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Participants

G. L. Ada
Walter and Eliza Hall Institute of Medical Research
Melbourne, Australia

Frank L. Adler
Division of Immunology
The Public Health Research Institute of the City of New York, Inc.
New York, New York

Jean-Louis Amiel
Institut Canceroligie et Immunogenetique
Hôpital Paul-Broussais
Villejuif, France

Bernard Amos
Department of Microbiology and Immunology
Duke University Medical School
Durham, North Carolina

Fritz Bach
Department of Medical Genetics
University of Wisconsin
Madison, Wisconsin

Glover W. Barnes
State University of New York at Buffalo
Buffalo, New York

Baruj Benacerraf
Department of Pathology
New York University School of Medicine
New York, New York

M. C. Berenbaum
Department of Immunology
Institute of Child Health
University of London
London, England

G. Biozzi
Centre de Recherches Immunologiques
Hôpital Broussais
Paris, France

Enzo Bonmassar
University of Milan
Institute of Pharmacology
Milan, Italy

William Boyle
Department of Microbiology and Immunology
Duke University Medical Center
Durham, North Carolina

Sam L. Clark, Jr.
Department of Anatomy
Washington University School of Medicine
St. Louis, Missouri

Charalambos Coutsogeorgopoulos
Roswell Park Memorial Institute
Buffalo, New York

Gustavo Cudkowicz
Roswell Park Memorial Institute
Buffalo, New York

James F. Danielli
State University of New York at Buffalo
Buffalo, New York

Marvin Fishman
Division of Immunology
The Public Health Research Institute, of the City of New York, Inc.
New York, New York

Audrey Fjelde
Roswell Park Memorial Institute
Buffalo, New York

Abdul Gaffor
Ohio State University
Columbus, Ohio

Dr. Garsa
University of Guelph
Division of Immunology
Guelph, Ontario, Canada

Leslie Glick
Roswell Park Memorial Institute
Buffalo, New York

Luigi Gorini
Department of Bacteriology and Immunology
Harvard Medical School
Boston, Massachusetts

T. S. Hauschka
Roswell Park Memorial Institute
Buffalo, New York

I. Helström
Department of Microbiology
University of Washington Medical School
Seattle, Washington

K. E. Helström
Department of Pathology
University of Washington Medical School
Seattle, Washington

Y. Hinuma
Roswell Park Memorial Institute
Buffalo, New York

George H. Hitchings
Research Director
Chemotherapy Division
The Wellcome Research Laboratories
Tuckahoe, New York

James Holland
Roswell Park Memorial Institute
Buffalo, New York

Kengo Horibata
Irvington House Institute and Department of Medicine
New York University School of Medicine
New York, New York

J. S. Horoszewicz
Roswell Park Memorial Institute
Buffalo, New York

Y. Iwasaki
Department of Surgery
University of Colorado School of Medicine and the Denver Veterans Administration Hospital
Denver, Colorado

Stephen R. Kaplan
Yale University School of Medicine
Clinical Pharmacology and Chemotherapy Research Center
New Haven, Connecticut

N. Kashiwagi
Department of Surgery
University of Colorado School of Medicine and the Denver Veterans Administration Hospital
Denver, Colorado

Aurelia M. C. Koros
Department of Microbiology
University of Pittsburgh School of Medicine
Pittsburgh, Pennsylvania

Maurice Landy
Laboratory of Immunology
NIAID,
National Institute of Health
Bethesda, Maryland

John P. Leddy
Department of Medicine
University of Rochester School of Medicine
Rochester, New York

Duncan McCollester
Columbia Presbyterian Medical Center
New York, New York

Lionel A. Manson
Wistar Institute
Philadelphia, Pennsylvania

Thomas Mao
Roswell Park Memorial Institute
Buffalo, New York

T. L. Marchioro
Department of Surgery
University of Colorado School of Medicine and the Denver Veterans Administration Hospital
Denver, Colorado

Gabor Markus
Roswell Park Memorial Institute
Buffalo, New York

Paul H. Maurer
Department of Biochemistry
Jefferson Medical College
Philadelphia, Pennsylvania

D. Metcalf
Roswell Park Memorial Institute
Buffalo, New York

Enrico Mihich
Roswell Park Memorial Institute
Buffalo, New York

Felix Milgrom
Department of Bacteriology and Immunology
State University of New York at Buffalo
Buffalo, New York

Malcolm S. Mitchell
Yale University School of Medicine
New Haven, Connecticut

Hans J. Müller-Eberhard
Department of Experimental Pathology
Scripps Clinic and Research Foundation
La Jolla, California

William Munyon
Roswell Park Memorial Institute
Buffalo, New York

Charles A. Nichol
Roswell Park Memorial Institute
Buffalo, New York

David Osoba
Ontario Cancer Institute
Toronto, Ontario, Canada

Kenneth Paigen
Roswell Park Memorial Institute
Buffalo, New York

Joy Palm
Swiss Institute for Cancer Research
Lausanne, Switzerland

Richmond T. Prehn
The Institute for Cancer Research
University of Pennsylvania
Philadelphia, Pennsylvania

Robert A. Philips
500 Sherbourne St.
Toronto, Ontario

Paul Pinchuck
Department of Biochemistry
Jefferson Medical College
Philadelphia, Pennsylvania

David Pressman
Roswell Park Memorial Institute
Buffalo, New York

William Regelson
Roswell Park Memorial Institute
Buffalo, New York

Arnold E. Reif
Tufts Department of Surgery
Boston City Hospital
Boston, Massachusetts

Fred Rosen
Roswell Park Memorial Institute
Buffalo, New York

Larry D. Samuels
Children's Hospital
Columbus, Ohio

Barbara Sanford
Pathology Department
Massachusetts General Hospital
Boston, Massachusetts

George W. Santos
Johns Hopkins Hospital
Baltimore, Maryland

Michael Schlesinger
Department of Experimental Medicine and Cancer Research
The Hebrew University Hadassah Medical School
Jerusalem, Israel

Robert S. Schwartz
Clinical Immunology Service
New England Medical Center Hospitals and the Department of Medicine
Tufts University, School of Medicine
Boston, Massachusetts

G. M. Shearer
Roswell Park Memorial Institute
Buffalo, New York

M. Michael Sigel
Department of Microbiology
University of Miami School of Medicine
Coral Gables, Florida

T. E. Starzl
Department of Surgery
University of Colorado School of Medicine and the Denver Veterans Administration Hospital
Denver, Colorado

Jaroslav Šterzl
Czechoslovak Academy of Sciences
Institute of Microbiology
Department of Immunology
Prague, Czechoslavakia

Morris N. Teller
90-16 68th Avenue
Forest Hills, New York

G. Jeanette Thorbecke
Department of Pathology
New York University School of Medicine
New York, New York

Thomas B. Tomasi, Jr.
Buffalo General Hospital
Buffalo, New York

J. J. Trentin
Division of Experimental Biology
Texas Medical Center
Baylor University
Houston, Texas

George Tritsch
Roswell Park Memorial Institute
Buffalo, New York

J. L. Turk
Department of Immunology
Institute of Dermatology
University of London
St. John's Hospital for Diseases of the Skin
London, England

Johathan W. Uhr
Irvington House Institute and Department of Medicine
New York University School of Medicine
New York, New York

Delta E. Uphoff
National Cancer Institute
Department of Health, Education and Welfare
Bethesda, Maryland

William Werkheiser
Roswell Park Memorial Institute
Buffalo, New York

Issac Witz
Roswell Park Memorial Institute
Buffalo, New York

Yasuo Yagi
Roswell Park Memorial Institute
Buffalo, New York

David Yohn
Roswell Park Memorial Institute
Buffalo, New York

Paul C. Zamecnik
The John Collins Warren Labroatories of the Huntington Memorial Hospital of Harvard University at the Massachusetts General Hospital
Boston, Massachusetts

Contributors

Numbers in parentheses refer to the pages on which the authors' contributions begin.

ADA, G. L., Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia (17)

ADLER, FRANK L., Division of Immunology, The Public Health Research Institute of the City of New York, Inc., New York, New York (177)

BERENBAUM, M. C., Department of Immunology, Institute of Child Health, University of London, London, England (217)

BIOZZI, G., Centre de Recherches Immunologiques, Hôpital Broussais, Paris, France I.N.S.E.R.M., Association Claude Bernard and C.N.R.S. (103)

BOYLE, WILLIAM, Department of Microbiology and Immunology, Duke University Medical Center, Durham, North Carolina (243)

CLARK, SAM L., JR., Department of Anatomy, Washington University School of Medicine, St. Louis, Missouri (141)

FISHMAN, MARVIN, Division of Immunology, The Public Health Research Institute of the City of New York, Inc., New York, New York (177)

GORINI, LUIGI, Department of Bacteriology and Immunology, Harvard Medical School, Boston, Massachusetts (167)

HELLSTRÖM, I., Department of Microbiology, University of Washington Medical School, Seattle, Washington (51)

HELLSTRÖM, K. E., Department of Pathology, University of Washington Medical School, Seattle, Washington (51)

HORIBATA, KENGO, Irvington House Institute and Department of Medicine, New York University School of Medicine, New York, New York (187)

*IWASAKI, Y., Department of Surgery, University of Colorado School of Medicine and the Denver Veterans Administration Hospital, Denver, Colorado (351)

KASHIWAGI, N., Department of Surgery, University of Colorado School of Medicine and the Denver Veterans Administration Hospital, Denver, Colorado (351)

MARCHIORO, T. L., Department of Surgery, University of Colorado School of Medicine and the Denver Veterans Administration Hospital, Denver, Colorado (351)

MAURER, PAUL H., Department of Biochemistry, Jefferson Medical College, Philadelphia, Pennsylvania (319)

MOUTON, D., Centre de Recherches Immunologiques, Hôpital Broussais, Paris, France I.N.S.E.R.M., Association Claude Bernard and C.N.R.S. (103)

MÜLLER-EBERHARD, HANS J., Department of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, California (311)

PINCHUCK, PAUL, Department of Biochemistry, Jefferson Medical College, Philadelphia, Pennsylvania (319)

PREHN, RICHMOND T., The Institute for Cancer Research, University of Pennsylvania, Philadelphia, Pennsylvania (265)

SCHLESINGER, MICHAEL, Department of Experimental Medicine and Cancer Research, The Hebrew University Hadassah Medical School, Jerusalem, Israel (281)

SCHWARTZ, ROBERT S., Clinical Immunology Service, New England Medical Center Hospitals, and the Department of Medicine, Tufts University, School of Medicine, Boston, Massachusetts (203)

STARZL, T. E., Department of Surgery, University of Colorado School of Medicine and the Denver Veterans Administration Hospital, Denver, Colorado (351)

*Present address: 2nd Surgical Department, Chiba University School of Medicine, Chiba, Japan.

ŠTERZL, JAROSLAV, Czechoslovak Academy of Sciences, Institute of Microbiology, Department of Immunology, Prague, Czechoslavakia (71)

STIFFEL, C., Centre de Recherches Immunologiques, Hôpital Broussais, Paris, France, I.N.S.E.R.M., Association Claude Bernard and C.N.R.S. (103)

TURK, J. L., Department of Immunology, Institute of Dermatology (University of London), St. John's Hospital for Diseases of the Skin, London, England (1)

UHR, JONATHAN W., Irvington House Institute and Department of Medicine, New York University School of Medicine, New York, New York (187)

ZAMECNIK, PAUL C., The John Collins Warren Laboratories of the Huntington Memorial Hospital of Harvard University at the Massachusetts General Hospital, Boston, Massachusetts (155)

Foreword

This is the report of the first of a series of symposia organized jointly by the Institute for Pharmacology of the University of Milan and by the School of Pharmacy of the State University of New York at Buffalo.

Pharmacology is an area which is deriving great benefit from developments in biophysics and biochemistry and from the somewhat pretentious offspring of these two disciplines, molecular biology. Great interest is also arising from developments in immunology, for the control of the immunological process is now becoming essential for a variety of developments in medicine and surgery. Both university centers therefore thought that Enrico Mihich's suggestion that our first joint symposium should be concerned with drugs affecting the immune process was most appropriate.

The present symposium was also sponsored by the Roswell Park Memorial Institute and by the Center for Theoretical Biology of the State University of New York at Buffalo.

Buffalo, New York J. F. DANIELLI

Preface

In recent years many drugs have been found to interfere both with antibody response and with transplantation immunity. At the same time substantial progress has been made in continuing efforts to clarify the mode of action of anticancer agents, many of which also inhibit the immunological response. Moreover, at the molecular level, basic knowledge has been gained on various processes regulating cell metabolism, particularly those involving the synthesis of specific proteins. The knowledge acquired in these areas should provide the basis for a clarification of various phases of the immunological response. Indeed, all these functions can be brought into better perspective by studies of the alterations caused by drugs. Yet, in spite of the existence of excellent reviews, there is little evidence to indicate that a great deal of interaction and interchange of ideas is occurring among specialists working in fields of research potentially related to immunological problems. A need was recognized, therefore, for a symposium to consider recent advances in immunology from the biological, biochemical, and pharmacological points of view. In planning the program for the meeting, Dr. D. B. Amos, Dr. G. H. Hitchings, and I sought to bring together specialists with different backgrounds and interests, hoping to stimulate interaction among proponents of various points of view. These proceedings are the result of this interchange and are based upon diverse, yet highly specialized, individual contributions. The papers and discussions should be of interest not only to investigators interested in the specific topics presented, but also to those who are aware of the importance of immunological phenomena in the development of new approaches to antineoplastic therapy and to clinicians studying problems of organ transplantation and therapy of autoimmune diseases. The basic mechanisms of the immune response, the expression of antigens in normal and tumor tissues, and the alterations of various phases of the immune response by drugs are discussed extensively.

The complexity of the problem becomes apparent in attempts to exploit the interrelationships between drugs and immunity. In the case of organ transplantation and therapy of autoimmune diseases one wishes to depress the immune response; in the case of cancer therapy one wishes to enhance this response.

Uncertainty persists as to whether there is any immunity to cancer in man, and, if so, as to why this immunity is not clincally evident. Yet it may already be inferred that resistance to tumors exists in cancer patients. Autologous serum and leukocytes can exert a growth-suppressive effect on autoimplants of tumor cells. The cancer patient is often resistant to re-implantation of his own tumor. Tumor cells are frequently found in the circulation even in the absence of metastases. In animals substantial evidence indicates that induced tumors possess antigens not present in normal tissues of the adult host. The question to be answered, therefore, is why the immunological response is so inefficient against primary tumors. Specific immunological conditions may be responsible for the growth of autochthonous tumors in spite of their antigenicity. The establishment of immunological tolerance, tumor growth enhancement by circulating antibodies, modulation of tumor antigens by antibodies, immunodepression by the tumor or by carcinogenic factors or immunoselection of less antigenic tumor cell lines, could each account for the ineffective host response observed.

Further studies may indicate whether it is possible to increase the efficiency of host defenses by the use of drugs. As is discussed in this volume, not all known antitumor agents are immunodepressant, at least as used therapeutically. Moreover, some agents may depress or stimulate the immunological reaction depending upon factors such as the proliferative response of lymphoid tissues and the relative antigen mass. Should tumor immunity be analogous to immunity against normal tissues, one would expect that some of the immunodepressant drugs would not affect such immunity beyond the period of induction of the host response. Most likely this period is passed by the time clinical therapy is instituted. Nevertheless, one should consider the possibility that cancer patients may become immunologically tolerant to their tumor as a result of drug treatments even beyond the initial period of tumor growth. New approaches pursued in cancer chemotherapy may yield compounds which are not primarily antiproliferative in action but which may affect tumor growth indirectly.

Further advances in tumor biology are necessary to evaluate the possibility of altering tumor differentiation and tumor-caused immunodepression. In particular, the possibility of increasing the expression of

antigen in tumor cells should be mentioned as an objective requiring clarification of basic cell regulatory phenomena.

The possibility of using drugs to "dissect" the immunological response and to alter it deserves continuing study. Recognition of antigen, cellular differentiation, cellular multiplication, antibody synthesis, specific and nonspecific phenomena occurring at the site of antigen localization may all be altered by drugs. Indeed, the immunological response provides the pharmacologist with unique systems for the study of drug selectivity at the cellular and molecular level.

I wish to take this opportunity to acknowledge with deepest gratitude the invaluable cooperation of Drs. D. B. Amos and G. H. Hitchings in arranging the program of the symposium. We are indebted to the National Science Foundation, the School of Pharmacy of the State University of New York at Buffalo, and the Roswell Park Memorial Institute for providing the support which made this conference possible. The help of Misses A. I. Mulhern, Rita Redmond, and others on our staff in dealing with technical and organizational matters and the prompt and efficient assistance of the staff of Academic Press are sincerely appreciated.

Buffalo, New York ENRICO MIHICH
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The Effect of Immunosuppressive Drugs on Cellular Changes after Antigenic Stimulation

J. L. TURK

Department of Immunology, Institute of Dermatology (University of London)
St. John's Hospital for Diseases of the Skin, London, England

Throughout the past decade there has been an increasing amount of work on the action of drugs on immunological processes. Many of the drugs which have been found to be active are compounds in current use as cancer chemotherapeutic agents. In fact, it has been logical to study this group of compounds, since many analogies exist between the rapid proliferation of cancer cells and the rapid proliferation of cells during the induction of an immune response. The analogy should not, however, be taken any further. Many workers have presumed that the action of these compounds on cancer cells and the immune response is identical. In other studies the action of drugs has not even been studied on proliferating cancer cells but on such conventional biochemical models as the rat liver or chicken bone marrow. The action of drugs on biochemical processes in such normal nonproliferating tissue is then extrapolated to its action on enzyme systems in proliferating cancer cells and finally to the immune response. The fault for this does not lie with those who made these original observations, but on subsequent workers who failed to investigate models nearer the true system and on the writers of review articles who have considered, without further investigation, that the action of a drug under physiological conditions in nondividing tissue is the same as that on dividing tissues under pathological conditions. A point that has to be emphasized is that drugs can act on a number of different biochemical processes at the same time. The final action of the compound on the system to be studied could be due, for instance, to its action at the same time on a number of different enzymes involved in different metabolic pathways. Another aspect of this problem is that

different enzyme pathways may exist in different species of animals to produce a final metabolic effect. An antimetabolite effective on one or more metabolic pathways could be an effective immunosuppressant agent in one species and not in another. Similarly, the dose of a particular drug, to be an effective immunosuppressant, can differ from one species to another, and also probably depends on the metabolic pathways affected.

It has become recognized over the past few years that immune mechanisms in mammals can be divided into two main types, cellular immunity (delayed hypersensitivity) and humoral immunity (circulating antibody). Although delayed hypersensitivity was once thought to be a part of the process of humoral antibody production, it is now realized that these are completely separate processes, originating in different areas of lymphoid tissue. Moreover, cellular immunity is known to be controlled by the thymus in the neonatal animal whereas it has recently been suggested that humoral immunity is controlled in the newborn rabbit by the appendix and Peyer's patch lymphoid tissue—possibly the equivalent of the bursa of Fabricius in the chicken (Cooper *et al.*, 1966b).

It is logical therefore to discuss the effect of immunosuppressant drugs on these two forms of immune response separately. Work over the past 4 years in the author's laboratory has been on the effect of immunosuppressant drugs on cellular immune reactions. It will therefore be left to other contributors to discuss the effect of these drugs on humoral antibody formation. Immunization with many antigens, especially bacteria, with or without Freund's adjuvant, produces both cellular immunity and humoral immunity in parallel. However, it is possible to gear the immune response of the animal more in the direction of cellular immunity than humoral immunity, so that one can study a process involving an almost pure state of delayed hypersensitivity. Examination of the draining lymphoid tissue during the days subsequent to painting a chemical sensitizing agent on the skin or the application of a homograft to the skin will show a picture that can be correlated in its early stages almost exclusively with delayed hypersensitivity (Oort and Turk, 1965; Turk and Heather, 1965). In our present study two such models have been used: the application of 0.2 ml of a 10% ethanol solution of the chemical sensitizer 2-phenyl-4-ethoxymethylene-5-oxazolone (oxazolone) to the ear of guinea pigs or the application of a C57BL skin homograft to the flank of C3H/He mice.

I. CELLULAR CHANGES IN LYMPHOID TISSUE FOLLOWING SENSITIZATION

The cellular changes which occur in lymphoid tissue during the development of delayed hypersensitivity have been described previously

(Turk and Stone, 1963; Oort and Turk, 1965; de Petris *et al.*, 1966). Specific changes may be seen in the draining lymph node within 48 hours after the application of oxazolone to the ear of the guinea pig. These consist of the enlargement of an area of the cortex which Dr. Oort of Leiden and I have described as the "paracortical area" of the lymph node. This area is situated between the true cortex and the medulla where the small lymphocytes are not so closely packed as in the true cortex. Enlargement of this paracortical area is associated with the proliferation of large numbers of large pyroninophilic cells within it. I prefer to call these cells immunoblasts as they are rapidly dividing cells, as many as 70% of which incorporate thymidine-^{3}H within 1 hour of its intravenous injection. They develop specifically as a result of an immunological stimulus, and they do not occur in the lymph nodes draining the primary application of a chemical sensitizing agent if the animal is in a state of specific immunological tolerance to this hapten (Turk and Stone, 1963; Turk, 1965). Up to the fourth day after sensitization, examination of the lymph node 24 hours after the injection of thymidine-^{3}H demonstrates the presence of equal numbers of both small lymphocytes and immunoblasts containing less than 50% of the label present in the immunoblasts 24 hours previously.

Enlargement of the paracortical area continues until the fourth day after sensitization, namely, until the day before the animals develop generalized sensitivity. At this time immunoblasts are also present in the paracortical areas at their highest concentration. The number of immunoblasts in the paracortical area of draining lymph nodes falls off rapidly during the fifth and sixth day after sensitization as the animals become increasingly more sensitive, and by the ninth day it reaches a level close to that found in normal lymph nodes. On the fifth day after sensitization, when the animal is already sensitive, the number of small lymphocytes in the draining node, which are labled with thymidine-^{3}H injected 24 hours previously, has risen to 10.5%. In contrast, the normal unsensitized lymph node contains approximately 4% labeled small lymphocytes 24 hours after injection of thymidine-^{3}H and the same proportion is found 3 days after sensitization, before the animal shows any signs of generalized sensitivity. The presence of the increased number of labeled small lymphocytes in the lymph node, 24 hours after the intravenous injection of thymidine-^{3}H, seems to be associated with the development of sensitivity, as also does the fall-off in the concentration of immunoblasts. This suggests the possibility that the immunoblasts begin to divide into cells morphologically resembling small lymphocytes at the time that sensitivity begins. It is about this time that lymph nodes begin to be able to transfer contact sensitivity adoptively in inbred guinea pigs and there is evidence that immunologically active cells have disseminated through-

out the body, so that the local lymph nodes are no longer necessary for the maintenance of the immune state (Turk and Stone, 1963).

A peak in the concentration of immunoblasts in the draining lymph node has also been found in the rabbit at 4 days after the application of a skin homograft (Scothorne and McGregor, 1955). However, these cells do not always reach a peak on the fourth day. In C3H/He mice which have received a skin homograft, immunoblasts do not reach a peak level in the draining lymph node until the seventh day after the graft has been applied. Dr. Oort of Leiden has made a similar observation on 020 mice which have received a renal homograft. The same mice, however, develop a peak in the immunoblasts in the draining lymph node 4 days after the application of a renal xenograft.

Germinal centers in the cortex and plasma cell proliferation at the corticomedullary junction and in the medullary cords do not appear to play any part in the development of delayed hypersensitivity (Turk and Oort, 1966). However, since the work of Miller (1961), Arnason *et al.* (1964), Cooper *et al.* (1966a), it must be accepted that delayed hypersensitivity reactions are under the control of the thymus. This is also shown by the fact that the paracortical areas of the lymph node do not develop in neonatally thymectomized mice (Parrott *et al.*, 1966). Moreover, immunoblasts do not appear in neonatally thymectomized mice in these areas in draining lymph nodes, following the application of oxazolone to the skin (Parrott and de Sousa, 1966).

The ultrastructure of cells in lymph nodes draining a site of application of oxazolone to the skin has been examined in the guinea pig (de Petris *et al.*, 1966). Immunoblasts can be recognized by their pale nuclei with one or more nucleoli and an abundant cytoplasm. The mitochondria have a distorted structure and scanty cristae. The main characteristic of these cells, however, is their high content of ribosomes which are present in clusters or polysomes. Endoplasmic reticulum is scanty or absent. A similar appearance has been described in lymph nodes draining a skin homograft (Binet and Mathé, 1962; André-Schwartz, 1964). Other cell types were seen, as well as typical small lymphocytes, and were particularly numerous on the fifth and sixth days after sensitization with oxazolone. These cells are intermediate in size between immunoblasts and small lymphocytes, and often contain many ribosomes grouped in polyribosome clusters. The mitochondria are dense and regular, more like those found in the small lymphocyte, and the nucleus, though sometimes intermediate between that of the immunoblast and the small lymphocyte, often resembles that of the small lymphocyte.

It has been suggested by Medawar (1965) that delayed hypersensitivity is initiated by a process of peripheral sensitization, in which lymphocytes

pass through a fixed antigenic site in the periphery. These cells would then pass to the regional lymph node where they would find the right milieu in the paracortical areas to proliferate. In humoral antibody production, a soluble antigen passes down into the lymph node and stimulates plasma cell proliferation at the corticomedullary junction. Thus the cellular pattern in the lymph node and the resultant immune response could be a direct result of the mode of presentation of antigen to the immunological mechanism.

II. EFFECT OF IMMUNOSUPPRESSIVE DRUGS ON THE SENSITIZATION PROCESS

The first thing that must be determined in any study of the effect of an immunosuppressant drug on delayed hypersensitivity is whether the effect is a true one on the central immunological mechanisms or whether in fact the drug has a nonspecific effect on the ability of the animal to manifest an inflammatory reaction. It is easy to determine whether the effect of an immunosuppressant on humoral antibody production is a central effect by estimating the amount of antibody present in the circulation. However, in the case of cellular immunity, many workers judge the effect of the drug by the final ability of the animal to manifest an inflammatory reaction in the periphery. Many nonspecific steps occur between the development of a state of cellular immunity and the final demonstration of delayed hypersensitivity in the periphery. This problem has been approached by seeing whether guinea pigs could be passively sensitized while being treated with immunosuppressant drugs (Turk, 1964a). It was found, for instance, that an 8-day course of methotrexate (5 mg per animal every other day), sufficient to block primary contact sensitization completely, did not affect the ability of a guinea pig to show full sensitivity following passive transfer with lymphoid cells. However, an 8-day course of 10 mg cyclophosphamide per animal dropped the ability of guinea pigs to show contact sensitivity by 50% after a similar passive transfer. This showed that methotrexate at this dosage had no effect on the nonspecific ability of the animal to manifest delayed hypersensitivity reactions, whereas cyclophosphamide was shown to have a peripheral effect as well as the more specific central effect described below.

The possible mode of action of immunosuppressant drugs on the central processes involved in the induction of sensitivity can be illustrated by the three experimental models which we have been studying over the past 4 years. These are the effect of cyclophosphamide and methotrexate on contact sensitivity to oxazolone in the guinea pig and the effect of thalidomide on homograft rejection in the mouse. It must be emphasized

that the effects described have valid implications only for the particular species of animal and the dose schedule studied. There is no evidence that these results could be extrapolated to the effect of the drug on a different species or after administration by a different dose schedule. These results must be taken only as illustrations of the possible effects that can be caused by immunosuppressive drugs on the cellular changes which take place during the inductive phase of delayed hypersensitivity.

A. Contact Sensitivity in the Guinea Pig

1. Cyclophosphamide

Cyclophosphamide appeared to act on the cellular events in lymph nodes in a number of ways. In the first, it prevented the outburst of large numbers of immunoblasts; 4 days after sensitization only 2.2% were found instead of the expected 10.6%. Those cells which did develop were swollen and distorted, and up to five times larger than those seen in the lymph nodes of sensitized guinea pigs which had not received the drug. Those immunoblasts which developed were able to incorporate thymidine-^{3}H to the same extent as normal immunoblasts. Cyclophosphamide did not, however, reduce the number of nonpyroninophilic cells (probably lymphoblasts) which incorporated thymidine-^{3}H 1 hour after intravenous injection (Turk, 1964b). Twenty-four hours after the injection of thymidine-^{3}H only 0.6% of small lymphocytes were labeled in unsensitized nodes instead of the 4% found in animals not treated with cyclophosphamide. In nodes draining the site of application of a chemical sensitizer given 5 days previously, only 1.75% of small lymphocytes were labeled compared with the 10.5% found in animals not treated with the drug.

Thus cyclophosphamide can be said to have three effects: (1) It prevents the differentiation of small lymphocytes into immunoblasts which occurs as a result of antigenic stimulation. (2) It has a toxic effect on the immunoblasts which develop. (3) It is an antimitotic agent.

The drug does not appear to affect DNA synthesis, as demonstrated by the normal incorporation of thymidine-^{3}H by those few immunoblasts which are able to differentiate as a result of stimulation with antigen.

2. Methotrexate

The effect of methotrexate on the train of events occurring in the lymph node following sensitization is very different from that of cyclophosphamide. Immunoblasts develop normally as in an animal not treated with the drug. However, there is no development of the specific population of new lymphocytes which can be detected by an increase in labeled

small lymphocytes 24 hours after thymidine-^{3}H, on the fifth day after sensitization. While the development of these new cells is blocked, methotrexate does not appear to block the normal turnover of small lymphocytes. Following treatment with methotrexate, labeled small lymphocytes form about 4% of the cells in imprints of both normal lymph nodes and those draining the area of application of the sensitizer. The grain count over small lymphocytes in treated and untreated animals 24 hours after thymidine-^{3}H is $\simeq$ 50% of that over immunoblasts 1 hour after thymidine-^{3}H. However, the grain count over immunoblasts 24 hours after thymidine-^{3}H is normally $\simeq$ 33% of that 1 hour after thymidine-^{3}H, whereas in animals treated with methotrexate it is definitely higher, $\simeq$ 50%.

Thus methotrexate appears to cause a block in the development of a specific population of small lymphocytes which is present in the draining node once the animal becomes sensitized, and also seems to slow down the division of immunoblasts into other immunoblasts (Turk and Stone, 1963).

Recently, Dr. Diengdoh and I have attempted to look for biochemical changes that occur within immunoblasts as a result of treatment of the animal with methotrexate, and which might give us a more precise lead to the mode of action of this drug in blocking the development of contact sensitivity in the guinea pig (Diengdoh and Turk, 1966). Whereas one could use classical biochemical techniques to assess the effect of a drug on an organ like the liver or on tumor cells in culture, where one is dealing with a relatively homogeneous cell population, the lymph node contains a mixed population of cells present in different areas with different functional potentials. For this reason it was thought that histochemical rather than biochemical techniques might lead to a better understanding of the processes occurring in lymphoid tissue as a result of an immunological stimulus.

It has been suggested from biochemical studies (Werkheiser, 1961) that methotrexate acts through its ability to bind the enzyme folic acid reductase which reduces dihydrofolic acid to tetrahydrofolic acid. Tetrahydrofolic acid is necessary for the conversion of uracil deoxyriboside to thymidine. If methotrexate acted in our system through its ability to bind folic acid reductase, one might have expected a detectable drop in the total body thymidine after 6 days' treatment with the drug, a course sufficient to block the development of contact sensitivity. This might then cause an increase in the specific activity of thymidine-^{3}H present in the immunoblasts, 1 hour after injection, and a resultant increase in the concentration of labeled thymidine incorporated into the DNA. No such increase was found; in fact, the grain counts over immunoblasts 1 hour

after the injection of thymidine-^{3}H were the same whether the animals had been treated with methotrexate or not.

We therefore decided to see whether any effect could be detected on protein synthesis by examining the ability of these cells to incorporate uridine-^{3}H as an indicator of RNA synthesis and leucine-^{3}H as an indicator of protein synthesis itself. A slight, though significant drop in the incorporation of both uridine-^{3}H and leucine-^{3}H was found when the animals were treated with methotrexate. There was a 60% drop in the grain count over immunoblasts as shown by radioautography, 1 hour after the intravenous injection of uridine-^{3}H, and a 56% drop in the incorporation of leucine-^{3}H.

We also looked at the effect of methotrexate on four enzymes which could be detected easily by histochemical means, namely glucose-6-phosphate dehydrogenase, adenosine triphosphatase (ATPase), alkaline phosphatase, and acid phosphatase. A marked increase in both glucose-6-phosphate dehydrogenase and alkaline phosphatase and a slight increase in ATPase activity was detected in the draining lymph nodes on the fourth day after the application of oxazolone to the skin. However, in sensitized animals treated with methotrexate, the activity of these enzymes remained at the same level as that found in normal nodes. When imprints of sensitized nodes were incubated *in vitro* with 100 μg/ml methotrexate added to the incubation medium, a similar reduction in activity was found. Methotrexate, however, had no effect on the increase in the number of small lymphocytes containing lysosomes, as assessed by acid phosphatase staining, and in the number of lysosomes per cell that occurs in the local lymph node as a result of sensitization with oxazolone (Diengdoh and Turk, 1965).

Glucose-6-phosphate dehydrogenase is one of the enzymes which take part in the pentose shunt pathway of carbohydrate metabolism and is involved in the metabolism of pentose sugars which are implicated in the synthesis of RNA. It seems possible therefore to speculate whether the main effect of methotrexate on immunologically active cells in guinea pig lymph nodes could be on RNA synthesis through the pentose shunt pathway. Probably the drug has little effect on folic reductase and DNA synthesis in this system. The effect on alkaline phosphatase and ATPase could be a primary effect or a result of a secondary effect on protein synthesis.

It is also of interest that Vogel *et al.* (1963) have found that *in vitro* both folic acid and methotrexate inhibited a number of dehydrogenases, including glucose-6-phosphate dehydrogenase. Folinic acid does not have this effect and, therefore, folic acid would not have this effect *in vivo* because it is readily reduced to the tetrahydrofolic derivative. These

authors believe that the effect seen is due to the chelation of zinc, a trace metal important at the active site of certain dehydrogenases, to the pteridine moiety of methotrexate or folic acid.

No changes have been found in the ultrastructure of cells in the draining lymph nodes between the fourth and sixth days after the application of oxazolone on the skin while the animals were being treated with methotrexate (de Petris *et al.*, 1966).

The failure to find an effect of methotrexate on immune mechanisms in the guinea pig that could be accounted for by the binding of folic reductase is of interest in relation to the work of Berenbaum (1964). In this work it was found that increased homograft survival could be produced by the use of what would have been a toxic dose of methotrexate. However, the toxic effects of methotrexate were inhibited by treating the animals with folinic acid 24 hours after each dose of methotrexate. It could be, therefore, that the toxic effects of methotrexate on other tissues was through its effect in binding to folic reductase, whereas its effect on immune mechanisms was on a totally different process.

B. The Homograft Reaction

The effect of immunosuppressive agents on the cellular changes which occur in lymph nodes during homograft rejection have been studied in the rabbit by Scothorne (1956) and André *et al.* (1962). Scothorne (1956) found that he could prolong the survival of skin homografts in rabbits by treatment with 10 mg cortisone injected systemically each day. However, although the grafts appeared healthy, they failed to heal into the graft bed and also to become vascularized. When graft survival was prolonged due to systemic treatment of the animal with cortisone, there was a significant reduction in the appearance of immunoblasts in the local lymph node; this could be taken as an indication of a reduced proliferation of lymphocytes. It is likely that the effect of cortisone was not a central effect on the graft but a peripheral effect, namely, an inhibition of the formation of granulation tissue between the graft and its bed. This would stop vascularization and, as a consequence, either absorption of antigen would be impaired or lymphocytes would fail to enter the graft and become sensitized in the periphery (Medawar, 1965).

André *et al.* (1962) also investigated the effect of 6-mercaptopurine and thioguanine on the cellular response in the draining lymph node during homograft rejection. Both these drugs reduced the number of immunoblasts present in the lymph node. However, in these experiments the graft healed in normally so that the effect of the drugs must have been a central one. It is of interest that the first sign that an animal had become

resistant to the drug and had started to reject its homograft was the appearance of immunoblasts in the regional lymph node.

More recently, we have been studying the effect of thalidomide on the homograft reaction in the mouse (Turk *et al.*, 1966). The immunosuppressive action of this drug is of special interest because it was found necessary to treat the donor graft with the drug either *in vivo* or *in vitro* as well as treating the recipient after grafting, to obtain a significant effect. The dose used was 25 mg/mouse/day to both donor and recipient starting 5 days before grafting (Hellmann *et al.*, 1965). As has been mentioned earlier, immunoblasts were present at highest concentration in the draining lymph node 7 days after the application of C57BL skin to C3H/He mice. On this day, there was an average of 100 immunoblasts in four adjacent microscopic fields, each measuring 125 μ diameter, in the paracortical area of the lymph node. If both the donor and the recipient were treated with thalidomide, the average number of immunoblasts dropped to 16 in four similar adjacent fields. If recipients alone were treated, the drop was only to 33 and if donors only were pretreated with the drug, the drop was only to 49 cells in the same area. It would therefore appear that thalidomide could have a peripheral effect on the graft as well as a central effect. Thalidomide has been found to acylate naturally occurring diamines (Fabro *et al.*, 1965). It could therefore be expected to bind onto proteins. The skin has been found to have a high content of thalidomide 6 days after the end of 28 days' treatment with the drug (Faigle *et al.*, 1962). There must be a significant amount of drug present in the skin taken for grafting after the donor has been treated for 6 days with the drug. The immune process could therefore be inhibited by an effect on lymphocytes passing through the graft as part of the process of peripheral sensitization. This could be caused by a direct immunosuppressive effect or by the drug blocking antigenic sites on the transplantation antigen. However, a more conventional explanation might be that small amounts of drug are released from time to time and pass down to the regional lymph node where it can exert a direct immunosuppressive effect on the differentiation of small lymphocytes into immunoblasts.

