandi, EL4.0 (see above), many HLA negative colon carcinomas (Koch and Momburg, 1989)

4.) β_2m and H-2 chains present, but no association. Association inducible with IFN-gamma. Examples: Bc2 fibrosarcoma, CMT lung carcinoma (Klar et al 1989)

An interesting posttranslational defect was observed in several cell lines in which class I surface expression could be induced with IFN-gamma. It was found that transfected H-2 genes were expressed in these cells but that the β2m and the H-2 heavy chains failed to associate. Association, which is required for transport into the Golgi, terminal glycosilation and further transport to the cell surface could be induced by the IFN (Klar et al 1989). These observations suggest that association of β2m and H-2 is not a spontaneous event but that cellular mechanisms are required.
These cells could be important for our understanding of the mechanisms involved in intracellular chain association. It will also be of interest to see whether the defect in association will have consequences for antigen processing and presentation to CTL.

4.) MHC ANTIGENS ON HUMAN TUMORS

A large percentage of human tumors of various origins have been found with deficient for HLA-A,B,C expression (in general 10-15%). The crucial question is whether the observed lack of HLA on the tumors led to a decrease of their immunogeneicity as was described for many murine tumor systems (see above). Therefore, several investigators have attempted to correlate the lack of HLA with pathological criteria. Some of these reports are summarized in Table 5. For some tumors, such as epidermal tumors, melanoma, mammary carcinoma, bladder carcinoma, B cell lymphoma, and colon carcinoma the loss of class I antigen was more frequently observed on the forms with increased malignancy. In studies where the case numbers were large enough, a statistical evaluation was performed. For the colorectal carcinoma (Momburg et al 1986) and B cell malignancies (Möller et al 1987) a significant statistical correlation between loss of class I and the malignant phenotype was observed.

These studies suggest that also in the human the loss of MHC class I antigens can contribute to their malignancy. In our large study on colon carcinomas no significant difference in the expression of HLA on metastasis and primary tumors was found. Also, in a preliminary follow up study of patients after surgical removal of the tumor no correlation between decreased HLA expression on the tumor and survival or relapse free time was evident (Stein et al 1988). These studies will have to be extended before a final evaluation is possible. For other tumors, such as cervical carcinoma, endometrical carcinoma, small cell lung carcinoma, neuroblastoma etc., no correlation was reported. For the aberrant class II expression on human tumors the situation is even less clear.

Table 5a. Correlation between deficient HLA-A,B,C expression on human tumors and the malignant phenotype

Epidermal tumors	(1) Turbitt and Mackie 1981
	(1) Holden et al 1984
Melanoma	(1) Ruiter et al 1982,1984
	(3) van Duinen et al 1988
Bladder carcinoma	(1) Walton et al 1986
B cell lymphoma	(2) Möller et al 1987
Larynx carcinoma	(2) Esteban et al 1989
Mammary carcinoma	(2) Sawtell et al 1984
Colon carcinoma	(2) Momburg et al 1986

Table 5b. Class II and human tumors

Melanoma	increased with metastasis	Bröcker et al 1984,1985 Zaloudik et al 1988
B cell lymphoma	decreased with high grade malignancy	(2) Momburg et al 1987
	decreased: poor survival	(3) Spier et al 1988

HLA-A,B,C and/or β_2m expression was determined by immunohistochemical staining. Correlation with the malignant phenotype was done by (1) comparison of malignant with benignant tumors; statistical evaluation in relationship to (2) the pathological grading, or (3) survival.

For the melanoma it was reported that class II antigens are more strongly expressed on highly invasive primaries and in metastatic legions (Ruiter et al 1985, Zalondik et al 1988). In contrast, for B cell lymphomas decreased class II expression was more frequently observed in tumors of high grade malignancy (Momburg et al 1987); and in the case of large cell B lymphomas poor survival of the patients appeared to correlate with loss of HLA-DR (Spier et al 1988).

In most of the studies on class I expression on human tumors monomorphic HLA reagents were used which did not differentiate between the individual HLA alleles. In analogy to the work with mouse tumors, where only certain H-2 alleles were found to be important for syngeneic tumor rejection (see Table 1), it is conceivable that an analysis of the human tumors with allele-specific antibodies could yield more information. In fact, Momburg et al (1989) were able to observe selective loss of HLA-A and B alleles with allele-specific antibodies in about 18% of colon carcinomas. Taken together with the 20% of colon carcinomas which have a defect in β2m expression (see above), the surprisingly high number of 38% of colon carcinomas emerges which display a class I defect. It has to be pointed out that a detailed analysis of selective expression of HLA antigens on tumors by immunohistochemical methods is hampered by the fact that so far only few suitable allele-specific HLA antibodies are available. Better reagents will be required for a more thorough analysis. The production of monoclonal HLA antibodies which are allele-specific has not been very successful, because after immunization of mice with HLA antigens usually antibodies against monomorphic HLA determinants are obtained. Therefore, we have used HLA transgenic mice for immunization because these mice are tolerant for the monomorphic HLA epitopes. Infact, we have observed that immunization of HLA transgenic mice with allo-HLA antigens lead to a very high frequency of monoclonal HLA antibodies against polymorphic determinants (Hämmerling et al 1989). It is hoped that this novel technique will not only establish a large panel of monoclonal HLA typing reagents, but that these antibodies will also be suitable for a detailed analysis of human tumors.

In conclusion, it is likely that also in human tumors changes in class I expression can influence their malignant properties. In the future a large number of tumors will have to be screened with allele-specific reagents. Only then will it be possible to assess the role of MHC antigens on human tumors in critical statistical evaluations and follow up studies.

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Immunological Analysis of Micrometastases and the Metastatic Phenotype of Human Tumors

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INTRODUCTION

Metastasis is the principle cause of death in the most common malignant diseases of man. Tumor progression and the acquisition of metastatic potential by cancer cells are ill-understood processes which evolve over years and for which adequate experimental models and commensurate in vitro systems do not yet exist. Thus it comes as no surprise that human tumors are capturing the center stage in tumor immunology, particularly with regard to metastasis. The acquisition of metastatic potential is only one of the many steps which tumor cells undergo before reaching the fully expressed malignant phenotype. The complex, extended process of tumor progression, can be divided into several different stages, generally denoted initiation, promotion and progression, and is thought to proceed by the stepwise acquisition of new characteristics (Farber and Cameron, 1980; Weinstein, 1988; Yuspa et al., 1988). Few human tumors lend themselves to the study of these stages better than cutaneous melanoma with its superficial location and pigmented nature. The first part of this chapter is concerned with the identification of molecular changes which accompany the transition from early sedentary melanoma to an advanced malignant lesion endowed with a distinct metastatic potential. The observation that de novo expression of several distinct cell adhesion molecules characterize this transition underlines the importance of novel cell-cell interactions in metastasis. One of the most critical stages in tumor progression is the manifestation of the metastatic phenotype in the formation of the earliest metastases, the micrometastases. The second part of this chapter is devoted to the identification and characterization of bone marrow micrometastasis of the most common solid tumors. These cells can be shown to be capable of proliferation and tumor formation; the demonstration of their prognostic relevance may pave the way for new therapeutic approaches to solid tumors.

PHENOTYPE OF HUMAN MELANOMA: THE IDENTIFICATION OF ANTIGENS CORRELATED WITH THE DEVELOPMENT OF METASTATIC POTENTIAL

A broad range of benign and malignant melanocytic lesions have been analysed by histopathologists, correlated to clinical outcome and ordered into a scheme which is proposed to reflect the development of metastatic melanoma (Fig.1; Clark et al., 1984; Herlyn and Koprowski, 1988). Proliferation of epidermal melanocytes gives rise to a benign melanocytic tumor, the common acquired nevus. Nevi which show architectural as well as cytologic atypia are known as dysplastic nevi and are generally held to be premalignant lesions (Green et al., 1985). Melanomas in contrast, are invasive malignant tumors. They

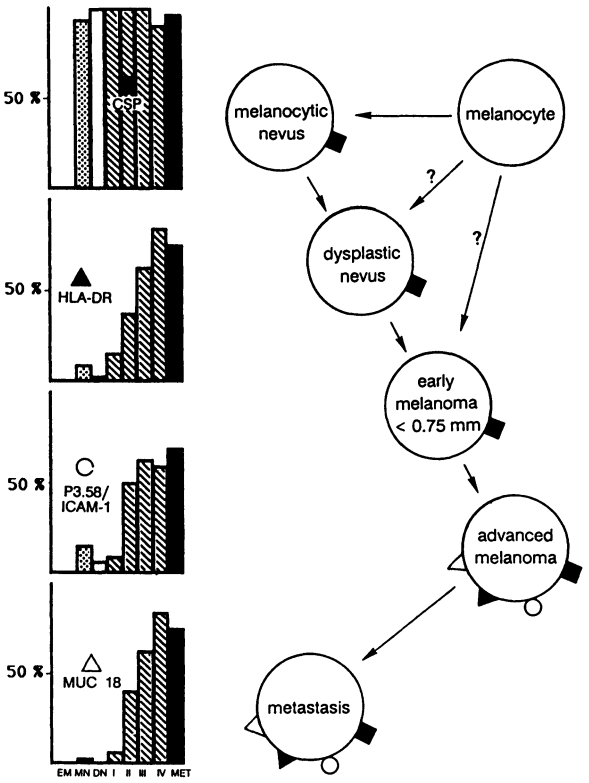

Fig. 1. Antigen phenotype of advanced human melanoma. The per cent reactive lesions is shown for MUC18, P3.58/ICAM-1, HLA-DR and CSP the melanoma chondroitin sulfate proteoglycan (Wilson et al., 1981). Lesions tested are EM (epidermal melanocytes), MN (common acquired nevus), DN (dysplastic nevus), MET (metastases) and primary tumors divided into 4 groups (I-IV) according to increasing thickness.

initially grow superficially in the dermis and during this radial growth phase remain very thin (≤ 0,75mm) showing little tendency to metastasize. In more advanced stages melanomas exhibit a vertical growth pattern, penetrating the basal membrane and invading the stratum reticulare. Since the probability of development of metastatic disease is linearly correlated with the vertical thickness of the primary tumor (Breslow, 1970), it is at the transition from the radial to the vertical growth phase where one should expect to find those phenotypic changes which reflect the acquisition of metastatic potential. In an attempt to identify such molecular changes, melanoma-reactive monoclonal antibodies (mAb) were tested for reactivity with benign melanocytic lesions and primary tumors staged according to tumor thickness (Bröcker et al., 1985; Holzmann et al., 1987; Johnson et al., 1989). A distinct reactivity pattern could be observed for each of the stages of melanoma development. Three antigens - HLA-DR, P3.58 and MUC18 - have been found which are expressed in a pattern which correlates the onset of vertical growth and with an increasing risk of metastasis (Fig. 1). While they are only rarely expressed on benign melanocytic lesions or on primary tumors in the radial growth phase, they are present at significant levels in tumors entering the vertical growth phase and generally increase in strength and frequency with increasing tumor thickness. Expression of these molecules may thus be directly or indirectly involved in the metastatic process itself.

HLA-Class II Molecules

The products of the HLA D region are constitutively expressed only by B lymphocytes and monocytes but they can be induced via lymphokines on other cell types. They are also frequently expressed on malignant cells derived from class II negative cells (Carrel et al., 1986). In melanoma, the expression of HLA-DR was found to be predictive for the early development of metastasis independent of tumor thickness (Brőcker et al., 1985). Little is known about the locus or allele specific class II expression in different stages of melanoma. Because of their role in T cell recognition, this prognostic significance raises the question of whether the immune system is involved in growth control and progression of melanoma.

P3.58/ICAM-1

In 1985 a mAb-defined cell surface glycoprotein of 89kD which is expressed selectively on advanced primary melanomas and metastases was described by Holzmann et al. (1985,1987). This antigen, called P3.58, was also found to be expressed in vivo on macrophages, vascular endothelium and in germinal centers. Since the expression of P3.58 could also be modulated on lymphocytes and melanoma cells by interferon-τ and tumor necrosis factor α (Holzmann et al., 1987), the antigen appeared to be involved in the function of the immune system. Indeed antibodies to P3.58 could partially inhibit antigen induced T cell proliferation and completely block lymphocyte-monocyte aggregation in vitro (Johnson et al., 1988a). Using a pool of four mAbs induced against the isolated and denatured antigen, Johnson et al. (1988b; 1989a) isolated cDNA clones encoding the P3.58 glycoprotein from a human melanoma lambda expression library. The sequence of the cDNA revealed that P3.58 was identical to the intercellular adhesion molecule ICAM-1 which had been cloned from myeloid and endothelial cells (Simons et al., 1988; Staunton et al, 1988). ICAM-1 is an adhesive ligand of the lymphocyte function associated molecule LFA-1 and is thought to strengthen adhesion between target and effector cells (Marlin and Springer, 1987).

Southern analysis on DNA from melanoma and autologous B lymphocytes indicates that the P3.58/ICAM-1 gene is neither rearranged nor amplified in melanoma. In addition, no qualitative differences have been found in the mRNA species expressed by melanoma and hematopoietic cells.

The MUC18 Antigen

Another antigen first detectable on primary tumors with a vertical diameter of 1mm is MUC18, a 113kD cell surface glycoprotein (Lehmann et al., 1987). MUC18 cDNA clones were isolated from a human melanoma cDNA expression library using mAbs produced against the denatured glycoprotein (Lehmann et al., 1989). Inspection of the derived amino acid sequence shows that MUC18 belongs to the immunoglobulin superfamily of cell surface molecules (Williams and Barclay, 1988). Among these molecules, MUC18 shows the greatest similarity to the neural cell adhesion molecules L1, amalgam, fasciclin II, NCAM, and myelin-associated glycoprotein, and to the human carcino-embryonic antigen CEA. All of these molecules have been shown to function as intercellular adhesion molecules and to be developmentally regulated during organogenesis (Dodd and Jessell, 1988; Benchimol et al., 1989). Based on its sequence similarity to these molecules, MUC18 may also be a developmentally regulated intercellular adhesion molecule.

Northern analysis reveals a single 3.3kb mRNA species and Southern analysis provides no indication that the MUC18 gene is rearranged in melanoma cells.

Possible Involvement of Melanoma Cell Surface Glycoproteins in Metastasis Development

The expression by melanomas of HLA-DR and P3.58/ICAM-1, two molecules instrumental in immune recognition by T lymphocytes, is associated with the development of metastatic disease. Particular combinations of antigens and class II molecules have been shown to induce suppression or a state of non-responsiveness (Baxsevannis et al., 1982; Hirayama et al., 1987) and thus a melanoma antigen restricted by HLA-DR molecules may induce preferentially CD4 suppressor-inducer cells. Metastatic melanoma cells can be in fact shown to exert a suppressive effect on the generation of autologous anti-tumor responses in vitro, an effect which is correlated with the level of tumor cell HLA-DR expression (Guerry et al., 1984; Taramelli et al., 1984). If HLA-DR molecules on melanoma cells do bias the immune response towards suppression, ICAM-1 would have a cooperative effect in this and its association with a poor prognosis would thus be explained. However, there are other ways in which ICAM-1 could potentially contribute to tumor progression of melanoma cells. MAb 7F7, directed against ICAM-1, has been shown to inhibit B lymphocyte binding of the complement component C3b (Schulz et al., 1988), suggesting that ICAM-1 may play a role in the cell surface mediated binding and subsequent inactivation of complement. In addition most primary melanomas are characterized by the presence of a mononuclear cell infiltrate consisting of LFA-1 expressing lymphocytes and monocytes. Expression of ICAM-1 by the tumor cells endows them with the ability to interact with these mobile mesenchymal cells. Depending on the strength of the ensuing heterotypic intercellular adhesion, the homotypic adhesion between tumor cells may be weakened thus facilitating the mobilization of tumor cells from the tumor parenchyma.

Although no data are yet available on the function of MUC18, it is structurally similar to a group of cell surface molecules which mediate cell adhesion through homotypic interaction. MUC18 expression in adult tissues appears limited to vascular smooth muscle (Lehmann et al., 1987). This leads to the speculation that MUC18 expression aides melanoma cells in their interaction with vascular elements and thereby facilitates their hematogeneous dissemination.

IMMUNOLOGICAL ANALYSIS OF MICROMETASTATIC CELLS

While the acquisition of the metastatic phenotype in primary tumors can be regarded as the incipient stage of the metastatic cascade, the establishment of single disseminated tumor cells in the extravascular space of distant organs signals the last phase of metastatic development. Tumor cells reaching this point have overcome formidable odds (Fidler and Hart, 1982). Not only have they completed the difficult tasks of penetration through different basal membrane layers but they have survived the turbulent vascular system and evaded immune destruction. The phenotype of these "founder" cells, which will eventually give rise to overt metastatic lesions, is clearly of great interest. Immunocytochemical techniques relying on mAbs have recently been successful in identifying these single disseminated tumor cells which have previously defied the most

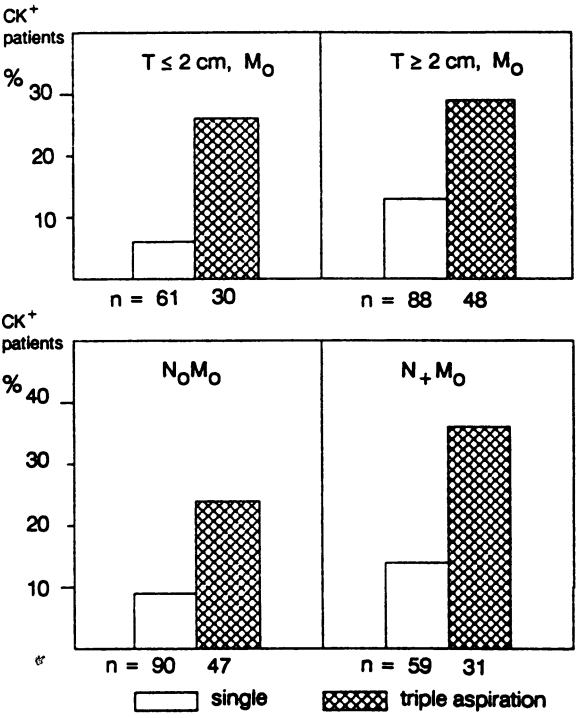

Fig 2. Incidence of breast cancer patients with CK+ cells in bone marrow. Correlation with tumor stage. Since the tumor cells are not homogeneously distributed in the marrow, multiple aspirations at different sites are required for efficient detection of tumor cells.

sophisticated physical diagnostic methods (Redding et al., 1983). Using mAbCK2 against cytokeratin 18 (Debus et al., 1982), Schlimok et al. (1986, 1987) could identify in the bone marrow, epithelial cells derived from tumors of breast, colorectum and stomach. These cells were present at a frequency of 10^{-4}-10^{-5} and, as demonstrated in Fig. 2, their incidence correlated with the tumor stage.

Since cytokeratin (CK) expression is conserved in transformed cells (Moll et al., 1982), anti CK mAb can be used to identify the tumor cells and they can then be investigated for the presence of antigens which are more heterogeneously expressed. The alkaline phosphatase anti-alkaline phosphatase staining method was combined with an ^{125}Iodine labeled antibody to examine the expression on CK+ cells of the proliferation associated markers epidermal growth factor receptor, transferrin receptor and the Ki-67 nuclear antigen. Using this double staining procedure the epithelial cells in bone marrow were found to express proliferation markers in approximately 50% of patients with advanced breast cancer. Since the Ki-67 nuclear antigen is restricted to cells moving through the cell cycle, this is strong evidence that these disseminated tumor cells are indeed proliferating (Gerdes et al., 1984; Funke et al., 1989a,b).

Considering the fact that a loss of HLA class I antigens is well documented in primary tumors and can in some cases be correlated with cellular dedifferentiation (Zuk and Walker, 1987), it was of particular interest to know whether the tumor cells in the bone

marrow expressed HLA antigens. As can be seen in Table 1, HLA class I negative, CK positive cells were observed in aspirates of 58% of all breast carcinoma patients tested. The lack of HLA class I antigens on disseminated tumor cells in the bone marrow is interesting in light of the studies of Wallich et al. (1985) who demonstrated that a highly metastatic murine lymphoma which had lost MHC class I expression could be converted to non-metastatic behavior by transfection of isolated class I genes.

Table 1. HLA Class I Antigen Expression on Cytokeratin Positive Tumor Cells in Bone Marrow of Breast Cancer Patients

HLA-class I positive[a]	HLA-class I negative	HLA-class I heterogeneous[b]
8/19 (42,1%)	5/19 (26,3%)	6/19 (31,6%)

a Defined with MAB W 6/32 in double staining procedure
b Presence of HLA^+ as well HLA $^-$ CK^+ cells

Functional Characteristics and Clinical Relevance of Micrometastases

While the phenotypic characteristics of the CK+ cells found in the patients' bone marrow were consistent with proliferating tumor cells, it was nevertheless important to try to show that they could in fact give rise to tumors. To this end, nucleated bone marrow cells were isolated by density centrifugation from breast and colorectal cancer patients and cultured in vitro. An expansion of epithelial cells, as verified by anti-CK staining, was obtained in 15 of 38 cultures and in one case a homogeneous CK^+ cell line could be established.

Tumorigenicity of these epithelial cells was tested by transplantation into nu/nu mice. Cultured cells obtained from two breast carcinoma patients and one colorectal carcinoma patient gave rise to tumors. Immunohistochemical analysis of these tumors revealed a typical histological pattern and indicated that the tumor cells expressed human HLA class I antigens. It is interesting to note that all three tumors were obtained with cells isolated from the bone marrow of patients who at the time, showed no clinically manifest metastases.

These observations raised questions about the relevance of these cells in vivo. Did patients with CK^+ cells in their marrow have a higher risk of relapse than patients without such cells? Clinical evaluation has thus far been possible in 85 colorectal carcinoma patients and in 102 patients with breast carcinoma. The median time of observation for both groups was approximately 30 months. In both groups, patients presenting with CK^+ cells at the time of primary operation had a distinctly higher risk of developing clinically overt metastasis. This is perhaps surprising in the case of the colorectal patients since the bone marrow is not a common site for metastases of this tumor. The relapse rate in the CK^+ breast cancer patients was three times higher and in the colorectal carcinoma patients two times higher ($p = 0.002$) than in the CK^- patients. Since these follow-up data were based on a single aspiration, i.e. a low sensitivity technique, the prognostic precision could well be increased by analysis of multiple aspirates. Whether patients with small primary

tumors or without regional lymph node involvement, particularly the axillary node negative breast cancer patients, are more prone to relapse when CK^+ cells are detected in their bone marrow remains to be seen.

Antibodies against membrane antigens which have been infused into patients have been shown to label epithelial cells in bone marrow (Schlimok et al., 1987). The fact that CK^+ cells can be examined in individual patients over time, opens up the possibility to monitor the effects of various therapies on this cell population and thus to correlate this with overall survival.

CONCLUDING REMARKS

Micrometastasis is a critical stage in tumor progression and these cells are ideal targets for immunotherapeutic attack. Not only is the tumor load minimal but in contrast to larger metastases they are not surrounded by basement membranes and have no extra vascular system. Therefore they are more easily accessible to macromolecular drugs like antibodies and antibody toxin conjugates. The identification of groups of markers uniquely characteristic either in a quantitative or qualitative sense, for early metastatic cells remains the ultimate goal of the phenotypic analyses of human tumors. As the studies reviewed here demonstrate, this problem is approachable through the analysis of the changing phenotypes in primary tumors as well as through the analysis of single disseminated tumor cells in the bone marrow.

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Basic Aspects of Immunotherapy of Human Tumors

H. Koprowski

INTRODUCTION

It is now almost 10 years since monoclonal antibodies (MAbs) opened new approaches to immunotherapy of cancer (Koprowski et al. 1978). During that time we have learned a great deal about antibody manipulation, antibody-tumor interaction *in vitro* and *in situ* and many other facts and factors related to antibody behavior. We have also realized the necessity to continue evaluating the existing tools of immunotherapy and to develop new ones.

In this brief review I shall try to present some of the more relevant observations which in my opinion may need to be taken into account when mapping pathways for the future of immunotherapy.

MANIPULATION OF MONOCLONAL ANTIBODIES: 1. ANTIBODY HETEROCONJUGATES

MAbs reacting with tumor associated antigens (TAAs) mediate destruction of cancer cells through action of effector cells which have receptors binding the Fc fragment of MAb. Recently it became feasible to produce antibody heteroconjugates (AHCs) consisting of an anti-CD3 T-cell antibody and anti-tumor cell target antibody. These AHCs were found to activate human cytolytic T cells (CTLs) and natural killer cells (Scott et al.1988). It was further found that cancer cells (target cells) coated with anti-target AHCs, which are also reactive with CD3 and CD 2.8 receptors of T cells, function as accessory with the T cells (Jung and Muller 1989) in inducing cytotoxic efforts against the tumors in question. This approach could have clinical applications in arming tumor-specific cytotoxic tumor-infiltrating CTLs after their *in vitro* expansion.

MANIPULATION OF MONOCLONAL ANTIBODIES: 2. IMMUNOTOXINS

Following first construction of immunotoxin with ricin A chain conjugated to TAA 17-1A (Gilliland et al. 1980) many experiments were conducted using either ricin or diptheria A chains as components of immunotoxins. The ricin toxin which was and is used preferentially as a component of immunotoxin is an extract of the castor plant (*Ricinus communis)* consisting of two 30 kDa subunits, an A chain (referred to as RTA) which inhibits protein synthesis (Ross et al. 1980) and a B chain which reacts with surface receptors of most mammalian cells permitting the entry of RTA (Endo and Tsurugi 1987).

Through conjugation of RTA to MAbs reacting with various receptors of lymphocytes, it was possible to kill selectively various subpopulations of malignant but also of normal lymphocytes *in vitro* (Jansen et al. 1982). Use of immunotoxins in treatment of solid tumor was limited to animal models (Manske et al. 1989) and limited clinical trials in melanoma patients (Harkonen et al. 1987) with unclear results. Since serious neurotoxic manifestations were observed in patients treated with RTA immunotoxin for breast cancer (Spitler et al. 1987) the future use of the immunotoxin may require modification of its structure.

MANIPULATION OF MONOCLONAL ANTIBODIES: 3. CHIMERIC ANTIBODY

Induction of anti-mouse antibody in the sera of patients after treatment with mouse MAb may be obviated by the construction of a mouse-human chimeric antibody (Morrison et al. 1984) through joining DNA containing the productively rearranged mouse variable light and heavy chain genes with the human constant region genes of the light and heavy chains (Figs. 1 and 2).

This approach has been successfully applied to several mouse MAbs reacting with various TAAs (Sahagan et al. 1986; Nishimura et al. 1987; Liu et al. 1987). In all cases, the chimeric antibody retains all the characteristics of the murine antibody but, in mediating destruction of cancer cells, the chimera containing human IgG1 was the most effective. As expected, the immune response to mouse globulin of patients receiving chimeric MAb was minimal or absent.

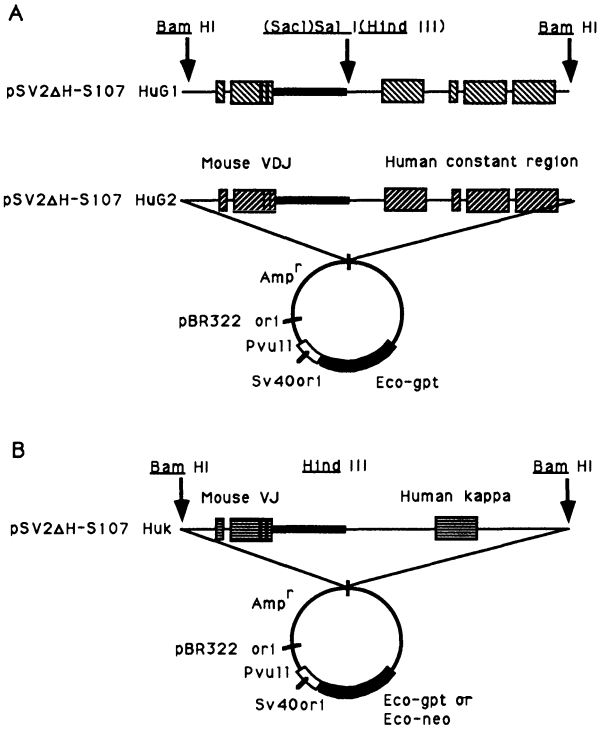

Fig. 1. Structure of chimeric gene. Insertion of cloned mouse V gene into plasmid containing human gene.

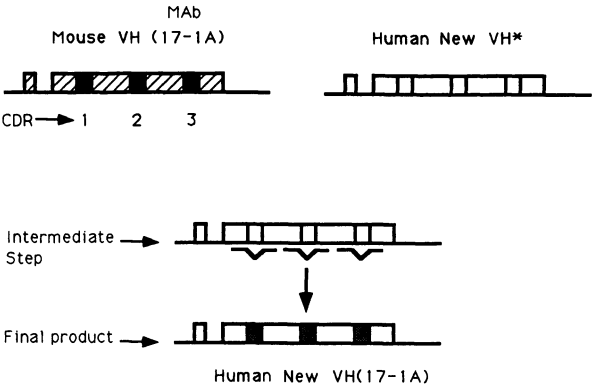

Fig. 2. Schematic presentation of formation of chimeras with heavy chain genes of mouse and human immunoglobulin. The 3 heavily-shaded areas represent the 3 CDR regions which determine antigen specificity of the mouse VH region. First step, anneal 3 oligonucleotides to the human gene. Encode the mouse VH 17-1A CDR regions as well as matching sequence either side to the human gene. Final step shows human gene now with the CDR regions from mouse VH 17-1A, demonstrating the binding specificity of 17-1a Ab. *Analyzed by x-ray crystallography.

One group (Reichmann et al. 1988) has "humanized" rat MAb in a different way. After constructing rat-human chimeric Ab genes, they converted by site-directed mutagenesis, the rat framework regions to encode human framework amino acids. After a minor adjustment of one amino acid, the humanized Ab retained the binding specificity of the native Ab.

Genetic engineering now makes it feasible to produce antigen-binding Ab with widely varying structures so that its function can be optimized for use in diagnosis and immunotherapy.

INTERNALIZATION OF MABS AND DESTRUCTION OF CANCER CELLS FROM WITHIN

Although most anti-TAAs MAbs, particularly those recognizing carbohydrate determinants, react only with antigenic sites at the cell surface, a small fraction of MAbs are internalized and are present on chromatin (Table 1).

Table 1. Intracellular distribution of MAbs in cells of different tumor lines

Antigen recognized by the MAb	Target cell	MAb molecules/cells	
		Cytoplasm	Chromatin
EGF	Carcinoid	180,000	2,100
Tumor-associated antigen			
Gastrointestinal cancer	Colorectal cancer	222,865	7,050
Melanoma	Colorectal cancer	32,000	9,250
Breast cancer	Breast cancer	175,840	27,530

The study of one such antibody shows that it can be recovered from chromatin in a non-degraded form and bound to 55 kDa protein localized in two HindII-digested fragments (Rakowicz-Szulczynska and Koprowski 1989). The MAb inhibits transcription of ribosomal RNA genes and interferes with growth of the target cells.

In another study nuclear uptake of ^{125}I-labelled MAb directed against TAA of gastrointestinal cancers resulted in specific [for the colorectal cancer (CRC) target cells] irreparable cell damage and cell death (Woo et al. 1989). The characteristic radiation damage of the chromosomes consists of chromosome breaks. No nuclear uptake was observed after exposing cells to the same radionuclide conjugated with an irrelevant MAb.

^{125}I radionuclide-associated with TAA-reactive MAb, which is internalized, was and is used in cancer immunotherapy (Woo et al. 1988). The results depend in large measure upon the vascularization of the cancer bed and the resulting accessibility of a large majority of tumor cells to intracellular penetration by radionuclide-MAb conjugates. If only a fraction of the tumor cell population is accessible to the MAb internalization, the chances are less that the tumor progression will be interrupted by radiation and cell death.

CANCER ANTIGENS AS VACCINES FOR HUMANS

Use of human cancer tissue as implants into healthy human subjects has a long history. Since the late 18th century, surgeons implanted mostly into themselves and occasionally into their colleagues, fragments of cancer tissue removed from a patient (cited by Altman 1986). Domagk, the discoverer of sulfa drugs, was probably the first who injected himself in the course of 10 years with "sterilized" extracts of cancer tissue obtained at autopsy in order to study anti-cancer immunity (Altman 1986; Domagk 1942). Although he did not develop cancer, it was clearly impossible to evaluate the immunological response to the cancer tissue.

Injection of cancer patients with sterilized cancer tissue obtained mostly from the patient's own tumor, is still practiced today. But the difficulties of evaluating a meaningful immunological response in this procedure, except for clinical follow-up, are enormous. The plethora of tumor-associated and normal tissue antigens in the inoculum prevents even a perfunctory search for an antibody or antibodies which would show any degree of specificity. The same reservations apply to the search for specificity in cellular response.

There are two ways to assure a specific response. The TAA can be cloned or an anti-idiotype carrying the internal image of the TAA can be produced and both could act as immunogens.

Let us first discuss the cloning of TAA. ME491 is a TAA which is expressed by cells of nevi and human melanoma at the early stages of malignancy (Atkinson et al. 1984). It is not expressed by normal melanocytes but is expressed by adenocarcinoma cells of the colon and prostate (Ernst et al. 1986). ME491 is membrane-bound glycoprotein detected intracellularly (see above) and at the cell surface. The molecular weight ranges from 30,000 to 60,000 with a single core protein

of 20,000 (Ross et al. 1985). A monoclonal antibody, MAb 491, reacting with the antigen permitted the detection of antigen secreted by mouse L-cell fibroblasts transfected with high molecular weight human DNA obtained from either Hela cells or cells of a human melanoma. Using secondary transformants of L cells it was possible, by probing Alu human repetitive sequences, to molecularly clone the ME491 gene (Hotta et al. 1988) (Table 2). After transfection with the cloned gene, the L cells express an antigen indistinguishable by Western blot analysis from ME491 expressed by melanoma cells. Nucleotide sequences of cloned cDNA to ME491 indicate a sequence of 272 amino acids. No structural homology between this sequence and other proteins has been so far reported. The ME491 gene was localized on human chromosome 12 in the region 12q12→12q14 (Hotta 1988).

Cells of murine melanoma B16 line could be transfected successfully with human genomic fragments encoding the ME491 antigen. The transfectants may be used effectively as target cells in immunological assays. Less successful were attempts to identify the immunogenic peptide of MAb 491 since, although two out of three peptide sequences corresponding to amphipathic helices induced antibody reacting with the corresponding peptide, it failed to react with the human melanoma cells which expressed the ME491 antigen.

Molecular cloning of another TAA presented a more challenging problem, as the antigen was found to be a member of a family of genes. The antigen in question, GA 733, is expressed by cells of adenocarcinomas of gastrointestinal tract and also by some adenocarcinomas of cervix, bladder and lung (Herlyn et al. 1986).

Monoclonal antibodies reacting with the antigen immunoprecipitated a 40 kDa glycoprotein from the surface of cancer cells. From the purified antigen it was possible to determine its partial amino acid sequence

Table 2. Recombinant DNAs for tumor-associated antigens.

Antigen	Expression	Cloning strategy	Reference
ME 491	Dysplatic nevi RGP 1º melanoma Colon Ca.; Prostate Ca.	Gene transfer ↓ Genomic clone ↓ Melanoma cDNA	Hotta, 1988
GA733-1	Pancreatic Ca. Placenta	Oligomer probe ↓ Genomic clone ↓ Placental cDNA	Linnenbach, 1989a
KSA	Several Carcinomas	Polyclonal Ab ↓ Lung Ca. cDNA	Strnad, 1989
		Oligomer probe ↓ Lung Ca. cDNA	Perez & Walker, 1989

(Linnenbach et al. 1989a). Based on this sequence information, an oligonucleotide probe was synthesized and used to screen a human genomic library (Linnenbach 1989a). The original genomic isolate was found to encode a protein with sequences similar to, but not identical with, antigen derived from colorectal cancer cells. Thus, GA 733 was found to be a member of a family of at least two genes; the gene identified first was referred to as GA 733-1. The predicted amino-acid sequence of GA 733-1 consisted of 323 amino acids with 4 potential N-linked glycosylation sites and a protein molecular mass of 35.7 kDa. The GA 733-1 gene was intronless and was much more strongly transcribed in cells of pancreatic carcinoma or placenta than in cells of colorectal carcinoma (Table 2). The GA 733-1 gene was found to be located on human chromosome 1 between 1p36 and 1q12 regions (Linnenbach et al. 1989b).

Two separate laboratories have isolated cDNA clones for a second member of the GA 733 gene family (Table 2). The MAb KS1/4, which has a specificity similar to that of the GA 733 MAb, was used to affinity purify antigen from the human lung adenocarcinoma cell line. The cDNA libraries constructed from these cells were screened by antigen expression with a polyclonal antibody that had been raised against affinity purified antigen (Strnad et al. 1989) or by an oligonucleotide probe that was based on partial protein sequence data (Perez and Walker 1989). The sequence of the cDNA clones predict a protein 314 residues in length with 3 potential N-linked glycosylation sites and a mass of 26.4 kDa identical with the sequence of GA 733 antigen purified from the SW 948 colorectal carcinoma cell line (Fig. 3). Thus, the second member of the GA 733 family, designated GA 733-2 (Linnenbach 1989b), is likely to be the same

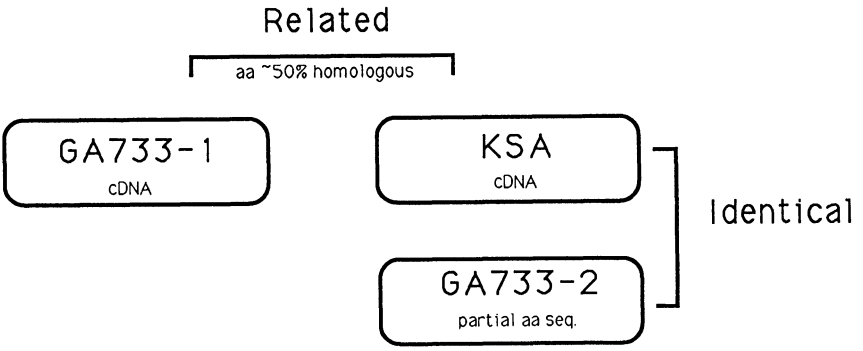

Fig. 3. GA 733 antigen gene family.

antigen as the one defined by the KS1/4 antibody Cloning of these antigens took too much time but it is now possible using the High-Efficiency COS cell expression system (Aruffo and Seed 1987) to clone the complete gene of the TAA. Even if tumor-associated genes are on hand, we are still a long way from their use as immunizing agents. One impediment is the problem of finding a suitable vector: bacterium, yeast, or a recombinant with a virus such as vaccinia, or several others. Only with the right vector will it be possible to investigate immune response to the specific TAAs and evaluate their role as meaningful tools in cancer immunology. Bearing in mind these reservations, one must today turn his attention to the study of the internal image of the antigen as expressed in an anti-idiotype.

INTERNAL IMAGE OF THE HUMAN TUMOR-ASSOCIATED ANTIGEN (TAA)

In the analysis of the results of passive immunotherapy of cancer patients with TAA-reactive MAb, some association between the clinical response to immunotherapy and appearance of an anti-idiotype to the TAA-MAb in sera of the patients seems to be observed in two separate clinical trials (Koprowski et al. 1984; Steinitz et al. 1988). These statistically insignificant observations gave rise, however, to the idea of producing an idiotypic cascade in cancer patients aiming at the induction of an Ab_3 after immunization with TAA-image carrying Ab_2. Induction of tumor-immunity by Ab_2 in experimental animals is the subject of only a few studies (Herlyn and Koprowski 1988) and the results are not very easy to evaluate. Since the initial aim of these studies was to rely on responses of the B-cell component to immunization with possible stimulation of the cells *in vitro*, it seemed proper to undertake such studies in cancer patients. The ultimate aim of these studies is to have patients produce their own antibodies which would mimic experimentally-produced TAA-reactive MAbs and ultimately control the patient's own tumor.

First of all, however, it was necessary to establish procedures to produce Ab_2 carrying the internal image of the TAA. Following production of two polyclonal goat Ab_2s reacting with two related TAAs of the cancer gastrointestinal tract and of the development of monoclonal Ab_2, it was possible to induce with these antibodies responses in immunized rabbits and/or mice (Table 3). These antibodies shared idiotypes with the Ab_1s and showed

Table 3. Properties of polyclonal and monoclonal Ab_2's

Anti-idiotype Ab_2		Ab_3		
	Percentage		Binding to:	
Species	Mimicking of TAA	Species	Ab_2	CRC cells
		Rabbit	+	+*
Goat polyclonal	± 26	Mouse	+	+
		Human	+	+*
Rat monoclonal	± 66	Rabbit	+	+**

* in purified serum immunoglobulin
** in unprocessed sera

identical specificity in binding to CRC cells (Table 3). These results were convincing enough to consider the two Ab_2s (Table 3) as carrying the "internal image" of the TAA and the decision was reached to undertake a clinical study with one of the Ab_2s. It was important however, to also investigate whether activation of the idiotypic cascade in the cancer patients would result not only in induction of Ab_2 but also in the formation of Ab_3. Peripheral blood mononuclear cells (PBMC) obtained from five patients with pancreatic cancer and injected once with TAA-binding MAb 17-1A were stimulated *in vitro* by MAb CO17-1A (Wettendorff et

al. 1989). The cells produced an Ab_2 binding to the Ab_1 which inhibited binding of Ab_1 to the polyclonal goat but did not bind to irrelevant MAb Ab_2 (Table 4) (Wettendorf et al. 1989).

Table 4. Responses of PBMCs obtained after treatment of cancer patients with C017-1A MAb

Stimulation of PBMCs	Ab_3 binding to: TAA	Ab_2	Goat IgG	% CRC	% Inhibition of binding by Ab_3 of: $Ab_2 \rightarrow Ab_1$	$Ab_1 \rightarrow$CRC
MAb C017-1A (Ab_1) or C017-1A purified antigen	+	+	-	25-40	10-30	30-60
Normal goat globulin	-	-	-	0-5	nil	± 10

Stimulation of healthy donors did not produce such antibodies and the patients' PBMC were nonreactive to stimulation by normal mouse globulin. The patients thus produced an Ab_2. Conversely, *in vitro* stimulation of PBMC of the five patients with either purified TAA or with polyclonal goat Ab_2 resulted in production of antibodies which bear all characteristics of Ab_3 (Wettendorff et al. 1989); they bind to the purified TAA and to the Ab_2, but do not bind to purified normal IgG (Table 4). These antibodies bind also to colorectal cancer cells but not to human cells of other origin; they inhibit binding of Ab_2 to Ab_1 and of Ab_1 to colorectal cancer cells (Table 4). The antibodies do not bind, or bind only minimally, to normal goat globulin. Based on these characteristics it can be safely assumed that these are Ab_3s and that treatment of pancreatic cancer patients with MAb 17-1A resulted in the induction of at least two components of the idiotypic cascade, an Ab_2 and an Ab_3.

Parallel to these studies, 30 patients with advanced metastases of colorectal cancer were immunized with a polyclonal Ab_2 produced by immunizing goats with MAb 17-1A (Herlyn et al. 1987). The patients developed an Ab_3 which could be detected in their blood between 6 and more than 239 days after immunization. The Ab_3 bound to Ab_2 (see above) and inhibited binding of MAb 17-1A to TAA. The production of Ab_3 represented 56 to 79% of the total immune response of all patients and one may conclude from the results of partial inhibition of binding of Ab_2 to Ab_1 by Ab_3, that Ab_3 shares some but not all Ab_1-related idiotypes identified by Ab_2 paratopes.

Because several of the patients were in terminal status it was difficult to evaluate the clinical response, although stabilization of tumor growth seemed to occur in seven of the patients.

Availability of two monoclonal antibodies, an Ab_1 reacting with TAA of gastrointestinal cancer and Ab_3 produced by immunization of mice with polyclonal anti-idiotype bearing internal image of the antigen (see

above), made it possible to compare nucleotide and amino acid sequences of the V region of the two MAbs. The most interesting observation (Caton et al. 1989) was that the two antibodies express identical H chain CDR3 amino acid sequences; as it is known the CDR3 region is encoded by the D (diversity) gene segment, which is the most variable region for different antibodies. The confirmation of findings was obtained through sequencing of another Ab_1 reactive with the same TAA as the first MAb. Again, the same CDR3 sequences were observed suggesting perhaps that specific sequences play an important role in defining specificity of the group of MAbs for the given TAA (Caton et al. 1989).

ENVOI

After 10-12 years of dealing with the many confounding problems of immunotherapy of human tumors, I sometimes feel frustrated about the slow pace of progress in this field.

In order not to develop "Selbstwertzweifel" or even "Minderwertigkeits-komplex" I refer you to the poem by Wilhelm Busch.

> "Früher, als ich unerfahren und bescheidener war als heute,
> Hatten meine höchste Achtung andere Leute,
> Später traf ich auf der Weide ausser mir noch andre Kälber
> Und nun schätz ich, sozusagen erst mich selber."

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T Cell-Mediated Tumor Regression in Experimental Systems

R.J. North, M. Awwad, and P.L. Dunn

INTRODUCTION

It was established in the 1950s and 60s (Old and Boyce 1964; Sjögren 1965; Klein 1966; Hellström and Hellström 1969) that murine tumors can possess transplantation rejection antigens capable of evoking an antitumor immune response in their syngeneic hosts. It was also established that antitumor immunity is cell-mediated. In the 1970s a great deal of effort led to the realization that, in spite of their possession of rejection antigens, immunogenic tumors are highly resistant to attempts to cause their regression by immunotherapeutic modalities that employ immunoadjuvants (Bast *et al.*, 1976). Consequently examples of successful active immunotherapy of immunogenic tumors are rare. In contrast, it has become almost routine to cause the regression of established murine tumors by adoptive immunization with immune T cells from immunized donors. The convincing success of adoptive immunotherapy serves to demonstrate that immunogenic tumors fail to undergo regression because of a shortfall in host effector lymphocytes, rather than because of some tumor-mediated anti-host mechanism that blocks the expression of immunity. It is perhaps time again, therefore, to work at discovering ways to stimulate the host itself to produce therapeutic numbers of effector lymphocytes, rather than to supply it with T cells from immune donors, or with tumor-infiltrating lymphocytes expanded in number *in vitro*. It will be argued here that, in order for anti-tumor immunity to be augmented to a therapeutic level, it is necessary, in the case of some tumors, to remove the negative regulatory influence of tumor-induced suppressor T cells. Indeed, it needs to be realized that removing the influence of suppressor cells is also a prerequisite for successful adoptive immunotherapy as practiced at present.

TUMOR GROWTH EVOKES THE GENERATION OF SUPPRESSOR T CELLS THAT DOWN-REGULATE CONCOMITANT IMMUNITY.

It has been shown in the case of several tumors (North 1985) that progressive tumor growth evokes the generation of a host concomitant immune response that eventually decays under the influence of suppressor T cells. Concomitant immunity was measured in terms of the acquisition of T cells capable of passively transferring antitumor immunity to appropriate recipients (North 1984a; North and Bursuker 1984; North and Dye 1985), or in terms of the acquisition of T cells cytolytic for tumor cells *in vitro* (North and Dye 1985). It was found that tumor-immune T cells were generated between about days 6 and 9 of tumor growth, but after this time they progressively disappeared from lymphoid tissues. This loss was associated with the progressive acquisition of dominant numbers of T cells with immunosuppressive ability. Such tumor-induced suppressor T cells, assayed for by their ability to suppress the expression of T cell-mediated

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adoptive immunotherapy in tumor-bearing recipients, were found to be of the L3T4 phenotype.

T CELL SUPPRESSORS OF CONCOMITANT IMMUNITY SUPPRESS ADOPTIVE IMMUNITY

It stands to reason that the presence in a host of suppressor T cells in the process of down-regulating a recipient's concomitant immune response would also down-regulate passively transferred donor immunity. Indeed, it seems not to be generally appreciated that successful adoptive immunotherapy of established tumors requires that the tumor-bearing recipient be treated with cyclophosphamide (Cy), or exposed to sublethal ionizing radiation, before immune T cells are infused (North 1982; 1984b). Otherwise, adoptive immunity is not expressed and tumor regression fails to take place. This rule also applies to adoptive immunotherapy of murine tumors with TIL (Rosenberg *et al*; 1986). The conclusion reached, therefore, is that a physiologic barrier exists in a tumor bearer which blocks attempts to cause regression of its tumor by adoptive immunotherapy.

Results generated in this laboratory point to tumor-induced suppressor T cells as being responsible for blocking the expression of adoptive immunotherapy. It was shown that the ability of irradiation (North 1984b) and Cy (North 1982) to facilitate the expression of adoptive immunotherapy could be negated by infusing the tumor-bearing recipients with T cells from donor mice in which concomitant immunity was in the process of undergoing T cell-mediated suppression. It was shown more recently (Awwad and North 1987) that the T cells that, on passive transfer, suppress adoptive immunotherapy are of the L3T4 phenotype, in that they are destroyed by treatment with anti-L3T4 mAb and complement. This is in keeping with the results of an ongoing study which shows that the barrier to adoptive immunotherapy can be removed by selectively depleting the tumor-bearing recipient of $L3T4^+$ T cells, but not of $Ly\text{-}2^+$ T cells. Experimental results showing this immunofacilitating action of anti-L3T4 mAb are presented in Fig. 1.

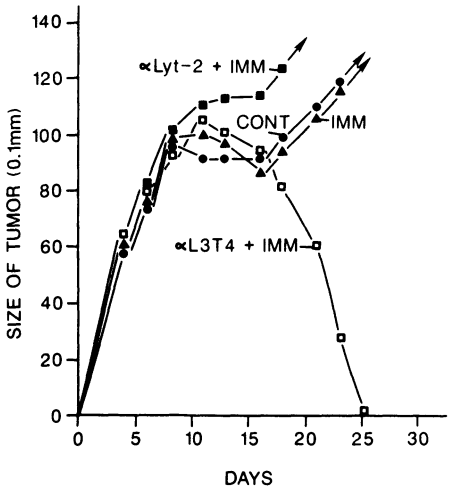

Fig. 1. Evidence that the barrier to adoptive immunotherapy can be removed by treating the recipient with anti-L3T4 mAb (αL3T4 + IMM). In contrast, infusion of immune T cells into untreated recipients (IMM), or into recipients treated with anti-Ly-2 mAb (αLy-2 + IMM) failed to cause tumor regression. All mice were thymectomized 2 weeks earlier and mAbs given 10

days earlier. Donor immune T cells were given on day 4. Means of 5 mice per group.

ELIMINATION OF SUPPRESSOR T CELLS CAN RESULT IN SPONTANEOUS TUMOR REGRESSION.

Given the evidence that failure of the host to generate enough effector T cells to cause regression of its immunogenic tumor is due, at least in some cases, to the tumor-induced production of a dominant number of suppressor T cells, it is logical to predict that selective removal of suppressor cells should result in tumor regression. This prediction was recently successfully tested in mice bearing the immunogenic L5178Y lymphoma. Three different agents were used to selectively eliminate suppressor T cells and cause tumor regression. They were anti-L3T4 mAb, Cy, and the antimitotic drug, vinblastine (VB). Anti-L3T4 mAb was chosen because the results of pilot studies showed that immunity to the L5178Y is mediated exclusively by $Ly\text{-}2^+$ T cells. In addition, the results of already published studies demonstrated (Awwad and North 1987) that suppressor T cells are exclusively of the L3T4 phenotype. Cy was chosen because of the general knowledge that suppressor T cells are Cy sensitive, and because the L5178Y lymphoma is highly Cy resistant.