III. CONCLUSIONS

The effect of various drugs on the cellular events which occur during the induction of delayed hypersensitivity are summarized in Fig. 1. It can be seen that a number of effects can be ascribed to many of the compounds studied. In most cases, however, it cannot yet be stated with certainty whether one effect is the cause of another. Evidence appears to exist for an effect of methotrexate on immunological mechanisms other than those described on other proliferating tissues. This emphasizes the

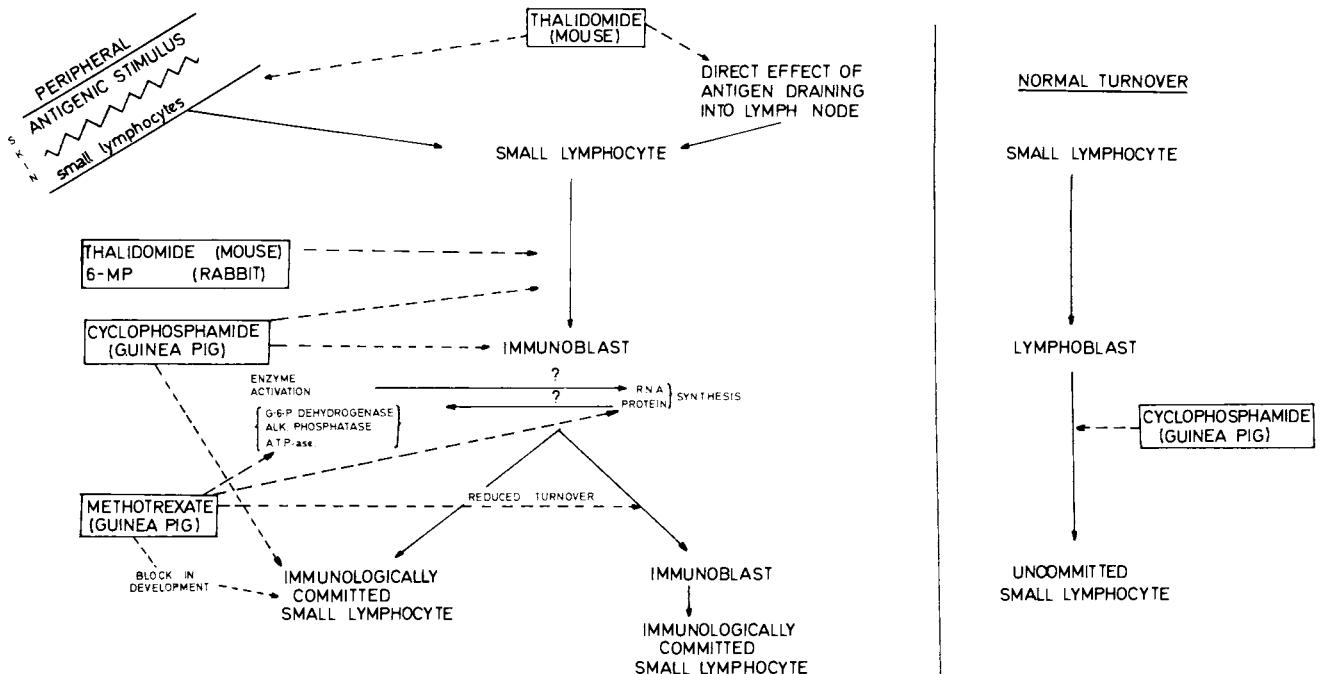

FIG. 1. Effect of immunosuppressive drugs on cell differentiation during the induction of delayed hypersensitivity.

point that caution is necessary in drawing conclusions about the action of a drug on one tissue from experiments on another.

A study of the mechanism of action of immunosuppressive drugs is a valuable tool in providing us with more information about the biochemical pathways involved in the mechanism of immunological reactions. At the same time we can learn more about the diverse action of these compounds on different tissues. It would appear that biochemical pathways vary not only from species to species, but from tissue to tissue, and this is well brought out by the different action of immunosuppressive drugs in different species as well as by the variation in their effect on different tissues in the same animal.

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DISCUSSION

DR. CLARK: In view of the hypothesis that in delayed hypersensitivity antigen-sensitive cells are stimulated peripherally at the site of the antigen, I wonder what is the state of the postcapillary venules in the draining lymph nodes? These vessels have been suspected of being the site where lymphocytes leave the bloodstream to enter the lymph nodes. If lymphocytes are coming from the periphery, presumably they would not use this route.

DR. TURK: We have not studied the postcapillary venules. We have, however, other data which are consistent with peripheral stimulation. The small lymphocytes in lymph nodes draining an area of application of a sensitizer have an increased number of acid phosphatase-staining granules in them which may be phagosomes containing antigen as well as hydrolytic enzymes. The hypothesis of peripheral sensitization in delayed hypersensitivity was suggested by Medawar (1965) *Brit. Med. Bull.* **21,** 97.

DR. MIHICH: You showed that there was a significant decrease of the immunoblast response in the regional lymph nodes of animals grafted with skin from donors treated with thalidomide, yet the survival of the graft was not prolonged. How do you explain this apparent inconsistency?

DR. TURK: I suspect one can have a drop of immunoblasts to one-third of those seen normally without obtaining any significant increase in graft survival. When one gets a drop to 10% of those seen normally, one begins to see increased graft survival, from a median of about 11 days to that of 15 days. One needs to treat both the donors and the recipients to get this, probably because the graft needs to have continuous treatment with the drug over a period of time not only when it is in the donor but also when it is in the recipient. A significant drop in immunoblasts in the lymph node is probably the most sensitive indication of a decreased immune response.

DR. ŠTERZL: I would estimate that in your preparations the number of cells incorporating tritiated thymidine after primary sensitization was higher than 1% of all lymphoid cells. In comparison, the highest proportion of antibody-producing cells during a secondary response is about 1–5% of all lymphoid cells. Since the identification of cells as immunoblasts was based primarily upon their ability to incorporate labeled precursors of DNA, I wonder whether all the metabolically active cells observed were responding specifically to the antigen. Could at least part of the proliferative activity found be related to a nonspecific response? The specificity of the response may not be necessarily proved by data obtained in tolerant animals, in which nonspecifically stimulating factors released after contact of sensitized cells with antigen [Dutton and Harris (1963) *Nature* **197,** 608] are absent.

DR. TURK: In lymph nodes draining a site of contact sensitization there is in fact a fantastic number of cells incorporating tritiated thymidine. On the fourth day, when this reaction is at a peak, one finds that up to 20% of the cells are in this state in the paracortical area of the lymph node. As early as 48 hours after sensitization, if one looks at a histological preparation, not at an imprint, one can observe that there are already 10% of blast cells in the paracortical area. As to the immunological specificity of this response, I think that the following experiment should be mentioned. Dinitrochlorobenzene (DNCB) and oxazolone were used as two noncross-reacting antigens. Animals were made tolerant to DNCB by a standard technique, namely by injecting a large dose of dinitrobenzene sulfonic acid i.v. on two occasions at a 2-week interval. Two weeks later, sensitization was attempted.

In the nontolerant animals, at 4, 5, and 6 days after DNCB sensitization, blast cells were about 9%, 6%, and 5% of all cells, respectively. In the tolerant animals they were only about 1% all the way through. The animals tolerant to DNCB were tested with oxazolone, and were capable of reacting to this antigen to the same extent as the nontolerant controls. Thus tolerance did not block the response to a noncross-reacting antigen.

DR. LANDY: Dr. Šterzl has in part asked my question but perhaps I can word it another way: Are the immunoblasts, which Dr. Turk describes as reaching such a great peak number on day 4 after skin sensitization with oxazolone or DNCB, specifically reactive with the sensitizing agent? The tolerance data discussed are very convincing evidence for the overall specificity of the phenomenon but in no way deal with the issue of the specific commitment of the enormous number of blast cells observed in the draining lymph node. Should these cells react *in vitro* with oxazolone or DNCB, this would assure beyond any doubt that they were specifically induced by the antigen.

DR. TURK: You are asking really whether we are demonstrating that the immunoblast response is the specific process in delayed sensitivity. All we can say is that this process is associated with the development of delayed sensitivity and that, in the absence of any other phenomenon, this is the only response that we can see bearing on immunity. It is true that we cannot prove that this process is the direct cause of delayed sensitivity. Nevertheless Sandy Stone and I observed that if the draining lymph nodes are taken out prior to the fourth day after sensitization, the sensitization process itself was blocked. Thus these lymph nodes are the site of the specific sensitization response. If these lymph nodes are taken out on the fourth and fifth day, and are transferred into other animals, they will carry on the sensitization process adoptively. I must agree that, to a certain extent, we are working by inference.

DR. SANTOS: I wonder whether dose-response experiments were performed with methotrexate and cyclophosphamide. Is it possible that the dose of cyclophosphamide used was relatively greater than that of methotrexate? Also, has Dr. Turk looked at the effect of methotrexate or cyclophosphamide given at different points in time, for example on day 4, when the large immunoblasts are present?

DR. TURK: The doses used were the minimum that would block delayed sensitivity, namely 5 mg every other day for methotrexate and 10 mg daily for cyclophosphamide in guinea pigs weighing about 450 gm. In the experiments described here, the dose schedule started 2 days before sensitization and continued throughout the experiment. In previous studies, however, complete block of the sensitization was observed also when the onset of the drug was delayed up to the fourth day after sensitization. If the drug was started between the fourth and eighth day, a partial block of sensitization was seen. Very little or no effect was noted when the drug was administered starting after the eighth day. Passive transfer of delayed sensitivity was performed by transferring the sensitized cells and skin testing at the same time. The reaction was evaluated 24 hours later. Therefore this system is different from the adoptive transfers usually done in inbred animals. In this system, animals which had been on methotrexate for 7–8 days showed unimpaired ability to be passively sensitized. A 50% drop in the ability to respond was always observed in animals treated with cyclophosphamide. My interpretation is that cyclophosphamide has an anti-inflammatory effect as well as an antiimmune effect. This may be true of a number of immunosuppressive drugs, including possibly 6-mercaptopurine, which are also acting peripherally on the ability of the animal to produce the inflammatory response associated with delayed hypersensitivity.

DR. SCHWARTZ: What happens to immunoblasts that are blocked in their development by methotrexate? Have you done any electron microscopic studies on immunoblasts from methotrexate-treated guinea pigs to determine what they look like?

DR. TURK: After treatment with methotrexate there is a decreased turnover of immunoblasts. Normally 24 hours after the administration of thymidine-^{3}H immunoblasts have a grain count one-third of that seen 1 hour after thymidine-^{3}H. In animals treated with methotrexate, the grain count, 24 hours after thymidine-^{3}H is increased to one-half of that found 1 hour after labeling, the same as that found in small lymphocytes. Some of the immunoblasts would seem to divide twice in a 24-hour period in normal sensitized animals not on the drug. However, when exposed to methotrexate, they would appear to divide on the average only once in this period of time. This may be also the reason why one does not detect any increased number of immunoblasts in that period after the fourth day peak. Electron microscopic studies in animals treated with methotrexate failed to reveal any difference in the immunoblasts, in the intermediate cells, or in the lymphocytes in lymph nodes draining areas of sensitization with oxazolone.

DR. BERENBAUM: Your observation that methotrexate inhibits three histochemical reactions *in vitro* is intriguing. Most people think that folic acid antagonists *in vivo* do not do anything important other than binding to folic reductase. It would be interesting to confirm your findings with orthodox enzymologic methods. Perhaps so much methotrexate sodium salt was added that the pH of the histochemical reagent mixture changed or the compound complexed with one or other of its constituents purely on a chemical basis and in a way irrelevant to its biological actions?

DR. TURK: The *in vitro* results of others parallel our *in vivo* results. Vogel *et al.* (1963) *Biochem. Biophys. Res. Commun.* **10,** 97 studied the effect of methotrexate on dehydrogenases in orthodox biochemical systems and found that the drug blocks dehydrogenase activity *in vitro.*

DR. WERKHEISER: I would like to make a comment about the comparative biochemistry of methotrexate. Most of the studies from which we have gained biochemical information on methotrexate have been in man, mouse, and rat, where this drug is exceedingly toxic and is a lethal agent to mucosal and marrow elements. In the guinea pig and in the rabbit, animals with a large cecum and a huge bacterial flora, this drug is not really toxic. The doses used in guinea pigs by Dr. Turk would have been more than lethal in other animal species. Presumably, this lack of toxicity in guinea pig and rabbit is due to the generation by the intestinal bacteria of tetrahydrofolate cofactor forms which protect the animals from the drug. In fact, treatment with folinic acid 24 hours after the administration of methotrexate will protect the sensitive animals. It may well be that the data obtained in studies of the effects of the drug on the immune response are related to secondary phenomena which are unmasked by eliminating the toxic response usually observed with this drug, which is related to the inhibition of folate reductase. A minor comment is concerned with the expectation of Dr. Turk that treatment with methotrexate will lower the body pool of thymidine. The pool sizes of thymidine are quite small. Methotrexate makes it impossible for the DNA synthesis to go on and to use the thymidine. Unless thymidine is recycled through the pool, DNA synthesis does not go on. I do not think one can reasonably expect a great reduction in the size of the body pool of thymidine by treatment with methotrexate.

DR. TURK: I agree with Dr. Werkheiser that in studying the action of immunosuppressive drugs one cannot extrapolate from one species to another. From studying the action of these drugs in one species one can only get an idea of how an immunesuppressive drug can act and at which levels in the sensitization process. This does not mean that the drug will have the same effect in another species, and, if effective, it may not be at the same dose level or at the same level in the sensitization process.

DR. BERENBAUM: Dr. Werkheiser mentioned that one may be able to show the immunological effects of methotrexate in the guinea pig with ease because this species is not very susceptible to the toxic action of the drug. However, it is quite easy to show immunological

suppression by methotrexate in sensitive species such as the mouse [Berenbaum and Brown (1965) *Immunology* **8,** 251] or dog [Thomas *et al.* (1962) *Blood* **20,** 112]. Brooke [(1960) *Transplant. Bull.* **26,** 453] and Meeker *et al.* [(1960) *Ann. N.Y. Acad. Sci.* **87,** 203] showed that rabbits, which were given moderately toxic doses of folic acid antagonists still produced normal titers of antibodies against bovine γ-globulin and sheep blood cells and rejected skin grafts at the normal rate. Even in the guinea pig one can increase the degree of immunosuppression obtainable with methotrexate by giving folinic acid [Berenbaum (1964) *Lancet* **2,** 1362]. Therefore, among various animal species there does not seem to be a good correlation between the ease with which immunosuppression may be produced and susceptibility to methotrexate toxicity. Other factors must be involved in the phenomena observed.

Specialized Cell Function in the Lymphoid and Reticuloendothelial Cell Series

G. L. ADA

Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia

There are many ways in which an immune response may be manifested, and the experimental approaches necessary to investigate the mechanism involved vary considerably in complexity. At first sight it might be thought that one of the simplest models of a naturally occurring immune response would be to study the induction of antibody formation after injecting a simple protein as antigen into an animal. In fact, it has proved to be exceedingly difficult to unravel the processes which take place. The knowledge we now have of the inductive process of antibody formation is reminiscent of the situation which existed 10 years ago in the field of virus multiplication. Some of the basic findings were then just emerging, such as the demonstration of the infective nature of viral ribonucleic acid (RNA) preparations. Similarly, key pieces of the jigsaw puzzle of immune induction are now beginning to appear. Two of the more important aspects which have emerged in the past few years and which should lead to some of the missing links are the indication not only that the immune response to a foreign substance involves a partnership of two different classes of cells but also that within each class of cells there are divisions of speciality. This article will largely be concerned about this involvement and a considerable proportion of it will be based upon results obtained in the past few years at the Walter and Eliza Hall Institute. It is not a comprehensive survey. The first and major approach has been to use antigen as a tool to study cells of the reticuloendothelial and lymphoid systems. Another line of investigation pursued has involved a comparison of the immunological potential of lymphoid cells from different animals and from

various tissues while developing means of isolating the different classes of lymphoid cells to allow a biochemical study of their properties.

I. DEMONSTRATION OF DIFFERENCES IN ANTIGEN HANDLING AMONG CELLS OF THE RETICULOENDOTHELIAL SYSTEM

Two reasonably successful methods have been applied to the study of antigen in the immune response in the past few years. The first approach is essentially to study the end product of an animal's overall response to antigen by determining the amount and characteristics of the antibody produced, following entry of antigen into the animal. This method has used antigens as diverse in properties as bacterial virus or synthetic compounds. These results will be described elsewhere in this Symposium.

The second method which attempts to investigate the mechanism whereby antigen causes induction of antibody formation is to chart the fate of each molecule or particle of antigen which enters the lymph node or spleen and to relate the induction process to the pattern of antigen distribution within cells of particular tissues. Each approach has its limitations, but to a great extent the two approaches complement each other. I will be dealing in some detail with this second aspect. The antigen chosen to study this aspect will determine very largely the success of the project, and some space is devoted to a consideration of the reasons for this.

A. Choice of Antigen

Several considerations should be borne in mind when choosing an antigen for tracer studies. One consideration is to choose a substance which will cause the formation of antibody for which there is a sensitive method of estimation. Techniques such as measuring the agglutination of tanned and antigen-sensitized red cells (Boyden, 1951), or the Farr test for the determination of antigen-binding capacity of serum (Farr, 1958), greatly increased the sensitivity of estimation of antibody and are applicable to many antigens. More sensitive methods for the estimation of antibodies to some antigens are available, notably neutralization of bacterial viruses (Uhr, 1964), or the bacterial immobilization test (Mäkelä and Nossal, 1962). Another important point is the dose of antigen which is to be administered. The fewer molecules or particles of antigen which are injected consistent with obtaining a particular effect such as detectable antibody synthesis, the less difficulty will be experienced in interpreting the information received. It is preferable to think in terms of molecules, rather than in milligrams of antigen. If it can be shown that antibody induction has occurred in a lymph node due to retention of antigen in that node at a level of 1–10 rather than 100–1000 molecules of

antigen per cell, this itself limits the number of ways in which antigen can be postulated to be acting.

A third major consideration which dictates the use of a particular antigen is the method to be used in tracing the antigen.

1. Detection of Antigen

a. Biological Properties. The infectivity of a virus and the enzymic activity or serological properties of a protein are methods which have been used with varied success to follow the fate of antigen in the host animal. Sensitivity of tracing has depended on the preparation used. Viruses may be estimated with great sensitivity if one particle is infectious; a method of assay for at least one enzyme, β-galactosidase, has been developed which allows the detection of one molecule (Rotman, 1961); some serological techniques such as passive cutaneous anaphylaxis are also very sensitive (Humphrey and White, 1964). However, none of these methods has allowed the detailed following of the molecules or particles of antigen within tissues. The direct immunofluorescent technique introduced by Coons *et al.* (1942) has made a most valuable contribution to our knowledge of the distribution of antigen after injection. The low sensitivity of the immunofluorescent technique is perhaps its major drawback. There is little information about the theoretical limit of sensitivity but Miller and Nossal (1964), in demonstrating the presence of antigen in the lymphoid follicles of rats found it necessary to inject a 2-mg dose of flagella for consistent results by the immunofluorescent technique. Only a fraction of a microgram of flagella needed to be injected to show the same distribution if radioautography was used.

b. Physical Properties. Macromolecules with specific morphological shape or size may be visually detected within tissues. Erickson *et al.* (1953) followed the distribution of tobacco mosaic virus in tissue sections by electron microscopy. More recently, Wellensiek and Coons (1964) and others have similarly followed the distribution of ferritin which, because of the density of its iron core, was readily identified in electron micrographs. The procedure is very sensitive but two aspects must be remembered. First, the iron portion and not the antigenic protein portion is detected by this procedure, and second, a section for electron microscopy will represent a small percentage of the total volume of a single cell. Particles will be detected with certainty only if the cells concerned contain on the average some hundred of particles.

c. Isotopic Markers. This has proved to be the method of choice for most work on antigen distribution (Campbell and Garvey, 1963), but an

absolute requirement is the availability of purified antigen. Five considerations dictated the choice of isotope. (1) Ease of estimation—gamma emitters are simpler to estimate than beta emitters and after measuring radioactivity the sample may be subsequently used for other purposes. (2) As radioautography will be the major technique used for studying the localization within tissue areas, a beta emitter must be used. Furthermore, the lower the energy of the beta emission, the better the resolution which will be obtained. (3) In order to obtain effective isotopic substitution, the isotope should be available as a carrier-free preparation—i.e., most atoms of the element in the preparation are radioactive. (4) The specific activity of an isotopically labeled substance is measured as the disintegration per unit of time per unit of weight of the substance. A protein labeled with 1 atom of iodine-125 (half-life 60 days) has the same specific activity as a protein labeled with 35 atoms of tritium (half-life, 12 years). Obviously, labeling of a protein to very high specific activity may only be achieved with an isotope of short half-life. (5) It is preferable that the isotope be introduced without changing the chemical properties of the antigen—e.g., biosynthetically.

No isotope satisfies all the criteria. Iodine-125 (satisfying 1–4) is the best compromise, with tritium (satisfying 2, 3, and 5) the next most favorable one. These criteria limit the type of substances suitable as antigens. The use of polysaccharides labeled to high specific activity has not yet been reported. The use of radioactive iodine has been restricted to proteins, natural or "synthetic," containing tyrosine or to a carrier substance substituted with iodinated haptenic groups. Synthetic antigens can be prepared which have a high concentration of tyrosine, and in at least one case labeling with both tritium and ^{125}I has been achieved (Humphrey, personal communication). They are, however, usually of low immunogenicity compared with many natural proteins.

2. *The Immune Response to Antigen*

Another major criterion which decides the choice of antigen is the type of immune response which is elicited following its injection. In our work several antigens have been injected into rats but I will refer mainly to three. (1) The soluble protein flagellin, from *Salmonella adelaide,* which is obtainable in pure form by the depolymerization of flagella particles. It causes a response consisting entirely of IgG (Nossal *et al.,* 1964a). (2) The particulate forms of this antigen were used, either flagella particles or a polymer which closely resembles flagella and can be readily made from flagellin. Both particulate forms give an early IgM response which is closely followed by IgG production. (3) A poor antigen, human serum albumin (HSA) which even in milligram doses in saline does not yield

detectable antibody formation in the rats although high titers of antibody are produced after injection with adjuvants.

B. Entry of Antigen into Tissues

1. Distribution Between Tissues

Antigen may be injected into an animal by various routes. Discussion will be limited to two—via the hind footpad, and intravenously. Injection via the hind footpad has been used particularly where it was required to study the entry and distribution of antigen into a draining lymph node, in this case the popliteal nodes. In untreated 10–12-week-old rats kept under hygienic conditions, these nodes did not contain any germinal centers resulting from previous infection, so that the morphological changes which occurred as a result of antigenic stimulation were accurately followed. The amount of antigen trapped by a node varied according to the antigen used. A disadvantage of footpad injection was the prolonged time of antigen drainage from the injection site so that the kinetics of antigen-trapping by a node were difficult to evaluate. Injection of antigen intravenously does not involve a prolonged drainage time.

Irrespective of the route of injection, the lymphoid system trapped only a small percentage of the injected antigen and the amount of antigen trapped was even less if a poor antigen such as HSA was used. For example, 4 hours after the injection of iodinated flagella into the hind footpads of rats, all lymph nodes plus spleen contained about 4% of the radioactivity injected. The thymus contained trace amounts only, (Ada *et al.*, 1964b).

Most antigen was trapped by tissues for which there was no strong evidence of intimate participation in the immune response, such as liver. Studies of the role of antigen in the induction of antibody formation were confined to tissues such as the lymph nodes and spleen, where it was known that antibody formation occurred, and therefore it was a reasonable assumption that the immediate fate of antigen within those tissues was of direct relevance to the problem of antibody formation.

2. Entry of Antigen into Lymph Nodes and Spleen

All lymphoid organs are a relatively heterogeneous collection of cells, and techniques for antigen distribution studies were required which allowed detection of antigen at least in areas of the node and preferably in individual cells. Three techniques were used—immunofluorescence; radioautography; and, to a lesser extent, biochemical fractionation of the tissue concerned. Radioautography was used to study the entry of antigen into lymph nodes and spleen.

In the popliteal lymph node, antigen was first detected in the circular sinus in the node and then in macrophages which lined the medullary sinuses, where the concentration of antigen reached a maximum level at about 2 hours after injection. At 2 hours, antigen also appeared in the primary lymphoid follicles which were present in the cortex of the node. The follicles have been shown to consist of a web of reticular cells which interdigitate between densely packed small lymphocytes. Antigen appeared to be between, rather than in, the lymphoid cells, as shown by radioautography in the light microscope (Nossal *et al.*, 1964b). Immunofluorescent techniques (White, 1963; Miller and Nossal, 1964) indicated quite clearly that most of the antigen was on processes between the lymphocytes.

Powerful immunogens such as flagella and flagellin were retained in both the medullary and follicular areas of lymph nodes for considerable periods of time. Particulate antigens showed this localization most dramatically. A soluble antigen such as flagellin was more readily diffusible throughout the node but after the first 24–48 hours, when most "background labeling" had disappeared from the node, specific retention was obvious in both the medullary macrophages and lymphoid follicles. HSA, a poor antigen, also readily diffused throughout the node, but cleared away very rapidly, leaving a small amount in medullary macrophages and trace amounts only in the follicles.

The entry of antigen into and distribution in the spleen after intravenous injection was more complicated than entry of antigen into lymph nodes (Nossal *et al.*, 1966). A few minutes after injection of radioiodinated polymerized flagellin, label was found within the red pulp and in a zone marginal to the white pulp. Within 1 hour after injection, label in the red pulp decreased and at later time points, the red pulp area was largely free of radioactivity. Label in the marginal zone external to the white pulp, however, increased. After 1 hour, antigen appeared to migrate from the marginal zone across the marginal sinus into the white pulp and was found at 2 days in the follicular web of germinal centers in the white pulp. Label was never found in the white pulp immediately surrounding the central arteriole. The results have been interpreted as a purposeful movement of antigen-carrying cells, rather than movement of antigen by itself.

3. Site of Trapping of Antigen in Tissues

What cell or cells were involved in the antigen-trapping process? What was the fate of antigen within this cell? The resolution in standard radioautographs of tissue sections containing ^{125}I was sometimes insufficient to establish with certainty the cell which contained the isotope. However, radioautographs examined in the electron microscope (high resolution

radioautography) showed not only which cell was involved but also the physical relationship of antigen to this cell (Mitchell and Abbot, 1965). When iodinated flagella was injected into the footpads of rats, antigen which localized in the medulla of popliteal nodes appeared first on the cell membrane of the medullary macrophages and was then rapidly engulfed by the cell so that at times later than a few hours after the injection much of the antigen was present in the subcellular components. Some was present in small vacuoles which appeared to be free in the cytoplasm but by 24 hours most antigen was in inclusions which appeared to be either phagosomes, lysosomes, or phagolysosomes. A different antigen distribution was found in the follicles; the antigen appeared to be present either on membranes of cells which were probably reticular cells or in or on narrow cellular processes which were between lymphocytes. It is inferred that these cellular processes were the arms of reticular cells. The processes themselves were relatively free of structures resembling lysosomes but sometimes contained vesicles. One form of reticular cell, the tingible body macrophage, which occurs in the lymphoid follicle (Flemming, 1885), was rarely found to contain antigen at any time after a single injection of antigen. Thus in the primary response, two types of reticular cells were shown to be involved directly in antigen trapping.

C. Mechanism of Antigen Trapping

Many workers have found that such factors as the size and the net electrical charge of the antigen injected into an animal influenced the trapping and retention of antigen in the lymph node. In our system, two particulate antigens, flagella and polymerized flagellin, after injection into the hind footpads of rats were trapped and retained in the popliteal and aortic lymph nodes to a greater extent than was soluble flagellin (Ada *et al.* 1964b). A study has been made of the effect of various procedures which, when applied to the animal to be injected or to the antigen, have affected not only the trapping and retention of antigen but also the site of localization of the antigen in the lymph node. Three results suggested that opsonins were involved in localization of antigen within the lymphoid follicles. When rat red blood cells were labeled with radioactive iodine and injected into the hind footpads of rats, the label was found in the medulla but not in the follicles of the popliteal nodes. The label was found both in the medulla and lymphoid follicles of the node when labeled chicken red blood cells were injected into rats. This difference in behavior was attributed to the fact that rats are known to contain a natural antibody to chicken red blood cells. If flagellin was injected via the hind footpads of rats previously immunized with this antigen or into rats to which rat antiflagellin antibody had been previously passively adminis-

tered, there was a two to five-fold increase in the retention of antigen in the draining lymph nodes. When the number of grains over the medullary and follicular areas in radioautographs of the popliteal nodes were counted, it was seen that most of the additional antigen retained in the nodes was present in the follicles (Nossal *et al.*, 1965b). If rat hemoglobin or rat serum albumin was labeled and injected via the hind footpads of rats, the small amount of these substances which was retained by the popliteal node was present in the medulla of the node. Rat γ-globulin, however, when labeled and injected into another rat, was localized in both the medulla and in the follicles (Ada *et al.*, 1964a). These early results prompted a more detailed study of the effect of various treatments to labeled antigen on the subsequent trapping of the antigen in the lymph nodes after footpad injection into rats.

1. Trapping of Modified Human Serum Albumin in the Lymph Nodes of the Rat

Human serum albumin (HSA) after injection into the hind footpads of rats was very poorly trapped and retained in the lymph nodes. Examination of radioautographs of sections of popliteal nodes showed that the antigen which was retained was present in the medullary macrophages. The rationale of the following experiments was to see whether the HSA could be treated in various ways to cause preferential localization in the follicles or medulla of the node.

a. Preferential Localization of Human Serum Albumin in the Lymphoid Follicles. Following the earlier results, a study was made of the fate of antigen in antigen-antibody complexes after injection into rats. Follicular localization of the antigen in the lymph nodes was consistently observed. HSA labeled with radioactive iodine was premixed with excess antibody, previously prepared by injection of HSA with complete Freund's adjuvant into other rats. After injection of the complex into rats, between ten and forty times more antigen was retained by the draining lymph node (Ada and Lang, 1966). It made little difference to the amount of antigen retained whether the antigen after mixing with antiserum was injected immediately or after standing for 24 hours. A similar but not so dramatic picture was obtained if the rat was injected with antiserum alone some hours before injection of the iodinated antigen. A more detailed examination showed that 1 hour after injection of the antigen-antibody complex, the medulla of the node was heavily labeled but the follicles only lightly labeled. At 24 hours both medulla and follicles were heavily labeled. About 3 days after injection the medulla was found to contain only trace

amounts of label, whereas the lymphoid follicles were heavily labeled and remained so for another 3–4 days.

Follicular localization of the antigen appeared to be because of the associated antibody, as antigen which previously had not localized in the follicles of the node was now strongly localized. This property of the antibody was not species specific—labeled HSA mixed with antibody to HSA made in the rabbit or in the mouse localized quite well in the follicles of the rat. HSA mixed with antibody made in the rat and injected into mice localized in the follicles of the mouse lymph nodes. Both the IgM and IgG fractions of rat anti-HSA antibody when mixed with labeled HSA caused follicular localization of the HSA in rat lymph nodes. The efficiency of the two types of antibody in causing this follicular localization, when compared on a molecular basis, has not been established.

Preliminary results have indicated that the net electrical charge of the antigen played an important role in follicular localization. HSA was modified so that the electrophoretic mobility of the protein at pH 8.2 was reduced—the modified HSA migrated with an electrophoretic mobility between that of β and γ-globulin. This material now strongly localized in the follicles as well as in the medulla. By contrast, rat γ-globulin was modified so that the electrophoretic mobility was between that of rat albumin and rat α-globulin. This modified globulin was trapped in the medulla but not in the follicles. Experiments are in progress to determine whether modified rat anti-HSA γ-globulin, after reaction with labeled, unmodified HSA will cause follicular localization of the HSA.

b. Preferential Localization of Human Serum Albumin in Medullary Macrophages of the Rat Node. An increased retention of labeled HSA in the lymph nodes of rats occurred if the HSA in dilute solution (0.1 mg/ml) was heated at 90°C. for 15 minutes before injection. A more dramatic effect was observed if a concentrated (10 mg/ml) solution of labeled HSA was heated so that flocculation of the protein occurred and the floccules were injected into rats. Compared with labeled but unheated HSA, a ten times greater amount of the flocculated labeled antigen was retained in the lymph nodes. Twenty-four hours after injection of the floccules, radioautographs of sections of the rat popliteal and aortic nodes showed that the trapped antigen was only present in the medulla of the node. Occasionally radioautographs demonstrated a higher number of grains over the lymphoid follicles and the cortex than was present as a general background, but localization of the antigen in these areas was minimal. This treatment, therefore, resulted in preferential localization of the antigen in the medullary macrophages.

In summary, modifications of HSA caused either by premixing HSA

with antibody or by direct physical or chemical treatment of HSA have resulted in increased retention of antigen in the draining lymph nodes. The distribution of antigen between the medulla and lymphoid follicles depended upon the particular treatment used.

c. Reaction of Antigen-Antibody Complexes with Cells. Evidence has been presented that the Fc fragment of the immunoglobulin molecule may be specifically involved when antigen-antibody complexes react with macrophages (Berken and Benacerraf, 1966). Bovine serum albumin when complexed with the Fab fragment on the Fab dimer prepared from anti-BSA antibody and injected into animals was eliminated more slowly from the blood than was bovine serum albumin complexed with intact anti-BSA antibody (Spiegelberg and Weigle, 1966). This result also implied that the Fc fragment of the antibody molecule might be involved in the reaction of antigen-antibody complexes with cells. Experiments are in progress to determine whether the Fc portion of the antibody molecule is involved in the reaction of antigen-antibody complexes with membranes of reticular cells in the lymphoid follicles of the node.

D. State of Antigen in Tissues

Radioautography, although an almost irreplaceable technique for this work, has limitations. Tissue sections or cells are usually fixed under conditions which allow the leaching out of molecules or fragments of antigen which are not bound to components in the cell (Mitchell, 1966). Techniques which allow retention in tissue sections of unbound and low molecular weight components have not yet been widely used. Radioautography, therefore, did not readily answer the following questions: To what extent was antigen free or bound to subcellular components in tissues? Could an accurate estimation of the amount of antigen present in the medulla and follicles of lymph nodes be obtained? An answer to this question was necessary before the role of antigen in the different areas of the node toward the formation of antibody could be evaluated. Was the premise that the grains in radioautographs were due to label attached to antigen valid? Could it be demonstrated that material associated with the isotope after extraction from the tissues was still specific antigen? Could techniques other than radioautography furnish evidence indicating the type of particle to which the antigen was bound? These questions were partly answered by biochemical procedures in association with radioautography.

1. Distribution of Antigen in Cellular Fractions

Although both spleen and lymph nodes are very heterogeneous in their cell population, a considerable amount of information has been

found out about antigen distribution by fractionating these tissues by standard biochemical procedures.

In brief, lymph nodes or spleen containing labeled antigen were homogenized in a Potter-type homogenizer and separated by differential centrifugation in sucrose into the following categories: (1) a nuclear debris fraction (8000 g/minute); (2) a large granule fraction (200,000 g/minute); (3) a cell supernatant fraction remaining after removal of (1) and (2). The amount of radioactivity in each fraction was then determined. In a typical experiment, the label was distributed as follows (Ada and Williams, 1966). At early time points after injection of iodinated flagella, most of the radioactivity in the nodes was recovered in the cell supernatant, but by 24–48 hours after injection, 50% of the radioactivity was present in the large granule fraction, and about 15% in the nuclear debris. By 3 days 70% of the radioactivity was in the large granule fraction, and for at least 2 months most radioactivity was recovered in this fraction. Over a period from 1 day to 2 months after injection of antigen, the proportion of radioactivity in the nuclear debris fraction increased slowly to about 20–25%. Most attention has been directed to the large granule fraction. This fraction would be expected to contain mitochondria, lysosomelike particles, pieces of membrane, perhaps some polysomes, and aggregates of these components.

2. *Isopycnic Centrifugation of Cellular Fractions*

To answer some of the questions posed above required separation of these components in the large granule fraction from each other. Small yields of relatively pure samples of particulate components such as lysosomes or mitochondria have been obtained from selected tissues—e.g., liver—by earlier workers, but the fractionation of whole tissues to obtain complete discrete fractions of each component in high yield has been very difficult. Some progress toward this end has been made by subjecting the large granule fraction of lymph nodes, removed at various times after footpad injection of antigen, to isopycnic centrifugation. The solute used to prepare the gradient was Urografin (Schering, A.G.).

Urografin appears to have a mild dispersive action and when applied to the large granule fraction of rat liver, kidney, spleen, or lymph node extracts, a better separation of the enzyme activities associated with the various components was achieved compared to the separation achieved on gradients made with either sucrose or dextran (Williams and Ada, 1967a,b). When a large granule preparation prepared from rat lymph nodes was placed on top or underneath a Urografin gradient (density range 1.065–1.200) and centrifuged to equilibrium, two main bands of opacity resulted. The upper band was diffuse and extended from density 1.108 to 1.144. The lower band was sharp and extended from density 1.174 to

1.186. Some types of particles were well separated. Potassium-dependent adenosine triphosphatase, regarded as a marker for cell membrane, peaked sharply at a density of 1.112. Two lysosomal enzymes, acid phosphatase and β-glucuronidase, peaked at densities 1.124 and 1.163 respectively, i.e., near the upper and lower extremities of the upper opacity band. Succinic dehydrogenase, a mitochondrial enzyme, was present in only small amounts in lymph nodes and the activity peaked at density 1.184, within the lower band of opacity.

Rats were injected with (1) heat-denatured HSA and the popliteal and aortic nodes removed 24 hours later when, judging from radioautographic evidence, all the antigen would be present in the medullary area; (2) an HSA-antibody complex and the popliteal and aortic nodes removed 3 days later when, judging from radioautographic evidence, the antigen would be present only in the lymphoid follicles. In each case, the large granule fraction from the lymph node was prepared, introduced on top of a gradient of Urografin, and centrifuged to equilibrium. In the first case, more than 95% of the radioactivity was present in a broad peak which encompassed the location of the two lysosomal enzymes, acid phosphatase and β-glucuronidase, in the gradient. If this fraction was submitted to cycles of freezing and thawing before centrifugation, most of the radioactivity was found to be in a nonsedimentable state. In the second case, mentioned above, 70% of the antigen sedimented as an antigen-antibody complex to the bottom of the gradient. The remaining 30% of the antigen was associated with lysosomes. The antigen which sedimented to the bottom of the gradient was considered to have been dissociated from the membrane by the action of Urografin. The sedimentation behavior of this antigen was unaffected by prior exposure to cycles of freezing and thawing, in contrast to the behavior of antigen present in lysosomes.

This technique was applied to a study of the proportion of labeled flagella in the medullary and lymphoid follicles of nodes at different times after injection of this antigen into rats. At all times up to 3 days after injection most antigen was found in the medulla of the node, the proportion in the lymphoid follicles ranging from 14 to 21% of the total antigen present.

3. State of Antigen in the Lymph Node

To what extent was isotope in the lymph node still associated with macromolecular material, and specific antigen?

a. Breakdown of Antigen. Within 24–48 hours after footpad injection into rats of iodinated antigen such as flagella or flagellin, most of the radioactivity injected was excreted in the urine if care was taken to

minimize retention of radioactive iodine in the thyroid. Most injected antigen was rapidly broken down. Rapid breakdown of antigen also occurred in the lymph nodes. After footpad injection of iodinated flagella, the level of radioactive low molecular weight material (assumed to be the breakdown product of antigen) in extracts of the popliteal and aortic lymph nodes reached a peak in the first 24–48 hours and then decreased. The maximum level reached was about 11% of the total radioactivity present, and at 10 days this had decreased to about 1% and remained low. When such nodes were fractionated and the proportion of radioactive, low molecular weight material was estimated in each fraction, the highest proportions were found in the cell supernatant and in lysosomes present in the large granule fraction.

b. Association of Radioactivity with Antigen in Vivo. This was tested by an indirect precipitin procedure (Ada and Williams, 1966). Lymph nodes from rats which had previously been injected with radioiodinated flagella or flagellin were fractionated, as described previously, and the different fractions solubilized by the use of mild alkali or detergent. Extracts containing isotope corresponding to about 100 pg of antigen were reacted with serum from a rabbit immunized against the original antigen. The amount of rabbit serum added was sufficient to bind at least 1 μg of antigen. The globulin in the added serum was then precipitated quantitatively by adding a slight excess of goat antiserum to the rabbit globulin. If the radioactivity was still associated with antigen (or presumably with an antigenic determinant), then the precipitate which formed after addition of all reagents was radioactive. The extent to which the radioactivity was found in the final precipitate was a measure of the proportion of the total radioactivity in the lymph extract associated with antigenic material. In view of the technical difficulties involved, the procedure probably yielded a minimum value. Most attention has been paid to the large granule fraction. In extracts of nodes from rats previously injected with flagella or flagellin, up to 80% of the radioactivity in the granule extract could be shown to be associated with specific antigen. Highest values were obtained with samples from rats given a second injection of antigen when it was known that there was increased follicular localization of the antigen. The results suggested that flagella and flagellin were retained in the lymphoid follicles of the node in such a manner that their antigenicity was preserved. It also seemed likely that some macromolecular material associated with isotope in the medulla of the node was also specifically antigenic. In separate experiments, no evidence was obtained that radioactive low molecular weight substances would still react with antiserum to the original antigen.

The mechanism of antigen removal from the follicles is not clear. The retention of antigens in the follicles varied considerably with the antigen used. Flagella was retained for a substantially longer period than flagellin, and flagellin in turn for a longer period than an HSA-antibody complex. The disappearance of antigen may not have been due to the movement of antigen-bearing cells, but rather to the ease with which these antigens were either removed by association with passing cells, or by enzymic attrition.

E. Antigen Localization in Tissues and Induction of Antibody Formation

Williams (1966) has described the behavior of injected antigen in rats during the first few weeks of life and has shown a close temporal relationship between the maturation of the antigen-trapping system and the ability to generate a prompt antibody response. In adult animals receiving the antigen dose used in our investigation, soluble antigens such as flagellin and HSA spread throughout the draining lymph node shortly after injection. Particulate antigens behaved differently and, at all stages after injection, localized specifically in macrophages and in follicles. The question of whether antigen, particulate or soluble, penetrated and was retained in lymphoid cells was examined in some detail in adult rats.

1. Does Antigen Directly Trigger an Antigen-Sensitive Cell?

Several possibilities existed. Antigen, without previous contact with reticular or phagocytic cells, might directly trigger the target cell and initiate the differentiation to plasma cells and antibody production. One or a few molecules (or particles) might be sufficient. The antigen might be in association only briefly with the cell or might penetrate the cell and remain in the cell during the differentiation cycle. According to the original template theory, the antigen was required to be present in the cytoplasm of the cell in relatively large amounts. This aspect was investigated by measuring the amount of labeled antigen present in antibody-producing cells. Initial indications demonstrated that there were few grains overlying plasma cells in radioautographs of sections from nodes removed from rats some days after injection of labeled antigen (Nossal and Ada, 1963). To investigate in detail, the technically simpler approach was the first adopted. Antibody-producing cells were isolated from lymph nodes a week or more after antigen injection. At this time most antibody-producing cells were mature plasma cells and there appeared to be little if any free, unphagocytosed antigen in the node.

Between 16 and 100 ng of ^{125}I-labeled flagellin were injected into rats and more than 200 antibody-producing cells individually examined at

times between 7 and 66 days later. After exposure to photographic emulsion for 60 days, the number of grains in the developed emulsion over these cells was found to be no higher than the background level. Calculations indicated that these cells could not contain more than 5 molecules of intact iodinated antigen (Nossal *et al.,* 1965a). A second approach was to examine closely cells at an earlier stage of differentiation, that is, very shortly after the initiation of the induction process (Nossal, 1966). Cells were obtained from single cell suspensions of lymph nodes or spleen or from thoracic duct lymph collected via an indwelling canula. Some antibody-producing cells from tissues were found to contain antigen, but most did not. Free cells in the lymph node never contained detectable antigen. Positive cells were more frequent in the secondary than in the primary response and frequently the antigen seemed to be present near the surface of the cell, as though it was merely adsorbed to the cell, possibly due to the presence of antibody. It was concluded that cells in the early inductive phase of antibody response could, but need not, contain more than 20 molecules of antigen.

McDevitt *et al.* (1966) have studied the fate of a synthetic polypeptide [(T,G)-A-L] labeled with iodine-125. After injection of this labeled substance, the distribution of radioactivity in lymph nodes of mice was found to be rather similar to the distribution of labeled flagella or flagellin in rat lymph nodes. The grain counts over specific antibody-containing cells were not significantly different from the background count. It was calculated that if the labeled (T,G)-A-L remained undegraded, it would have been possible to detect the presence of fifteen molecules of this substance in a cell.

Two studies using ferritin as an antigen gave conflicting results. The presence of this substance was determined by visual examination of electron micrographs of cell sections. Wellensiek and Coons (1964) claimed that during the secondary response, the ferritin entered into plasma blasts and was handed down to progeny cells so that a mature plasma cell might contain 12,000 antigen molecules. De Petris and Karlsbad (1965) failed to confirm these results using the same antigen. In both studies, ferritin molecules were not found in cells following the primary injection.

It seemed from these studies that the antibody-forming cell might need only a few (<5) molecules of intact antigen to be induced to differentiate and produce antibody or that antigen needed to be "processed" by other cells—cells of the reticuloendothelial system—before being an effective inducer. While the first possibility cannot be excluded at present, the second seems the more likely, in view of other work (see later contributions to this Symposium), and immediately raised the question of the relative importance of the two sites of antigen trapping.

2. *Relative Importance of Medullary and Follicular Localization of Antigen in Immune Induction*

There is evidence, some indirect, which implicates the role either type of localization may play in immune induction. Several workers have made a study of the immunogenicity of tissue fractions from animals receiving an injection of antigen. A notable example was the work of Franzl and Morello (1965) who studied the immunological properties of phagocytosed antigen. A mouse spleen subcellular fraction possessing sedimentation characteristics of "lysosomes" was isolated at various times after injection of antigen (sheep red blood cells) and injected into normal or sensitized mice. This "lysosomal" fraction, when prepared 1–12 hours after antigen administration, initiated both primary and secondary antibody responses and possessed the priming capacity in a manner analogous to whole antigen, when administered to mice. The "lysosomal" fraction, prepared 1–3 days after antigen administration to mice, evoked only the secondary antibody reaction and would partly sensitize mice, whereas "lysosomal" fractions, prepared 4–7 days after antigen administration, were immunologically inactive but would sensitize very effectively.

It seemed likely that the lysosomal fraction of Franzl and Morello would contain cell membrane as well as lysosomes. If the assumption was made that after injection, the distribution of injected red blood cells in the mouse spleen followed closely the distribution of labeled flagella in rat spleen, the following sequence might have occurred. After administration, there would be a rapid uptake of red blood cells into the red pulp, reaching a peak within 12 hours, after which the uptake would decrease, red blood cells would be almost absent by 2 days. By contrast, antigen bound to membrane in cells present in the follicular web would reach a maximum by 2 days. One interpretation, therefore, of this finding is that antigen present in macrophages in the red pulp, the lysosomal-bound antigen, could initiate at least a primary response and possibly a secondary response. Antigen bound to membranes of cells in the follicular web might generate a secondary antibody response and be able to sensitize animals. Thorbecke in a series of investigations (for summary see Cohen *et al.*, 1966), has studied the development of antibody-forming cells in the white and red pulp of spleen. With protein or bacterial antigens, production of both IgM and IgG occurred in, and antibody cells were present in, both white and red pulp. Using red blood cells as antigen, both the white and the red pulp were active as before, but only the white pulp when removed 2 days after antigen injection developed antibody-forming cells on cultivation for 2 days. Reexposure of the tissue fragments to the antigen showed that the white pulp usually contained the greater proportion of sensitized cells. This again may relate to the retention of antigen in

the white compared to the red pulp. This work supported the concept that the immune induction might take place in both the red and white pulp but formation of sensitized cells occurred in the white pulp.

Diener and Nossal (1966) have studied the immune response in the toad, *Bufo marinus* after injection of labeled flagella. Nothing resembling the antigen-trapping web of rat lymph node follicles was observed nor were there any germinal centers in the jugular bodies. There was a good primary response featuring a prolonged synthesis of mercaptoethanol-sensitive antibody and little or no evidence of a secondary response, suggesting that the absence of immunological memory was due to the lack of the follicular-type antigen-trapping web, with resulting germinal centers.

One result indicated that antigen localized in the medulla of the rat lymph node could induce antibody formation (Lang and Ada, in preparation). A dilute solution of labeled HSA (100 μg/ml) was heated (90°C, 15 minutes) and injected into the hind footpads of rats. As described previously, this antigen localized exclusively in the lymph node medulla. Although there was a high level of antigen retention in the lymph nodes, detectable antibody formation did not occur. Rats were therefore injected with either labeled HSA (unheated, 10 μg) and endotoxin (40 μg) or labeled, heated HSA (10 μg) and endotoxin. Antibody to HSA was detected only in the serum of the second group of rats. Three weeks after the first injection of antigen, both groups of rats were injected in the hind footpads with 10 μg HSA. Rats originally receiving heated HSA and endotoxin showed a secondary response whereas rats originally receiving HSA and endotoxin still did not produce detectable amounts of antibody. In these experiments, the administration of endotoxin was effective only if injected within 8 hours of the antigen injection. The pattern of antigen localization in the popliteal and aortic lymph nodes was not demonstrably altered by the administration of endotoxin.

These results point to the importance of medullary localized antigen in immune induction. It is not known whether antigen localizing in the lymphoid follicle may also play a role. This is more difficult to establish as it has not been possible so far to obtain antigen localization exclusively in the lymphoid follicle.

F. Concluding Remarks to Section I

Studies using isotopically labeled antigens have led to much clarification of the role of reticuloendothelial cells in the lymphoid system. Three types of cells in this class of cells have been studied. First there is the medullary macrophage. This cell has a rather broad specificity in dealing with antigenic material entering into the nodes. In our experiments, all

iodinated materials which were injected were taken up to a lesser or greater extent by these cells. There is no doubt that opsonization plays a role here, but it also seems that some substances, for example, denatured HSA, need not be opsonized in order to be taken up by these cells. Particulate antigen was trapped by the lymph node more effectively than soluble antigen. As antigen was found in lysosomal-like particles in these cells, it is perhaps natural that one should think of this cell primarily as a "scavenger," whose main function was to completely destroy ingested material. This is too simple an explanation. A significant proportion of the antigen was stored or only partly broken down. Detailed biochemical knowledge of antigen handling in this cell is required.

Retention of antigen on the surface of the reticular cell in the lymphoid follicle involved a greater specificity than phagocytosis by medullary macrophages. Complexing of antigen with opsonin or antibody caused follicular localization. In a natural infection, it may be the most important mechanism whereby follicular localization of antigen occurs. Possibly the Fc fragment of the immunoglobulin molecule is specifically involved in this reaction. The net electrical charge of the antigen or antibody may determine whether follicular localization occurs, and it is interesting to speculate that this is the underlying mechanism of this phenomenon. On this basis, recognition of the antigen-globulin complex would be mediated by charge relationships between cell membrane and globulin. Association with globulin would be the means whereby an antigen with unfavorable surface charge was converted to a state which allowed follicular localiza- of the antigen to occur.

Finally, there is the tingible body macrophage which is present in the lymphoid follicle and is particularly prominent after a secondary response. A major role of this cell seems to be the ingestion of broken-down cellular material, particularly during degradation of a germinal center (Swartzendruber and Congdon, 1963). In high resolution radioautography, antigen has been seen near but rarely in or on this cell after a primary injection of the antigen. There seems to be no anatomical reason why antigen should not come into contact with this cell, so at present the simplest interpretation is that this cell has no specific mechanism for the recognition of antigen or of globulin-coated antigens. All three cell types are involved in the immunological process but only the medullary macrophages and the reticular cell in the lymphoid follicle appear to be active in antigen handling. Much remains to be done in elucidating the exact function each plays in immunological reactions in the tissues.

II. SPECIALIZATION OF CELLS OF THE LYMPHOID SYSTEM

Differences in the antigen-handling properties of cells of the reticuloendothelial system have been of interest to relatively few laboratories.

Lymphocyte function is being studied by an ever-increasing circle of workers and it is not feasible in an article of this length to attempt to consider adequately all the different facets. Those pertaining to the immune response to an antigen will be discussed and particular reference will be made to a technique which is being developed for the separation of cells.

Advances in knowledge of specific properties of individual cells have depended on methods for the isolation and observation of these cells. Three techniques in particular have made a major contribution. The first was a microdrop technique in which individual cells were teased out from the appropriate tissue and manipulated into microdrops sufficiently large to allow nutrition for cell function but small enough to prevent antibody dilution beyond a detectable level (Nossal and Lederberg, 1958).

The second was the introduction by Jerne and Nordin (1963) of the hemolytic plaque technique. Animals were immunized with red blood cells of a suitable species, usually sheep, and at various times thereafter lymphoid tissue was teased into a cell suspension, mixed with a suspension of the cells originally used as antigen, and incorporated into an agar layer in a Petri dish. After incubation, the mixture was overlaid with guinea pig complement. On further incubation, plaques were observed which had formed because of lysis of red blood cells in areas adjacent to cells which were producing antibody. The technique as originally devised largely measured IgM production due to the much greater efficiency of this antibody in reacting with complement to cause cell hemolysis (Borsos *et al.*, 1964). Modifications have since been introduced which allow IgG production to be measured independently of IgM production (Dresser and Wortis, 1965; Šterzl and Řiha, 1965). The third method was the fluorescent antibody technique.

A. Antibody Production by Single Cells

1. One Cell–One Antibody Phenomenon

It has now become clear that the majority of cells capable of forming antibody produce antibody with a specificity which is directed against only one of a mixture of antigens which may be injected. This was first established by the work of Nossal and colleagues. When rats were immunized with two, three, or four unrelated antigens, most cells tested formed detectable amounts of antibody of only one specificity, even though each animal formed approximately equal amounts of antibody to all antigens injected. Even the 2% of cells which appeared to form two antibodies formed one in great excess of the other. This finding was challenged by Attardi *et al.* (1959), who immunized rabbits with unrelated bacterial viruses and found that about 20% of the antibody-forming cells isolated from the lymph nodes draining the injection site were capable of

neutralizing approximately equal numbers of each strain of virus. This discrepancy between the two sets of results has not been resolved. The fluorescent antibody technique has also been used in similar studies (Coons, 1958; White, 1958), and cells producing antibody of more than one specificity were not detected; as mentioned previously, the sensitivity of this technique might not be sufficient to detect the production by a cell of small amounts of antibody of a second specificity.

Jerne (1965), shortly after his introduction of the plaque technique, made a similar study of this problem. Mice were injected with sheep and rabbit red blood cells. There was no cross reaction between the antigens involved. Results showed that the two antigens did not compete for the same cells and favored the interpretation that there were, in fact, two kind of predetermined cells present before stimulation. One kind was prepared to react when stimulated by the sheep red blood cells and the other kind when stimulated by the rabbit red blood cells. Nakano and Braun (1966) studied the location of antibody-forming cells in spleen fragments and concluded that different populations of cells reacted against two different antigens.

This aspect has since been examined more stringently by assessing the number of cells reacting to two different antigens during the development of clones of cells in spleens. Sheep and chicken red blood cells were injected intravenously into mice and the mice were irradiated 3 days later. A second dose of the two species of red blood cells was injected 7 days after irradiation. Colonies of cells appeared in the spleen and the mice were killed at times between 10 and 13 days after irradiation. Cell clones were isolated and studied by plating on agar for the assay of both IgM and IgG antibodies produced against each antigen (Celada and Wigzell, 1966).

It had already been demonstrated by Playfair *et al.* (1965) that transmission of specificity among antibody-forming cells was rigidly vertical. Celada and Wigzell investigated the distribution of the four types of plaque-forming cells found in such colonies. The figures so obtained were compared with values calculated on the basis that a given cell or clone of cells produced antibody of a single specificity. The observed results were close to expected values, and these workers concluded that: (1) plaque-forming cells against the two antigens propagated in a clonal manner within spleen colonies; and (2) the production of two antibodies of different specificities by a single cell was an event which occurred in less than 1% of cells in the investigated system. As this later demonstration was carried out on the progeny of single cells – i.e., the descendants did not express different traits from that of the individual cell – it was considered by these authors that a single cell had only the genetic information to synthesize antibody with a specificity directed against one antigen.

Hiramoto and Hamlin (1965) hyperimmunized guinea pigs with human γ-globulin, a protein known to contain two antigenic determinants. Animals producing antibodies which reacted with papain-digested antigen, as demonstrated by two bands in immunoelectrophoresis, were killed and spleen sections examined to see if individual cells contained antibodies to both fragments of the γ-globulin molecule. Antibodies were detected using an indirect, paired, fluorescence technique. Almost half of the positive cells contained antibodies directed against both antigenic determinants. This result provides perhaps the strongest evidence yet obtained of the multipotency of immunologically competent cells.

2. *The Production of Different Macromolecular Forms of an Antibody by a Single Cell*

Could a single cell produce antibodies which, although they express the same specificity toward an antigen, are produced in different molecular sizes? Antibodies exist in several main types—IgG has a molecular weight of about 150,000, IgM has a molecular weight of about 1×10^6, and IgA exists in sizes which vary between these two values. In most immune responses the main serum antibodies are IgG and IgM. It has been commonly found that in model systems of antibody production a single injection of macromolecular particulate antigen will lead to the formation of IgM and later IgG antibodies (Bauer and Stavitsky, 1961; Uhr and Finkelstein, 1963). It is known that both these types of antibody are made by plasma cells of varying degrees of maturity. Nossal *et al.* (1964c) used a microdrop technique to establish whether at any stage during the immune response cells could be found which produced both IgG and IgM. Rats were injected with flagella from *Salmonella adelaide* under conditions which resulted in the appearance of IgM, followed by IgG.

The antibody produced was characterized by its sedimentation pattern in sucrose gradients, by its susceptibility to 2-mercaptoethanol treatment, and by the use of an antiserum which would distinguish between the two types of antibodies, IgG and IgM. Rats were killed at various time intervals after the antigen injection, the draining nodes teased, and individual cells containing antibody detected and isolated. The cells were broken, releasing antibody into the medium, and the volume of fluid in the microdrop measured. This volume was split into four portions which were then tested for antibody content after (1) no treatment, (2) treatment with mercaptoethanol, (3) treatment with rabbit antirat IgG serum, or (4) treatment with both mercaptoethanol and the antiserum. In the first few days after injection when only IgM antibody was detected in the serum, only IgM antibody was detected in individual cell extracts. At a later stage when only IgG antibody was present in the serum, cells tested con-

tained only IgG, but at a time when both IgG and IgM antibody were in the serum, 17 cells out of a total of 144 cells tested were found to contain both of these types of antibody by the above criteria. No morphological differences between the cells producing these two types of antibody were found and though it seemed likely that most cells produced only IgM or IgG at a given time, there were some cells in which IgM production was followed by production of IgG – i.e., these cells had the ability to produce sequentially different heavy chains showing the same specificity toward one antigen. A cell need not necessarily pass through a stage of IgM production before IgG is produced as, after injection of monomeric flagellin into rats, only IgG was found in detectable amounts in the serum (Nossal *et al.*, 1964b).

Other workers have found that different cells produce these two types of antibody after an antigenic stimulation, e.g., Stavitsky *et al.* (1965) studied the response by the rabbit to intravenous injection of diphtheria toxin in complete Freund's adjuvant, and found that different cells in the spleen were producing different species of antibody. Large mononuclear cells in the red pulp of the spleen were predominantly associated with IgM synthesis, whereas plasma cells in the nonfollicular white pulp were predominantly associated with the synthesis of IgG. The data of Celada and Wigzell (1966) did not permit a clear-cut decision on this aspect. IgM and IgG clones were found to show similar growth kinetics. There was a small degree of association of IgM and IgG plaque-forming cells directed against sheep red blood cells, but no association against the anti-chicken red blood cell system. The results argued against the widespread occurrence of IgG and IgM production within an individual cell.

In summary, therefore, a single cell could make both these types of antibody but this, in practice, was the exception rather than the rule.

3. *Does a Cell Produce Different Varieties of the One Class of Immunoglobulin?*

Preparations of immunoglobulins obtained from the serum of a normal individual are heterogeneous, as recognized by their antigenic behavior and by their detailed chemical composition. The light chains, which are common to all classes of human immunoglobulin, occur in two different molecular forms, κ and λ chains. They are completely different antigenically (Fahey, 1963) and share no common tryptic peptides (Putnam and Easley, 1965). In humans they are readily obtained in pure form as Bence-Jones proteins. Both light and heavy chains may also be characterized by their possession of genetically determined antigenic determinants known as allotypic markers. In humans, this is the Inv marker on the light chain and the Gm system of markers on the heavy chains. Rabbits

also possess allotypic markers on the light and heavy chains. The production of antibody chains of different antigenic specificity by cells has been studied extensively with the fluorescent antibody technique, using specific sera against isolated and well-characterized light and heavy chains containing the appropriate antigenic specificity. Pernis and Chiappino (1964) studied the distribution of κ and λ chains in cells of spleens from adult humans. In the red pulp, plasma cells were found to react with antiserum to either one or the other light chain. No correlation was seen between the morphology of the cell and the antigenic type of light chain that it contained. In the follicles of both spleen and lymph node, cells were present which appeared to contain both types of light chains. In contrast, Bernier and Cebra (1964), using an improved immunofluorescent technique, found individual cells to contain only one type of light chain, that is either the κ or λ. In addition, they found separate localization in cells of the α and γ chains, the heavy chains of IgA and IgM, irrespective of the organ studied. This apparent discrepancy between the results will be discussed later.

Lymphoid cell specialization has also been generally observed when the presence in cells of allotypic markers has been studied. Colberg and Dray (1964) examined the production of allotypic A4 and A5 markers in homozygous and heterozygous rabbits. The fluorescent antisera were found to react specifically with the cytoplasm of plasma cells and intrinsic cells in the germinal center. In the heterozygotes identical cellular localization of the two allotypes A4 and A5 were found. This work was not confirmed. Pernis *et al.* (1965) studied the distribution of allotypic variants of the light chain present in animals heterozygous at the B locus and found them to be strictly localized in separate lymphoid cells. Cebra *et al.* (1966) have since found that the allotypic markers Aa^1 and Aa^2 found on heavy chains were separately localized in cells of the Aa^1/Aa^2 heterozygous rabbits. The results found earlier by Colberg and Dray were considered suspect due to the method of staining which was used and the fact that the cells which double stained for both A4 and A5 markers were found in the germinal centers of spleen and of lymph nodes. This was also the site of cells found by Pernis and Chiappino to stain for both major antigenic types (κ and λ) of light chains. One explanation for the prevalence in germinal centers of cells which appeared to contain different immunoglobulins is based on our experiments discussed earlier and is as follows. Germinal centers in lymphoid tissue occur as a result of antigenic stimulation and thus would contain antigen associated with antibody of different classes and subclasses. Upon making imprints or sections of such areas, these antibodies may have contaminated adjacent cells, thus resulting in the double staining.

There are further differences in immunoglobulin composition which have not been revealed so far by antigenic analysis of the type described above. In IgG isolated from normal animals or humans, the light chain may be divided into ten bands on gel electrophoresis, irrespective of the type specificity of the light chain (Cohen and Porter, 1964). A similar resolution of the heavy chain has since been accomplished by disc electrophoresis in polyacrylimide gels in the presence of urea (Reisfeld and Small, 1966). This separation depends largely upon variation in the amino acid sequence at the N terminal ends of the chains. It remains to be seen to what extent cells are restricted to the synthesis of a single light and heavy chain by this criteria.

The evidence from analysis of the products of myeloma cells shows that, in general, only one kind of heavy and light chain are formed per cell. Until it is more clearly established that myeloma proteins are structurally analogous to monoclonal antibodies, it is important to establish whether a similar homogeneous pattern occurs after stimulation by a known antigen. Presumably this question could be settled by labeling the antibody produced by single cells, adding carrier globulin, and carrying out electrophoresis of the isolated light and heavy chains.

B. Methods for Cell Separation

Techniques for the study of single cells are essentially of an analytical value and their potential is consequently limited. The biochemical analysis of, for example, protein and nucleic acid metabolism in a cell can rarely be studied adequately by such techniques. Separative procedures which serve the dual function of analytical and preparative roles need to be developed for this purpose. The task is a formidable one but the evidence is now clear that cell separation techniques can be devised which will serve both purposes. In this concluding section I wish to refer to the work of my colleague, Dr. K. D. Shortman, who has developed two techniques which are proving to be of increasing potential, initially at the biological level.

1. Separation of Lymphocytes

Two physical parameters of lymphocytes have been exploited to effect separation of one type from another, namely, size and density.

Separation based on the size differences among cells has been accomplished by passing the cells slowly through a column of 60–90 μ siliconed glass beads at 4°C, in a balanced salt solution containing 16% serum (Shortman, 1966). If filtration was repeated till only 10% of the cells remained, the final filtrate contained pure (>99.9%) undamaged, viable

small lymphocytes. Large and medium lymphocytes, plasma cells, macrophages, and any damaged cells remained in the column. "Lymphocytes" active in 19 S antibody formation (Jerne plaque test) were completely removed and the level of DNA synthesizing cells was reduced seventyfold.

Although this technique yielded one morphological category of cells in a pure state, the other cell classes were enriched only slightly. In addition, no separation of small lymphocytes into functional classes was possible. This was, however, accomplished by a technique based on density differences.

A preparation of lymphocytes was distributed in a density gradient of albumin (15–29% w/w, pH 5.1), in a balanced salt solution, and centrifuged to equilibrium at 3700 *g* for 1 hour at 4°C; great care was needed to avoid artifacts due to aggregation and association of cells, to irregularities in the gradient, to the production of streaming effects and sedimentation of cells against the tube walls. In this way a complete density distribution profile was obtained for all cells in a given population. The position of a peak was reproducible to within a density difference of ± 0.0003 g/cm^3 from one run, on one day, to another.

Lymphocytes from blood, thoracic duct lymph, lymph nodes, and thymus have been analyzed by this technique. The following findings applied to cells from each source. Large, medium, and small lymphocytes were considerably displaced from each other, the larger cells being, in general, least dense. However, each morphological class itself shows density heterogeneity, distinct peaks being obtained. For example, small lymphocytes from rat thymus may be found in up to six distinct density types. Even cells of identical volume as well as morphology may be further subdivided in the density gradient.

2. *The Demonstration of Biological Activity in Separated Lymphocytes*

The ability of lymphocytes from chick blood to initiate a graft-versus-host reaction is assayed by the formation of pocks on the chorioallantoic membrane of chick embryos (Szenberg and Shortman, 1966). This technique has a reproducibility of $\pm 8\%$. Lymphocytes from chicken blood were submitted to isopycnic centrifugation as described above and the fractions tested for their ability to initiate a graft-versus-host reaction. Some degree of cell-to-cell association reduced resolution in these early experiments, but the following points were established. Activity was found in fractions where small lymphocytes were concentrated, including fractions completely lacking large or medium cells. Although activity could clearly be attributed to small lymphocytes, not all small lymphocytes were active.

III. CONCLUDING REMARKS

Antibodies even with a single specificity of reaction with antigen occur in a bewilderingly great variety of forms due to differences in size of their polypeptide chains, their amino acid composition, and amino acid sequence. The picture emerging from the study of antibody production by single cells *in vivo* indicates very clearly that cells are committed to the almost exclusive production of one of the very large number of possible antibody molecules. Is this simply genetic restriction? If not, at what stage is the restriction invoked? There are already sufficient indications in the work reported above to show that cells have a greater potential than is normally expressed. Some cells can make antibody to more than one antigen, even when the antigenic determinants are present on different molecules. When a macromolecule containing two antigenic determinants was presented to an animal, a high proportion of cells were found to produce antibody directed against each specificity. Some cells can make at one time more than one type of immunoglobulin heavy chain, and it may well be found that one cell may make more than one type of light chain. Herzenberg and Warner (1966) have in fact described a mouse myeloma protein which carried the antigenic specificities of two classes of immunoglobulin—i.e., the gene products of two loci. In *in vitro* experiments it may be found that less restriction on the cell occurs. There is probably some reason why *in vivo* the potential of the cell is restricted during antibody production.

Necessity is the mother of invention, and techniques have recently been devised which allow individual functioning cells to be detected, enumerated, and their proliferation studied. These techniques utilize the ability of heavily X-irradiated animals to act as a nursery for antigen-primed cells or for antigen-sensitive cells introduced with antigen (Kennedy *et al.*, 1965; Playfair *et al.*, 1965; Brown *et al.*, 1966). Hopefully, these procedures will allow the identification of the target cell, i.e., the precursor to the antibody-forming cell and a much closer analysis of antigen-mediated cell differentiation. Techniques of cell separation will play an important part in these advances.

Finally, the detailed action of antigen in priming the target cell still remains to be determined. Does intact antigen act by contact at the cell membrane? How important is the role of modified antigen? Where and how does modification take place? These are some of the questions which need to be answered.

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DISCUSSION

DR. TRENTIN: A few years ago Dr. Fahlberg and I presented, at the M. D. Anderson Symposium on Immunology in Houston, an experimental model for determining the antibody-producing ability of clonally derived lymphoid cells. This was based on repopulating the hemopoietic and lymphoid systems of lethally irradiated mice with a single clone of cells derived from a single bone marrow-induced spleen colony. Becker and McCulloch have shown such colonies to be of clonal origin, arising from a single bone marrow stem cell.

The hemopoietic and lymphoid systems of mice were first eliminated by supralethal whole body irradiation, and replaced by a low enough dose of marrow cells to give separate and discrete, easily dissectable spleen colonies. Before these became confluent, they were separately dissected out and each colony was injected into a single lethally irradiated mouse. After the secondary host had undergone confluent hemopoietic and lymphoid repopulation from the clonally derived cells, its bone marrow and lymphoid cells were harvested and "expanded" by transfusion into a group of sixteen lethally irradiated mice. After these mice had undergone confluent hematopoietic and lymphoid repopulation, they were chal-

lenged with three antigenically distinct *Salmonella* group vaccines. Each surviving mouse responded to all three antigens, a finding supporting some sort of an instructional mechanism of antibody response rather than the theory of clonal selection.

We have more recently repeated this experiment in collaboration with Dr. David Weiss of the University of California, using T6 chromosome-marked donor bone marrow to verify the assumption that the lymphoid system of the tested mice was derived from the injected marrow, rather than by host lymphoid regeneration. We extended the study using sheep red cells and bovine serum albumin antigens, since the response to *Salmonella* could conceivably represent a secondary response. We also tested some secondary as well as tertiary hosts to allow less time for possible mutation of the transplanted clones.

Because of the low colony-forming unit content of most spleen colonies, and the consequent low percentage of survival of irradiated mice injected with a single spleen colony, we used pooled colonies from a single spleen containing from four to thirteen colonies. This is still compatible with the intended test of the clonal selection theory, since the probability of selecting from all possible antigens one that matches any of four to thirteen randomly selected monospecific clones remains negligibly low.

Our recent experiments using cells with the T6 chromosome marker establishes beyond doubt that the lymphoid repopulation of most of the recipient mice takes place with cells derived from marrow stem cells of donor origin (Table I). The mice repopulated with four to thirteen spleen colonies responded to all three of the antigens as well as did appropriate controls.

Barring an unprecedentedly high rate and selective nature of mutation of lymphoid cells or their precursors, the data suggest that antibody-forming cells are immunologically pluripotent rather than monoordained.

DR. LEDDY: Dr. Trentin, I wonder whether you have any information as to the number of immunoglobulin classes that might have been produced by the mice that received presumably a single clone of lymphoid cell or hematopoietic cells with potential for lymphoid differentiation. The work on myeloma or plasmocytoma in mice and man would lead us to believe that if one has a single clone of cells, perhaps one would have only a single immunoglobulin class.

DR. TRENTIN: We have not done studies of this kind.

DR. BENACERRAF: I would like to address myself to two aspects of Dr. Ada's talk. Stimulated by the studies of Dr. Ada and Dr. Nossal, we have also investigated the fate of antigen in local lymph nodes. We used hapten-proteins and hapten-polyamino acid conjugates labeled with ^{3}H with the label in the hapten, as well as particulate suspensions such as carbon and saccharated iron oxide. Thus antigenic and nonantigenic materials were injected. We were concerned with the property of the antigen which leads to its localization in primary and secondary follicles. It appears that both antigenic and nonantigenic materials can be found localized in these anatomical sites primarily because of physicochemical properties which allow them to be adsorbed on the surface of macrophages previous to phagocytosis. The following nonantigenic materials were found to localize in germinal centers: saccharated iron oxide, colloidal carbon, and nonantigenic polyamino acid polymers bearing haptens. In the latter case this was achieved if antibody to the hapten was present in the animal to opsonize this material. There is therefore in these follicles a trapping mechanism for phagocytizable material which causes the localization at these sites of material capable of being taken up usually by macrophages. Although the arrest of these materials at these sites does not seem to be related to the presence or absence of antigenic determinant, I agree with Dr. Ada that this process may constitute a very important mechanism in the induction of the immune response as it may provide a site where lymphocytes may come into close contact with trapped antigenic material.

TABLE I

ANTIBODY PRODUCTION BY MICE WITH CLONALLY REPOPULATED HEMOPOIETIC AND LYMPHOID SYSTEMS

Mouse No.[a]	Preantigen Treatment 850 r and:	No. antigen injections before testing	Antibodies present against	Reciprocal of antibody titer at intervals after last immunization				T6 cells/total mitotic cells counted (cellularity of tissue)[b]		
				4 days	7 days	5 weeks	8 weeks	Bone marrow	Spleen	MLN[c]
1	4 colonies		SRBC	320	160	2560	2560	28/28	33/33	12/12
	(1.4×10^7 viable cells)	5	BSA	40	40	80	0	(good)	–	(moderate, little
			Salmonella "O"	0	0	40	0			or no EMH)
2	7 colonies		SRBC	5120	10,240	5120	5120	69/69	54/54	9/9
	(7.7×10^6 viable cells)	5	BSA	40	80	80	0	(good)	(good, some	–
			Salmonella "O"	160	320	320	0		EMH)	
3	13 colonies		SRBC	6400	5120	5120	5120	52/53	50/51	4/5
	(8.8×10^6 viable cells)	5	BSA	200	80	40	0	(moderate)	–	–
			Salmonella "O"	400	160	160	0			
4	9 colonies		SRBC	2560	5120	5120	2560	51/51	lab accident	13/13
	(1.4×10^7 viable cells)	5	BSA	0	40	40	0	(moderate)	(good, little	–
			Salmonella "O"	0	40	40	0		EMH)	
5	1.5×10^6 B.M.+1.2×10^6		SRBC	0	0	1280	–	50/50	37/37	no mitoses
	viable spleen and MLN	4[d]	BSA	0	0	0	–	(moderate, few	(moderate-	(moderate, very
	cells from 2nd re-		*Salmonella* "O"	0	0	0	–	myelocytic cells)	good, some	small amount
	cipient which had re-								EMH)	EMH)
	ceived 7 colonies from									
	1st recipient									
6	1.6×10^6 B.M.+4.7×10^6		SRBC	–	0	2560	–	30/30	50/50	1/1
	viable spleen cells from	4[d]	BSA	–	0	0	–	(very poor)	(moderate,	(moderate-good
	2nd recipient which		*Salmonella* "O"	–	0	0	–		much EMH)	little or no EMH)
	had received 7 colonies									
	from 1st recipient									

7	1.6×10^6 B.M.+4.7×10^6 viable spleen cells from 2nd recipient which had received 7 colonies from 1st recipient	5[e]	SRBC	200	320	10,240	–	40/47 (moderate)	38/39 (poor-moderate, much EMH)	13/14 (moderate, some EMH possible)
			BSA	0	0	40	–			
			Salmonella "O"	0	0	0	–			
8	1.6×10^6 B.M.+4.7×10^6 viable spleen cells from 2nd recipient which had received 7 colonies from 1st recipient	5[e]	SRBC	320	320	10,240	–	2/40 (moderate)	0/67 (poor, much EMH)	1/6 –
			BSA	0	0	40	–			
			Salmonella "O"	40	40	40	–			
9	4 colonies (6.1×10^6 viable cells)	5	SRBC	3840	2560	–	–	Animal died unexpectedly		
			BSA	40	0	–	–			
			Salmonella "O"	60	0	–	–			

[a] Mice Nos. 1, 2, 3, 4, and 9 are secondary hosts; Nos. 5, 6, 7, and 8 are tertiary hosts (see text).
[b] Histologically determined by H. and E. sections.
[c] EMH = extramedullary hematopoiesis. MLN = Mesenteric lymph node.
[d] Fourth injection given 4 weeks after third, and 5 days previous to third bleeding.
[e] Fourth injection given 4 weeks after third, fifth given 1 week after fourth (4 days before third bleeding).