It was found (Awwad and North 1988) that depleting mice of $L3T4^+$ T cells by infusing them on day 9 of tumor growth with anti-L3T4 mAb resulted after a short period of delay, in complete tumor regression and in long-term host survival. It was shown, in addition, that treating the tumor bearer with anti-Ly-2 mAb, or with both mAbs, resulted in a significant increase in the rate of tumor growth, and in a much shorter host survival time. These results, plus the finding that tumor regression in mice treated with anti-L3T4 mAb was mediated by an augmented number of $Ly\text{-}2^+$ effector T cells, justifies the conclusion that removal of $L3T4^+$ suppressor T cells enabled $Ly\text{-}2^+$ effector T cells to expand to a therapeutic number.

In the case of Cy-induced regression of the L5178Y lymphoma it was necessary to give a single 100mg/kg dose of the drug during the early stages of tumor growth. This resulted, after a 7-10 day delay, during which time the tumor grew to a relatively large size, in complete tumor regression (Awwad and North 1989). A key additional finding was that the therapeutic action of Cy failed to occur in mice that had been made immunoincompetent. Again, the ability of Cy to cause tumor regression was completely negated by infusing the host, immediately after giving Cy, with $L3T4^+$ T cells from a donor bearing a well established L5178Y tumor. Cy-induced regression of the L5178Y lymphoma resulted in long-term host survival.

The decision to attempt to use VB to cause immunologically mediated regression of the L5178Y lymphoma was prompted by our failure to cause regression of an advanced L5178Y lymphoma with Cy. It has been shown by others (Mokyr and Baker 1986) that Cy can cause regression of a large MOPC-315 plasmacytoma. In the case of this tumor, however, immunity is aided by the ability of Cy to cause a substantial reduction in tumor burden by direct cytotoxic action. No such reduction occurs in case of the L5178Y lymphoma, because it is highly Cy resistant. Therefore, it was decided that a small dose of VB be given to attempt to cause a reduction in tumor burden, prior to giving Cy to destroy suppressor T cells. As it turned out treatment with Cy was not needed, because treatment with VB alone resulted in complete regression of a large L5178Y lymphoma (Fig. 2).

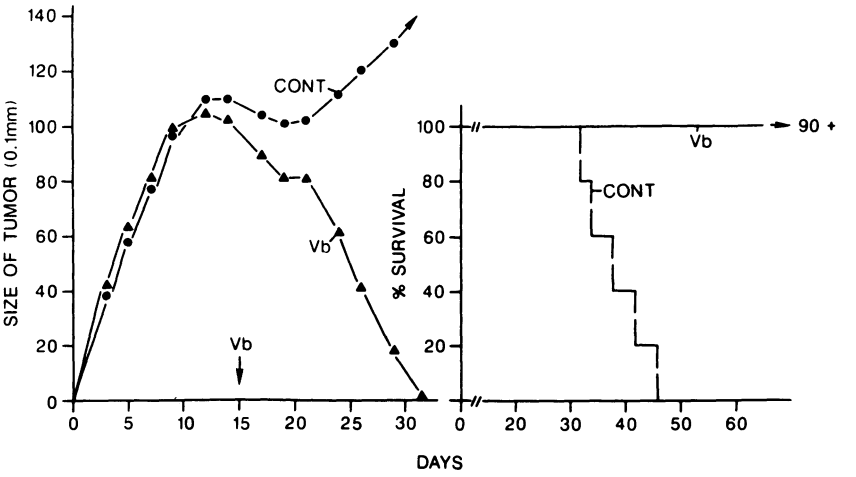

Fig. 2. Therapeutic effect of giving mice bearing a 15 day L5178Y lymphoma a single 100μg dose of vinblastine (Vb). The primary tumor and liver metastases underwent complete regression (left panel), resulting in long-term host survival (right panel). Tumor regression failed to take place in T cell-depleted mice (result not shown). Means of 5 mice per group.

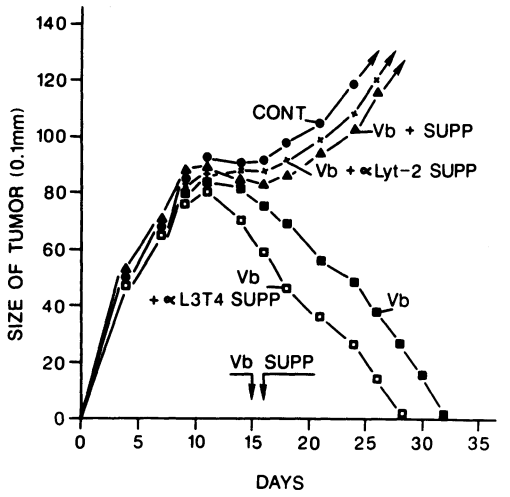

Fig. 3. Therapeutic effect of vinblastine against an advanced L5178Y lymphoma (Vb) was reversed by infusing the host with splenic T cells from a donor bearing a 15 day tumor (Vb + SUPP), but not if the donor T cells were first treated with anti-L3T4 mAb and complement (Vb + αL3T4 SUPP). Means of 5 mice per group.

However, VB was therapeutic only if the tumor was growing in immunocompetent mice. Thus VB-induced regression was immunologically mediated, and the results of additional experiments revealed that the immunity was mediated by Ly-2$^+$ T cells. The therapeutic action of VB could be negated, however, by infusing the VB-treated host with L3T4$^+$ T cells from a donor with an advanced tumor (Fig. 3). These and other results (to be published) show that VB is therapeutic against an advanced L5178Y lymphoma because it destroys actively replicating suppressor T cells, and spares resting Ly-2$^+$ effector T cells. Presumably, these effector T cells, on release from suppression, rapidly undergo activation in response to tumor antigen.

CONCLUSION.

In spite of an apparent reluctance these days to be enthusiastic about models of T cell suppression, the physiologically convincing, functional evidence summarized here, showing that L3T4$^+$ suppressor T cells play a central negative regulatory role in the immune response to immunogenic tumors, cannot be ignored. These suppressor T cells are generated progressively after a tumor grows beyond a certain size, and their selective elimination results in the generation of an increased level of immunity and in tumor regression. Published evidence indicates, moreover, that these suppressor T cells represent a barrier to successful adoptive immunotherapy with immune T cells.

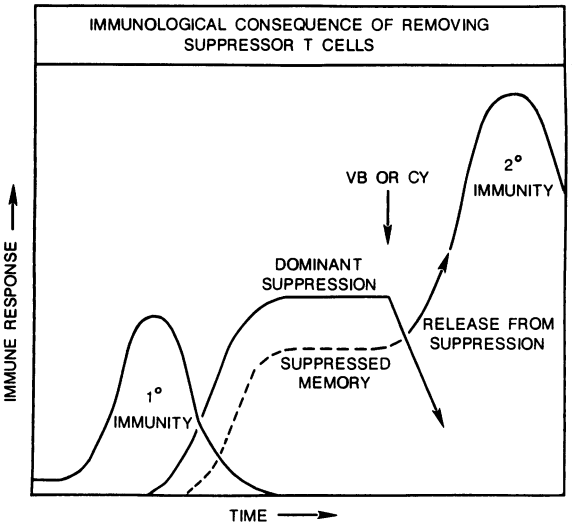

Fig. 4. Diagrammatic representation of the immunotherapeutic consequences of removing suppressor T cells from a tumor bearer whose concomitant immune response (IMMUNITY 1°) has been negatively regulated. It is suggested that the decay of concomitant immunity is associated with conversion of effector T cells into suppressed, resting memory T cells. Removal of replicating suppressor T cells by treatment with vinblastine (VB), or cyclophosphamide (Cy), releases memory cells from suppression and allows them to give rise to an augmented secondary immune response (IMMUNITY 2°) that is driven by an adequate quantity of tumor antigen.

These findings about immunity to tumors should be viewed in conjunction with published results (Pearce *et al.*, 1989; Quigley *et al.*, 1989) of physiological studies of induced unresponsiveness to allografts in rats where unresponsiveness was shown to be passively transferred with replicating $CD4^+$ suppressor T cells. Again it has been shown (Hill *et al.*, 1989) that susceptibility of certain strains of mice to leishmaniasis is caused by the production of dominant numbers of $CD4^+$ suppressor T cells.

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Apoptosis in Monoclonal Antibody-Induced Tumor Regression

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INTRODUCTION

A variety of cell surface molecules are involved in growth control of normal and malignant cells and transmit lateral or vertical signals regulating cell replication. It is the purpose of our experiments to define such molecules and their function. Therefore, we raised monoclonal antibodies in mice against the malignant human B cell line SKW6.4 and tested them for growth inhibition of SKW6.4 cells *in vitro*. The following article describes the activity of a selected monoclonal antibody, anti-APO-1, which induces growth inhibition of SKW6.4 and a variety of other cells by induction of programmed cell death (apoptosis).

RESULTS AND DISCUSSION

After repeated injection of $1x10^7$ SKW6.4 cells (once per week, four times) into Balb/c mice, spleen cells were used for fusion (Köhler and Milstein 1975) and the resulting hybridoma supernatants screened for growth inhibition of SKW6.4 cells in 200 μl cultures. Between 10 - 15,000 hybridomas were screened and one monoclonal antibody, named anti-APO-1 (IgG 3, κ) was selected because it had the most drastic effect on growth of SKW6.4 cells. By Scatchard analysis we determined that the antibody recognized about 40,000 epitopes on SKW6.4 cells with high affinity ($K_D = 1.9x10^{-10}$). Anti-APO-1 precipitated a protein antigen of a molecular weight of 52,000 which we called APO-1. We also saw coprecipitation of a minor 25 kD band which is either a breakdown product of APO-1 or another noncovalently associated protein. Anti-APO-1 showed characteristic effects on SKW6.4 cells in culture and induced a process which morphologically appeared like programmed cell death, apoptosis. Apoptosis is the most common form of death in eukaryotic organisms (Duvall and Wyllie 1986). It is observed in embryogenesis and metamorphosis, whenever old tissue is replaced by new tissue, in tissue atrophy, and in tumor regression. Morphologically it is characterized by fragmentation of the nucleolus, segmentation of the nucleus, condensation of the cytoplasm, zeiosis (membrane blebbing), and biochemically by a fragmentation of genomic DNA into multimers of 180 bp by activation of an endonuclease which cuts the DNA between the nucleosomes (Wyllie 1980). We found apoptosis in various malignant T and B cell lines after incubation with anti-APO-1 for 1 - 24 hrs. Such cells show a reduced growth rate. In fact, 1 - 10 ng/ml of anti-APO-1 reduce growth of SKW6.4 cells and other malignant T and B cells *in vitro* by over 95%. A variant of SKW6.4 cells selected by prolonged growth *in vitro* under antibody pressure with anti-APO-1 did not undergo apoptosis even at concentrations of anti-APO-1 which were about 25,000 times higher than used for the wildtype SKW6.4 cells.

We next wanted to investigate the signal requirements for anti-APO-1 and found that crosslinking of the APO-1 antigen is necessary for apoptosis to be induced. This is also supported by the fact that 4 - 5 times less anti-APO-1 antibody is needed for induction of apoptosis when protein A as an Fc-binding crosslinker is mixed to the culture. In addition, anti-APO-1 immobilized on beads was perfectly capable of inducing apoptosis. This indicates that anti-APO-1 is not internalized for induction of apoptosis. These data suggest that the APO-1 antigen when triggered by the anti-APO-1 antibody delivers a signal which leads to the induction of the endogenous suicide process.

We also investigated the representation of the APO-1 antigen on normal cells and found that APO-1 is absent from the majority of resting T and B lymphocytes. However, after activation of T lymphocytes with PMA/PHA and Interleukin-2 the APO-1 antigen appears on the cell surface after about one day of activation. Only a slight additional increase of expression of APO-1 on the cell surface is observed on day 6 of activation. Day 1- and day 6-activated T cells, however, show a considerable difference in sensitivity for induction of apoptosis by anti-APO-1. Whereas hardly any apoptosis occurs on day 1-activated T cells, apoptosis is induced on day 6-activated T cells. Likewise, the majority of resting B cells is negative for APO-1 expression. B cells activated with pokeweed mitogen, however, express the APO-1 antigen and after incubation with the anti-APO-1 antibody cease to secrete IgM, probably due to induction of apoptosis. Apart from activated lymphocytes, the APO-1 antigen is expressed in a restricted fashion on a variety of normal tissues. Thus, as an example APO-1 is found in a basolateral distribition on epithelial cells of the colon mucosa. Interestingly, a number of colon carcinomas have lost APO-1 and others show a strong expression of APO-1.

Apart from the above cells, we tested a number of *in vitro* cell lines for the expression of APO-1 and the induction of apoptosis by anti-APO-1. We found that the human B cell lines SKW6.4, CESS, and BJAB were positive as well as the human T cell lines Jurkat, Molt, and CCRF-CEM. The human myeloid cell line U937, the gibbon T cell line MLA-144 and the mouse T cell line EL4 were negative. The cell lines of the above panel which express APO-1 also underwent apoptosis after incubation with anti-APO-1. The effects of anti-APO-1 *in vitro* prompted us to test whether the antibody was also effective *in vivo*. Although BJAB was the least anti-APO-1 sensitive of our *in vitro* B cell lines we selected this cell line for our *in vivo* experiments because it was the only line that grew to a large tumor in nu/nu mice. BJAB carries about 15,000 APO-1 antigens on the cell surface. Approximately five weeks after injection of $5x10^7$ BJAB cells into nu/nu mice BJAB tumors had reached a diameter of about 1 - 2.5 cm. The tumor carrying animals were then injected once intravenously with 500 μg of anti-APO-1 or a similar amount of isotype matched binding or non-binding control antibody, respectively. Experiments using radiolabelled antibodies indicated that anti-APO-1 and binding control antibodies localized in the tumor tissue while non-binding antibodies only showed background localization. Control antibodies did not have any effect on the BJAB tumors in the nu/nu mice. However, anti-APO-1 led to complete tumor regression in most animals between 5 and 14 days after injection of the antibody. Histological thin sections of tumors before complete regression showed that anti-APO-1 had also induced apoptosis *in vivo*.

In conclusion, therefore, our experiments showed that the APO-1 antigen is associated with growth and differentiation of human lymphocytes. The anti-APO-1 antibody induces growth inhibition and programmed cell death (apoptosis) of activated normal human lymphocytes and B and T cell tumors. Further experiments indicated that these processes can also be induced in a number of non-lymphoid tumors. The effect of anti-APO-1 is complement independent, and a transduction of the APO-1 mediated trans-membrane signal depends on efficient APO-1 crosslinking. Finally, a single intravenous injection of anti-APO-1 induces a rapid regression of a large tumor mass of the human Burkitt-like B cell lymphoma BJAB in nu/nu mice.

Future experiments are aimed at defining the physiological role of APO-1 and establishing a molecular understanding of growth inhibition and the mechanism of apoptosis. Furthermore, it is apparent that anti-APO-1 might be used as a diagnostic and putative therapeutic tool in oncology.

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V Preventive and Therapeutic Manipulations

Vaccination

An Introduction to Vaccines: An Old Problem in a New Setting

E.S. Lennox

The story of modern vaccine development begins with Jenner 200 years ago and today smallpox has been completely eradicated. This unique and remarkable achievement has been accomplished in part because vaccinia meets so well the requirement of a good vaccine (Table 1). No less important, however, has been the mobilisation of social, political and economic resources around the world coordinated by the World Health Organisation. The will to do the job and an effective strategy coupled to an almost ideal vaccine made this possible.

Table 1. Requirements for a good vaccine

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* Safety
* Efficacy
* Economy
* Ease of administration
* Stability under a wide variety of conditions

In contrast, rabies for which Pasteur provided the original vaccine about 100 years ago is still with us, mostly as a problem in developing countries. Many other diseases for which the causative agents are known also are with us still. For rabies one of the major problems is that inexpensive vaccines are not yet available nor are they easy to administer, requiring repeated injections over a few weeks after exposure (Vodopija 1988).

With regard to other pathogens e.g. Plasmodium falciparens which causes malaria, although much is known about the pathogen and its proteins, antigen variation of crucial proteins expressed in various life cycle stages seems to be a major block to an effective vaccine (Perrin et al 1988).

The large variety of organisms some with complex life cycles against which humans (and animals) need protection, the need to protect in a very wide range of social and economic situations, the need to protect infants even though they may carry passive antibodies from the mother, the need to protect a population highly polymorphic in HLA antigens and hence immune response potential - these present problems which will require all the cleverness and techniques presently available for the invention and development of new types of vaccines.

In particular Table 2 highlights the events and technologies which are having a profound impact in the development of vaccines and which are the subject of this symposium. The last 3 items are particularly important in this context.

Table 2. Events leading to modern vaccines

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* Identification and isolation of infectious agents
* Identification of a protective antigen
* Understanding of events leading to immune state
* Recombinant DNA technology
* Monoclonal antibody technology

For both viruses and bacteria, the main point of entry is via mucosal surfaces particularly lung, gastrointestinal tract and urogenital surfaces. While the immunology of these surfaces shares many properties of the immune system reached by the usual methods of injection of antigen, it does have its own central immune system (Russell and Mestecky 1988) that sends properly prepared cells to these surfaces. It is the detailed unscrambling of this particular system which will give better guidance to the design of antigen properties and modes of presentation which will make an impact on vaccine design. For example it is already known that live and heat killed cholera and salmonella are very different in their ability to protect when given as an oral vaccine (Dougan et al 1988).

In Table 3 are shown the kinds of material now available for vaccines. Recombinant DNA technology has played a dominant role in making available antigens known to be important in establishing immunity and in providing sequence information for peptide vaccines. A very good example is in providing material for a hepatitis B vaccine (Zuckerman 1988).

Table 3. Kinds of vaccines

* Related animal viruses
  - vaccinia, rotavirus.

* Attenuated viruses
  - polio, rabies, influenza,varicella

* Mutant bacteria
  - salmonella auxotrophs

* Mutant viruses
  - proteinase activation mutant of paramyxovirus

* Subunits
  - derived from virus by dissociation and purification
  - recombinant origin

* Vaccinia vector

* Yeast 60 nm virus like particles

* Peptides

While the earliest hepatitis B vaccine was made by virus derived particles shed into the serum of carriers and was successful, the inherent danger of serum derived proteins opened the opportunity for replacement by gene cloning and expression in yeast of the protective protein. This is just one example of 'subunit vaccines'.

Another major contribution of rDNA technology is in the preparation of vaccinia virus derivatives as vectors for proteins derived from other viruses (Hruby D.E 1988). Vaccinia is ideal for the following reasons:

1. It is a virus whose safety is known.

2. It has a very large genome with a capacity to have large portions of foreign DNA inserted.

3. It presents antigen in a highly polymeric hence immunogenic form.

4. It is easy to grow, relatively inexpensive and stable.

Monoclonal antibody technology has played three important roles in vaccine design:

1. In immunopurification of viral components.

2. Identification of epitopes related to essential function of the pathogen, i.e. by defining sites where binding inactivates infectivity.

3. In selecting serological mutant viruses as possible vaccine candidates.

Table 4 draws attention to some of the difficulties that arise in preparing good vaccines even when the causative agent is well known and can be cultivated.

Table 4 Problems to be solved

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* Neutralization in vitro
  - poor correspondence with protection to natural infections
  - examples: HA and F proteins in measles
    G and F proteins in PIV, RSV
  - need for proper animal models
* Antigenic variation
  - especially malaria, trypanosomes, influenza
* Immunization of mucosal surfaces
* Poor immunogenicity (subunit vaccines)
* T vs B immunity
* Protection of polymorphic population
  - peptide vaccines
* Passive protection
  - high affinity human monoclonal antibodies needed

For example, since in vitro assays are a simplification of the natural infection process, they may identify by antibody neutralization several proteins involved in infection but fail to indicate that it is essential to neutralize more than one protein to block natural infections. An example are the G and F proteins of measles where anti G or anti F antibody can each neutralize in vitro activity of the virus but to block infection a vaccine must raise neutralizing antibodies to both the hemagglutinin (G) as well as to the cell fusion inducing protein (F). Other examples occur with the parainfluenza virus (PIV) and respiratory syncytial virus (RSV) (Murphy et al 1988).

Antigenic variation is an especially difficult problem with malaria and trypanosomes where many of the proteins that might be used to raise protective immunity undergo rapid variation. It is possible that knowledge of the amino acid sequences of the many proteins that might be protective could yield peptides common to many isolates of the parasites. While they do not normally act as protective immunogen they might be induced to do so by presentation as peptides coupled to suitable carriers. This will of course require extensive testing in suitable animal systems.

Since the entry of most pathogens is via the mucosal surfaces, much attention is being paid now to design of vaccines that elicit immunity in those surfaces (Russell and Mestecky 1988). To do this effectively will require a much better understanding of the special aspects of the immune system that relate to these surfaces. Meanwhile practical approches are being discovered, for example it has been shown that living salmonella are much better at giving mucosal immunity than heat killed ones (Dougan et al 1988).

Since Salmonella may be useful as a cloning recipient for vaccine components this also suggests that it could be used in a similar way as vaccinia as a universal carrier but in this case capable of inducing protective immunity to organisms that come in via the oral route. Similar vectors would have to be found for other mucosal surfaces.

The current understanding of the role of major histocompatibility antigens in antigen presentation reveals what might be a problem in preparing peptide vaccines that would be capable of inducing immunity widely in a population highly polymorphic for these MHC antigens. A peptide that elicits good T cell response because it binds well to a specific MHC class II protein (to give help to the antibody response) or to a specific MHC class I protein (to raise T cytolytic cells) might be too specific in its binding to a given allelic form of the protein to be universally useful. None the less, there are indications that peptides can be found (Rothbard J, this Symposium) that bind well enough to the various allelic MHC molecules to make it possible that they would have wide applicability as peptide vaccines. This will of course need testing.

Up to this point I have emphasised the use of agents to induce active immunity. However, the use as therapeutic agents of injected antibodies or appropriately activated T cells should not be forgotten.

The use of antibodies for human prophylaxis or therapy, unless they be of human origin is limited by their immunogenicity. While the making of human monoclonal antibodies is occasionally successful, the methods available do not lead to the large range of specificities, affinities and biological properties of monoclonal antibodies available from mice and rats. This situation has led to the searching for other ways of developing 'humanised' antibodies by using the methods of rDNA. (Bruggemann and Neuberger 1988).

In addition, as is discussed elsewhere at this Congress, beginnings have been made in preparing transgenic mice with portions of the human immunoglobulin genome as well as SCID mice reconstituted with human lymphoid cells. Both of these may yield ways of selecting human antibodies of desired specificity and high affinity.

In summary, there are now a large number of precision tools made available for preparing modern vaccines. The need for well designed vaccines for a large variety of infectious agents will draw on all of these skills.

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High Degeneracy of HLA DR-Peptide Interactions: Possible Ramifications for Vaccine Design

J.B. Rothbard, C.M. Hill, J. Hickling, and R. Busch

INTRODUCTION

The demonstration that both MHC class I and class II molecules bind fragments of protein antigens (Babbitt 1985, Buus 1986, Watts and McConnell 1986) and that the formation of MHC-peptide complexes is a necessary requirement for T cell recognition (Schwartz 1985) has led to the postulate that the differential capacity of each allele to bind peptides is the molecular basis of genetic differences in immune responsiveness (Guillet 1987, Buus 1987). The results of the binding studies supported experiments demonstrating that the specificity of the cellular immune response to an immunogen varies between different strains of mice. Consequently, any peptide based vaccine must be composed of a suffiently large cocktail of peptides to be widely effective. However, these conclusions were based principally on the binding studies using purified murine class II MHC molecules and consequently only a limited number of alleles were examined.

The development of a fluorescent assay for binding of peptides to HLA-DR proteins on the surface of intact cells in this study has permitted a direct examination of the specificity of the interactions between peptides and many different DR alleles (Busch, submitted). The rapid, simple, and quantitative binding assay involves flow cytometric analysis of Epstein-Barr transformed B cell lines stained with fluoresceinated streptavidin following incubation with a biotinylated peptide. Binding of the biotinylated peptide required surface expression of human class II molecules, and was inhibited by an anti-DR monoclonal antibody as well as the unbiotinylated natural determinant. Rates of association and dissociation of the peptide were low, and the peptide bound only approximately 1% of the DR molecules expressed on the cell surface. When assayed on twenty-two different DR-homozygous B cell lines, the peptide was shown to bind each line to varying extents. The ramifications of these results on vaccine design will be discussed.

MATERIALS AND METHODS

Peptides

The T cell determinants used represented residues 307-319 from influenza hemagglutinin (HA 307-319; Rothbard 1988), residues 280-293 from the major capsid protein of human papillomavirus 16 (L1 280-293; unpublished data), residues 17-29 from the matrix protein of influenza virus (Mat 17-29; Rothbard 1988) and residues 3-14 from the 19 kDa protein of *Mycobacterium tuberculosis* (Tub 3-14; Lamb 1988). Lysines were substituted with arginines, and, where necessary, the α amino group was acetylated to prevent nonspecific biotinylation. The analogs were synthesized on an Applied Biosystems (Foster City, CA) 430A peptide synthesizer and biotinylated (sulfosuccinimidyl-6-(biotinamido)hexanoate, Pierce) either at the ε amino group of a lysine substituted at the amino terminus (Mat 17-29 and L1 280-293) or at an unacetylated α amino group (HA 307-319 and Tub 3-14). The biotinylated peptide was purified by reversed phase chromatography and analyzed by amino acid analysis, fast atom bombardment mass spectrometry, and reaction with dimethylaminocinnamaldehyde (McCormick 1970).

Cells

The B-lymphoblastoid cell lines used, and their DR types were MAJA (DR1 Dw1; HLA-A2,3, B35, C4, Bw6, DQw1, DP4), LWAGS (DR1 Dw20), EFI-ND (DR 10BR), PGF (DR15 Dw2), WT18 (DR16 Dw21), WT20 (DR17 Dw3), PRIESS (DR4 Dw4), AL10 (DR4 Dw10), JHF (DR4 Dw13), PE117 (DR4 Dw14), HIN-ND (DR4 Dw15), KOZ (DR9), MANN (DR7 Dw7), DBB (DR7 Dw11), IDF (DR11 Dw5), HERLUF (DR12 Dw H), OLL (DR8 Dw8.2), DAUDI (DR13b Dw19), ARNT (DR13a Dw18), WT52 (DR14b Dw9), and AZL (DR14a Dw16). The class II negative mutant B-lymphoblastoid cell line, RJ 2.2.5 is derived from RAJI cells (HLA-A3, 19; B51, 35; C3,4; Bw4, 6; Accolla). Transfected L cells 5-3.1 (expressing DR1, Rothbard 1988) and Q/Q1H-L (expressing DQw1) were trypsinized 12 hours before use in binding assays.

Binding assay

3×10^5 cells were incubated with biotinylated peptide (50 μM) at 37°C for 4 hr, followed by fluoresceinated streptavidin (4.22 μg/ml; Calbiochem) at 4°C for 30 min. When greater sensitivity was required, staining of cells incubated with biotinylated peptide was carried out using successive layers of fluoresceinated avidin D (10 μg/ml, Vector Labs), biotinylated anti-avidin D (10 μg/ml, Vector Labs), and again fluoresceinated avidin D. Each incubation was followed by two washes at 4°C with 0.1 % bovine serum albumin in Dulbecco's phosphate buffered saline. Stained cells were subjected to flow cytometry on a FACScan analyser (Becton-Dickinson Immunocytochemistry Systems, Mountain View, CA). In inhibition experiments, cells were coincubated with biotinylated peptide and varying amounts of either the purified anti-DR monoclonal antibody, L243 (Lampson 1980), or the natural determinant, HA307-319.

RESULTS

A T cell determinant from influenza hemagglutinin (HA; residues 307-319), which is recognized by a HLA-DR1 restricted T cell clone (Rothbard 1988), was assayed for its ability to bind Epstein-Barr virus transformed human B lymphocytes homozygous for HLA-DR1. These cells were chosen because they were homozygous, well characterized cell lines that express unusually high levels of HLA class II proteins. Because of concerns of high background fluorescence and a low specific signal, the peptide was not directly fluoresceinated. Instead, it was conjugated to a biotinyl group to take advantage of the amplification that can be obtained by using multiply fluoresceinated streptavidin.

When the DR1-homozygous B cell line, MAJA, was incubated with the peptide, stained with fluoresceinated streptavidin, and analysed for green fluorescence by flow cytometry, the cell surface fluorescence was approximately five times higher than in the absence of peptide (Fig. 1A). The signal was two orders of magnitude less intense than that obtained by indirect immunofluorescence with a monoclonal antibody specific for a determinant present on many human class II molecules (Lampson 1980).

If the fluorescent signal observed with this peptide on MAJA cells reflects the interaction of the HA determinant with HLA-DR1, a B cell line deficient in DR expression should fail to generate a fluorescent signal. RJ 2.2.5, a variant of the B cell line, RAJI (Accolla 1983), does not express MHC class II proteins because of a mutation in a regulatory protein required for class II expression. The mutation does not appear to affect the expression of MHC class I proteins (Hume 1987) or any other cell surface molecules. When RJ 2.2.5 cells were used in the assay, the fluorescence in the presence of peptide was indistinguishable from background (Fig. 1B). This demonstrated that the fluorescent signal on the DR1-homozygous B lymphoblastoid cells was specific and strongly suggested that the peptide bound to class II proteins or other proteins that are coregulated. However, the possibility that the peptide binds to other cell surface proteins expressed on MAJA but not on RJ 2.2.5 cells could not formally be eliminated, even though the cell surface of the class II deficient mutant should closely resemble that of the DR-homozygous line.

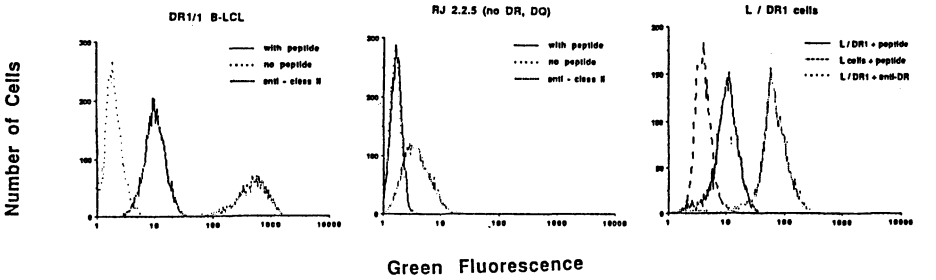

Fig. 1. Binding of a biotinylated analogue of HA 307-319 to cell surfaces. *A*, Binding of the HA307-319 analog to the DR1-homozygous B cell line, MAJA (———). Surface expression of class II MHC proteins was shown by indirect immunofluorescence using the anti HLA-D region antibody, TAL 31.1(·····). Background fluorescence in the absence of biotinylated peptide was determined by incubation only with streptavidin (· · · · ·). *B*, Failure of the HA analogue (———) to bind to the class II-deficient B-cell line, RJ 2.2.5, above background (· · · · ·). *C*, Binding of the biotinylated HA peptide to L cells transfected with HLA-DR1. Transfectants (clone 5-3.1) (———) or untransfected L cells (- - -). The level of staining for DR (.....) on the transfected L cells was about ten times lower than on B-lymphoblastoid cells.

L cells transfected with genes encoding α and β chains of human class II molecules were used to demonstrate that the peptide interacts with DR1. When L cells transfected with DR1 (Rothbard 1988) were used in the assay, the fluorescent signal again was approximately one per cent of that seen with the anti-class II antibody. However, because the fluorescent signal obtained with the antibody was much lower than on the B cell line, the signal obtained with peptide was only slightly above background. To distinguish more clearly the specific fluorescence from that seen on untransfected L cells, the single incubation with fluoresceinated avidin was replaced with successive layers of avidin, biotinylated anti-avidin, and again avidin, and the cells were incubated overnight with large amounts (100 μM) of biotinylated peptide. A distinct fluorescent signal was observed when the HA peptide was incubated with cells transfected with DR1, but absent on normal L cells (Fig. 1C). In contrast, the fluorescence on L cells expressing DQw1 was indistinguish-able from that of untransfected cells. This result proves that the peptide interacts with HLA-DR1, but not with DQw1 within the detection limits of the three layer assay.

Further evidence that the cell surface fluorescence on the DR1-homozygous B cell line was due to the interaction between the peptide and DR1 was that the signal could be modulated by coincubating the cells with an anti-DR monoclonal antibody, L243 (Lampson 1980). Increasing amounts of antibody progressively reduced the fluorescent signal (Fig. 2A). However, complete inhibition of the signal with the antibody was not achieved because an antibody concentration that was saturating could not be obtained without aggregating the cells. Nevertheless, the reduction of the fluorescent signal closely paralleled the saturation of cell surface DR1 by the antibody (Fig. 2B), supporting the conclusion that the fluorescent signal is mainly due to formation of a peptide-MHC class II complex. However, the possibility that a small fraction of the fluorescent signal is due to binding of non-MHC moieties could not be eliminated by this method.

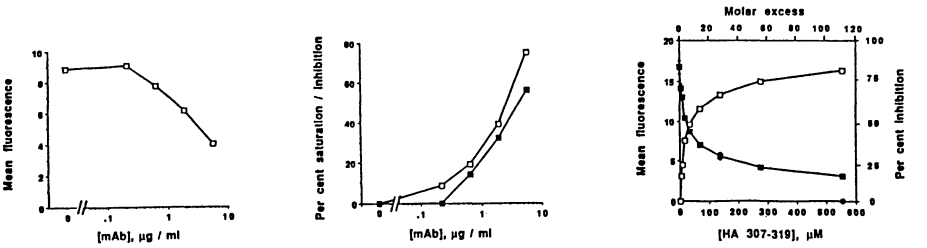

Fig. 2. Inhibition of peptide binding by an anti-DR monoclonal antibody and the natural determinant, HA307-319. *A*, peptide binding to MAJA cells in the presence of varying amounts of protein A-sepharose purified L243 anti-DR antibody. *B*, Comparison of the reduction in fluorescent signal by L243 (■) and the fraction of DR molecules bound by antibody (□). *C*, Inhibition of peptide binding (5 µM) to MAJA cells by varying concentrations of HA 307-319. Both the fluorescence (■; scale on left) and the fractional reduction in fluorescent signal (□; scale on right) are shown. RJ 2.2.5 cells incubated with biotinylated peptide and competitor gave no signal (●).

The natural determinant also was shown to compete with the biotinylated peptide, demonstrating that both occupy the same site on the cell surface. Half inhibition could be obtained at a sevenfold molar excess of the competitor over 5 µM biotinylated peptide(Fig. 2C). As the concentration of the competitor was increased, the fluorescence decreased, reaching 80 % inhibition at a molar ratio of 110. The molar excess of competitor could not be increased further because of the limited solubility of the unbiotinylated peptide.

The ease of the assay and the availability of many DR-homozygous B cell lines allowed us to examine the ability of the biotinylated HA307-319 analogs to bind to many cell lines expressing different DR types (Fig. 3). Remarkably, even though the fluorescent signal significantly varied between the twenty-two cell lines examined, detectable fluorescence distinct from background was present in each case with the exception of the class II deficient cell line (Fig. 3A). When peptide binding was corrected for the variations in HLA-DR expresssion (Fig. 3B) by dividing the fluorescence obtained with peptide by that obtained with the anti-DR monoclonal antibody (Fig. 3C), significant differences between cell lines remained, which allowed classification of the cells into groups having haplotypes with high (DR14a Dw16, DR13a Dw18, DR13b Dw19, DR16 Dw21) low (DR17 Dw3, DR9, DR4 Dw15), or intermediate (all others) capacity for associating with the HA307-319 analog.

Even the lowest levels of cell surface fluorescence detected in this experiment are likely to represent a specific interaction between the biotinylated peptide and cell surface DR. DR4 Dw15 expressing cells, which bound the biotinylated HA analogue most weakly, presented the natural determinant to a HA307-319 specific T lymphocyte clone as well as the autologous restriction element, DR1 Dw1, which binds the biotinylated peptide at intermediate levels (Fig. 3C and Rothbard 1989). Furthermore, the signal on DR4 Dw15 expressing cells was inhibited by 50% in

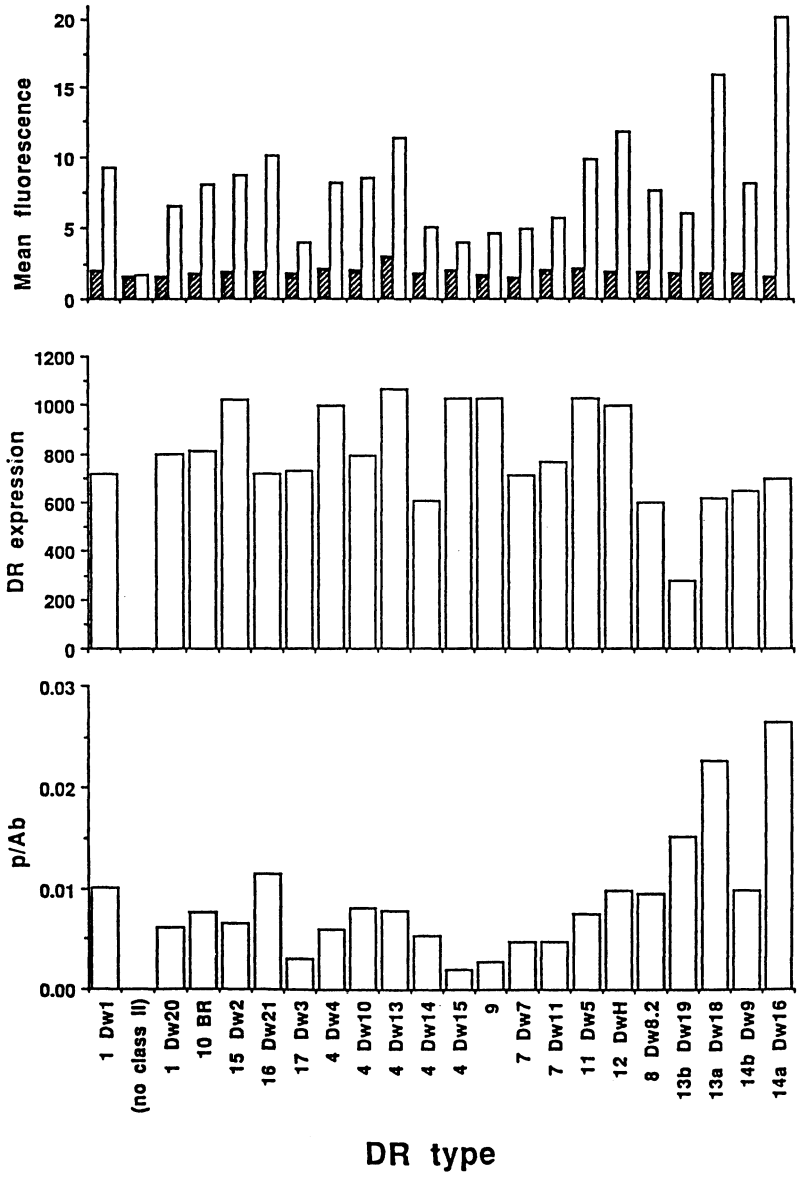

Fig. 3. Binding of the HA peptide to B cell lines homozygous for different DR alleles. *A*, Mean fluorescence on cell lines homozygous for the DR types indicated, when incubated with peptide (□) and without peptide (▨). *B*, Mean fluorescence on the same cells stained with directly fluoresceinated L243 anti-DR monoclonal antibody. *C*, Relative peptide binding (from panel *A*) divided by relative antibody binding (from panel *B*).

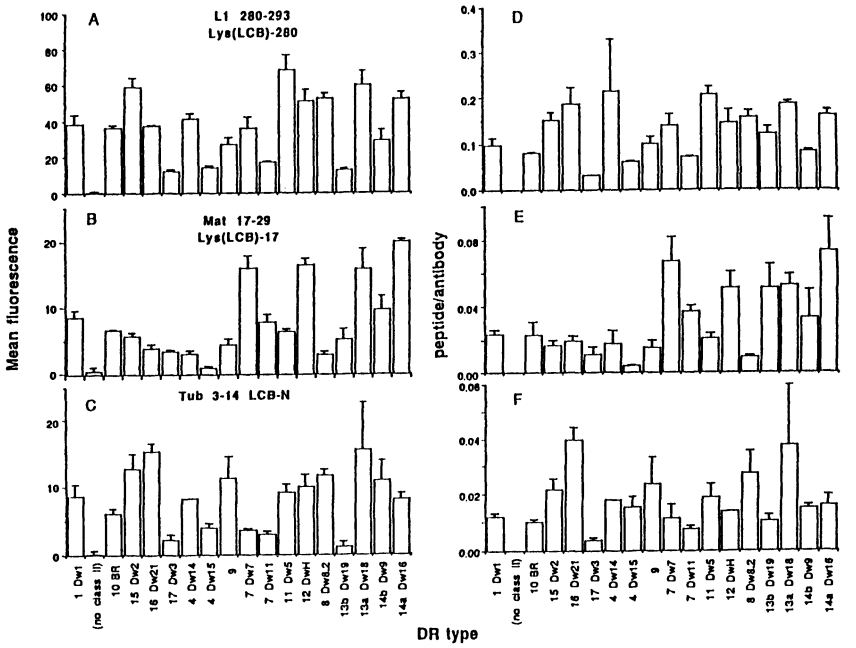

Fig. 4. Binding of three different T cell determinants to DR-homozygous B cell lines. Biotinylated analogues of human papillomavirus type 16 L1 280-293 (*A, D*), influenza matrix 17-29 (*B, E*) and *M. tuberculosis* 19 kDa 3-14 (*C, F*) were used in the three layer cell surface binding assay. *A-C*, Binding expressed as mean fluorescence minus background. *D-F*, Peptide fluorescence divided by fluorescence obtained with fluoresceinated L243 anti-DR antibody. LCB, long chain biotin.

the presence of amounts of L243 anti-DR antibody which also halved the fluorescent signal on the DR1 Dw1 homozygous cells.

In order to examine whether the degeneracy of binding of the HA peptide was typical of T cell determinants, three other determinants were examined for binding to multiple B cell lines using the sensitive three layer assay. Like the HA307-319 peptide, none of the analogs bound to the class II deficient B cell line, RJ 2.2.5, but each gave fluorescent signals on almost all DR expressing B cell lines tested (Fig. 4). The only exceptions to this were the interaction of the influenza matrix peptide with DR4 Dw15 and that of the peptide from the mycobacterial 19 kDa protein with cells expressing DR13b Dw19. There were further quantitative differences in the ability of each peptide to bind to cells expressing different alleles, and the pattern of reactivity was different for each peptide. This suggests that the differences in fluorescence levels between cell lines are not due to differences in nonspecific binding or in the proportion of each DR allele available for peptide binding.

DISCUSSION

A fluorescent assay for direct binding of long chain biotinylated peptides to HLA-DR molecules on cell surfaces has been developed. The evidence for the specificity of the fluorescent signal was that the level of cell surface fluorescence correlated with the amount of HLA-DR expression, and that the signal was significantly reduced by competition with either the unbiotinylated peptide or a monoclonal anti-DR antibody. Little, if any, of the fluorescent signal on B-lymphoblastoid cells appeared to be due to binding to either DQ or DP (E. Long, personal communication) class II proteins because no fluorescent signal distinguishable from background was detected when L cells expressing these molecules were assayed. Binding to class I MHC proteins also probably did not contribute significantly to the fluorescent signal, both because the class II negative, class I positive (Accolla 1983) B cell line, RJ 2.2.5, expresses each of the class I alleles found on MAJA cells with the exception of HLA-A2, yet failed to bind the HA peptide, and because the anti-class I antibody, W6/32, known to block peptide-MHC class I interactions (J. Hickling, personal communication), did not inhibit the fluorescent signal (R. B., unpublished data). The failure to obtain quantitative inhibition using either unbiotinylated peptide or anti-DR antibody appeared to be due to the technical limitations of the assay, such as peptide solubility or agglutination of cells by antibody, rather than nonspecific binding of a small proportion of the biotinylated peptide to the cell surface.

The kinetics of peptide binding to cell surface HLA-DR molecules, as measured in this assay, was similar to that observed with purified class II molecules in detergent solution (Buus 1986, T. Jardetzky, personal communication). In each case, the rates of association and dissociation were low, and binding was nonstoichiometric. This suggests that the peptide-MHC complex detected in the indirect fluorescent assay is formed by direct association of the peptide with the class II molecule on the cell surface, justifying the use of a simple kinetic model for the interaction. Consistent with this hypothesis, inhibitors of endocytosis and reagents that increase endosomal pH failed to affect the fluorescence, indicating that peptide binding, as measured in this assay, does not require internalization of the peptide or its interaction with the class II molecule in a low pH environment. However, the inability of the cell surface binding assay to detect intracellularly bound peptide does not imply that intracellular events are unimportant in antigen processing. Recently, Ceppellini et al. (1989) have reported binding of radioiodinated matrix 17-29 peptide to cell surface DR proteins. In their assay, peptide binding appears to be complete in 45 minutes but is much slower after glutaraldehyde fixation of the cells, suggesting that an intracellular association step may be involved in the binding reaction measured by the radioactive binding assay.

When four different biotinylated peptides were examined for binding to many different DR homozygous B cell lines, each peptide appeared to be capable of binding to the vast majority of DR expressing cell lines while none of them bound to class II deficient cells. There were quantitative differences in the ability of each peptide to bind to cells homozygous for different alleles. At present, the possibility that at least some of the peptides studied interact with supertypic specificities in some haplotypes cannot be eliminated. However, assuming that the second DR chain is expressed at similar levels in different B cell lines, the significant differences observed between cell lines sharing a common supertypic specificity (for example, the different DR4 Dw subtypes, which share the DRw53 β chain) are probably due to differential binding of DRβ1 allelic variants.

The ability of a peptide to bind multiple DR alleles might be a general feature of peptide recognition by MHC class II proteins. In addition to the four examples of direct binding in this paper, two other cases of a peptide binding to multiple DR alleles have been shown indirectly. A peptide from the malarial circumsporozoite protein was recognized by T cell lines from donors representing seven different DR specificities (Sinigaglia 1988). A second example was the unusual degeneracy of an individual T cell clone to a tetanus toxoid peptide, which recognized the determinant in the context of 10 different DR alleles (A. Lanzavecchia, personal communication).

When different peptides were examined for binding to many B cell lines, the fluorescence patterns generated by different peptides were dissimilar. This suggests that, although each peptide studied appears to bind to each allele, the alleles are to some degree selective for different peptides. Consequently, mechanisms involving determinant selection at the level of peptide-MHC interactions might still be important in determining immunodominance. At physiological antigen concentrations, the differences in the capacity of each allele to bind peptide may be much greater than at the relatively high concentrations required to observe peptide binding in this assay. A further increase in selectivity might occur if peptides with different abilities to bind a particular class II allele compete with each other for binding, as is likely to occur in a natural infection. Nevertheless, the surprising ability of peptides to bind to many different DR alleles indicates that MHC restriction of T cell recognition probably arises as much from MHC-T cell receptor interactions as from different capacities of MHC proteins to bind peptide.

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T and B Cell Recognition of Hepatitis B Viral Antigens: Implications for Vaccine Design

D.R. Milich

INTRODUCTION

The clinical consequences of HBV infection are extremely variable. It is likely that nonviral, host factors are involved in the pathogenesis of hepatocellular injury since the hepatitis B virus is not directly cytopathic for hepatocytes (Barker 1973). It has been suggested that variation in immune responsiveness to HBV infection may, at least partially, account for the diversity of clinical syndromes including fulminant, acute, chronic active and chronic persistent hepatitis as well as the asymptomatic chronic carrier state (Dudley 1972). Furthermore, chronic hepatitis B virus infection has been epidemiologically linked with the development of hepatocellular carcinoma (HCC) (Beasley 1984). This association of HCC and chronic HBV infection accounts for the high overall incidence of HCC, making it one of the most frequent fatal malignancies, particularly in the Far East and in sub-Saharan Africa (Brechot 1987). It is hoped that worldwide HBV vaccination will eventually eliminate this common human cancer (Popper 1988).

In the absence of an inbred animal model of hepatitis B virus (HBV) infection, several laboratories have chosen to study the murine immune response to HBV-encoded proteins as immunogens as opposed to an infectious agent. This article reviews the immunogenicity, the fine specificity of T- and B-cell recognition of HBV antigens, and the genetic influences that regulate these responses. It is anticipated that this approach will increase our understanding of immune-mediated viral clearance mechanisms during HBV infection, and may provide the framework for the design of second-and third-generation HBV vaccines.

The specific serological marker of HBV infection is the hepatitis B surface antigen (HBsAg) which is present as the envelope in the intact virion and as free circulating filamentous and spherical 22-nm subviral particles. The HBsAg is composed of a major polypeptide, P25, and its glycosylated form, GP28. Additional polypeptides of higher molecular weight (P39/GP42 and GP33/GP36) have recently been identified (Stibbe 1983; Hermann 1984). The larger polypeptides share the 226 amino acids of P25 (S region) at the C terminus and possess additional residues at the N terminus. The pre-S(2) region consists of 55 residues N-terminal to the S region (Machida 1984), and the pre-S(1) region consists of 119 residues N-terminal to the pre-S(2) region (Hermann 1984). Herein, HBsAg particle preparations are designated by virtue of the highest molecular weight polypeptide present (i.e. HBsAg/P39, HBsAg/GP33 and HBsAg/P25).

The nucleocapsid of the HBV is a 27-nm particle composed of multiple copies of a single polypeptide (P21), and the intact structure exhibits hepatitis B core antigenicity (HBcAg). A nonparticulate form of HBcAg designated HBeAg may be present in the serum during HBV infection.

RESULTS AND DISCUSSION

Immunogenetics of the Humoral Response to HBV Envelope Antigens.

Early studies indicated marked strain variation in antibody production after immunization with HBsAg/P25 (Milich 1982, 1983). Studies in H-2 congenic and intra-H-2 recombinant strains identified high ($H\text{-}2^{d,q}$), intermediate ($H\text{-}2^{a} > H\text{-}2^{b} > H\text{-}2^{k}$); and nonresponder ($H\text{-}2^{f,s}$) phenotypes (Milich 1984). When recombinant HBsAg particles containing the larger molecular weight polypeptides became available, studies were extended to examine genetic influences on the immune responses to pre-S sequences.

Antibody production to the pre-S(2) region after immunization with HBsAg/GP33 is also H-2 restricted, however, the hierarchy of response status differs from that of the S region (Milich 1985a) (Table 1). It was also notable that the pre-S(2) region was significantly more immunogenic than the S region in terms of primary antibody production in vivo. Additionally, immunization with HBsAg/GP33 was capable of bypassing nonresponse to the S region in HBsAg/P25 nonresponder B10.S mice. The immune response to the pre-S(1) region of HBsAg/P39 is also influenced by H-2-linked genes, but again the hierarchy of response status differs from the responses to the S and pre-S(2) regions (Milich 1986a) (Table 1). HBsAg/P39 immunization elicited anti-pre-S(1)-specific antibody in all strains, and furthermore elicited anti-S and anti-pre-S(2) responses in all strains including an S-specific response in the "nonresponder" B10.S strain, and S and pre-S(2)-specific responses in the "nonresponder" B10.M strain. These data indicated that distinct H-2-linked genes influence S, pre-S(2), and pre-S(1)-specific antibody production in vivo.