Another question which has been introduced by Dr. Ada's paper is the production by single cells of antibodies of two unrelated immunological specificities. This problem has been studied by many investigators and has been reexamined recently in our laboratory in collaboration with Dr. I. Green, using antigens bearing two types of noncross-reactive determinants on the same molecule. These studies were made using a combination of immunofluorescence and radioautographic techniques and using as antigen dinitrophenylated proteins capable of inducing the formation of antibodies against the DNP group and the carrier molecule. Several hundred lymph node cells producing antibodies were examined 1 month after immunization. In no case did we find cells that were producing antibodies both to the hapten and to the carrier protein. To study this problem further, we took advantage of the fact that the immune response to DNP poly-L-lysine in guinea pigs is under the control of a dominant gene and that the nonresponding guinea pigs lacking the gene can nevertheless be induced to form anti-DNP-PLL antibodies by combining it with a foreign albumin which acts as a carrier molecule. Such animals produced both anti-DNP antibodies and antibodies against the carrier albumin. Studies of the lymph nodes of these guinea pigs revealed that these two types of antibodies with different immunological specificities were produced by different cells. In no case did we find antibodies against a hapten and against the carrier molecule in the same cell.

DR. ADA: I think Dr. Benacerraf's work and ours generally agree as far as localization of antigen is concerned. I would like to stress that natural antigens which are found to be localized in the follicular reticulum of the lymph node have been taken there or trapped there by an antibody. It may be that the fundamental factor which is involved in this trapping mechanism is the charge of the antibody relative to the charge on the reticulum cell surface.

DR. HINUMA: In relation to Dr. Ada's and Dr. Benacerraf's comments, I would like to mention some of our data on the establishment of cell clones which produce at least two types of immunoglobulins *in vitro* [(1967) *Proc. Soc. Exptl. Biol. Med.* **124,** 107]. Immunofluorescent studies of smears from cell lines which were derived from human leukemia and Burkitt lymphoma demonstrated that specific immunoglobulin staining occurred in the cytoplasm of most but not of all cells. Since these observations suggested a possible heterogeneity of the cell lines with respect to immunoglobulin production, we have tried to clone some of these cells.

The cell lines used were the 64-10 and LKID lines derived from the buffy coat of peripheral blood of patients with leukemia, and two cell lines derived from Burkitt lymphoma which were designated SL-1 and P-3 Jijoye. These cell lines were cloned by a semisolid agar procedure which has been developed in our laboratory very recently [(1966) *Cancer* (in press)]. The medium used was Eagle's minimum essential medium plus 20% fetal calf serum and with addition of 0.4% and 0.3% bacto-agar for the base and seed layers respectively. Five milliliters of the base agar were solidified in a plastic petri dish with 60 mm diameter. Two milliliters of seed agar were poured onto the base layer; 100 cells/ml or 10 cells/ml had been suspended in this seed layer. The seeded agar plates were incubated in a 5–7% CO_2 incubator. Within 7–10 days after plating, colony formation of cells could be observed very easily under low power magnification. The cloning efficiency varied between 10 and 80%, depending upon the cell line used. For each cell line it was independent of the initial cell density. A dilution effect on the number of colonies formed was observed, providing evidence that each colony arose from a single cell. Well-isolated colonies were transferred by Pasteur pipettes to a regular liquid medium. Production of heavy chains of immunoglobulin in the cell of the parent culture and in the cells of randomly selected clone cultures was determined by immunofluorescence. Fluorescein isothiocyanate-conjugated monospecific goat antiserum against IgA, IgM, and IgG was used. Acetone-fixed cell smears were stained with each of these fluorescent antibodies.

The parent LKID cell line and all of the four clones tested were stained with both anti-IgG and anti-IgA. The cells of the parent culture of the P-3 Jijoye line were stained with both anti-IgG and anti-IgM. Two out of five clones tested in this cell line were stained with both anti-IgG and anti-IgM. However, the remaining three clones were stained only with anti-IgM and not with anti-IgG. Thus it appears that the parent cell line is heterogeneous, as measured by these parameters. The eleven clones derived from the other two cell lines studied were stained only for one type of immunoglobulin. These results strongly suggest that at least two different immunoglobulins may be produced by a single cell.

DR. UHR: I wish to direct my comments to two aspects of Dr. Ada's interesting presentation. The first deals with the relationship of antigen localization to the induction of the antibody response. Clearly here the major problem is the relevancy of a particular localization pattern to the antibody response. It is generally agreed that a very small fraction of injected antigen acts as immunogen, hence the burden of proof will always be on the investigator to show that a particular population of antigen molecules is concerned with inducing a particular aspect of the antibody response. You discussed and have reported upon one approach to this problem, which is to use procedures which affect the antibody response such as induction of tolerance, use of passive antibody, denaturation of antigen, etc., and see whether they affect antigen localization. I want to ask you about the converse. Have you found any procedures which do *not* affect the antibody response but which alter the pattern of antigen localization? This is another approach to finding out which aspects of the localization pattern are irrelevant, and, therefore, by exclusion, which are relevant. In particular, have you studied the effect of thoracic duct drainage of lymphocytes on antigen localization? With very good immunogens such as bacteriophage ϕX in the rat, 5 days' drainage of thoracic duct lymphocytes prior to immunization does not significantly affect the late primary antibody response. Also, have you looked at the effect of prior injection into the footpad of reticuloendothelial blocking agents?

I have a final comment on the part of your presentation which dealt with the question of whether antigen is present in antibody-forming cells. You stated that a maximum of four molecules of flagella might be present in antibody-forming cells. This finding excludes of course an instructive role for antigen in the determination of the specificity of antibody formation. It does *not* exclude, however, the possibility that a very small number of antigen molecules, perhaps one, acting as depressors may need to be in contact with antibody-forming cells for a period of time after their initial stimulation.

DR. ADA: Williams [Williams, G. M. (1966) *J. Immunol.* 11, 467[found that after about 5 days of drainage from the thoracic duct, localization of flagellan antigens in the lymph nodes was modified due to the absence of opsonins. As for your second point, all I can say is that, with an agent such as flagella or flagellin, the same percentage of antigen is trapped by the draining lymph node over quite a large dosage range, say from less than a microgram to a milligram. The trapping mechanism is rather inefficient. We have not done a specific experiment in which we have used blocking agents before giving the antigen. In regard to your final comment, about the number of molecules of antigen present in the cell which is actually making antibody, this applies to cells which varied in their morphology from blasts to mature plasma cells. This aspect was studied in a series of experiments in which between 10 to 16 ng of flagellin, labeled with ^{125}I, were given to rats; at 5 and 10 days after injection the lymph nodes were teased out and single cells were examined for their ability to produce specific antibody against flagellin particles. Radioautography was carried out on individual cells for the period of the half-life of these isotopes, which was approximately 60 days. When these slides were examined, considering the background levels of radioactivity, the half-life of the isotope and the relation between the number of grains in the emulsion and the amount of isotope in cells underneath the emulsion, no extra grains over

the cells compared with background levels were found. If there had been one extra grain over these cells compared to background levels, this would have corresponded to approximately four molecules of intact labeled antigen per cell. At the moment we are trying to increase the specific activity of the antigen in our system and this may help us to answer the final question posed by Dr. Uhr.

DR. MAURER: In reference to your last point, Dr. Ada, I wonder how many determinants flagellin has and whether each determinant has tyrosine associated with it?

DR. ADA: We do not have information regarding the intimate structure of the flagellin molecule. At the moment we are treating the molecule with cyanogen bromide and studying the resulting fragments to see where the tyrosine residues are and whether in fact the one or two tyrosine residues which are labeled with radioactive iodine form part of an antigenic determinant.

DR. CUDKOWICZ: You mentioned that by density gradient centrifugation you could separate peaks of small lymphocytes. Did you test them for specialized functions? Was the ability to induce graft versus host reactions diffused throughout the gradient or was it concentrated in a peak?

DR. ADA: This particular work, which was carried out by Dr. Shortman and Dr. Szenberg, was presented at the New York Academy of Sciences meeting early this year. As you know, the small lymphocyte and the white cells in chicken blood are capable of initiating a graft versus host reaction on the chorioallantoic membrane of the chick embryo and this was previously attributed to medium or large lymphocytes. When these cells were subjected to gradient centrifugation, Dr. Shortman found that there was one large peak of large lymphocytes which was at the top of the gradient and one peak of medium-sized lymphocytes which was slightly down, and then at least three different peaks of small lymphocytes. The plaque-forming cells were present in a part of the gradient where there were virtually only small lymphocytes. The second interesting point was that not all the small lymphocytes were active in this system. They are carrying out work with a more refined technique to separate antibody-forming cells from other cells in lymph taken 3–4 days after an injection of sheep red blood cells into the footpads. The preliminary results show that they can obtain an almost complete separation of such cells from small lymphocytes. This technique has a very great potential.

Allogeneic Inhibition and Its Possible Relation to Cell-Bound Immunity in Vitro

K. E. HELLSTRÖM and I. HELLSTRÖM*

Departments of Pathology and Microbiology
The University of Washington Medical School, Seattle, Washington

Snell was the first to report that homozygous mouse lymphomas of C57BL origin grew better when transplanted into syngeneic C57BL recipients than into "semisyngeneic" F_1 hybrids between C57BL and unrelated strains (Snell, 1958; Snell and Stevens, 1961). It was not possible to influence the tumor take frequency in F_1 hybrid hosts by sensitizing them against possible histocompatibility antigens specific for homozygous cells (Snell and Stevens, 1961).

We obtained similar results during studies on isoantigenic variation in mouse lymphomas. Isoantigenic variant lines which had been selected from an A×A.SW F_1 lymphoma for compatibility with the A or A.SW parental strains and which lacked detectable H-2 isoantigens from the other parent, grew better in the (syngeneic) strain of selection than in various F_1 hybrids, including A×A.SW F_1. The original A×A.SW F_1 line, from which the variants had been selected, grew equally well in A×A.SW F_1 as the variants did in their parental strain of selection. Homozygous lymphomas, originating in strains A, A.SW, C57BL, C57L, and C3H grew deficiently when transplanted to F_1 hybrids as compared to syngeneic recipients, and the same was true for a series of sarcomas and carcinomas. To detect the deficient growth in F_1 hybrid mice, small numbers of cells had to be inoculated, as a rule below 10^6 cells. Since a detailed account of these findings has been published recently (Hellström, 1966a), they need not be further presented here.

To characterize the preferential growth of transplanted tumors in syngeneic as compared to F_1 hybrid recipients, we introduced the term syngeneic preference (Hellström and Hellström, 1965). The growth inhibition in the hybrids was called allogeneic inhibition. These two terms were introduced to avoid the use of previously employed terms such as "F_1 hybrid effect" or "hybrid resistance" since we felt that allogeneic

inhibition might be due to another mechanism than that of hybrid resistance (Cudkowicz, 1965).

We have performed a series of *in vitro* experiments to investigate the mechanisms of allogeneic inhibition (Hellström *et al.*, 1964; Hellström and Hellström, 1965, 1966). The Möllers, at the Karolinska Institutet in Stockholm, who studied cellbound immunity phenomena *in vitro*, have also obtained results that contribute to our understanding of the phenomenon (Möller, 1965; Möller and Möller, 1965). I shall summarize these findings and also discuss whatever possible connections there may be between allogeneic inhibition and cellbound immunity phenomena as studied with mouse cells cultivated *in vitro*.

I. ATTEMPTS TO DEMONSTRATE ALLOGENIC INHIBITION IN VITRO

It was thought that allogeneic inhibition of homozygous tumor cells in F_1 hybrid recipients was due to one of three mechanisms: (1) "general" differences between homozygous and F_1 hybrid hosts related to their metabolism, endocrine status, etc., or "hybrid vigor"; (2) an immunological reaction* performed by immune cells of the F_1 hybrid recipients and directed against hypothetical isoantigens which would be specific for homozygous as compared to F_1 hybrid cells; (3) a previously unknown mechanism for which the relevant point of attack would be the fact that homozygous cells grafted to F_1 recipients would meet H-2 isoantigens of the other partner of the F_1 cross, which would be foreign to them.

As it was published in a series of papers (Hellström and Hellström, 1965; Hellström *et al.*, 1964, 1965; Bergheden and Hellström, 1966), we have attributed allogeneic inhibition to the third possibility for the following reasons: (1) Neutralization of tumor growth *in vivo* could be detected subsequent to *in vitro* target cell exposure to F_1 hybrid lymphocytes in the presence of phytohemagglutinin (PHA). The neutralized growth was detected as a lower-take frequency of transplanted tumor cells in preirradiated syngeneic recipients, if the tumor cells had been exposed to allogeneic or F_1 hybrid lymphocytes as compared to syngeneic lymphocytes used in the controls (Bergheden and Hellström, 1966). X-Irradiation of the admixed lymphocytes did not abrogate their inhibitory capacity. Furthermore, the normal lymphoid cells could be replaced by X-irradiated intact or ultrasonicated allogeneic lymphoma cells which were also effective in neutralizing target cell outgrowth (Bergheden and Hellström, 1966).

* By "immunological reaction" we mean a classic immunological reaction involving specific antibodies or lymphoid cells specifically sensitized toward target cell antigens.

(2) Untreated "semisyngeneic" F_1 hybrid lymphocytes were cytotoxic to homozygous normal or neoplastic cells cultivated *in vitro* in the presence of PHA. The F_1 hybrid lymphocytes were cytotoxic to approximately the same degree as the allogeneic lymphocytes (Möller, 1965; Hellström *et al.*, 1967). (3) Cell-free extracts prepared from tumor or normal spleen or liver tissues, containing particulate H-2 isoantigenic material, reduced the number of tumor cells growing *in vitro* if the extracts contained H-2 isoantigens foreign to the tumor target cells (Hellström *et al.*, 1964). (4) Lymphoma variant lines, selected from an A× A.SW F_1 lymphoma for compatibility with the A or A.SW parental strains and only containing detectable H-2 antigens specific for the strain of selection, produced cytopathogenic changes in petri dish cultures of sarcoma and carcinoma cells if they contained H-2 antigens foreign to the explanted cells and were added together with PHA (Hellström *et al.*, 1965; Hellström and Hellström, 1966). (5) Ultrasonication of the lymphoma cells did not destroy their cytotoxicity (Hellström and Hellström, 1966). (6) The cytopathic effect of ultrasonicated allogeneic lymphoma cells could be abrogated, if the H-2 antigens of the added cellular material which were foreign to the targets were neutralized with antiserum (Hellström and Hellström, 1966).

It has been suggested that lymphocyte-mediated, cellbound immunity reactions, as studied with mouse cells *in vitro,* might be explained in the same way as allogeneic inhibition, namely by target cell exposure to other cells containing H-2 isoantigens which are not represented on the targets (Möller, 1965; Hellström and Möller, 1965). This suggestion was based on experiments in which mouse neoplastic or normal cells were cultivated *in vitro* and exposed to allogeneic lymph node cells derived from sensitized or untreated donors. If lymphocytes from untreated donors were used, either PHA or rabbit antimouse serum had to be added to produce a cytotoxic effect with allogeneic or F_1 hybrid lymphocytes. Both PHA and the rabbit serum agglutinated the lymphocytes to the targets (Möller, 1965). If sensitized lymphocytes were used, the same degree of agglutination was detected in the absence of PHA and rabbit antiserum. It was postulated that cell killing by sensitized lymphocytes as well as by untreated lymphocytes added together with PHA were both due to the same mechanism: target cell exposure to the foreign H-2 isoantigens of the added lymphocytes (Möller, 1965). According to this postulate, the role of sensitization would be to establish antibody receptors at the lymphocyte surfaces by which these cells attach to the targets in the same way as when PHA is added.

To approach the question whether similar mechanisms are responsible for allogeneic inhibition and for cellbound immunity reactions as studied with mouse cells *in vitro,* and to clarify these mechanisms to some extent, we have lately developed a so-called colony inhibition technique

which offers considerable advantages in comparison to earlier methods used for the demonstration of allogeneic inhibition and cellbound immunity reactions *in vitro* (Hellström, 1966). This technique is a modification of a method previously developed by Hellström and Sjögren for the *in vitro* demonstration of isoantigens and specific tumor antigens by measuring the plating efficiencies of appropriate tumor cells subsequent to their incubation with antibodies and complement (Hellström and Sjögren, 1965).

A recloned Moloney virus-induced leukemia of A/Sn origin, YAA-C1-C3, and a methylcolanthrene tumor of A.SW origin, MWE, were used as target cells. Both tumor lines were kept by serial transplantation in syngeneic hosts.

Allogeneic sensitized lymph node cells were harvested from mice which had been injected intraperitoneally and subcutaneously on both sides of the back with a crude tumor cell suspension carrying the target cell H-2 antigens. One to four such injections were given at 1–2-week intervals; 3–10 days after the last immunizing dose, cervical, axillar, inguinal, mesenteric, and retroperitoneal lymph nodes were dissected out and suspended by pressing them through a 60-mesh stainless steel screen into Eagle's tissue culture medium. Lymphoid cells from syngeneic A/Sn mice immunized against the methylcholanthrene-induced sarcoma MWE of A.SW origin were used as controls. The isoantigens and tumor-specific antigens of this tumor do not cross react with those of YAA-C1-C3, according to transplantation tests. Nonimmune allogeneic and syngeneic lymphocytes were used in other experiments. Finally, tests were made in which lymphoma variant cells were used instead of lymphocytes. These cells were derived from the LNSF lymphoma (of A×A.SW F_1 origin), had been selected for compatibility with the A or A.SW parental strains and contained only H-2 antigens of A or A.SW origin, respectively. The variants were harvested after 1–5 passages in A×A.SW F_1. The unselected LNSF line of origin, which has H-2 antigens from both A and A.SW, was used as well.

Tissue cultures were prepared from transplanted YAA-C1-C3 or MWE tumors and maintained *in vitro* during 7–60 days. They were trypsinized and the cells diluted in conditioned Eagle's medium with 15% fetal calf serum, the concentration being 1000 YAA-C1-C3 and 2000 MWE cells/ml respectively. Four milliliters of the cell suspensions were added to each of 2–3 fifty mm-Falcon plastic petri dishes. After incubation overnight at 37°C in CO_2 atmosphere, when the cells had attached to the petri dish surfaces, the medium was replaced by 0.5 ml of a suspension containing 2×10^6–4×10^7 lymphoid cells/ml and 0.3 ml Wellcome PHA diluted 1 : 20. After 45 minutes' incubation at 37°C,

each petri dish received 4 ml of Eagle's medium containing 15% fetal calf serum. The number of colonies growing out was counted 3–4 days later after staining with 0.1% crystal violet.

As shown in Table I, YAA-C1-C3 cells were sensitive to allogeneic lymphoma variant cells of the LNSFS (A.SW-compatible) type, in spite of the fact that both the LNSFS cells and the syngeneic (LNSFA) cells

TABLE I

PLATING EFFICIENCY OF YAA-C1-C3 (OF A/Sn ORIGIN) AFTER EXPOSURE TO INTACT OR ULTRASOUND-DISINTEGRATED LNSFS (A.SW-COMPATIBLE) LYMPHOMA VARIANT CELLS

No. of lymphoid cells added	Ultrasound disrupted (+ or −)	Syngeneic LNSFA added	Allogeneic LNSFS added	
		Colonies/plate	Colonies/plate	Reduction (%)
10^7	−	66,62,	42,36	40
5×10^6	−	70,66	42,38	41
10^6	−	92,96	66,86	19
5×10^6	+	100,86	50,46	48
10^6	+	99,98	70,74	25
5×10^6	−	38,40	22,24	41
10^6	−	58,38,59	48,50	6
5×10^6	+	40,45	17	60
10^6	+	56,52	42,43	21
10^7	−	30,25	8,16	60
5×10^6	−	30,26	15,12,17	46
10^6	−	32,36,29	31,28	8
5×10^6	+	34,22,26	11,15,12	54
10^6	+	42,33,34	25,24,21	36

used in the controls were harvested from A×A.SW F_1, this procedure ensuring that normal immunologically competent cells were not responsible for the findings. Table II shows that the unselected "semisyngeneic" LNSF line from which the variants were derived was equally effective as LNSFS in reducing colony formation. Since analogous findings were made with the A.SW-compatible sarcoma MWE (Table III), they cannot be attributed to a nonspecific toxicity of, e.g., the LNSFS line, which was the allogeneic source of lymphoma cells in the YAA-C1-C3 experiments. Table IV summarizes the experiments performed so far on

YAA-C1-C3 and MWE exposed to lymphoma variant cells of either the syngeneic (controls), allogeneic, or "semisyngeneic" type.

TABLE II

PLATING EFFICIENCY OF YAA-C1-C3 (OF A/Sn ORIGIN) AFTER EXPOSURE TO INTACT OR ULTRASOUND-DISINTEGRATED LNSF LYMPHOMA CELLS (A × A.SW F_1 ORIGIN)

No. of lymphoid cells added	Ultrasound disrupted (+ or −)	Syngeneic LNSFA added	Allogeneic LNSF added	
		Colonies/plate	Colonies/plate	Reduction (%)
10^7	−	14,15,16	2,2	86
5×10^6	−	10,12	5,2,4	68
5×10^6	+	10,12	4,4	64
10^7	−	28,24	8,7	71
5×10^6	−	26,28	16,20	33
10^6	−	36,26	28,29	8
5×10^6	+	30,24	3,7	82
10^6	+	22,26	19,33	0
10^7	−	11,15,14	7	46
5×10^6	−	16,17	9,13	33
10^6	−	14,22,17	17,21	0
5×10^6	+	14,11,18	6,6,7	58
10^6	+	26,21	20,15	26

As appears from Tables I–IV, ultrasonication of the lymphoma cells did not abrogate their ability to reduce colony formation. In fact, ultrasonicated cells were, in several experiments, more effective than intact cells in this respect (Table IV).

Tables V and VI show that allogeneic lymphocytes added to YAA-C1-C3 target cells, instead of lymphomas, were approximately as effective in reducing the numbers of target cell colonies as were the lymphoma variant cells. Sensitization of the lymphocyte donors *in vivo* did not increase their colony reductive capacity in the experiments performed so far, three of which are presented in Table V. However, a great difference between sensitized and untreated lymphocytes is observed if PHA is excluded from the systems; only the sensitized lymphocytes reduce colony formation in the absence of PHA and this reduction is of the same degree as in the presence of PHA (unpublished findings). As shown in Table VI, disintegration of untreated lymphocytes with ultrasound did not abrogate colony inhibition; PHA was added in all these experiments.

The findings presented indicate that allogeneic inhibition of trans-

planted tumors *in vivo* is, at least in part, due to exposure of the grafted cells to foreign H-2 isoantigens (or material closely associated with these

TABLE III
PLATING EFFICIENCY OF MWE (OF A.SW ORIGIN) AFTER EXPOSURE TO INTACT OR ULTRASOUND-DISINTEGRATED LNSF (OF A × A.SW F_1 ORIGIN) OR LNSFA (A-COMPATIBLE)

No. of lymphoid cells added	Ultrasound disrupted (+ or −)	Syngeneic LNSFS added		Allogeneic LNSFA or semisyngeneic LNSF added	
		Colonies/plate	Type	Colonies/plate	Reduction (%)
2×10^7	−	16,13	LNSF	4,7	63
10^7	−	20,18		6,12	53
5×10^6	−	30,32		26,28	13
10^6	−	48,40		43,41	5
10^7	−	65,40	LNSFA	36,34	33
5×10^6	−	40,46		46,45	0
5×10^6	+	50,56		32,35	32
10^6	+	56,60		64,66	0
2×10^7	−	20,21,16,18	LNSFA	6,6,2,4	76
2×10^7	−	19,20	LNSFA	8,14	44
2×10^7	−	37,28	LNSFA	20,18	39
5×10^6	+	20,24	LNSFA	8,10	60
5×10^6	−	80,68	LNSF	46,38	43
10^6	−	82,88	LNSF	88	0
10^6	+	94,74	LNSF	80,64	14

antigens). The strongest support for this idea comes from the demonstration that ultrasonicated allogeneic or F_1 hybrid lymphoma cells can reduce colony formation *in vitro,* as shown in this paper, and that the cytotoxic effect of disintegrated allogeneic lymphoma cells can be abrogated if their foreign H-2 isoantigens are specifically neutralized with isoantiserum (Hellström and Hellström, 1966). Therefore, our working hypothesis is that a close target cell contact with other cells is growth inhibitory (or cytoxic), when the surfaces of the two types of cells differ from each other, as is the case when the targets lack H-2 isoantigens present on the cells in their close neighborhood. In order to study which

is the role of the H-2 isoantigens themselves in this respect, experiments are being performed in which tumor target cells are exposed to different cell fractions and the ability of each fraction to give colony inhibition is compared with its content of H-2 antigens.

TABLE IV
PERCENT REDUCTION IN COLONY NUMBERS AFTER ADDITION OF LYMPHOMA VARIANT CELLS WITH FOREIGN H-2 ANTIGENS

Number of cells added	Intact cells	Sonicated cells
H-2 antigens of A.SW origin to A-compatible YAA-C1-C3[a]		
10^7	60.6 ± 3.8	Not tested
5×10^6	41.8 ± 5.5	51.1 ± 6.3
10^6	13.3 ± 7.1	31.1 ± 5.0
H-2 antigens of A origin to A.SW-compatible MWE		
5×10^6	30.8	51.0
10^6	2.5	21.3

[a] Seven to thirteen experiments were made with each dose. Mean reductions ± S.R. is given.

It may be speculated that the mechanisms proposed for allogeneic inhibition play a role during normal growth surveillance by eliminating aberrant cells whose isoantigenic equipment would mismatch that of cells surrounding them in a given tissue, without need for a classical immunological reaction by immunologically competent cells. Such speculations may be premature, however, particularly since our findings concern only H-2 isoantigens so far. If the mechanism proposed for allogeneic inhibition is proved to be of general importance for growth surveillance, then another mechanism will have to be postulated by which cells can escape from the control elicited by allogeneic inhibition, in order to explain the finding that chimeras can be established between cells with different isoantigenic equipment. However, at least two routes of escape from allogeneic inhibition are known. First, large tumor cell inocula are not sensitive to allogeneic inhibition as detected *in vivo* but grow in syngeneic and semisyngeneic recipients as well (Hellström, 1966a). Second, cell populations may be adapted to exposure to foreign isoantigens. By propagating an A tumor line in either A×A.CA F_1 or A×A.SW F_1 hybrid hosts for several passages, it was possible to isolate sublines of it with increased capacity to grow in A×A.CA F_1 hybrids, respectively, as com-

pared to a control tumor line maintained in A hosts (Hellström, 1966b).

The present demonstration that intact or ultrasonicated lymphoma cells were approximately as effective as normal lymphocytes in inhibiting colony formation *in vitro,* is compatible with the postulate by Möller that cellbound immunity reactions, as studied with mouse cells *in vitro,*

TABLE V

PLATING EFFICIENCY OF YAA-C1-C3 (OF A/Sn ORIGIN) AFTER EXPOSURE TO LYMPHOCYTES OR LYMPHOMA CELLS OF VARIOUS ORIGIN

Type cells added	Number of colonies in			
	Number	Experiment group	Control group	Reduction (%)
Sensitized lymphocytes	10^7	48,38	64,60	31
ACA anti-A in experiments	5×10^6	24,38	72,68	56
A anti-A.SW in controls	10^6	66,56	62,74	10
	0	–	64,58	–
Sensitized lymphocytes	10^7	1	30,15	96
ABY×CBA anti-A in experiments	5×10^6	11,12	23	50
A anti-A.SW in controls				
Sensitized lymphocytes	10^7	20,19	30,26	30
A.CA anti-A in experiments	5×10^6	8,19	30,34	58
A anti-ASW in controls	10^6	32,19	37,42	35
Untreated lymphocytes	2×10^7	5,3	22,24	83
ACA in experiments	10^7	8,4	12,24	74
A in controls	5×10^6	10,12	32,28	52
Untreated lymphocytes	10^7	7,10	14,18	47
A.SW in experiments	5×10^6	7,6	18,14	59
A in controls	10^6	14,18	16,20	0

at least in part are due to target cell contacts with the "foreign" surface of added allogeneic lymphocytes. However, before any conclusions can be drawn, more information is needed on this point. The colony inhibition technique described seems to be well suited for such studies.

Finally, it should be pointed out that the possibility prospected, namely that allogeneic inhibition is due to *in vivo* target cell exposure to F_1 hybrid cells carrying foreign histocompatibility antigens, does not exclude the possibility that other mechanisms may also operate *in vivo* and that they may differ in different systems and be of either an immunological or a nonimmunological nature.

TABLE VI
PERCENT REDUCTION IN COLONY NUMBERS AFTER ADDITION OF INTACT AND DISINTEGRATED NORMAL A.SW LYMPHOID CELLS TO YAA-C1-C3 (A-COMPATIBLE) TARGET CELLS

	Number of lymphoid cells added	Percent reduction[a]
Intact cells added	10^7	63.6 ± 4.0
	5×10^6	55.5 ± 1.5
	10^6	18.7 ± 4.4
Disintegrated	5×10^6	50.6 ± 4.3

[a] Mean reduction ± S.R. is given.

II. SUMMARY

Lymphomas, carcinomas, and sarcomas originating in homozygous hosts grow better when transplanted into syngeneic mice than in F_1 hybrids between the syngeneic and an allogeneic strain. The growth inhibition in F_1 hybrids is called allogeneic inhibition. It was attributed to exposure of grafted homozygous cells to those H-2 antigens in the F_1 hybrids which are foreign to these. Findings were presented that support the postulate that lymphocyte-mediated immune reactions against allogeneic mouse cells cultivated *in vitro* are, at least partly, due to the same mechanism as that of allogeneic inhibition.

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DISCUSSION

DR. CUDKOWICZ: I would like to limit my discussion to the part of Dr. Hellström's paper concerned with *in vivo* parent-to-hybrid tumor transplantation. I have been studying for a few years F_1 hybrid resistance to grafts of normal parental hemopoietic tissue, a phenomenon which resembles at first glance what Dr. Hellström has called allogeneic inhibition or syngeneic preference. An extensive discussion of hybrid resistance has been reported recently [Cudkowicz (1965). *In* "Isoantigens and Cell Interactions" (J. Palm, ed.), pp. 37–56, Wistar Inst. Press, Philadelphia, Pennsylvania]. In brief, hemopoietic cells of inbred parental strain mice do not grow, or grow deficiently, upon transplantation into adult F_1 hybrids. The phenomenon of incompatibility is restricted to combinations of parental donors homozygous for the *H-2*^{b} allele and of F_1 recipients *H-2*^{b} heterozygous. This constitutes strong evidence for genetic control of hybrid resistance and for association with *H-2* in linkage group IX. Subsequent studies have established that the hybrid resistance locus, designated by the symbol *Hh-1*, is closely linked with the D subregion of *H-2*, but separable through meiotic crossing-over from the TLa locus (thymic-leukemia antigen) on the one side, and from the subregions C and K of *H-2* on the othe side. The sequence of the named loci is TLa (D *Hh-1*) C K, with a crossover frequency of 1.51 for the interval TLA-D [Boyse, E. A. *et al.* (1965). *Intern. Symp. Immunopathol., Monaco*], 0.21 for the interval D-C, and 0.36 for the interval D-K [Stimpfling, J. H. *et al.* (1965). *Genetics* **51**, 831]. The relative positions of *Hh-1* and of the components of the D subregion of *H-2* are still to be determined.

The deficient growth of parental hemopoietic grafts in F_1 hybrids is best explained by a specific destructive reaction mounted by the heterozygous host against homozygous donor cells, similar to the homograft reaction. Such a mechanism cannot account, however, for the failure to grow of parental tumor cells in F_1 mice which has been described by Dr. Hellström as allogeneic inhibition, on the basis of *in vivo* and *in vitro* experiments. In the latter case, the deficient growth of parental tumor grafts would be due to a property inherent to, and to a reaction of, the transplanted cells rather than to a reaction of the host. Hence, allogeneic inhibition and hybrid resistance are entirely different phenomena.

Recently, I studied the growth of transplantable lymphoma cells from *H-2*^{b} homozygous mice (carrying, therefore, the genetic determinant of hybrid resistance) into several types of F_1 hybrid mice heterozygous either at known histocompatibility *(H)* loci or at subregions of *H-2*. Such an experiment was conceived to detect both hybrid resistance and allogeneic inhibition, and to compare their properties under conditions as similar as possible. On the basis of experience with normal cells, hybrid resistance to parental lymphoma cells was expected to occur only in F_1 hybrids heterozygous at the D subregion of *H-2*, irrespective of heterozygosity at other sites. Since hybrid resistance is dependent upon host reactivity, the growth of the parental lymphomas was evaluated also in previously X-irradiated F_1 hosts. Moreover, since infant F_1 mice are temporary susceptible to the growth of parental marrow, the growth of the lymphomas was compared in F_1 mice of appropriate genotype at the age of 20 days or less, and in their counterparts after weaning age. In this way I hoped to be able to distinguish allogeneic inhibition from hybrid resistance by means of genetic analysis and by the independence of the former from host age and irradiation.

Two unrelated, radiation-induced lymphomas of C57BL/10 (B10) mice, namely, lymph-

omas S1033 and S1043, were kindly supplied by Dr. G. D. Snell of the Jackson Laboratory. Minimal numbers of cells from these lymphomas were grafted into isogenic and F_1 hybrid recipients. The lymphomas were highly strain specific and the hybrid mice were offspring from congenic-resistant parents. Hence, the F_1 mice employed were heterozygotes only for chromosome segments containing either the genes *H-1* and *H-4*, or *H-2*, or subregions of *H-2*.

The results of a typical experiment are presented in Table I. They are consistent with those obtained in three additional repetitive experiments. Lymphoma cells of both tumors grew without detectable impairment in most types of F_1 hybrids and killed the majority of them, except in the case of F_1 mice which were heterozygotes for the D subregion of *H-2*. In these resistant hybrids the growth of parental cells was deficient so as to fail to kill a a large proportion of recipients. Results presented in Table II indicate that (B10×B10.A) F_1 hybrids became resistant to the cells of lymphoma S1033 after weaning age but were susceptible at the age of 14–18 days. Furthermore, the resistance was abrogated in adult (B10×B10.A) F_1 mice by exposure to 500 r of X-irradiation. Therefore, according to three criteria, genetic, host age, and radiosensitivity, the phenomenon of hybrid resistance to parental hemopoietic cells applies also to the two lymphomas studied. The grafted cells were capable of optimal growth in the presence of foreign allelic H-1, H-2 (components of subregion C and K), and H-4 isoantigens, a condition which should have generated allogeneic inhibition.

Failure of the two lymphomas investigated to exhibit allogeneic inhibition has prevented me from comparing it directly with hybrid resistance. Nevertheless, the results warrant the conclusion that the properties of hybrid resistance are markedly different from the properties of allogeneic inhibition reported by Dr. Hellström, even when hybrid resistance is tested against tumor cells. The findings also indicate that not all tumors are subjected to allogeneic inhibition. Could Dr. Hellström tell us if he were to agree that allogeneic inhibition, when detectable *in vivo*, represents a special case rather than the rule?

DR. HELLSTRÖM: I think we agreed at the Wistar Institute Symposium last year that syngeneic preference and hybrid resistance are different entities [see discussion following the paper by Hellström (1965). *In* "Isoantigens and Cell Interactions," (J. Palm, ed.) Monograph, Vol. 3, pp. 79–91, Wistar Inst. Press, Philadelphia, Pennsylvania]. As you mentioned, hybrid resistance seems to be best explained by a homograft reaction in the F_1 hybrid hosts against antigens specific for homozygous cells; it is radiation sensitive; it is confined to cells carrying the $H\text{-}2^b$ allele. The findings presented by you on two C57BL10 lymphomas indicate also to me that you have demonstrated that these tumors are sensitive to hybrid resistance. On the other hand, you could not demonstrate any allogeneic inhibition similar to what we have described, and you suggested that allogeneic inhibition is not a general phenomenon but rather a special case, detectable with isoantigenic variant sublines, selected from a particular A×A.SW F_1 lymphoma used by ourselves. Since I did not present a detailed description of our *in vivo* findings today but mostly commented on our approach to the mechanisms of allogeneic inhibition, as this has been done in tissue culture experiments, I may recapitulate findings previously published by me this year [Hellstrom, K. E. (1966). *Intern. J. Cancer* **1**, 349–359]. I have there described allogeneic inhibition, defined (solely) as deficient growth of parental strain tumor cells in F_1 hybrid hosts, with a total of seven different lymphomas (A.SW, A, C57BL, C57L, C3H), four carcinomas (C3H, A.SW, A.CA, A.BY), four sarcomas (C57BL, A.CA, C57L). In addition, it was detected with fifteen A.SW-compatible and four A-compatible variants, selected from the A×A.SW F_1 lymphoma LNSF. Our finding of deficient growth of the homozygous tumor cells in F_1 hybrid hosts confirmed and extended previously published findings by Snell [(1958) *J. Natl Cancer Inst.* **21**, 843–877] who described this for several C57Bl leukemias, for one

TABLE I

ROLE OF HETEROZYGOSITY AT THE D REGION OF *H-2* IN THE RESISTANCE OF F_1 HYBRID MICE TO GRAFTED CELLS OF PARENTAL LYMPHOMAS S1033 AND S1043

Host strain	Heterozygosity	Lymphoma S1033[a]		Lymphoma S1043[b]	
		No. dead at 90 days	Percent dead ± SE	No. dead at 90 days	Percent dead ± SE
B10 controls	None	22/23	95.6 ± 4.3	15/18	83.3 ± 8.7
B10 × B10.A	D C K of *H-2*	5/12	41.6 ± 14.2	4/15	26.6 ± 11.4
B10 × H-2I	D C of *H-2*	4/10	40 ± 15.5	–	–
B10 × H-2H	C K of *H-2*	10/10	100	20/20	100
B10 × B10.129(23M)	*H-1, H-4*	–	–	9/10	90 ± 9.5

[a] 100 nucleated cells injected into the tail vein.

[b] 200 nucleated cells injected into the tail vein.

TABLE II

ROLE OF AGE AND EFFECT OF RADIATION ON THE RESISTANCE OF (B10 × B10.A) F_1 HYBRID MICE TO 100 GRAFTED CELLS OF PARENTAL LYMPHOMA S1033

Host			No. dead at 90 days	Percent dead ± SE
Strain	Age	Irradiation		
B10 controls	14–31 days	–	21/21	100
B10 × B10.A	14–18 days	–	12/13	92.3 ± 7.4
B10 × B10.A	21–31 days	–	4/10	40 ± 15.5
B10 controls[a]	Adult	500 r	19/19	100
B10 × B10.A	Adult	–	4/9	44.4 ± 16.3
B10 × B10.A	Adult	500 r	24/28	85.7 ± 6.6

[a]For adult, non-irradiated B10 controls see Table I.

C3H leukemia, and for one A sarcoma. In addition, the Mollers have demonstrated deficient growth of C57BL sarcoma lines in F_1 hybrids [Möller, E (1964). *J. Natl. Cancer Inst.* **37,** 979–989], and also killing of homozygous normal or tumor cells of various strains (C57Bl, A.SW, A, C3H) after *in vitro* contact with F_1 hybrid lymphocytes and PHA [Möller, E. (1965). *Science* **147,** 873–879; Möller, G. and Möller, E. (1965). *Nature* **208,** 260–263; Möller, E. (1966). On humoral and cellular factors in cytotoxic reactions against allogeneic normal and neoplastic cells. Thesis, Univ. of Stockholm]. I think all those data taken together strongly support our view that homozygous tumor cells are inhibited by F_1 hybrid cells *(in vivo* and *in vitro)* also in cases where the tumor cells do not contain the $H\text{-}2^b$ allele.

As regards the interpretation of our data, our main reason to attribute them to cell surface differences between homozygous and F_1 hybrid cells and not to immunological killing of homozygous cells by F_1 hybrid lymphocytes is the consequence of our *in vitro* findings. I have great difficulty in understanding how ultrasonicated F_1 hybrid lymphoma cells can kill homozygous target cells by an active immunological mechanism, and how this killing can be abrogated if the foreign H-2 antigens of the added sonicated cells are specifically covered by isoantiserum. However, I am open for all suggestions on this point.

To comment on your Table II, I can only say that we have observed a deficient growth of homozygous tumor cells in F_1 hybrids also, when these were young and when they were X-irradiated, if we compared tumor growth in similarly irradiated syngeneic and F_1 hybrid recipients. We also have observed that X-irradiated F_1 hybrid lymphoid cells could inhibit the outgrowth of parental strain target cells in so-called neutralization experiments [Bergheden and Hellström (1966). *Intern. J. Cancer* **1,** 361–369].

DR. CUDKOWICZ: I would like to call your attention to the fact that two unrelated C3H adenocarcinomas studied by A. C. Wallace [(1965) *Nature* **207,** 309] did not exhibit allogeneic inhibition in F_1 hybrid hosts, although the experimental conditions were set for the detection of this phenomenon. In addition, D. Oth *et al.* [(1965) *Compt. Rend. Soc. Biol.* **159,** 2231] could not confirm the properties of allogeneic inhibition with a C3H and an inbred Swiss tumor. Hence, six unrelated tumors, three of C3H, two of C57BL10, and one of Swiss origin, do not share with your tumors the allogeneic inhibition reaction. I still think, therefore, that you may be dealing with special cases or with small and temporary differences of growth which do not lend themselves to generalization. In fact, you are working in most cases either with variant tumors of F_1 hybrid origin selected in one of the parents, or with tumors of strain A mice.

DR. HELLSTRÖM: Since we have studied a great many tumors for growth in homozygous and F_1 hybrid hosts and found virtually all of them to grow worse in the hybrids than in the syngeneic recipients, I am rather reluctant to look upon allogeneic inhibition as an exceptional case, detected only with parental strain-compatible isoantigenic variant lines [Hellström (1966). *Intern. J. Cancer* **1,** 349–359]. Snell's findings, which in fact were made before ours, also support my point of view [Snell, G. D. (1958) *J. Natl. Cancer Inst.* **21,** 843–877; Snell, G. D., and Stevens, L. C. (1961) *Immunology* **4,** 366–379].

DR. TURK: I would like to bring this down to a more fundamental level and ask what you think is the actual interaction between these cells during the reaction in which one cell type with an antigenic and genetic difference is having a cytopathic effect on another cell type? Although you do not believe that there is any immunologic reaction going on, have you studied this phenomenon at the subcellular or at the biochemical level? I must say that I find it rather difficult to believe that an antigen can be cytotoxic per se. I have always accepted antibodies as being cytotoxic.

DR. HELLSTRÖM: Our main reason for believing that allogeneic inhibition is due to target cell exposure to foreign antigens is that cell death in our tissue culture experiments could

be abrogated if the foreign antigens were specifically covered with isoantibodies. I agree that it might be difficult to believe an antigen to be cytotoxic. However, what we consider to be an isoantigen may be a very fundamental component of the cell surface. Our findings may be equally difficult to understand, but not more so than are the cell surface reactions which are responsible for the specific aggregation of cells from different species of sponges, or for contact inhibition. We have not studied the mechanisms of allogeneic inhibition at the subcellular level except for recent work in cooperation with Dr. Dan Motet at the Karolinska Institutet in Stockholm which suggest that the cell fraction which is inhibitory in our CI experiments is that containing the cell membranes.

DR. HAUSCHKA: Dr. Hellström's review of allogeneic inhibition was really enlightening, especially because of the new data he presented today on inhibition of plating efficiency by allogeneic antigens and failure to inhibit after coating these antigens with a specific antiserum. This seems to support his theory considerably. From Dr. Cudkowicz's discussion it is clear that hybrid resistance is a quite distinct phenomenon. It is under known genetic control, it is observed with tissues of hemopoietic origin, it operates in certain but not in other genotypes of F_1 hybrids, and is abrogated by irradiation of the F_1 host. It seems important to emphasize this today since the fundamental differences between hybrid resistance and allogeneic inhibition have not always been recognized. For instance, they have been regarded as "identical inhibition phenomena" recently by Simonsen [*Transplantation* **4**, 354 (1966)]. Now, the issue I would like to take with Dr. Hellström is not aimed at his beautiful and vast data but at the broad biological interpretation of these findings. Regarding the biological significance of allogeneic inhibition, the Hellströms and also George Klein have speculated as follows, if I may simplify and abbreviate what they have said: Allogeneic inhibition is a nonimmunological "surveillance mechanism" which eliminates mutants with different surface characteristics from the soma, even within a given tissue and without recourse to orthodox immunological interference. In their view, this is not only a model for growth control but also for the elimination of potential cancer cells recognized as foreign by cell contact before they can get hold of the host. This recognition occurs through H-2-like substances on the cell surfaces. I would like to consider first some conservative alternate interpretations of Hellström's data and second some apparently irreconcilable facts. And again I want to emphasize that this is simply an issue of interpretation rather than a questioning of the data. We do not know whether the smaller size of Hellström's tumors grown in the F_1 hybrids involves fewer tumor cells or less stroma or both. Is the stroma-eliciting capacity of tumor cells in a foreign environment possibly impaired? This question could be easily answered by using Chalkley's three pointer method, which is an objective way of scanning histologic sections and finding out how often the three corners of a triangle in the ocular coincide with the stroma and how often they fall on the tumor tissue proper. Perhaps a difference could be found in a double-blind comparison of this type. The cells of any primary tumor, or of a tumor transplanted in the inbred strain of origin are no doubt selected for optimal competitive growth in their syngeneic environment. It is a well-established fact that if you transplant small cell numbers into hybrid hosts which are "semiforeign" to the syngeneic cells then there has to be adaptation and reselection before the tumor can attain optimal growth in its new host environment. Hellström himself finds that such selection does occur upon retransplantation in the allogeneic F_1 environment. In the *in vitro* experiments of Hellström, the tumor cells are incubated with an average excess of 1000 spleen cells to one tumor cell. The incubation period is, I think, 48 hours or 3 days. Now if the spleen is syngeneic, it cannot compete with the tumor cells for metabolites because of the selection that occurred in the original host. If the spleen is allogeneic it may very well do so, even after irradiation with 1000 r. Such spleen cells would continue to synthesize RNA and proteins and other molecules withdrawing nutrilites

from the tumor cells and inhibiting them thereby in a nonspecific way. The *in vitro* demonstration of allogeneic inhibition, at least in the earlier work, has required addition of phytohemagglutinin. Phytohemagglutinin is known to be a mitogenic growth stimulant of hemopoietic cells rather than of other cell types, including tumor cells. Therefore, phytohemagglutinin would exaggerate the hypothetical metabolic advantage of the foreign spleen cells which I have just mentioned over the tumor cells and this seems as valid an explanation as Hellström's also acceptable assumption that phytohemagglutinin works by promoting closer surface contact between target cells and spleen cells. But this ought to be checked out with phase contrast microscopy, and then it should also be shown whether or not cortisone, which counteracts allogeneic inhibition, breaks up the close cell contact. Both these questions should be reexamined. Today we were told of some painstaking *in vitro* experiments which constitute a more critical approach than the incubation experiments with allogeneic spleen. Plating efficiency of tumor cells exposed to allogeneic H-2 antigen before and after reaction with specific antisera was compared. The results are convincing. I cannot knock a hole into them at this moment. However, there still remain a number of biological phenomena irreconcilable with the assumption that allogeneic inhibition is a universal biological surveillance mechanism. The first of these comes from Dr. Klein's laboratory at the Institute of Tumor Biology, in Stockholm. In *Nature,* 1956, the Kleins and Revesz reported allogeneic stimulation, not allogeneic inhibition, when they inoculated a few A-strain tumor cells mixed with a large excess of DBA tumor cells into nonimmunized A-strain mice. Exposure of the A cells to the foreign antigens of the DBA cells should have caused inhibition, but it did just the opposite; they grew better; they grew in a situation where they otherwise would not have grown. Now let us look at the chimeras, both natural and experimental. Among natural chimeras, I would like to mention Giblet's hermaphrodite of Scandinavian ancestry. This case, reported in the *American Journal of Human Genetics* in 1963, showed a generalized tissue mosaicism. One eye of the patient was brown, the other was hazel. The leukocytes were about half XX the other half XY. Skin cells cultured from the left side of the abdomen were XX, from the right side of the abdomen were XY. A laparotomy was performed and on the left there was a normal ovary, on the right an ovotestis. A very careful screening was done for human isoantigens, 23 in all, of the patient's father, mother, and of the patient herself, who had two populations of red cells. From this study it became apparent that in the red cells both of the paternal gene combinations for two separate isoantigenic systems were expressed and must have come in through two separate sperms. This patient was MS and NS for the MNSs system, and cDE and cde for the Rh system. One of these combinations was inherited with the paternal X-carrying sperm, the other with the Y-carrying sperm. Since this is a euploid mosaic having 46 chromosome XX and 46 chromosome XY cells, the best interpretation is to assume that two spermatozoa of different genotypes combined with two female gametes, one being the egg proper and the other a polar body. In spite of this life-long allogeneic symbiosis, there was no inhibition of one cell type by the others. In Marmoset monkeys, the frequent twinning and vascular anastomoses between twins of unlike sex produce many XX, XY mosaics within which the two component cell types maintain their equilibrium. Beatrice Mintz has achieved many successful fusions between mouse blastulae of diverse genotypes. These grow up into healthy composite mice of normal longevity having components of both parental genotypes. Two-membered mammalian cell cultures, for example, cells from a human papilloma mixed with Chinese hamster kidney cells *in vitro* and human papilloma cells mixed with the hyperdiploid Ehrlich ascites tumor have continued to show about equal proportions of mitoses of both karyotypes after prolonged cultivation in Goldstein's laboratory at Roswell Park Memorial Institute. These cultures were mixed because we wanted to achieve somatic hybridization between man and mouse. We failed, but in Oxford,

Harris achieved such a somatic hybrid between mouse cells and human cells, confirmed by Ford's chromosome identification. Now, somatic hybridization is a well-known fact; it was achieved first by Barski, then by Ephrussi and since then in several other laboratories. In our own work here we have typed (together with Spencer, Amos, and Ephrussi) the H-2 antigens of several somatic mouse hybrids. The somatic hybrids showed a distinct growth advantage over the parental mouse strains from which they were derived, and all the H-2 antigens introduced by the two different parental cell strains were codominant in the hybrid cells. So, where is the allogeneic antagonism between different H-2 substances in these hybrids? One should not disregard the successful propagation of numerous tumors in embryonated chicken eggs where the bird stroma fails to inhibit the mammalian cells, nor the growth of heterologous tissues in the anterior chamber of the eye or in the brain. Finally, and I think most convincing, there is the irradiated feeder layer technique developed by Puck and associates and now in very general use. This seems to be one of the most effective ways to clone single normal and malignant cells, with a high plating efficiency regardless of antigenic species differences. Here we actually observe allogeneic stimulation without which some of these single cells could not grow at all, despite the supposedly inhibitory foreign antigens of the feeder layer with which they must have some contact. All these facts then detract somewhat from the general biological claim for allogeneic inhibition as a model for growth control. This is not to disregard contact inhibition as an important and real phenomenon. But in contact inhibition as we know it, syngeneic normal cells stop growing and inhibit one another as soon as they have formed a contiguous layer on glass. Here we have in fact syngeneic inhibition, while with the irradiated feeder layer one observes allogeneic stimulation. Would it not be wise for the Hellströms to reinterpret their painstaking and fruitful experiments, for which I have a very high regard, in line with all these seemingly obvious contradictions? If these are accommodated, then "allogeneic inhibition" and "syngeneic preference" might be replaced by more suitable terminology.

DR. HELLSTRÖM: I wish to thank you very much for your detailed comments and I agree with you that allogeneic inhibition and hybrid resistance seem to be different phenomena, which shall be kept apart. This was in fact the reason for us to introduce the term "allogeneic inhibition," and I may point out that this term (as well as syngeneic preference) is entirely descriptive, referring to the finding of a deficient growth of transplanted homozygous tumor cells (not only those of $H\text{-}2^b$ origin) in F_1 hybrid recipients.

You started by mentioning that the smaller growth of our tumors in F_1 hybrids may either involve fewer tumor cells or less stroma or both. This may be true. However, by decreasing the cell numbers inoculated, we got an absolute difference in tumor takes: for example 75% of A.SW mice inoculated with ten cells of an A.SW-compatible tumor line got tumors while none of A×A.SW F_1 recipients did [Hellström, K. I. (1966). *Intern. J. Cancer* **1**, 349–359]. I quite agree with the criticisms you gave on the early experiments of the Möllers and ourselves, where tumor cells were mixed with spleen or lymph node cells *in vitro*. However, the same criticism is not valid for our recent experiments, as those presented today, where sonicated lymphoma cells were added to our target cell cultures and gave inhibition of colony formation [cf. also Hellström *et al.* (1965). *Nature* **208**, 458–460].

Your second point was that there are a number of biological phenomena which might seem to be irreconcilable with our interpretation of allogeneic inhibitions. I had no time to comment on this point in my talk, but discussed it somewhat in my manuscript. There are known routes of escape from the mechanisms we have postulated for allogeneic inhibition; thus cells can be selected which are less sensitive to it [Hellström, K. E. (1966). *Exptl. Cell Res.* **42**, 189–192]. I do not think that the findings mentioned by you exclude our explanation of allogeneic inhibition more than the fact that cancer is a fatal disease excludes the (proved) existence of tumor-specific antigens. Whether "our" mechanisms of allogeneic

inhibition have any role for normal growth surveillance or not is entirely unknown, and I, therefore, agree with you that we might have to be careful with speculations, particularly before we know whether target cell exposure to other cells carrying foreign antigens than those determined by locus H-2, is growth inhibitory or not.

The Effect of Immunosuppressive Drugs at Various Stages of Differentiation of Immunologically Competent Cells

JAROSLAV ŠTERZL

Czechoslovak Academy of Sciences, Institute of Microbiology, Department of Immunology, Prague, Czechoslovakia

In this review I would like to discuss the use of metabolic inhibitors in studies of the mechanism of antibody formation rather than to summarize completely all the data available on the effects of immunosuppressive drugs; this was done extensively by Schwartz and André (1962), Šterzl (1962), Hitchings and Elion (1963), Schwartz (1965), Floersheim (1965), and others.

For a better understanding of the problem to be discussed, I shall briefly characterize the theories of antibody formation as they were known at the time the inhibitors of nucleic acid synthesis were introduced into immunological studies, i.e., at the beginning of 1958.

The idea that the antigen induces biochemical changes in cells *de novo,* for example, the synthesis of specific nucleic acid which then directs antibody formation—an indirect instructive effect of antigen—was supported by some data from our laboratory.

Antibodies were isolated in the form of nucleoproteid from spleen cells taken shortly after immunization (Šterzl, 1954). After transfer of these nucleoproteids to newborn rabbits, antibodies were detected in the serum of recipients (Šterzl and Hrubešová, 1956). The demonstration that nucleic acids are involved in the synthesis of antibodies led us to investigate the effects of 6-mercaptopurine (6-MP), an inhibitor of nucleic acid synthesis (Šterzl and Holub, 1957). Similarly, the effects of this drug on the synthesis of antibodies was studied by Schwartz *et al.* (1958).

An alternate hypothesis to explain antibody formation was proposed by Talmage (1957), and Burnet (1959). This hypothesis assumed not only that the lymphoid cell has genetically determined capacity to form antibodies (developed in the course of evolution), but also that in some of these cells the potentiality to form antibodies is spontaneously expressed (formation of the so-called natural antibodies – Burnet, 1959, pp. 54 and 95). According to this original clonal selection hypothesis, the antigen does not act as a real inductor but only initiates a quantitative proliferation of the cell responding to the antigen and already producing a small amount of the corresponding antibody.

Metabolic inhibitors were used not only to select the most effective agents in depressing immune responses but also to study the effects of various types of inhibitors on different phases of the immune response so as to test the validity of the two theories of antibody formation mentioned above. The aim of these studies was to determine whether antibody induction is necessarily linked to cell proliferation or whether mitotic division of cells might be inhibited without preventing the differentiation process of immunologically competent cells into antibody-producing cells. Some data indicated that a certain antagonism exists between these two processes, i.e., differentiation and cell division (Swann, 1958).

In studies on antibody formation it is extremely difficult to separate cell differentiation from cell division. The increase of antibodies during the exponential phase of the response, namely, during 48 hours after administration of antigen, results predominantly from mitotic divisions. Therefore any substance which inhibits proliferation would lead to a depression of the antibody response; indeed, this was found using substances with antimitotic activity (Berenbaum, 1960; Dutton and Pearce, 1962; Schwartz and André, 1962).

To distinguish the effect of metabolic inhibitors on the process of cellular proliferation from their influence on biochemical processes during differentiation, we used a model in which cellular proliferation was limited considerably. The effect of metabolic inhibitors was studied using transfers of isolated spleen cells from normal adult rabbits, mixed with antigen *in vitro* and administered intraperitoneally to newborn animals (Šterzl, 1957). In this system the transferred cells are allogeneic and have a very limited lifetime: as early as 4 days after transfer the cells cannot be stimulated by antigen (Šterzl, 1958) because they are being destroyed by the homotransplantation reaction of the recipient. Under these experimental conditions, differentiation of the transferred cells into antibody-producing cells occurs without demonstrable mitotic division (Holub, 1960, 1962). Differentiation of the transferred cells without mitosis was

also described by Gowans *et al.* (1963) and Ford *et al.* (1966). Thus multiplication of cells during differentiation is not essential. This idea is also supported by the fact that cells X-irradiated *in vitro* before transfer with up to 350 r do not lose their potentiality to differentiate into antibody-producing cells (Harris *et al.*, 1959).

Another reason favoring the use of metabolic inhibitors with known mechanism of action in studies of antibody formation is a quantitative one. The number of cells competent to respond to an antigen in primary responses appears to be 1 per 10^6 lymphoid cells. At the peak of a primary response this number is increased to one cell per 1000 lymphoid cells. In a secondary response one cell per 100 produces the corresponding antibody. In such a mixed cell population the specific changes cannot be analyzed by common biochemical methods. Using metabolic inhibitors, however, some conclusions can be reached about the processes which take place in the course of differentiation.

To describe the sequential stages of differentiation of immunologically competent cells, the terms X-cell, Y-cell, and Z-cell are used in this review as was done previously by Sercarz and Coons (1962), and by Šterzl (1962). The X-cell, the immunologically competent cell, is defined as a cell which possesses the potentiality to respond specifically to an antigen but which is not yet engaged in any specific response. The Y-cell, the immunologically activated cell, is the cell which arises as a consequence of the specific action of the antigen on the X-cell, which has the capacity to multiply, and which is responsible for the immunological memory (secondary response). The Z-cell, the antibody producing cell, is the result of the terminal differentiation of immunologically functioning cells (X and Y) and develops after the Y-cell has been stimulated by a second contact with the antigen.

I. DEFINITION OF THE INDUCTIVE PHASE BY THE USE OF METABOLIC INHIBITORS

One of the first aims pursued was the delimitation of the inductive phase. This term is used to define the period shortly after injection of antigen when formation of antibodies cannot be detected either in the serum or in the lymphoid tissues. It was difficult, however, to conclude unequivocally that the inductive phase is fully qualitatively distinct from the later, productive phase. It was objected that the inductive period is only apparently negative for antibodies since the small amount of antibodies formed may be below the level of sensitivity of the methods used for their detection. However, in our reviews (Šterzl, 1960b, 1963, 1965b) we concluded that the inductive phase differs from the productive

phase because it is more sensitive to the action of some hormones (Berglund, 1962), to X-irradiation (Dixon *et al.*, 1952; Taliaferro *et al.*, 1952), and to vitamin deficiency (Axelrod and Pruzansky, 1955).

Even these differences might be quantitative in character; however, the sensitive phase could correspond to the small number of immunologically active cells, the resistant phase to their quantitative increase resulting from antigenic stimulation.

Other experiments supported the real existence of an inductive phase of the antibody response: in tissue cultures, spleen cells isolated from nonimmunized animals and stimulated with antigen *in vitro* do not differentiate into antibody-producing cells. If the cells are isolated 48 hours after immunization of the donor, then those already producing antibodies prolong their function in tissue cultures. The cells isolated from normal donors survive in tissue culture in good condition for at least 24–48 hours; the cells mixed after 48 hours of cultivation with antigen *in vitro* and transferred intraperitoneally to newborn rabbits ("tissue culture" *in vivo*) differentiate into antibody-forming cells (Fig. 1, Šterzl, 1959a,b).

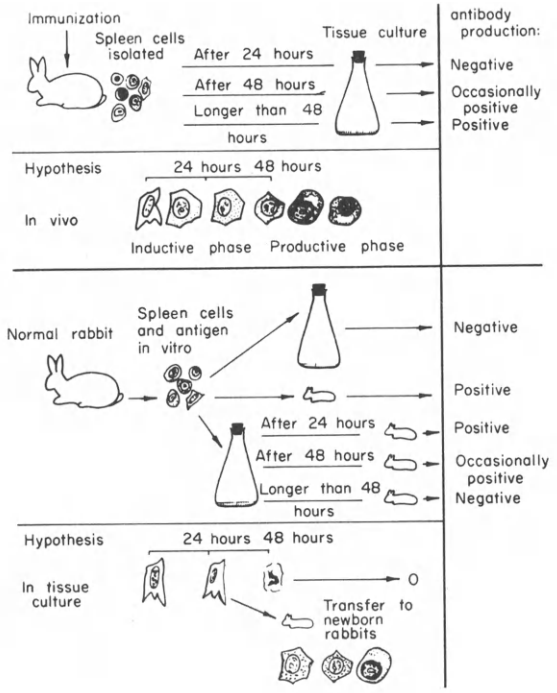

FIG. 1. Differentiation between inductive and productive phase in studies on antibody formation by isolated cells in tissue culture *in vitro* and "in tissue culture" *in vivo* (by transfer of cells into newborn animals) (Šterzl, 1959a,b).

Evidently the tissue culture environment does not enable the induction of processes leading to antibody synthesis which have metabolic requirements different from those of the antibody production proper.

The assumption that the inductive phase is not a period during which functional cells are multiplying was further supported by experiments in which various quantities of cells were transferred into nonreactive recipients (Šterzl, 1960a). In most experiments 50×10^6 spleen cells, mixed with antigen *in vitro,* were transferred and the first antibodies were detected in the serum of the recipient on the third day. Should the multiplication of cells start immediately after transfer, their number would increase sixteen to thirty-two-fold by the time the first antibodies appear. Should the detection of antibody in the serum of the recipient be dependent only upon the number of antibody-forming cells, an increase in the quantity of transferred cells would result in a significant shortening or a complete elimination of the inductive phase. Such experiments were carried out, and instead of 50×10^6 spleen cells, $500–1000 \times 10^6$ cells were transferred. No shortening of the inductive phase could be demonstrated, however (Fig. 2). These results are in accordance with observations by Makinodan and Albright (1963). Transfers of increasing num-

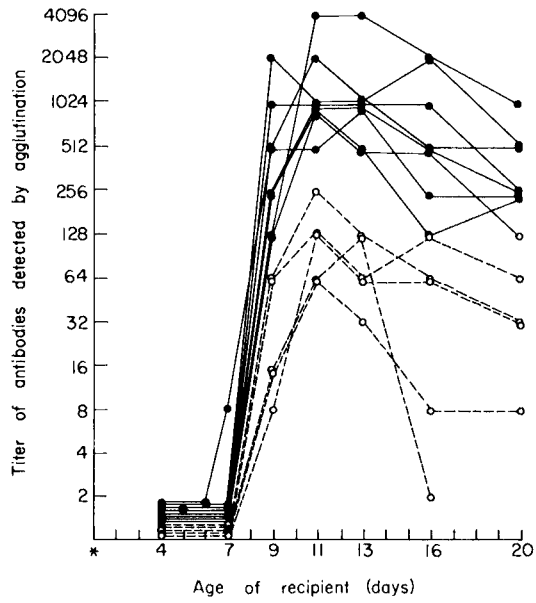

FIG. 2. Antibody formation following transfer of various amounts of spleen cells mixed with *Brucella suis* antigen *in vitro*. Transfer of 50×10^6 cells i.p. to newborn rabbits (dashed line), 500×10^6 cells (full line). (Šterzl, 1960a.)

bers of isolated cells were also used in experiments with metabolic inhibitors.

The influence of 6-mercaptopurine was studied at different times after the transfer into newborn rabbits of isolated spleen cells mixed with antigen *Brucella suis in vitro* (Šterzl, 1960c,d). Administration of 6-MP began either simultaneously or on the second or third day after spleen cell transfer. When the administration of the inhibitor was started simultaneously, or 24 hours after cell transfer, the antibody formation was in most cases completely inhibited (Figs. 3, 4). When the 6-MP injections were started later on, e.g., 48 or 72 hours after transfer, the effect

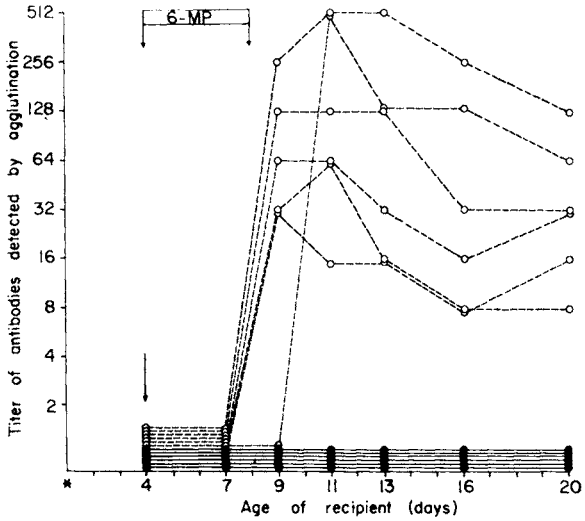

FIG. 3. The effect of 6-MP (0.5 mg/100 gm body weight for 5 days) on antibody formation by transferred cells (50×10^6) mixed with *Br. suis* antigen *in vitro* when 6-MP is administered starting on the day of cell transfer (solid line). Controls (dashed line) are recipients receiving the cells only.

of 6-MP was considerably weaker or absent (Figs. 4, 5). When cells obtained from a donor already producing antibodies were transferred into newborn rabbits and 6-MP was administered simultaneously, no inhibition of antibody formation was found (Fig. 6). From these results we concluded that 6-MP interferes mainly with processes taking place shortly after the administration of antigen, i.e., during the inductive phase of the response. The processes affected by the drug most likely involve the synthesis of new nucleic acid. Frisch *et al.* (1962) came to similar conclusions in the case of 6-thioguanine and LaVia *et al.* (1960) in that of β-3-thienylalanine. In another series of experiments (Jarošková *et al.*,

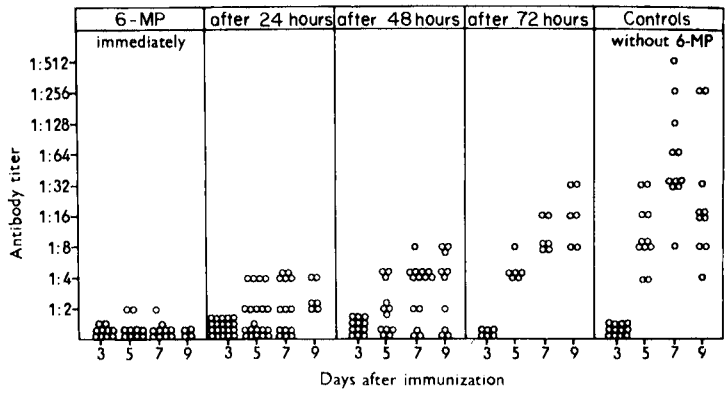

FIG. 4. Inhibition of antibody formation by 6-MP (0.5 mg/100 gm body weight) administered at various intervals after transfer of spleen cells (50×10^6) into newborn rabbits.

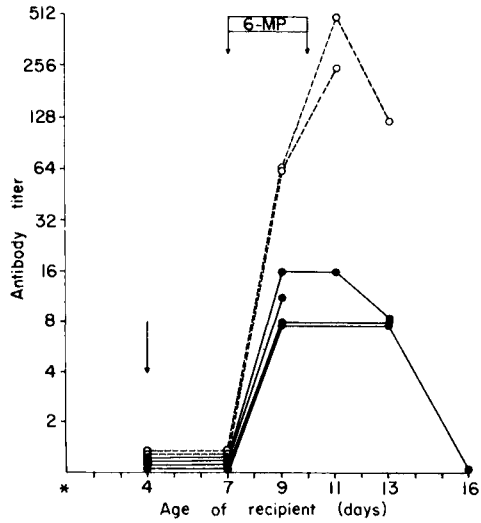

FIG. 5. Antibody formation by isolated spleen cells from normal donor mixed with *Br. suis* antigen *in vitro*. 6-MP (0.5 mg/100 gm) administered for 5 days starting 72 hours after transfer of cells (solid line). Controls receiving the same cell suspension without 6-MP treatment (dashed line).

1966), increased quantities of cells were transferred simultaneously with the administration of 6-MP. If the functions of 6-MP were simply cell-number dependent, the action of the drug might be decreased by an increase in the number of transferred cells. Instead of 50×10^6 spleen cells, 500×10^6 cells were transferred; the inhibitory effect of 6-MP was not overcome, however (Fig. 7).

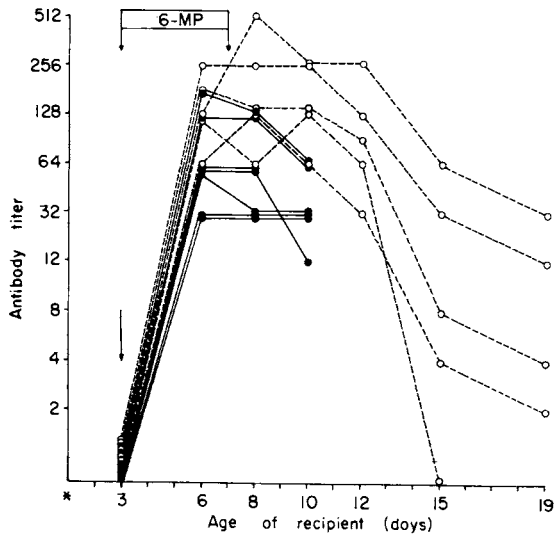

FIG. 6. Transfer of spleen cells (50×10^6) from immunized donors to recipient, newborn rabbits. 6-MP administration (0.5 mg/100 gm for 5 days) was started on the day of transfer (solid line). Controls not treated with 6-MP (dashed line).

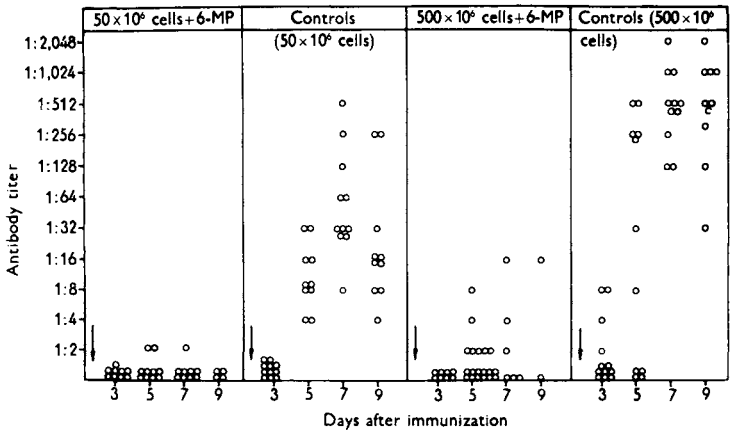

FIG. 7. Transfer of different amounts of spleen cells to newborn rabbits and inhibition of antibody formation by the simultaneous injection of 6-MP (0.5 mg/100 gm).

The findings described above are in good agreement with data on cell dynamics during antibody response. In fact, using the plaque-forming technique, it was found that 24 hours after antigen injection the number of antibody-producing cells is not increased (Jerne, 1965; Šterzl *et al.*, 1965). By the same quantitative technique it was detected that *in vivo*

the highest increase in the number of antibody-producing cells is reached between the third and fourth day. Data on the incorporation of thymidine-^{14}C into central cells in plaques led to the conclusion that the main increase in the number of antibody-producing cells is a result of mitotic divisions (Šterzl *et al.*, 1965, 1966).

Studies of the effect of the time of administration of various inhibitors on antibody formation gave additional information. It is known that X-irradiation is effective only before the administration of antigen (Taliaferro *et al.*, 1952; Dixon *et al.*, 1952; Simić *et al.*, 1965). The similar time-dependent action of radiomimetic agents such as dichlorethylensulfide, nitrogen mustard, and derivatives has been reviewed by Schwartz (1965). Also busulfan has the same time-dependent effect as X-irradiation (Berenbaum, 1962b). It seems that drugs functioning before antigen administration have a direct lethal effect on immunologically competent cells. On the contrary, most of the nucleic acid inhibitors are effective only if administered with the antigen or shortly after it (Berenbaum, 1961; Frisch and Davies, 1962a).

Using the model of cell transfer, we investigated the action of various types of inhibitors on antibody formation. The degree of inhibition and the toxic effects of each compound are shown in Table I. Summarizing these results, it is possible to conclude that none of the compounds inhibiting only mitotic division (colchicine, actinomycin C) depresses antibody formation, not even when these compounds are given in toxic doses. Similarly, none of the metabolic inhibitors influencing DNA synthesis (6-azathimine, 5-fluorouracil, alkylating agents, and aminopterin) affects antibody formation under the conditions used. This suggests that the synthesis of DNA is not a necessary step in the induction of the immune process. The most effective inhibitors were purine analogs, such as 6-mercaptopurine and 6-thioguanine. Similar results, namely that purine but not pyrimidine analogs are effective, were obtained by Nathan *et al.* (1961) and Frisch and Davies (1962b).

Additional data on the role of DNA synthesis during the induction phase of the antibody response were obtained using mitomycin C. The mode of action of this substance resembles that of alkylating agents (Berrah and Konetzka, 1962). Indeed this antibiotic acts directly on the structural characteristics of DNA molecules (Kuroda and Furuyama, 1963). Mitomycin C had no inhibitory effect on antibody formation when administered to young rabbits immunized with sheep red cells in doses of 0.5, 1, and 2 mg/kg (Jarošková *et al.*, 1966; Table II).