Table 1. Influence of H-2 Genotype on the Humoral Response to HBsAg Particles of Varied Composition

			Specific Antibody Titer (1/dilution)		
Immunogen	Strain	H-2	S	pre-S(2)	pre-S(1)
HBsAg/P25	B10.D2	d	81,920	0	0
	B10	b	20,480	0	0
	B10.BR	k	5,120	0	0
	B10.S	s	0	0	0
	B10.M	f	0	0	0
HBsAg/P33	B10.D2		40,960	10,240	0
	B10		10,240	40,960	0
	B10.BR		1,280	2,560	0
	B10.S		640*	10,240	0
	B10.M		0	0	0
HBsAg/P39	B10.D2		81,920	5,120	640
	B10		20,480	40,960	10,240
	B10.BR		5,120	1,280	2,560
	B10.S		5,120*	10,240	1,280
	B10.M		10,240*	1,280*	10,240

Adapted from Milich (1986a).
*Represents an antibody response to a specific region of HBsAg that is not observed when the strain is immunized with that same antigen (ie, B10.S is nonresponsive to the S region when immunized with HBsAg/P25).

T Cell Recognition of Pre-S Regions of HBsAg can Circumvent Nonresponse

The ability of HBsAg/GP33 immunization to bypass S region nonresponder status in the B10.S strain and HBsAg/P39 immunization to bypass S and pre-S(2) region nonresponder status in the B10.M strain, suggests that although these strains lack an S-specific T cell response they must possess pre-S-specific T cells that can help B cell clones specific for S as well as pre-S region determinants. The T cell responses after HBsAg/GP33 immunization were regulated by H-2-linked genes, and correlated with in vivo anti-pre-S(2) antibody production such that B10 > B10.D2 > B10.S > B10.BR > B10.M (Milich 1985b). This hierarchy of T cell response status differed from that observed after HBsAg/P25 immunization indicating that distinct H-2-linked genes can influence S and pre-S(2)-specific T cell responses. However, since S and pre-S(2) region determinants exists on the same polypeptide (GP33), T helper (Th) cells specific for a determinant on one region are capable of providing functional help to B cell clones recognizing a determinant on the other region.

After HBsAg/P39 immunization in the B10.M strain, only HBsAg/P39 induced a proliferative T cell response in vivo and not HBsAg/P25 or HBsAg/GP33, indicating an exclusive pre-S(1)-specific T cell response. A summary of T and B cell responses after HBsAg/P39 immunization of three representative H-2-congenic strains is shown in Fig. 1. The B10.D2 strain is responsive at the T cell level to the S and pre-S(2) regions,

and possibly the pre-S(1) region (not determined). The B10.S strain is only responsive to the pre-S regions at the T cell level. The B10.M strain only recognizes the pre-S(1) region at the T cell level. However, all strains produce antibody specific for each of the regions due to the fact that T cell recognition of a single region is sufficient to provide help for multiple B cell specificities present on the three regions of HBsAg. For these reasons it has been suggested that pre-S sequences be included in future generation HBsAg vaccines (Neurath 1984; Milich 1985a).

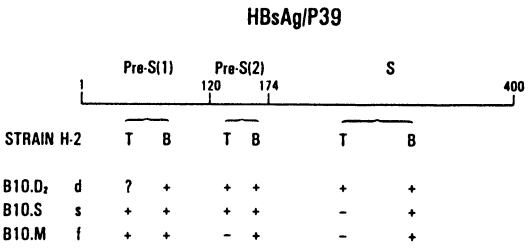

Fig 1. Summary of T-cell proliferative responses (T) and in-vivo antibody production (B) of H-2 congenic murine strains immunized with HBsAg/P39. A plus in the T column represents significant, dose-dependent T-cell proliferation and Il-2 production. A plus in the B column represents significant antibody production after secondary immunization

Fine Specificity of Antibody Recognition of the Pre-S Regions of HBsAg.

Additional studies have been aimed at identifying T cell and B cell (antibody) recognition sites within the pre-S regions of HBsAg. These studies have been greatly facilitated by the fact that the pre-S regions of HBsAg possess continuous (Milich 1985a; Neurath 1984) as opposed to discontinuous or conformational antibody determinants unlike the S region, which requires intact disulfide bonds for full antigenicity (Vyas 1972). Two synthetic peptides derived from the pre-S(2) region sequence elicit antibodies crossreactive with the native pre-S(2) region, and bind antibodies raised to the native protein. Denoting the amino terminus of the pre-S(2) region as residue 120, synthetic peptides p120-145 (Neurath 1984) and p133-151 (Okamoto 1985) were shown to bind human antibodies elicited by HBV infection. Further analysis using a combination of truncated synthetic peptides and monoclonal antibodies revealed that the murine antibody response to the pre-S(2) region is predominantly focused on residues 133 through 143, and two distinct but overlapping epitopes were identified as p133-139 and p137-143 (Milich 1986b). Sera from HBV-infected patients also bind p133-145 and the constituent overlapping epitopes within this sequence. A number of pre-S(1)-specific antibody binding sites recognized by murine and human sera have also been recently elucidated including p1-21 (Neurath 1985), p32-53, p41-53, p94-105, and p106-117, (Milich 1987a).

Influence of T-Cell Fine Specificity on B-Cell Fine Specificity

We have identified a number of T cell recognition sites within the pre-S(1) region (Milich 1987a) and pre-S(2) region (unpublished observation) of HBsAg. The specificity of T cell recognition is dependent on the H-2 haplotype of the responding strain. Identification of these T cell and B cell recognition sites within the pre-S(1) and pre-S(2) regions of HBsAg permitted examination of the influence of T cell fine specificity on antibody fine specificity. This was accomplished by examining the ability of distinct peptide-primed T cell populations to provide functional T cell help for a series of B cell specificities on HBsAg. Two pre-S(1)-specific T cell determinants were chosen (i.e. p12-21, p94-117), which induced only minimal antibody responses. This allowed priming of T helper (Th) cells with peptides and subsequent determination of in vivo antibody production after challenge with a suboptimal dose of

HBsAg/P39 in the same animal, as opposed to performing transfer experiments. This approach requires the memory T cells primed by immunization with peptide to be recalled by challenge with native particles, indicating the relevance of the synthetic T cell site to the native molecule. Using this protocol it was demonstrated that priming with a single synthetic peptide, p12-21, elicited Th function resulting in in vivo antibody production to p16-27, p133-140, p135-145 in the pre-S region and group- and subtype-specific determinants in the S region (Milich 1987a). Similarly, priming with p94-117 elicited Th function resulting in in vivo antibody production specific for p32-53, p94-105, p106-117, p133-140, p135-145 in the pre-S region, but did not prime antibody production to S region determinants. These results indicate that T cells primed to a single determinant are sufficient to provide functional help to multiple B cell clones, which recognize distinct epitopes on a complex, particulate antigen.

Note that the pre-S(1)-specific, T cell recognition sites, p12-21 and p94-117, primed antibody production specific for unique as well as common B cell determinants. For example, p94-117 primed an anti-p32-53 response, whereas, p12-21 did not. This data provides strong evidence that the fine specificity of the Th cell can influence the fine specificity of the antibody produced. Berzofsky (1983) has proposed a T cell-B cell reciprocity circuit in which B cell immunoglobulin receptor-antigen-Ia interactions may limit T cell specificity, which in turn limits B cell specificity. In the context of this hypothesis, the B cell clone specific for the p32-53 epitope may present the P39 polypeptide in the context of Ia in such a way as to be recognized by the T cell clone(s) specific for p94-117, but not by the p12-21-specific T cell clone(s), and therefore will not receive the necessary Th cell signals from p12-21-primed T cells.

Immunogenicity of the HBV Nucleocapsid

Envelope and nucleocapsid-specific cellular immune responses have been suggested to be important in virus elimination and the attendant hepatocellular injury (Eddleston 1974; Mondelli 1982), and vaccination with both antigens has been reported to protect against HBV infection (Gerety 1979; Murray 1984) Comparative studies of murine antibody production revealed that anti-HBc responses were significantly greater (at least 80-fold) than anti-HBs responses in all strains tested (Table 2). The influence of H-2-linked genes on the anti-HBs response is apparent, and no nonresponder strains have been identified. The comparative magnitudes of the anti-HBc and anti-HBs responses, and the lack of nonresponsiveness to HBcAg are consistent with the human immune responses to these HBV antigens.

Table 2. Comparison of Primary Antibody Responses After Immunization with HBsAg and HBcAg

Strain*	H-2	Anti-HBs (titer)	Anti-HBc (titer)
B10	b	256	40,960
B10.D2	d	1,024	81,920
B10.S	s	0†	163,840
B10.BR	k	32	163,840
B10.M	f	0†	20,480
$C_3H.Q$	q	2,048	327,680
Balb/c	d	1,024	327,680

*Groups of 5 mice from each strain were immunized with 4.0 μg of HBsAg or HBcAg in complete Freund's adjuvant, and pooled sera were analyzed by solid-phase radioimmunoassay for IgG antibodies of the indicated specificities at day 24. Data are expressed as the reciprocal of the highest serum dilution to yield 4 times the counts of preimmunization sera (titer).

†The $H\text{-}2^s$ and $H\text{-}2^f$ haplotypes are nonresponsive to HBsAg after secondary immunization.

Studies of the murine cellular response to HBcAg revealed that HBcAg was an extremely efficient immunogen at the T cell level as measured by the in vivo dose required to induce T cell sensitization (1.0 μg), and the minimal in vitro concentration required to elicit IL-2 production from primed-T cells (0.03 ng/ml) (Milich 1987b). This degree of T cell immunogenicity is approximately 100-fold that observed for HBsAg subviral particles (Milich 1985c). Similarly, studies of the human T cell response to HBcAg also suggest that HBcAg is an efficient T cell antigen (Ferrari 1986).

Examination of the fine specificity of T cell recognition of HBcAg revealed that HBcAg-specific T cells from a variety of strains recognized multiple but distinct sites within the HBcAg/HBeAg sequence (Milich 1987b) (HBeAg lacks the C-terminal 34 residues of HBcAg). Each strain recognized a predominant T cell determinant, and the fine specificity of this recognition process was dependent on the H-2 haplotype of the responding strain: For example, H-2^a strains recognized p120-131, H-2^b strains recognized p129-140, H-$2^{f,q}$ strains recognized p100-120, and H-2^d mice recognized p85-100 predominantly (Milich 1987b, 1988a) (see Fig. 2). This murine model predicts that a human outbred population would exhibit similar complexity, and individuals may recognize distinct T cell sites in the context of their HLA genotype.

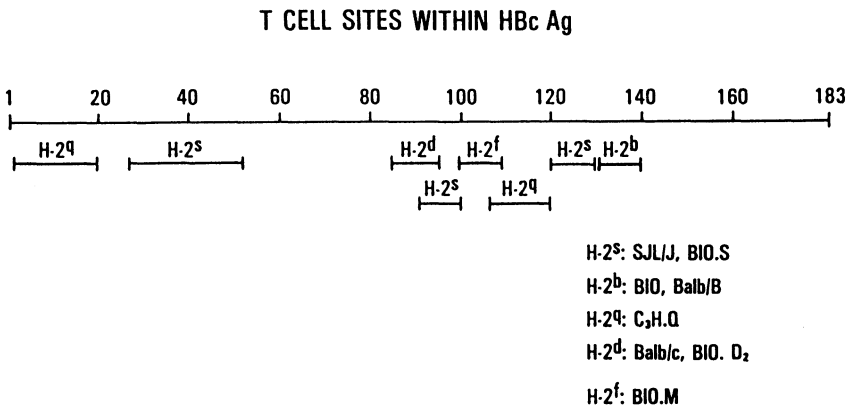

Fig 2. Localization of T-cell sites within the HBcAg/HBeAg sequence using sunthetic peptide analogs. The indicated strains were immunized with HBcAg and T cells cultured with a panel of synthetic peptides from the HBcAg/ayw sequence and IL-2 production determined. Peptides that activated HBcAg-primed T cells were then used for immunization in the reciprocal experiment to determine the ability to prime HBcAg-specific IL-2 production.

To determine the functional ability of synthetic T cell sites to prime Th cells and induce antibody production in vivo, the HBcAg-specific peptide 120-140 and N-terminal (p120-131) and C-terminal (p129-140) fragments were chosen for study in the B10.S and B10 strains (Milich 1988a). Mice were primed with peptide and challenged with a suboptimal dose of HBcAg, and serum anti-HBc measured. In the B10.S strain, p120-140-primed and p120-131-primed mice produced IgG, anti-HBc efficiently 7 days after the challenge, whereas, p129-140-primed mice did not. Similarly, priming with p120-140 elicited anti-HBc in the B10 strain. In contrast to the B10.S strain, the C-terminal p129-140 contained the active T cell site. These results were consistent with the T cell proliferation (Tp) results and indicate a concordance between Tp and Th cell fine specificities.

Induction of HBcAg Antibody Responses via a T cell Independent Pathway

Although HBcAg is an efficient T cell immunogen, this characteristic alone cannot explain the extremely high levels of antibody produced after primary immunization with HBcAg. The nonparticulate form of HBcAg, namely HBeAg is crossreactive with HBcAg at the T cell level, but is a relatively poor immunogen in mice in terms of in vivo antibody production (Milich 1988b). Therefore, the ability of HBcAg to activate B cells directly was examined by immunizing Balb/c euthymic and Balb/c athymic (nude) mice with a mixture of HBcAg and HBsAg. HBcAg was able to induce antibody production in athymic mice, whereas the comparably sized HBsAg particle was not immunogenic in athymic Balb/c mice (Milich 1986c). The T cell independence required that the HBcAg be particulate because denatured HBcAg and nonparticulate HBeAg were not immunogenic in athymic mice. The immunogenic effects of an antigen that possesses both T cell-independent and T cell-dependent characteristics may be synergistic in the presence of competent T cells. This property of HBcAg may explain its enhanced immunogenicity in the mouse model and during HBV infection.

Ability of HBcAg-specific T Cells to Prime Antibody Production to HBsAg: Intermolecular/Intrastructural T Cell help.

Due to the marked immunogenicity of HBcAg and the observation in the influenza system that matrix-specific T cells could elicit hemagglutinin-specific antibody production (Russell 1979), the ability of HBcAg-primed T cell to function as Th cells for antibody production to envelope (HBsAg) epitopes was examined (Milich 1987c). B10.S mice primed with HBcAg and challenged with a mixture of HBcAg and HBsAg/P39 produced no anti-HBs, however, mice challenged with virions produced anti-S, anti-pre-S(2), and anti-pre-S(1)-specific antibodies. To confirm the T cell nature of this effect, the identical experiment was performed using the synthetic T cell recognition site, p120-140, as the priming antigen. The results obtained were similar to those using native HBcAg as the priming antigen. This result indicated that HBcAg-primed T cells could function to help anti-envelope antibody production to multiple epitopes, and the Th cell activity did not require that HBcAg and HBsAg be present on the same molecule (intermolecular), but did require that they be within the same particle (intrastructural) (Milich 1987c).

Production of antibodies specific for the S, and pre-S regions occurs during resolving acute HBV infection, but not during the acute phase or subsequently in chronic asymptomatic infection (Milich 1987d). However, a subset of chronically infected HBV patients has been reported to produce only anti-pre-S(1) in the absence of anti-pre-S(2) or anti-S antibodies, which correlated with seroconversion from HBeAg to anti-HBe status and viral clearance (Takai 1986). Anti-pre-S(1) production in the absence of anti-pre-S(2) and anti-S production may be mediated by Th cells specific for HBcAg/HBeAg determinants.

Although antibodies to HBcAg do not prevent infection, the fact that HBcAg/HBeAg-specific Th cells can elicit anti-envelope antibodies, which are virus neutralizing (Gerety 1979; Itoh 1986; Thornton 1987; Emini 1989), may explain the reported ability of HBcAg vaccination to protect against HBV liver disease (Murray 1984). Furthermore, since HBcAg/HBeAg-specific Th cells were shown to induce anti-S antibody production in S region nonresponder mice, this represents another mechanism of circumventing HBsAg nonresponsiveness (Milich 1987c). This observation can be applied to vaccine development. The HBcAg may be used as a T cell carrier for HBsAg or other pathogen-specific antigens by coupling the antigens either chemically or by recombinant DNA technology. Alternatively, synthetic HBcAg/HBeAg Th cell epitopes may be coupled or genetically engineered into HBsAg particles or polypeptides as discussed in the next section.

A Totally Synthetic HBV Immunogen

Because the p120-140 sequence of HBcAg was shown to encompass distinct Th cell recognition sites for B10.S and B10 mice, the ability of p120-140, coupled directly to

a synthetic B cell epitope, to act as a T cell carrier moiety was examined (Milich 1988a). The B cell epitope chosen was the pre-S(2) region peptide p133-140, which was previously shown to represent a dominant antibody binding site within the pre-S(2) region (Milich 1986b). The unconjugated p133-140 sequence of the pre-S(2) region is nonimmunogenic. B10.S, B10 and B10.BR mice were immunized with a composite peptide composed of residues 120-140 from the HBcAg sequence and residues 133-140 from the pre-S(2) region of the envelope designated c120-140-(133-140). The B10.S and B10 strains produced antibody to the envelope B cell epitope (p133-140), which was highly crossreactive with native HBsAg/GP33. The B10.BR strain does not recognize the p120-140 HBcAg sequence at the T cell level, and predictably this strain was a nonresponder to immunization with c120-140-(133-140). To confirm that the predicted sites within the composite immunogen were functioning as T cell recognition sites, c120-140-(133-140)-immunized mice were evaluated at the T cell level as well. B10.S, c120-140-(133-140)-primed T cells responded to c120-140, the N-terminal fragment, p120-131, and to native HBcAg. B10, c120-140-(133-140)-primed T cells were activated by c120-140, the C-terminal fragment, p129-140, and native HBcAg. The B cell epitope (133-140) and native HBsAg/GP33 were non-stimulatory in both strains. B10.BR, c120-140-(133-140)-primed T cells were nonresponsive to the entire antigen panel (Milich 1988a). These results demonstrate the feasibility of constructing complex synthetic immunogens representing multiple proteins of a pathogen, and capable of engaging both T and B cells relevant to multiple native antigens.

SUMMARY

This experimental model offers a unique opportunity to study the genetic, cellular, and molecular basis for variable immune responsiveness to HBV-encoded antigens. The linkage between the MHC and the regulation of immune responsiveness to HBsAg in mice has been extended to the human immune response by reports of an association between HLA phenotype and low to nonresponsiveness to recent HBsAg vaccines (Walker 1981; Craven 1986). The murine model has provided a means of studying the immunogenicity of the pre-S regions of HBsAg and has elucidated the independent H-2-linked genes regulating antibody production to pre-S and S region determinants. The ability to circumvent genetic nonresponsiveness has implications for the design of future HBV vaccines. The murine model afforded the opportunity to examine the ability of HBcAg to activate B cells directly, and to prime Th cells capable of eliciting anti-envelope antibody production. These observations have potential clinical relevance, and may explain the ability of HBcAg vaccination to protect against HBV infection. The murine system has also facilitated the mapping of T cell and B cell recognition sites within HBV proteins, which at least conceptually enhances the prospects for development of a synthetic HBV vaccine.

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Vaccinia Virus Vectors: Applications to Vaccines

B. Moss

INTRODUCTION

The usefulness of vaccinia virus, a member of the poxvirus family, for the prevention of smallpox was established by Edward Jenner in the 18th century. The global deployment of this vaccine resulted in the eradication of smallpox approximately 10 years ago. The potential use of vaccinia virus as a vector to produce live recombinant vaccines against other infectious dieases has received considerable attention. While a recombinant vaccine of this type has yet to be licensed, vaccinia virus vectors have proven to be extraordinarily valuable tools for determining the targets of humoral and cell-mediated immune responses.

CONSTRUCTION OF RECOMBINANT VACCINIA VIRUSES

Poxviruses are large DNA viruses that replicate in the cytoplasm of infected cells (Moss 1985). Their unique ability to replicate outside of the nucleus is partly due to the encoding of enzymes for both transcription and replication within the viral genome. In order for vaccinia virus to express genes from other microorganisms, it is necessary to use poxvirus transcriptional signals. In addition, since vaccinia virus mRNAs are not spliced and transcription occurs in the cytoplasm, genes that have continuous open-reading-frames or cDNA copies of mRNAs must be employed. The large, approximately 185,000 base pair genome, makes it most convenient to insert genes by recombinational methods (Mackett et al. 1982; Panicali and Paoletti 1982). Special plasmid insertion vectors that contain vaccinia promoters, unique restriction sites for gene insertion, and flanking vaccinia virus DNA that directs recombination to a non-essential site within the vaccinia genome have been constructed (Mackett et al. 1984). After insertion of the gene and propagation of the plasmid in *Escherichia coli*, the plasmid is used to transfect tissue culture cells that have been infected with vaccinia virus. Since recombination is not very efficient, only about 0.1% of the progeny have the desired DNA insert. Although, gene specific procedures such as DNA hybridization alone or antibody binding may be used to pick the recombinant virus plaques, more efficient selection and general screening methods have been developed. These include thymdine kinase negative selection (Mackett, et al. 1984), β-galactosidase co-expression (Chakrabarti et al. 1985; Panicali et al. 1986), and neomycin (Franke et al. 1985) and mycophenolic acid selection (Boyle and Coupar 1988; Falkner and Moss 1988). By employing rapid new methods

of genetic engineering involving oligonucleotide synthesis and polymerase chain reaction coupled with efficient methods of isolating recombinant vaccinia viruses, the desired constructs may be obtained in a matter of weeks.

Both the level and timing of expression are affected by the choice of vaccinia virus promoters. The majority of genes have been expressed using a compound promoter (P7.5) containing both early and late transcriptional signals providing for synthesis during the entire growth cycle (Cochran et al. 1985). Higher expression can be obtained using vectors with strong late promoters (Falkner and Moss 1988; Patel et al. 1988). However, as will be discussed later, antigens that are derived from proteins that are expressed late may not be presented well in association with MHC class I molecules. The recent successful transfer of the *lac* repressor system from *E. coli* to vaccinia virus (Fuerst et al. 1989) provides further regulation that may be particularly useful for the expression of toxic genes.

The origin and pedigree of vaccinia virus strains are difficult to determine because of the long history of their use. Those strains most widely employed during the World Health Organization smallpox eradication campaign were the New York City Board of Health strain, the Lister strain, and the Temple of Heaven strain. The latter was used primarily in China. For laboratory studies, the WR derivative of the New York City Board of Health strain has become a standard among virologists and has the advantage of being mouse adapted. However, because of its passage history and neurovirulence for animals, it is unlikely to be used as a human vaccine.

DETERMINATION OF TARGETS OF HUMORAL AND CELL MEDIATED IMMUNITY

Genes from virtually any source may be expressed in avian or mammalian cells by a vaccinia virus vector. Moreover, proteins that are normally made in eukaryotic cells will be processed and transported appropriately. Thus, there are numerous examples of expressed proteins that have been glycosylated and transported to the plasma membrane and some that have been transported to the nucleus. Cells infected in vitro with a recombinant vaccinia virus can be used for screening of complex mixtures of antibodies. Alternatively, animals inoculated with the live vaccina virus recombinant will frequently make a good antibody response to the expressed protein (as well as to vaccinia proteins). In this way polyclonal or monoclonal antibodies (Yilma et al. 1987) can be generated without purifying a protein.

Vaccinia virus-infected cells may be lysed by cytotoxic T lymphocytes (CTL) in an MHC class I restricted manner. Moreover, the target of such CTL may be a foreign protein expressed by a recombinant vaccinia virus (Bennink et al. 1984). Thus, at least during the early stages of vaccinia infection, processing of proteins and association with MHC molecules occurs in an apparently normal manner. Because of the possiblility of defects in antigen presentation

late in infection (Townsend et al. 1988), it may be best to use an early or a compound early/late promoter rather than a late promoter for expression. With the latter caveat, vaccinia virus has proven to be extremely versatile for CTL studies. For example, with this system, virtually all influenza proteins have been shown to be CTL targets (Bennink et al. 1987; Bennink et al. 1986; Yewdell et al. 1985). Since the same recombinant vaccinia virus may be used to infect different cells, specificty for MHC molecules may readily be determined in mouse or human systems. Alternative procedures involve the use of recombinant vaccinia virus for CTL priming in animals or for stimulating CTL precursors in vitro.

ANIMAL PROTECTION STUDIES

Since recombinant vaccinia viruses induce both humoral and cell-mediated immunity, the resistance of such immunized animals to infection may be determined by challenge. Protection of experimental animals ranging from the chimpanzee to the mouse and against a variety of DNA and RNA viruses has been achieved (Moss and Flexner 1987). In some cases, the vaccination elicited a neutralizing antibody response whereas in others only cell mediated immunity was demonstrated. The vaccine is usually administered by intradermal inoculation similar to the procedure used for smallpox. Other routes including oral, intranasal, intraperitoneal and intravenous also have been used. Protection against rabies virus has been achieved by administration of the recombinant vaccinia virus by the oral route and its efficacy as a wild-life bait vaccine is under evaluation (Blancou et al. 1986).

STUDIES OF HUMAN IMMUNODEFICIENCY VIRUS (HIV)

Human acquired immunodeficiency syndrome (AIDS) is caused by HIV-1, a member of the retrovirus family. The expression of HIV-1 proteins by vaccinia virus vectors (Chakrabarti et al. 1986; Hu et al. 1986; Kieny et al. 1986) has provided considerable information relevant to vaccines . The envelope protein is composed of two glycosylated polypeptides, gp120 and gp41, which are formed by proteolytic cleavage of the gp160 precursor. The precursor has been expressed by recombinant vaccinia viruses and was correctly processed into subunits and inserted into the plasma membrane (Chakrabarti, et al. 1986). Moreover, the protein was biologically active and caused fusion to occur between cells infected with the recombinant vaccinia virus and uninfected cells bearing CD4, the HIV receptor (Lifson et al. 1986). This result demonstrated that no other HIV proteins are needed for this process. Recent sucrose gradient, cross-linking and polyacrylamide gel electrophoretic analyses by my co-workers Patricia Earl, Robert Doms, and Sekhar Chakrabarti have demonstrated that the HIV-1 and HIV-2 envelope proteins synthesized by recombinant vaccinia viruses exist in a natural oligomeric form.

The polymerase of HIV is expressed as the C-terminal half of a large polyprotein that is made from a *gag-pol* mRNA by ribosomal frame-shifting. Vel Karacostas in my laboratory has demonstrated that both *gag* and polymerase

proteins are formed when the *gag-pol* genes are expressed by a recombinant vaccinia virus. Interestingly, most of the reverse transcriptase was found in the cell culture medium. Further analysis, in collaboration with Matthew Gonda and Kunio Nagashima of the National Cancer Institute revealed immature and mature particles, closely resembling HIV, that had budded from the plasma membrane (Karacostas et al. 1989). Thus, assembly and maturation of HIV-like particles can occur in the absence of either infectious RNA or HIV envelope proteins. Such particles, with the addition of envelope proteins, may represent a safer immunogen than inactivated HIV.

Recombinant vaccinia viruses have been used to identify mouse and human CTL targets against several HIV-1 genes including *env* (Koenig et al. 1988; Plata et al. 1987; Takahashi et al. 1988; Walker et al. 1987), *gag* (Nixon et al. 1988; Walker, et al. 1987), and reverse transcriptase (Walker et al. 1988). Surprisingly, relatively high numbers of circulating CTL are present in HIV infected individuals. Whether such CTL are playing a vital, although ultimately ineffective, role in combating HIV is uncertain. Walker and co-workers at the Massachusetts General Hospital have succeeded in establishing long term human CTL clones directed to the reverse trancriptase. In order to map the target epitopes of these CTL clones, Charles Flexner and I constructed a panel of recombinant vaccinia viruses containing truncated *pol* genes. Fine epitope mapping was then achieved with a set of overlapping 25-mer peptides (Walker et al. 1989). Another panel of recombinant vaccinia viruses expressing truncated *env* genes, constructed by Patricia Earl in my laboratory, have been used by Scott Koenig, also at the National Institute of Allergy and Infectious Diseases, for mapping human CTL epitiopes.

Recombinant vaccinia viruses expressing HIV *env* have been shown to stimulate largely isolate-specific neutralizing antibody in monkeys (Earl et al. 1989). Although chimpanzees that were immunized with a similar recombinant vaccinia virus developed proliferative and cytotoxic T cells to HIV *env*, the animals were not protected against an HIV challenge (Hu et al. 1987; Zarling et al. 1987). A more complicated immunization schedule has resulted in the induction of neutralizing antibody in one human volunteer (Zagury et al. 1989). Phase 1 testing of a recombinant vaccinia virus expressing *env* is in progress in the United States.

SAFETY OF RECOMBINANT VACCINIA VIRUSES AS LIVE VACCINES

Vaccinia virus was considered to be a highly effective and relatively safe vaccine against smallpox. Indeed, the global use of this vaccine resulted in the eradication of the disease. Nevertheless, there are risks associated with the use of vaccinia virus. Perhaps most important is the progressive spread of the vaccinia infection in immunodeficient individuals. The genetic engineering of vaccinia virus to reduce this risk is therefore of considerable importance. Athymic nude mice have been used as an immunodeficiency model. The intraperitoneal injection of only 100 plaque forming units of a mouse adapted strain of vaccinia virus results in 100% mortality (Moss and Flexner 1987).

Attenuation of vaccinia virus was achieved by two different approaches. In the first, certain replication competent deletion mutants were shown to spare nude mice inoculated with as much 100 million plaque forming units (Kotwal et al. 1989). The second approach involved the insertion of lymphokine genes into vaccinia virus. Nude mice were resistant to 100 million plaque forming units of recombinant vaccinia virus expressing interleukin 2 (Moss and Flexner 1987). The decreased mortality was associated with rapid clearance of the virus expressing ithe lymphokine (Ramshaw et al. 1987).

SUMMARY

Recombinant vaccinia viruses have proven to be extremely useful for determining the targets of CTL and are being developed as candidate vaccines for medical and veterinary purposes. The ability to stimulate both humoral and cell mediated immunity, economy of manufacture, heat stability, and ease of delivery are attractive features of a live recombinant vaccine.

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Peptide Binding to MHC Class II Molecules: Applications to Vaccine Design

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INTRODUCTION

Much effort is currently being devoted to develop safe, cheap and effective vaccines to viral, bacterial and parasitic pathogens.
The engineering of vaccines has been greatly facilitated by recent advances in gene technology and our increased understanding of how T cells recognize antigens. It is now clear that T cells recognize protein antigens as peptides bound to major histocompatibility complex (MHC) molecules (Moller 1987). Immune responsiveness of the individual is therefore determined in large part by the aminoacid sequences of the highly polymorphic MHC gene products, which correlate with the ability of the immunogenic peptide to bind in vitro to the particular MHC molecule (Buus et al. 1987). The shortcoming of this is that many peptides have limited abilities to bind to or be presented in association with different HLA antigens, presenting a potential barrier to the future development of synthetic vaccines.
In this symposium, through the work we have done on malaria sporozoite vaccine development, I should like to discuss new strategies for identifying pathogen-derived sequences, whose characteristics offer possible solutions to the problem of MHC restriction of the immune response for peptide vaccines.

SPOROZOITE MALARIA VACCINE

The basis for an anti-sporozoite malaria vaccine is to prevent sporozoites from entering hepatocytes or to destroy infected hepatocytes prior to release of merozoites (Nussenzweig and Nussenzweig 1989). Although the mechanisms that mediate protective immunity against malaria parasites are not yet entirely clear, it is likely that class II (Spitalny et al. 1977) and class I MHC-restricted T cells (Schofield et al. 1987, Weiss et al. 1988) as well as B cells (Zavala et al. 1987) are involved. Initial efforts to develop a malaria vaccine had however focused only on the induction of antibodies directed against an immunodominant B cell epitope, (NANP)3, located within the repeat region of the circumsporozoite (CS) surface protein (Zavala et al. 1985). Preliminary vaccination trials with vaccines containing the repeated sequence gave unsatisfactory results (Herrington et al. 1987, Ballou et al. 1987, Etlinger et al. 1988); this underlines the importance of identifying specific epitopes that stimulate T cell-mediated immunity to sporozoites.

T cell epitopes in the CS protein

We have searched for T cell epitopes outside the repetitive NANP sequence that could be incorporated into new vaccines.
We started by testing the in vitro proliferative response of peripheral blood mononuclear cells (PBMC), obtained from both immune and non-immune donors, to CS protein-derived peptides wich were predicted to contain T cell epitopes. Peptides derived from at least 3 different regions of the CS protein (102-122, 325-341 and 378-398) could stimulate human PBMC to proliferate. Most of the individuals responded to the peptide corresponding, with the exception of two cysteine-alanine substitutions, to residues 378-398 of the CS protein, while none of the 16 donors tested responded to the repeat region of the CS protein (Sinigaglia et al. 1988a).

A peptide binding to different alleles of a HLA class II molecule

The highly restrictive MHC patterns obtained in early studies with malaria peptides in murine systems suggested that MHC restriction may pose a major problem in the selection of epitopes for inclusion in a synthetic vaccine and that several T cell recognition sites may be required to ensure responsiveness in a high percentage of vaccine recipients (Good et al. 1988). We therefore wished to know the spectrum of HLA antigens with which each T-cell epitope-containing peptide could associate. We established an indirect functional assay for peptide binding to MHC molecules that allowed us to determine for each peptide the spectrum of HLA-encoded immune response genes (Kilgus et al. 1989). The assay is based on the proliferative response of a given T cell clone to a "test peptide", and asks whether other peptides can specifically inhibit this reaction by competing with the "test peptide" for a single binding site on the MHC restriction element of the antigen-presenting cells (APCs) in culture. Initially we used two T cell clones, one that recognized peptide 325-341 of the CS protein in association with DRw11(5), and one specific for peptide 260-273 of the P.falciparum blood stage p190 protein associated with DRw6 molecules. We found that Ala-substituted CS 378-398, which we named CS.T3, was the only peptide able to compete with the binding of the stimulator peptides to DR5 and DRw6. CS.T3 was also the only peptide out of the five tested to bind to DR1 using a direct binding assay on soluble DR1 molecules (T. Jardetsky, Harvard University, personal comunication).

Using T cell clones fom unprimed donors to characterize the CS.T3 epitope

In a second step, we examined the ability of the CS.T3 peptide to induce primary T-cell responses and to induce T-cell clones able to respond to the native protein in the presence of appropriate APCs. We therefore challenged PBMC of 20 malaria non-immune donors with the peptide in vitro. From the stimulated cells of 8 donors, close to 300 peptide-specific T cell clones were derived. Analysis of the restriction specificity of the clones revealed that the

peptide could be recognized in combination with at least 8 different DR molecules including DR1, 2, 4, 5, 6, 7, 9 (Sinigaglia et al. 1988b) and 8 (Sinigaglia et al. 1989) (Fig.1). T cell clones recognizing CS.T3 peptide in association with several DR antigens also responded in vitro to the parasite-derived CS protein as well as to a recombinant polypeptide containing the CS.T3 sequence with the native protein's cysteine residues (Guttinger et al. 1988, Kilgus et al. 1989). The primary T-cell responses to the peptide we selected by using the competition assay therefore indicate the ability of the peptide to induce memory T cells that can be boosted by subsequent infections with the pathogen (Sinigaglia et al. 1989). An important observation is that this region is invariant in different parasite isolates (Guttinger et al. 1988, Caspers et al. 1989); thus if the CS.T3 peptide does function to induce responses against the parasite, the protection it offers will not be limited to only a small subset of malaria isolates.

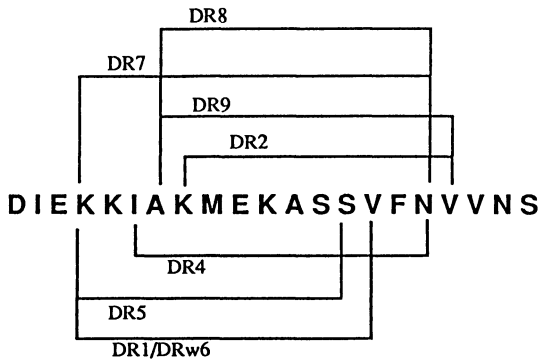

Fig. 1. Definition of determinants in peptide CS.T3 recognized by T-cell clones restricted to different DR alleles (Sinigaglia et al. 1988b).

Construction of a synthetic vaccine

We next constructed a synthetic immunogen by coupling the CS.T3 peptide to the B cell determinant (NANP)3. Mice from several strains which were normally unable to respond to the NANP repeat did produce antibodies to the repeats, as well as to sporozoites, when injected with the CS.T3-(NANP)3 conjugate (Sinigaglia et al. 1988b). Although C57BL/6 T cells could recognize $(NANP)_n$, T cells from the other strains did not respond to the repetitive sequence alone (Togna et al. 1986, Good et al. 1986). The fact that all strains tested responded to (NANP)3 coupled to CS.T3, in addition to confirming that CS.T3 was indeed a helper T cell site, showed that the CS.T3 sequence is also recognized in association with many different mouse Ia molecules. These results have clear implications with respect to the development of a completely synthetic malaria vaccine. Since the synthetic T cell determinant CS.T3 was capable of priming T cell help for antibody production to the B cell determinant (NANP)3 and since antibody reacted with the native CS protein, this system provides evidence that

synthetic T and B cell recognition sites can be combined to yield a functional malaria immunogen. Such a vaccine, obviating the requirement to conjugate the B cell site to a heterologous protein carrier, should elicit T and B cell memory relevant to the pathogen.

Structural requirements for the interaction between CS.T3 and class II molecules

The CS.T3 peptide is recognized in association with at least 8 different DR antigens (possibly more, as we lack information about the other DR alleles). Although there is evidence that some peptides can bind to more than one MHC allele, this was the first evidence for such permissive association. The structural basis for this broad binding capacity to DR molecules is unclear. We initially proposed the possibility that the binding of the peptide is largely if not exclusively dependent on DRα chain, which shows limited allelic polymorphism (Sinigaglia et al. 1988b). However this possibility seems to be excluded by our more recent analysis of the fine specificity of the interaction of CS.T3 with DRw11(5) and DRw6. Using a series of peptides truncated at either the N or C-terminus of the CS.T3 sequence we have determined the binding profile for the two DR molecules (Table 1). The critical residues required for interaction with DRw11(5) are contained within residues 380 and 391 and for interaction with DRw6 within residues 381-392. Thus distinct, although closely overlapping, regions of the peptide were involved in the binding to two different class II alleles.

Table 1. Effect of CS.T3 truncations on binding to DRw11(5) and DRw6

CS Peptide	Aminoacid sequence	Binding to DRw11(5)	DRw6
378-398	DIEKKIAKMEKASSVFNVVNS	1[a]	15
379-398	IEKKIAKMEKASSVFNVVNS	0.7	12
380-398	EKKIAKMEKASSVFNVVNS	2	15
381-398	KKIAKMEKASSVFNVVNS	>2000	67
382-398	KIAKMEKASSVFNVVNS	>2000	>2000
383-398	IAKMEKASSVFNVVNS	>2000	>2000
378-396	DIEKKIAKMEKASSVFNVV	7	5
378-395	DIEKKIAKMEKASSVFNV	2	8
378-394	DIEKKIAKMEKASSVFN	32	15
378-393	DIEKKIAKMEKASSVF	28	17
378-392	DIEKKIAKMEKASSV	35	100
378-391	DIEKKIAKMEKASS	160	>2000
378-390	DIEKKIAKMEKAS	>2000	>2000

[a] Concentration (μM) of truncated peptide required for 50% inhibition of antigen presentation (Kilgus et al. 1989).

As a another approach to the analysis of the specificity of the interaction between CS.T3 and different DR alleles, we have synthesized a large number of analogues of CS.T3 with a single non-conservative amino acid substitution at each position of the 380-396 sequence and examined their capacity to bind DRw11(5) and DRw6. Binding to DRw11(5) was significantly affected by substitutions at positions 383, 384, 386, 387 and 388, while substitutions at position 389, 390 and 392 abrogate binding to DRw6 (Kilgus, Trzeciak, Gillessen et al. manuscript in preparation). Therefore different residues are involved in the interaction with the two closely related DR molecules. This again would suggest that different alleles of the same MHC molecule may actually recognize different structures of the CS.T3 peptide.

MHC restriction and malaria vaccines

Genetic control of immune responsiveness to experimental antimalarial vaccines was found in several studies using inbred mice. Only mice carrying the I-A^b class II MHC allele, for example, can generate specific antibodies when immunized with NANP polymers (Del Giudice et al. 1986, Good et al. 1986); similarly, only mice bearing I-A^k responded to a yeast-derived recombinant P.vivax protein (Nardin et al. 1989). The implication of these findings was that if the same situation occurred in humans an antimalaria vaccine might be effective in only a small proportion of individuals.
Our studies have however indicated that the 378-398 region of the malaria CS protein is a promising vaccine candidate. It binds to and is presented by a large number of MHC class II proteins of mice and men and immunization of mice with CS.T3 induced helper T cells which could be boosted by immunization with whole sporozoites (Sinigaglia et al 1989). Studies with human populations have also shown that there are epitopes in other malaria vaccine candidates which are seen by a large proportion of individuals. (Rzepczyk et al. 1988 and Sinigaglia et al. 1988c). In line with these findings we have recently evaluated 18 T cell determinants, derived from different proteins, for the ability to bind several class II molecules (DR1, DR2, DR4, DRw11(5), DRw6(14), DR7 and DRw52b). We found that 2 of such epitopes were shown to bind to varying extent to all 7 DR alleles examined (Guttinger M, manuscript in preparation). This observation is encouraging for vaccine design as it would suggest that epitopes binding multiple allelic forms of the same class II molecules might also be found in other pathogen-derived sequences.

CONCLUSIONS

Collectively the data presented here indicate a strategy for the development of human vaccines. The screening of potential vaccine candidates can be carried out in vitro and their ability to activate T cells can be tested by in vitro priming of PBMC. In most cases, as exemplified by the present results, peptides binding to MHC class II molecules should also be able to induce T cell activation.

The possibilities of studying in vitro interactions between peptides and HLA class II molecules and of priming human T cells in vitro may facilitate the identification of T cell determinants to be incorporated in effective vaccines potentially directed against any human pathogen.

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Expression and Secretion of Repertoires of VH Domains in *Escherichia Coli:* Isolation of Antigen Binding Activities

E.S. Ward, D.H. Gussow, A. Griffiths, P.T. Jones, and G.P. Winter

INTRODUCTION

The production of monoclonal antibodies using hybridoma technology (Köhler and Milstein, 1975) represents a landmark for the use of antibodies as reagents for medicine and biology. It is now possible to use recombinant DNA methods to isolate and genetically manipulate antibody genes, and the resulting immunoglobulins or immunoglobulin fragments can be efficiently expressed in mammalian or bacterial hosts (for a review, see Morrison et al., 1988).The immunoglobulin molecule consists of a string of domains, each domain consisting of about 100 amino acid residues. The constant domains carry the effector functions, such as complement mediated lysis and ADCC. The antigen binding site is fashioned by both heavy (VH) and light (VL; Vκ or Vλ) chain variable domains, as demonstrated by the solved crystallographic structures of antibody in association with antigen (Amit et al.,1986; Sheriff et al., 1987; Colman et al.,1987) or hapten (Satow et al, 1986). Variable domains have been pasted onto constant domains (Morrison et al.,1984; Boulianne et al., 1984; Neuberger et al.,1985) and hypervariable loops (CDRs) onto the underlying β-sheet framework of variable domains (Jones et al.,1986; Verhoeyen et al.,1988; Riechmann et al.,1988a). Grafting CDRs has been used to humanise rodent antibodies and one such antibody used in the successful treatment of 2 patients with non-Hodgkins lymphoma (Hale et al.,1988; Riechmann et al.,1988a).

As the variable domains of an antibody confer the affinity and specificity of binding, their isolation and characterisation are prerequisites for both structure-function analyses and medical applications. There is therefore a need for methods of rapidly generating and screening banks of variable domains for desired binding specificities. The expression and secretion of antibody variable domains using *E.coli* as a host represents a convenient way of cloning and analysing binding activities, and is generally much faster than the route using mammalian expression systems.

We have been analysing the interactions of the anti-lysozyme antibody D1.3 with antigen (Amit et al., 1986), and have expressed the VH and VL domains individually, or in association as an Fv fragment by secretion into the *E. coli* periplasm (Skerra and Plückthun, 1988). Both the Fv fragment and the VH domain can be purified. They both bind antigen with a high affinity. This observation led us to generate repertoires of VH domains for the expression of binding activities in *E. coli.* To generate these VH repertoires two approaches have been used; (1) rearranged VH genes have been cloned from antibody producing cells of immunised mice using the polymerase chain reaction (PCR), (2) the gene encoding the VH domain of the anti-lysozyme D1.3 antibody has been extensively mutated. For both repertoires, VH domains with desired binding specificities have been identified and, in some cases, purified and characterised kinetically.

METHODS

Vectors

For expression of the VH domain (VHD1.3) of the D1.3 antibody, the vector pSW1-VHD1.3 was constructed by cloning the VHD1.3 gene into a pUC19 vector (Yanisch-Perron et al., 1985) with a synthetic oligonucleotide encoding a pelB signal sequence (Better et al.,1988) (Fig. 1). For expression of both domains, the vector pSW1-VHD1.3-VKD1.3 was constructed by cloning the Vκ domain and pelB signal into pSW1. For cloning and expression of the VH repertoire, the vector pSW1-VHPOLY was built by cloning a restriction enzyme polylinker sequence to replace the body of the VHD1.3 gene. This vector was further adapted (pSW1-VHPOLY-TAG1) by ligating a synthetic oligonucleotide encoding a peptide tag (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu-Asn) from c-myc (Evan et al., 1985; Munro and Pelham, 1986), to the C-terminal end.

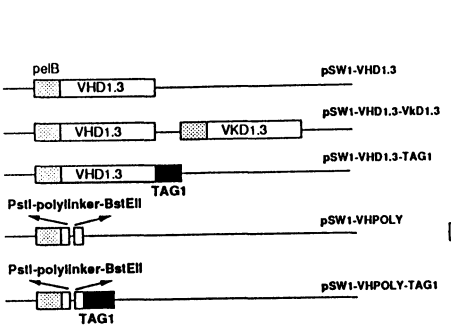

Figure 1. Expression vectors for secretion of immunoglobulin fragments from *E. coli*.

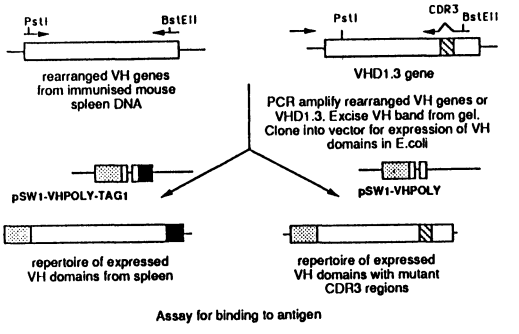

Figure 2. Strategy for the cloning of VH repertoires into *E. coli* expression vectors.

PCR amplification of mouse DNA (see Fig. 2)

Balb/c mice were hyperimmunised with hen egg-white lysozyme (100 µg antigen i.p. day 1 in complete Freunds adjuvant and 50 µg antigen i.v. day 35 in incomplete Freunds adjuvant, kill day 39) or similarly with keyhole limpet haemocyanin (KLH). DNA was prepared from the spleen (Maniatis et al., 1982) and the rearranged mouse VH genes were amplified using PCR (Saiki et al., 1985; Orlandi et al., 1989) using the primers VH1FOR-2 (5' TGA GGA GAC GGT GAC CGT GGT CCC TTG GCC CC 3') and VH1BACK (5' AGG T(C/G)(C/A) A(G/A)C TGC AG(G/C) AGT C(T/A)GG 3') and conditions described in Ward et al., 1989.

PCR mutagenesis (see Fig. 2)

The VHD1.3 gene cloned into M13mp19 was amplified with a mutagenic primer based in CDR3 and a primer based in the M13 vector backbone (5' AAC AGC TAT GAC CAT G 3'). The mutagenic primer 5' GGA GAC GGT GAC CGT GGT CCC TTG GCC CCA GTA GTC AAG NNN NNN NNN NNN CTC TCT GGC 3' (where N can be any of the four bases T, C, G or A) hypermutates the central 4 residues of CDR3 (Arg-Asp-Tyr-Arg).

Cloning and expression of antigen binding activities

PCR amplified DNA was digested with PstI and BstEII (encoded within the amplification primers) and fractionated on an agarose gel. A band of about 350 bp was extracted and cloned into the M13VHPCR1 vector (Orlandi et al., 1989) for sequencing or into the pSW1-VHPOLY or pSW1-VHPOLY-TAG1 vector for expression. The recombinant plasmids were transformed into *E. coli* BMH71-18 (Gronenborn, 1976), colonies selected on TYE plates (Miller, 1972) with 1% glucose (GLU) and 100 μg/ml ampicillin (AMP), and toothpicked into 200 μl 2 x TY (Miller, 1972), AMP, GLU in wells of ELISA plates. Colonies were grown and induced as in Ward et al., 1989.

Western blotting

As in Towbin et al., 1979: supernatant (10 μl) from the cultures was subjected to SDS-PAGE (Laemmli, 1970) and the proteins transferred electrophoretically to nitrocellulose. The VH domains were detected via the peptide tag with the 9E10 antibody (Evan et al., 1985; Munro and Pelham, 1986), using horse radish peroxidase conjugated rabbit anti-mouse antibody and 4-chloro-1-naphthol as the peroxidase substrate.

ELISA

Wells of ELISA plates (Falcon) were coated with antigen in phosphate buffered saline (PBS) overnight (3 mg/ml lysozyme or 50 μg/ml KLH), then blocked with 2% skimmed milk powder in PBS for 2 hr at 37 °C. Bacterial supernatants were screened for binding activities as in Ward et al., 1989. For competition ELISA, the binding of VHD1.3 tagged with the c-myc peptide (Fig. 1) was assayed in the presence of dilutions of untagged VH domain (VH1).

Purification of Fv and VH domains binding to lysozyme

500 ml cultures were grown and induced as above, and the supernatant passed through a 0.45 μm filter (Nalgene), then down a 5 ml column of lysozyme-Sepharose (Riechmann et al., 1988b). After washing with phosphate buffered saline (PBS), the Fv fragment or VH domains were eluted with 50 mM diethylamine, neutralised with 1/10 volume 1M Tris-HCl pH 7.4, and analysed by SDS-PAGE.

Affinity for lysozyme

The purified D1.3 Fv fragment and VH domains were titrated with lysozyme using fluorescence quench (Perkin Elmer LS-5B Luminescence Spectrometer) to determine their affinities of binding. The stoichiometry of binding of the VHD1.3 domain was measured by fluorescence quench titration (to yield the total number of lysozyme binding sites), and by amino acid hydrolysis (to yield the total amount of protein). The kinetics of lysozyme binding were determined by stopped-flow (HI Tech Stoped Flow SHU) at 20 °C under pseudo-first order conditions with binding sites in 5-10-fold excess over lysozyme . The number of binding sites was determined by fluorescence quench titration with lysozyme in excess (Ward et al.,1989).

RESULTS

Expression of D1.3 Fv and VH in *E. coli*

The Fv fragment of the D1.3 antibody was expressed in *E. coli* using the plasmid construction shown in Fig.1. Secreted Fv could be purified with a yield of about 10 mg/litre culture. SDS polyacrylamide gel electrophoresis of the pure Fv showed 2 bands of about 14 kDa. N-terminal sequence determination after transfer of the 2 separated bands onto PVDF membranes (Matsudaira, 1987; Fearnley et al., 1989), showed that the pelB leader had been cleaved correctly. The VH domain of the D1.3 antibody was also expressed and secreted using the construct lacking the D1.3 Vκ gene (Fig. 1), and could be purified using lysozyme sepharose chromatography. The yield of the VH domain was much lower than that of the Fv, suggesting that for the Fv, the Vκ may prevent VH aggregation and/or stabilise the folding.