Actinomycin D was used to test whether the process of antibody induction depends on the formation of new messenger RNA. In fact, it is generally accepted that the inhibitory effect of this antibiotic reflects the

TABLE I
ACTION OF METABOLIC INHIBITORS ON ANTIBODY FORMATION

Type of inhibitors	Compound	Dose (mg/100 gm body weight)	Inhibition of antibody formation[a]	Toxicity[b]
Pyrimidine antagonists	5-Fluorouracil	2.0	0	+
		1.0	0	0
	5-Bromouracil	5.0	0	0
		2.0	0	0
	6-Azauracil	1.0	0	0
		0.5	0	0
	6-Azauridine	50.0	0	+
		5.0	0	0
	6-Azathymine	2.0	0	0
		1.0	0	0
Purine antagonists	6-Mercaptopurine	1.0	+	+
		0.5	+	0
		0.05	±	0
	Buthiopurine	8.0	+	+
		4.0	+	0
		2.0	±	0
		1.0	±	0
	6-Thioguanine	0.1	+	+
		0.05	+	0
		0.02	+	0
		0.01	0	0
	6-Thioguanosine	0.5	+	+
		0.1	+	0
Folic acid antagonist	Aminopterin	0.15	0	+
		0.05	0	0
		0.025	0	0
		0.01	0	0
Inhibitors of mitotic division	Colchicine	0.2	0	+
		0.05	0	0
	Actinomycin C	0.002	0	+
		0.001	0	0

TABLE I (continued)

Type of Inhibitors	Compound	Dose (mg/100 gm body-weight)	Inhibition of antibody formation[a]	Toxicity[b]
Polyfunctional alkylating agents	TS - 160 (trichlorethylamine)	0.5	−	+
		0.2	0	0
		0.1	0	0
	Endoxan (Cytoxan)	5.0	0	+
		2.5	0	+
		2.0	0	+

[a]Antibody formation was estimated in newborn rabbits to which spleen cells isolated from nonimmunized adult rabbits and mixed with *Brucella suis* antigen *in vitro* were transferred.

Agglutinating antibodies were estimated at 3, 5, 7, and 9 days after transfer and were statistically evaluated using the Wilcox test. In comparison to controls, the effect of antimetabolites in 20 experimental animals per group were classified as complete inhibition (+), inhibition in 50% of the animals (±), and no significant inhibition (−).

[b]Toxic effects in more than 50% of experimental animals were classified as +, and no significant toxic effects as 0.

TABLE II

ANTIBODY RESPONSE OF RABBITS IMMUNIZED WITH ERYTHROCYTES AND INJECTED SIMULTANEOUSLY WITH DIFFERENT DOSES OF MITOMYCIN C[a]

Mitomycin C	Antibody response on following days after immunization:		
	5	7	9
50 μg/100 gm	8, 2	32, ex	16, ex
100 μg/100 gm	0, 0, 0	16, 8, 32	16, 8, 32
200 μg/100 gm	2, 4, 4, 4, 16, 8, 4, 2, 4	4, 4, 16, 16, 128 128, 4, 4, 8	32, ex, 16, 16, 64, 8, ex, 4, ex
Controls	0, 0, 4, 8, 2, 2, 2,	2, 64, 128, 32, 4 4, 2,	8, 16, 128, 8, 8 8, 8

[a]The values indicated are the reciprocal of the hemagglutinating titers in individual animals; ex = expired.

block of RNA synthesis (Reich *et al.*, 1962). In the experiments with actinomycin D, as was done in those with mitomycin C, 10-day-old rabbits were injected with 1 ml of 10% suspension of sheep red blood cells.

Actinomycin D was administered once or twice in quantities up to toxic doses (100–200 μg of actinomycin D per kilogram of body weight) (Table III). In these experiments inhibitory effects on antibody formation were not seen in accordance with the data obtained by Geller and Speirs

TABLE III
ANTIBODY RESPONSE OF RABBITS IMMUNIZED WITH ERYTHROCYTES AND INJECTED SIMULTANEOUSLY WITH DIFFERENT DOSES OF ACTINOMYCIN D[a]

Actinomycin D	Antibody responses on following days after immunization:		
	5	7	9
5 μg/100 gm	0, 8, 4,	16, 8, 16	32, 8, 4
10 μg/100 gm	32, 4, 4, 32, 8, 2	128, 32, 8, 32, 8, 16	64, 32, 4, 128, 8, ex
20 μg/100 gm	4, 2, 16, ex, ex,	8, 64, 16, ex, ex	ex, ex, 64, ex, ex
2×10 μg/100 gm	4, 4, 16, 4, 8, ex, 4, 4, 4,	16, 8, 16, 64, 32, ex, 16, 32, 4	2, 4, 8, 64, ex, ex, ex, 32, 8
Controls	2, 4, 4, 4, 4, 8, 8, 8, 4, 16, 32	4, 4, 16, 8, 4, 16, 16, 32, 32, 128	4, 16, 16, 32, ex, 8, 8, 32, 128

[a]The values indicated are the reciprocal of the hemagglutinating titers in individual animals; ex = expired.

(1964). Wust *et al.* (1964) found that the antibiotic causes only a delayed onset of antibody response, not a complete inhibition. In contrast, other authors report suppression of the immune response by actinomycin D (Nathan *et al.*, 1961; Uhr, 1964; Cirković and Simić, 1964; Speirs, 1965; Muschel *et al.*, 1966). A possible explanation for our negative results is the high toxicity of actinomycin D in young rabbits. The dose which can be used may not be sufficient to block the synthesis of new RNA in the specific target cells. Another possibility to consider is that in conventionally reared animals the antigenic stimuli from intestinal bacterial flora and food immunizes the animals shortly after birth against the antigens present in sheep red blood cells (Šterzl *et al.*, 1965). Thus the long-life messenger RNA might be already formed (Nossal and Mitchell, 1963) and would not be affected by actinomycin D. Therefore, the influence of actinomycin D on the development of the immune response was studied in sterile piglets reared on antigen-free diet. Actinomycin D was injected repeatedly using doses of 200 μg or 100 μg simultaneously with large doses of sheep red blood cells. Under these conditions, in control ani-

mals a significant number of antibody-forming cells were detected by the plaque-forming technique as early as the third day after the administration of antigen. In animals treated with the antibiotic, no antibody-forming cells were detected (Table IV). This proves that actinomycin D inhibits the primary response, probably by blocking new nucleic acid synthesis.

TABLE IV
EFFECT OF ACTINOMYCIN D ON THE PRIMARY RESPONSE IN STERILE PIGLETS[a]

Antigen dose		Complement only	With anti-IgG serum
10 ml conc. suspension of Srbc i.v. (10^{11})		44, 44	56, 62
10 ml conc. suspension of Srbc i.v. (10^{11}) + Actinomycin D i.p.	2×100 μg	0	0
	2×200 μg	0	0
	3×200 μg	0	0

[a]Average number of plaque-forming cells per 10^8 lymphoid cells 60 hours after antigen injection.

The results discussed do not support the hypothesis that synthesis of DNA and proliferation of cells functionally preformed occur during the early stages after antigen administration. The studies with metabolic inhibitors lead to the conclusion that the induction period is a real phase of the antibody response during which antigen induces metabolic changes *de novo*. Because of the effective suppression of antibody induction caused by actinomycin D, 6-mercaptopurine, and 6-thioguanine, it is concluded that at least one of the steps in the induction of antibody synthesis is dependent upon the synthesis of new ribonucleic acid.

II. THE INFLUENCE OF METABOLIC INHIBITORS ON ANTIBODY PRODUCTION

The evaluation of all the possible actions of metabolic inhibitors during antibody production is not simpler than it is during the inductive phase. In cells producing antibodies, the synthesis of new messenger RNA may occur; also, as mentioned above, cells already producing antibodies do multiply in the productive period. During this proliferative phase metabolic inhibitors which are not effective during the induction period may have a significant influence on the quantity of antibody synthetized. Thus the fact may be explained why most of the substances inhibiting mitotic division were found to be effective in depressing antibody synthesis

(Berenbaum, 1960). These findings led to the conclusion that there is a correlation between the ability of agents to inhibit antibody production and their ability to inhibit tumor growth (Berenbaum, 1962a). Similarly, Schwartz and André (1962) concluded that ". . . it is clearly evident that inhibitors of cell growth suppress antibody formation."

In studies of antibody production, a system in which further proliferation of antibody-forming cells is limited consists of the cultivation of isolated lymphatic cells *in vitro*. In this system, after the addition of various amounts of 6-MP (1, 10, 100 mg/ml) to the culture medium, no changes in the amount of antibody produced were found (Šterzl, 1963). These results support the conclusion that 6-MP does not inhibit the process of antibody production proper.

Antibody production is affected in tissue culture as well as *in vivo* also by those metabolic inhibitors which have direct influence on the end steps of proteosynthesis. For example, L-phenylalanine causes a marked inhibition of antibody synthesis in both the rat and the rabbit (Ryan and Carver, 1964).

By the addition of nucleic acid inhibitors such as actinomycin D to cells producing antibodies in tissue cultures, it is possible to estimate the lifetime of messenger RNA in these cells (Svehag, 1964; Uhr, 1964). In other experiments the action of inhibitors was studied in cell explants taken from animals only after the second antigenic stimulus. Not only RNA inhibitors such as 8-azaguanine (Dutton *et al.*, 1958), but also DNA inhibitors such as 5-bromouracil deoxyriboside were effective in tissue culture. These findings can be explained only as being related to an inhibition of cell proliferation during the secondary response and to a toxic action on explanted cells.

III. THE INFLUENCE OF INHIBITORS ON THE PREPARATION FOR THE SECONDARY RESPONSE

In contrast to the primary response, the preparation for the secondary reaction depends on the persistance of a certain type of cell (Y-cells) and on cellular proliferation. Therefore, the preparation for the secondary response cannot be studied in time-limited experiments, in tissue culture, or by the transfer of isolated cells into nonisogenic recipients. The results of experiments *in vivo* depend on the animal species used because the rate of cell proliferation, and the sensitivity to inhibitors, are characteristic for each species. The existence of species-specific differences may explain the contradictory results published on the influence of inhibitors on the preparation of the secondary response.

In our experiments 6-mercaptopurine was used in doses of 50 and 75 mg per kilogram of body weight in four groups of mice. The first group

was given 6-MP only during the primary response, the second only during the secondary response, the third during both primary and secondary responses; control animals received only the primary and secondary doses of antigen. The effect of 6-MP is best manifested on the formation of hemolytic antibodies: 6-MP inhibits the onset of antibody formation even if administered during the secondary response. This is in accordance with results obtained by LaPlante *et al.* (1962) with 6-MP, and by Frisch *et al.* (1962) with 6-thioguanine. If 6-MP is injected only during the primary

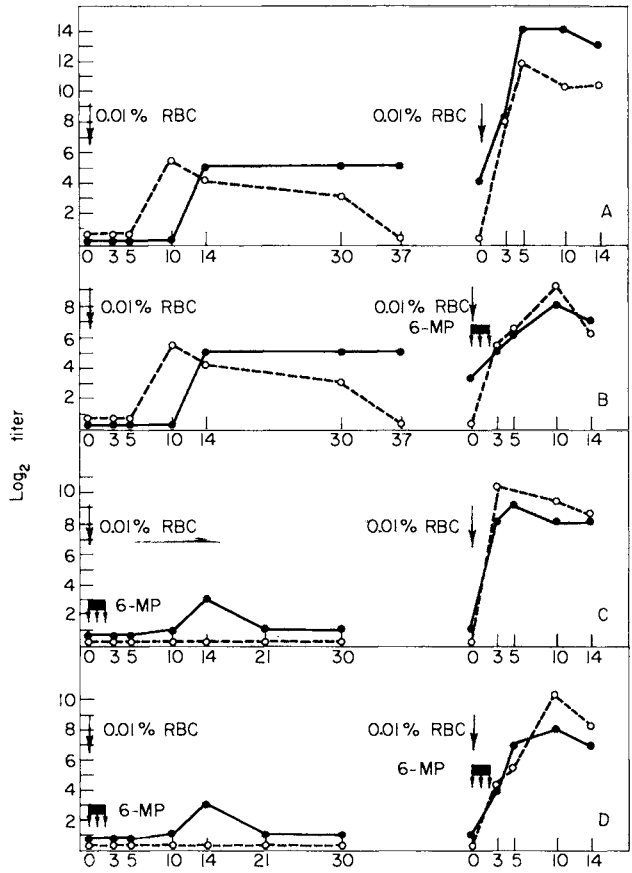

FIG. 8. The effect of 6-MP on the primary and secondary reactions of antibody formation. Mice immunized and revaccinated intravenously with 0.5 ml of a 0.01% suspension of sheep red blood cells (RBC); 6-MP injected subcutaneously on three successive days in amounts of 50 mg/kg body weight. A, no injection of 6-MP; B, 6-MP injected during the secondary response; C, 6-MP injected during the primary response; D, 6-MP injected during both primary and secondary reactions. Abscissa: days; Ordinate: $\log_2$ titers estimated by hemagglutination (solid line) or by hemolysis (dashed line).

response, the onset and development of the secondary response is not inhibited at all (Figs. 8, 9). Identical results (i.e., typical onset of the secondary response after inhibition of the primary response by 6-thioguanine in mice) were demonstrated by Fontalin *et al.* (1966). A typical secondary response after inhibition of the primary response by chloramphenicol was detected in rabbits by Weisberger *et al.* (1964). It is

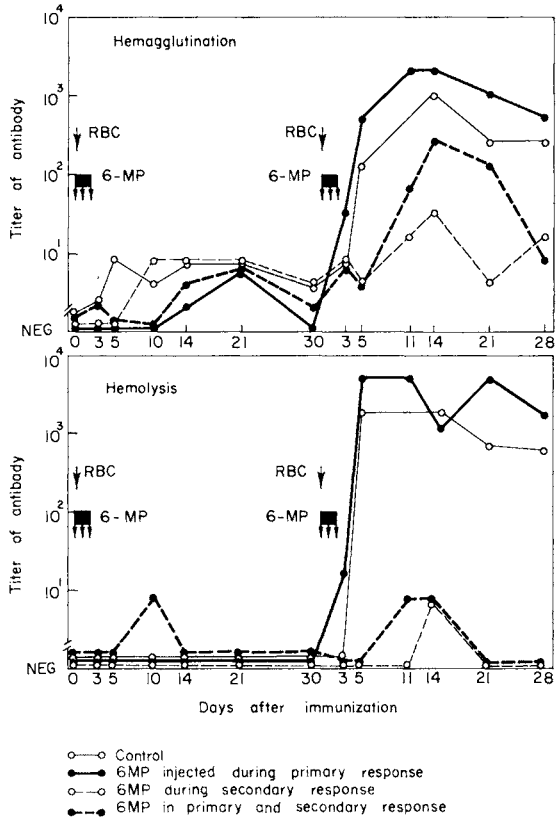

FIG. 9. The effect of 6-MP on the primary and secondary reaction in experiments similar to those shown in Fig. 8.

known, however (Talmage *et al.*, 1951), that X-irradiation, which inhibits the primary antibody response, also depresses the secondary reaction. We assumed that the different actions of 6-MP and X-irradiation represented additional evidence for a two-stage induction of antibody formation: the first phase, namely, the differentiation of X-cells into Y-cells, or "priming," would be a part of the primary response conditioning the preparation of the secondary reaction; this stage is sensitive to X-

irradiation. In contrast, the further stage of induction, namely, the transformation of Y-cells into Z-cells, is a part of both the primary and the secondary response, and in both cases is sensitive to nucleic acid inhibitors such as 6-MP (Fig. 10).

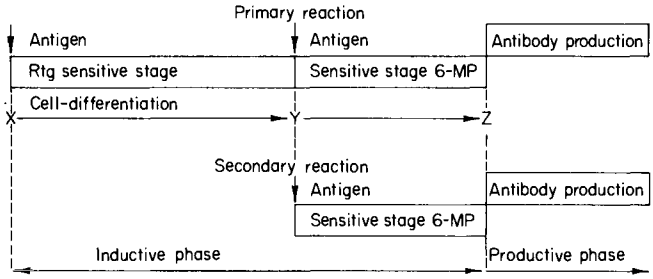

FIG. 10.

These conclusions were reinvestigated and it was found that in mice, similarly to the effect of 6-MP, X-irradiation does not eliminate the preparation for the secondary response. In rabbits, on the contrary, 6-MP and X-irradiation have approximately the same effect and both inhibit the secondary response (Jilek and Šterzl, 1967). The suppression of the preparative processes for the secondary response in rabbits given 6 mg of 6-MP per kilogram per day was observed also by Cruchaud (1966). This suggests that the differences observed in these two species of animals occur as a consequence of the different regenerative and proliferative activities of cells injured by the inhibitors or by X-irradiation. Differences in metabolic activity in rabbits and mice are evident: for instance, the half-time of γ-globulin in rabbits is 4.6–5.7 days, whereas in mice it is only 1.9 days (Dixon *et al.*, 1953).

In some experiments the preparation for the secondary response was inhibited by the administration of inhibitors during the primary response, when cyclohexamide (Cooney and Bradley, 1962) or chloramphenicol were the agents used. The conclusion can hardly be accepted that only the process of "priming" (i.e., the change of X-cells into Y-cells) is affected (Cruchaud and Coons, 1964). Our experiments support the view that the proliferation of immunologically activated Y-cells is essential in the preparation of secondary response.

We suppose, furthermore, that during the proliferation of Y-cells, compartment genetic changes occur which are the basis for the synthesis of antibodies of different character (19 S, 7 S) (Šterzl *et al.*, 1966). The hypothesis that changes appear during proliferation in the Y-cells compartment can be supported by an analogy. In hemoglobin synthesis the

shift from fetal to adult hemoglobin occurs in the same cell line; in fact, in the same cell both types of hemoglobins can occasionally be demonstrated (Kleinhauer *et al.*, 1957). It is assumed that a certain number of cellular divisions during differentiation are necessary for such a shift (Baglioni, 1963).

Assuming that the switch from 19 S to 7 S antibody occurs during the proliferation of Y-cells, different effects of metabolic inhibitors on 19 S and 7 S antibody formation may be explained. Different sensitivity to X-irradiation, namely low inhibition of 19 S antibodies and very effective suppression of 7 S antibodies, was demonstrated by Smith (1964), Smith and Robbins (1965), and Svehag and Mandel (1964). Similar effects were observed by Sahiar and Schwartz (1964), Smith and Robbins (1965), and Borel *et al.* (1965) in the case of 6-mercaptopurine. Indeed, while this drug prolongs the production of 19 S antibody, it abolishes the synthesis of 7 S antibodies. The same effect was obtained by the use of other inhibitors such as methotrexate (Blinkoff, 1964) or cyclophosphamide (Santos and Owens, 1966). The likeliest explanation for these observations is that 19 S and 7 S antibodies do not result from an identical process of differentiation and that 7 S antibodies are apparently formed only following a proliferative activity of cells.

The use of metabolic inhibitors provides basic information on the differentiation of immunologically competent cells which is not expressed by antibody formation: the data obtained prove the existence of immunologically activated cells (Y-cells) which by proliferating prepare the secondary response.

IV. THE INFLUENCE OF INHIBITORS ON THE SECONDARY RESPONSE

The effects of metabolic inhibitors on the secondary response may elucidate whether the induction of the secondary reaction in already sensitized, immunologically activated cells (Y-cells, memory cells) is completely distinct from the processes occurring during the primary response.

As mentioned in Section III, it was possible to inhibit significantly the secondary response by the administration of 6-mercaptopurine during the first days after the second dose of antigen. The same result was obtained by LaPlante *et al.* (1962) who used 6-mercaptopurine during the secondary response to protein antigen.

After a method of induction of the secondary response *in vitro* had been worked out (Michaelides and Coons, 1963), the influence of metabolic inhibitors was studied at different stages of development of the secondary response. O'Brien and Coons (1963) found that after the induction of the secondary response, the antibody-producing cells start to proliferate and

that this process may be inhibited by 5-bromodeoxyuridine; the maximum effect was obtained by giving the antimetabolite on the fourth day after the antigenic stimulus. Similar results were published by Dutton and Pearce (1962) who showed the effects of DNA inhibitors on cells explanted 2 days after the secondary stimulus *in vivo*. However, the addition of chloramphenicol at various intervals after the stimulation by antigen produced progressively less complete suppression of the secondary response the later the drug was added (Ambrose and Coons, 1963). Since, according to some authors (Ishihama *et al.*, 1962) chloramphenicol inhibits the function of messenger RNA in protein synthesis, the same stage of induction seems to be affected in the secondary as in the primary response. These results support the hypothesis that the inductive phase consists of two stages, the first occurring only during the primary response, the second having the same features both in the primary and in the secondary response. The essential feature of this second stage would be the synthesis of new ribonucleic acid.

V. STUDIES ON IMMUNOLOGICAL INHIBITION (TOLERANCE) USING METABOLIC INHIBITORS

The possibility of evoking immunological tolerance in adult animals by long-term injections of a large excess of antigen was observed and described repeatedly (Glenny and Hopkins, 1924; Felton and Ottinger, 1942; Chase, 1946; Taliaferro and Taliaferro, 1951; Johnson *et al.*, 1954; Šterzl, 1954; Dixon and Maurer, 1955). In newborns or fetuses it was found that the immunological inhibition could be induced by high doses of antigen much more easily than in adults. The immunological inhibition obtained in young animals (tolerance) was considered to be a phenomenon completely different from the immunological inhibition obtained in adults (Medawar, 1960). The possibility of inducing immunological tolerance in adults by appropriate doses of antigen suggests that the same mechanism is operating in both cases. The main difference between young and adult animals is that in newborns the number of immunologically competent cells is much smaller than in adults. The favorable relationship between number of cells and antigen dosage can be the explanation for the easier establishment of tolerance in newborns.

The first observation on the possible use of antimetabolites in the induction of tolerance was presented by Schwartz and Dameshek (1959). They reported the immunological inhibition obtained in rabbits injected concomitantly with human serum albumin and 6-MP. These results were repeated with different antigens in various species of animals, as reviewed in detail by Schwartz (1965). Certain differences were seen in the potentiality of inducing immune tolerance to transplantation antigens ad-

ministered simultaneously with 6-MP, when tolerance was tested by the transfer of homotransplants from the antigen donor (Fig. 11). We did not observe the induction of tolerance to a significant degree (Šterzl, 1960d). On the contrary, in similar experiments other authors (e.g., Uphoff, 1961) reported the induction of tolerance.

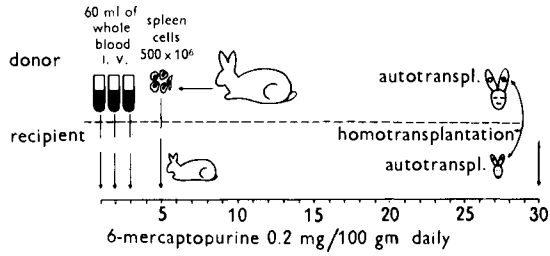

FIG. 11. Experiments designed to investigate whether large doses of donors' tissue antigens in addition to treatment with 6-MP for 15 days result in the induction of tolerance. Tolerance was tested by a skin homograft performed 15 days after the last injection of 6-MP.

The immunological inhibition of antibody formation (tolerance) is usually thought to occur in one of three possible ways: (1) by preventing the access of antigen to a site of immunological response; (2) by the absorption of newly formed antibodies by the excess of antigen (treadmill phenomenon); (3) by a failure to respond induced in immunologically competent cells (damage of cell or intracellular blockage of the antibody-forming mechanism). The last assumption is in accordance with most experimental data. Burnet (1959) explains acquired tolerance as an allergic damage resulting from the elimination of predetermined cells after contact with the corresponding antigen. Medawar (1959) and Lengerová (1962) assume that the antigen acts at the stage of the stem cells by preventing the normal maturation of reactive cells, and that this leads to the state of immunological tolerance (heterogeneous model). On the other hand, Michie and Howard (1962), on the basis of experimental data, assume that the onset of tolerance is possible at all stages of cell differentiation if antigen is present in excess (homogeneous model).

Based upon previous experiments (Šterzl and Trnka, 1957), and recent ones (Šterzl, 1966), we propose an alternate mechanism of tolerance. We observed that increasing the dose of the primary antigenic stimulus in newborns increases the number of antibody-forming cells. None of the doses tested was found to induce an immediate inhibition of antibody formation. These animals were reinjected 3 weeks later with the same antigen. The number of cells producing antibodies after the secondary stimulus was significantly lower in animals stimulated with maximum

doses of antigen during the primary reaction, namely in those animals that produced the maximum number of antibody-forming cells during the primary reaction (Tables V and VI). On the basis of the results obtained, we concluded that acquired tolerance may be considered to be the result of terminal exhaustive differentiation. The majority or perhaps all of the

TABLE V

ANTIBODY FORMATION IN YOUNG RABBITS IMMUNIZED AFTER BIRTH WITH DIFFERENT DOSES OF SHEEP RED BLOOD CELLS (SRBC) AND REVACCINATED 3 WEEKS AFTER THE PRIMARY IMMUNIZATION

Dose of Srbc in primary immunization	Average number of plaque-forming cells per 10^8 lymphoid cells		
	8 days after primary stimulus	5 days after secondary stimulus with 2×10^9 Srbc	
	Developed by complement	By complement only	By anti-IgG + complement
0	0	2,585	732
1 ml 0.01% Srbc (2×10^6 cells)	0	2,735	532
1 ml 0.1% Srbc (2×10^7)	3	14,070	102,433
1 ml 1% Srbc (2×10^8)	9	1,788	7,810
1 ml 10% Srbc (2×10^9)	35	978	1,445
1 ml conc. Srbc (2×10^{10})	46	66	166

cells competent for the given antigen in the newborn are stimulated and, in the presence of excess of antigen, the immunologically activated cells (Y-cells) differentiate directly without proliferation into short-lived antibody-producing cells. In contrast to what happens in animals after the injection of a small dose of antigen, which stimulates the proliferation of activated cells and prepares the secondary reaction, in animals injected in the primary reaction with a large dose, the second dose of antigen stimulates only a very small number of immunologically competent cells and results in immunological inhibition.

One of the presumptions in the terminal exhaustion hypothesis is that after the first antigenic stimulus, proliferation of cells in the Y compart-

ment does not occur. Therefore, we studied the influence of 6-mercaptopurine administered at various times after the injection of sheep red blood cells in newborn rabbits. The influence of 6-MP on Y-cell proliferation was tested by the intensity of the secondary response. It was demonstrated that if antigen is administered together with 6-MP and the primary response is completely inhibited, the secondary response is detected.

TABLE VI
ANTIBODY FORMATION IN STERILE PIGLETS IMMUNIZED AFTER BIRTH WITH DIFFERENT DOSES OF SHEEP RED BLOOD CELLS (SRBC) AND REVACCINATED 3 WEEKS AFTER THE PRIMARY IMMUNIZATION

Dose of Srbc in primary immunization	Average number of plaque-forming cells per 10^8 lymphoid cells		
	10 days after primary stimulus	5 days after secondary stimulus with 2×10^8 Srbc	
	Developed by complement	By complement only	By anti-IgG + complement
0	0	0	0
10 ml 0.01% Srbc (2×10^7 cells)	0	0	0
10 ml 0.1% Srbc (2×10^8)	0	0	0
10 ml 1% Srbc (2×10^9)	22	2,640	21,600
10 ml 10% Srbc (2×10^{10})	89	1,180	1,660
10 ml conc. Srbc (2×10^{11})	389	0	800

On the contrary, the administration of 6-MP 24 or 48 hours after antigen injection inhibits completely the secondary response; if the drug is administered 3, 4, or 5 days after the first administration of antigen, a significant inhibition of the secondary response is still obtained (Fig. 12). Since we know from experiments with cell transfer (see Section I) that the administration of 6-MP later than 48 hours after antigen injection does not inhibit the induction of antibody response, it is obvious that in these experiments the effects of 6-MP administered 72 or 96 hours after antigen are not on the metabolic processes occurring during the induction period,

but on the proliferation of immunologically activated cells. The results obtained with 6-MP support our conclusions that the immunological inhibition is not a primary phenomenon but that it takes place during the process of antibody formation as a consequence of the inhibition of proliferation and the subsequent exhaustion of activated Y-cells.

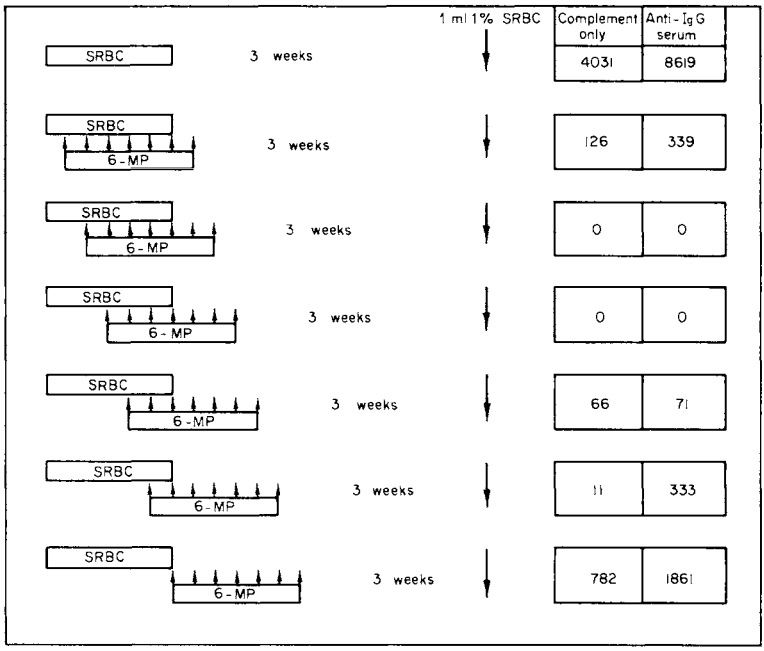

FIG. 12. The influence of daily doses of 6-MP (3 mg/kg) started at different days after antigen injection (sheep red blood cells, SRBC). Antibody response expressed by the number of plaque-forming cells detected in the presence of complement only (19 S) or of complement with anti-IgG serum (7 S).

VI. UNITARIAN CONCEPT OF IMMUNE REACTIONS BASED ON THE MODEL OF IMMUNOLOGICAL CELL DIFFERENTIATION

The building of a hypothesis of immunological cell differentiation has some importance because it allows the testing of the hypothetical scheme by new experiments and with all contemporary methods available. The central core of the proposed concept is the following one: the result of antigen injection, namely, the potential character of the immune status leading to secondary response or to immunological tolerance is the consequence of the intensity of the activated Y-cells' proliferation. This proliferative activity is influenced by the quantity and character of the antigen and by factors regulating the metabolism of proliferating cells,

e.g., factors from the internal milieu of the organism, nucleotides, hormonelike substances regulating the proliferation of lymphatic tissue (Metcalf, 1966), or drugs introduced into organisms as metabolic inhibitors.

A primary response is the conversion of X-cells into immunologically activated Y-cells. Nucleic acid inhibitors do not seem to influence this cell differentiation. During this stage, irritation of the cellular surface of immunologically competent cells by the respective antigen changes them into quickly multiplying Y-cells. This is a process similar to the nonspecific action of phytohemagglutinin. Only the later phase, namely, the change of activated Y-cells into antibody-producing Z-cells, is connected with the formation of specific nucleic acid, and is blocked by actinomycin D and 6-mercaptopurine. According to the quantity and quality of the primary antigenic stimulus, different results may be expected (see Fig. 13). A small dose of antigen injected may act as a true primary stimulus in newborns or in sterile animals fed nonantigenic diet and may result in the X-Y transformation only, called "priming" (Fig. 13,1a). The proliferation ($Y_0 \ldots Y_n$) in the compartment of activated Y-cells is the basis for the secondary response: during the proliferation of the Y-cells some changes might occur ("genetic shift"), for example, between generations Y_4 and Y_5. Such a change may determine the new character of the antibody which would be produced if the $Y_5 - Y_n$ cells come into contact with an additional dose of antigen. If a sufficient amount of antigen is present, the completed primary reaction could occur without the proliferation of the Y-cells compartment ($X\text{-}Y_0\text{-}Z_0$) (Fig. 13,1b).

After one dose of antigen, to the already described possibilities (the "priming" and the completed primary reaction without proliferation of the Y-cell), additional and more complicated situations are to be expected, namely, the activation of Y-cells, their proliferation, and the contact with persisting antigen, leading to a mixed primary and secondary reaction (Fig. 13,1c).

A secondary response, according to the differentiation model presented, is a result of the antigenic stimulation of Y-cells which had proliferated after the first antigenic stimulus (Fig. 13,2). Under the effect of the second stimulation, Y-cells differentiate terminally into antibody-producing Z-cells. If the antigen stimulates the group of cells ($Y_0 - Y_4$) which have not yet gone through the supposed genetic shift, the secondary response consists mostly of 19 S antibodies. Only the cells which have undergone the genetic shift, namely, Y_5 to Y_n, develop into 7 S antibody-forming cells ($Z_5\text{-}Z_n$). The different actions of inhibitors on 19 S and 7 S antibodies indicate that cell proliferation has a role at this stage.

The activation of the Y-cells by the antigen leads to the synthesis of messenger RNA. Therefore, this process, similar to that occurring in a complete primary response ($Y_0 - Z_0$), is inhibited by the same nucleic acid analogs.

Immunological inhibition (tolerance) is supposed to be the result of the terminal exhaustive differentiation of immunologically competent cells following the restricted proliferation of the Y-cells compartment (Fig. 12,3). The restriction of Y-cells proliferation can be achieved by the injection of a large amount of an antigen which has low nonspecific irritative (proliferative) action so that most of the activated Y-cells differentiate immediately into antibody-forming Z-cells without proliferation. After the death of the Z-cell population (half-life of the Z-cell is between 2 and

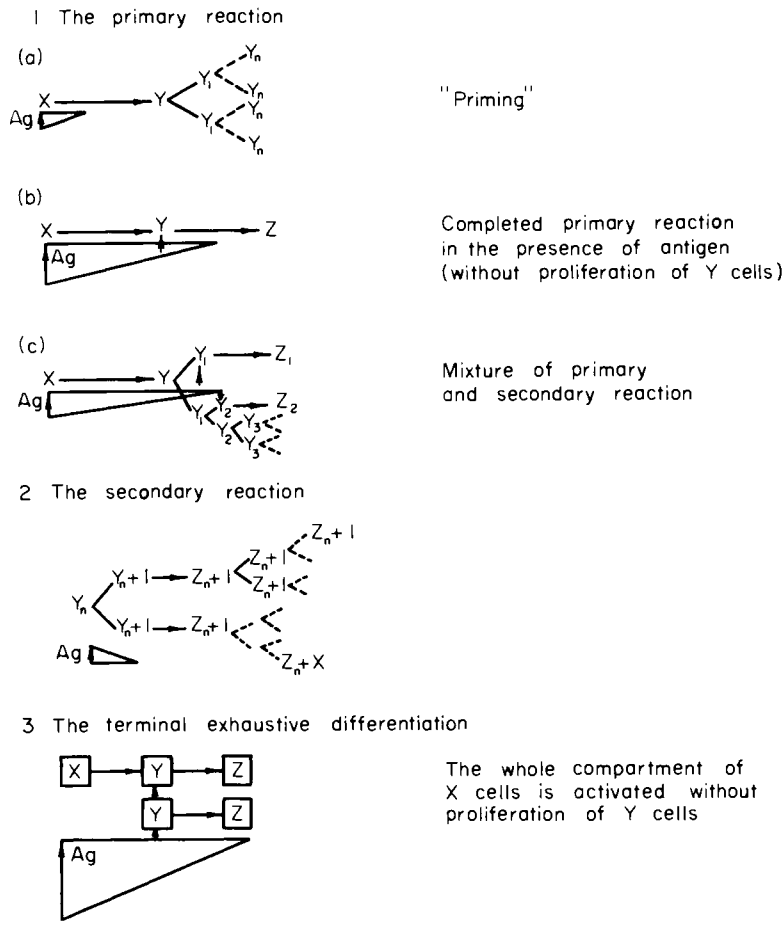

FIG. 13. The unitarian concept of immune reactions.

3 days), only a small number of X-cells is available for a subsequent stimulation by the antigen—not more than one X-cell per 10^6 lymphoid cells. If some additional external influence, for example, treatment with metabolic inhibitors, prevents the multiplication of the activated Y-cell, then the state of the tolerance develops much more easily.

I have described different stages of differentiation of immunologically competent cells subjected to the action of metabolic inhibitors. I would like to express my expectation that a more profound knowledge of the biochemical events leading to these differentiation stages, and the development of specific inhibitors interfering with them, will eventually permit the full control of the immune processes; namely, their stimulation or inhibition.

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DISCUSSION

DR. THORBECKE: I have done some studies together with Drs. Hurlimann and Wakefield on the effect of 6-mercaptopurine and a few other immunosuppressants, including colchicine, on priming for a secondary response and have obtained results strikingly similar to those reported by Dr. Šterzl [(1966) *Symp. Germinal Centers, Bern,* to be published]. We have used alum-precipitated bovine γ-globulin as the antigen given in the footpads of young rabbits. When we started to give daily injections of 6-MP either before or a few days after antigen administration, we could show very striking effects on the secondary response, and these appeared to be independent of complete inhibition of the primary response. There was, however, a difference with your results which I think may be related to the persistence of antigen. When we started the drug, say on day 3 after the primary response, stopped it around day 14 and then immediately reinjected the antigen, we noted a striking reduction of the booster response. However, if we waited a week after stopping the drug before the challenge was given, we observed a recovery of the ability of the animal to make a secondary response. You apparently did not observe such a recovery, and it looks as though the persistence of antigen beyond the period of drug treatment may be important for this recovery. We have also interpreted these observations as indicating that the drug may inhibit a phase of proliferation which is needed for priming; indeed, histologically we find that the drug inhibits cell proliferation in the lymphoid tissue. We find that there appears to be a good correlation between the effect on "priming" and the inhibition of germinal center formation in the draining lymph nodes. We feel that this represents supporting evidence for the role of these germinal centers in producing "primed" cells for a secondary response.

DR. AMIEL: We have been interested in the relationships between the timing of 6-MP administration and the effects on priming for a secondary response. During the experiments of bone marrow grafting in mice, we have seen that blood transfusion from the marrow donor strain prevents the subsequent take of the bone marrow grafted after lethal irradiation of the recipient. The secondary response is not prevented by lethal doses of irradiation. We tried to prevent the secondary response by various treatments of the recipient mice with 6-MP given at different times in relation to the blood transfusion. The drug was given for 2 weeks before the first injection of blood, or after the last injection of blood (in this case bone marrow was grafted 2 weeks after the treatment) or during the transfusion. Mice receiving concurrently blood transfusion and 6-MP for 2 weeks were not committed to a secondary response, after lethal doses of irradiation, when they accepted the bone marrow grafts. I think that this is in good accordance with the results of Dr. Šterzl and Dr. Thorbecke.

DR. ROSEN: I was interested in the experiment reported by Dr. Šterzl in which he was not able to block antibody synthesis with actinomycin D. Using a somewhat different system, namely, the induction of enzymes in rat liver by glucocorticoids, we have observed effects that are dependent on the dose of this toxic antibiotic that is used as well as the period of observation. Thus the induction of two enzymes (tyrosine-α-ketoglutarate transaminase and tryptophan pyrrolase), which respond maximally within 5 hours after the intraperitoneal administration of cortisol, can be completely blocked when doses of actimomycin D that are twice the LD_{50} (400 μg/kg) are given concomitantly with the steroid. Attempts to block the induction of enzymes (serine dehydrase and alanine-α-ketoglutarate transaminase) that respond slowly over a period of several days to cortisol administration requires daily treatment with small doses of actinomycin D. Under these circumstances, the induction of these enzymes by cortisol is not impaired, and there is actually a stimulation in enzyme activity produced by the antibiotic [(F. Rosen *et al.* 1964) *Science* **146,** 661]. We think that the dose

of actinomycin D required to inhibit RNA synthesis in rat liver limits the observation period to not more than 8 hours; beyond this time secondary actions of the antibiotic appear and the results are difficult to interpret. Similar considerations apply to the use of two antibiotics which inhibit protein synthesis, cycloheximide and puromycin. In order to attribute their action mainly to impaired protein synthesis, the period of observation following their administration must be relatively short and the doses used greater than the LD_{50}.

DR. ŠTERZL: I think that this is a very important point not only in the case of inhibitors of nucleic acid synthesis but also in general. A similar example is provided by the action of colchicine: this drug is very toxic for most laboratory animals (for instance, rabbits) and in doses which are tolerated it causes enhancement of antibody formation [Tanaka and Coons (1956) *Bull. N. Y. Acad. Med.* **32,** 171; Jaroslow and Taliaferro (1966) *J. Infect. Diseases* **116,** 139]. In the hamster, however, which tolerates this drug even in massive amounts, nearly lethal doses of colchicine resulted in a considerable reduction of the antibody response [Hirata and Redlich (1962) *Proc. Soc. Exptl. Biol. Med.* **109,** 628].

DR. KOROS: Dr. Šterzl, you mentioned that during the response you studied, a large per-percentage of the cells were in proliferation. Could you please tell me what percentage you estimate were actually proliferating?

DR. ŠTERZL: There are differences according to the experimental model. In mice injected with sheep red blood cells the number of antibody-forming cells estimated by the hemolysis plaque technique starts to increase after a 24-hour delay, and reaches a maximum at day 4. We injected thymidine-^{14}C at 5-hour intervals after antigen injection and followed the number of cells labeled in the center of the plaques. In the early stages of antibody appearance (48 hours after antigen injection) only 25% of antibody-forming cells were found to incorporate thymidine. The largest proportion of cells differentiate at that stage without undergoing mitosis. If thymidine is administered at the time of the highest numerical increase of antibody-forming cells, i.e., between the third and fourth day after the immunization, then more than 75% of antibody-forming cells incorporate thymidine. This suggests that most of these cells develop through mitosis [Šterzl *et al.* (1965) *Mol. Cellular Basis Antibody Formation, Proc. Symp., Prague, 1964,* pp. 534–553; Šterzl *et al.* (1966) *Nature* **209,** 416]. Different results were obtained with cells cultivated in diffusion chambers. Normal lymphoid cells (5×10^7) mixed with antigen (sheep red blood cells, 1×10^8) were introduced in a diffusion chamber which is inserted intraperitoneally. Thymidine-^{14}C was introduced in the diffusion chamber at 5-hour intervals. After 5 days 50–100 cells forming antibodies were detected in the population of 5×10^7 lymphoid cells. About 90% of cells among antibody-forming cells were not labeled. So, if you ask how many cells developed and entered the antibody-forming cells compartment through proliferation, it is very important to define the system and stage of the response you are referring to.

DR. KOROS: I am very interested in this question because we have performed similar experiments [Koros, A. M. C., Fuji, H., and Jerne, N. K. (1966) *Federation Proc.* **25,** 305; Koros, A. M. C., Fuji, H., and Jerne, N. K. (1966) *Proc. 9th Intern. Congr. Microbiol., Moscow* Abstr. p. 594] except that we do *in vitro* pulse labeling of the plaque-forming cells with tritiated thymidine. If spleen cells are taken, for example, from mice on day 3 after primary stimulation, 55% of plaque-forming cells are labeled by only a 30-minute pulse, whereas no more than 5% of nonplaque forming cells are labeled. You seem to find almost 100% of plaque-forming cells labeled by day 4.

DR. ŠTERZL: I agree, day 3 after antigen injection is already the time when most of the cells involved in the immune response go through proliferation. The difference between your point of view and ours is that we do not expect mitosis to be absolutely necessary for the differentiation of antibody-producing cells, as suggested by the experiments in diffusion chambers.

DR. KOROS: My interpretation would be that the approximately 90% antibody-producing cells you find labeled on day 4 originated through proliferation.

DR. ŠTERZL: Even so, you cannot reach this conclusion in the system you use, which is based upon the explanation of cells 3 or 4 days after immunization and their short exposure *in vitro* to labeled nucleic precursors. This gives you only a slight reflection of the incorporating activity. One should do the experiment by starting to give the label at 0 time and by treating the animals continuously or giving many injections in short intervals, as we do. Then you could take cells at various times and ask what percentage of antibody-forming cells developed through proliferation.

DR. SCHWARTZ: Dr. Šterzl has raised a most important point. During the primary response, immunologically competent cells are certainly dividing, but the question is whether or not this cellular division is an essential part of the primary immune response. My first question has to do with the very interesting table in which you listed various families of metabolic antagonists. I noticed that compounds which were more or less specific inhibitors of DNA synthesis, namely the pyrimidine antagonists, had no effect on antibody formation in your transfer system. This implies, although it certainly does not prove, that DNA synthesis is not an essential feature for antibody formation in your system. Would you agree with that, Dr. Šterzl?

DR. ŠTERZL: In the nonproliferating system, we did not observe inhibition of antibody synthesis with some pyrimidine antagonists (e.g., 5-fluorouracil) and some antibiotics (e.g., mitomycin) which inhibit mostly DNA synthesis. In contrast, inhibition is seen with 6-mercaptopurine. This is indirect evidence that DNA synthesis is not an essential feature for the development of antibody-forming cells. The data on thymidine-^{14}C incorporation provide the direct evidence in this regard.

DR. SCHWARTZ: My colleague, Dr. Walter Tannenberg, has also been very much interested in the question of whether or not antibody-forming cells, as detected in Jerne's plaque-forming system, are dividing and what their rates of division are. He showed that although these cells incorporated tritiated thymidine, which was given by a constant infusion rather than by pulse labeling, the rate of incorporation by plaque-forming cells was the same as that of similar lymphocytes not forming plaques. The interpretation of these results is that although there may be proliferation during the primary response, it is not an essential feature of antibody synthesis at that time [Tannenberg, W. (1966) *Nature* (in press)].

DR. ŠTERZL: In our experiments we are studying the true primary response in germ-free animals. I wish to mention that under normal nonsterile conditions as early as the fifteenth to twentieth day of life most of the animals have some cells producing antibody against sheep red blood cell antigen which developed under the influence of food antigens or cross-reacting polysaccharides of intestinal bacteria. Studying the primary response in germ-free animals reared on nonantigenic diet, we detect an average of 100–500 antibody-forming cells per 1×10^8 of inactive lymphoid cells. By means of thymidine-^{14}C incorporation experiments, we have some evidence that during this true primary response immunologically competent cells can differentiate into antibody-producing cells without proliferation.

A Study of Antibody-Containing Cells in the Course of Immunization

G. BIOZZI, C. STIFFEL, and D. MOUTON

Centre de Recherches Immunologiques, Hôpital Broussais, Paris, France
I.N.S.E.R.M., Association Claude Bernard and C.N.R.S.

Our knowledge of the mechanism of antibody production has progressed rapidly in the past few years with the development of new methods designed to approach the problem at the cellular level. The serum antibodies are the final product of a process of globulin synthesis which takes place in specialized cells of the lympho-plasmocytic series.

The methods presently available for the study of antibody-producing cells are designed to investigate the morphology and the kinetics of these cells in the course of immunization. The present work deals chiefly with the kinetic study of the number of antibody-producing cells. The results obtained with the different methods which have been developed for such types of investigation will be compared and discussed.

The detection of the active cells among the cell population isolated from the lymphoid tissue of immunized animals is based on specific antigen-antibody reactions. In this respect the different methods fall into two categories since they depend upon either of the two following phenomena:

(1) The antibody produced by the active cells *in vitro* is released and interacts with the antigen in the surrounding medium.

(2) The antibody-producing cells contain a certain amount of antibody on their surfaces, which specifically binds the corresponding antigen.

The methods based on (1) require that the cells be alive and supplied with all the nutritional and environmental factors essential for the antibody synthesis *in vitro*. For the methods based on (2) it is immaterial whether the cells are living or not, provided that the antibody persists on

the cell surface. Both methods can be applied to the study of single cells isolated in microdrops or to the detection of active cells in large populations of lymphoid cells.

The microdrop method developed by Nossal (1958, 1962) and Attardi *et al.* (1959) has provided much useful information chiefly concerning the morphology of the antibody-producing cells and the specificity of the antibodies produced by each cell. Nevertheless, the technical requirements of this method limit its application to a relatively small number of cells which is insufficiently representative of the immunological response of the whole lymphoid system in the course of immunization.

The detection of active cells in large populations of lymphoid cells can be easily undertaken with methods based on the localized hemolysis plaque formation in gel (Bernovska *et al.*, 1963; Ingraham, 1963; Jerne and Nordin, 1963; Ingraham and Bussard, 1964). However, these methods as originally described can detect only cells producing hemolytic antibodies.

The phenomenon called immunocytoadherence (ICA) served as the basis for a simple and quantitative method which has been devised to detect and count the cells producing antibodies among large populations of lymphoid cells (Nota *et al.*, 1964; Biozzi *et al.*, 1965, 1966). A similar technique has been used by Zaalberg (1964).

Both the method of hemolysis plaque formation and that of ICA can be applied to the quantitative investigation of the kinetics of antibody-producing cells. It seems interesting, therefore, to compare the results obtained with these two methods.

The technique of hemolysis plaque formation in gel is well known: it consists essentially of the immunization of animals with heterologous red cells, and the incubation at 37°C of their lymphoid cells together with the red cells used as antigen suspended in a nutrient medium thickened by a gel. The active cells release antibodies which, in the presence of complement, produce easily identifiable plaques of pericellular hemolysis. This method has been adapted to the study of cells producing antibody against bacterial polysaccharides (Halliday and Webb, 1965; Landy *et al.*, 1965) or simple haptens (Merchant and Hraba, 1966) using red cells coated with these substances. Bacteria sensitive to bactericidal action of antibody and complement can also be used instead of red cells with the plaque-forming method (Schwartz and Braun, 1965).

The method using ICA is based on the specific binding of a particulate antigen at the surface of the cells containing the corresponding antibody. This phenomenon has been well established by using bacteria as antigens (Reiss *et al.*, 1950; Moeschlin and Demiral, 1952; Hayes and Dougherty, 1954; Mäkelä and Nossal, 1961). However, for quantitative studies of

large populations of cells, it is preferable to use heterologous red cells as antigens because they can be distinguished much more easily than bacteria in fresh preparations. The heterologous red cells adhere strongly *in vitro* to the lymphoid cells containing the corresponding antibody on their surfaces and give rise to the formation of characteristic "rosettes." The number of "rosettes" can be easily counted in a hematocytometer. This method can also be applied to the study of cells producing antibody against soluble antigens or haptens by fixing these to the red cells.

The ICA technique has been extensively described in preceding articles (Nota *et al.*, 1964; Biozzi *et al.*, 1966) and consists essentially of the following procedures.

Ice-cold buffered saline is used for the isolation of lymphoid cells from spleens or lymph nodes and is prepared by mixing 1/3 volume of 0.15 *M* phosphate buffer pH 7.2 with 2/3 volume of 0.15 *M* NaCl. In preceding works, Hank's solutions were used. Although the results of ICA are the same in both cases, the cellular suspension is more homogeneous in buffered saline.

The lymphoid tissue is cut into small pieces and the cells dissociated by pressing the fragments gently in a glass tube with a loosely fitting piston. The preparation is filtered through a fine stainless steel mesh. The cells are washed twice with large volumes of cold buffered saline and resuspended in a small volume (2–4 ml) of the same solution.

The centrifugation of cells is made with the minimum centrifugal force required to sediment the cells in 7 minutes. The cell suspension is passed through a syringe needle of 0.4 mm until an homogeneous single cell suspension is obtained. The number of nucleated cells is counted in a hematocytometer.

To perform the ICA test, 6×10^6 lymphoid cells are mixed with 24×10^6 washed sheep erythocytes in a final volume of 1 ml of buffered saline containing 5% of homologous normal serum previously absorbed with sheep erythrocytes. ICA can be obtained also with erythrocytes from other animal species (Biozzi *et al.*, 1966).

The mixture of lymphoid cells and erythrocytes is made in glass test tubes (60×12 mm) closed with rubber stoppers. The whole procedure is carried out at 0°C. The tubes are left standing overnight at 4°C, and during this time the cells sediment at the bottom of the tube, thus facilitating their adherence. Thereafter, the cells are resuspended by rotating the tubes upside down at 16 revolutions per minute for 10 minutes. The number of "rosettes" is counted in a hematocytometer.

A "rosette" consists of a nucleated cell with five or more erythrocytes strongly adhering to its surface. Usually a greater number of erythrocytes are sticking around the surface of the cell. The number of nucleated cells

in each preparation is checked again after hemolysis of red cells with an aqueous solution of 1% acetic acid. The percentage of "rosettes" is calculated from the number of nucleated cells actually found in the preparation. All cell preparations are set up in duplicate and the percentage of "rosettes" is calculated by averaging the values of four readings made by four different investigators. The percentage of "rosettes" is usually calculated from the study of about 5×10^4 lymphoid cells. When the percentage of "rosettes" is very low, proportionally larger numbers of cells are examined. The standard deviation of "rosette" counts made by four different investigators in the same preparation is about 13 per 100. The variability in the number of "rosettes" found in several preparations from the same spleen or lymph-node is about 20 per 100. On the whole, the experimental error of the ICA method is $\pm$ 25 per 100.

I. COMPARISON BETWEEN THE RESULTS OBTAINED WITH THE ICA AND THE HEMOLYSIS PLAQUE FORMATION TECHNIQUES

Such a comparison is made possible by evaluating the data obtained by different investigators using mice immunized intravenously with similar doses of sheep erythrocytes. Under these experimental conditions the ummunological response takes place principally in the spleen.

Reported in Table I is the number of active cells found by the "rosette" and plaque methods in the spleen of mice before and after immunization. Both methods detect a background of active cells in the spleen of nonimmunized animals. The number of cells forming "rosettes" in the spleen of normal mice is about 1000 times higher than that of cells giving hemolysis plaques. At the peak of the response after primary immunization, this difference is reduced to about ten to twenty times.

The active cells found in nonimmunized animals are believed to synthesize the small amount of natural antibody to sheep red cells present in the serum of adult mice. This supposition is substantiated by the lack of either plaque or "rosette"-forming cells in the lymphoid tissue of newborn animals (Šterzl *et al.*, 1965; Rowley and Fitch, 1965; Hechtel *et al.*, 1965; Biozzi *et al.*, 1966). Immunization increases markedly the number of active cells in the spleen, the maximum value being reached on the fourth day for plaques and on the fifth to seventh day for "rosette" formation.

It is clear that the "rosette" method detects a larger number of active cells than that of hemolysis plaque formation, particularly in nonimmunized animals. Such a difference between the two methods has been confirmed recently (Shearer, 1965; Berenbaum, 1966; Zaalberg *et al.*, 1966).

TABLE I

NUMBER OF PLAQUE-FORMING CELLS AND "ROSETTE"-FORMING CELLS IN THE SPLEEN OF NORMAL MICE, AND OF MICE IMMUNIZED INTRAVENOUSLY WITH 10^8 SHEEP ERYTHROCYTES

Method	Authors	Number of positive cells per 10^8 spleen cells[a]		Time of peak response, (days after immunization)
		Normal mice	Immunized mice peak of response	
Hemolysis plaque formation	Jerne *et al.* (1963, 1965),	18–220 (mean 80)	60,000–216,000 (mean 110,000)	4
	Šterzl and Mandel (1964), Šterzl *et al.* (1965)	55–65	34,000–90,000	4
Immunocytoadherence "rosette" formation	Biozzi *et al.* (1966) and this article	20,000–80,000 (mean 50,000)	about 1,000,000	5–7

[a] The number of nucleated cells isolated from the spleen of normal mice is about 1.15×10^8 (calculated for 20 gm mice with spleen weighing 85 mg).

Preceding experiments have shown that the specificity of ICA in immunized animals is identical to that of serum antibodies (Biozzi *et al.*, 1966). Furthermore, as will be shown below, the treatment of lymphoid cells from immunized animals with antibodies directed against homologous immunoglobulins completely inhibits "rosette" formation. These observations demonstrate that the formation of "rosettes" is mediated by specific antibody present at the surface of the lymphoid cell.

The question then arises whether the "rosette"-forming cells are the antibody-producing cells or whether at least a fraction of them consists of cells passively sensitized by cytophilic antibody. The latter phenomenon might occur only *in vivo* because the ICA test is carried out in the cold to prevent the cellular synthesis of antibody (Helmreich *et al.*, 1962). As a matter of fact, it is well established that macrophages from the peritoneal cavity are able to fix passively anti-red cell antibodies with consequent "rosette" formation (Boyden, 1964; Berken and Benacerraf, 1966; Biozzi *et al.*, 1966; Howard and Benacerraf, 1966). Nevertheless, the cellular fixation of cytophilic antibody put in evidence by the "rosette" test appears to be limited to macrophages while lymphocytes and polymorphonuclear leucocytes are not involved (Boyden, 1964).

Other experiments have shown that cells isolated from spleen or lymph nodes cannot be passively sensitized *in vitro* under the experimental conditions used for the ICA test (Nota *et al.*, 1964; Biozzi *et al.*, 1966; Howard and Benacerraf, 1966; Zaalberg *et al.*, 1966).

These findings indicate that the macrophages contained in the lymphoid tissue (spleen and lymph nodes) either behave differently from those of the peritoneal cavity as far as the fixation of cytophilic antibody is concerned, or are destroyed, damaged, or lost during the procedures of cell isolation and washing followed for the ICA test.

The study of cells becoming passively sensitized *in vivo* is more interesting for the purpose of the present investigations, as the ICA method is used to detect antibody-containing cells in immunized animals.

Passive immunization of mice and guinea pigs with large amounts of homologous antibody against sheep red cells does not cause any increase of the number of "rosette"-forming cells (over the normal background) in the lymph nodes. In the spleen only a few cells can occasionally become passively sensitized while a large number of macrophages from the peritoneal cavity fix the antibody giving "rosette" formation (Biozzi *et al.*, 1966). Lack of passive sensitization of spleen cells *in vivo* has also been observed by Zaalberg *et al.* (1966).

Useful information about the significance of "rosette"-forming cells in the organism can be obtained by performing experiments of active immunization in which the immunological response is limited only to a

restricted region of the lymphoid system (Biozzi *et al.*, 1966). A suitable immunization of mice with sheep red cells injected intravenously restricts the antibody production to the spleen (Rowley, 1950; Biozzi *et al.*, 1960). The distribution of "rosette"-forming cells in mice immunized in such a way is shown in Fig. 1A.

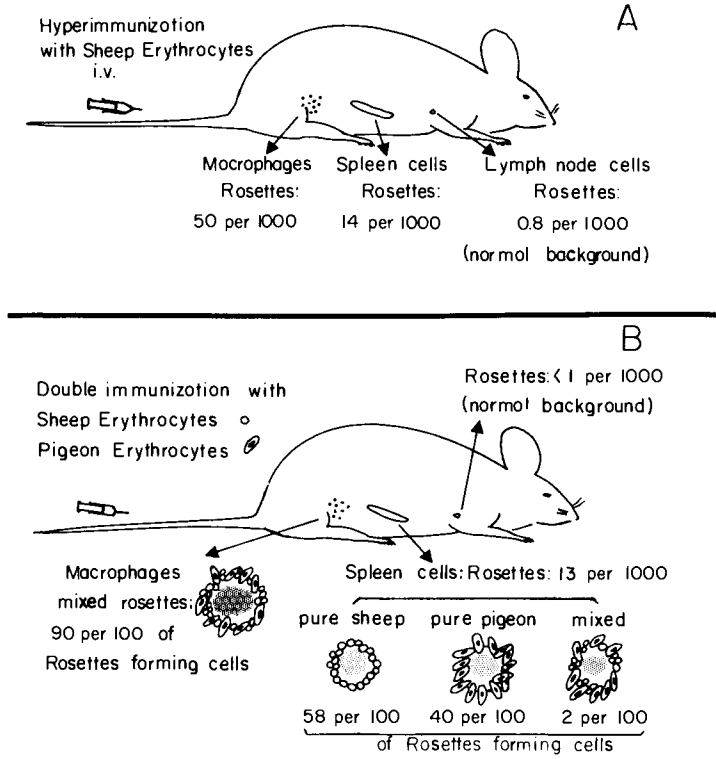


FIG. 1. Localization of "rosette"-forming cells in mice. A. Mice immunized intravenously with sheep erythrocytes. Titer of serum agglutinins = 1:30,000. B. Mice immunized intravenously with sheep and pigeon erythrocytes. Titer of serum agglutinins = 1:20,000 against sheep erythrocytes and 1:30,000 against pigeon erythrocytes.

The spleen contains a high percentage of "rosette"-forming cells (14 per 1000 of all nucleated spleen cells) while the percentage of "rosette"-forming cells in the lymph nodes is not modified. The normal background of "rosette"-forming cells in the spleen and lymph nodes of nonimmunized mice is less than 1 per 1000 (ranging from 0.2 to 0.9 per 1000). This finding indicates that in spite of a high concentration of serum antibodies (agglutinin titer 1/30,000) the cells of the lymph nodes do not become passively sensitized. On the contrary, many of the macrophages from

the peritoneal cavity fix cytophilic antibody as revealed by their ability to form "rosettes" (the cell population from the peritoneal cavity is constituted of about 60 per 100 macrophages, 35 per 100 lymphocytes, and 5 per 100 polymorphonuclear leucocytes).

Experiments of double immunization confirm that the spleen cells which form "rosettes" are involved in active antibody production while the peritoneal macrophages are passively sensitized by cytophilic antibodies produced in the spleen (Biozzi *et al.*, 1966). An example of double immunization experiments is presented in Fig. 1B. Mice are immunized by the intravenous route with a mixture of sheep and pigeon erythrocytes. The erythrocytes of these two animals can be easily distinguished (sheep erythrocytes $4 \times 4\ \mu$; pigeon erythrocytes $7 \times 13\ \mu$, nucleated) and do not share any immunological cross reactivity in the mouse.

This double immunization produces a high titer of serum agglutinins against both sheep and pigeon erythrocytes. The ICA is studied by adding a mixture of sheep and pigeon erythrocytes to lymphoid cells or macrophages from the immunized mice. When only sheep or pigeon erythrocytes adhere to a single mouse cell, the "rosette" is a "pure rosette." When both sheep and pigeon erythrocytes adhere to the same mouse cell, the resulting "rosette" is a "mixed rosette." The results presented in Fig. 1B show that 98 per 100 of the "rosette"-forming cells found in the spleen produce "pure rosettes" while about 90 per 100 of the rosettes formed by the peritoneal macrophages are of the "mixed" type.

Under our experimental conditions, the large majority of antibodies are produced in the spleen. These antibodies are released in the circulation and sensitize passively the macrophages of the peritoneal cavity which fix cytophilic antibodies irrespective of their immunological specificity, giving rise to "mixed rosettes." The finding of a large majority (98 per 100) of "pure rosettes" in the spleen shows that the phenomenon of passive sensitization of spleen cells does not occur within the spleen itself. It seems reasonable to assume that if the spleen macrophages (or any other spleen cell) were passively sensitized by antibodies synthesized by other spleen cells, they would produce "mixed rosettes" similarly to the macrophages from the peritoneum. The number of 'rosette"-producing cells in the lymph nodes of mice submitted to the double immunization is not increased. This fact confirms the results in Fig. 1A showing a lack of passive sensitization of the lymphoid tissue by circulating antibodies.

These experiments have been repeated using as antigens a mixture of sheep and human (A group) erythrocytes which can be distinguished by virtue of their different size. The results obtained are similar to those presented in Fig. 1B (Biozzi *et al.*, 1966).

Other experiments with double immunization have been carried out in guinea pigs using mixtures of erythrocytes from sheep and pigeon or from sheep and human (A group). The guinea pigs were immunized in their footpads in such a way as to involve both the regional lymph nodes and the spleen in antibody production. From 90 to 96 per 100 of the "rosettes" found in the spleen or lymph nodes were "pure rosettes" whereas 60 to 80 per 100 of the "rosettes" formed by macrophages from the peritoneal cavity were of the "mixed" type (Biozzi *et al.*, 1966).

The results of the double immunization experiments performed in mice and guinea pigs indicate that the large majority (more than 90 per 100) of "rosette"-forming cells found in the spleen or lymph nodes are actually antibody-producing cells because they give rise to "pure rosettes." The small percentage (less than 10 per 100) of "mixed rosettes" found in the lymphoid tissue can be regarded as formed by either passively sensitized cells or double antibody producers. The percentage of cells able to synthesize two antibodies of different specificity in animals immunized with two particulate antigens such as bacteria or viruses, varies according to different authors from about 2 per 100 (Nossal, 1962) to 15–20 per 100 (Attardi *et al.*, 1959) of the active cells.

Applying the hemolysis plaque formation method to the study of spleen cells from animals immunized with two different erythrocytes (sheep and chicken), various investigators reported concordant results showing that the large majority of active cells produce antibody of single specificity, the double producers being absent or very few (less than 1 per 100) (Friedman, 1964a; Nota *et al.*, 1964; Playfair *et al.*, 1965; Celada and Wigzell, 1966). These results are in agreement with the data obtained with the ICA method which detects 2 per 100 of cells containing two antibodies in the active cells from mouse spleen and from 3 to 10 per 100 in the cells from lymph nodes of the guinea pig (Biozzi *et al.*, 1966). It is impossible to establish whether this small percentage of cells represents double producers or cells passively sensitized inside the lymphoid tissue by cytophilic antibodies produced by other cells. Even accepting the latter possibility, the reliability of the ICA method for the quantitative detection of antibody-producing cells is not invalidated. In fact, the "rosette" number would give a maximal overestimation of antibody-producing cells of less than 10 per 100; this value is well within the standard error of the technique.

The results reported above demonstrate that the ICA method can be correctly applied to the quantitative study of the antibody-producing cells contained in the spleen and lymph nodes of immunized animals. The hemolysis plaque formation method is also aimed at the same purpose. How can one explain the different results which were obtained by the "rosette" and plaque methods as shown in Table I? Two reasons

can be responsible for such a discrepancy, one related to the quality and the other to the quantity of antibodies produced by each cell. The method of hemolysis plaque formation can detect only cells releasing hemolytic antibodies whereas any kind of antibody present on the cell surface can produce the ICA, which is simply based on an antigen-antibody reaction.

It is probable that the hemolysis plaque formation method detects only or mainly cells producing 19S antibodies which are highly hemolytic (Talmage *et al.*, 1956; Robbins *et al.*, 1965). Cells producing 7S antibodies with lower or no hemolytic activity would escape detection. The existence of cells synthesizing nonhemolytic antibody has been proved by the increase in the number of hemolysis plaques occurring as a consequence of the addition of antiimmunoglobulin serum to the preparation (indirect hemolysis plaque method) (Dresser and Wortis, 1965; Jerne *et al.*, 1965; Šterzl and Riha, 1965; Friedman and Young, 1966; Ingraham *et al.*, 1966).

The number of plaque-forming cells detected by the direct and indirect methods is the same during the earlier ascending phase of the primary response, until the fourth to fifth day after immunization. Afterward, the number of active cells revealed by the direct method drops rapidly whereas the decrease of the number of plaque-forming cells detected by the indirect method is slower. At the fifteenth day after immunization, the spleen contains about ten times more cells detectable by the indirect than by the direct method.

The difference in the quality of antibodies produced by each cell can only partially explain the discrepancy between the number of antibody-producing cells detected by the "rosette" and by the plaque methods. Another important factor is related to the quantity of antibodies produced by each cell. It seems reasonable to suppose that a larger quantity of antibodies should be produced by the cell in order for them to diffuse in the gel and give a plaque of hemolysis, than that required to agglutinate the erythrocytes on the surface of the cell. Moreover, the ICA can be produced also by killed cells whereas the enzyme machinery of the living cell must be preserved to synthesize the amount of antibody necessary to give a plaque of hemolysis (Ingraham and Bussard, 1964).

Recent findings by Cunningham (1965) demonstrate that the method of hemolysis plaque formation in gel is not sensitive enough to reveal cells producing small amounts of hemolytic antibodies. This author has described a more sensitive method of hemolysis plaque formation in thin layer which detects about three times more antibody-producing cells than the classical technique. Moreover, using polysaccharide-sensitized erythrocytes, Cunningham's thin layer method can reveal as many as 10,000 hemolysin-producing cells which completely escape detection by the classical plaque method.

From the facts discussed above, it appears that the "rosette" method is more sensitive than the plaque method in the detection of antibody-producing cells. The higher sensitivity of ICA permits a better understanding of the data reported in Table I, namely, of the greater discrepancy between the number of plaques and "rosettes" found in normal than in immunized animals. It can be supposed that a large majority of lymphoid cells in normal animals produce too small amounts of natural antibodies to give plaques of hemolysis, but enough to form "rosettes." After immunization, the amount of antibodies produced by each cell increases, consequently a larger population of cells reaches the threshold of detection by the plaque technique.

The fact that the gap between the "rosette" and plaque methods is greater in normal than in immunized animals provides additional proof against the passive fixation of antibodies on spleen cells.

II. APPLICATION OF THE ICA METHOD TO THE STUDY OF CELLS PRODUCING ANTIBODIES AGAINST HAPTENS*

The principle of the method is to perform the ICA test using red cells coupled with soluble antigens or haptens. Different antigens or haptens coupled with heterologous or homologous erythrocytes can be used successfully. In the present article, a model experiment carried out in guinea pigs immunized with the dinitrophenyl hapten will be described.

Guinea pigs were immunized with 1 mg of highly conjugated dinitrophenyl-bovine-γ-globulin (DNP-BGG) emulsified with complete Freund's adjuvant and injected in the hind footpads. A second injection was given 1 week later. At different periods after the second injection the number of "rosette"-forming cells was determined in the regional (popliteal and inguinal) lymph nodes using sheep red cells coupled with the DNP hapten. The conjugation of DNP with sheep erythrocytes was performed by the method of Ling (1961) as described by Bullock and Kantor (1965), using 1,3-difluoro-4,6-dinitrobenzene.

The results summarized in Table II show that lymph node cells from guinea pigs immunized with DNP-BGG produce a large number of "rosettes" when mixed with sheep erythrocytes bearing DNP on their surface. The background level of "rosette" formation by cells from normal guinea pigs is a little higher for sheep erythrocytes coupled with DNP (1.4 per 1000) than for native sheep erythrocytes (less than 1 per 1000). The augmentation of the percentage of "rosette"-forming cells in the lymph nodes of immunized animals represents a considerable increase in the total number of antibody-producing cells since the size of the regional lymph nodes is much larger in immunized than in normal animals.

*These experiments have been performed in collaboration with Dr. R. Binaghi.

The titer of serum antibodies against DNP increases markedly after immunization as measured by the agglutination of sheep erythrocytes coupled with DNP.

TABLE II
INCREASE IN THE NUMBER OF CELLS FORMING "ROSETTES" WITH SHEEP ERYTHROCYTES COUPLED WITH DNP IN THE LYMPH NODES OF GUINEA PIGS IMMUNIZED WITH DNP-BGG

Days after second injection	Number of "rosettes" per 1000 lymph node cells	Titer of serum agglutinins against sheep erythrocytes coupled with DNP
Nonimmunized	1.4	< 1/10
6	12	1/4000
8	20	1/3000
13	10	1/2000

III. INHIBITION OF ICA BY HETEROLOGOUS ANTIBODIES AGAINST IMMUNOGLOBULINS*

The phenomenon of ICA is postulated to be mediated by specific antibodies present at the surface of the "rosette"-forming cells, because the immunological specificity of the ICA is the same as that of serum agglutinins (Biozzi *et al.*, 1966). This interpretation is substantiated by the results obtained in experiments of inhibition of the ICA by antibodies against the immunoglobulins. The phenomenon of suppression of bacterial adherence by antiserum protein antibodies has been observed also by Mäkelä and Nossal (1961).

Theoretically, all the cells producing the different classes of antibodies should give "rosette" formation because the ICA is dependent only upon the specific binding capacity of the antibody which is a fundamental characteristic of all the types of antibody molecules. The inhibition of ICA by antibody specifically directed against each class of immunoglobulins permits the determination of the type of immunoglobulin produced by the cell.

In the present article, we shall discuss only the inhibition of the "rosettes" formed by lymphoid cells of guinea pig, immunized with sheep erythrocytes, which is produced by rabbit antiserum against guinea pig immunoglobulins.

*These experiments have been performed in collaboration with Dr. R. Binaghi.

Guinea pigs were immunized with 3×10^9 washed sheep erythrocytes emulsified with complete Freund's adjuvant and injected in the hind footpads. Fourteen days after immunization, the regional lymph nodes (popliteal and inguinal) were removed and the lymphoid cells isolated. With one part of the cell suspension, the number of "rosette"-forming cells was determined in the usual way (untreated preparation). Aliquots of 2×10^7 lymphoid cells were incubated for 30 minutes at room temperature with the final concentrations of rabbit antibodies against the guinea pig immunoglobulins or of the Fab fragment common to all the immunoglobulins (Table III). Thereafter, the cells were washed and the number of "rosette"-forming cells was determined by the usual technique. Control experiments were performed with normal rabbit serum added at the same concentration used for the rabbit antiserum. In these experiments Hank's solution without homologous serum was used instead of buffered saline.

The antiserum against guinea pig immunoglobulins was prepared in rabbits immunized with purified guinea pig anti-DNP antibodies obtained as described by Oettgen *et al.* (1965).

Rabbit immune serum antiguinea pig Fab fragment was prepared by immunization with the Fab fragment of normal guinea pig γ_2-globulin prepared according to the method of Nisonoff *et al.* (1960).

The data presented in Table III show that 14 days after immuniza-

TABLE III

INHIBITORY EFFECT OF RABBIT IMMUNE SERUM AGAINST GUINEA PIG IMMUNOGLOBULINS ON THE FORMATION OF "ROSETTES" BY LYMPH NODE CELLS FROM GUINEA PIGS IMMUNIZED WITH SHEEP ERYTHROCYTES[a]

Treatment of preparation	Number of "rosettes" per 1000 lymph node cells	Percentage of "rosettes" inhibited
Untreated	8.8	–
Anti-guinea pig immunoglobulins[b]		
0.24	3	66
2.4	0.6	93
24	0.6	93
Anti-guinea pig Fab[b]		
0.2	6.8	23
2	0.9	90
20	0.1	99

[a] Normal rabbit serum added at the same concentration used for immune serum did not affect the "rosette" formation. Both normal and immune rabbit serum were absorbed with sheep and guinea pig erythrocytes.

[b] μg *N* Ab/ml.

tion, the percentage of "rosette"-forming cells in the regional lymph nodes rises to 8.8 per 1000 lymphoid cells. Pretreatment of the lymphoid cells with both rabbit antisera inhibits the ICA phenomenon. A concentration of 2 μg N/ml of antibody suffices to bring back the number of "rosettes" to the normal background level found in nonimmunized animals (less than 1 per 1000). Data not shown in the table indicated that pretreatment of the lymph node cells with normal rabbit serum at the same concentration used for the rabbit antiserum does not affect the number of "rosette"-forming cells.

The inhibitory effect of the anti-Fab antiserum is particulary strong since it causes an almost complete suppression of "rosette" formation. Therefore, a fraction of the normal background of "rosette"-forming cells should also be inhibited. To clarify this point, the effect of the Fab antiserum was studied with cells from spleen and lymph nodes of nonimmunized guinea pigs. The technique used was the same as for the preceding experiment. The results of these experiments are reported in Table IV.

The incubation of cells from spleen or lymph nodes of normal guinea pigs with rabbit antiserum against guinea pig Fab fragment strongly reduces the number of "rosette"-forming cells, whereas the normal rabbit serum is inactive. This finding indicates that the "rosette"-forming cells found in nonimmunized animals are probably involved in the production of the low level of natural antibodies against sheep erythrocytes which are present in the serum of adult guinea pigs.

This assumption is corroborated by experiments showing a total absence of "rosette"-forming cells in lymphoid tissues from newborn mice, in which antibody synthesis is still lacking (Biozzi *et al.*, 1966).

IV. KINETICS OF THE APPEARANCE OF "ROSETTE"-FORMING CELLS IN THE SPLEEN OF MICE IMMUNIZED INTRAVENOUSLY WITH SHEEP ERYTHROCYTES

A. Primary Immunization

Experiments were carried out in adult male Swiss mice (20–25 gm) immunized intravenously with sheep erythrocytes. At different times after immunization, the total number of "rosette"-forming cells in the spleen was calculated from the percentage of "rosettes" and the number of spleen cells recovered from the whole spleen. The titers of agglutinins and hemolysins in the serum were also measured. These values were established using pooled spleen cells and sera from groups of five mice. The data reported in the figures represent the average values from two or three groups of five mice each.