Affinity measurements using fluorescence quench titrations (Perkin Elmer LS-5B luminescence spectrometer) and stopped flow kinetics were carried out with the purified Fv and VH (Table 1). The affinity of the Fv fragment (3 nM) is similar to that of the parent antibody (2 nM). The affinity of the VH domain for lysozyme was determined as < 40 nM by fluorescence quench and as 19 nM by stopped-flow. Thus the affinity of the VH domain is only approximately 10-fold weaker than the complete antibody. The stoichiometry of binding of the VH domain was determined as 1.2 mole of lysozyme per mole of VH, suggesting an equimolar complex.

Table 1.Binding affinities of immunoglobulin fragments for lysozyme

	Stoichiometry	Affinity nM	kon $M^{-1}s^{-1}$	koff s^{-1}	koff/kon nM
Fv-D1.3	n.d.	3	0.8×10^6	n.d.	n.d.
VHD1.3	1.2	<40	3.8×10^6	0.075	19
VH1	n.d.	<15	n.d.	n.d.	n.d.
VH3	n.d.	n.d.	2.9×10^6	0.036	12
VH8	n.d.	n.d.	3.3×10^6	0.088	27

Generation and sequence analysis of a VH domain repertoire from mouse spleen cells

The PCR was used to amplify rearranged heavy chain genes from spleen DNA of a mouse which had been hyperimmunised with hen egg lysozyme. Following gel purification, the amplified DNA was cloned into an M13 vector, and 48 clones sequenced. Each of the 48 clones were found to have unique CDR3 sequences, and VH genes corresponded to all but 2 of the mouse heavy chain gene families as defined in Kabat (Kabat et al., 1987). A summary of the D- and J-segments found in these 48 clones is given in Table 2.

Table 2. Usage of VH gene, D segment and J-region families in the VH repertoire

VH genes		D segments		J regions	
Family	Number	Family	Number	Family	Number
IA	4	SP2	14	JH1	3
IB	12	FL16	11	JH2	7
IIA	2	Q52	5	JH3	14
IIB	17			JH4	14
IIIA	3				
IIIB	8				
IIIC	1				
VA	1				

Characterisation of VH domain binding activities from the repertoire

Amplified DNA from a mouse immunised with lysozyme was ligated into an expression vector which incorporates a C-terminal tag to facilitate detection of expressed VH domains (Figs. 1 and 2), and used to transform *E. coli*. To analyse the expression of these amplified VH domains, 17 clones were grown and induced for expression, and culture supernatants fractionated on a polyacrylamide gel. Western blotting of this gel showed that 14 of these clones secreted VH domains of the expected size. Two thousand colonies were toothpicked in groups of 5 into wells of ELISA plates, and the supernatants tested for binding to lysozyme coated plates. Twenty one supernatants were shown to have lysozyme binding activity. As a control, the supernatants were tested for binding to keyhole limpet haemocyanin (KLH), and 2 supernatants identified with KLH binding activity. VH domains were also derived by amplification and cloning of VH genes from spleen DNA of a mouse which had been hyperimmunised with KLH. From 2000 clones analysed, 14 showed KLH binding activity in an ELISA, whereas a single clone had binding affinity for lysozyme.

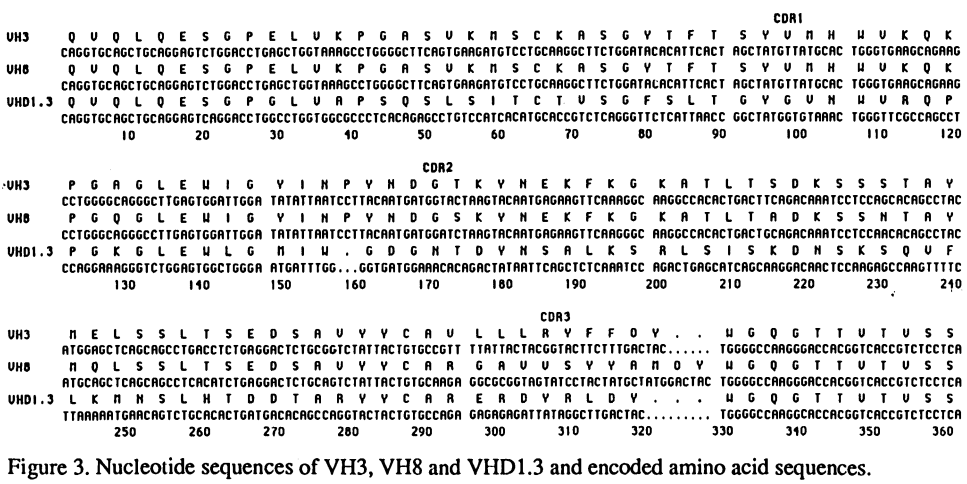

Figure 3. Nucleotide sequences of VH3, VH8 and VHD1.3 and encoded amino acid sequences.

Two of the genes corresponding to VH domains (VH3 and VH8) with anti-lysozyme affinities were sequenced, and both are different from VHD1.3 (Fig. 3). They belong to the same VH family (Kabat II) and D-segment family (FL16). The J-segments of the 2 domains are different (JH2 and

JH4), as are the CDR3s (Fig. 3). VH3 and VH8 were purified by affinity chromatography and their affinities measured using stopped flow kinetics and found to be 12 nM and 27 nM respectively (Table 1). The specificity of VH3, VH8 and the VHD1.3 domains was checked for binding to 4 other protein antigens (glyceraldehyde-3-phosphate dehydrogenase, KLH, insulin and cytochrome c), foetal calf serum, milk powder and microtitre plate plastic. No binding was observed.

In conclusion, it appears that VH domains can be rapidly isolated from mouse spleen DNA using PCR, and that 2 of these anti-lysozyme VH domains bind specifically with affinities of about 20 nM.

Mutagenesis of D1.3 VH to Generate a Repertoire

The PCR was used with a partially degenerate primer to randomly mutate the 4 central residues of the third CDR of the D1.3 VH. The amplified products were cloned and analysed by DNA sequencing. The mutant genes were subsequently ligated into the expression vector pSW1-VHPOLY and transformed into *E.coli*. Recombinant clones were toothpicked in pools of 5 into the wells of ELISA plates, grown up and induced for expression and secretion of VH domains. Culture supernatants from a total of 2000 clones were analysed for binding to lysozyme and KLH; 19 supernatants showed lysozyme binding activity and 4 had KLH binding activity.

To determine whether it is possible to generate VH domains of higher affinity than the parent domain (D1.3 in this case) using this mutagenesis approach, one of the mutant VH domains (VH1) which binds to lysozyme was selected for further analysis. VH1 was purified using affinity chromatography, and shown to compete with wild type D1.3 VH in a competition ELISA. Furthermore, fluorescence quench titrations indicate that this VH has an affinity which is less than 15 nM, and therefore significantly better than that of the parent VH. Sequence analysis shows that CDR3 of VH1 is completely different to that of D1.3, and the 4 central residues Arg-Asp-Tyr-Arg are replaced by Thr-Gln-Arg-Pro.

DISCUSSION

In previous work, separated heavy and light chains were identified with antigen (Fleischmann et al., 1963) or hapten binding activities (Utsumi and Karush, 1964), but the affinities were poor with no evidence for binding by single chains (Jaton et al., 1968) rather than dimers (Edmundson et al., 1984). Thus the observation that the VH domain of the D1.3 antibody binds lysozyme with high affinity in a 1:1 complex is novel.

In the D1.3 antibody, lysozyme makes extensive interactions to both domains. The VH domain makes 9 hydrogen bonding interactions, whereas the Vκ makes only 3. Binding of lysozyme buries about 300 A^2 of Vκ domain to solvent, and 400 A^2 of the VH domain (Amit et al., 1986; Chothia, unpublished results). Despite these interactions, the Vκ domain appears to make only a small net contribution to the energetics of binding. As the VH domain competes with the Fv fragment of the D1.3 antibody for lysozyme binding (not shown) it presumably binds antigen in a similar way as the antibody. It is possible, however, that the whole surface of interaction might reorientate slightly, perhaps by rocking on side chains to create a new set of contacts (Chothia et al., 1983), or that the loops of the VH domain could adjust to binding of antigen (Getzoff et al., 1987).

The report (Orlandi et al.,1989) in which PCR and 2 "universal"primers have been used to clone VH genes from cDNA of mouse hybridomas has now been extended to the cloning of a VH gene repertoire from mouse spleen DNA. Although from the analysis of the sequences of 48 VH domains the repertoire appears diverse, we cannot yet rule out the possibility of a systematic bias due to our choice of primers or hybridisation conditions.

The VH domains have been cloned and expressed in *E. coli*, and specific binding activities of high affinities (in the range of 20 nM) isolated. Immunisation increases the frequency of antigen specific clones, but is not essential. Thus PCR cloning of VH domains, followed by expression and secretion in *E. coli* may provide a convenient alternative to hybridoma technology. We are currently developing additional screening formats, using different vector systems, to facilitate the isolation of specific binding domains. In addition, it should be possible to reconstruct complete Fv fragments starting from a given VH domain, by screening a repertoire of Vκ domains for binding to the selected VH. These Vκs have been generated using PCR and Vκ specific primers with mouse spleen DNA.

The monomeric nature of the domain suggests that these molecules might be useful reagents for the blocking of canyon sites on viruses (Rossman et al., 1985); these canyon sites are too small to allow an Fv fragment to penetrate. Their small size should also faciltiate NMR analysis and high resolution epitope mapping.

An alternative route to the production of antigen specific VH domains is by random mutation of selected regions of an existing VH domain. For this approach, we have used PCR to randomly mutate the 4 central residues of the third hypervariable loop of the D1.3 VH. CDR3 was selected as it is the site of V-D-J recombination, and therefore the most variable region in both length and sequence of an antibody combining site. From a library of D1.3 derived VH domain, with 'randomised' CDR3s, most of the VH domains lose affinity for lysozyme; this is not surprising as CDR3 of this VH forms multiple contacts with lysozyme (Amit et al.,1986). A number of mutants have however been identified which retain high affinity for lysozyme, and one of these (VH1) binds with higher affinity than the parent VH domain. The CDR3 residues of this domain are completely different to those of D1.3 VH, and Pro99 (numbering as in Kabat et al., 1987) may kink the antigen binding loop. In addition, VH domains with affinity for KLH have also been identified from this D1.3 derived library; thus we believe that this approach may be extended to other binding activities to allow us to analyse the fine specificity of VH domains, and thus evaluate their utility in therapy.

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V Preventive and Therapeutic Manipulations

Transplantation

Prospects for Transplantation

P.J. Morris

INTRODUCTION

Over the past 35 years clinical transplantation has exploded from its tentative and experimental beginnings to become a major clinical discipline. Renal transplantation has become the treatment of choice for most causes of end-stage renal failure; cardiac and liver transplantation the preferred treatment for certain end-stage diseases of these organs; and bone marrow transplantation the favoured approach in the management of acute leukaemia in remission. Furthermore, the early results of combined heart and lung transplantation in conditions such as cystic fibrosis, and of transplantation of the endocrine pancreas to arrest the vascular complications of insulin dependent diabetics, are encouraging.

Indeed the current state of transplantation might lead one to suppose that most of the problems of transplantation had been resolved. This is certainly not true and although current immunosuppressive protocols have reduced the early losses of allografts from acute rejection, there is still a steady attrition of grafts from chronic rejection. Furthermore the complications of the immunosuppressive therapy are not inconsiderable, and include infection, cancer, hypertension and other manifestations of cardiovascular disease which is now the major killer after transplantation.

Where then does the future of transplantation lie? It is fair to say that in general the technical aspects of organ transplantation have been overcome. But rejection still remains the major unresolved problem and so a better understanding of the immune response to an allograft is essential if approaches to immunosuppression are to be made more specific, and indeed if the holy grail of transplantation, namely immunological tolerance, is ever to be achieved.

IMMUNE RESPONSE TO AN ALLOGRAFT

A simplified version of the immune response to an antigen is shown in Fig. 1. Antigen is processed and presented to the Th cell by the antigen presenting cells and this presentation is MHC restricted. Similarly in the allograft response, foreign histocompatibility antigens (incompatibility for which is the sine qua non of the allograft response) are processed and presented by host APC to host Th cells. But in addition it seems likely that foreign histocompatibility antigen may

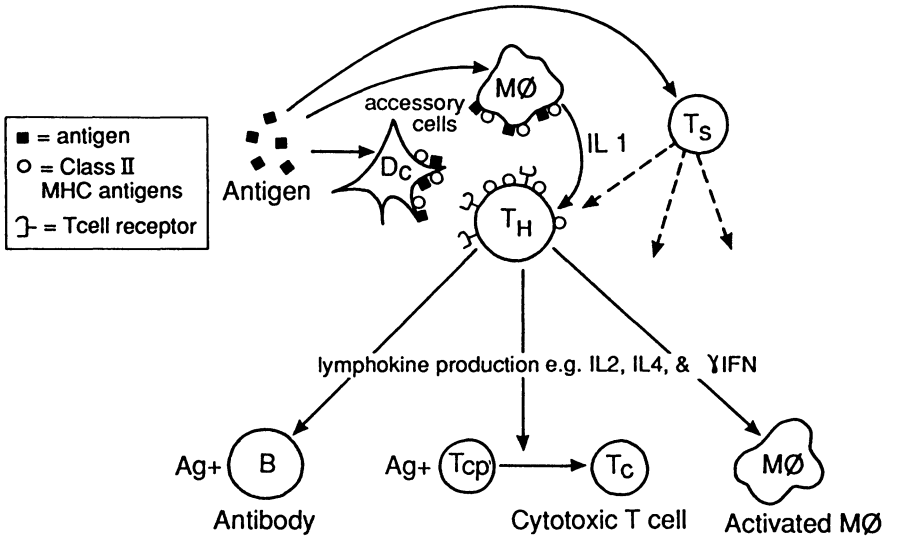

Figure 1. The immune response to an antigen (reprinted with permission - Dallman and Morris, 1988).

be recognised directly either within the graft itself or after the passage of donor dendritic cells to the spleen by host Th cells [Larsen et al 1989]. This conflicts with our understanding of antigen presentation in that the recognition of antigen cannot be HLA restricted in that situation. One has to postulate that the T cell receptor on host T cells is able to recognise some relatively public epitope of the allogeneic histocompatibility antigen, perhaps as expressed on the dendritic cell, which would be compatible with the relatively high frequency of T cells recognising a given histocompatibility antigen. Again the interaction between the cells involved in the immune response is controlled by an evergrowing family of cytokines, the various roles of which are just beginning to be defined.

The effector arm of the response comprises a specific response, both humoral and cellular, and a non-specific response involving macrophages and cytokines. The role of antibody in rejection is controversial, other than in the antibody mediated damage of a graft in the sensitised recipient. The key cell in the specific cellular response is also uncertain, for there is evidence that both the CD4+ Th cell or the CD8+ Tc cell of themselves may be sufficient to effect tissue damage of an allograft [Dallman & Morris 1988]. Another part of the immune response which is poorly defined is the role of suppressor cells. There is no doubt that suppressor cell phenomena appear to be real, and that cells, usually T cells, can adoptively transfer suppression from a host bearing a long surviving allograft to syngeneic hosts challenged with a fresh graft [Hutchinson 1986]. But it is not known whether these suppressor cells are a special subpopulation of T cells or T cells (of either CD4 or CD8 phenotype), which behave as effector cells or suppressor cells depending on the environment and the presence of appropriate lymphokines. The latter seems the most likely scenario.

CURRENT IMMUNOSUPPRESSION

Today cyclosporine provides the back-bone of all immunosuppressive protocols, but the most appropriate use of cyclosporine remains uncertain [Morris 1988]. For although the introduction of cyclosporine in the early eighties has led to improved survival of all organ allografts this has not been achieved without cost. The most important side effect associated with cyclosporine has been nephrotoxicity, and it is because of this that various protocols which allow the use of lower doses of cyclosporine have been introduced. These include triple therapy (low dose azathioprine, prednisolone and cyclosporine), double therapy (low dose azathioprine and cyclosporine), sequential therapy (antilymphocyte/antithymocyte globulin (ALG or ATG) or a pan T monoclonal antibody with azathioprine and prednisolone followed by the introduction of cyclosporine when good renal function is established), and conversion therapy (cyclosporine initially with later conversion to azathioprine and prednisolone). Nevertheless it must be said that there is no evidence at this time that one protocol is superior to another in terms of graft survival, although the relative freedom from complications with the triple therapy protocol has made it increasingly popular [Morris 1989]. Furthermore it does not seem that low doses of cyclosporine necessarily lead to the disappearance of nephrotoxicity and it now seems likely that an immunosuppressive dose of cyclosporine is also nephrotoxic. New drugs with immunological properties similar to those of cyclosporine are also being investigated with the emergence of promising experimental data, e.g. FK-506, rapamycin, and 15-deoxyspergualin.

The treatment of acute rejection remains high dose steroids, while ALG or a pan T monoclonal antibody is used for steroid resistant rejection. Again there is no evidence as yet that a pan T monoclonal antibody (OKT3 is the most widely used) is superior to a heterologous antilymphocyte/thymocyte globulin in the treatment of acute rejection although obviously the monoclonal antibody has the theoretical advantage of being a standard reagent [Cosimi 1988].

HISTOCOMPATIBILITY ANTIGENS AND MATCHING

Matching for the major histocompatibility complex (MHC) in man (HLA) does improve graft survival, but the effect is less striking with current immunosuppressive protocols which include cyclosporine. The striking influence of matching for the class II MHC antigens, HLA-DR, first demonstrated in 1978 in patients treated with azathioprine and prednisolone [Ting & Morris 1978], is still apparent today but the effect, at least in the short and medium term, is less apparent in first grafts although strongly so in second grafts [Table 1 and Opelz 1989]. Matching for the whole HLA complex is in general impractical in cadaver transplantation without extensive national and international organ sharing schemes, because of the extensive polymorphism of the system. But matching for HLA-DR alone is attractive in that it simplifies matching to the extent that it could be applied to heart and liver transplantation where prolonged preservation which allows organ sharing, is not possible. Indeed, recently a beneficial influence of matching for HLA-DR in cardiac transplantation has been reported [Festenstein et al 1989]. There is also some evidence that matching for the public specificities DRW52/53 influences the outcome of renal allografts, which if true, would further simplify matching.

Table 1. The influence of matching for HLA-DR in recipients of cadaveric grafts treated with cyclosporine in Oxford. (First grafts - p=0.14; regrafts - p=0.03).

HLA-DR Mismatches	1 year cadaveric graft survival (%) First graft (n)	Regrafts (n)
0	81 (79)	85 (23)
1	78 (134)	66 (36)
2	73 (83)	54 (28)

Another advancing area has been the recognition over the past 10 years of different types of antibodies which occur in patients awaiting renal transplantation as a result of transfusions, pregnancies, or a failed previous graft. Not all of these antibodies are directed at HLA and many are autoantibodies which, although reacting with donor cells in the crossmatch, are not damaging to a subsequent graft. As knowledge in this area has increased it has allowed renal transplantation in many patients who would otherwise be considered unsuitable for transplantation [Taylor et al 1989].

STRATEGIES FOR IMPROVED IMMUNOSUPPRESSION

The passenger leucocyte (dendritic cell)

That leucocytes carried over in the donor organ might be important was first recognised in experimental models in the early seventies, but as clinical trials directed at removing these passenger leucocytes from kidneys before grafting were on the whole unconvincing, interest waned. However in recent years there has been renewed interest following the demonstration that these passenger leucocytes are a highly specialised accessory cell, the dendritic cell, which is a potent inducer of allogeneic reactions in vitro even in very small numbers [Austyn et al 1983; Steinman et al 1986]. There is also increasing experimental evidence in vivo that in certain models these cells are of key importance in the induction of the allogeneic reaction. Of relevance is the recent demonstration in a cardiac allograft model that dendritic leukocytes migrate from the grafted organ in the first few days after transplantation to the spleen where they associate with Th cells [Larsen et al 1989]. This would suggest that induction of the immune response against an organ allograft may occur first in the spleen. Thus renewed efforts are being directed at defining the precise function of these cells in the allograft reaction as well as at methods designed at their deletion or functional inactivation in tissue allografts.

Monoclonal antibodies

As we increase our understanding of the complex cellular interactions involved in the allograft reaction, the availability of monoclonal antibodies to various lymphoid cell populations has not only helped define the different populations of cells involved in rejection but also allows manipulation of the reaction. So far the major clinical use of

monoclonal antibodies in clinical transplantation has been the use of a pan T monoclonal antibody (OKT3) directed against CD3, both to treat steroid resistant rejection and prophylactically in the prevention of rejection [Cosimi, 1988]. Other pan T monoclonal antibodies, directed against molecules other than CD3, have been much less effective suggesting that it is the close association of CD3 with the T cell receptor (TcR) which is important. Of relevance here is an encouraging early report of the clinical efficacy of a monoclonal antibody against the TcR [Wonigeit et al 1989].

However, pan-T monoclonal antibodies are not particularly specific in their effect, although providing a more specific reagent than the polyvalent anti-lymphoid sera used hitherto. The use of antibodies against CD4+ (Th) or CD8+ (Tc/s) cells represents a greater degree of selectivity in suppression. For example an anti-CD4 antibody in the mouse (anti L3T4) will produce a striking prolongation of survival of skin allografts [Cobbold et al 1984] as well as heart allografts [Madsen et al 1987]. In contrast an anti-CD8 antibody (anti-Lyt2) does not prolong survival of cardiac allografts in the mouse unless the animal is sensitised in which case it is more effective than the anti-CD4 antibody [Madsen et al 1989 and Table 2]. This would also suggest that, at least in this model, the CD4+ Th cell is the primary cell involved in first set rejection of a vascularised organ allograft, while the CD8+ Tc cells is the major cell involved in a second set rejection, both of which are relevant to future clinical application.

Table 2. Balb/c hearts grafted into naive or sensitised CBA recipients, treated with an α-CD4 (YTS 191.1) or an α-CD8 (YTS 169.4), antibody for 2 days before and 10 days after transplantation.

Treatment	Survival in days (Range) Naive Recipient	Sensitised Recipient
Nil	10 (9-11)	4 (3-6)
α-CD4	>100 (21->100)	10 (4-15)
α-CD8	13 (10-14)	20 (12-58)

Antibodies directed against activation antigens expressed on lymphocytes have the potential of being even more selective in that an antibody directed against T cells expressing such antigens would react only with cells that in the early stages of the immune response to a graft were recognising and responding to that graft. Such an approach has been shown to be appropriate using monoclonal antibodies against the IL2 receptor (IL2R) both in rat cardiac and renal allograft models [Kupiec-Weglinski et al 1986; Tellides et al 1989]. Experiments from my own laboratory however have clearly demonstrated the variation in immunosuppressive effect depending on the experimental model and the epitope on the light chain of the IL2R against which the antibody is directed but not to the antibody subclass [Tellides et al 1987]. It would appear that only antibodies which block the IL2 dependent proliferation of activated lymphocytes are active in vivo. There has been a most encouraging report of the prophylactic use of an IL2R antibody in clinical renal transplantation [Soullilou et al 1987], and of relevance is that this antibody had the same characteristics that would have been predicted as being necessary from the above rat transplant model.

The use of monoclonal antibodies in clinical transplantation is still in its infancy but obviously the potential for manipulating the immune response to a graft is enormous. But the difficulties lie not only in understanding the relevance of the various cellular interactions involved in graft rejection, but in determining the functional activity of antibodies directed against different epitopes on the same target antigen.

INDUCTION OF TOLERANCE

Tolerance to an allograft remains the ultimate goal of all transplantation biologists, but it has proved an elusive goal in the adult animal. Although tolerance to an organ allograft in the rodent has been produced in a variety of models, using active or passive enhancement, antilymphocytic globulins or monoclonal antibodies, and drugs such as cyclosporine, the mechanism by which this phenomenon is induced and maintained remains uncertain. Nevertheless, the report of 3 patients who had received no immunosuppressive drugs from 1 to 6 years after transplantation, strongly suggests that they had acquired tolerance to their renal allografts following total lymphoid irradiation before transplantation, as also demonstrated by donor-specific unresponsiveness in vitro [Strober et al 1989]. The possible mechanisms of this specific unresponsiveness are clonal deletion, clonal anergy and active suppressor mechanisms, the latter two appearing to be the more likely.

Antigen pretreatment as a method of inducing tolerance is of considerable interest because of the well recognised transfusion effect in clinical transplantation, which has provided considerable impetus to the study of antigen-induced suppression especially in allograft models both in the rat and the mouse. It has been clearly shown over many years that pretreatment with donor antigen in the form of whole blood or the various components of blood expressing either class I or class II MHC can induce specific suppression of the response against a subsequent vascularised organ allograft. Cells or micelles expressing class I MHC alone are effective, but not soluble antigen [Foster et al 1989; Spencer & Fabre 1987]. Indeed specific suppression against the whole allogeneic MHC of a subsequent cardiac allograft can be induced by pretreatment with a single class I MHC gene product in the mouse [Madsen et al 1988]. The induction of this operational tolerance is dependent on the strength of the MHC difference, the antigen dose, and the route of administration of the antigen. Tolerance has also been induced to soluble antigens in the adult mouse with anti-CD4 monoclonal antibodies [Cobbold et al 1986] and more recently to cardiac allografts, where a very potent form of specific immunosuppression has been induced with antigen pretreatment in the form of whole blood under the umbrella of anti-CD4 therapy, a protocol which has attractive clinical possibilities [Madsen, Morris & Wood - in preparation]. A current clinical trial of the administration of cryopreserved donor bone marrow following an induction course of ALG after transplantation in an attempt to produce donor specific unresponsiveness is promising [Barber et al 1989].

Nothing is known about the processing or recognition of histocompatibility antigen that leads to suppression of the aggressive response to the graft, be it by the induction of suppressor cells or clonal anergy. Certainly in many models it is possible to actively transfer tolerance with T cells from the animals bearing long surviving

grafts to syngeneic recipients which allows a subsequent naive graft to survive [Hutchinson 1986]. These T cells may be of the $CD4^+$ or $CD8^+$ phenotype, and it seems likely that they are not a specialised population of T cells, but cells which given the appropriate environment act as suppressor cells. There is also some evidence in a rat renal allograft model that there may be a hierarchy of suppressor cells, similar to that described by Dorf and Benaceraff [1984] in a delayed hypersensitivity model in the mouse [Rodriguez et al 1989]. Nevertheless it must be said that although the phenomenon of suppressor cells is widely accepted, there remains a healthy scepticism because of the inability to demonstrate a specific T cell population responsible for suppression. Recent data from our own laboratory in an allograft model of tolerance has suggested that there are specific cytotoxic T cells in the non-rejected graft but inadequate production of IL2 [Dallman et al 1989]. Similar data showing that tolerance in the adult animal can be explained on the basis of a lack of IL2 has also been provided in a model of unresponsiveness to MLS in the mouse [Rammensee et al 1989] and H-2k in transgenic mice [Morahan et al 1989]. These data all suggest the mechanism of specific suppression is one of clonal anergy.

Nevertheless for the present it would seem imperative that the mechanisms by which tolerance to an allograft is induced and maintained (it is unlikely that a single mechanism is operative) are defined before any serious attempts at clinical trials can be contemplated.

THE FUTURE

The future of organ transplantation lies in the development of more specific immunosuppression, for the technical aspects of vascularised organ transplantation no longer represent a problem. In the case of bone marrow transplantation the graft versus host reaction remains a major obstacle to the widespread application of transplantation between non-HLA identical siblings, which would allow more effective treatment of leukaemia with chemotherapy and/or total body irradiation. Advances in this area have been directed at better suppression of the GVH reaction or deletion of T cells from the bone marrow.

As newer approaches to immunosuppression evolve the possibility of transplanting pancreatic islets or fetal pancreas early in the course of insulin-dependent diabetes would be feasible, provided that it can be firmly established that successful transplantation of the endocrine pancreas would prevent the development of the microangiopathic complications of the disease. Even further away is the possibility that embryonal neuronal tissue might be transplanted to correct the neurological defects of conditions such as Parkinson's or Alzheimer's disease. However not only is more knowledge of the immunological behaviour of neuronal transplants required, but also a better understanding of the underlying neurophysiological and biochemical defects of the conditions which might be amenable to treatment by transplantation.

Thus the future of transplantation is at an exciting period of its history, for the means by which a further understanding of the immune response to an allograft and its suppression are now available, and as the complex cellular interactions of the allograft reaction are unravelled so too will the direction of immunosuppression, and even the means for the induction of tolerance, be indicated.

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Antigen, Cytokines, and the Regulation of the Allograft Response

R.G. Gill and K.J. Lafferty

INTRODUCTION

The key to understanding the allograft response is understanding the immune response to MHC antigens. This may appear to be a truism, but in fact is a concept that still is not fully understood. Our main aim here is to discuss the roles that antigen and cytokines play in both the activation and the expression of the allograft response. Special interest will be devoted to the role of helper cells, or what we call helper cells, in allograft immunity.

MHC ANTIGENS IN ALLOGRAFT IMMUNITY: THE TRANSPLANTATION PARADOX

Classical View of Allograft Immunity

According to the classical view of allograft immunity, MHC antigen is an inherently strong immunogen, and binding of this antigen drives T cell activation (Medawar 1963). Although allograft rejection clearly represents a response to transplantation antigens, there has been a nagging problem with this view of allograft immunity. Attempts to isolate and characterize transplantation antigens have found them to be quite weak immunogens (Snell 1957; Batchelor 1978). This observation leads us to a *transplantation paradox*: allograft immunity represents a violent response to antigens which are inherently weak immunogens. We later will show that solution of this paradox comes from understanding the role of antigen presentation in immune induction.

The Problem of Xeno-reactivity

Another problem with the classic concept of allograft immunity relates to the issue of xenogeneic responses. Studies of the graft-versus-host reaction (GVHR) found that the intensity of this reaction actually *decreased* as the phylogenetic separation between donor and recipient increased (Lafferty 1969). The species specificity of the GVHR also is reflected by the in vitro correlate of the allograft response, the mixed lymphocyte reaction (MLR) (Woolnough 1979). Data shown in Table 1 below illustrates this point. MLR activity within the species, or alloreactivity, is very strong. Xenoreactivity between concordant species (rat versus mouse) also is quite strong. However, as the phylogenetic difference between the responder and stimulator increases (guinea pig versus mouse), the reactivity actually disappears.

This lack of reactivity between discordant species can be interpreted by suggesting that the allogeneic response is high due to a high precursor frequency for allogeneic antigens but that guinea pig cells do not respond to mouse stimulator cells due to a low frequency of xenoreactive cells. This interpretation is invalid; limiting dilution analysis demonstrated that the precursor frequency of xenoreactive cells was comparable to the frequency of alloreactive cells (Woolnough 1979). These results comprise another paradox: there is a high precursor frequency for xenoantigens, but xenoantigens are weak immuogens. This problem of xenoreactivity further confounds the classic notions of allograft immunity and T cell activation by demonstrating that antigen recognition alone cannot account for T cell activation, either in vivo or in vitro.

Table 1. Species specificity of the mixed lymphocyte reaction

Responder Lymph Node	P815 Stimulators	Log Cytotoxic Units/Culture			
		----Source of CS Added-----			
		none	Mouse	Rat	Guinea-pig
Mouse	γ-irradiated	5.5	--	--	--
Rat	γ-irradiated	5.2	--	--	--
Guinea-pig	γ-irradiated	<3.9	<3.7	--	5.3
Mouse	UV-irradiated	<3.9	5.8	6.2	<3.9

Lymph node cells ($2x10^6$) from the indicated species were activated against $2.5x10^5$ P815 mouse tumor cells($H\text{-}2^d$). Five days after primary activation, cytotoxic activity was measured against P815 target cells. Cultures were supplemented where indicated with 0.1 ml of Con-A stimulated spleen cell supernatants (CS) from the indicated source. P815 stimulator cells were either γ-irradiated (S+) or metabolically inactivated with ultraviolet (UV) irradiation (S-).

Resolving the transplantation paradox: The two-signal hypothesis

The solution to these problems was provided by the proposition that two signals were required for T cell activation (Lafferty 1975). Signal one (antigen) was provided by binding of the T cell receptor and signal two was provided by an inductive molecule, or *costimulator*, produced by the metabolically active stimulator cell. Cells capabale of elaborating the costimulator will have a stimulatory (S+) phenotype, whereas cells which present the same antigen but do not produce the costimulator will have a non-stimulatory phenotype (S-). Since the S+ phenotype is normally expressed only by cells of lymphoreticular origin, we can see why leukocytes provide the major source of tissue immunogenicity. The role of MHC in this process is that of a control structure; that is, release of the costimulator is triggered by engagement of MHC on the surface of an S+ cell (Lafferty 1983).

This theory provides a solution to the transplantation paradox. The immunogenicity of MHC antigen is not due to its inherent antigenic structure, but rather to its *functional* role as a control stucture for the release of the costimulator. This model

predicts that MHC antigens will only be immunogenic while on the surface of metabolically active S+ cells; isolated MHC antigens or MHC antigens on the surface of S- cells will not be immunogenic (Talmage 1977; Lafferty 1980). The significant contribution of the two-signal hypothesis to transplantation biology is the notion that alloantigen alone is *not* the barrier to allografting.

This theory also explains the paradox of xenoreactivity. The species specificity of the GVHR and MLR results from the species specificity of the second signal, or costimulator. Data shown in Table 1 illustrates this point. Whereas guinea pig cells do not respond to mouse stimulator cells, this response can be restored by adding back the costimulator activity in the form of Con A-stimulated spleen cell supernatants. The species specificity of the costimulator is shown by the finding that guinea pig factors will reconstitute the guinea pig response to mouse cells whereas mouse factors will not.

Theory of allograft immunity: the passenger leukocyte concept

The two-signal model for T cell activation has a profound influence on the how we view allogenic interactions. This model predicts that active (S+) APCs within the graft will provide the major source of tissue immunogenicity and that depletion of S+ cells from the graft prior to transplantation will facilitate allograft survival (Lafferty 1983). A variety of tissue pretreatment techniques aimed to eliminate donor APCs have resulted in successfful transplantation of allogeneic tissues, including pancreatic islet allografts (Gill 1989). Such pretreated grafts do express recognizable alloantigens; rejection of established cultured allografts is readily triggered by challenging the the host with lymphoid (S+) donor-type cells (Talmage 1976; Bowen 1981).

The two-signal model explains much of alloreactivity by emphasizing the role that cytokines play in the direct activation of graft-specific T cells. However, this model ignores the role of the indirect pathway of antigen presentation of graft antigens by host antigen presenting cells (APC).

THE ROLE OF HELPER CELLS IN ALLOGRAFT IMMUNITY

A common question concerning the fate of tissue allografts which have been pretreated to reduce immunogenicity is the following: if cultured allografts express transplantation (especially MHC) antigens, then why can't graft antigens be shed and processed by host antigen presenting cells. This presentation then should lead to the activation of helper T cells which in turn prime cytotoxic cells that cause rejection. That is, why doesn't this indirect pathway work? There is in vitro evidence that MHC antigen can be shed and processed by responder APCs to activate helper T cells which then promote the activation of CD8 cytotoxic T cells (CTL) (Golding 1984). There also is evidence that this pathway can play a role in allograft immunity (LaRosa 1987). However, the fact that appropriate tissue pretreatment can result in long-term survival of allografts in non-immunosuppressed recipients provides empirical evidence that this indirect pathway of alloantigen presentation is very inefficient in vivo. This issue brings our attention to the general issue of T cell help in allograft immunity.

Class II MHC antigen is not inherently immunogenic

To address the issue of T cell help, we first must address the role of MHC antigens in the generation of 'helper' T cells. Eloquent genetic studies determined that MHC antigens could be divided into the two groups now known as class I and class II MHC antigens (Bach 1976; Bach 1978). This work led to an alternate two-signal model that placed emphasis on the role of class II MHC antigen in immune induction (Bach 1976). This model proposed that class II MHC recognition activated helper T cells which in turn provided the second signal, notably IL-2, which led to the activation of the CTL. However, it is now clear that neither class I *nor* class II antigens are strong immunogens.

The issue of class II MHC immunogenicity was elegantly examined recently by Markmann et al (1988). These investigators transfected I-E$^-$ recipient mice with a class II I-E gene under control of the insulin promoter (ins-I-E), leading to tissue-specific I-E expression in pancreatic islet β cells. They then transplanted fetal pancreas from the ins-I-E mice into I-E$^-$ parental strain mice. Despite high expression of the transgene on the β cell surface, such fetal pancreas grafts did not trigger alloimmunity (Markmann 1988). Further, immunizing recipient mice with I-E$^+$ spleen cells (S+ cells) triggered prompt rejection of the ins-I-E graft, indicating that the transfected fetal pancreas could serve as a target for immune destruction. What these experiments clearly demonstrate is that class II MHC antigen is not inherently immunogenic and so is not sufficient for the generation of helper T cells.

The requirement for T cell help in allograft immunity

MHC antigen, either class I or class II, is not the barrier to allografting. Tissue immunogencity is provided by active APCs within the graft. However, we still are faced with the problem of why indirect antigen presentation by the host does not trigger the rejection of cells bearing allogeneic MHC antigens. We also are faced with a related problem: The rejection of established cultured islet allografts depends on the function of CD8 T cells (Prowse 1983); but the rejection of islet allografts is CD4 T cell-dependent (Shizuru 1987; Hao 1987). That is, islet allograft rejection requires helper cells. This leaves us with an important problem; the helper pathway as described above does not appear to be a major pathway of allograft immunity and yet T cell help is required for islet allograft immunity. We propose that the answer to this problem lies in studying the nature of T cell 'help', or more specifically, the nature of T-T collaboration.

The role of cytokines in the expression of allograft immunity

To address the issue of T cell help in allograft immunity we first need to discuss the role of cytokines in the expression, or effector phase, of the rejection process. Under appropriate conditions pancreatic islet tissue, bearing foreign class I or class II MHC antigens, can be grafted to recipient animals where it will survive and function. This tissue is destroyed by the adoptive transfer of immunity with primed CD8 CTLs (Prowse 1983). However, this process is sensitive to cyclosporine A (CsA), suggesting that islet allograft rejection is lymphokine-dependent (Prowse 1985). This CsA effect can be overcome if the antigen

density on the surface of the target islet is increased by treatment of the tissue with γ-IFN prior to grafting (Hao 1989).

We interpreted these results to mean that the T' cells are producing cytokines, such as γ-IFN, which up-regulate the antigen expression of the graft and so facilitate cytotoxic activity. Thus cytotoxic activity and lymphokine production act synergistically in the process of islet graft rejection. This means that the characteristics of antigen-triggered lymphokine release from T' cells will influence graft rejection. The regulation of lymphokine release from T' cells may provide a solution to the helper problem stated above.

T-T collaboration in the regulation of lymphokine release from T'cells

The dependence of graft rejection on lymphokine production provides a solution to the problem of T cell help. The triggering of lymphokine release from T' cells can be mathematically modeled (Gill 1987). Simply stated, the release of lymphokine from an alloreactive T cell can be represented by the following equation:

$$T' + nA \rightarrow lk$$

where A represents the antigen-bearing target cell and n is the order of reaction - the number of target hits required to trigger the T' cell into lymphokine (lk) production. We assumed that n would have a value of 1. However, experimental evidence has shown that T' cells with the same apparent specificity can be either first order (n=1) or second order (n=2) depending on the culture conditions (Gill 1987). This is shown schematically in figure 1 below.

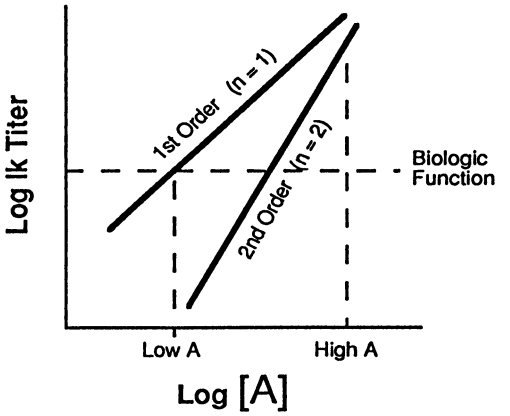

Figure 1. *Hypothetical plot of antigen-triggered lymphokine release used to determine the order of lymphokine release from activated T cells (T') in vitro. A plot of log lymphokine (lk) titer versus antigen concentration (log A) will generate a straight line with a slope n, which is the order of reaction and defines the number of target cells required to trigger lymphokine release from a single T' cell (Gill 1987).*

First order T cells are triggered to release lymphokine by one productive interaction with the target cell. In the case of the second order T cell, the response threshold is above that reached

by a signal from one interaction with the target cell; two consecutive signals are required to trigger the T' cell.

We would predict that this feature of lymphokine release would have tremendous impact on islet allograft rejection which, as stated earlier, is a lymphokine-dependent event. At high antigen concentration, both the first order and the second order T' cell can be expected to exert biologic function. However, as antigen expression/density becomes limiting, only the first order T' cell can be expected to function (Figure 1). This means that with allografts such as pancreatic islets where the availability of antigen is low, a first order T cell will be necessary to trigger lymphokine production, and triggering lymphokine production will be essential for the cytotoxic cell to kill the graft.

What regulates the type of T' cell (first or second order) generated? Recent evidence shows that a form of T-T collaboration determines the order of the T' cell generated (Gill 1988) (Table 2). When T cells are generated against either a class I or a class II disparity alone, the ensuing T' cells are second order for lymphokine triggering. When activated against a mixture of class I and class II MHC-disparate stimulator cells, the ensuing T' cells also are second order. However, activating against F1 stimulator cells, where the class I and class II MHC alloantigens are presented on the same APC, results in a first order T' cell . Further, the generation of the first order T' cell requires the metabolic function of the APC (Table 2, Gill 1988).

Table 2. The generation of first order T' cells requires T-T collaboration.

Stimulator Cell	MHC Disparity	n (order of reactivity)[a]
B6.C-H-2^{bm1} (bm1)	Class I	No Response
B6.C-H-2^{bm12} (bm12)	Class II	1.9 ± .03
bm1 + bm12	Class I + Class II	1.9 ± .06
(bm1xbm12)F1	Class I x Class II	1.1 ± .05
UV-(bm1xbm12)F1[b]	Class I x Class II	1.9 ± .09

[a] C57Bl/6 responding lymph node cells were activated against the indicated stimulator cells and triggered for lymphokine (IL-3) release with bm12 target cells. Results are shown ± SE of 4 experiments.
[b] F1 stimulator cells were metabolically inactivated with ultraviolet irradiation (UV) prior to activation. The response was restored by supplying exogenous factors in the form of supernatants from Con-A activated spleen cells.

The requirement for linked antigen presentation can be explained by either of two models (Figure 2). One model suggests that the APC focuses the two interacting cells (CD4 and CD8) together, allowing the passage of signals between them. According to this model the APC plays a passive role in mediating T-T collaboration. An alternative model has the APC playing an active role in mediating T-T collaboration; one cell, the 'helper' cell, interacts with the APC, altering the way that this APC presents

antigen to a subsequent T cell. The fact that the metabolic function of the APC is required to mediate the form of T-T collaboration described above leads us to favor the latter view of T-T collaboration; that is, that T-T collaboration is mediated indirectly via the APC.

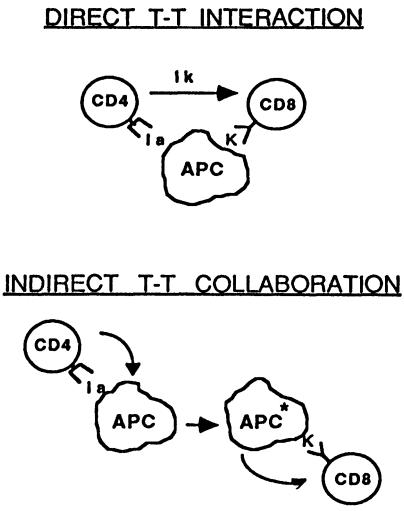

Figure 2. *Alternative models of T-T collaboration. The predominant view of T-T collaboration involves a direct T-T interaction whereby the CD4 T cell interacts with the CD8 T cell, probably by the release of soluble factors. This view implies a passive role for the APC. Another view of T-T involves an active role for the APC. In this model, one T cell is required to deliver an activating signal to the APC (APC --> APC*) which in turn delivers a signal to another T cell. This view implies that T-T collaboration is mediated via the APC.*

T-T collaboration for the rejection of cultured islet allografts

The same form of T-T collaboration appears to operate for triggering the rejection of established, class I MHC-disparate islet allografts (Gill, unpublished observations). C57Bl/6 (B6) recipient mice were grafted with class I MHC-disparate B6.C-H-2^{bm1} (bm1) islet grafts which had been pretreated for 7 days in 95% oxygen culture to eliminate tissue immunogenicity (Bowen 1980). Thirty days after grafting, recipient mice were immunized with live spleen cells from a variety of donors to determine the cellular stimuli necessary to trigger rejection of the established graft. Rejection of the bm1 graft was not triggered by challenge with donor-type (bm1) spleen cells (0/6) or by challenge with a mixture of bm1 plus class II MHC-disparate B6.C-H-2^{bm12} (bm12) spleen cells (0/5). However, challenge with (bm1xbm12)F1 spleen cells triggered acute rejection of the established bm1 grafts (7/7). These results demonstrate a form of T-T collaboration necessary for islet allograft immunity. Further, the requirement for linked presentation of class I (bm1) and class II (bm12) alloantigens indicates an essential role for the APC in mediating T-T collaboration. Metabolic inactivation of the F1 cell with UV irradiation prevents rejection, even in recipient animals primed against the class II (bm12) antigens (Gill, unpublished data).

This last finding supports the notion that T-T collaboration requires the active participation of the APC and so favors an indirect pathway of T-T collaboration (Figure 2).

CONCLUSION

These results provide a solution to the question of T cell 'help' in allograft immunity. There *is* a helper pathway, but it is not the classic helper pathway whereby lymphokines produced by one T cell are used for the activation of other T cells (Bach 1976; Cantor 1975; Wagner 1978). We propose that T-T collaboration is *not* due to a direct T-T interaction but rather is indirectly mediated by the APC. This model requires that interacting T cells recognize the same APC in vivo. Thus APCs of the graft play an essential role in allograft immunity both for the direct activation of graft-specific T cells and for the mediation of T-T collaboration resulting in T' cells which are efficient mediators of immune destruction.

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Mixed Chimerism and Transplantation Tolerance

D. H. Sachs, Y. Sharabi, and M. Sykes

INTRODUCTION

Reconstitution of lethally irradiated mice with MHC mismatched allogeneic bone marrow leads to allogeneic reconstitution and specific tolerance. Such reconstitution does not, however, provide a workable approach to achieving transplant tolerance for two reasons: 1) if mature T cells are not removed from the allogeneic inoculum, graft-versus-host disease (GVHD) ensues, and can be lethal; and 2) if mature T cells are removed from the allogeneic bone marrow inoculum the animals engraft allogeneically but are relatively immunoincompetent when examined late after reconstitution (Zinkernagel 1980). This immunoincompetence is presumably due to a failure of the newly maturing T cells in such animals to find appropriate presenting cells capable of presenting foreign antigens in the context of thymic MHC products. The new T cells develop in a host thymus, but the presenting cells, like the T cells, are derived from the allogeneic donor. Singer and colleagues (1981) have shown that mature T cells from such animals are in fact competent in vitro if allowed to react with antigen-presenting cells of appropriate host MHC type. Consistent with this finding, they showed, and we have confirmed (Ildstad 1985), that the reconstitution of such animals with a mixture of T-cell-depleted host and donor bone marrow cells leads to survival of both lymphohematopoietic lineages, and spleen cells from such animals are immunocompetent (Fig. 1) and specifically unresponsive in vitro to both donor and host MHC.

On the basis of these findings, this laboratory has attempted over the past few years to use mixed bone marrow reconstitution as a means of producing immunocompetent chimeras tolerant to tissue grafts of the donor type (Ildstad 1985). We have demonstrated that reconstitution of lethally irradiated B10 recipients with a 3:1 mixture of B10.D2 and B10 T-cell-depleted (TCD) bone marrow leads to long-term mixed chimerism, full immunocompetence and specific, long-term tolerance to B10.D2 skin grafts (Ildstad 1985, 1984). We present here some data of possible relevance to the mechanism of the tolerance induced by this procedure, and describe a recent extension of this work which permits induction of long-term mixed chimerism following a non-lethal preparative regimen.

MECHANISM OF TOLERANCE IN MIXED CHIMERAS

There are two major theoretical mechanisms by which the induction of specific transplantation tolerance is generally envisioned: 1) Clonal deletion or clonal inactivation of cells destined to react with the graft, a process generally thought to occur for self antigens during thymic T cell maturation (Sprent 1988); and 2) Continuous suppression of the activity of such alloreactive cells once they are released into the periphery, a process which has been speculated to occur on the basis of anti-idiotypic or antigen-

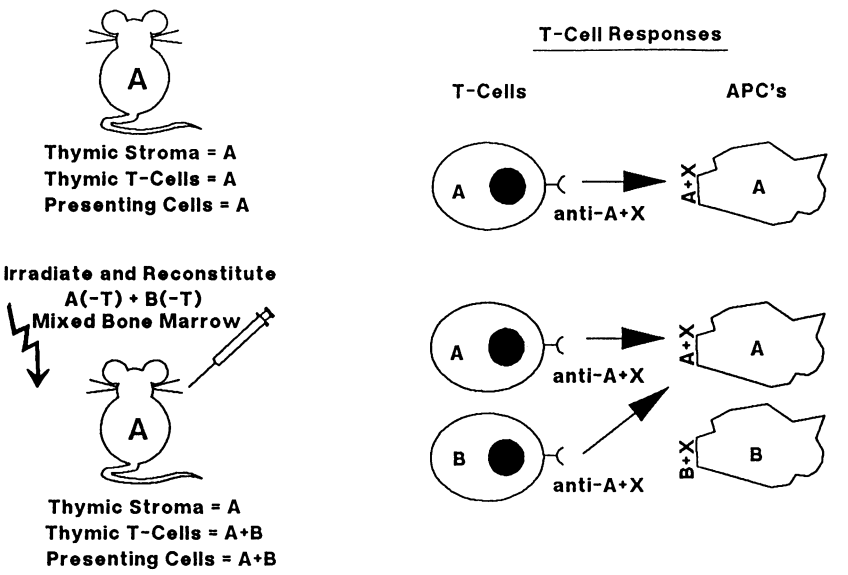

Fig. 1: Lethal irradiation followed by reconstitution with a mixture of syngeneic plus allogeneic TCD bone marrow results in stable, mixed lymphohematopoietic chimeras. Because the thymic stroma is of type A, T cells derived from both A and B marrow precursors require A presenting cells, which are also present. The animals are therefore immunocompetent.

specific suppressor cells (Batchelor 1984; Dorsch 1982). A third possibility combining some features of both of these mechanisms involves the elimination of activated cells by a non-specific mechanism. In this case one might nevertheless achieve specific tolerance if the only cells activated at the time of elimination were those that were alloreactive for the donor, at a particular time point after reconstitution.

Mixed bone marrow reconstition appears to avoid alloreactivity in both the graft-versus-host (GVH) and host-vs-graft (HVG) directions, leading to mutual tolerance among newly developing T cells of donor and host (Ildstad 1985). In addition, an anti-GVHD effect of TCD syngeneic marrow against mature allogeneic T cells has been observed when non-TCD allogeneic spleen cells were added to the mixed bone marrow inoculum (Ildstad 1986). Previous studies from this laboratory have focused on the mechanism of this anti-GVHD effect, and have shown that the TCD syngeneic marrow is the major source of the marked suppressive activity which develops in the spleens of mice at early time points (8 to 21 days) following irradiation and reconstitution with TCD mixed marrow (Sykes 1988). This suppressive activity was measured by the inhibition of generation of cytotoxic T cells in vitro; it was not antigen-specific, and was attributed to cells most similar to the natural suppressor (NS) cells described in other developing lymphohematopoietic tissues (Holda 1985). Thus the

mechanism of GVHD suppression appeared to be most consistent with the third possibility above.

Theoretically, such a mechanism could also lead to elimination of HVG reactivity acutely, if such an alloreaction were also occurring at the same time following mixed reconstitution. However, while acute elimination of mature donor T cells with GVH potential might be sufficient to prevent GVHD, it is difficult to understand how such an event, if transient in nature, could prevent the generation of new host T cells reactive against the donor, which would be expected to develop continuously in the host thymic environment. If no other mechanism existed to prevent such cells from developing or being activated, they could subsequently suffice to reject the graft, and yet our mixed chimeras remained fully tolerant for many months following reconstitution.

In order to determine whether or not a suppressive mechanism might explain this long-term tolerance, we examined late (more than 8 weeks) mixed chimeras for evidence of suppression in the same assay used to detect early NS, inhibition of CTL generation in vitro. Spleen cells from such long-term, stable mixed chimeras of the type (B10+B10.D2 -> B10) were mixed with each of the primary cultures shown in Fig. 2, and the percent suppression of CTL generation in each culture was measured. The fraction of animals showing > 40% inhibition of CTL generation is shown for each genetic combination.

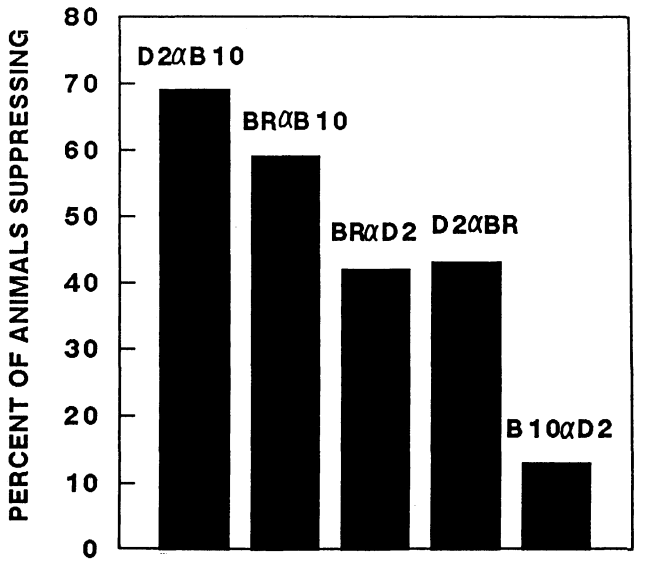

Fig. 2: Suppression of CML generation by spleen cells from long-term mixed chimeras.

As seen in this figure, suppression consistent with NS activity was still present at this late time point, but was considerably weaker than in the early chimeras. The least inhibition of CTL generation observed was that directed against the HVG reaction (ie. B10 anti-B10.D2), providing no evidence for suppression as the mechanism of maintenance of tolerance.

In addition, we have found that mixed chimeras are particularly susceptible to breaking of donor-specific tolerance by the administration of fresh host speen cells (Sykes 1988). A dose of 20 x 10^6 B10 spleen cells was sufficient to break tolerance in mixed chimeras, as evidenced both by a fall in chimerism (Fig. 3) and a loss of donor-specific allogeneic skin grafts. This dose had no effect on the tolerance of completely allogeneic chimeras which had received donor T cells in the original reconstituting inoculum. Thus, these studies also failed to provide evidence for the involvement of an active suppressive mechanism in the maintenance of tolerance in mixed chimeras.

Clonal deletion (or inactivation) of host cells with potential anti-donor reactivity therefore appears to be the most likely explanation for the long-term tolerance of mixed chimeras. Although we have no

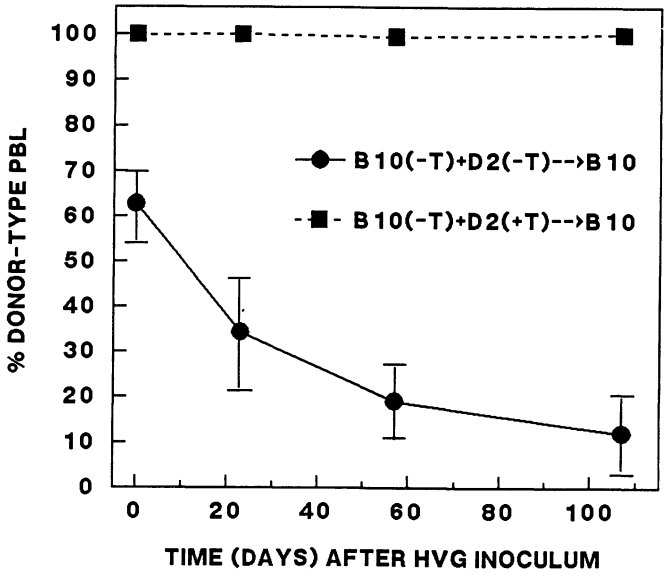

Fig 3: Effect on chimerism of inoculation of long-term chimeras with fresh, normal B10 spleen cells.

data bearing directly on this mechanism, we would speculate on the basis of data from other models (Sprent 1988) that this deletion is most likely to occur in the host thymus, and involve entry into that thymus of donor bone marrow-derived cells. Studies designed to eliminate donor-derived cells from long-standing mixed chimeras by treatment with specific antibodies are in progress, and may help to clarify the role of persistent donor cell populations in this process.

INDUCTION OF MIXED CHIMERISM BY A NON-LETHAL PREPARATIVE REGIMEN

The major limitation of bone marrow transplantation as a means of inducing specific transplantation tolerance is the toxicity of total body irradiation (TBI) used to abrogate host resistance and permit

donor bone marrow cell engraftment, and this same limitation would apply to our protocol for producing mixed chimeras. We have therefore recently extended this model by substituting treatment with anti-T cell monoclonal antibodies (mAbs) for a major portion of the TBI (Sharabi 1989). These studies were based on a report by Cobbold et al. (1986), showing that such treatment is capable of permitting allogeneic bone marrow engraftment, although only transient engraftment was observed unless high doses of TBI were also administered.

Using a preparative regimen of anti-CD4 plus anti-CD8 mAbs on day -6, 300 R TBI and allogeneic bone marrow on day 0, we observed transient engraftment of allogeneic marrow elements, confirming the results of Cobbold et al. (1986). We next examined the lymphoid tissues of animals treated with these mAbs and found that mature $CD4^+$ and $CD8^+$ T cells were eliminated rapidly from the peripheral compartments, but remained in the thymus in considerable numbers, coated with mAbs but not eliminated (Sharabi 1989). We therefore added 700 R of local irradiation to the thymus to the preparative regimen. This triple regimen (mAbs, 300R TBI and 700R thymic

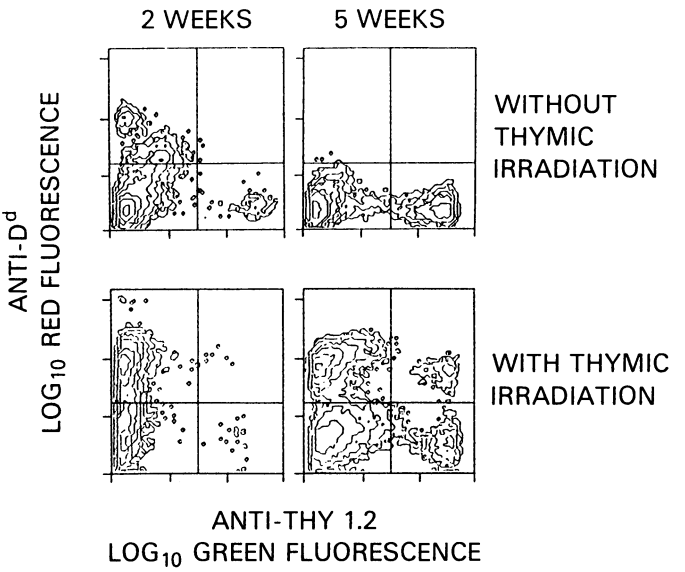

Fig 4: Time course of reconstitution of B10 mice with B10 (host) and B10.D2 (donor) lymphoid cells following preparation with mAbs and 300R TBI, without or with 700R thymic irradiation.

irradiation), was found to permit long-term engraftment of allogeneic bone marrow elements in 70% of recipients given allogeneic marrow on day 0. By 2-color flow microfluorometry, it was found that the engrafted animals became true mixed chimeras, demonstrating a mixture of host and donor lymphoid cells in all lymphoid compartments (Sharabi 1989). In addition, control animals to which allogeneic bone marrow was not administered showed syngeneic reconstitution and long-term survival, demonstrating that the preparative regimen was in itself non-lethal.

The course of lymphoid reconstitution (Fig. 4) in these animals was indicative of the mechanism by which thymic irradiation led to long-term chimerism. Because anti-T cell mAbs were still circulating in the animals at the time of bone marrow administration, the intial effect of the procedure was to produce mixed chimeras lacking mature T cells of both host and donor types. Without thymic irradiation, the returning peripheral T cells were found to be only of host type, and presumably contained mature T cells which had been spared in the thymus. On the other hand, following the addition of thymic irradiation, both host and donor type mature T cells returned with a similar time course, both presumably resulting from T cell precursor which matured in the host thymus.

The mixed chimeras produced by this non-lethal regimen were indistinguishable by flow microfluorometry from mixed chimeras produced by the lethal regimen, but were considerably healthier. Both demonstrated specific tolerance to donor-type skin grafts and prompt rejection of third party grafts. Studies of the mechanism by which tolerance is maintained in this model are in progress.

CONCLUSIONS

Production of mixed (donor plus host) chimerism provides an effective means of inducing specific transplantation tolerance while maintaining immunocompetence. The mechanism of tolerance induction appears to involve clonal deletion or inactivation since no evidence for active suppression was observed, and since the tolerance was relatively easy to obliterate by administration of host type spleen cells. The major complications of the original methodology used to produce mixed chimeras were related to the toxicity of the lethal TBI required to permit allogeneic engraftment. It appears that in vivo treatment with mAbs to mature T cell subsets may be an effective substitute for at least part of the TBI, permitting the establishment of mixed chimerism by a non-lethal preparative regimen.

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Pathology and Immunopathology of Rejection and Graft-Versus-Host Disease in Bone Marrow Transplantation

R. Storb

INTRODUCTION

Unlike transplantation of solid organs, successful marrow grafting, undertaken as therapy for hematological diseases, requires the crossing of a double barrier consisting of host-versus-graft and graft-versus-host reactions (GVHD). Host-versus-graft reactions may cause failure of the transplant, an often fatal complication. GVHD may follow successful engraftment, and is associated with life-threatening infections. GVHD and graft failure are mutually related problems, along with relapse of the underlying disease for which the transplant was carried out.

Factors governing the fate of marrow grafts include the immunosuppressive effects of the conditioning regimens used for the recipient, the degree of histocompatibility between donor and recipient, the composition of the cells in the graft, and the nature of postgrafting immunosuppression. This report will review graft failure and GVHD, along with recurrence of the underlying disease.

GRAFT FAILURE

A common problem in patients given marrow grafts for aplastic anemia has been graft failure, occurring despite pregrafting immunosuppression with high doses of cyclophosphamide and the use of marrow from HLA-identical siblings (Bacigalupo et al 1986; Champlin et al 1984; Devergie and Gluckman 1982; Hows et al 1982; Niederwieser et al 1988; Ramsay et al 1983; Storb et al 1984). Two factors have been shown to presage rejection: positive in vitro tests of cell-mediated immunity (indicating reaction of host lymphocytes against antigens on donor cells before transplantation), and a low number of transplanted donor marrow cells ($<3 \times 10^8$/kg). Studies in experimental animals have indicated that immunity of recipient against donor is the result of transfusion-induced sensitization. Apparently, certain mononuclear cells in the transfused blood products cause sensitization of the recipient against minor non-HLA antigens of the donor not suppressed by the pretransplant immunosuppressive conditioning programs. These findings have been confirmed by the fact that graft failure is rare when transplants are carried out in patients who have not received transfusions. We conclude that, in patients with aplastic anemia, immunologic mechanisms involved in graft failure are primarily induced by previous blood transfusions.

Many studies involving the use of more intensive pretransplant immunosuppression are being carried out to attempt to avoid rejection in aplastic anemia patients who have received multiple transfusions. Varying combinations of cyclophosphamide with total body irradiation (TBI), total lymphoid irradiation, thoracoabdominal irradiation, and

buffy coat cells from the marrow donor are being explored. All of these programs have resulted in decreased rejection rates with increases in survival to 60-70%. Most of the conditioning programs have associated risks, however: irradiation may cause cancer, and buffy coat cells increase the risk of chronic GVHD. Because of these risks as well as the persisting (although decreased) possibility of rejection, emphasis should be placed on preventing rather than on overcoming sensitization, and this is best achieved by carrying out transplantation before transfusions have been administered. If transfusions can not be avoided, they should be depleted of white cells before being used.

In leukemia patients transplanted with marrow from HLA-identical sibling donors, graft failure is rare. Poor graft function is observed occasionally and may be due to drug toxicity, or it may be the result of viral infection, e.g., cytomegalovirus. Graft function usually recovers after discontinuing incriminating drugs and/or after recovery from the viral infection.

True failure of engraftment and graft rejection in leukemia patients have been seen in two settings: transplantation with marrow from donors who are not HLA-identical (Beatty et al 1985), and transplantation with marrow (either HLA-identical or -nonidentical) which has been depleted of T cells (Martelli et al 1988; Martin et al 1987). Apparently certain T cells in the marrow inoculum are needed to destroy the host cells which are responsible for rejection of the graft.

Experimental studies in the canine model have shown that marrow graft failure and rejection depend upon the particular donor-recipient combination: radiation-insensitive Ia-positive non-T cells have been implicated in destruction of DLA-nonidentical grafts, and host T cells appear to be involved in rejection of DLA-identical grafts (Storb and Deeg 1986). Canine studies have also indicated that pretreating the recipient with monoclonal antibodies directed against Ia or anti-leukocyte adhesion molecules and raising the dose of TBI from 9.2 to 18.0 Gy are successful in overcoming resistance to mismatched grafts. And, pretreating the recipient with antithymocyte globulin (ATG) is effective in preventing failure of matched grafts.

In human trials, intensified conditioning programs, including the addition of total lymphoid irradiation, have decreased the risk of graft rejection but have caused an increase in regimen-related toxicities negating any survival advantage. Studies are underway adding antibodies directed at host immune cells (such as ATG, anti-Ia antibodies, and anti-leukocyte adhesion molecule antibodies) to the pretransplant conditioning regimens. Animal studies, however, have indicated that antibodies are not uniformly successful in overcoming graft rejection, so further efforts are directed at coupling certain monoclonal antibodies to short-lived radioactive isotopes to increase their effectiveness. It is conceivable that the T cells in the marrow graft which cause GVHD may be different from those which help overcome graft rejection, and clinical trials are under way using antibodies with the specific aim of removing from the marrow cells which cause GVHD while leaving other immune cells thought to be necessary for successful engraftment. Finally, the role of hemopoietic growth factors in establishing "permanent" hemopoiesis is also being explored.

ACUTE GVHD

Acute GVHD results from infusing immunologically active, genetically disparate marrow into an immunologically suppressed host unable to

reject the donor cells. GVHD is the result of cytotoxic T cells in the donor marrow attacking targets in the host's skin, liver, and gastrointestinal tract. HLA-nonrestricted cytotoxic cells and cytokines may also be involved. The clinical and histological characteristics of the disease are well-known (Sale and Shulman 1984; Santos et al 1985; Storb and Thomas 1985; Storb 1986; Sullivan et al 1987; van Bekkum and Lowenberg 1985).

Of the patients receiving marrow grafts from HLA-identical siblings or from HLA phenotypically matched family members, 20-50% develop significant acute GVHD (Storb 1987). GVHD incidence is 60-80% in patients who are genotypically identical with their donors for one HLA haplotype but differ for 1-3 HLA loci on the nonshared haplotype (Beatty et al 1985; Hansen et al 1987), and the increase in GVHD incidence is seen whether or not the difference on the second haplotype involves an HLA-A, -B (class I) or -D/DR (class II) locus.

Increasing patient age has been shown to be associated with an increasing incidence of acute GVHD. Preceding pregnancy of the marrow donor also seems to be a risk factor for acute GVHD, while other characteristics such as cytomegalovirus infection, donor/recipient sex mismatch, and certain donor or recipient HLA antigens, remain controversial as risk factors. Patients suffering clinically significant acute GVHD are twice as likely to die than those who experience only mild or no GVHD (Clift et al 1987; Storb and Thomas 1985). Death in these cases is often due to infections: GVHD may set the stage for this by increasing the postgrafting immunodeficiency and by creating portals of entry through lesions in skin and intestinal tract. In patients with aplastic anemia, it has been shown that prevention of infection by laminar airflow room isolation and decontamination helps to lessen the incidence and to delay the onset of acute GVHD, thus increasing survival (Storb and Thomas 1985); the mechanism by which this is accomplished remains unknown, but perhaps decontamination prohibits a process by which infections cause lymphocytes to secrete lymphokines, which otherwise would increase the expression of class II histocompatibility antigens on tissue cells such as gut epithelium, making them targets for GVHD.

GVHD prevention is undertaken most often with immunosuppressive drugs (Santos et al 1985; Storb and Thomas 1985; Storb 1986, 1987; van Bekkum and Lowenberg 1985). Unlike recipients of solid organ transplants, marrow graft recipients do not need immunosuppression for an indefinite period of time and it often can be discontinued as early as 3-6 months after grafting. Exclusion of immunosuppressive drugs causes an unacceptably high incidence of acute GVHD, however (Sullivan et al 1986). During the 1970s, a frequently used drug to prevent GVHD was methotrexate. In the late 1970s, a new immunosuppressive agent, cyclosporine, became available, but clinical trials failed to show significant advantages of cyclosporine over methotrexate in regard to GVHD and survival (Storb et al 1988). However, subsequent randomized trials have confirmed the superiority of a combination of methotrexate and cyclosporine over either drug used alone in preventing acute GVHD and improving survival (Storb et al 1986a, 1986b, 1989). Unfortunately the toxicities of both drugs often necessitate dose reductions, thereby impairing their efficacy in GVHD prevention. And, even though acute GVHD has been lessened, chronic GVHD continues to be seen, and for some diseases (acute nonlymphoblastic leukemia) the relapse risk may be increased. The use of prednisone with methotrexate and/or cyclosporine may further improve GVHD prevention but this remains to be proven in prospective trials (Forman et al 1987; Santos et al 1987). Studies combining ATG and methotrexate with or without prednisone have decreased the incidence of acute GVHD without improving survival.

An alternative method of preventing GVHD is the removal of T cells from the marrow with monoclonal antibodies used either for immunoadsorption or for targeting lytic activities of complement or toxins (Martelli et al 1988; Martin et al 1987), or with selective agglutination to soybean lectin, or through E-rosetting and elutriation. Each of these techniques reduces the number of infused T cells by 1-3 logs (Apperley et al 1986; Butturini and Gale 1988; Hale and Waldmann 1986; Martelli et al 1988; Martin et al 1986, 1987; Storb 1987), resulting in the removal of most differentiated cells involved in generating GVHD and in the return of the immune system to an early prenatal state. New stem cell-derived immune cells are thought to accept the host antigenic environment as "self" and become tolerant. For patients given HLA-identical marrow, a significant reduction in acute and possibly also chronic GVHD has been seen if T cell depletion of at least 1.5 logs is achieved, regardless of which depletion technique is used. However, this improvement in GVHD incidence was accompanied by significant increases in graft rejection (from <1% to 10-20% in HLA-identical and from 5% to 35% in HLA-non-identical recipients of T cell depleted marrow), presumably caused by host immune cells which survived the conditioning regimen and whose continued survival is possible due to the absence of GVHD. Also, many studies have shown an increase in the risk of leukemic relapse following T cell depletion. No improvement in survival will be realized in patients given T cell depleted marrow until those problems can be alleviated, since graft failure and leukemic relapse nearly always result in death. This is likely to be accomplished with improved pretransplant conditioning regimens, possibly using antibody isotope conjugates, leading to significantly better elimination of both malignant and immune cells of host type. It is also possible that the T cells which cause GVHD are different from those which promote engraftment and induce "graft-versus-leukemia" effects. Increased knowledge of the precise role played by lymphocytes in mediating these diverse immune functions may result in the development of strategies to specifically eliminate GVHD without disrupting engraftment and graft-versus-leukemia reactions.

The most common agents used to treat acute GVHD are prednisone, ATG, cyclosporine, and monoclonal antibodies directed against T cells, with responses received being variable (Storb and Thomas 1985; Storb 1986). As more monoclonal anti-T cell antibodies are developed, reagents may be identified that specifically interact with those cells that are actively involved in acute GVHD. Improved therapeutic effect may be achieved by coupling such antibodies to toxins such as the Ricin A chain.

CHRONIC GVHD

Twenty-five to fifty percent of all patients surviving at least one year after transplant develop chronic GVHD (Santos et al 1985; Storb and Thomas 1985; Sullivan et al 1987). Clinical manifestations of this disease resemble those seen in systemic collagen vascular diseases and include skin lesions, keratoconjunctivitis, buccal mucositis, esophageal and vaginal strictures, intestinal abnormalities, chronic liver disease, generalized wasting, and pulmonary insufficiency. Chronic GVHD is often seen in patients who experienced acute GVHD and in older patients. If untreated, chronic GVHD has a poor prognosis and most patients become disabled or die. Prednisone given for 9-12 months either alone or together with procarbazine, cyclophosphamide, or azathioprine is capable of reversing many of the signs and symptoms of the disease. Early treatment to prevent disability and joint contractures is advisable. A prospective trial

showed better survival among patients treated with prednisone and placebo than among those given prednisone and azathioprine (Sullivan et al 1988a). A more recent study combining cyclosporine with prednisone has improved survival in patients with high-risk chronic GVHD (Sullivan et al 1988b). Approximately one-half of the treated patients survive with Karnofsky performance scores of 100% and an additional one-fourth survive with scores of 80-90%. In 50% of the patients, therapy can be stopped after 9-12 months. One-fourth of affected patients die, mainly with infections from encapsulated gram-positive bacteria due to the impaired immunologic status characteristic of chronic GVHD (Atkinson et al 1982).

GVHD AND RECURRENCE OF MALIGNANCY: "GRAFT-VERSUS-LEUKEMIA EFFECT"

It is thought that some of the apparent cures seen after marrow grafting for hematologic malignancies may be due to a "graft-versus-leukemia effect" directed at transplantation antigens and perhaps also at leukemia-associated antigens present on leukemic cells (Okunewick and Meredith 1981). Significantly higher leukemic recurrence rates are seen in patients experiencing acute or chronic GVHD than in those without GVHD (Weiden et al 1981). Similarly, as already discussed, recipients of T cell depleted marrow experience less GVHD but have a higher incidence of leukemic relapse than those given non-depleted marrow (Apperley et al 1986; Butturini and Gale 1988; Hale and Waldmann 1986; Martelli et al 1988; Martin et al 1986, 1987), and the increase in relapse outweighs any advantage gained from the reduction in GVHD. It appears that certain T cells in the donor marrow are capable of eliminating residual host leukemic cells. Attempts at inducing GVHD for the purpose of decreasing the risk of leukemic relapse and improving survival have not been successful to date, since increases in severe GVHD and infections have offset the lessened incidence of relapse (Sullivan et al 1989).

Results in mice imply that certain cells in the grafted marrow specifically react with surface antigens expressed on leukemic cells, while other cells react more broadly with transplantation antigens. This suggests that a graft-versus-leukemia effect can be distinguished from GVHD (Truitt et al 1987). Attempts have been made to verify these findings in man. In one study, clones of donor-derived lymphocytes were isolated which showed spontaneous cytotoxicity against host leukemic cells but not against activated host T lymphocytes (Hercend et al 1986). Cells of this type might have an antileukemic effect without causing GVHD, and it can be inferred that measures to prevent GVHD without increasing the risk of leukemic relapse might be possible. Also, clinical attempts are underway to parallel findings in the murine model, expanding in vitro T cells sensitized to tumor antigens before infusing them along with IL-2 into tumor-bearing hosts (Cheever et al 1981; Greenberg et al 1981).

IMPROVEMENTS IN THE PRETRANSPLANT CONDITIONING PROGRAMS

Most conditioning programs for marrow grafting have involved chemotherapy and TBI. While many variations of this basic program are being explored, the limits of nonhemopoietic toxicity have been rapidly reached, and, barring unexpected developments, no further improvements can be anticipated.

The most effective way to destroy host immune cells along with the underlying disease, e.g., leukemia, would be through agents which specifically interact with these cells. The approach which may come

closest to this ideal is the use of monoclonal antibodies. Monoclonal antibodies injected in vivo can concentrate on target cells; however, their effect is limited, in part because cells, though coated by antibody, may not be killed by it. Studies are in progress involving antibodies linked to toxins, e.g. the ricin-A chain, to produce more effective cell kill. Another way to use monoclonal antibodies is to attach them to a radioactive isotope with a short half-life and a short pathway (Appelbaum et al 1988, 1989). In this way, cells expressing the target antigen will be killed as well as close-by neighboring cells which may be antigen-negative, while cells which are farther away, e.g., normal nonhemopoietic tissue cells, would not be affected.

Initial experiments in a canine model have shown appropriate radiolabelled antibodies to localize preferentially in marrow and spleen, and, to a lesser extent, also in lymph nodes. The amount of isotope in the marrow compared to other organs, e.g., liver, may achieve ratios of 20:1 or better (Bianco et al in press). Such radiolabelled antibodies are capable of producing fatal marrow aplasia which can be reversed by grafting cryopreserved autologous marrow eight days later, at a time when very little radioactivity is left. Combinations of chemotherapy, TBI, and radiolabeled antibody can now be explored for their efficacy in preparing dogs for grafts of T cell depleted marrow. The results of these experiments will prepare the stage for clinical application of this technique. Refinements of this approach, particularly the use of high-energy beta-emitting isotopes with short linear energy transfer, are likely to produce less toxic but more effective conditioning regimens, not only providing better eradication of malignant disease but also improving the problem of graft failure. Better conditioning regimens will permit a broader application of T cell depletion to prevent GVHD, thus extending marrow grafting to include more HLA-nonidentical and unrelated patients.

SUMMARY

Ideally, prevention of acute GVHD should result in decreased mortality from infections and reduced risk of chronic GVHD without compromising hematopoietic engraftment or increasing the risk of leukemic relapse due to the absence of a graft-versus-leukemia effect. Thus, the net effect of GVHD prevention should be improved patient survival.

None of the current approaches at GVHD prevention measure up to this ideal, although postgrafting immunosuppressive therapy comes closest. The technique of T cell depletion is very effective in reducing the risk of GVHD, but for this technique to become universally applicable, conditioning programs must be developed which are better able to deal with the problems of graft failure and recurrent malignancy. Whether this can be achieved with systemic chemotherapy and total body irradiation is doubtful. Innovative approaches such as monoclonal antibodies, either administered alone or linked to short-lived radioactive isotopes with short linear energy transfer, promise to result in less toxic but more effective programs, not only providing better eradication of malignant disease, but also improving the problem of graft failure. Perhaps, however, it will not be possible to kill the last leukemic cell by any form of chemoradiation therapy, and some graft-versus-leukemia effect may still be needed. It might be possible in the future to separate lymphocytes causing the graft-versus-leukemia effect from those causing GVHD and use them in attempts at therapy. In the meantime, most transplant teams continue to use postgrafting immunosuppression to prevent and treat GVHD with resultant steady improvement in survival.

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Graft Host Interactions Following T-Cell Depleted Marrow Transplants

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INTRODUCTION

Over the last 20 years, allogeneic marrow transplants have emerged as a potentially curative therapy for a series of lethal congenital disorders of hematopoiesis and immunologic function, and certain genetic disorders of metabolism, and constitute a treatment of choice for aplastic anemia and for patients with acute leukemia who have failed an initial remission. They are also the only curative approach available for the treatment of chronic myelogenous leukemia.

Unfortunately, because of the severity of graft vs. host disease (GvHD) encountered in recipients of HLA-mismatched transplants, the true potential of an allogeneic marrow transplant has only been realized for the small proportion of individuals who have an HLA matched sibling. Even among recipients of HLA matched sibling marrow grafts, the incidence of moderate to severe acute graft vs. host disease has ranged from 40-60%. Furthermore, this complication has directly contributed to peritransplant mortality in 15-35% of individuals depending upon their age. While new chemoprophylactic regimens, particularly the combination of cyclosporine and methotrexate, have significantly reduced the incidence of severe acute GvHD and its associated mortality in HLA matched individuals, these regimens have not prevented severe or lethal graft vs. host reaction among recipients of HLA disparate marrow and have had little or no effect upon the incidence or severity of chronic GvHD.

In 1980, we introduced a technique for depleting alloreactive T-cells from human bone marrow which permitted transplants of HLA haplotype disparate marrow into children with lethal congenital immune deficiency without risk of significant acute GvHD (1,2). Subsequently, several other techniques of T-cell depletion have also been explored in clinical trials (3,4). Taken together, these studies have demonstrated that certain methods of T-cell depletion can prevent or abrogate both acute and chronic graft vs. host disease even in HLA haplotype disparate recipients. However, this benefit has been significantly counterbalanced by an increase in the incidence of graft failure or rejection, and, among patients transplanted for leukemia, a variable increase in the incidence of post transplant relapse. In this report, I will present some of our recent studies of the interactions between donor and host cells which may foster or restrict the potential of a T-cell depleted marrow transplant to achieve durable engraftment, reconstitute hematopoietic and immunologic function and prevent leukemic relapse.

The results to be cited are based on studies of a series of 209 patients transplanted at our center, of whom 82 received HLA non-identical marrow grafts for SCID (30 patients) or leukemia (52 patients) and 127 received HLA matched transplants for leukemia. Patients with leukemia were prepared for transplantation with hyperfractionated total body irradiation (HFTBI) (1320-1440r, 10-15r/min) followed by cyclophosphamide (60mg/kg/day x 2). Leukemic patients receiving HLA matched transplants received no drug prophylaxis for graft vs. host disease in the post transplant period. Among the 52 patients with leukemia transplanted from haploidentical parents or siblings, 19 received high dose cytosine arabinoside ($3g/m^2$ q 12 h x 8 doses) rather than cyclophosphamide. Of these 52 patients, 40 also received immunosuppressive agents administered during the first 30 days post transplant to facilitate engraftment. The agents included: Cyclosporine (9 patients), prednisone alone (13 patients), or antithymocyte globulin and prednisone (18 patients). Of the 30 patients who received HLA haplotype disparate parental grafts for SCID, 23 received no cytoreductive treatment prior to their first graft. The other 7 patients were prepared for their primary graft with cyclophosphamide and either cytosine arabinoside (2 patients) or busulfan (5 patients); an additional 7 patients who failed to engraft following their first transplant were treated with cyclophosphamide coupled with busulfan (2 patients), ATG (4 patients), or cytosine arabinoside (2 patients) prior to a secondary transplant.

All patients received transplants of marrow depleted of T-cells by a modification of the technique of Reisner et al (2) as previously described (5). The final SBA^-E^- marrow graft was infused after an initial infusion of an irradiated (3000r) SBA^+ fraction used to supply radioresistant cells such as macrophages which might promote engraftment and bind any host antibodies which might react against the SBA^-E^- marrow inoculum.

Engraftment was documented by cytogenetic analysis, and, in mismatched cases, by HLA serotyping of marrow and peripheral blood leukocytes. In sex identical, HLA matched donor recipient pairs, identification of donor cells was based on clinical and quinacrine banding characteristics of donor and host chromosomes. The diagnosis and grading of GvHD was based on the clinical criteria of Glucksberg et al (6) and the pathologic criteria of Slavin and Woodruff (7). Techniques used to monitor numbers of hematopoietic progenitors and clonable T-lymphocytes in the T-cell depleted marrow inoculum (8), hematopoietic and immunologic reconstitution (10), manifestations of tolerance (11,12), and the cellular interactions potentially contributing to graft rejection (9,13,14) are previously reported.

GRAFT HOST TOLERANCE FOLLOWING T CELL DEPLETED MARROW TRANSPLANTS

When human marrow is fractionated by SBA lectin agglutination and E rosette depletion (2), a 2.8-3.0 $\log_{10}$ reduction in the number of clonable T cells is regularly achieved (15). With this degree of T cell depletion, GvHD can usually be prevented, even in HLA-haplotype disparate recipients. As shown in Table 1, the incidence

Table I

INCIDENCE OF ACUTE AND CHRONIC GvHD FOLLOWING SBA^-E^- T-CELL DEPLETED MARROW GRAFTS

TYPE OF TRANSPLANT/ INDICATION	PTS. N.	GRAFT FAILURE OR REJECTION	1° OR 2° DURABLE ENGRAFTMENT	GRADE OF ACUTE GvHD% 0 1 2 3 4	CHRONIC GvHD%
HLA-MATCHED, LEUKEMIA	127	19	106	88 7 5 0 0	0
HLA, NON-IDENT., LEUKEMIA	52	16	35	84 8 8 0 0	6
HLA-NON-IDENT., SCID	30	4	26	93 7 0 0 0	0

of grade II acute GvHD observed among 108 leukemic patients durably engrafted with SBA^-E^- marrow for HLA-matched donors has been 5%; grade III-IV acute GvHD and chronic GvHD have not been observed. Similarly of 35 durably engrafted leukemic patients and 26 SCID patients engrafted with HLA disparate marrow, grade II-III GvHD has been observed in only 3 cases and mild transient chronic GvHD in 2 cases. Among the leukemic recipients of HLA matched marrow, GvHD has been observed exclusively in those patients who have received doses greater than 10^5 clonable T cells/kg (16). The threshold dose for GvHD in recipients of HLA mismatched marrow recipients also appears to be in this range.

The tolerance which develops following an SBA^-E^- T cell depleted graft has been most extensively studied in children with SCID, who have received transplants from their HLA A,B,D haplotype disparate parents. Pretransplant cytoreduction is not routinely used in these cases. As a consequence, these patients develop a unique state of chimerism: the functional T cells emerging after transplant are of donor origin while other blood cells, including macrophages and B cells, are of host origin. In these cases and in patients who are full chimeras, Keever et al (11) have documented that engrafted donor T cells are fully reactive to third party allogeneic cells but exhibit no response to host cells either in mixed lymphocyte culture or in CML assays. When the donor's own

T cells have been mixed with the engrafted donor T cells either at the time of priming with irradiated cells from the non-donor parent or at the time effector cells are added in CML assays, their alloreactivity to host cells has not been reduced, suggesting that the tolerance observed is not primarily based on the development of suppressor cells (11). This is further supported in recent studies by Rosencrantz et al (12). Using limiting dilution techniques, they have found that the frequency of host specific allocytotoxic cells in these patients is low, and comparable to the frequencies of autocytotoxic cells observed in normal individuals. Cells specifically suppressive to these host specific cytotoxic populations are also detected, and may play a role in restricting the reactivity of the small number of mature clonable T cells administered in the SBA^-E^- marrow graft. However, the low frequency of these suppressor cells suggests that their regulatory role is limited. Thus, the tolerance appears to be predominantly based on the deletion of host reactor clones. This contrasts with a suppressor-based mechanism which has been hypothesized to be pivotal to the control of GvHD in recipients of unmanipulated marrow grafts (17).

MARROW GRAFT FAILURE OR REJECTION

A central obstacle limiting a broad and successful application of T cell depleted marrow transplants is their susceptibility to rejection. In our series of patients transplanted for leukemia, 15% of grafts administered to HLA matched recipients and 30% of grafts given to HLA disparate recipients have failed or been rejected despite preparation with supralethal doses of total body irradiation and cyclophosphamide (5). Bone marrow manipulations associated with different techniques of T cell depletion could contribute to graft failures through several mechanisms including: loss of stem cells, depletion of accessory cells required for hematopoietic progenitor development or removal of certain T cells important for down-regulating residual host effectors capable of initiating graft rejection. In our series, comparisons of SBA^-E^- T cell depleted transplants which failed with those inducing durable engraftment and hematopoietic reconstitution have revealed no significant differences in the doses of marrow progenitors (ie CFU_{GEM}, CFU_{GEMM} or BFU_E) or clonable T cells administered (9). Thus, graft failures have not been ascribable to deficiencies in the marrow inoculum itself. Rather, studies from our own and other groups suggest that differences in the characteristics of host resistance systems or their capacities to survive preparatory cytoreduction probably contribute most to the graft failures observed.

In murine and canine models, at least two mechanisms of marrow graft resistance have been described: 1. immune rejections mediated by cytotoxic T cells sensitized to alloantigens displayed on donor hematopoietic cells (13,19) and 2. a primary resistance to hematopoietic grafts which does not require prior sensitization, is T cell independent (and fully expressed in T cell depleted nude and SCID mice) and is likely mediated by NK cells (20-22). The studies of Kernan et al (13) and Sondel et al (23) provided the

first clear evidence suggesting a role for host T lymphocytes in rejections of human marrow. In leukemic recipients failing HLA non-identical SBA⁻E⁻ marrow grafts, Kernan et al (13) have now repeatedly demonstrated the emergence of host type CD8+ Leu 7- CD3+ T cells which are both cytotoxic and myelosuppressive against donor cells. In each case studied, these T cells have been shown to be specifically reactive against single class I HLA determinants unique to the donor. To date, preferential reactivity against HLA A versus B specificities has not been observed. The capacity of these host cells to discriminate the subtle differences between donor and host has been emphasized by the recent studies of Yang et al (24), of 1 patient in our series who rejected a marrow graft from an HLA serologically identical MLC compatible unrelated donor. In this case, isoelectric focusing of class I HLA antigens revealed that the donor and recipient possessed different variants of HLA BW44. The host T cells isolated directly from the blood at the time of graft rejection exhibited donor specific cytotoxicity and, further, specifically lysed a series of cell lines sharing the donor's HLA BW44.1 but not cells bearing the host BW44.2. It is well recognized that alloreactive cytotoxic T cells generated in vitro can distinguish the IEF variants of class I HLA determinants (25). This case suggests that subtle class I disparities detected only by IEF may also constitute significant targets for rejection in vivo.

Rejections of HLA matched T cell depleted marrow grafts are also associated with the emergence of host type CD3+, CD8+ Leu 19- T cells. In our experience, these cells have not exhibited donor specific cytotoxicty but have specifically inhibited the growth of donor type myeloid colony forming progenitors in vitro (14). The phenotype of the CD8+, CD3+ cells, which are Leu 7+ is also consistent with a suppressor rather than a cytotoxic T cell. However, HLA restricted and donor specific cytotoxic host T cells can be generated from cell lines established from these circulating host T cell populations (26-28). Therefore, it remains to be determined whether the donor specific-colony inhibition which we have consistently detected in patients rejecting HLA matched grafts reflects distinctive interactions between minor alloantigen reactive host type suppressor T cells and donor hematopoietic progenitors, possibly leading to the release of cytokines such as TNF capable of colony inhibitory activity, or small populations of cytotoxic T cells not detected in CML assays but capable of lysing progenitors detected in the more sensitive colony inhibition assays.

The minor alloantigens stimulating these host T cells are as yet poorly defined. In our series, SBA⁻E⁻ marrow grafts from male donors have been markedly more susceptible to failure or rejection than grafts from female donors (actuarial risks of 27% and 3% respectively)(9), suggesting the possibility that the H-Y antigen may be important. Indeed, Goulmy et al (26) and Voogt et al (27) of the Leiden group have isolated and propagated H-Y specific, HLA restricted cytoinhibitory and cytotoxic T cells from the blood of female patients rejecting HLA matched marrow grafts. Recently, Kernan et al (28) have also detected such cells in patients

rejecting matched grafts in our own series, confirming their findings. Rejections based on responses to other minor alloantigens, such as the HA antigens recently identified by Goulmy et al (29) are also likely, but have not as yet been reported.

The contribution of host NK cells to graft failure or rejection in leukemic patients who have been prepared with total body irradiation and cyclophosphamide is controversial. NK cell populations and their function recover to normal levels within 18-21 days post transplant (30). However, cytogenetic analyses indicate these cells are usually of donor origin (30). Furthermore, in our series, the appearance of these cells is a consistent prognosticator of durable engraftment (9). In contrast, for patients with SCID, who have not been prepared for transplantation with chemotherapy or radiation, the presence of normal NK cell functions prior to transplant has been the most consistent predictor of graft failures (31). Thus, of 18 patients prospectively examined, 10/10 patients deficient in NK function achieved immediate engraftment and immunologic reconstitution, compared with 1/8 patients with normal NK cell activity ($p < 0.001$). Thus, these studies suggest that in man, as in mouse, NK cells may contribute to graft resistance, particularly in individuals genetically depleted of T lymphocyte functions. However, in man as in mouse, preparative immunosuppression with agents such as cyclophosphamide may abrogate this form of graft resistance.

ANTI-LEUKEMIC ACTIVITY OF T CELL DEPLETED MARROW GRAFTS

The anti-leukemic effects of an unmanipulated marrow allograft may be based on any or all of a series of factors, including the intensity of this cytoreductive regimen used (32), the type and duration of drugs administered to prevent or treat GvHD (33,34), the reaction of donor T cells against alloantigens probably expressed on normal and leukemic host cells (GvHD-inducing antigens) or alloantigens selectively expressed at high density on leukemic cells (35,36,37), or the action of other effector systems which can lyse leukemic cells, such as activated NK cells or macrophages which develop from donor marrow progenitors.

In our series, recipients of HLA matched SBA^-E^- marrow grafts do not receive post transplant chemoprophylaxis against GvHD, yet only 5% develop grade II acute GvHD and chronic GvHD has not been observed. Therefore, the potential antileukemic effects of GvHD, its prophylaxis and its treatment have been eliminated. Despite this, the incidence of relapse following transplants for AML in first remission has not been increased, and increments in the incidence of relapse following transplants for CML have been observed only in older patients, patients who would be less likely to survive to be at risk for relapse following an unmanipulated graft (5). Accordingly, we have begun to explore alternative mechanisms of leukemia resistance which might be differentially expressed in recipients of T cell depleted grafts who do not relapse, so as to identify alternative strategies whereby the advantages of the T cell depleted graft can be preserved without compromising its anti-leukemic activity.

In an analysis of several series of patients transplanted with CAMPATH-1 treated T cell depleted marrow, Hale et al (4) observed that patients who ultimately relapsed initially exhibited too delayed and often incomplete hematopoietic reconstitution. Because of this finding, they hypothesized that the central role of the T cell in an allograft is to suppress host resistance, thereby permitting full engraftment of donor hematopoietic cells which can then compete effectively with residual host leukemic cells for nutrients and space within the microenvironment of the host.

We have also observed significant delays in hematopoietic reconstitution in patients who have subsequently relapsed in our series. Furthermore, in a study of our patients transplanted for chronic phase CML, Offit (38) also found that patients who were mixed chimeras with a higher proportion of cytogenetically normal host cells in the marrow in the early post transplant period were at markedly higher risk for subsequent relapse. Such chimeric states were also particularly common in older CML patients (> 32 years), patients who, in our series, are at particular risk both for rejection (9) and relapse (5). Thus, our studies tend to support the concept that the quality of engraftment may play an important role in preventing leukemic relapse. However, we question whether rapid expansion of donor hematopoietic cells alone is sufficient to prevent the reemergence of leukemic cells. Rather, our studies have led us to hypothesize that the establishment of cell systems capable of active suppression of residual leukemic clones is also necessary and to suggest that activated NK cell populations may be of particular importance.

NK cells and activated NK (or LAK) cells induced by IL-2 are capable of lysing K562 and Daudy cells respectively, emerge early after either an unmodified or $SBA^{-}E^{-}$ marrow graft in almost all cases (30). However, in prospective analyses of our CML patients, Hauch et al (39) have observed that in a proportion of patients, the IL-2 activated NK cells generated as early as 18 days post transplant are also able to lyse CML cell targets isolated prior to transplant from the HLA matched recipient. The risk of subsequent relapse for such patients is low (20% at 3 years). In contrast, patients whose LAK cells fail to lyse host CML cells are at high risk of subsequent relapse (66% at 3 years). ($p < 0.03$). Furthermore, the CML lytic activity detected in the host post transplant is strongly correlated with the level of LAK cell mediated lysis of host CML cells which can be generated from the donor's own LAK cells. Such studies, if confirmed, suggest the possibility that donor cell systems other than alloreactive T cells may exert an anti-leukemic activity of biologic significance. The genetic or acquired characteristics which distinguish donors of LAK cells which are or are not able to lyse host CML cells, however, remain to be defined.

In summary, our studies have continued to demonstrate that $SBA^{-}E^{-}$ marrow grafts are sufficiently depleted of T cells to prevent GvHD in both HLA matched and HLA disparate recipients. The

tolerance developed by donor T cells developing within the host is largely the result of a deletion of host reactive clones, but low frequencies of both host-specific cytotoxic cells and suppressors of their activity can be detected. The principal obstacle to the success of these grafts is rejection, which in leukemic patients is likely mediated by CD8+ donor specific cytotoxic or suppressor cells which survive preparatory cytoreduction. Lastly, we hypothesize that the major role of the T cell in preventing leukemic relapse may not be to directly inhibit or kill residual host leukemic cells, but rather to facilitate the engraftment and regeneration of donor hematopoietic progenitors and particularly progenitors of LAK cells and other cells capable of lysing host leukemic cells early in the post transplant period.

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Rapamycin: A New and Highly Active Immunosuppressive Macrolide with an Efficacy Superior to Cyclosporine

B.M. Meiser, J. Wang, and R.E. Morris

INTRODUCTION

In 1975, as part of a program at Ayerst Research Laboratories in Montreal to screen for non-polyene antifungal antibiotics, Dr Sehgal's group (Sehgal 1975) discovered that a fermentation product of the fungus *Streptomyces hygroscopicus* inhibited *Candida albicans* and dermatophytes (Vézina 1975; Baker 1978; Singh 1979). The active anti-fungal principle was isolated and determined to be a novel 31-membered macrolide lactone with a FW of 914.2 and the molecular formula $C_{51}H_{79}NO_{13}$ (Swindells 1978). The antibiotic was named rapamycin (RPM) since the streptomycete had been isolated from a soil sample collected during an expedition of caves on Easter Island (Rapa nui).

Although RPM was found to be minimally toxic in rodents [LD50's (mg/kg): mouse 597 i.p.; >2500 p.o. and rat >1600 i.p.; >1600 p.o.], long-term toxicity studies in dogs revealed that RPM caused hypoplasia of lymphoid tissues. RPM's effects on the immune system were confirmed in studies in rats in which RPM was shown to inhibit T-cell dependent autoimmune diseases and IgE-like antibody formation (Martel 1976). These immunosuppressive effects discouraged RPM's development as an antifungal antibiotic. Other than investigations of its anti-tumor activity (Houchens 1983; Eng 1984), RPM was not regarded as a serious drug candidate.

The immunosuppressive antifungal antibiotic cyclosporine (CsA) was discovered about the same time as RPM (Borel 1976). In contrast to RPM, CsA's immunosuppressive activity was considered an asset rather than a liability. As a consequence of CsA's development and clinical use, the practice of transplantation was transformed.

An understanding of CsA's mechanism of immunosuppressive action enabled Fujisawa Pharmaceutical to search for and discover another fungal product with immunosuppressive activity: FK506 (Kino 1987) which has been shown to prolong organ allograft survival (Thomson 1988, Morris 1989). The discovery that FK506 was a macrolide with structural similarity to RPM (Tanaka 1987) stimulated a renewed interest in the immunosuppressive properties of RPM (Sehgal, Calne; personal communication).

We recently reported for the first time that RPM prolongs survival of mouse and rat cardiac allografts (Morris and Meiser 1989). Subsequently, Calne et al (1989) reported their own independent results showing that RPM prolongs rat cardiac and pig renal allograft survival.

This communication extends our original work and reaffirms that RPM is a more active immunosuppressant than CsA in the rat cardiac allograft model.

MATERIALS AND METHODS

Lewis (LEW) rat recipients of heterotopic abdominal Brown Norway (BN) rat heart grafts (Meiser and Morris 1989) were treated daily under light ether anesthesia either i.p. or p.o. with sterile normal saline, RPM (generously provided by Wyeth-Ayerst Research Laboratories, Princeton, NJ) in 2% carboxymethyl cellulose in water or CsA (i.v. formulation) diluted in sterile saline daily according to the schedules shown in Table 1. Graft survival time was defined as the post transplant day on which the heart graft had lost all contractile activity as determined by palpation through the abdominal wall and as confirmed by visual inspection at laparotomy.

RESULTS AND DISCUSSION

The results of our experiments are shown in Table 1. Comparison of the graft survival times for the saline-treated control group with the graft survival times for the RPM- or CsA-treated groups show that both drugs, regardless of the route of administration, were able to prolong graft survival with the doses we used.

Table 1: Prolongation of BN -> Lewis heart graft survival by administration of rapamycin (RPM) and cyclosporine (CsA)

Treatment	Dose (mg/kg)	Route	Schedule	Individual survival times (days)	Group survival times (days) Mean ± 95% confidence limit	Median	p values[a]
Saline	-	i.p.	daily	6,7,7,7,7,7,7	7±0.35	7	
CsA	0.75	i.p.	1-14	10,10,11,11,13	11±1.52	11	0.009
RPM+ CsA	1.5+ 0.75	p.o.+ i.p.	1-14	25,26,31,33,46	32.2±10.4	31	0.009
RPM	1.5	p.o	1-14	9,10,11,12,15	11.4±1.03	11	0.009
RPM	1.5	i.p.	1-14	>76,>76,>77,>77,>78	>76.8±1.04	>77	0.008
CsA	1.5	i.p.	1-14	27,31,37,39,39	34.6±6.67	37	
RPM	3	p.o.	1-14	15,22,22,29,31	23.8±7.9	22	0.009
RPM	3	i.p.	1-14	>174,>179,>182,>182,>183	>180±4.6	>182	0.009
CsA	3	i.p.	1-14	26,43,46,69,89	54.4±30.6	46	
RPM	12	i.p.	(-5)-(-1)	>44,>44,>50,>50,>50	47.6±4.1	>50	

[a] *Mann-Whitney U-test (one tail)*

To evaluate the influence of different routes of administration on the immunosuppressive effect of RPM, we compared the graft survival times of animals treated with identical doses of RPM either p.o. or i.p.. Animals treated with a dose of 1.5 mg/kg i.p. had graft survival times (median survival time [MdST] >77d) that were significantly greater than the graft survival times of animals that were treated with the same dose p.o. (MdST=11d). The same route-dependent difference in graft survival was seen in the 3 mg/kg RPM treatment group: RPM given by the i.p. route produced a MdST greater than 182 days; p.o. administration resulted in a MdST of 22 days. This difference in MdST's may be explained by the fact that the RPM used was in the form of the pure compound and was not formulated for oral administration. Appropriately formulated RPM should increase RPM's bioavailability, thus minimizing the route-dependent differences in RPM's immunosuppressive efficacy.

A comparison of the graft survival times between the orally treated 1.5 mg/kg and 3 mg/kg treatment groups showed that RPM's immunosuppressive effect is dose dependent (MdST=11d and 22d, respectively).

To compare the immunosuppressive potencies of RPM and CsA, graft survival times for animals treated with the same dose of either drug and administered by the same route (i.p.) were compared. While the MdST's of the CsA treated rats at a dose of 1.5 and 3 mg/kg were 37 and 45 days respectively, grafts in rats treated with 1.5 mg/kg of RPM survived over 77 days and grafts in rats treated with 3 mg/kg of RPM survived more than 182 days. Even though RPM was administered for only 14 days, all the grafts in both RPM treatment groups continue to beat well. While RPM induces long term survival after short term treatment, CsA given at the same dose can only prolong graft survival for a relatively short period after cessation of treatment. This indicates that the immunosuppressive efficacy of RPM is far superior to that of CsA.

Since we wanted to investigate the interaction between RPM and CsA, we treated a group of animals with both drugs. We chose for this purpose doses and administration routes that resulted in similar survival times when the drugs were given separately. While the MdST's of grafts in animals that were treated with 1.5 mg/kg RPM p.o. or with 0.75 mg/kg CsA i.p. were 11 days in each group, combined treatment with these RPM and CsA doses resulted in a MdST of 31 days. This shows that the drugs are not antagonistic and that a combination of RPM and CsA is at least additive. Combined treatment with RPM and CsA may, therefore, enable each drug to be used at low, non-toxic doses without sacrificing overall immunosuppressive efficacy.

Since we had found that RPM given i.p. at a low dose (1.5 mg/kg) for only 14 days post-transplantation resulted in a profound prolongation of graft survival, we wondered whether pretreatment with RPM would be immunosuppressive. Even though we used a relatively high RPM dose (12 mg/kg) treatment with RPM for only five days before transplantation resulted in a MdST for this group of greater than 50 days with all these grafts

continuing to contract vigorously. It has been shown by Homan et al (1981), that pretreatment of rat renal allograft recipients with a similar dose of CsA (10mg/kg p.o.) administered for a longer period of time (for 14 days before transplantation) cannot prolong graft survival.

In conclusion, RPM is clearly a more active immunosuppressant than CsA when given post-transplant by the i.p. route. Furthermore, unlike CsA, RPM can even prolong allograft survival if given for a few days preoperatively. The doses of RPM that are needed to produce toxicity in rodents are extremely high compared to the doses of RPM that enable the indefinite survival of cardiac allografts. Since CsA is known to have a very narrow therapeutic index in humans, and since the safety of RPM in humans is unknown, it was valuable to learn that both drugs could be used in combination at low doses to produce a net immunosuppressive effect that was at least additive.

Hidden within the structure of many novel natural products is often a very useful pharmacological effect waiting to be appreciated. Now that RPM's potential as an immunoregulatory molecule has been uncovered, we must learn as much as possible about RPM's effects on the immune system. Through this aquisition of knowledge, we may gain the power to use RPM to control the immune system more precisely, effectively and safely.

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V Preventive and Therapeutic Manipulations

Immune Intervention

IL-2 Receptor-Directed Immunotherapy: A Model of Immune Intervention

T. A. Waldmann, C. K. Goldman, R. Bamford, J. Burton, and R. Junghans

INTRODUCTION

The field of clinical immunology began almost two centuries ago when Jenner introduced a form of immune intervention, vaccination, with cow pox as a means of protecting against small pox. This form of immune intervention directed at prevention of disease culminated in the complete elimination for the first time of a major human disease, small pox. In conjunction with classic public health measures, immunization as a form of immune intervention has played a dominant role in the prevention of infectious disease. Similarly, with the development of monoclonal antibodies, flow microfluorometry, and modern molecular genetics, immunological approaches are playing a major role in diagnosis.

Immune intervention is also playing an increasing role in therapy, although clearly we have not achieved the potential provided by the great specificity of the immune system. A series of antigen non-specific strategies have been developed that include the use of immunosuppressive agents directed at the amelioration of autoimmune diseases and the replacement of elements of the immune system, such as the immunoglobulins that are not produced normally in patients with immunodeficiency disorders.

An examination of the form of severe combined immunodeficiency disease (SCID) associated with adenosine deaminase (ADA) deficiency gives us a classic example of the interplay between fundamental advances and progress in immune intervention. Early on, this disorder was recognized as a profound combined deficiency of cell-mediated and humoral immunity. Subsequently, Giblett and coworkers (1972) demonstrated that approximately 40% of patients with the autosomal recessive form of SCID have a deficiency of the enzyme ADA involved in the conversion of adenosine to inosine. Furthermore, the gene for ADA has been cloned and the nature of the defect at the molecular level in different families defined (Orkin et al. 1983; Adrian et al. 1984). Successful approaches have been reported that take advantage of these insights concerning pathogenesis. Initially in studies performed by Gatti, Good, and associates (1968), an ADA-deficient SCID patient was the recipient of the first bone marrow transplant from a genotypically HLA identical sibling that led to complete immunological reconstitution. With the discovery of the defect in ADA deficiency, there have been efforts directed toward enzymatic replacement. Hershfield, Buckley, and their coworkers

(1987) have had considerable success in treating children with ADA deficiency by injecting bovine ADA modified by conjugation with polyethylene glycol. Finally, SCID secondary to ADA deficiency represents an excellent candidate for gene therapy in the near future. The disease, as we have discussed, can be cured by bone marrow transplantation. The enzyme defect has been identified, and the gene encoding ADA has been cloned and introduced into a retroviral expression vector (Kantoff et al. 1986). Furthermore, the gene in retroviral expression vectors has been reintroduced into ADA-deficient lymphocytes.

In another area, dramatic progress has been achieved in defining the various growth and differentiation factors and effector molecules associated with many of the major hematopoietic lineages and B and T lymphocytes. A series of human interferons, at least seven interleukin molecules, four or more colony-stimulating factors, and a series of growth factors now available through recombinant DNA technology permit clinical trials to define their value in the treatment of neoplastic, autoimmune, and immunodeficiency states. Furthermore, it is possible to synthesize antagonists to these agents and to synthesize agonists that would have useful pharmacokinetic properties.

Finally, a major goal of the clinical immunologist is to develop antigen-specific strategies for immune intervention. The use of specific antigen as a method of inducing suppression has been employed successfully for allergic desensitization. The observation that specific antibody will suppress the immune response to the relevant antigen has been applied clinically to prevent Rh(D) sensitization to susceptible pregnant women and thus to prevent the development of erythroblastosis fetalis. In another area, tumor-infiltrating lymphocytes are being expanded in vitro with interleukin-2 (IL-2) and are being used in cancer therapy (Rosenberg et al. 1988). Finally, there has been a major effort to exploit the capacity of monoclonal antibodies to specifically identify molecules on the surface of tumor cells and lymphocytes for immune intervention. To illustrate the potential of such monoclonal antibody therapy, particularly lymphokine receptor-directed approaches, I will for the remainder of my presentation focus on an area of special interest in my own laboratory—the receptor for the lymphokine IL-2 as a target for immune intervention.

The hybridoma technique of Köhler and Milstein (1975) has rekindled interest in the use of antibodies as agents to treat cancer patients. Many in vitro studies have shown selective high-affinity binding of monoclonal antibodies to tumor cells. However, such monoclonal antibodies have to date been relatively ineffective in the therapy of human malignancies, with only 23 partial and 3 complete remissions reported in 185 patients among 25 clinical trials (Catane and Longo 1989). There have been a number of explanations for this observed low therapeutic efficacy. One of the primary factors is that many of the monoclonal antibodies employed are neither cytocidal nor cytostatic against human neoplastic cells. Furthermore, in most cases the antibodies were not directed against a vital structure present on the surface of malignant cells, such as a growth factor receptor required for tumor cell proliferation. In an attempt to circumvent

these problems, we are using the anti-Tac monoclonal antibody, an antibody that is directed against the receptor for IL-2. This receptor is expressed on adult T-cell leukemia (ATL) cells but not resting cells (Waldmann 1986; Waldmann 1989a,b). The use of the anti-Tac monoclonal antibody to treat patients with leukemia or with an autoimmune disease, as well as those receiving allografts, depends on a series of observations. The body defends itself against foreign invaders, such as bacteria and viruses, by a defense system that involves antibodies and thymus-derived lymphocytes (T cells). The success of this response requires that T cells change from a resting to an activated state.

The sequence of events involved in the activation of T cells begins when resting T cells encounter a foreign pathogen. A very small number of these T cells possess on their surface specific proteins, termed antigen receptors, which can recognize and bind a stimulating foreign antigen. The antigen-stimulated activation of these resting T cells induces the synthesis of the 15,500 molecular weight (15.5-kDa) IL-2 lymphokine molecule (Morgan et al. 1976; Smith 1980). To exert its biological effect, IL-2 must interact with specific high-affinity membrane receptors. Resting cells do not express high-affinity IL-2 receptors, but they are rapidly expressed on T cells after activation with antigen or mitogen (Waldmann 1989a,b). Progress in analyzing the structure, function, and expression of human IL-2 receptors was greatly facilitated by our production of an IgG_{2a} mouse monoclonal antibody (anti-Tac) that blocks the binding of IL-2 to its receptor, preventing T-cell proliferation (Uchiyama et al. 1981a).

We have used the anti-Tac monoclonal to define the structure of the IL-2 receptor, to molecularly clone the gene encoding the Tac peptide of this receptor, to define those functions that require an interaction of IL-2 with its receptor, and to analyze aberrant receptors in disease. Furthermore, we have used this antibody in a program of IL-2 receptor-directed therapy of patients with leukemia and lymphoma.

STRUCTURE OF THE MULTISUBUNIT IL-2 RECEPTOR

The IL-2-binding receptor peptide identified by the anti-Tac monoclonal on phytohemagglutinin (PHA)-activated normal lymphocytes is a 55-kDa glycoprotein (Leonard et al. 1982, 1983) that is sulfated and phosphorylated on a serine residue (Shackelford and Trowbridge 1984). A series of issues were difficult to resolve when only the 55-kDa Tac peptide was considered. Specifically, most cells display two classes of receptors, one with an affinity of 10^{-11} M and another with an affinity of 10^{-8} M. The Tac peptide was shown to participate in both the high- and low-affinity forms of the IL-2 receptor. Isolation of cDNAs encoding the Tac peptide did not provide an explanation for the great difference in affinity between high- and low-affinity receptors. In addition, the amino acid sequence of the Tac peptide deduced from the cloned cDNA revealed a very short (13 amino acid) cytoplasmic domain that is too short to independently transduce receptor signals to the nucleus (Leonard et al. 1984; Nikaido et al.

1984). Furthermore, it had been shown that certain cells not expressing Tac, including large granular lymphocytes (LGLs), which are precursors of natural killer (NK) and lymphokine-activated killer (LAK) cells, could be activated to become efficient killers by IL-2 (Grimm and Rosenberg 1984; Ortaldo et al. 1984). Finally, the cell line MLA-144 was Tac negative yet manifested 4,000 IL-2-binding sites with intermediate affinity. These observations led us to consider the possibility that the high-affinity IL-2 receptor was not a single peptide but rather a receptor complex that included the Tac peptide as well as novel non-Tac peptides. In studies initially presented at the Sixth International Immunology Congress in July 1986, we utilized radiolabeled IL-2 in cross-linking to define the size of the IL-2 receptor peptides on various cell lines, including MLA-144 (Waldmann et al. 1986; Tsudo et al. 1986, 1987a). In these studies, we employed ^{125}I-labeled IL-2 that had been cross-linked using the bifunctional agent disuccinimidyl suberate. We identified a 70/75-kDa IL-2-binding protein on the MLA-144 cell line. The binding of IL-2 to this peptide was blocked by excess unlabeled IL-2 but not by the anti-Tac antibody, confirming the presence of a novel 70/75-kDa IL-2-binding protein (p75). When a variety of T-cell lines were examined for IL-2 binding and were subjected to IL-2 cross-linking studies, we demonstrated a correlation between the affinity of IL-2 binding and the IL-2-binding peptides expressed (Tsudo et al. 1987a). In these studies, cell lines bearing either the p55 Tac or the p75 peptide alone manifested low- or intermediate-affinity IL-2 binding, whereas cell lines bearing both peptides manifested both high- and low-affinity receptors. In light of these observations, we proposed a multichain model for the high-affinity IL-2 receptor. In this model an independently existing p55 or p75 peptide would create low- or intermediate-affinity receptors, whereas high-affinity receptors would be created when both receptors were expressed and noncovalently associated in a receptor complex. In independent studies, Sharon et al. (1986) proposed a similar model.

Kinetic binding studies with IL-2 have provided an interesting perspective on how the two separate IL-2-binding proteins cooperate to form the high-affinity receptor. Each chain reacts very differently with IL-2, with distinct kinetic and equilibrium binding constants. The on-and-off rate for IL-2 binding to the Tac protein is rapid (5-10 sec), while the on-and-off rate for IL-2 binding to the p70/75 protein is markedly slower (>20 min) (Lowenthal and Greene 1987; Wang and Smith 1988). The kinetic binding data obtained when high-affinity receptors are analyzed show that the association rate of this receptor depends on the fast-reacting p55 Tac chain, whereas the dissociation rate is derived from the slow-reacting p75 chain. Because the affinity of binding at equilibrium is determined by the ratio of the dissociation constant and the association rate constant, this kinetic cooperation between the low- and intermediate-affinity ligand binding sites results in a receptor with a high affinity for IL-2.

Evidence suggests a more complex subunit structure that involves peptides in addition to the p55 and p75 IL-2-binding peptide. With the use of coprecipitation analysis, radiolabeled IL-2 cross-linking procedures, binding by insolubilized IL-2, and flow cytometric resonance energy transfer measurements, as well as other techniques,

a series of peptides of molecular weight 22,000, 35,000, 40,000, 75,000 (non-IL-2 binding), 95,000-105,000, 135,000, and 180,000 have been associated with the two IL-2-binding peptides (Szöllösi et al. 1987; Saragovi and Malek 1987; Herrmann and Diamantstein 1987). Further studies, including molecular cloning followed by expression of the peptides in concert in cells not expressing IL-2 receptors, will be required to define which of these peptides plays a meaningful role in the multisubunit IL-2 receptor.

DISTRIBUTION OF IL-2 RECEPTORS

The majority of resting T cells, B cells, or monocytes in the circulation do not display the 55-kDa peptide of the IL-2 receptor. However, most T and B lymphocytes can be induced to express this IL-2 receptor peptide (Uchiyama et al. 1981a; Depper et al. 1984; Waldmann et al. 1984a). Furthermore, IL-2 receptors identified with the anti-Tac monoclonal antibody have been detected on activated cells of the monocyte/macrophage series, including cultured monocytes, Kupffer's cells of the liver, cultured lung macrophages, and Langerhans' cells of the skin (Herrmann et al. 1985; Holter et al. 1987).

Rubin and coworkers (1985) demonstrated that activated normal peripheral blood mononuclear cells and certain lines of T- or B-cell origin release a soluble form of the IL-2 receptors into the culture medium. Using an enzyme-linked immunosorbent assay with two monoclonal antibodies that recognize distinct epitopes on the human IL-2 Tac receptor, they showed that normal individuals have measurable amounts of IL-2 receptors in their plasma and that certain lymphoreticular malignancies are associated with elevated plasma levels of this receptor. The determination of plasma levels of IL-2 receptors appears to provide a very valuable noninvasive approach to the analysis of both normal and disease-associated lymphocyte activation in vivo.

With the use of specific monoclonal antibodies, the presence of the p75 IL-2-binding peptide has been demonstrated on circulating CD8 but not CD4 T cells (Tsudo et al. 1989). Furthermore, this peptide is expressed along with the 55-kDa Tac peptide on activated B and CD4 and CD8 lymphocytes. In addition, it is expressed on certain circulating cells that do not express the Tac antigen. LGLs not expressing Tac can be stimulated by IL-2 to enhance NK cell activity and to generate the cytotoxic LAK cells that can lyse NK cell-resistant tumor targets (Grimm and Rosenberg 1984; Ortaldo et al. 1984). Using cross-linking methodology with radiolabeled IL-2, we demonstrated that normal LGLs and leukemic LGLs from all individuals tested expressed the p75 IL-2-binding peptide but did not express the Tac peptide (Tsudo et al. 1987b). Flow cytometric analysis with anti-p75 monoclonal antibodies confirmed that this peptide is expressed in Leu-19$^+$ NK cells (Tsudo et al. 1989).

DISORDERS OF IL-2 RECEPTOR EXPRESSION IN MALIGNANT DISEASE AND AUTOIMMUNE DISORDERS

In contrast to the lack of Tac peptide expression in normal resting mononuclear cells, this receptor peptide is expressed by a proportion of the abnormal cells in certain forms of lymphoid neoplasia, in select autoimmune diseases, and in individuals rejecting allografts (Waldmann 1989a,b). That is, a proportion of the abnormal cells in these diseases express the Tac antigen on their surface. Furthermore, the serum concentration of the soluble form of the Tac peptide is elevated. In terms of the neoplasias, certain T-cell, B-cell, monocytic, and even granulocytic leukemias express the Tac antigen. Specifically, virtually all of the abnormal cells of patients with human T-cell lymphotrophic virus I (HTLV-I) express the Tac antigen (Waldmann et al. 1984b; Uchiyama et al. 1985). Similarly, a proportion of patients with cutaneous T-cell lymphomas, including the Sézary syndrome and mycosis fungoides, express the Tac peptide (Waldmann et al. 1984b; Schwarting et al. 1985). Furthermore, the malignant B cells of virtually all patients with hairy cell leukemia and a proportion of patients with large and mixed cell diffuse lymphomas are Tac^+ (Korsmeyer et al. 1983). The Tac antigen is also expressed on the Reed-Sternberg cells of patients with Hodgkin's disease and on the malignant cells of patients with true histiocytic lymphoma (Schwarting et al. 1985). Finally, a proportion of the leukemic cells of patients with chronic and acute myelogenous leukemia are Tac^+ (Armitage et al. 1986; Yamamoto et al. 1986). In addition to these Tac-expressing leukemias and lymphomas, there are certain leukemias (e.g., acute lymphoblastic leukemia and LGL leukemia) that do not express the Tac peptide but do express the p75 peptide of the IL-2 receptor.

Autoimmune diseases may also be associated with disorders of Tac antigen expression (Diamantstein and Osawa 1986). Some mononuclear cells in the involved tissues express the Tac antigen, and the serum concentration of the soluble form of the Tac peptide is elevated. Such evidence for T-cell activation and disorders of Tac antigen expression appears in patients with rheumatoid arthritis and systemic lupus erythematosus, certain patients with aplastic anemia, and individuals with HTLV-I-associated tropical spastic paraparesis. Finally, the T cells that recognize foreign histocompatibility antigens following allograft implantation as well as those involved in graft-vs-host disease express the Tac antigen.

IL-2 RECEPTOR AS TARGET FOR IMMUNE INTERVENTION

The scientific basis for therapeutic trials using agents to eliminate the IL-2 receptor-expressing cells is the observation that T cells in patients with certain lymphoid malignancies express IL-2 receptors identified by the anti-Tac monoclonal antibody, whereas normal resting cells and their precursors do not. Such agents could theoretically eliminate Tac-expressing leukemia cells or activated T cells involved in other disease states, while retaining the Tac^- mature normal T cells and their precursors that express the full repertoire of antigen receptors for T-cell immune responses. The agents that we

have used include (a) unmodified anti-Tac monoclonal antibody; (b) toxin conjugates of anti-Tac (e.g., A chain of ricin toxin, *Pseudomonas* exotoxin [PE], and truncated PE [PE40]); (c) IL-2 truncated toxin fusion proteins (e.g., IL-2 PE40); (d) α- and β-emitting isotopic (e.g., ^{212}Bi and ^{90}Y) chelates of anti-Tac; and (e) hybrid "humanized" anti-Tac with mouse light- and heavy-chain variable or hypervariable regions joined to the human constant κ light-chain and IgG_1 or IgG_3 heavy-chain regions.

We have performed a clinical trial to evaluate the efficacy of iv administered anti-Tac monoclonal antibody in the treatment of patients with ATL (Waldmann et al. 1985, 1988). None of the nine patients treated suffered any untoward reactions, and only one, a patient with anti-Tac-induced clinical remission, produced antibodies to the mouse immunoglobulin or to the idiotype of the anti-Tac monoclonal antibody. After anti-Tac therapy, three of the patients had a temporary mixed, partial, or complete remission lasting 1, 5, and over 8 months, respectively (Waldmann et al. 1988).

These therapeutic studies have been extended by the examination of the efficacy of toxins coupled to anti-Tac to selectively inhibit protein synthesis and viability of Tac$^+$ ATL lines. The addition of anti-Tac antibody coupled to PE inhibited protein synthesis by Tac-expressing HUT-102-B2 cells, but not that by the acute T-cell leukemia line MOLT 4, which does not express the Tac antigen (FitzGerald et al. 1984).

The initial PE-anti-Tac conjugate was hepatotoxic when administered to patients with ATL. Functional analysis of deletion mutants of the PE structural gene has shown that domain I of the 66-kDa PE molecule is responsible for cell recognition, domain II for translocation of the toxin across membranes, and domain III for ADP-ribosylation of elongation factor 2, the step actually responsible for cell death (Hwang et al. 1987). A PE molecule from which domain I has been deleted (PE40) has full ADP-ribosylating activity but extremely low cell-killing activity when used alone because of the loss of the cell-recognition domain. The PE40 was produced in *Escherichia coli*, purified, and conjugated to anti-Tac. The anti-Tac PE conjugates inhibited the protein synthesis of Tac-expressing T-cell lines but not that of lines not expressing Tac.

IL-2-PE40, a chimeric protein composed of human IL-2 genetically fused to the amino terminal of the modified form of PE40, was constructed to provide an alternative (lymphokine-mediated) method of delivering PE40 to the surface of IL-2 receptor Tac$^+$ cells (Hwang et al. 1987; Lorberboum-Galski et al. 1988). The IL-2-PE40, a cytotoxic protein, was produced by fusion of a cDNA-encoding human IL-2 gene to the 5' end of a modified PE40 gene that lacks sequences encoding the cell-recognition domain. The addition of IL-2-PE40 led to the inhibition of protein synthesis by the toxin moiety of IL-2-PE40 when added to human cell lines expressing either the p55 or p75 or both IL-2 receptor subunits. The receptor internalization was much more efficient when high-affinity receptors composed of both units were present. IL-2-PE40 is a powerful reagent for studying IL-2 receptor interactions and for analyzing pathways of human immune response and

its regulation. This chimeric protein toxin is being evaluated as an agent for IL-2 receptor-directed therapy in rodents with IL-2 receptor-expressing transplantable leukemias, with different forms of autoimmune disease, as well as in primates receiving allografts.

The action of toxin conjugates of monoclonal antibodies depends on their ability to be internalized by the cell and released into the cytoplasm. Anti-Tac bound to IL-2 receptors on leukemic cells is internalized slowly into coated pits and then endosomic vesicles. Furthermore, the toxin conjugates do not pass easily from the endosomic vesicles to the cytosol, as required for their action on elongation factor 2. To circumvent these limitations, alternative cytotoxic reagents were developed that could be conjugated to anti-Tac and that were effective when bound to the surface of Tac-expressing cells. In one case, it was shown that ^{212}Bi, an α-emitting radionuclide conjugated to anti-Tac by use of a bifunctional chelate, was well suited for this role (Kozak et al. 1986). Activity levels of 0.5 μCi or the equivalent of 12 rad/ml of α radiation targeted by ^{212}Bi-labeled anti-Tac eliminated greater than 98% of the proliferative capacity of the HUT-102 cells, with only a modest effect on IL-2 receptor-negative lines. This specific cytotoxicity was blocked by excess unlabeled anti-Tac but not by human IgG.

In parallel studies, the β-emitting ^{90}Y was chelated to anti-Tac with the use of the chelate 1(2)-methyl-4-(P-isothiocyanatobenzyl) diethylenetriamine-pentaacetic acid, which did not permit elution of the radiolabeled yttrium from the monoclonal antibody (Kozak et al. 1989). Rhesus monkeys receiving a xenograft of a cynomolgus monkey heart showed a marked prolongation of xenograft survival after the administration of ^{90}Y-labeled anti-Tac (Cooper et al. 1988). Thus, ^{212}Bi-labeled anti-Tac and ^{90}Y-labeled anti-Tac are potentially effective and specific immunocytotoxic agents for the elimination of Tac-expressing cells.

Prior attempts at anti-tumor therapy in humans with mouse monoclonal antibodies have been limited in their effectiveness by their ineffective recruitment of host effector mechanisms and by the neutralization of the mouse monoclonals by the development on the host of human anti-mouse antibodies. To circumvent these problems we have used genetic engineering to prepare hybrid "humanized" anti-Tac with mouse light- and heavy-chain variable (chimeras) or hypervariable regions (hyperchimeras) joined to the human constant κ light-chain and IgG_1 or IgG_3 heavy-chain regions (C. Queen, R. Junghans, and T.A. Waldmann, manuscript in preparation). In the hyperchimeric humanized form the entire molecule is "humanized" with human constant and variable framework regions with the exception of the small segments of the mouse complementarity regions (CDRs) which are critical to the specificity of epitope binding. In addition, a molecular model of the murine anti-Tac was used to identify the amino acids that, although outside the CDRs, had a high probability of interacting with the CDRs or antigen surface. Such residues could have an effect on antigen binding and were therefore retained in constructing the antibody.

It is hoped that these humanized anti-Tac antibodies will lack the T-helper cell recognition domains required for the production of human

anti-monoclonal antibodies. The hyperchimeric antibodies to different antigens studied by Hale (1988) and by LoBuglio and their coworkers (1989) elicited either no or only modest anti-globulin responses. The chimeric and hyperchimeric anti-Tac antibodies maintained their high binding affinity ($3\text{-}9 \times 10^{-9}$) for Tac-expressing cells and preserved their abilities to inhibit antigen- and mixed leukocyte-induced T-cell proliferation. Furthermore, the IgG_1 chimeras and hyperchimeras manifest a new activity of antibody-dependent cell-mediated cytotoxicity (ADCC) with human mononuclear cells that was absent in the parental mouse anti-Tac (R. Junghans, T.A. Waldmann, and C. Queen, manuscript in preparation). With this new ADCC activity it is hoped that there will be a substantial improvement in the performance of the antibody in vivo that should translate into an increase in the therapeutic efficacy.

In addition to their use in the therapy of patients with ATL, these IL-2 receptor-directed approaches are being evaluated for their ability to eliminate IL-2 receptor-expressing T cells in other clinical states, including certain autoimmune disorders. In these disorders, the lymphocytes infiltrating the affected organs express the Tac antigen, and the soluble form of the IL-2 receptor in the serum is elevated. Appropriate anti-IL-2 receptor antibodies were shown to suppress murine diabetic insulitis, lupus nephritis, experimental allergic encephalomyelitis, and adjuvant arthritis (Diamantstein and Osawa 1986; Kelley et al. 1988). With these encouraging results in animal models, IL-2 receptor-directed therapy is being initiated in patients with autoimmune disorders.

Monoclonal antibodies that recognize the IL-2 receptor have also been used to inhibit the graft-vs-host reaction and organ allograft rejection. Antibodies to the p55 IL-2 receptor (Tac) were shown to inhibit the proliferation of T cells to foreign histocompatibility antigens expressed on the donor organ and to prevent the generation of cytotoxic T cells in allogeneic cell cocultures (Uchiyama et al. 1981b; Depper et al. 1983). Volk and coworkers (1986) demonstrated that the acute graft-vs-host reaction across a strong major histocompatibility barrier in mice can be suppressed by AMT-13, a monoclonal antibody directed against the 55-kDa IL-2 receptor on activated mouse lymphocytes. Furthermore, in studies by Kirkman and coworkers (1985), the survival of cardiac allografts was prolonged in some cases to indefinite survival in rodent recipients treated with an anti-IL-2 receptor monoclonal antibody. In parallel studies, the administration of anti-Tac for the initial 10 days after transplantation prolonged the survival of renal allografts in cynomolgus monkeys (Shapiro et al. 1987). Furthermore, Bacha and coworkers (1988) have achieved prolongation of allograft survival and suppression of delayed-type hypersensitivity with genetically engineered diphtheria toxin linked to the NH_2-terminus of human IL-2. However, unmodified anti-Tac did not lead to a prolongation of graft survival in heterotopic cardiac xenografts in which rhesus monkeys received cardiac xenografts from cynomolgus donors. In contrast, animals receiving ^{90}Y-labeled anti-Tac showed a prolongation of graft survival from a value in untreated animals of 6-8 days to a mean graft survival of 40 days in animals receiving a dose of radioactivity that had acceptable toxicity (Cooper et al. 1988). In light of these encouraging results, human recipients of cadaver donor renal allografts are

receiving different anti-IL-2 receptor monoclonal antibodies as adjunctive immunotherapy (Soulillow et al. 1987; Shapiro et al. in press). Antibody treatment has been well tolerated, and 50 of 53 recipients treated retain a functioning allograft.

In summary, our present understanding of the IL-2/IL-2 receptor system opens the possibility for more specific immune intervention strategies. The IL-2 receptor may prove to be an extraordinarily versatile therapeutic target. The clinical applications of anti-IL-2 receptor-directed therapy represent a new perspective for the treatment of certain neoplastic disease and autoimmune disorders and for the prevention of allograft rejection.

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Cytokine Therapy of Patients with Cancer

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INTRODUCTION

The immunotherapy of cancer has been based on the expectation that immunologically recognizable differences could be demonstrated between tumor tissues and normal tissues (Lotze, 1988). The mechanism by which tolerance to tumors and nominal tumor antigens occurs in the host is unknown but presumably is similar to findings based on recognition of soluble and alloantigens with either thymic or peripheral tolerization (Schwartz, 1989; Nossal, 1989). The role of the tumor itself in inducing immune suppression is still poorly understood. The development over the last five years of successful immunotherapies using cytokines for the treatment of cancer in murine models, as well as man has now provided an opportunity to test certain postulates regarding the immune effects of these treatments. This has perhaps been best demonstrated in treatment with Interleukin-2 (IL-2) based regimens (Mulé, 1984, 1987). As shown in Table 1, a variety of effects have already been demonstrated concerning the role of host immune elements in the response to tumor. Questions regarding the mechanism by which the host fails to respond to the tumor, as well as critical questions regarding the antigens recognized, presumed requirements for antigen processing, and the nature of the specific T-cell receptors remain important unanswered questions.

Table 1. Postulates For Demonstrating Immune Effect of Cancer Treatment with Cytokines

	Demonstrated For IL-2	
	Mouse	Man
1. Regression Of Tumor	+	+
2. Infiltration Of Tumor By Lymphoid Cells	+	+
3. Immunosuppression Abrogates Effective Therapy	+	+
4. Persistent Regression (Immunity) For Prolonged Periods	+	+
5. Immunity Transferrable With Immune Cells To Syngeneic Host	+/-	-
6. Identification Of Mechanism By Which Host Fails To Respond	+/-	-
7. Identification Of Antigen(s) Recognized	-	-
8. Demonstrate Requirement For Antigen Processing	-	-
9. Identification Of Unique T Cell Receptor(s)	-	+/-
10. Transfer Of Unique T Cell Receptor To Naive Cells Confers Activity	-	-

NCI SURGERY BRANCH IMMUNOTHERAPY TRIALS

The availability of recombinant IL-2 as well as other cytokines including TNF-alpha, interferon-alpha, GM-CSF, and IL-4 allowed the evaluation of a variety of different immunotherapy regimens. The initial demonstration that high-dose IL-2 therapy was capable of mediating tumor regression when used alone (Rosenberg 1985a, 1987, Lotze 1985, 1986) as well as in combination with the adoptive transfer of IL-2 activated peripheral blood mononuclear cells, (Rosenberg, 1985a, 1987) also referred to as lymphokine activated killer cells (LAK) (Lotze 1980, 1981, Grimm 1983) prompted a variety of other treatment protocols. A recently updated summary of current immunotherapy trials in our branch is shown below in Table 2.

Table 2. NCI-Surgery Branch Immunotherapy Trials

Treatment	No. Pts.	CR	PR	Notes
High Dose IL-2	130	4	18	100,000 U/kg TID
IL-2/LAK	177	14	30	0.4-1.4x10^{12} Cells
IL-2 + TNFα	38	1	3	TNF 50-350$\mu g/m^2$x3
IL-2 + IFNα	113	7	25	1-6x10^6U/m^2 qd-TID
IL-2 + TILs	66	P	P	1.1-4.7x10^{11} Cells
Monoclonal Antibody $\pm$ IL-2	32	0	0	Antibodies 9.2.27,L6, 17.1A, B72.3
IL-2 + Cyclophosphamide	19	0	2	10-50 mg/kg Cyclophos.
GM-CSF	19	0	0	3-100μg/kg/dx21d
IL-4	24	P	P	Escalating Dose 1-20μg/kg QD-TID
Adjuvant Therapy IL-2, IL-2/LAK	60	-	-	

Adopted from Rosenberg 1989a, 1989b. P, pending followup.

Further, the demonstration of the presumably active host component, namely antigen reactive T-cells, has been pursued both in murine and human trials (Rosenberg, 1988) with the adoptive transfer of tumor infiltrating lymphocytes (TILs). Attempts to combine Interleukin-2 with other cytokines, as well as monoclonal antibodies are also in progress. Significant responses have been noted in many of these IL-2 based protocols. The use of Interleukin-4 (IL-4) as an immunotherapeutic reagent is being explored (see below). These treatments were primarily developed in patients with widespread metastatic disease from a variety of different neoplasms. Objective responses have been noted in patients with renal cell carcinoma, malignant melanoma, colorectal carcinoma, and non-Hodgkin's lymphomas. Based on murine studies (Mulé, 1984) which indicated an improvement in response with low tumor burden, adjuvant protocols were initiated approximately three years ago in patient's with resected stage II melanoma, as well as in

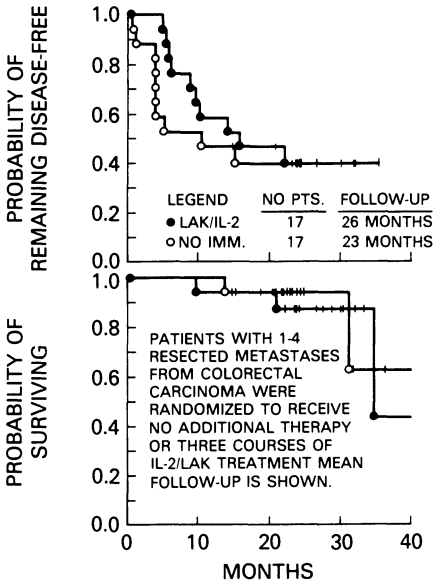

FIG. 1. ADJUVANT THERAPY FOLLOWING RESECTION OF HEPATIC METASTASES

patient's following hepatic metastesectomy for colorectal carcinoma though too few patients (less than 20 in any treatment arm) have been accrued to draw any firm conclusions. disorders. An update of our adjuvant protocol following the resection of hepatic metastases is shown below.

INTERLEUKIN-2 STUDIES

The administration of recombinant Interleukin-2 to patients with cancer has been associated with a variety of side effects, including renal dysfunction (Belldegrun, 1986), hemodynamic changes including hypotension and mild myocardial depression (Lee, 1989), decrease in the ability of polymorphonuclear leukocytes to follow chemotactic gradients (Jablons, 1989c) which may be associated with an increased incidence of line-related infections, profound reversible cholestatic jaundice (Fisher, 1989) as well as the development of a leaky capillary syndrome (Cotran, 1988). Many of these effects appear to be related to the secondary elaboration of a variety of other cytokines. We have been able to detect gamma interferon (Lotze, 1985) as well as tumor necrosis factor and IL-6 (Jablons, 1989a) in the sera of patient's receiving Interleukin-2 therapy. Interleukin-6 appears to act as a circulating hormone mediating many of the early events noted following the

acute inflammatory response, including the stimulation of acute phase plasma proteins (Baumann, 1987). It has been detected in the sera of patient's following tumor necrosis factor infusion (Jablons, 1989a; Brouchaert, 1989), as well as following elective surgery (Shenkin, 1989) and in the tumor bearing state (Jablons, 1989b). Some of this information is summarized below in Table 3.

Table 3. Circulating IL-6 In Sera Of Patients

Treatment	No. Patients	Mean Peak IL-6 (HGF) Units/ML (Range)
TNFα ($2\text{-}3\times10^2\mu g/m^2$)	6	232 (140-310)
IL-2 (10^5U/kg)	2	63 (30-95)
IL-2 (10^4U/kg)	2	<20
IFNα (3×10^6U/kg)	2	<20
Normal Individuals	10	<5
Cancer Patients	17	11, Sarc; 8, GI; 12, RCC

Serum samples were measured in the B9 hybridoma growth factor assay (Jablons 1989a, 1989b). Activity in all cases was neutralized by a specific rabbit anti IL-6 antibody; Sarc, sarcoma; GI, GI malignancies; RCC, renal cell cancer.

Evidence of approximately 20-30% response rate in patient's with metastatic melanoma and renal cell carcinoma have been demonstrated (Rosenberg, 1985b, 1987) and have been confirmed by others (West, 1987; Fisher, 1988). Of great interest immunologically is the finding of large activated T-cells and expression of activation antigens including class II molecules at the site of tumor regression. (Cohen, 1987; Rubin 1989) This has largely been noted in the setting of biopsied lesions from patients with cutaneous sites of metastatic disease, as shown below.

Table 4. Responding Lesions Excised Following Immunotherapy Have A T Cell Infiltrate and Express DR

Lesion	No. Pts.	CR/PR	Mean Score T Cell Infiltrate	Mean Score DR Expression
Response	12	6	2.6	2.5
No Response	14	1	1.4	0.4

T Cell infiltrate was graded as none, 0; minimal, 1; 2; diffuse, 3; DR expression was graded as minimal (,25%), 0; moderate (50% cells), 1; most (75%) cells); or strong (100% cells), 3. Response of lesions was judged by decrease in size; clinical response was based on conventional criteria for complete response (CR) and partial response(PR).

Recently, we have begun treatment of patient's with advanced cancer with combinations of IL-2 and alpha interferon based on murine studies showing synergy of these reagents. Patients on our protocols received alpha interferon at a dose of up to 6×10^6U/m^2 once to thrice daily in combination with up to 6×10^6U/m^2 of IL-2 again administered thrice daily (Rosenberg, 1989a). In our initial studies, objective responses were seen in between 17-41% of patients with the highest responses observed in those receiving the highest doses of these agents. Further development of combination cytokine therapy predicated on these results would appear promising.

INTERLEUKIN-4 STUDIES

Interleukin-4 was initially described as a B-cell stimulating factor and has subsequently been shown to have effects on cells of many lineages, including T-cells, fibroblasts, and monocytes. It has recently been cloned in the mouse and the murine sequence led to the subsequent isolation of a human IL-4 cDNA clone from human T-cells. The human IL-4 gene occurs as a single copy in the genome and is found on chromosome 5 (Arai, 1989). Although studies in the mouse (Mulé 1987, 1989) showed that it was capable of regulating murine lymphokine activated killer cell activity, causing induction of LAK activity by itself as well as synergistically with IL-2, it was found to be primarily suppressive of IL-2 induced LAK activity in man (Kawakami, 1989). It does promote the growth of human T-cells following antigen or mitogen activation (Spit, 1987) and promotes the growth of tumor infiltrating lymphocytes cytotoxic for human autologous melanoma (Kawakami, 1988). Based on its ability to expand T-cells in vitro, we initiated a clinical protocol evaluating the role of IL-4 approximately one year ago. A total of 24 patients have been placed on protocol to date. Initial studies using single daily administration of IL-4 at doses of 1-30 ug/kg demonstrated an alpha distribution of approximately eight minutes and a beta clearance of approximately 48 minutes. This was demonstrated in a biologic assay specific for IL-4 measuring the induction of CD23 on malignant B-cells (Custer, 1989). At the highest total cumulative daily doses of IL-4 (20 ug/kg given thrice daily) toxicities have been mild to moderate and include sinusitis, headache, apparent development of a leaky capillary syndrome, and renal dysfunction which are also characteristic of treatments employing IL-2. Interestingly, it does not cause major hypotension as has been observed with IL-2. Recent studies have demonstrated that introduction of the IL-4 gene into tumor cells is associated with regression mediated by an inflammatory infiltrate composed of eosinophils and macrophages (Tepper, 1989). Future studies will examine the antitumor role of IL-4 alone and in combination with IL-2 in clinical protocols.

FUTURE DIRECTIONS/PROLOGUE FOR THE 90'S

The last five years have seen substantial progress in our understanding of basic immune mechanisms and these have been applied with enthusiasm in the treatment of patients with cancer. We end this decade with clear cut evidence that cytokine therapy can be associated with tumor responses and indeed these reagents have been applied to the treatment of a variety of other hematopoietic disorders, as well. Recombinant cytokines with potential therapeutic use are listed below in Table 5. The subsequent development of these reagents alone or in combination will certainly occupy much of the next decade. Studies combining cytokine treatment with chemotherapy or radiation therapy (Cameron, 1990) are in progress.

Table 5. Recombinant Cytokines With Potential Therapeutic Use

Name	Human Trials	Molecular Weight(kD)	Clinical Use	Reference
IL-1	+	15.17	Cancer	Longo, unpub.
IL-2	+	15	Cancer	Lotze, 1985
IL-3	-	25	Hematopoiesis	Donahue, 1988
IL-4	+	21.5	Cancer	Lotze, unpub.
IL-5	-	45-60	Parasitic Diseases	Lopez, 1988
IL-6	-	19-30	Cancer	Mulé, unpub.
IL-7	-	25	Cancer, immunodeficiency	Morrisey, 1989
GM-CSF	+	14-34	Hematopoiesis	Groopman, 1987
G-CSF	+	19.6	Hematopoiesis	Gabrilove, 1988
M-CSF	+	45	Cancer	Cameron, unpub.
TNFα	+	17-60	Cancer	Blick, 1987
IFNα	+	17.5-21	Cancer	Merigan, 1988
IFNβ	+	18.5	Cancer	Rinehart, 1987
IFNγ	+	40-60	Cancer	Maluish, 1988
EPO	+	18-34	Erythropoiesis	Eschbach, 1987
TGF-β	-	25	Wound healing	Sporn, 1983

IFN, interferon;EPO, erythropoietin; TNF,tumor factor; kD, Kilodalton.

The role of the adoptive transfer of cells including tumor infiltrating lymphocytes appears to be a promising approach (Rosenberg, 1988) both in demonstrating the mechanism by which antitumor effects occur but also as potential vehicles for proteins encoded by transduced genes. We have begun studies using the neomycin resistance gene introduced into tumor infiltrating lymphocytes using incompetent murine retroviral vectors. (Culver, 1989; Kasid, 1989). The introduction of other cytokine genes, as well as receptors possibly important in antigen recognition and trafficking will undoubtedly follow as important studies in both mouse and man. Further, as noted above, the direct introduction of cytokine genes into tumor targets as a mechanism to induce local immune responses as a form of active immunotherapy (as has been evaluated with IL-4) are interesting alternative approaches. The introduction of other cytokines, including tumor necrosis factor or IL-2 (Bubenik, 1988) are other possible avenues of research.

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Modulation of Autoimmune Responses with Normal Polyspecific IgG for Therapeutic Use

G. Dietrich, F. Rossi, and M.D. Kazatchkine

Intravenous immunoglobulins for therapeutic use (IVIg) are preparations of polyspecific IgG obtained from large normal plasma pools. IVIg have primarily been used as substitutive therapy for primary and secondary antibody deficiencies (Morell and Nydegger 1986). Since the early observations of a beneficial effect of IVIg in idiopathic thrombocytopenic purpura (ITP) (Imbach et al. 1981) ; Schmidt et al. 1981 ; Newland et al. 1983), the use of IVIg has been extended to a number of autoimmune disorders. Infusions of IVIg have resulted in clinical improvement and/or decrease in autoantibody titer in autoimmune neutropenia (Bussel et al. 1983), hemolytic anemia (Mc Intyre et al. 1985), autoimmune red cell aplasia (Mc Guire et al. 1987), myasthenia gravis (Gajdos et al. 1984), the chronic equivalent of Guillain Barré syndrome, chronic inflammatory demyelinating polyneuropathy (CIDP) (Vermeulen et al. 1985), Kawasaki's disease (Furosho et al. 1984) in women with recurrent abortions and anticardiolipin antibodies (Carreras et al. 1988), and in patients with autoantibodies to procoagulant Factor VIII (FVIII) (Sultan et al. 1984).

Several mechanisms of action of IVIg in autoimmune disorders have been postulated (Table 1).

Table 1. Proposed mechanisms of action of IVIg in autoimmune disorders

Reversible blockade of Fc receptors on cells of the reticuloendothelial system.
Fc-dependent down-regulation of autoantibody production by B cells.
Generation of specific suppressive T cells.
Anti-idiotypic suppression and interference with network regulation of autoimmunity.

Reversible inhibition of Fc receptor function of cells of the reticuloendothelial system is one of the mechanisms that may be operative in peripheral autoimmune cytopenias in which the effect of IVIg is often only transient. Evidence for Fc receptor blockade *in vivo* comes from the observation of an increased half life of infused anti-RhD-coated autologous erythrocytes in patients with ITP who had been treated with IVIg (Fehr et al. 1982). In addition, *in vitro* studies have shown that exposure of normal monocytes to IgG at 37°C results in the loss of the ability of the cells to bind antibody-coated platelets (Schmidt et al. 1981). Inhibition of Fc receptor function cannot however explain the suppressive effect that IVIg may have on autoantibody responses and the long term autoantibody suppression that had been observed in several autoimmune disorders.

We have proposed that suppression of autoimmune responses by IVIg may be dependent on the presence in IVIg of specific anti-idiotypes against autoantibodies and, in a broader sense, on the restoration by pooled normal IgG of a functional network of connected antibody interactions similar to that which prevents the expression of autoimmune pathogenic clones in normal individuals (Rossi et al. 1989). This manuscript briefly summarizes some of the evidence supporting the latter hypothesis and speculates on the source of anti-idiotypes in IVIg. The concept was derived from the observation of patients with anti-FVIII autoantibodies in whom infusion of IVIg has resulted in a decrease in autoantibody titer by 80-95 % of pretreatment titer (Sultan et al. 1984). The rapid effect of IVIg that was seen within the first 36 hours of infusion suggested a direct interaction between infused IgG and patients' autoantibodies. The prolonged suppressive effect of IVIg that has lasted for now over 5 years in one of the patients, indicated that IVIg also interfered with autoantibody synthesis.

Four lines of evidence obtained in vitro with anti-FVIII and with other autoantibodies, indicate that IVIg contain anti-idiotypes against idiotypic determinants expressed by autoantibodies from patients with autoimmune diseases :
1) $F(ab')_2$ fragments from IVIg inhibit the binding and/or the functional interaction of autoantibodies with autoantigens. $F(ab')_2$ fragments from IVIg neutralized the anti-FVIII activity of $F(ab')_2$ fragments prepared from patients' IgG (Sultan et al. 1984) ; $F(ab')_2$ fragments from IVIg were aslo found to inhibit the binding of IgG autoantibodies from patients with other autoimmune diseases to the relevant autoantigen, including thyroglobulin, DNA, intrinsic factor (Rossi and Kazatchkine 1989), a target autoantigen for IgM autoantibodies of CIDP (Van Doorn et al. 1989), and the cytoplasmic neutrophil autoantigen which is recognized by autoantibodies in the plasma from patients with Wegener's granulomatosis (Jayne et al. 1989). In all instances, inhibition of autoantibody activity by IVIg was dose-dependent following a bell-shaped curve with maximal inhibition occurring at a specific molar ratio between patients' IgG or $F(ab')_2$ and $F(ab')_2$ from IVIg (Table 2). The optimal inhibitory ratio between patients' and therapeutic antibodies was specific for each patient and each antibody specificity tested. Autoantibodies from some patients were not inhibited by IVIg in vitro. In the case

Table 2. Inhibition of autoantibody activities by $F(ab')_2$ fragments from IVIg *

Autoantibody	Inhibition of autoantibody activity (%)	moles/moles
Anti-FVIII:C	100	0.450 (0.450- 1.800)
Anti-thyroglobulin	61	0.090 (0.090- 5.730)
Anti-DNA	76	0.280 (0.035- 0.560)
Anti-peripheral nerve	100	0.015 (0.005- 0.023)
Anti-intrinsic factor	36	6.670 (0.417-13.250)
Anti-neutrophil cytoplasmic antigen	52	0.137 (0.069- 1.100)

Footnotes on the next page.

* Sandoglobulin^{R}
$F(ab')_2$ fragments, IgM or IgG containing autoantibody activity were incubated with $F(ab')_2$ fragments from IVIg for 1h at 37°C and overnight at 4°C. The Table shows the maximal inhibition of autoantibody activity that was observed and the molar ratio between autoantibodies and $F(ab')_2$ from IVIg at which maximal inhibition was achieved. Parentheses indicate the range of molar ratios between autoantibodies and $F(ab')_2$ from IVIg that were tested.

of anti-FVIII autoantibodies the lack of inhibition of autoantibody activity by IVIg in vitro correlated with the lack of suppressive effect of IVIg in vivo (Rossi et al. 1988).

2) $F(ab')_2$ fragments with autoantibody activity are specifically retained on affinity columns of Sepharose-bound $F(ab')_2$ fragments from IVIg. Results of affinity chromatography of IgG or $F(ab')_2$ autoantibodies on insolubilized $F(ab')_2$ fragments from IVIg (Sultan et al. 1984 ; Rossi et al. 1988 ; Rossi and Kazatchkine 1989) are summarized in Table 3. The finding of an increase in specific autoantibody activity in acid-eluted fractions from the columns as compared with the loaded material, indicated that some antibody species in IVIg were specifically capable of high affinity interactions with the variable region of anti-FVIII, anti-thyroglobulin, anti-DNA and anti-intrinsic factor autoantibodies. These experiments demonstrated the presence in IVIg of anti-idiotypes against autoantibodies and/or that of anti-idiotypes against IVIg in patients' autoantibodies.

Table 3. Chromatography of IgG or $F(ab')_2$ fragments containing autoantibody activity on Sepharose-bound $F(ab')_2$ fragments from IVIg *

	Loaded		Acid-eluted	
Autoantibody	Amount (µg)	specific autoantibody activity	Amount (µg)	specific autoantibody activity
Anti-FVIII:C	9000	0.98 BU/mg	73	38.09 BU/mg
Anti-thyroglobulin	1850	1.26 U/mg	153	18.20 U/mg
Anti-DNA	2750	0.31 U/mg	85	10.79 U/mg
Anti-intrinsic factor	1207	3.22 U/mg	17	4.32 U/mg

* Sandoglobulin^{R}
Specific anti-F VIII:C activity was expressed as Bethesda Units (B.U.) per mg of protein. Specific anti-thyroglobulin, anti-DNA and anti-intrinsic factor activities were expressed in arbitrary units (U) per mg of protein.

3) IVIg contain no detectable anti-allotypes against the Gm-1 (3), Gm-1 (4), Gm-1 (1), Gm-1 (17), and Km 1 allotypic specificities that are most commonly expressed in the $F(ab')_2$ region of human IgG (Rossi et al. 1988).

4) IVIg share anti-idiotypic specificities against autoantibodies with heterologous anti-idiotypic antibodies. The data were derived from experiments using rabbit anti-idiotypic antibodies (anti-T44

antibodies) raised against the antithyroglobulin autoantibody from a patient with autoimmune thyroiditis. $F(ab')_2$ fragments from anti-T44 antibodies were found to inhibit the binding of IVIg to insolubilized anti-thyroglobulin $F(ab')_2$ autoantibodies (Dietrich and Kazatchkine 1989). In addition, the fraction of anti-thyroglobulin autoantibodies that bound to IVIg-Sepharose columns expressed the T44 idiotype, whereas autoanti-thyroglobulin antibodies from the effluent of the columns and immunoglobulins from healthy individuals failed to express the idiotype (Table 4). The idiotype recognized by IVIg and by anti-T44 antibodies was expressed by autoantibodies from 8 of 9 patients with autoimmune thyroiditis, but not by IgG from healthy individuals containing anti-thyroglobulin activity indicating that IVIg contain anti-idiotypes against an immunodominant, cross-reactive, disease-associated idiotype of anti-thyroglobulin autoantibodies.

Table 4. Expression of the T44 idiotype in the retained and fall-through fractions of anti-thyroglobulin autoantibodies and of normal IgG upon affinity chromatography on Sepharose-bound $F(ab')_2$ fragments from IVIg *

	Expression of the T44 idiotype	
	acid eluate	fall through
Anti-thyroglobulin antibodies from patients with autoimmune thyroiditis	+++ (8/9)	-
IgG from normal individuals	- (0/5)	-
IVIg	+	-

* Sandoglobulin^R. The presence of the T44 idiotype was assessed in the acid-eluted $F(ab')_2$ fragments and in the effluent of the columns by direct binding of anti-T44 antibodies to insolubilized $F(ab')_2$ fragments. $F(ab')_2$ fragments from patients' IgG did not contain detectable antibodies against rabbit Fc gamma fragments.

Cross-reactive idiotypes are often shared between human autoantibodies of given specificities (Delves et al. 1984 ; Fong et al. 1986 ; Levfert 1984 ; Williams et al. 1968). These idiotypes may represent priviledged targets for therapeutic anti-idiotypic manipulation of autoimmune responses. IVIg contain anti-idiotypes against cross-reactive idiotypes of anti-thyroglobulin autoantibodies (Table 4) and anti-FVIII autoantibodies (Sultan et al. 1987) from patients with autoimmune diseases. IVIg also contain anti-idiotypes against autoantibodies contained in IVIg itself, as indicated by affinity chromatography experiments (Table 5).

Table 5. Affinity chromatography of IVIg on Sepharose-bound $F(ab')_2$ from IVIg *

	Specific autoantibody activity (U/mg)	
	Loaded material	Acid-elutable fraction
Anti-thyroglobulin	0.0945	0.9625
Anti-DNA	0.1120	0.5395

* Sandoglobulin Specific anti-thyroglobulin and anti-DNA activities were expressed in arbitrary units/mg of IgG.

IgG autoantibodies are present in IVIg as they are present in individually tested plasmas from healthy subjects. It is possible that IVIg also contain small amounts of autoantibodies expressing characteristics of disease-associated antibodies, as suggested by the finding in IVIg of low concentrations of antibodies expressing the T44 idiotype (Table 4). Thus, IVIg represent a complex mixture of idiotypes and anti-idiotypes expressed by normal and disease-associated autoantibodies. The finding of anti-idiotypes against autoantibodies appears relatively specific of large pools of IgG since the anti-idiotypes are only inconstantly found in the IgG fraction from individually tested normal donors that may contribute to the pool.

Several reasons may explain the preferential expression of anti-idiotypic specificities against autoantibodies in pools of IgG from large numbers of donors. The expression in IVIg of anti-idiotypic specificities that are not detectable or only poorly represented in the plasma from individual donors, may result from the additive contribution of anti-idiotypes from each donor participating in the pool, just as functional antibody activity may be expressed by polyclonal antibodies or a mixture of monoclonal antibodies but not by monoclonal or oligoclonal antibodies. Recent evidence (Gronski et al. 1988) indicates that increasing numbers of donors contributing to a pool of IVIg, result in increased numbers of $F(ab')_2$-$F(ab')_2$ dimers in the preparation. Formation of dimers reflects the expression of anti-idiotypic specificities in IVIg directed against idiotypes expressed by immunoglobulins of the pool. It is conceivable that some donors are "higher contributors" of anti-idiotypes to the pool than others, e.g. individuals who recovered or are in remission from autoimmune disease. Thus, anti-idiotypes against anti-DNA (Abdou et al. 1981), anti-acetylcholine receptor (Dwyer et al. 1983), anti-fibrinogen (Ruiz-Arguelles 1988), anti-FVIII (Sultan et al. 1987) and anti-peripheral nerve (Van Doorn et al. 1989) autoantibodies have been found in the serum of patients in remission from the diseases characterized in their acute phase by the presence of these various autoantibodies. The anti-idiotypes could be directed against cross-reactive idiotypes shared by autoantibodies of similar specificity from different patients or against "regulatory idiotypes" expressed by autoantibodies differing both in their specificity and patients' origin. We have obtained preliminary evidence for such "polyvalent anti-idiotypes" in experiments in which IgG antibodies obtained from the plasma of patients who recovered from ITP, CIDP or anti-FVIII autoimmune disease, were found to inhibit autoantibody activity in prerecovery sera from the patients and to inhibit the function of autoantibodies of other specificities, e.g. anti-DNA and anti-thyroglobulin autoantibodies. In addition, we have recently shown that IVIg contain anti-idiotypes against polyspecific monoclonal IgM autoantibodies secreted by EBV-transformed B cells from normal individuals(F. Rossi et al., manuscript in preparation). These data suggest that circulating IgG participates in the regulation of the repertoire expressed by natural polyspecific antibody-secreting B cells. Anti-idiotypes against such IgM antibodies may represent regulatory idiotypes participating to a highly connected compartment of B cells in normal individuals (Coutinho et al. 1984).

The finding in IVIg of anti-idiotypes against IgG autoantibodies from patients, IgG autoantibodies present in low amounts in normal individuals, and against multireactive antiself IgM antibodies from healthy individuals, supports the concept of the existence of a functional network (Jerne 1984) regulating autoimmune responses in

man. A better understanding of the source of anti-idiotypes or of connected antibody specificities which determine the beneficial effect of IVIg on autoimmune response, may contribute to prepare pools of IgG that would be more selectively designed for immunointervention in autoimmune diseases.

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Biological Response Modification of "Physiological" Doses of Recombinant Cytokines

Ch. Huber and G. Gastl

I. Problems in Clinical Cytokine Research

Problems in clinical cytokine research relate to specific features of these mediators and to our somewhat limited knowledge of their biologic properties. Cytokines exhibit pleiotropic activities. It is neither possible to predict which effect will be the predominant one when a given cytokine is applied in vivo, nor to foresee the in vivo specificity of a single cytokine. All cytokines interact with each other in a network-like fashion. As a consequence it is unclear whether a biologic response is caused subsequent to application of an exogenous cytokine or by subsequent stimulation of the endogenous cytokine network. Most cytokines represent local tissue hormones and are not easy to detect in the bloodstream. There is growing evidence that at least for biological responses to certain cytokines, bell-shaped dose response curves might exist. The mode of action of most cytokines in human disease is still poorly understood. In particular it is not established whether cytokines exert their antineoplastic activity by direct interaction with tumor cells or indirectly by enhancing host antitumor responses.

Thus, it is not surprising that a simple approach does not exist to define the optimum use of cytokines in clinical trials. This contribution will deal with different strategies to define cytokine doses for therapeutic studies. For the sake of brevity the examples given will be restricted to the type I and type II interferons.

II. Two Different Approaches to Define Cytokine Doses for Clinial Trials

There are two different and to some extent complementary strategies for defining therapeutic dose levels for clinical cytokine studies:
One is the conventional approach which first defines maximum tolerated doses (MTD) in phase-I studies and then uses the MTD to determine clinical efficacy in phase II studies. Main reservations against solely basing the decision about dose levels on this approach stem from two considerations: MTD is per definition associated with high toxicity. Moreover, there are many documented examples of bell shaped dose response relationships. It is therefore an unclear apriori whether a MTD is always an optimum antineoplastic or biologic response modifying dose. The other approach to define cytokine doses for therapy studies uses the definition of biologic active doses (BAD). Difficulties with this approach relate to the problem of selecting appropriate biologic response parameters which relate to clinical efficacy.

III.Parameters to Define BAD of Recombinant Cytokines

Two different types of parameters can be selected to measure biologic activity of cytokines: parameters which relate to the presence of viable tumor cells or parameters which relate to host defence mechanisms with potential antineoplastic activity. The methods used to assess tumor burden in clinical cytokine studies are clinical measures of neoplastic lesions and/or biochemical assessment of tumor associated or tumor specific products. Limitations of this approach stem from the fact that clinical assessment is time-consuming; in sensitive tumors it may take several months to achieve remission and very few tumors such as the apudomas or myelomas can be followed by biochemical evaluation of their tumor specific products. This approach, which still is the only established basis to define clinically effective doses, is therefore not easy to use and can rarely be applied to titrate cytokine doses in individual patients.

Evaluation of biologic parameters which relate to antineoplastic host defence mechanisms represent the other option to define BAD. They relate to the assessment of innate or adaptive immune mechanisms with particular emphasis on macrophages, NK-cells, T-cells and cytokines. To approach such a definition of BAD clinical investigators have either studied cellular features of peripheral blood cells or various seric markers.

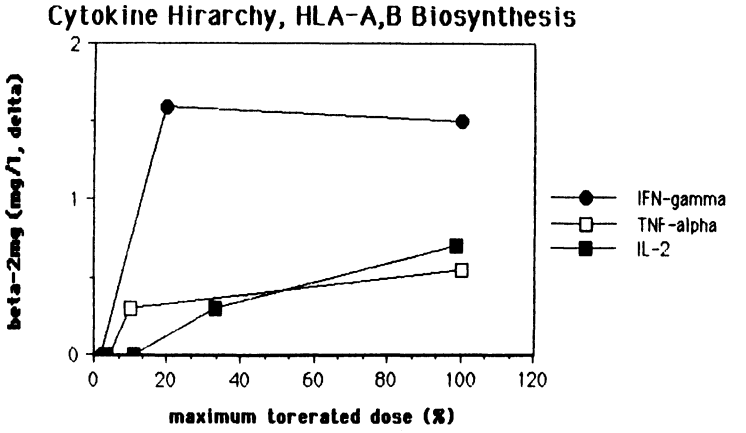

Fig.1. Induction of beta-2 microglobulin by various doses of human recombinant cytokines

Cellular parameters studied so far in great detail have been numbers and composition of white blood cells, functional assessment of their cytolytic potential or capacity to produce oxygen-radicals, expression of activation markers such as HLA-DR antigen or IL-2 receptor, biochemical assessment of cytokine dependent enzymes such as the 2'5'-OAS and, more recently, the expression of various genes relating to functional activation of leukocytes. Both Northern blot or dot-blot analyses and in situ hybridisation techniques are increasingly used to study mRNA levels of the above mentioned genes, or of genes which relate to proliferative activity such as the c-myc, Ki 67, p 53 or IP-10. By defining biologic activity solely on the basis of analyses of circulating blood cells, one has to make sure that application of cytokines does not alter tissue distribution of circulating blood cells. This possibility became increasingly unlikely after demonstration of cytokine-dependent induction of adhesion molecules of the LFA and the ICAM family. Our own recent studies strongly supported the view that type I and type II interferons as well as TNF-alpha dramatically alter the composition of circulating blood-cells: we also observed that it is only the cytokine-activated leukocyte which marginates and is not further assessable in the blood stream (Aulitzky WE, in press). Seric markers were also implied to study biologic activity of various cytokines. In principle, any product of a cytokine dependent gene which is released in measurable concentrations into the blood stream and has a reasonable stability and half-life can be used for such analyses. So far the products of the interferon-dependent genes, 2'5'OAS, beta-2 microglobulin, which represent the non-polymorphic chain of HLA-A,B antigens, kynurenic acid, a tryptophane degradation product, neopterin, a product of the pterin biosynthetic pathway and more recently the product of the Mx-gene as well as induction of other cytokines were measured. For most of these markers commercially available immunoassays and more recently cDNA probes enabling large scale measurement are available.

IV.Use of the Seric Markers Beta-2 Microglobulin and Neopterin for Definition of BAD:

During the last years we have studied preclinical and clinical aspects of the use of beta-2 microglobulin (Nachbaur 1988, Niederwieser 1988) and neopterin (Huber 1984, Schoedon 1986, Schoedon 1987, Troppmair 1988, Gastl 1987) for definition of BAD. Beta-2 microglobulin represents the non-polymorphic chain of HLA-A,B antigens. It is essential after assembling with the polymorphic heavy chain for the expression of the functional HLA-A,B antigen complex. This then represents the crucial target structure for cytotoxic T-lymphocytes. Biosynthesis of beta-2 microglobulin is under stringent control of type I and type II IFNs. We recently showed that the constitutive expression of the functional HLA-A,B antigen complex in certain resting tissues is too low to make them susceptible for lysis by cytotoxic T-lymphocytes (Niederwieser 1988). Interferon-induced enhancement of expression of HLA-A,B heavy chain also increased the release of beta-2 microglobulin from the surface of keratinocytes which at the same time became susceptible to

lysis by allospecific cytotoxic T-lymphocytes (Niederwieser 1988). In vivo application of IFN-gamma, TNF-alpha and IL-2 all lead to increasing beta-2 microglobulin levels (Nachbaur 1988). A clear hierarchy, however, exists between the various cytokines tested in vivo. As shown in Figure 1 IFN-gamma was by far most potent and non of the other cytokines in concentration up to the maximum tolerated dose were optimal.

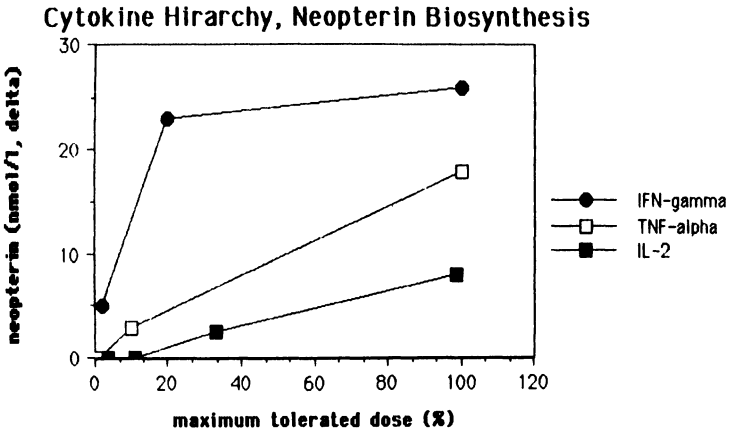

Fig. 2. Induction of Neopterin by Various Concentration of Human Recombinant Cytokines

Neopterin, a 2-amino-4-oxy-6-(D-erythro-1',2',3'- trihydroxypropyl) pterin is derived from the first intermediate of the pterin biosynthetic pathway. The physiological endproduct of this pathway is tetrahydrobiopterin, an essential cofactor of neurotransmitter synthesis. We recently showed that macrophages stimulated with interferon-gamma and to a lesser extent with IFN-alpha or IFN-beta, release large amounts of this compound (Huber 1984). Subsequently we unravelled the causes underlying this primate specific phenomenon: firstly, IFNs activate the key-enzyme of this pathway and secondly macrophages lack the activity of the first enzyme downstream of dihydroneopterin triphosphate (Schoedon 1986, Schoedon 1987). As a consequence, IFN-activated macrophages

synthethize large amounts of dihydroneopterin triphosphate which cannot be further degraded. Dihydroneopterin accumulates and is than released after dephosphorylation. Neopterin is a stable molecule which because of its small size can easily penetrate tissue barriers. It can be readily measured by commercially available radioimmunoassays in body fluids. Serum neopterin responses were studied in clinical studies involving IFN-alpha, IFN-gamma, TNF-alpha,IL-2 or stimulation with antigens or MTP-PE (Troppmair 1988). Examples for induction of in vivo neopterin biosynthesis by IFN-gamma, TNF-alpha and IL-2 are shown in Fig.2 As demonstrated, maximum induction was only achieved with IFN-gamma.

V.Comparison of Therapeutic Efficacy of BAD with MTD in the Treatment of Human Malignant Disease

We have previously defined BAD of IFN-alpha using the neopterin marker in patients with hairy cell leukemia (HCL) and in chronic myelogenous leukemia (CML). Therapeutic efficacy of BAD in the range of 0.5 to $1x10^6$ units per day was subsequently compared with that of a MTD of $5x10^6$ units per day (Gastl 1987, Gastl 1989). Results in HCL patients indicated that both dose levels were comparable in terms of antineoplastic efficacy but that the BAD was almost free of toxicity.

BAD of rIFN-gamma were also determined in patients with progressive metastasizing renal cell cancer (Aulitzky Wo, in press). As little as 100mcg applied once a week was active as demonstrated by measurement of beta-2 microglobulin and neopterin serum levels. This dose also induced complete or partial responses in 6 of 20 patients studied. Median duration of responses is now +14 months and toxicity of the BAD was minimal,

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V Preventive and Therapeutic Manipulations

Origins and Elements of Immune Systems

The Evolution of the Immune System: An Introduction

S. Brenner

How the immune system arose in evolution has been an enigma. It seems to have burst forth in the vertebrates, complete from the start, with a panoply of special cells and special molecules, and with no clear forerunner in earlier phylogenetic forms. This is most remarkable, in view of the elaborate cellular basis of the immune response, with the specific roles of B and T lymphocytes, their interactions with each other and, especially for T cells, interactions with other cells in the body. The immune system is unlike muscle, where we can trace the motile apparatus and its attendant molecules, myosin and actin, all the way back to simple unicellular eukaryotes. The one component that can be followed in phylogeny is phagocytosis which connects to the feeding mechanism of unicellular organisms. Here there is cellular continuity in evolution and the macrophages in vertebrates and similar cells in invertebrates must have been the platform on which the immune response was built. These and related cells collaborate with T cells in antigen presentation and also recognise immunoglobin molecules bound to foreign material. But while they participate in the immune response they are passive agents and do not contain or generate immunological memory, which remains the novel feature of immunity.

Defence against other organisms and their products is very common in Nature. Bacteria defend themselves against foreign DNA molecules, such as viruses, by restriction enzymes, and plants produce a whole range of enzyme inhibitors as a result of tissue damage. Many organisms make antibacterial peptides and there is a vast range of antibiotics, toxins, and bioactive agents produced by organisms either to defend themselves or to enhance their selective value. None of these is a good model for immunity. There have been attempts directly to investigate immune phenomena in invertebrates. These have ranged from the injection of cockroaches with antigens to the study of incompatibility in tunicates. The latter may well turn out to be a good model of histocompatibility in vertebrates. In fact, it is at the molecular level that we now do have evidence for precursors of the components of the immune system. Thus there are molecules in insects which resemble the CAM's of vertebrates and moreover play a similar role in cell surface interactions. The elementary domain of the immunoglobin family of proteins probably goes far back in evolution. Furthermore, the recombination mechanisms underlying the joining of genes is probably closely related to that displayed by genetic elements involved in transposition of various sorts and going all the way back to microorganisms. In bacteria, for example, flagellar variation in Salmonella is due to gene rearrangement. It seems likely that as we come to know more about the molecular components of the immune system, more connections will be revealed.

In considering the evolution of the immune system it is useful to see how it has exploited certain biological themes which are common to other biological systems. Two examples are considered here.

It is said that offence is the best means of defence, but if the defender is attacking an offender which is a component of a biological system, it had better ensure that it does not commit suicide in the process and that it can separate its identity from that of the offender. Recognition of self is central to all systems of defence. Thus bacteria defend themselves against foreign DNA of other bacteria by restriction enzymes which cut the foreign DNA and thereby lead to its destruction. Each enzyme recognises a distinctive short sequence of DNA and one mode of defence might be to exclude these sequences from the host DNA. However, this would clearly impose a serious constraint, and instead, such bacteria contain a modifying enzyme which methylates the recognition sequence on their own DNA, thereby marking it as self and protecting it from the cleaving enzyme. This is not entirely foolproof, because a certain fraction of the incoming DNA molecules escape digestion and win the race for modification and, in this way, make a Trojan entry into the genome of the bacterium. Thus the theme of self, so important in immunology, arises very early and is here implemented in a different way.

Another important theme is diversity. The astounding feature of the immune system is the vast range of molecular recognition that it covers. It has been said that if one takes a compendium of organic chemistry, such as Beilstein, an immune system would be capable of making an antibody with reasonable specificity to each and every compound there. But organic chemistry is not a closed subject and there will be further volumes of Beilstein with chemicals that do not exist today, and our statement will continue to be true of these as well. This is the Beilstein paradox: how can the immune system know about the future? Like other biological systems, the immune system achieves the illusion of total knowledge by near-total ignorance; it simply provides a set of elements which provide a large set of combinations such as to cover everything, plus a refining mechanism, in the form of subsequent mutations, to improve that which is already found to be on the lower rungs of the right ladder. Natural selection only ensures that the ensemble of germ line genes is the best set suitable for that organism in its environment. I said near-total ignorance because, echoing the previous discussion, this system must exclude from the repertoire those combinations which recognise self, or else auto-destruction will follow. But what sort of a recognition landscape is required that continues to cover the world while that of self is excluded? The answer is that the recognition structures of the antigens cannot be at too elementary a level but must involve combinations of elements such as sequences of aminoacids or, better still, 2-dimensional configurations of aminoacid residues as found on the surface of proteins. Thus not too many holes will be punched by self-recognition, and coverage is probably enhanced by a requirement for strong interaction for exclusion from the set. We find a similar principle displayed by populations of organisms. Thus the first aphids of the year hatch in the spring and grow as parthenogenetic females and presumably contain the most successful genotypes of that year. At the end of the season, haploid males are produced which fertilize the females and so reshuffle all the genotypes to produce a wider array of combinations. These eggs survive the winter, to hatch the next season and so produce that year's crop of parthenogenetic females. The set of winter eggs are analogous to the immunoglobulin germ line genes - while the parthenogenetic females are the analogue of lymphocyte clones. In both systems, a good "knowledge" of present

conditions does not prejudice the future and "ignorance", the capacity to deal with unknown environments, is retained.

The immune system is one of the triumphs of evolution. It has been elaborated by embedding one Darwinian process within another. Like the Russian doll, the universe of genome evolution contains within itself the microcosm of somatic speciation of the elements of the immune system.

Protein-Protein Interactions and Immune Recognition

P.M. Colman

INTRODUCTION

Specific interactions between proteins are fundamental to many biological phenomena. It is widely held that selection for a particular function has shaped the interacting molecules to provide the appropriate degree of specificity. Furthermore, in most cases there is a sense of 'uniqueness' about a particular interaction in which one structure is associated with only one function.

It can be argued that the immune system does not fit this paradigm. Its specificity, at least at the level of primary responses, is generated in the absence of antigen, and its capacity to respond to new synthetic molecules is remarkable. There is also a clear degeneracy in the immune response, whereby one antibody may service a number of antigens.

The protein structure data base has been studied by many investigators in search of common themes relating to protein folding, secondary structure interactions, and quaternary interactions between independently folded subunits of protein oligomers. Some of these data are drawn together here in an examination of the extent to which antibodies reiterate or elaborate these themes. Similarities in the amino acid sequences of T cell antigen receptors with the variable domains of antibodies suggest, but do not prove, common principles.

Some of the material presented here has been reviewed and cross-referenced in more detail elsewhere (Colman 1988).

IMMUNOGLOBULIN FOLD

The basic folding unit of the 100 amino acid immunoglobulin domain is an antiparallel β-sandwich of seven strands, labelled A through G. Variable domains differ from constant domains in the insertion of two additional strands after C, C' and C''. Strands ABED (C'') form one face of the sandwich and strands GFC (C') the other. The two faces or sheets are

joined through the intra-domain disulphide, and associate with their strand directions approximately parallel to each other.

This structure is typical of many examples in the data-base of so-called 'aligned' packing of β-sheets and is therefore in itself unremarkable.

In variable domains the three complementarity determining regions (CDRs) are on the loops between strands B and C, C' and C'', and F and G.

Fab QUATERNARY STRUCTURE

Non-covalent interaction between V domains and between C domains generate functional Fab fragments and provide further examples of interactions between β-sheets.

The second common mode of association between β-sheets is the so-called 'orthogonal' packing motif, where the strand directions in the two sheets are at right angles, instead of being nearly parallel. Classic orthogonal packing is observed between the ABED faces of CL and CH1 domains in Fab fragments, and between CH3 domains in Fc fragments. In terms of known structures, then, there is nothing remarkable about the pairing of domains in the Fab constant module.

The variable module, however, does not conform to known structure types (Chothia et al 1985). The interaction between the GFCC' faces of the VL and VH domains is neither aligned not orthogonal, but rather shows strand directions in the two sheets inclined at 55° to each other. The key structural arbiters of this peculiar interaction appear to be on strands G and C' where the regular β-structure is interrupted by bulges on each strand. The sequence signatures of these bulges are also found in the α and β chains of the T-cell antigen receptor.

Not only are the strand directions in the interacting sheets aberrant, but the domination of amino acids from the outside strands (G and C') in the contact surface is a further uncommon feature.

In all known Fab structures the pairings of variable domains adhere to this pattern, but significant differences are observed from one structure to another. Although these differences are small, their effect is to modulate the positions of the three CDRs on light and heavy chains with respect to each other. Such modulations will clearly effect the antigen binding surface of the antibody.

The underlying cause of the small differences in VL-VH pairing is the participation of the CDRs in the interface (Davies and Metzger 1983). Approximately one quarter of that interface derives from hypervariable sequences in the V domains, the remainder being invariant or conservatively exchanged across V region sequences. Apparently the invariant part of the interface is able to accommodate slight rearrangements caused by the variable contributors to the interface.

There is yet no observation of one Fab molecule displaying two different forms of VL-VH packing, but for Bence-Jones proteins, VL-VL interactions are seen to depend on crystallisation conditions.

One manifestation of the problem is seen in attempts to predict the structure of CDR surfaces from sequence data. Some success is enjoyed in getting the local structure of the individual loops correct, but sometimes there are large errors in predicting the relative positions of the heavy and light chain CDRs (Chothia and Lesk 1987; Chothia et al 1986, 1989).

INTERFACES

Protein-protein interfaces are characterised by contacts between stereochemically complementary surfaces over an area of at least 600 A^2 on each surface. Interactions between V domains in the V-module and C-domains in the C-module are typical examples, and the structures of known Fab-antigen complexes also conform (see below).

It is clearly of some interest to know whether all elements of the interface participate equally in determining the binding energy of the complex, or indeed are some of the contacts actually repulsive. The tools for extracting this information from known structures are still quite primitive. In a recent application to antibody-lysozyme structures (Novotny et al 1989), two of three studies yielded results consistent with known affinity constants, and further implied that only a subset of the 15 or so amino acids on each side of the interface contribute significantly to the binding energy.

In some cases it is recognised that interfaces between protein subunits can be modulated by ligand or environment, such as in oxygen binding to haemoglobin, Zn ion binding to insulin or the variation in VL-VL interactions referred to above. These modulations take the form of semi-rigid body movements of the proteins across the interface, triggered by small conformational changes in amino acid side chains near or at the interface.

There are also well-documented cases of conformational changes attending protein-protein complex formation (e.g. Greenblatt et al 1989).

Another type of protein-protein interface is observed in the tennous crystal contacts made by protein molecules with each other in a crystalline environment. Even though these interactions are typically very weak and involve much less than the 600 A^2 of buried surface area seen in biological complexes and referred to above, they can nevertheless cause small conformational changes in the protein as evidenced by studying a particular protein in 2 different crystal forms where the lattice contacts are non-identical (Wlodawer et al 1987).

The picture that emerges is one where the dynamic properties of protein molecules appear to play a role in generating complementary surfaces for complex formation.

ANTIBODY-ANTIGEN COMPLEXES

The available data (Amit et al 1986; Colman et al 1987; Sheriff et al 1987) suggest that antibody-protein interactions are appropriately viewed in the wider context of protein-protein interactions. In all cases the interfaces are greater than 600 A^2, being in the range 680-880 A^2 (Tulip et al 1989) and stereochemical complementarity is observed.

No data are available to directly address the question of antigen-induced conformational changes in antibodies since no high resolution structure of an Fab in its liganded and unliganded forms is available. In one system at least (Bentley et al 1989) work is well advanced towards that end.

Predictions of the conformations of the CDR loops (Chothia et al 1986, 1989) suggest in most cases no large-scale changes in their local structures.

Since different Fab structures show slightly different VL-VH pairing (Lascombe et al 1989) one cannot demonstrate an effect of antigen on this interface without knowledge of liganded and unliganded forms. The case that can be put in support of such changes occurring draws on the demonstrated variability of the VL-VH interface, and the large surface area of interaction between the V-module and antigen. If it is to be argued that some 200 A^2 of interaction in the VL-VH interface, which involves the CDRs, can modulate the remaining 600 A^2 of that interface, then presenting antigen with a surface of some 800 A^2 may equally cause small VL-VH displacements. Such displacements could reposition the CDR loops by up to 3-4A and serve to optimise the fit with antigen.

Any flexibility in the CDR surface will facilitate cross reactions of antibodies with unrelated antigens, and there is clearly a limit to the extent to which the immune system's specificity can be so compromised. The main advantage of an interaction with antigen through a nonrigid CDR surface is one of economy, because it implies a structurally-based amplification of the antibody repertoire. The magnitude of structural changes inducible in antibody by antigen will determine the balance between specificity and economy.

Two different monoclonal antibodies to influenza virus neuraminidase have now been observed to bind a common site on the neuraminidase (Colman et al 1989) although the two antibody CDRs show no sequence homology. We may expect in this and similar cases to observe bi- or multi-functional amino acids participating in interface contacts in a variety of ways; e.g. a glutamine side chain as hydrogen bond donor, or acceptor, or a threonine side chain interacting through hydrophobic, or hydrophilic contacts. Considerations of this type indicate how the 'image' of an antigen can become 'degraded' en route to antiidiotype antibodies.

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Evolutions of the Immune System

L. Du Pasquier and J. Schwager

INTRODUCTION

Determining the relative importance of the elements of the immune sytem is a major task to which comparative studies may help first in pointing to key elements and second in suggesting their phylogenetic origin. These comparative analyses are complicated by many difficulties due to the following facts. 1) The immune system coevolved with the other physiological systems of the organism and its evolution should not be considered as the evolution of a single independent entity. 2) With respect to the history of the system one has a very incomplete and perhaps misleading view of the phylogeny of the Animal Kingdom. 3) The immune system with what we know of its genetic multiplicity is going to be a complex evolving unit with probably an enormous amount of flexibility enabling some of its elements to evolve in a few generations perhaps as fast as in the history of the species, not to mention its somatic evolution during ontogeny.

The purpose of this short overview is to present essential elements without which there would be no immune system (as we understand it) characterized by specific recognition events, and then to present possible models to actually witness the evolution of some of the essential elements in the amphibian of the genus Xenopus: Immunoglobulins, Major Histocompatibility Complex and lymphocytes submitted to the constraints of ontogeny and polyploidization.

ESSENTIAL ELEMENTS OF THE IMMUNE SYSTEM

Figure 1 shows the animal kingdom and figure 2 the vertebrate classes with some essential elements of the immune system (data from reviews by Du Pasquier 1989 and Kaufman 1989). Data on the phylogeny of the T cell receptor are available only in mammals. In the absence of convincing evidence of a vertebrate-like immune system in invertebrates, one is obliged to lower one's standards and be satisfied by similitudes at the level of principles rather than by homologies at the level of the genes and functions. Figure 1 is meant to suggest that some key principles of the immune system can be detected as far back as in primitive diploblastic invertebrates. Yet this figure is not a representation of the evolution of the immune system, but more a chart where the origin of its elements can be traced back. Some of these elements, involved in the immune system of Vertebrates may have had precursors serving a totally different purpose in Invertebrates. Figure 1 should also remind everyone of the relative position of Vertebrates in the ani-

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mal kingdom: They represent only 1 phylum among 21. The multiplicity of phyla (figure 1) reflecting as many fundamentally different organization plans suggest that at the level of the "immune systems" one may encounter fundamental differences as well. This is certainly the case, for instance, in Arthropod humoral responses (ref. in Reinisch and Litman 1989).

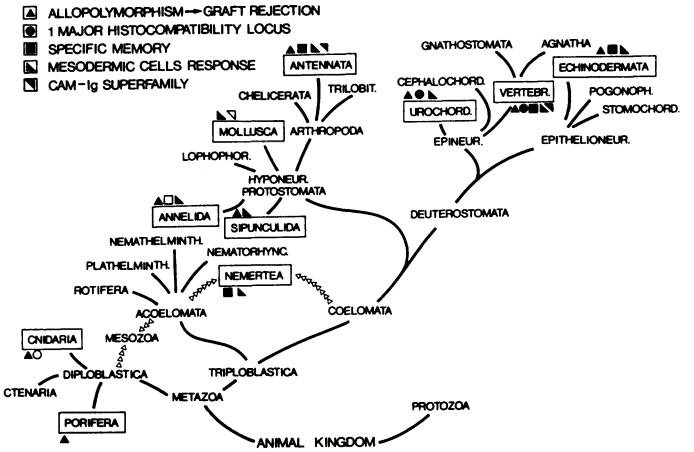


Fig. 1A. Elements of the immune system in the animal kingdom.

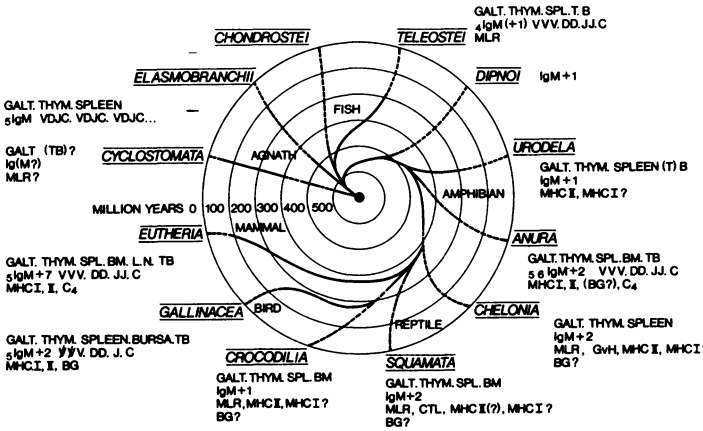


Fig. 1B. The immune system of vertebrates. GALT: gut associated lymphoid tissue; 5IgM + 7: pentameric IgM and 7 non-μ isotypes. MLR: mixed leukocyte reaction; GvH: graft versus host reaction. VVV DD JJ C: multiple V D and J segment, respectively, in the Ig locus associated to a single C. ΨV: pseudogene. Data from reviews by Du Pasquier 1989, Kaufman 1989, Reynaud et al 1989, Kokubu et al 1988b, Litman et al, this volume). C_4: Fourth component of complement.

An interesting feature is the specific memory encountered in an immune response which means that self-non self discrimination is achieved by the specific recognition of the various non-self determinants and not only soley by self recognition. Specific memory

seems to have been demonstrated convincingly in some grafting experiments performed in invertebrates, even in some very primitive ones such as Nemertea, triploblastic animals whose position as coelomate or acoelomate is still debated. There thus seems to be a correlation between the existence of specific memory (i.e. of specific receptors) and true mesoderm (and possibly that of a coelome). In many phyla the differentiation of the mobile lymphocyte-like mesodermic cells involved in phagocytosis, graft rejection, seems to be mediated by another conserved feature which is the interaction of these cells with endoderm derived tissues (e.g. thymocytes interacting with the thymus epithelium or B cell precursors interacting with the epithelia of the Bursa or Peyer's patches). In fact, gut associated lymphoid tissue is present in all vertebrates and its analogue (and perhaps homologue) can be found in invertebrates (again down to the Nemertea level). These constant features led us to speculations with respect to the nature and evolution of the receptors expressed by such tissues.

We suggest that the two different layers of the primitive diploblastic invertebrates, the ectoderm and the endomesoderm have been submitted to different external environments explaining the early evolutionary divergence of two sets of recognition molecules. Indeed the ectoderm is preferentially exposed to interactions with allopolymorphic determinants, whereas the endoderm is not. Both are subject to contact with pathogens and presumably parasites. The endoderm deals with the recognition and digestion of the food. Given the relationship between the endoderm and the mesoderm in triplobastic individuals, where the walls of the archenteron give rise to mesoderm, we now postulate that the mesoderm-derived cells (the lymphocytes or their equivalent) have inherited preferentially the expression of the endodermic receptor. This set is now submitted to a new selective fully internal environment. Receptors selected because of interactions at the ectodermic level would correspond to the precursors of MHC non Ig-like domain. These could act as polymorphic ligand and as receptors. This would confer to primitive MHC molecules a receptor role as already proposed (Kaufmam 1989).

Receptors selected because of interactions at the endomesoderm level would be the Ig-TCR precursors characterized by the evolution of the V domians. The endoderm-mesoderm interactions preserved as a suitable microenvironment for "lymphocyte" differentiation would be defined in vertebrates by the appropriate expression of class I and class II molecules involved in the positive selection of T cells.

The relationships of primordial recognition molecules with Ig, T cell receptors or MHC molecules of today are difficult to establish. However, recently several proteins belonging to the N-CAM-Ig superfamily (Edelman 1987, Williams and Barclay 1988) have been identified in invertebrates (Harrelson and Goodman 1987, Seeger et al. 1988) giving some support to the idea that the molecules achieving recognition in the immune system evolved from a primitive set of cell interaction systems mediated by like-like recognition principles (self recognition). Among these molecules, fasciclin II seems to be more related to N-CAM than to Ig and could already be far away from a potential Ig type. Like N-CAM, it consists of five constant domains, and has no V domain. Both molecules are involved in interaction events in ectodermic cells. The protein amalgam is probably more related to a real chain of an Ig molecule. Encoded by a single exon it consists in three Ig-like domains, the distal one looking like a V domain. The comparison that we made with Xenopus V_H genes areas revealed in addition to highly conserved features in the cystein, an interesting

particularity. In the position of the CDR1 a palindrome CAGCTCNNNGAGCTG is present where in all CDR1 of Xenopus V_H genes, a very similar palindrome AGCTANNTAGCT or AGCTNNAGCT has been localized (Schwager et al. 1989). No other palindrome of this type can be found in the rest of the molecule. Interestingly, with respect to the previous speculations about tissue specificities, amalgam is first and essentially expressed by mesodermic cells.

Table 1

Conservation and Variation in the Vertebrate Immune system

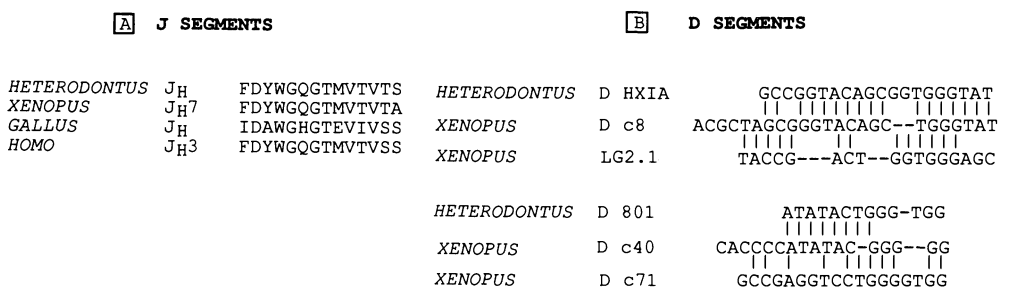
Conserved	Non-Conserved
LYMPHOID SYSTEM	
NK cells	Thymus
GALT, fetal or embryonic liver	Spleen
B lymphocytes, Plasma cells	Lymph nodes
MLR responders ("T")	Bursa of Fabricius
Interleukins	Bone Marrow
Endoderm-Mesoderm interactions	
COMPLEMENT	
Alternative pathway	Classical pathway
MHC	
Class II α + β chains	Ubiquitous MHC classical class I
Polymorphism	BG
MLR	MHC I polymorphism
IMMUNOGLOBULINS	
H + L IgM (polymeric)	Ig gene organization
Transmembrane region	Combinatorial use of
V_H gene structure	VDJ segments
J + D	Somatic mutations
Enhancer like seq.	Non μ isotypes
Recombination signal sequences	High antibody diversity
Rearrangement	Promoter octamer +
+ N diversification	heptamer
Allelic exclusion	
When other isotypes present:	
4 domains constant H chains	
Switch region	
T CELL RECEPTOR	
(αβ, γδ : data only in mammals and birds)	

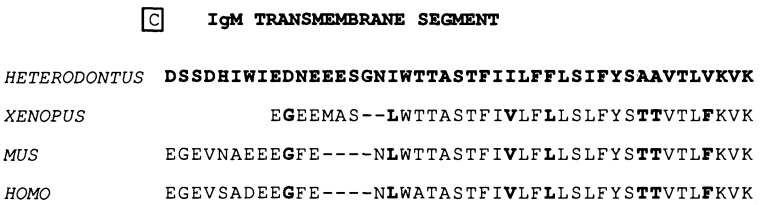
An element is considered conserved when found in all the species so far studied.

The "mammalian" immune system with its procession of cell types and subtypes, gene families etc., contains many elements that have not been detected at all in invertebrates. Therefore, the question may arise whether they appeared "suddenly" or in a "step wise" manner during the phylogeny of vertebrates. Among elements that may have been conserved between invertebrates and vertebrates are the interleukin and complement related molecules found in echinoderms (reviewed in Du Pasquier 1989). Only two subphyla of vertebrates are represented today, the Agnaths and the Gnathostomes. This means that we probably have to our disposal a very poor sampling of the possible evolutionary trends of vertebrates. Already, between the two remaining subphyla, profound differences can be found suggesting that major steps have been acquired during the phylogeny of the vertebrates themselves. It is probably not without good reason that the study of Agnath Ig and lymphocytes has been difficult. The structure of its Ig seems to differ markedly from gnathostomes' Ig and in fact remains undetermined. Similarly, its lymphocyte populations exhibit characteristics either of B cells alone, and of B cell like "T" cells involved in mixed lymphocyte reactions: effector cells seem to bear Ig on their surface. Agnath have a complement system but seem only to use the alternative activation pathway. Within Gnathostomes the immune system appears much more homogenous and many of its mammalian elements are present in most classes: immunoglobulin of the μ type, complement, lymphocyte heterogeneity detected in all classes, MHC class II and I have been detected as far back as the level of amphibians.

Compared to all the possible defence and immune systems of its 20 companion phyla, the immune system of gnathostomes seems to form a fairly homogenous unit of coevolved elements, where the building blocks are very conserved, even if their organization and usage may differ in an interesting manner. Yet, as can be seen from Figure 1b, there is still room for some major differences between chondrichthyes and the rest of the gnathostomes, especially in terms of MHC expression. Table 1 represents the conserved and non-conserved elements of the immune system derived from the comparisons of all known vertebrate immune systems. More details are available for the Ig genes (Fig. 2 presents some remarkable sequence conservations among D, J and transmembrane segments). Such conservation can reflect a multiple selection: at the amino acid level, at the DNA level and at both. An example of conservation at the amino acid level is that of the C Terminus of IgM C_H4 exon, where a strong conservation is seen from shark to man, but where the DNA homology is not very strong. Other examples are J segments. An example of conservation at the DNA level is the surprising finding that the 39 bp long J_H4 sequence of Xenopus shares 31bp with the human Kappa deleting element (fig. 2) in its complementary orientation, whereas human J_H segments only share ca 23bp with their own Kappa deleting element. An example of conservation at both levels is the transmembrane region of IgM. This hydrophobic region is composed of amino acids for which the genetic code is very degenerated. Yet the conservation at the DNA level is high (85% between Xenopus and human). The reasons for these conservations are easy to understand at the protein level. At the DNA level these conserved sequences might be important in alternate splicing procedure, rearrangements, etc. Among other interesting homologies, the recent comparisons of Xenopus IgM heavy chain domains and Heterodontus light chains with mammalian sequences have revealed a few unexpected results. First, the Ig domain seems to be able to evolve independently from each other. Second, an Ig domain of Xenopus can bear as much homology to a mammalian Ig domain as to a TCR domain or a class II Ig like domain (Schwager et al 1988a).

Third, the Heterodontus light chain C region shows homology at the amino acid level with some Ck and λ sequences and interestingly with the λ_5 sequence, a light-chain like gene found in mammalian pre B cells (i.e. primitive) (Kudo et al 1987) but whose role is not yet clear. Finally, the gene of the same Heterodontus light chain shows more homology to a TCR ß chain than to the light chain (Shamblott and Litman 1989). It seems therefore that the different categories of molecule may depend on a few residues to acquire their specific features; the rest of the molecule may vary within the frame allowed by the superfamily, and oscillate from a TCR to a class II Ig-like domain. These homologies do not necessarily mean affiliation between the molecules, but may be allowed convergences.





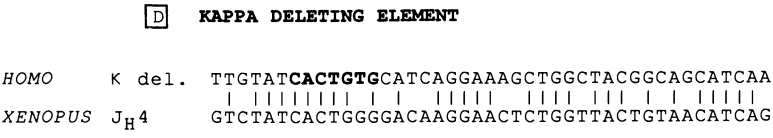


Fig. 2A. Alignments of J segment amino acid sequences from various vertebrates. Fig. 2B. Alignment of D segment nucleotide sequences from various vertebrates, and alignment of CDR2 from Xenopus V_H gene LG2.1 suggesting the "D-ness" of CDR2 (from Du Pasquier 1989, Hsu et al 1989 and unpublished observations). Fig. 2C. Alignment of IgM transmembrane segments, amino acid sequences (unpublished observations, and Kokubu et al. 1988b). Bold letters in Xenopus, Mus and Homo correspond to conserved residues in those three vertebrate classes. Fig. 2D. Alignment of the Xenopus J_H4 coding segment with the complementary sequence of the human Kappa deleting element (human sequence from Siminovitch et al. 1985).

EVOLUTION OF THE IMMUNE SYSTEM IN *XENOPUS*

So far, all the above mentioned comparisons were rather static and to gain more insight into the possible evolutions of the immune system it is now appropriate to switch to a particular model, the amphibian *Xenopus* where the immune system is submitted still in 1989 to evolutionary pressures lacking in mammals. How are the previously defined conserved elements going to behave when some of them become limited, or limiting, or on the contrary, redundant? For instance, lymphoid cells are limiting during the early ontogeny of *Xenopus* and genes are redundant in polyploid *Xenopus*.

Xenopus, a cold-blooded vertebrate, has an immune system very homologous to that of mammals. However, due to the peculiar ontogeny of the anuran with its metamorphosis, the immune system is confronted with situations unknown to mammals, which gears its ontogenetic evolution in an interesting manner. The tadpole immune system differs from the adult by a different antibody repertoire expression, by the lack of classical MHC class I expression on cells, by a different MHC class II tissue distribution and by a less efficient T cell function (Du Pasquier et al. 1989, Flajnik and Du Pasquier 1989).

Let us take only one example, that of antibody diversity. The fact that *Xenopus* antibody diversity appears restricted compared to mammals is probably due to three coevolved characteristics: small numbers of lymphocytes available early in ontogeny when the functional immune system differentiates (the number of a few 10^4 B cells present at the onset of immune competence in *Xenopus* probably does not represent a lower limit because there exists another genera of Pipidae, such as *Hymenochirus*, whose tadpoles are five times smaller than in *Xenopus*), lower diversity of the pool of V genes, and the apparent lack of sensu stricto somatic mutations (i.e. in CDR1 and CDR2 as opposed to N diversification and joining imprecision). All these three characteristics coevolved under the double selective pressure of the necessity of aquiring an immune system early during ontogeny and the impossibility to waste many lymphocytes. The same constraints may be responsible for another peculiarity of the immune system of *Xenopus*, the lack of ubiquitous bona fide class I molecules during larval life. The economy argument applied to B cells, can also apply to T cells in the sense that in the undesirability of wasting T cells, the tadpole immune system has developed a pool of T cells only restricted to class II. The cellular bottle neck seems not to have engendered major changes in the structure of the Ig locus which is remarkably mammalian like with a multipliciy of elements (Fig. 3 for an updated description of the heavy chain locus of *Xenopus*). Yet, multiplicity does not necessarily mean diversity, not only because of a possible lack of expression due to cellular limitations, but also because of the nature of the multiple elements themselves. Many genes share CDR sequences and therefore do not contribute to as much diversity as might be expected from their number (Schwager et al. 1989). Multiplicity of genetic elements is indeed a characteristic of the *Xenopus* Ig locus: it contains perhaps 100 V_H genes and more J_Hs segments than in mammals, its promoters are characterized by the presence of multiple octamers. This may reflect what we could call an "insurance". In a system that cannot afford wastage, and therefore, cannot rely on chance somatic mutations to improve its repertoire, it may be useful to have many selected elements that insure an efficiency of the system. Many selected J and D can be a good alternative to the lack or paucity of somatic mutations. Multiple

octamers may increase the rate of transcription, even at low temperatures (a feature that could matter in a cold blooded vertebrate). The variation in octamer numbers is particularly interesting since the shark Heterodontus does not show any octamer-like sequence in the putative promoter area (Kokubu et al. 1988b). They may also confer differential expression of V_H elements phylogenetically.

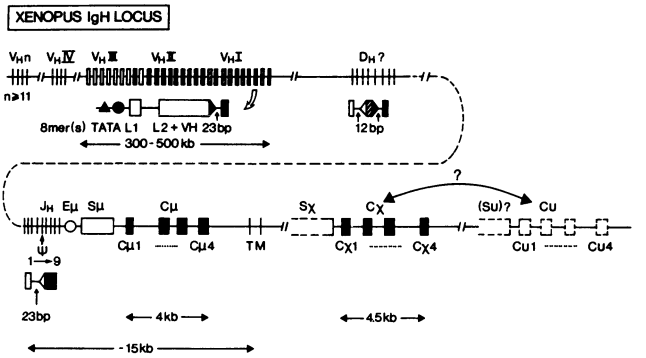


Fig. 3. The Xenopus immunoglobulin heavy chain locus. Eμ: enhancer like sequences, Sμ: switch region. Cμ, χ, υ: genes of IgM, IgX and IgY. Data from Schwager et al 1988b, Hsu et al 1989, Haire and Litman, personal communication.

POLYPLOIDY AND THE IMMUNE SYSTEM IN XENOPUS

Polyploidy, in providing duplicate genetic information might have been an important factor in vertebrate evolution. Xenopus, which comprises bisexual species of several different ploidy levels, represents a model for an experimental approach to the many problems associated with a polyploid condition (Kobel and Du Pasquier 1986). Polyploidy in Xenopus is accompanied by an increase in cell size and a decrease in the size and weight of the animals. The fate of duplicated loci can be interpreted as the result of interplays between various elements and various selection pressures. One constant selection pressure on an immune system is to have cells producing only one type of Ig per cell. This pressure led to allelic exclusion in polyploid animals even when they have 3 times more Ig genes/cell. Therefore, in a situation where one has at the same time unicommittment of cells, reduction in the number of cells and increase in the number of genes, one can expect 1) a reduction of clone size, 2) the silencing or deletion of certain genes or their transformation in pseudogenes, 3) a different mode of gene usage. In X. ruwenzoriensis (108 chromosomes), antibody diversity to DNP is larger than in X. laevis (36 chromosomes) and thus the species seems to have taken advantage of the duplication of the V gene loci, even though their lymphocyte number is smaller than that of X. laevis. This suggests that the low diversity of X. laevis (when compared to mammals) is not in fact limited in an absolute manner by the cell numbers. It could express at least as many genes as X. ruwenzoriensis since it has more cells. What the differences between the two species may mean is simply that X. laevis presents a better coadaptation with a larger clone size. It probably exploits its cellular and V gene potential without waste, whereas X. ruwenzoriensis, a more recently appeared species, may still be in the process of sorting out genes.

The comparison of antibody diversity and MHC expression in this species shows again, like the ontogenetic studies, that if there is something to sacrifice, it might be something in the T cell fraction. At least this is one way of interpreting, during the various polyploidization steps detected in Xenopus, why the MHC shows a strong evolutionary tendency towards disomic inheritance. Not expressing all the constituting haplotype can result in a proportionally smaller pool of restricted T cells, leaving enough space for B cells in the total lymphoid compartment.

CONCLUSION

Trying to define conserved key elements by comparative studies, and exploiting natural experimental situations where immune system elements are placed under variable pressures such as the ontogeny of Xenopus and the effect of polyploidization, have revealed evolutionary tendencies that may be of general interest: 1) The recognition of non-self as a means of discriminating self from non-self because of its presence in several phyla (although we do not know whether these are cases of convergence or filiation); 2) The conservation of endoderm-mesoderm interaction in the genesis of mature lymphocytes with specific recognition capacities; 3) The fact that multiplicity does not mean diversity at any price; 4) An evolutionary privileged selection of B cell versus T cells not to mention the quasi ubiquity of allo-recognition, the presence of molecules of the N-CAM-Ig family in invertebrates and vertebrates, subject treated in more detail elsewhere in this Volume.

Approaching the immune system through the biological significance of each of its elements may help focus on the necessity of these elements and when in a medical context, a problem of disability or replacement arises, it may be useful to remember some of these phylogenetic considerations.

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Allorecognition in Colonial Tunicates: Parallels with and Tangents from Vertebrate Immunity

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INTRODUCTION

With every amazing new advance in immunology it is curious that soon thereafter it is followed by the profound announcement - usually by a distinguished immunologist - that we have just witnessed the "end" of our gathering of essential knowledge in the field. This is not a peculiarity of immunologists - the end of molecular biology was announced over a decade ago. In the eyes of the announcer, it may be that the principles just discovered outline a path sufficient for his/her sighting of the entire future of the field. Such talented visions, however, are not easily transferred, and so the rest of us continue to look into phenomena poorly understood, or novel systems that promise new phenomena, in order to try to see more clearly.

It is our view that many such possible areas of investigation hold the potential for real biological advances in immunology - the kind that may change thinking in several fields. For example, human diseases have been described in incredible clinical detail, but for very few is their (immuno?) pathogenesis understood. The advent of a model for the direct experimental analysis of normal, and pathological human development (McCune et al. 1989) ought to allow investigations of the role of the human immune system in disease, and thereby likely will uncover undiscovered elements and functions of the immune system. For another example adhesion molecules other than antigen receptors belong to gene families that not only govern immune cell-cell interactions and homing, but also include members that have crucial roles in phases of invertebrate development (Gallatin et al. 1987; Holzmann and Weissman 1989; Siegelman and Weissman 1989).

Thus we come to the themes of the evolution of those genes currently used in the vertebrate immune system, and in parallel - whether mechanistic or semantic - the structures and functions in invertebrates that involve allorecognition and defense against infection, the hallmarks of vertebrate immune systems. Here we present some aspects of the genetics and biology of natural allorecognition phenomena in a highly complex metazoan invertebrate - the tunicate - and draw at least <u>semantic</u> parallels with vertebrate immune functions.

From first principles it should be obvious that allorecognition, particularly that of MHC by T cells, did not arise as a novelty of vertebrate immune recognition. Transplantation of tissues and organs -the main route for discovery of the MHC - is simply not a physiological consequence of vertebrate life. A phenomenon called <u>colony specificity</u> in colonial species of marine invertebrates is a daily occurrence in the wild with apparently powerful selective value -the phenomenon exists in several genera separated by several

hundred million years. This transplantation in the wild occurs in an accessible animal model wherein the functions studied are physiological, and in which it is possible to use the tools of genetics, biochemistry, and cell biology.

A SHORT LIFE HISTORY OF COLONIAL TUNICATES

The colonial tunicate Botryllus schlosseri are found as subtidal organisms throughout the world, and in all places undergo a genetically defined naturally transplantation in the wild, wherein individuals or colonies meet, then fuse or reject, the fusion resulting in establishment of a parabiotic multi-individual colony (for reviews, see Burnet 1971; Scofield et al. 1982). The fusion event occurs initially within genetically identical members of the same colony. The colony is established when a single individual gives rise to 1-3 asexually-derived genetically identical buds, which themselves give rise to buds every 7-10 days, each bud sending out extracorporeal blood vessels which fuse with the parent colony blood vessels within the overlying gelatinous tunic.

Botryllus schlosseri has two distinct developmental pathways, sexual and asexual, which lead to indistinguishable adult forms. Following gamete fusion the fertilized egg develops through the usual chordate intermediates of morula, blastula, and gastrula to give rise to a chordate larval tadpole complete with notochord, neural tube, and segmented musculature, a form resembling primitive vertebrates. The tadpole "hatches" from the parent (zooid), it swims away until it attaches to a free subtidal surface, and then undergoes a remarkable metamorphosis in which it loses its chordate characteristics (tail, notochord, neural tube, segmented musculature) and becomes a sessile invertebrate oozoid. The oozoid has already begun the process of asexual development, giving rise by budding to 1-3 new blastozooids, each connected to the parent zooid by a body stalk and extracorporeal blood vessels. The new blastozooids grow, and 7-9 days later vascular circulation to the parent zooid suddenly ceases and the parent zooid degenerates, a phenomenon called takeover.

Colony Specificity - A Genetically Controlled Transplantation Reaction

When two individual zooids, or two colonies of the same botryllid species meet on a surface the tunics touch, change their state at the point of contact, and the tips (broadened ampullae) and sides of the two blood vessels touch. The vessels and therefore the individuals then either fuse, or reject. Fusion occurs as described above for the asexual expansions of a colony, whereas rejection is unique. An initial vascular anastomosis is formed, but is rapidly plugged by inflammatory blood cells both within the blood vessel and in the perivascular tunic. As the intensity of the inflammatory reaction develops, cytotoxic cells and other blood cells, some (the morula cells) capable of pinching off blood vessels, stream out into the tunic between blood vessels (Scofield et al. 1982; Scofield and Nagashima 1983). Combined with the continual stress on the nascent vascular connection by to-and-fro extensions and contractions of these vessels, the connections starts to separate, resulting in

hemorrhage and further loss of vascular connection. Following this inflammatory process, a repair process takes place, building a barrier between the two individuals or colonies. This reaction is, in fact, transplantation in the wild. Unlike iatrogenic transplantation in vertebrate species, this represents a genetically controlled, biologically important common occurrence for colonial tunicates such as Botryllus schlosseri. The genetic basis for this colony specificity resides in a single highly polymorphic gene locus (haplotype) called the Fu/HC (fusibility/histocompatibility) locus, and the rules of "histocompatibility" in this species differ significantly from MHC-dependent graft rejection in vertebrates. As proposed by Watanabe and Oka, and later confirmed by us, two individuals sharing no alleles reject (e.g., AB and CD), whereas genetically identical individuals, or individuals sharing both alleles (e.g.., AB and AB) at the Fu/HC locus, or even individuals sharing one but not the other (e.g. AB and BC) meet and fuse within 24 hours after contact. Thus it appears that Botryllus species are capable of self/non-self recognition of allelic determinants at this Fu/HC locus; the rapid reactions involved in colony specificity appear to follow the rules that ejection ensues from a failure in self recognition.

The Colony Resorption Phenomenon

What happens to colonies which share one, but not both alleles, which fuse blood vessels and thereafter share a common vasculature? Does fusion resulting in chimerism provide a long-term selective advantage for the individuals that outweighs the costs of fusion (Buss 1981, 1982; Grosberg and Quinn 1986; Buss and Green 1985)? Not in all cases. We find that these chimeras do not, in fact, survive or grow as well as their nonfused, genetically identical colony-mates. AB-AC type fusions usually end in death of one of the two, followed by resorption of its body elements but at least temporary maintenance of the extended vasculature through the area demarcated by the fused colonies (Rinkevich and Weissman 1987; Taneda et al. 1985; Watanabe 1962; Saito and Watanabe 1982; Karakashian and Milkman 1967). This colony resorption phenomenon is limited to individuals which are not genetically identical, since two independent genetically-identical isolates from a single parent colony will meet, fuse, and give rise by asexual budding to growing colonies (Rinkevich and Weissman 1987). Colony resorption appears to be genetically controlled. When multiple subclones of (e.g. from colony 1 are each allowed to fuse with subclones from colony 2, the direction of resorption (e.g., colony 2 by colony 1) is invariant. That is, all subclones of colony 2 are resorbed following fusion with colony 1.

We have found that at least two and probably multiple gene loci (haplotypes?) govern this colony resorption phenomenon: One determinant is in or linked to the Fu/HC locus, wherein differences at just one of two alleles (say AA-AB), or two-way differences (e.g., AB-AC), leads to poor growth, (unilateral or bilateral), and colony resorption (Saito and Weissman 1989). With the advent of successful methods for laboratory mariculture of Botryllus schlosseri (Boyd et al. 1986; Grosberg 1982; Berrill 1937; Milkman 1967; Sabbadin 1960) we have been able to carry out AB x AB self crosses, and identify AA, AB, and BB progeny which vary at other gene loci. In pairs of Monterey Botryllus selected genotypically for one-way "recognition," e.g. AA-AB, AA is resorbed preferentially (Rinkevich et al. in preparation).

The resorption of Fu/HC homozygotes by Fu/HC heterozygotes in a laboratory setting might contribute to the impressive Fu/HC polymorphism in the wild, along with an Fu/HC-linked fertilization preference of eggs with sperm bearing non-shared Fu/HC alleles (Scofield et al. 1982; Oka and Watanabe 1957). The resorption of Fu/HC homozygotes by heterozygotes is also reminiscent of the "rejection" of bone marrow transplants of Hh-1 homozygotes by irradiated Hh-1 heterozygotes in mice, a self/nonself recognition apparently enforced by cells bearing NK-cell associated antigens (Cudcowicz 1968; Sentman et al. 1989).

Colony Resorption also occurs between nonidentical pairs that share both Fu/HC alleles, indicating that loci in addition to the Fu/HC haplotype may be involved. For example, in the offspring of the ABxAB cross we obtained several AA homozygotes that were allowed to develop independent multi-individual colonies by asexual budding, and then subclones from these colonies were placed pairwise for analysis of colony resorption. In this case all subclones from colony P21R resorbed all subclones from colonies P32R, P111R, and P94R. All subclones from colony P32R resorbed all subclones from colonies P111R and P94R; and all subclones from P111R resorbed all subclones of P94R (Rinkevich and Weissman, in preparation 1989). Thus a genetically-defined, non Fu/HC resorption hierarchy of P24R > P32R > P111R > P94R exists, whether the subclones are tested early or late in the growing season. The number of loci involved in colony resorption is not yet determined, but it is impressive that multiple loci exist to prevent the establishment of permanent parabiotic unions in Botryllus schlosseri.

Consequences of Botryllus Allorecognition

What are the likely selective forces which allow the emergence of colony specificity and colony resorption? Colonial tunicates in their adult form are sessile surface feeders, and as such are involved in great inter- and intra- specific competition for subtidal surfaces such as rocks, seaweed, or marine wharfs (Grosberg 1982, 1981). Successful domination of a feeding surface by multi-individual colonies effectively prevents colonization of that surface by other competitives, and should even be effective in allowing colony survival following attacks by predators that do not necessarily kill all individuals in a colony. Even the rejection reaction does not appear to compromise the ability of Botryllus species to survive, but it does limit the eventual feeding surface each rejecting colony may dominate. Because the rejection reaction results in a barrier to subsequent ampullar contacts, the two allogeneic colonies appear to reach an equilibrium state with each other, although they may in fact retreat from the various contact sites in a nonrandom fashion. When new tadpoles land on tunics of established colonies, they soon lift-off and die (Rinkevich et al. 1988).

How do the phenomena of colony specificity and colony resorption, both defined (wholly or in part) by gene products of the Fu/HC haplotype, interact in the economy of Botryllus existence? Do they represent the interstices of two competing forces of natural selection, the result being a metastable, but necessary "truce"? or

might they represent two stages of an interacting, programmed ontogeny which is highly beneficial for the species? We believe a potential answer favoring the latter comes from a consideration of population genetics: Botryllus schlosseri are enormously polymorphic at this locus, with estimates of at least 30 alleles coming from our analysis of Botryllus schloserri present in the Monterey Bay Marina alone (Scofield et al. 1982). Eggs are fertilized by sperm broadcast into the sea from others of the same species, and therefore the progeny from a single colony fertilized by many different sperm will virtually never be identical at the Fu/HC locus. However, 50 percent of the offspring should share one but not the other allele (50 percent are AX, AY,...AN, and 50 percent are BX, BY,...BN, etc.) When the sexually reproduced Botryllus tadpole hatches and swims away from the parent colony there is a limited distance it swims before it reaches a free surface, where it stops, adheres, undergoes a rapid degeneration of the tail and notochord, undergoes 180° rotation of its body, and begins life as oozoid. Because of the limited distribution of these organisms, settlement within a particular area is biased toward cosettlement of siblings simultaneously hatched. Even if cosettlement were random, there is a significant probability that siblings sharing an allele will land on adjacent surfaces, especially early in the growing season when a few surviving founder colonies dominate a particular microarea. Thus the progeny of a single egg-bearing parent have a great opportunity for early fusion on a particular surface, and perhaps dominating that surface. Reinforcing this sibling cosettlement bias Grosberg and Quinn (1986), have recently demonstrated a non-random cosettlement of kin on the surface near a parental colony, a phenomenon which they propose is due to genes which are at or are very tightly linked to the Fu/HC locus. Thus siblings sharing one allele at this locus "sense" each other, preferentially cosettle, and therefore should give rise to higher than random frequencies of sib-fusions. The net result of limited dispersal of Botryllus tadpoles and selective Fu/HC linked sibling cosettlement would be to increase very greatly the probability that the gene pool from at least the egg parent shall be dominant in a microenvironment near that parent. If the resultant fusion chimeras could live together in a mutually beneficial way over a long period, one might worry that the reproductive unit of these multi-individual multi-origin colonies would be the gene pools represented by all of the individuals contributing to the colony. In fact, the colony resorption phenomenon should remove most of the potential heterogeneity in the genomic pool by sexual maturity by elimination of one of the two colonies; and we and other are now testing whether potential gametic progenitors present in the bloodstream are also eliminated by the surviving colony during colony resorption. In any case, the sibling cosettlement, fusion, and resorption with retention of the vascular network in the tunic of the resorbed individual provides an important potential, but temporary advantage for the genetic material of the egg-bearing parent in that microenvironment. It is also interesting to note that sibling cosettlement linked to Fu/HC (Grosberg and Quinn 1986) implies some recognition of self Fu/HC elements at a distance from the original enclosed colony, a phenomenon reminiscent of olfactory recognition in vertebrates (Yamazaki et al. 1986).

Thus in contemporaneous protochordate species one finds well-established systems which appear to be analogous to the vertebrate MHC in terms of important functional characteristics. Botryllus species have a somatic histocompatibility system which governs at least the rapid fusion and rejection of self or non-self individuals in colonies in the colony specificity phenomenon; and imposed on that

is another histocompatibility-like system which appears to be involved in the colony resorption phenomenon, perhaps returning the fused chimeras to a genetically defined reproductive unit of only one of the partners. It is yet to be determined whether this histocompatibility system in botryllid species also has the capacity of self plus X identification of cells infected by microorganisms; that is, does this system have anything to do with immune defenses? At a second level genes at or near the Fu/HC locus give rise to products that apparently can be sensed at a distance for the cosettlement of Fu/HC allele sharing larvae, as shown by Grosberg and Quinn. While this sensing device is not yet linked in any known fashion to the attraction of mates for one another, the precedent for dispersement of pheromone-like substances for self/non-self, individual specific identification and behavior might have developed from this primitive advantage for co-settlement and fusion. Finally, at a different level, and apparently in contrast to vertebrates, Botryllus species do determine to a very great degree fertilization preference on the basis of allele expression at the Fu/HC locus/haplotype, providing a powerful selective force for the development and maintenance of Fu/HC polymorphism in these species (Scofield et al. 1982).

Are Colony Specificity, Colony Resorption, and Fertilization Preference All Controlled by an MHC, or an MHC-like System?

If we assume that contemporaneous Botryllus species are very similar to paleozoic Botryllus species for these important biological characteristics relating to the Fu/HC region, then individuals participating in the speciation events leading to the origin of vertebrates might very well have had in place a highly polymorphic MHC-like system, and elements capable of sophisticated allele specific recognition of these polymorphic gene products. Whether these systems will prove only to have been analogous in function, and not homologous in structure, should be testable in the future using techniques of monoclonal antibody detection of allelic products, protein chemical purification and sequence analysis of these products, and molecular genetic methods for obtaining these genes for comparison with contemporaneous vertebrate members of the MHC-Ig gene superfamily. At that point one can attempt to define similarities in the general structure of the gene products and perhaps their actual gene sequences. Should such homologies exist, one could then begin delving into direct analysis of the relative rate of stability and drift of polymorphic alleles within these populations of Botryllus schlosseri, and in comparison with closely related botryllid species, whether solitary or colonial. And also, one can hope to use the same reductionist approaches to identify elements which recognize Fu/HC polymorphic gene products, to assess whether they are homologous to various elements utilized in vertebrate self and allorecognition, i.e., the multimolecular complexes involved in T cell antigen receptors. Finally, by close observations of biological phenomena genetically controlled by genes at or linked to the Fu/HC locus, and the gene loci encoding recognition elements of the Fu/Hc system, one might pick up other clues about the multiple potential functions of this very interesting family of genes - clues which might change our thinking about some of the primary functions of histocompatibility systems in general.

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Elements of the Human Immune System: Studies of Mature Lymphoid Cells Following Xenotransplantation to Scid Mice

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INTRODUCTION

Three recent studies (Mosier *et al.* 1988; McCune *et al.* 1988; Kamel-Reid and Dick 1988) have reported transfer of elements of the human immune system to immunodeficient strains of laboratory mice. These experiments demonstrate the feasibility of using xenotransplanted animals to dissect interactions between cellular elements of the immune system, and these chimeric animals provide important new models for the study of human infectious diseases, autoimmune disorders, and human malignancies. The results obtained in these systems also challenge some of our current concepts about barriers to xenotransplantation.

While these models have introduced a new era of human biological studies, each has some deficits which indicate that they are but the first steps in what is likely to be a rapidly evolving field. We will review our studies on the transfer of adult human peripheral blood leucocytes to mice with severe combined immunodeficiency (Bosma *et al.* 1983) to create what are now termed hu-PBL-SCID mice. We will also compare our results to those obtained following transfer of human fetal lymphoid tissue to SCID mice (McCune *et al.* 1988) or human bone marrow cells to *nude.xid.beige* mice (Kamel-Reid and Dick 1988). Finally, we will summarize preliminary experiments demonstrating the utility of hu-PBL-SCID mice in the study of AIDS and Epstein-Barr virus (EBV)-associated B cell lymphomagenesis.

SURVIVAL AND FUNCTION OF MATURE HUMAN PBL IN SCID MICE

While it might be expected that the injection of peripheral blood mononuclear cells into immunodeficient SCID mice would lead to lethal graft-versus-host disease (GVHD) mediated by xenoreactive human T cells, an experiment designed to test this widely held premise yielded the opposite result (Mosier *et al.* 1988). SCID mice survive following the intravenous or intraperitoneal injection of as many as 100 x 10^6 PBL with no clinical or histological evidence of GVHD. Intraperitoneal injection,

but not intravenous injection, leads to engraftment of human cells. Human lymphoid and myeloid cells are found in SCID recipients not only in the peritoneal cavity, but also in the spleen, lymph nodes, Peyer's patches, and peripheral blood (Fig. 1).

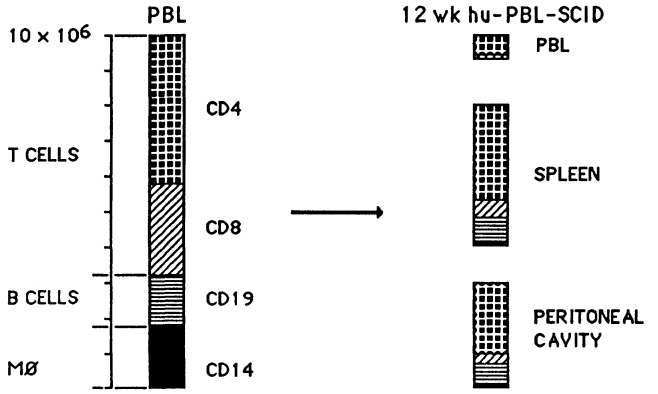

Figure 1. Survival of human lymphocytes and monocyte/macrophages (MØ) in SCID recipients at 12 weeks following reconstitution with 10 x 10^6 PBL intraperitoneally. The filled patterns represent the cell phenotypes determined by monoclonal antibody staining and flow cytometry analysis, and the heights of the bars represent the recovery of human cells estimated by *in situ* hybridization for human DNA sequences.

By 12 weeks post-reconstitution, hu-PBL-SCID mice appear to be stable for human cell content and immunoglobulin production (see below), with the exception that numbers of human MØ continue to decline. Both T and B lymphocytes engraft in hu-PBL-SCID mice, and survival of CD4 T cells appears to be favored over CD8 T cells. At earlier time points after reconstitution, up to 20% of human T cells express IL-2 receptors, and analysis of cell surface phenotype is more diffficult because sites of human cell engraftment also contain activated mouse cells which appear to consist primarily of NK-like cells.

Human lymphoid and myeloid cells not only survive in hu-PBL-SCID mice, they reconstitute a range of human immune function. Most strikingly, human immunoglobulin synthesis is established shortly following cell transfer (Fig. 2).

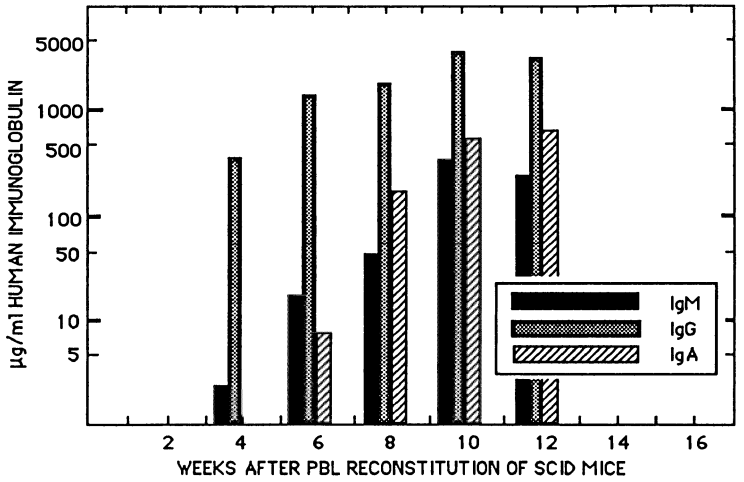

Figure 2: Spontaneous secretion of human immunoglobulins IgM, IgG, and IgA following reconstitution of SCID mice with 50 x 10^6 PBL. The levels of human immunoglobulin remain stable at 12 or more weeks post-transplantation.

The pattern of human isotype production in hu-PBL-SCID mice is noteworthy. Human IgG production is established first, followed by IgM secretion. This suggests early engraftment of long-lived memory B cells which have already switched to IgG, followed by expansion of perhaps smaller numbers of primary B lymphocytes. As will be noted below, it is easier to obtain secondary antibody responses than primary antibody responses from hu-PBL-SCID mice, which may reflect this differential engraftment of memory versus primary B cells.

A number of immune responses have been investigated in hu-PBL-SCID mice. The results of these studies are summarized in Table 1. Immunization of reconstituted mice with trinitrophenyl (TNP)-tetanus toxoid leads to a strong anti-tetanus antibody response and a weak anti-TNP response. Virtually all of the anti-tetanus antibody is IgG, indicating a secondary response. Primary immunization of hu-PBL-SCID mice with TNP coupled to hemocyanin also leads to a weak response consisting of 100-500 ng/ml of anti-TNP antibody. These results parallel those of spontaneous IgM and IgG secretion, and may reflect either preferential engraftment of memory versus primary B cells or different requirements for antigen-presenting cells in the secondary versus primary antibody response. Inducible antibody responses do indicate function of helper T cells, B cell precursors to antibody-forming cells, and probably antigen-presenting cell function of human macrophages. It is possible that

secondary antibody responses are stronger because antigen-specific B cells can subsume the role of antigen presentation.

Table 1. Summary of the human immune functions of hu-PBL-SCID mice.

	B CELLS	T CELLS	MØ
I° Ab	+	+	+/-
II° Ab	+++	++	?
IgM	++	+	+/-
IgG	+++	++	?
PHA		+ - +/-	+/-
MLR		+ - +/-	+/-
CTL		-	?
DTH		ND	

Evidence for persistence of human T cell function other than help for antibody responses is less strong. We observe *in vitro* proliferative responses of human cells recovered from the peritoneal cavity to stimulation by PHA or allogeneic, irradiated PBL, but these responses are generally weak and require the addition of syngeneic, irradiated filler cells. We have been unable to recover alloreactive cytotoxic T lymphocytes from these cultures. These results may reflect a transient immunosuppression associated with growth of human T cells, a suppressive response of the SCID recipient, or a general shortage of human macrophage function.

COMPARISON OF HUMAN TO MOUSE LYMPHOID OR MYELOID TRANSPLANT MODELS

Properties of each of the three recently developed models for studying human hematopoietic cell grafts in immunodeficient mouse recipients are summarized in Table 2. It should be emphasized at the outset that two of these models were designed to study early hematopoietic development, and both have shown very encouraging results in that area. The observations of Kamel-Reid and Dick (1988) on the preparation of the recipient for human myeloid precursor engraftment are important; use of the NK-cell deficient *nude.xid.beige* strain and 300R irradiation were required to see evidence of myeloid precursors. Administration of human IL-3 and GM-CSF was without apparent effect. These observations suggest that murine NK cells remain a partial barrier to xenotransplantation, and probably explain the failure to engraft myeloid precursors in the hu-SCID model (McCune *et al.* 1988). Low dose irradiation has recently been shown to improve engraftment of mature human PBL (Mosier *et al.*, unpublished observations), and it is likely that NK-deficient mice will be preferable to SCID mice as recipients in future experiments.

Table 2. Comparison of the three human → mouse xenotransplantation models.

	hu-PBL-SCID	SCID-hu	BM-NIH.III
donor tissue:			
	adult PBL	fetal thymus fetal liver fetal LN	adult bone marrow
recipients:			
	SCID mice irr. SCID mice nu.xid.bg mice	SCID mice	nu.xid.beige mice
	[SCID = T-, B-, NK+	nu.xid.bg = T-, B-, NK-]	
human cells engrafted:			
	T lymphocytes B lymphocytes MØ	thymocytes T precursors B lymphocytes (LN graft only)	myeloid precursors
human functions established:			
	IgM, IgG synthesis Ab responses weak T proliferative responses	IgG synthesis (LN) ? ?	?
duration of engraftment:			
	>18 months	3 months→12 months	1 month
HIV infection:			
	yes	yes	?
primary utility:			
	mature lymphoid function autoimmune diseases	lymphoid and hematopoietic precursors	hematopoietic precursors

In the SCID-hu model of McCune *et al.* (1988), maturation of human T cell precursors in the thymus graft is well-established. Some of the T cells recirculate to the spleen or peripheral blood, but reconstitution of mouse lymph nodes is not seen. No inducible function of these T cells is reported, but SCID-hu mice do show increased resistance to *Pneumocystis carinii* infection. Human immunoglobulin synthesis occurs spontaneously only when fetal lymph nodes are implanted, which would appear to reflect

activity of already mature T and B lymphocytes. The SCID-hu model thus has provided a tool for studying the generation of human T cells from precursor cells present in fetal liver, and for the survival of lymphoid elements present in mid-gestational lymphoid tissue. The rate at which T lymphocytes are produced once the thymic graft is repopulated has not been reported.

The survival of hu-PBL-SCID mice in the absence of obvious GVHD suggests that one premise dictating the use of fetal tissue for reconstitution, the attempt to avoid xenogeneic GVHD mediated by mature T cells, was incorrect in practice. The ability to quickly and easily inject human PBL or purified lymphoid subpopulations into SCID mice and observe human immune function for prolonged periods of time makes the hu-PBL-SCID model ideal for a range of studies that are otherwise impossible. Nonetheless, survival and function of human myeloid lineage cells is relatively poor in this model, and it is likely that second-generation models will present features found in all three current models.

STUDIES OF DISEASE IN hu-PBL-SCID MICE

A major incentive in the development of the hu-PBL-SCID mouse was to provide a small animal model for AIDS research. We have now completed studies (Mosier *et al.* 1989) showing that hu-PBL-SCID mice can be infected either with cell-free human immunodeficiency virus (HIV) or with HIV-infected syngeneic T lymphoblasts. Virus can be cultured from the spleen, peripheral blood, and lymph nodes of infected mice, and *in situ* hybridization confirms infection of human cells in the SCID spleen. These HIV-infected hu-PBL-SCID mice will be an important addition to research on the cause and prevention of AIDS, and therapeutic trials are already underway in this model.

An unexpected benefit of the development of this model has been the discovery of spontaneous outgrowth of human B cell lymphomas with a sporadic Burkitt's lymphoma-like appearance in hu-PBL-SCID mice receiving human PBL from EBV-seropositive donors. Cells from individuals not exposed to EBV do not give rise to tumors. These lymphomas appear within 5-12 weeks of transplantation, and their incidence is directly proportional to the number of PBL transferred. Information about these B cell lymphomas is summarized in Table 3.

Table 3. Properties of EBV-associated B cell lymphomas in hu-PBL-SCID mice.

surface phenotype:
CD19$^+$, CD20$^+$, CD5$^-$, surface Ig$^+$
EBV nuclear antigen (EBNA)-1$^+$, EBNA-2$^-$, latent membrane protein (LMP)$^-$

immunoglobulin secretion
monoclonal IgM or IgG, biclonal in some mice

DNA studies
c-myc rearrangements in 80% of tumors
EBV internal repeat sequences in 100% of tumors
restricted and repeated use of same immunoglobulin V_H genes

Frequency of pre-malignant B lymphocytes
1 in 2-4 x 10^6 B cells

Multiple hu-PBL-SCID mice given cells from one donor have developed independent tumors with the same *c-myc* rearrangement and expression of the same V_H gene. These results strongly suggest that a pre-existing malignant precursor, having both latent EBV infection and a *c-myc* $\rightarrow$ Ig rearrangement, exists in peripheral blood cells prior to transfer to SCID recipients. The appearance of lymphomas in SCID recipients indicates loss of a normal regulatory mechanism that controls the growth of these aberrant B lymphocytes. These observations provide the basis for an important new model for human B cell lymphomagenesis, ironically for a tumor type that is becoming increasingly frequent in AIDS patients (Pelicci *et al.* 1986). The hu-PBL-SCID model thus may contribute not only to studies of HIV pathogenesis in AIDS, but also to one of the major complications of AIDS.

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The Regulation of Lymphopoiesis by Interleukin-7

Ch.S. Henney

This laboratory has been interested for some time in the early events of lymphocyte differentiation and has specifically sought to define soluble factors associated with lymphopoiesis.

One avenue of approach, which has proven successful, was suggested by the work of Whitlock and Witte (1), who demonstrated that the addition of a bed of stromal cells to bone marrow cultures led to the appearance of a rapidly proliferating population of lymphoid cells, with the phenotypic characteristics of B cell precursors. It soon became apparent that the requirement of stroma in these cultures could be replaced by supernatants harvested from stromal cells (2). We initially (2) termed the factor present in such supernatant, and which was responsible for pre B-cell proliferation, lymphopoietin-1, and later, when the full array of biological activities mediated by the factor became apparent, Interleukin-7 (IL-7). This talk presents a brief summary of the molecular biology of IL-7 and what we believe to be its physiological role.

IL-7 Cloning and Protein Structure:

Murine bone marrow stromal cells were transfected (3) with the plasmid pSV3neo, containing the transforming sequences of SV40. The transfected adherent cells were removed with trypsin and cloned by limiting dilution. The transfected stromal cells were then used to prepare a cDNA library which was ligated into a direct expression vector and transformed into Escherichia coli. Transformants were plated to provide about 1000 colonies per plate. The resulting colonies were harvested and each pool used to prepare plasmid DNA for transfection into COS-7 cells. The supernatants were analyzed for biologically active IL-7 (the ability to cause the proliferation of a pre-B-cell population). Approximately 750,000 recombinants were screened before a pool yielding IL-7 was identified. A single cDNA clone was eventually identified from this pool that encoded biologically active murine IL-7. The isolated cDNA had a single open reading frame, 462 base pairs (bp) long, preceded by a 548 bp 5'. non-coding region. The open reading frame was followed by a 3' non-coding region, consisting of 579 bp containing a consensus polyadenylation site and terminating with a string of 15 adenines.

The amino terminus was identified by sequence analysis of murine IL-7 protein purified to homogeneity from the supernatant of the transformed

stromal cell line. Murine IL-7 was found to contain a 25 amino acid leader sequence, followed by 129 amino acids, predicting a protein of 14.9 kDa molecular weight. There were two potential N-linked glycosylation sites at amino acids 69 and 90; glycosylation of these sites presumably accounts for the fact that native IL-7 migrates as a protein of 25 kDa. Murine IL-7 contains six cysteine residues. The fact that reduction with 2-mercaptoethanol causes loss of biological activity suggested that intramolecular disulphide bonds plays a role in the activity, although the location of these bonds has not yet been determined. Murine IL-7 cDNA was used to probe a human cDNA library prepared from a line hepatoma cell line (4). A highly homologous human cDNA was isolated, that, when expressed in COS cells, directed the synthesis of a protein capable of supporting the proliferation of both human and mouse bone marrow and enriched populations of pre-B cells from both species. In keeping with the observed biological activity, human and murine IL-7 amino acid sequences showed a strong degree of homology (60%) with all six cysteines conserved. Interestingly, however, human IL-7 contained a 19-amino acid insert (residues 96-114) that was not present in murine IL-7. The insert was the result of an additional exon in the human IL-7 genome, but did not appear to be related to the biological activities of the IL-7 molecule.

Biological Activities of IL-7:

(a) On cells of B-cell lineage:

The earliest identified cells committed to the B cell lineage are pro-B (also called pre-pre B) cells which contain the heavy and light chain genes in the germ line configuration. These cells do not express the B-lineage antigen, B 220, and express only low levels of the T-lineage antigen, Thy-1. Pro-B cells differentiate into B220+ pre-B-cells, which rearrange and express cytoplasmically the immunoglobulin heavy chain genes. Following rearrangement of light chain genes, these cells give rise to mature B cells which express surface immunoglobulin.

B-cell populations exhibiting the characteristics of pro-B, pre-B and B cells were prepared from long-term marrow cultures (pro-B and pre-B cells) and from peripheral blood (B cells) and examined for their responsiveness to IL-7. IL-7 caused proliferation of both pre-B and pro-B cells, but not of mature B cells (Table 1). This activity is unique; no other previously defined growth factor, including IL-2 and IL-4 cause the proliferation of pro- or pre-B-cell populations.

Table 1

Response of B Lineage Cells to IL-7

	Unfractionated bone marrow	+++
	Long term bone marrow cultures	+++
Pro-B	slg$^-$220$^-$	++
Pre-B	slg$^-$ 220+	++++
B	slg+ spleen	-
	slg+ lymph node	-
	slg+ marrow	-
	slg+ and anti-μ	-

Primary target cell: *cμ+ slg$^-$ 220+ large pre-B cell*

It was interesting to note that B cells did not proliferate to an IL-7 stimulus even in the presence of anti-μ, which distinguishes IL-7 from IL-4. Furthermore, in cultures maintained in IL-7, no evidence of differentiation (gene rearrangement; surface markers) of either the pro-B- or pre-B-cell compartments was observed. The IL-7 signal to cells of the B lineage thus appears to be solely proliferative.

(b) On cells of the T-cell lineage:

Somewhat to our surprise, IL-7 was found to be mitogenic towards adult resting thymocytes, and to be co-mitogenic in the presence of the plant lectins phytohemagglutinin (PHA) and concanavalin A (Con A). In direct comparisons with IL-2 and IL-4 (two other lymphokines known to induce thymocyte proliferation) IL-7 shared with IL-2 the ability to induce thymocyte proliferation in the absence of mitogens (although IL-7 was only one-tenth as active as IL-2 on a weight basis); IL-4 was not mitogenic under similar circumstances. In the presence of PHA, all three growth factors greatly enhanced thymocyte proliferation (5). The proliferative response of thymocytes to IL-7 was not affected by the presence of antibodies specific for IL-2 or IL-4, nor was increased IL-2 or IL-4 specific mRNA, or the respective proteins, seen in thymocyte cultures stimulated with IL-7. These results strongly indicate that IL-7 acts directly on thymocytes and not by inducing the production of other known T-cell growth factors (5). To examine further the thymocyte populations responsive to IL-7, a CD4$^-$CD8$^-$ cell population, the least differentiated population of adult T cells, was isolated by negative selection from adult thymus. A further CD4$^-$CD8$^-$cell population was obtained from a 13-day fetal thymus, whose cells are

predominately of this phenotype. Both of these $CD4^-CD8^-$ cell populations proliferated in response to IL-7 even in the absence of added co-mitogen. Thus cells at the earliest stages of intrathymic differentiation were stimulated to proliferate by IL-7 (5).

In summary, IL-7, like IL-4 and IL-2, has proved to have a much broader array of lymphoproliferative effects than previously supposed. Thus, although IL-7 was cloned by following its activities on pre-B cells, it has also been shown to exhibit a T-cell orientation. The signals delivered by IL-7 are, however, selective; not all lymphocytes are responsive. Amongst cells of the B lineage, the responsive cells all display immunoglobulin light chains in their germ line configuration. Following rearrangement and the display of surface IgM, the cells no longer respond. Whether this reflects loss of the IL-7 receptor on differentiation remains to be established. Amongst T cells only $CD4^-CD8^-$ thymocytes, the least differentiated cell in the T-cell lineage, responds directly to IL-7. Other, mature, T cells do not proliferate in response to IL-7 unless activated by antigen or mitogen. Some quiescent T cells do, however, appear to show increased IL-2 receptor display following IL-7 stimulation; the first indication that IL-7 can deliver differentiative as well as proliferative signals.

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Expression of T Cell Receptor Associated Proteins During Human T Cell Development

D. Campana, E. Coustan-Smith, L. Wong, and G. Janossy

CD3 EXPRESSION DURING HUMAN T CELL ONTOGENY

T cells express membrane T cell receptor (TCR) heterodimers (TCR$\alpha\beta$ or TCR$\tau\delta$) which are associated with a set of monomorphic proteins recognized by CD3 monoclonal antibodies (MAbs). When CD3 MAbs are applied to suspensions of unpermeabilized infant thymocytes 50%-75% of cells are heterogeneously labeled (Lanier et al., 1986): approximately 50% of these are weakly positive ($CD3^{dim}$) and 50% are strongly $CD3^+$ ($CD3^{bright}$; Fig. 1). The remaining cells do not show membrane CD3 (mCD3) expression. However >90% of these $mCD3^-$ cells exhibit CD3ε and CD3δ molecules in the perinuclear area when the samples are re- investigated in cytocentrifuge preparations (Furley et al., 1986; Campana et al., 1987). The $mCD3^-$,cytoplasmic CD3 $(cCD3)^+$ thymocytes include the highly dividing immature large blasts strongly terminal deoxynucleotidyl transferase positive (TdT^+), $CD7^+$, and heterogeneously $CD2^+$ and $CD5^+$ (Bradstock et al., 1980; Campana and Janossy, 1988).

These findings are reinforced by the observations in thymic acute lymphoblastic leukemia (T-ALL) blasts. In our series, only 19 out of 70 T-ALL cases were $CD3^+$ when studied in cell suspension. However when the 70 cases were reinvestigated on cytocentrifuge preparations they were invariably $cCD3^+$ (Janossy et al., 1989). The synthesis of CD3 molecules is strongly associated with the T lineage. In fetal and postnatal bone marrow (BM) TdT^+ B cell progenitors, myeloblasts, normoblasts and megakaryocytes are $cCD3^-$ (Campana et al., 1987). Likewise, >500 samples of non-T cell acute leukemia investigated in our laboratory did not show CD3 expression (Janossy et al., 1989). The practical implication of these data is that the detection of cCD3 is a reliable marker for establishing the T lineage association of leukemic blasts (Janossy et al., 1989).

We investigated the possibility that immature T cells reside in the fetal liver before migrating to the thymus at around the 10th wk of gestation (Lobach et al., 1985). No CD3 staining was detectable when suspensions of fetal liver samples from the 7th to the 10th wk of gestation were examined (Campana et al., 1989). However when the same samples were reinvestigated on cytocentrifuge preparations, $CD3^+$

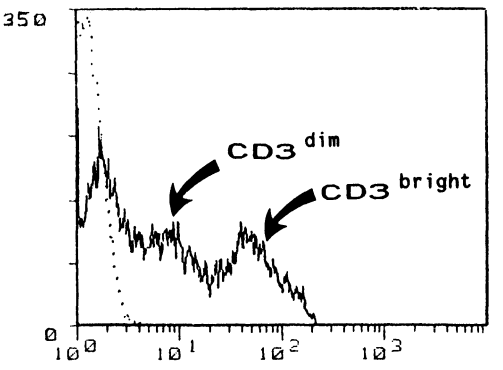

Fig.1 *Membrane CD3 expression of infant thymocytes.* The shape of the flow cytometry profile suggests two distinct cell populations rather than a progressive acquisition of membrane molecules.

cells were seen, representing 0.6%-5% of the total mononuclear cells. These cells were CD7$^+$, CD45$^+$ and a proportion of them (18%-78%) was also CD8$^+$ (Campana et al., 1989; D Campana, unpublished observations). In all the samples these cells were invariably negative for CD2, CD5, CD1, CD4, CD16, class II and TdT (Campana et al., 1989). Another characteristic of these immature T cells is their high proliferative activity demonstrated by the expression of the nuclear antigen Ki67 in >90% of these cells (Campana et al., 1989). Ki67 is found during the active phases of the cell cycle (Gerdes et al., 1984). In samples obtained from the 10th to the 20th wk of gestation mCD3$^+$ cells were present but 32%-78% of cCD3$^+$ cells still lacked detectable mCD3. Membrane CD3$^+$ cells showed a decline in proliferative activity and <10% were Ki67$^+$ (Campana et al., 1989). In conclusion, the synthesis of CD3ε chains and their accumulation in the rough endoplasmic reticulum is an early event during ontogeny which does not depend on the thymic environment. Cytoplasmic CD3$^+$ cells actively proliferate outside the thymus and the expression of mCD3 is associated with a decline in proliferative activity. Extrathymic cCD3$^+$,mCD3$^-$ cells have now also been found in avians (Coltey et al., 1989).

EXPRESSION OF TCR PROTEINS IN HUMAN FETAL TISSUES

MAbs to common determinants of the TCR proteins have become available (Table 1; reviewed by Triebel and Hercend, 1989) and the detailed investigation of the TCR expression during human T cell ontogeny and differentiation is now feasible. We initiated such investigation by testing the TCR protein status of the cCD3$^+$,mCD3$^-$ cells found in the fetal liver from 7th to the 10th wk of gestation and showed that these cells did not express TCRβ, as detected by βF1 MAb, or TCRτδ chains as detected by TCRδ-1 and δTCS1 MAbs, confirming the immaturity of such cells (Campana et al., 1989). Second we applied the same Abs to fetal liver samples obtained the from the 10th wk to the 20th wk of gestation. In these samples βF1$^+$ cells were seen, ranging from 0.1% to 9% of the mononuclear cells, and WT31$^+$ cells were also seen (0.1%-7%; Campana et al., 1989). In all the fetal liver samples .lm1 studied cells labeled by TCRδ-1 and δTCS1 were absent (<0.01% mononuclear cells) or represented a minority population when compared to βF1$^+$ or WT31$^+$ cells (Campana et al., 1989).

Table 1. *MAbs to human TCR proteins* (see also review by Triebel and Hercend, 1989).

Mab	Reactivity	Source
WT31	TCRαβ	Tax
BMA-031	TCRαβ	Kurrle
βF1	TCRβ	Brenner
αF1	TCRα	Ip
TCRδ-1	TCRδ	Brenner
11F2	TCRτδ	Borst
δ-TCS1	Vδ1	Ip
BB3	Vδ2	Moretta
Ti-τA	Vτ9	Hercend

Fetal thymic samples were analysed from the 10th to the 20th wk of gestation. From the 10th wk of gestation the majority of thymocytes had detectable TCRβ chains, the percentage of positive cells ranging from 49%-79% (Campana et al., 1989). These TCRβ$^+$ cells were always CD3$^+$ and included mCD3$^+$,WT31$^+$ thymocytes. A proportion of βF1$^+$ cells (1%-10%) appeared to express free cytoplasmic TCRβ chains in the perinuclear area, without mTCR expression (Campana et al; 1989; Campana et al., submitted). Recently a MAb (αF1) directed against an epitope of the human TCRα chain has become available (Henry et al, 1989). We applied this reagent to fetal thymic cytocentrifuge preparations obtained from the 10th to the 20th wk of

gestation and observed that 50%-80% of thymocytes in all samples studied reacted with αF1 MAb (Campana et al., submitted). These cells included the $mCD3^+$ and $WT31^+$ cells, while a proportion of thymocytes (1%-5%) appeared to express TCRα exclusively in the perinuclear area (Campana et al., submitted). Thus TCRα chains are synthesized early in human thymic ontogeny and no samples containing exclusively TCRβ chains were found. The expression of TCRα in early fetal liver samples remains to be investigated.

In fetal thymic samples cells labeled with TCRδ-1 and δTCS-1 MAbs represent <1% of thymocytes, even at the 10th wk of gestation (Campana et al., 1989). The absence of a TCRγδ peak at the earliest stages of thymic ontogeny is somewhat unexpected, considering the findings in avians (Chen et al., 1988) and rodents (Itohara et al., 1989) where there is an early wave of TCRγδ bearing cells soon after outnumbered by TCRαβ cells. The lack of these events in humans is paralleled by other differences between species such as the lack of a predominant TCRγδ population amongst human gut intraepithelial lymphocytes (Trejdosiewicz et al., 1989), in contrast to the observations in the mouse (Goodman and Lefrancois, 1988).

An additional feature of thymic ontogeny is the emergence of nuclear TdT at around the 18-19th wk of gestation (Bodger et al., 1984). The absence of TdT at earlier stages of development indicates that in the generation of diversity of TCR genes several mechanisms must be preferentially employed at the different develomental steps leading to the successful synthesis of TCR proteins even in the absence of TdT.

TCR EXPRESSION IN INFANT THYMUS

The analysis of postnatal thymus (10 samples) with Abs to TCR and CD3 proteins as well as to nuclear TdT enabled the identification of distinct cell populations representing successive maturative stages (Fig. 2). Immature $cCD3^+$,TdT^+,$mCD3^-$ include cells with no TCR expression (10%-25% of thymocytes) and cells labeled by TCRα or TCRβ MAbs exclusively in the perinuclear area (5%-15%; Campana et al., 1989; Campana et al., submitted). As expected, $mCD3^{dim}$ thymocytes reacted with both βF1 and αF1. Most of these cells are TdT^+ but a proportion of thymocytes (5%-30%) $mCD3^{dim}$,TdT^- is also seen (Campana et al., submitted).

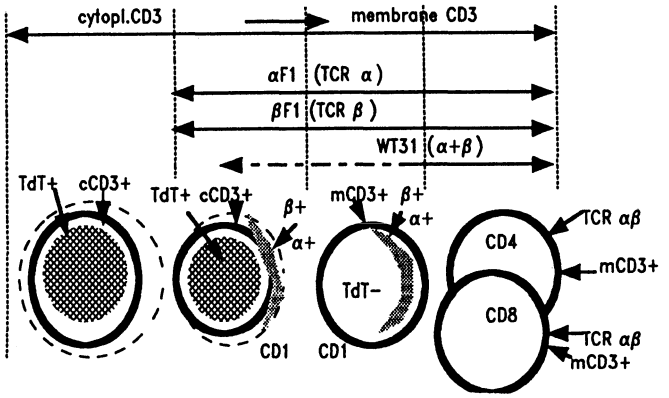

Fig.2 *The putative development of human T cells in the thymus.* This scheme is based on phenotypic observations (see in text) and observations in animals.

Most of the $CD3^{dim}$ are $CD1^+$ and simultaneously express CD4 and CD8 (Lanier et al, 1986). The remaining cells are mature $mCD3^{bright}$,TdT^- thymocytes reacting with αF1 and βF1 MAbs and expressing either CD4 or CD8 (Campana et al., 1989).

In conclusion, these observations suggest a model of TCRαβ cell differentiation in which CD3 molecules (CD3ε, CD3δ and, probably, CD3τ) are synthesized first and accumulated in the cytoplasm of immature, highly dividing T cells, while they are in the process of rearranging their TCR genes. This is followed by the addition of TCRα or TCRβ chains (whichever gene is rearranged productively first). The subsequent step is represented by the dim expression of the TCRαβ/CD3 complex on the cell membrane: these cells are likely to be the target in some of the processes of thymic selection followed by the dramatic increase in CD3/TCRαβ membrane expression and the loss of spontaneous proliferation.

Thymic TCRτδ bearing cells lack class II molecules and the majority (>80%) are $mCD3^{bright}$. A proportion of these cells in both fetal and infant samples (20%-45%) is $CD1^+$ indicating their thymic origin (Campana et al., 1989). $TCR\tau\delta^+$ cells (<10%) with TdT positivity can occasionally been observed. The lack of reagents which specifically react with all TCRτ chains has hampered the full elucidation of the phenotypic stages of TCRτδ assembly.

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Immunobiology of HLA

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Volume 1, **Histocompatibility Testing 1987,** is the result of the Tenth International Histocompatibility Workshop. Over 360 laboratories worldwide participated in collaborative projects, including the biochemical analysis of HLA antigens, identification of polymorphic HLA determinants detected by T-lymphocyte clones and a comprehensive analysis of Southern Blot with HLA-DNA probes. The detail of the Joint Reports from the individual workshop components, with the accompanying figures, tables and pictures of gels from the biochemical studies, as well as Southern Blots, constitute an "Atlas for Factors of the HLA System."
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Together, the two volumes provide up-to-date authoritative information on the molecular biology of the HLA system and serve as a useful handbook on the state of the art of basic research on HLA and the application of clinical histocompatibility testing.

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Realm of Tolerance

Proceedings of a meeting in memory of Professor Milan Hašek, October 5–8, 1988 in Ommen/Amsterdam

Foreword by M. Boiron

1989. XIX, 314 pp. 72 figs., 60 tabs. Hardcover
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Any professional concerned with immunology will be interested in this book dedicated to the memory of Milan Hašek, former director of the Prague Institute of Experimental Biology and Genetics. Prof. Hašek was a congenial scientist and most amiable person – a personal friend of almost all leading immunologists around the world. He was displaced from his post of director in 1970, yet had a lasting impact upon his students and the group known as the Prague School of Immunogenetics.
The topics covered in the contributions range from tolerance, immune network, and immunogenetics to the immunology of bacterial and viral infections. The are written by 27 of Prof. Hašek's former coworkers who emigrated to western contries around or after 1968 and became well-known and distinguished scientists in the field. The papers include their personal reflections of the Prague Institute, their impressions upon arriving abroad and their interesting experimental work since then. The book also provides a complete bibliography of their publications after leaving Czechoslovakia.

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