TABLE IV

INHIBITORY EFFECT OF RABBIT IMMUNE SERUM AGAINST GUINEA PIG FAB ON THE FORMATION OF "ROSETTES" BY CELLS FROM SPLEEN AND LYMPH NODES OF NONIMMUNIZED GUINEA PIGS

	Number of "rosettes" / Number of spleen cells examined	Number of "rosettes" per 1000 spleen cells	Number of "rosettes" / Number of lymph node cells examined	Number of "rosettes" per 1000 lymph node cells
Normal lymphoid cells; untreated preparation	65 / 138,000	0.47	43 / 103,000	0.42
Lymphoid cells pretreated with 1/10 dilution of serum from normal rabbits	46 / 153,000	0.30	60 / 140,000	0.43
Lymphoid cells pretreated with 1/10 dilution of serum from rabbits immunized against guinea pig Fab[a]	1 / 209,000	0.0048	3 / 290,000	0.01

[a] The rabbit immune serum contained 260 μg N Ab/ml. Both normal and immune rabbit sera were absorbed with sheep and guinea pig erythrocytes.

An equivalent number of animals were used to measure the background of "rosette"-forming cells before the primary or secondary immunization. The titer of natural agglutinins and hemolysins in normal mice is less than 1:10.

Figure 2 represents the primary response observed in mice immunized with 10^8 sheep erythrocytes. The normal background of "rosettes" found in nonimmunized mice is indicated on the ordinates at zero time. The number of "rosette"-forming cells in the spleen starts rising immediately after immunization, reaching its maximum on the fifth day. Afterward, it decreases progressively to reach the normal background level on the ninetieth day.

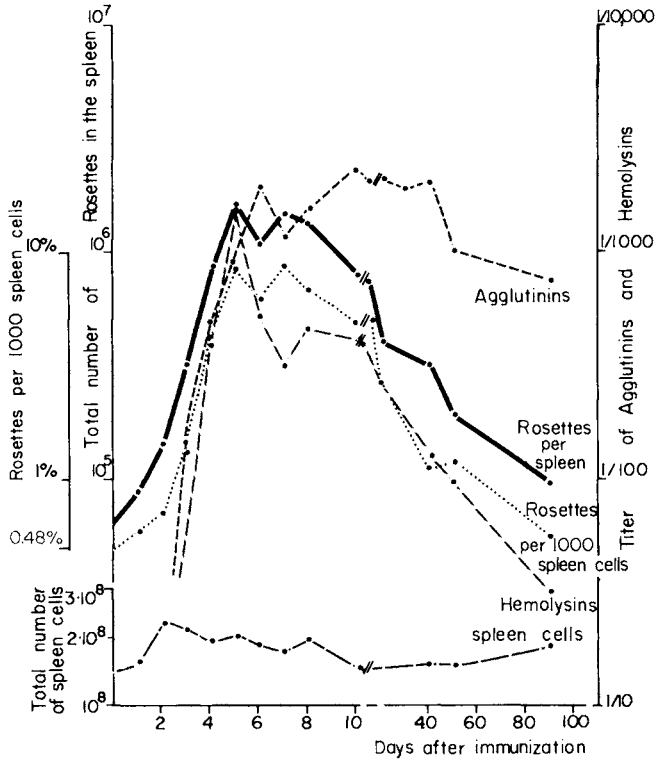


FIG. 2. Primary response. Number of "rosette"-forming cells in the spleen and titer of serum antibodies in mice immunized intravenously with 10^8 sheep erythrocytes.

The very rapid increase in the total number of nucleated spleen cells following the antigen injection should be noted. On the second day, the spleen cell population is increased by about 10^8 cells. This phenomenon persists only during the early ascending phase of the immunological response and is accompanied by a corresponding increase in the weight of the spleen.

The titer of serum antibodies is not increased until the second or third day. This lag period may reflect the fact that the cellular synthesis of antibodies precedes their release. Thereafter, the rise of serum antibodies follows the increase of the number of "rosette"-forming cells.

The number of "rosette"-forming cells is expected to be correlated with the level of agglutinins and not with that of hemolysins since the ICA is based upon an agglutination phenomenon. In fact, the hemolysin titer drops rapidly while the number of "rosette"-forming cells is still very high. Nevertheless, a dissociation is also evident between the agglutinins, which persist at their maximum level for at least a month, and the number of "rosettes" in the spleen, which decreases progressively.

Three months after immunization, when the number of "rosette"-forming cells has almost returned to the normal background level, the agglutinin titer is still about a hundredfold higher than the mean titer of natural agglutinins. This observation affords additional evidence against the possibility of passive sensitization of spleen cells by serum antibodies.

The reason for the discordance between the rate of decline of the number of "rosettes" in the spleen and that of the agglutinin titer is not clear. It seems that this phenomenon is not related to a redistribution of the antibody-forming cells into sites other than spleen, since the maximum level of extrasplenic localization of "rosette"-forming cells corresponds to the peak of "rosettes" found in the spleen (see Table V). It is possible that the rate of antibody synthesis at the cellular level is greater during the late phase of the immunological response. In this case, a smaller number of active cells could sustain a high titer of serum antibody. The prevalent class of antibody produced in the course of immunization could also be a determining factor because the circulating half-life of 19S immunoglobulins is shorter than that of 7S immunoglobulins; therefore, a smaller number of cells producing 7S antibody is required to maintain the serum level of 7S antibody. Other factors, such as antibody affinity, can also play a role in this phenomenon.

During the descending phase of the immunological response, a similar discrepancy between the number of hemolysin-producing cells and the level of serum hemolysins has been observed by several investigators using the technique of hemolysis plaque formation (Landy *et al.*, 1965; Möller, 1965; Rowley and Fitch, 1965; Šterzl *et al.*, 1965; Bourgeois and Paraf, 1966).

B. Secondary Immunization

The results obtained in the study of the secondary response are presented in Figs. 3 and 4. The experiment reported in Fig. 3 was carried out

in mice which received a primary intravenous immunization with 10^8 sheep erythrocytes. Forty-five days later, the mice were reinjected intravenously with the same number of sheep erythrocytes. At the time of the secondary immunization, the cellular and humoral effects of the first dose of antigen were still persisting, since the number of "rosette"-forming cells in the spleen was above the normal background and the agglutinin titer was considerably higher. (These values are indicated at zero time on the ordinate axis in Fig. 3.)

TABLE V
DISTRIBUTION OF "ROSETTE"-FORMING CELLS AMONG DIFFERENT TISSUES OF MICE IMMUNIZED INTRAVENOUSLY WITH SHEEP ERYTHROCYTES

Degree of Immunization	Number of "rosettes" per 1000 nucleated cells isolated from: Spleen	Lymph nodes[a]	Bone marrow	Blood leucocytes[b]
Days after primary immunization (10^8 sheep erythrocytes i.v.)				
5	8.5	0.5	–	1.4
12	5	1.2	0.2	0.1
20	2.6	1	0.3	0.1
60	1.2	3	0.1	0.2
90	0.6	0.8	–	–
Days after secondary immunization (10^8 sheep erythrocytes i.v. 45 days apart)				
3	17	1.9	1	9.2
4	14.4	0.9	1.2	5
5	19	2	–	–
7	15	1	–	–
10	6.7	2.2	–	–
20	3.3	0.8	–	–
Nonimmunized controls	0.5	0.8	0.2	0.1

[a] Total number of lymph node cells isolated = about 3×10^7 per mouse.
[b] Total number of leucocytes isolated = 1.5×10^7 per mouse.

The second injection of sheep erythrocytes produces a rapid increase in the number of "rosette"-forming cells in the spleen which reaches a peak on the third day. The maximum number of "rosettes" observed during the secondary response is about twofold higher than that seen during the primary response (see Fig. 2). Such a booster effect is also evident for the agglutinin production, whereas the secondary hemolysin response is similar to the primary one.

In the experiment reported in Fig. 4, the mice received a primary intravenous immunization with 10^5 sheep erythrocytes. Such a low dose of antigen did not produce a cellular or humoral response. In fact, groups of mice tested on the fourth, eighth, and eighteenth day after antigen administration showed no increase in the number of "rosette"-forming cells in their spleens and their serum titers of agglutinins and hemolysins was lower than 1:10. These mice were reinjected with the usual immunogenic dose of 10^8 sheep erythrocytes 25 days after the first subimmunogenic dose and showed a secondary response of "rosette"-forming cells in the spleen, similar to that of mice preimmunized with the dose of antigen capable of giving a primary response. In this experiment the increased

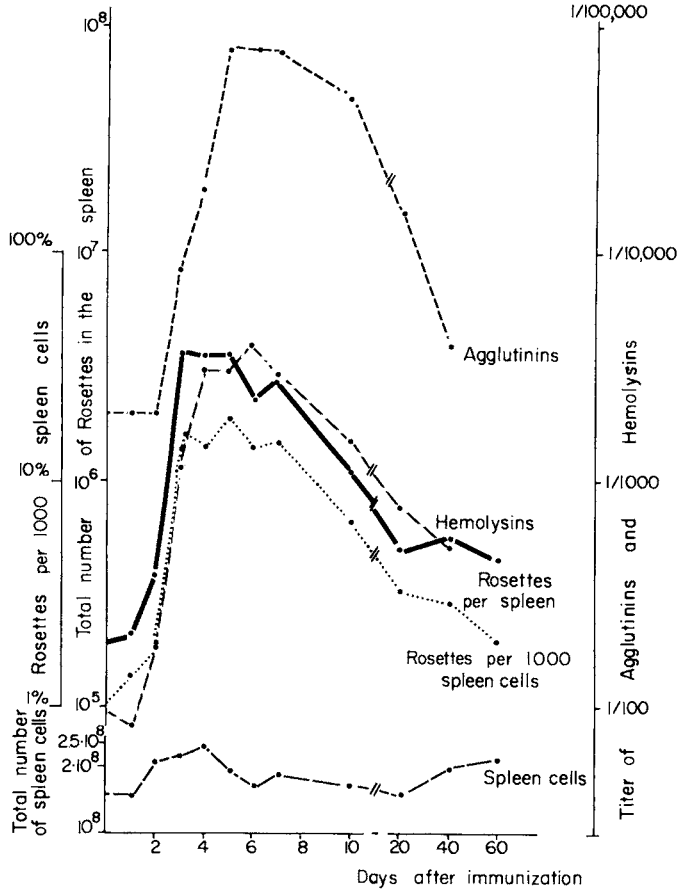


FIG. 3. Secondary response. Number of "rosette"-forming cells in the spleen and titer of serum antibodies in mice immunized intravenously with sheep erythrocytes: 10^8 and 10^8 sheep erythrocytes 45 days apart.

secondary antibody production was evident for both hemolysins and agglutinins.

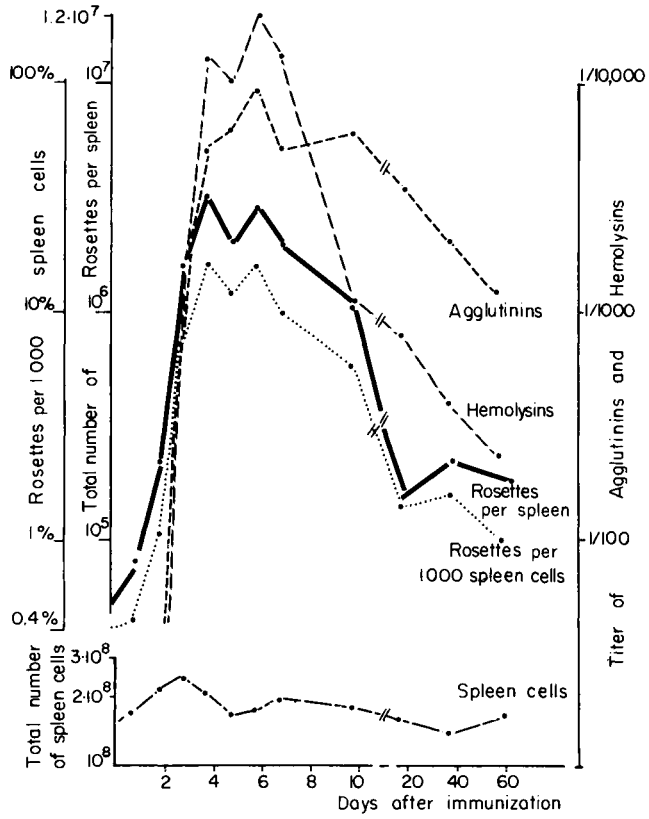


FIG. 4. Secondary response. Number of "rosette"-forming cells in the spleen and titer of serum antibodies in mice immunized intravenously with sheep erythrocytes: 10^5 and 10^8 sheep erythrocytes 25 days apart.

The differences in the secondary response between agglutinins and hemolysins (see Figs. 2, 3, and 4) can be explained by considering the class of antibody produced. The hemolysins are chiefly 19S immunoglobulins which show a secondary response only when appropriate amounts of antigen are injected and the two antigen injections are conveniently spaced (Hege and Cole, 1966a). In this connection it should be mentioned that the antigenic determinants inducing hemolysins may be different from those inducing agglutinins (Šterzl and Riha, 1965).

It should be noted that the total number of spleen cells shows the same rapid increase after either the primary or the secondary immunization.

Table V shows the distribution of "rosette"-forming cells in various tissues at different times after primary and secondary immunization. The data of Table V were obtained in the same animals used for the experiments reported in Figs. 2 and 3.

The number of "rosette"-forming cells in extrasplenic tissues was determined following the same technique used for the spleen. The blood leucocytes were obtained by mixing equal volumes of heparinized blood with a 4% solution of Dextran 500 (M.W. 370,000 Pharmacia Uppsala, Sweden) in saline. The mixture was left standing at 37°C for 30 minutes. The leucocyte-rich supernatant was separated from the erythrocyte sediment. The leucocytes were washed twice with buffered saline, then the ICA test was performed in the usual way.

The results obtained show that the spleen is the principal organ containing the "rosette"-forming cells. A definite increase in the number of "rosettes" formed by blood leucocytes was found during the early phase of the secondary response. These findings are in agreement with the results of Hullinger and Sorkin (1963) and Sorkin (1964).

Estimating at 1.5×10^7 the total number of white blood cells of the mouse, it can be calculated that about 20,000 leucocytes are able to form "rosettes"; this figure represents about 1 per 100 of the "rosette"-forming cells found in the spleen at the corresponding time of the primary response. At the peak of the secondary response, the total number of "rosettes" formed by the circulating leucocytes accounts for about 4 per 100 of the "rosettes" found in the spleen. The contribution of lymph nodes is more difficult to evaluate in an absolute manner because the total number of lymph node cells is unknown. Even assuming that the number of lymph node cells is equal to that of spleen cells, the extrasplenic location of "rosette"-forming cells is relatively low, especially during the early phase of the primary response.

Using the technique of hemolysis plaque formation, different investigators have found a variable number of active cells located in areas of the lymphoid system not directly involved in the principal immune response (Friedman, 1964b; Kearney and Halliday, 1965; Möller, 1965). It should be stressed that the dose of antigen and the route of injection are predominant factors determining the distribution of antibody-forming cells in the various areas of the lymphoid system.

C. Effect of Adjuvant

The effects of a bacterial adjuvant on the same parameters of the immune response studied for the primary reaction are shown in Fig. 5. The adjuvant used was a heat-killed suspension of *Corynebacterium parvum* (*C. parvum*). This microorganism produces a strong adjuvant

effect on antibody production (Neveu *et al.*, 1964). When the *C. parvum* is injected intravenously, the adjuvant effect on antibody production is associated with a marked increase in the number of spleen cells (Biozzi *et al.*, 1966). The phagocytic function of the reticuloendothelial system is also strongly increased by the *C. parvum* (Halpern *et al.*, 1963).

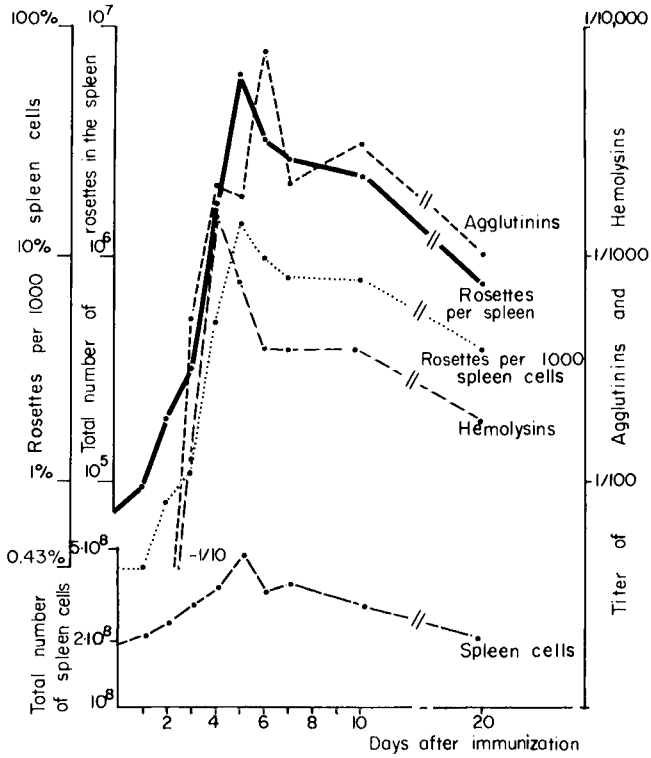


FIG. 5. Effect of the bacterial adjuvant *C. parvum* on primary response. Number of "rosette"-forming cells in the spleen and titer of serum antibodies in mice immunized intravenously with 10^8 sheep erythrocytes, 4 days after *C. parvum*.

The experiment represented in Fig. 5 was carried out in adult mice injected intravenously with 0.5 mg of *C. parvum* suspended in saline (the dose of *C. parvum* is expressed in dry weight of bacterial bodies). Four days later, the mice were immunized intravenously with 10^8 sheep erythrocytes.

The comparison of the data reported in Fig. 2 and Fig. 5 shows that the treatment with *C. parvum* increases both the maximum level of "rosette"-forming cells in the spleen and the peak agglutinin titer by about threefold. The hemolysin production is not enhanced by the adjuvant.

The injection of *C. parvum* produces a considerable increase in the number of spleen cells during 4 days preceding the immunization. This effect persists afterward. The number of spleen cells on the fifth day after immunization is about threefold above the normal value.

D. Analysis of the Kinetic Study on "Rosette" Formation During the Immunological Response

From some of the results obtained, it seems reasonable to assume that the cells which form rosettes in the spleen of nonimmunized animals (normal background) are not the same cells which respond to the antigenic stimulation giving rise to the clones of "rosette"-forming cells after immunization (target cells). In fact, the level of the normal background is the same in normal mice, in mice treated with adjuvant, and in mice immunized with a subimmunogenic dose of sheep erythrocytes. Nevertheless, the intensity of the immunological response after antigen stimulation is different (see Figs. 2, 4, and 5).

Various investigators, using the hemolysis plaque-forming method, obtained some evidence suggesting that the plaque-forming cells of normal animals are not the target cells for hemolysin production (Šterzl *et al.*, 1965; Hege and Cole, 1966b; Rowley and Fitch, 1966).

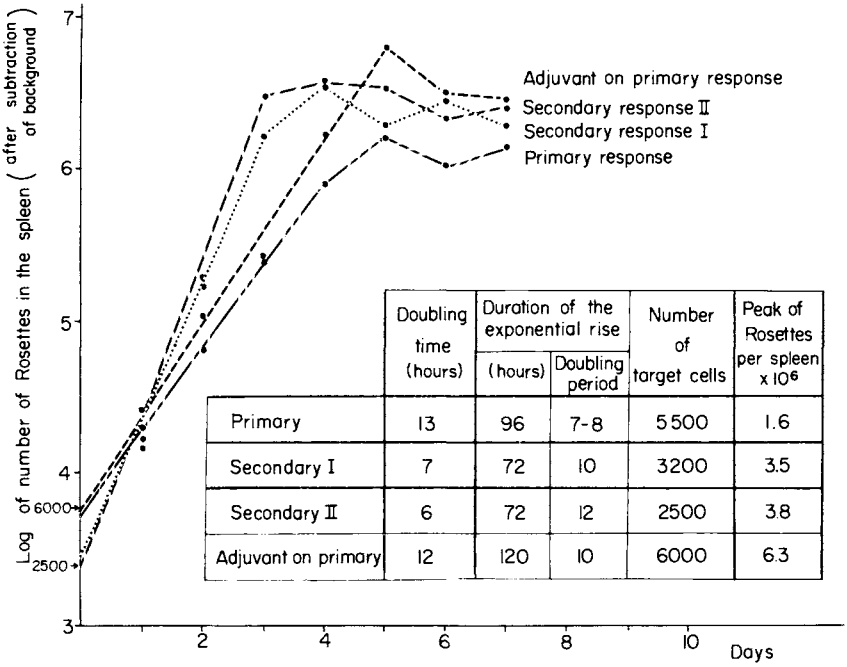
If this assumption is correct, the level of the normal background of "rosette"-forming cells should interfere with the exact appreciation of the initial rise of "rosette"-forming cells seen at the onset of the immunological response. Therefore, the curves of Figs. 2, 3, 4, and 5 were corrected by subtracting the corresponding values of the normal background and are represented in Fig. 6.

The curves shown in Fig. 6 indicate that the rise in the number of "rosette"-forming cells in the spleen follows an exponential function during 3–5 days after immunization.

Therefore, the mean doubling time of "rosette"-forming cells can be calculated on the exponential portion of each curve. This doubling time is 13 hours during the primary response. Treatment with adjuvant does not appreciably modify the doubling time (12 hours), but it prolongs the exponential phase of the response until the fifth day, thus leading to a higher peak of "rosette"-forming cells. On the other hand, the doubling time of "rosette"-forming cells is considerably shorter during the secondary response (6–7 hours). This faster rate is responsible for the higher peak of "rosettes" reached by the third day of the secondary response.

The doubling times of "rosette"-forming cells calculated above can be compared with the data obtained by other investigators using methods based upon thymidine incorporation in antibody-forming cells during the

immunological response (Schooley, 1961; Nossal, 1962; Nossal and Mäkelä, 1962).



	Doubling time (hours)	Duration of the exponential rise (hours)	Doubling period	Number of target cells	Peak of Rosettes per spleen x 10^6
Primary	13	96	7-8	5500	1.6
Secondary I	7	72	10	3200	3.5
Secondary II	6	72	12	2500	3.8
Adjuvant on primary	12	120	10	6000	6.3

FIG. 6. Cytodynamics of "rosette"-forming cells in the spleen of mice immunized intravenously with sheep erythrocytes. Doses of sheep erythrocytes: Primary, 10^8; Secondary I, 10^5 and 10^8 25 days apart; Secondary II, 10^8 and 10^8 45 days apart; adjuvant 4 days before, 10^8.

Assuming that the rate of production of "rosette"-forming cells is constant from the beginning of the immunological response, it is possible to extrapolate the number of "target cells" initially stimulated by the antigen. In this way, it can be calculated that the number of "target cells" is somewhere between 2500 and 6000 (Fig. 6). The dose of sheep erythrocytes used in the present experiments produces a maximal cellular and humoral immunological response (Jerne *et al.*, 1965; Dietrich, 1966). Consequently, the maximum number of cells in the mouse spleen capable of responding to the antigens of sheep erythrocytes can be estimated to range between 2500 and 6000.

This estimation is in agreement with the results obtained by Kennedy *et al.* (1965) and Playfair *et al.* (1965) using other methods.

The data presented in Fig. 6 are compatible with the following hy-

TABLE VI

PRINCIPAL ASPECTS OF THE IMMUNE RESPONSE CALCULATED FROM THE RESULTS OF KINETIC STUDIES OF "ROSETTE"-FORMING CELLS IN MICE IMMUNIZED WITH SHEEP ERYTHROCYTES

Immunization	Number of "rosette" forming cells			Peak titer of serum agglutinins	Doubling time of "rosette" forming cells during exponential rise (hours)	Duration of the exponential rise		Number of target cells per spleen	Total number of cells per spleen $\times 10^8$	
	Before primary or secondary immunization per spleen $\times 10^3$	Peak response after immunization				Hours	Doubling periods		Before primary or secondary immunization	Peak response after immunization
		Per spleen $\times 10^6$	Per 1000 spleen cells							
Primary 10^8 sheep erythrocytes i.v.	67	1.6 (fifth day)	8.5	1/2,500	13	96	7–8	5,500	1.4	2.3 (second day)
Secondary I 10^5 and 10^8 sheep erythrocytes 25 days apart	50	3.5 (fourth day)	16	1/9,000	7	70	10	3,200	1.4	2.3 (third day)
Secondary II 10^8 and 10^8 sheep erythrocytes 45 days apart	200	3.8 (third day)	19	1/78,000	6	72	12	2,500	1.5	2.5 (fourth day)
Adjuvant on primary[a]	78	6.3 (fifth day)	13.6	1/8,000	12	120	10	6,000	2	4.8 (fifth day)

[a] 0.5 mg of *C. parvum*, 4 days before immunization.

pothesis: the number of target cells initially stimulated by the antigen is the same in the primary or secondary response and after treatment with adjuvant. Starting from this constant level of target cells (2500–6000), the primary response is characterized by an exponential rise of antibody-forming cells with a doubling time of 13 hours lasting about 100 hours (7–8 doubling periods). The action of the adjuvant consists of prolonging the duration of the exponential rise of antibody-producing cells to about 120 hours (10 doubling periods). The secondary response is characterized by a shorter doubling time of antibody-producing cells (6–7 hours) persisting exponentially for only 72 hours (10–12 doubling periods).

The salient points resulting from the kinetic study of "rosette"-forming cells in the course of immunization are summarized in Table VI.

The results of the present experiments do not give any information on the nature of the mechanism involved in the rise of antibody-forming cells after immunization, namely, whether it is due to a process of cellular multiplication or differentiation. Possibly both mechanisms are involved. In this connection, it should be stressed that the antigen stimulation produces a remarkable increase in the total population of spleen cells which is due, very probably, to a process of cell multiplication. The number of cells in a normal mouse spleen is about 1.4×10^8. Two or three days after antigen injection, the number of spleen cells has almost doubled. The antigenic stimulation produces an increase of about 10^8 spleen cells, only 10^6 of which are engaged in specific antibody production. It is therefore most likely that different cell lines are induced into multiplication by the antigen. Recently, Tannenberg (1966) has observed a marked incorporation of thymidine into spleen granulocytes after antigen injection. It may be mentioned that these findings are compatible with the theory that the stem cells of the lymphoid tissue have plenipotential capacity (Albright and Makinodan, 1965).

V. SUMMARY

In vitro the antibody-containing cells isolated from animals immunized with heterologous erythrocytes are able to fix specifically at their surface the corresponding erythrocytes, leading to the formation of "rosettes." This phenomenon has been designated as immunocytoadherence (ICA) and can be elicited by either antibody-producing cells or cells passively sensitized by antibodies.

Lymphoid cells isolated from spleen or lymph nodes of mice and guinea pigs passively immunized with homologous antisheep erythrocyte immune sera do not form "rosettes" *in vitro*. On the contrary, the

macrophages from the peritoneal cavity become passively sensitized as shown by their ability to form "rosettes."

Experiments of active immunization of mice and guinea pigs with sheep erythrocytes show that when the immunological response is limited to a region of the lymphoid tissue (spleen or lymph nodes), only the cells isolated from that region are able to form "rosettes," whereas the rest of the lymphoid tissue is inactive. Macrophages from the peritoneal cavity become passively sensitized by antibody produced in the lymphoid tissue.

Using a mixture of sheep and pigeon erythrocytes as antigen, it is possible to establish whether a cell contains antibody of a single specificity ("pure rosette") or of two specificities ("mixed rosette"). When animals are submitted to this double immunization, from 90 to 98 per 100 of the "rosettes" formed by the cells of spleen or lymph nodes are "pure rosettes." On the contrary, the majority of "rosettes" formed by peritoneal macrophages are of the "mixed type."

These results demonstrate that the ICA method can be correctly applied to the quantitative detection of antibody-producing cells in the lymphoid tissue of immunized animals. For this purpose, the ICA method is much more sensitive than the method based upon hemolysis plaque formation.

The ICA method can be applied to the detection of cells producing antihapten antibodies by using erythrocytes coupled with the corresponding hapten.

The phenomenon of "rosette" formation is mediated by the antibodies present at the surface of the "rosette"-forming cells. Inhibition of ICA can be obtained by pretreating the cells with heterologous antibody specifically directed against the immunoglobulins produced by the cells. This inhibition phenomenon can be used to recognize the class of immunoglobulins produced by each cell.

A low level (less than 1 per 1000) of "rosette"-forming cells is found in the spleen and lymph nodes from nonimmunized animals (normal background).

The kinetics of appearance of antibody-forming cells has been studied by the ICA method using cells from the spleen of mice immunized intravenously with an optimum dose of sheep erythrocytes. After primary immunization, the number of "rosette"-forming cells in the spleen increases rapidly, reaching the peak on the fifth day. At this time, the spleen contains about 1.5×10^6 "rosette"-forming cells (8.5 per 1000 spleen cells). The exponential rise of the number of "rosette"-forming cells lasts for 4 days and occurs according to a doubling time of 13 hours.

In mice treated with a bacterial adjuvant (*C. parvum*) the exponential

rise of "rosette"-forming cells in the spleen lasts for 5 days and occurs according to a doubling time of 12 hours. On the fifth day the spleen contains about 6.3×10^6 "rosette"-forming cells (13.6 per 1000 spleen cells).

During the secondary response the exponential rise of "rosette"-forming cells in the spleen lasts for 3 days and occurs according to a doubling period of 6–7 hours. On the third day the spleen contains about 3.5×10^6 "rosette"-forming cells (16–19 per 1000 spleen cells).

The kinetics of appearance of "rosette"-forming cells indicates that the spleen of a mouse contains about 2500–6000 target cells able to respond to the antigens of sheep erythrocytes.

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DISCUSSION

DR. SHEARER: For almost 2 years we have been working with the ICA phenomenon, or cluster formation by agglutinin-releasing cells, and our results agree, in general, with those presented by Dr. Biozzi.

Figure 1 illustrates graphically the appearance of cluster-forming cells (solid line) and of plaque-forming cells (dashed line) in the spleens of (C3H × C57BL) F_1 mice following immunization with a single injection of 5×10^8 sheep red cells. The shaded area in the figure represents the number of cluster-forming cells (with the 95% confidence interval) in spleens of nonimmunized control mice. The corresponding values for plaque-forming cells are not in-

immunized control mice. The corresponding values for plaque-forming cells are not indicated in the figure because they were lower than 100 per spleen. The important points to be made are: (1) peak numbers of cluster-forming cells are observed on the sixth day after immunization, while peak numbers of plaque-forming cells occur on the fourth day; (2) on the days of peak responses, the number of cluster-forming cells exceeds the number of plaque-forming cells by a factor of three to four; (3) from the fifth day onward, the number of cluster-forming cells remains elevated, while the number of plaque-forming cells decreases steadily; (4) the number of cluster-forming cells in nonimmunized control mice exceeds the number of plaque-forming cells by a factor of about one thousand. These kinetic findings suggest that plaque-forming and cluster-forming cells correspond to two distinct populations.

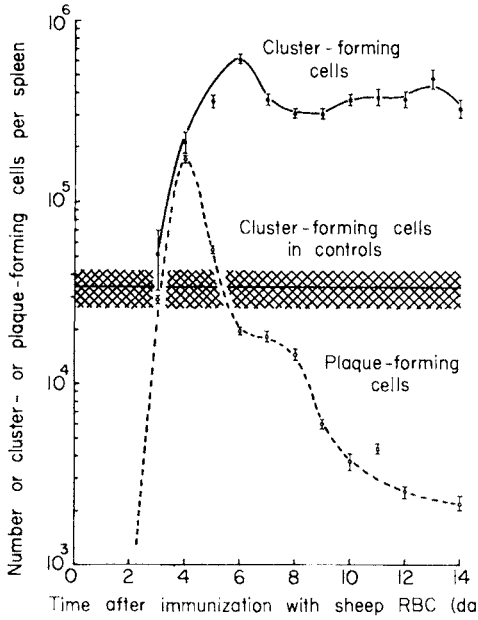

FIG. 1. The appearance of plaque-forming and cluster-forming cells in the spleens of (C3H × C57/BL)F_1 mice following a single intraveinous injection of 5×10^8 washed sheep RBC.

Whereas Dr. Biozzi has incubated his mixtures of immune spleen cells and sheep red cells overnight at 4°C, we have chosen to work at 37°C for the reasons illustrated in figure 2. In four separate experiments the number of detectable clusters appeared to increase with time of incubation up to a maximum at 1¼ hours. Upon further incubation at 37°C, there was, however, a gradual decrease in the number of detectable clusters. The disappearance of clusters could be prevented by transferring the spleen-sheep red cell mixtures to a water bath containing melting ice. If the mixtures were maintained in melting ice from the beginning of the incubation period, no clusters were formed for 2½ hours. Further incubation, however, of the same mixtures overnight at 8°C yielded a number of clusters comparable with the peak numbers obtained at 37°C. It seems, therefore, that temperature of incubation is not a very critical factor, provided that the time of peak cluster formation for any given temperature is known (see Fig. 2).

Before specific cluster formation *in vitro* can be considered an assay for agglutinin-forming cells, one has to exclude that cluster formation results from passive sensitization or antibody coating of spleen cells not actually engaged in the synthesis of antibody. Dr. Biozzi has clearly demonstrated that cytophylic antibody, as described by Boyden and

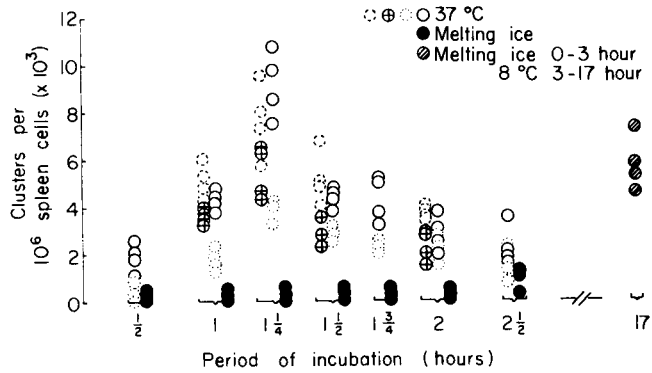

FIG. 2. The effect of incubation time with sheep RBC and of temperature on the number of clusters formed by spleen cells *in vitro*. The cells were harvested from (C3H × C57/BL) F_1 mice immunized with 5×10^8 sheep RBC 6 days previously.

Sorkin [(1960) *Immunology* **3**, 272], does not account for specific cluster formation by immune spleen cells. We have investigated the possibility that cluster-forming cells are coated by agglutinating antibody other than the classical "cytophylic antibody." We have found that, indeed, nonimmune spleen cells can acquire the ability of cluster formation with sheep red cells upon exposure *in vitro* to immune mouse-anti-sheep red cell serum or to the supernatant of a spleen cell suspension taken from an immune mouse. Some of our experimental results are presented in Table I. The cluster-forming ability of passively sensitized spleen cells is lost, however, after repeated washings of the cells in Eagle's medium, a property which may facilitate the distinction between cluster-forming cells coated by antibody and those actively producing antibody. Table II illustrates the effect of repeated washings

TABLE I

CLUSTER FORMATION *in Vitro* BY SPLEEN CELLS FROM NONIMMUNE (C3H × C57/BL)F_1 MICE PASSIVELY SENSITIZED BY ANTIBODY

Addition to Incubation Mixture[a]	Clusters per 10^6 spleen cells
Nonimmune spleen supernatant	200
Immune spleen supernatant	4000
Nonimmune mouse serum	500
Immune mouse serum	21,800
Immune mouse serum and three washings	700

[a] Basic incubation mixture kept at 37°C for 75 minutes; 5×10^6 spleen cells in Eagle's medium; 1% sheep red blood cells by volume. Additional components were added to the spleen cell suspension before or with the sheep red blood cells.

on the number of cluster-forming cells in spleens of mice actively immunized against sheep red cells. The washings decreased the number of clusters by about one-third or one-fourth. This suggests that part of the cluster-forming cells found in unwashed spleen cell suspensions could have been passively sensitized. Therefore, we feel that the enumeration of antibody-forming cells by the cluster technique should always be performed on washed spleen cells.

TABLE II
CLUSTER FORMATION *in Vitro* BY SPLEEN CELLS FROM (C3H × C57/BL)F_1 MICE ACTIVELY IMMUNIZED WITH SHEEP RED BLOOD CELLS[a]

	Clusters per 10^6 cells	
Experiment No.	Nonwashed	Washed
1	5900	3600
2	5100	3900
3	4200	2900
4	5000	3700

[a] Basic incubation mixture as in Table I except for three washings of spleen cells in Eagle's medium before incubation with sheep red blood cells.

DR. BIOZZI: I want to discuss the possibility that *in vitro* spleen cells could be passively sensitized. We have not been able to see this phenomenon under our experimental conditions. In our technique for immunocyto adherence (ICA), the lymphoid cells are always washed three times and resuspended in buffer with 5% homologous serum. Under these conditions the passive sensitization does not occur. It is possible that, without washing or with incomplete washing, some antibody will persist adsorbed on the cell surfaces, but this is a trivial phenomenon. Other investigators have observed some degree of passive sensitization *in vitro*, but their experiments were performed in the absence of homologous serum. The presence of homologous serum is important because normal globulins may eventually de-adsorb the antibody from the surface of the lymphoid cell. The essential point that I would like to stress here is that passive sensitization of lymphoid cells does not occur *in vivo*. Therefore, the method of immunocytoadherence (ICA) can be correctly applied to the quantitative study of antibody-producing cells from immunized living animals. As far as your results of passive sensitization *in vitro* are concerned, I would like to ask you whether the cells were incubated in the absence of normal serum. Also, the temperature of incubation is an important factor, and I think your experiments were done at 37°C and not in the cold.

DR. SHEARER: We preincubated the nonimmune spleen cells with immunized mouse serum, then removed the serum and took the treated cells, resuspended them in Eagle's medium, and incubated them with sheep red cells in the same serum-free medium at 37°C. Only complete clusters were recorded.

DR. BIOZZI: Probably the reason why you observed passive sensitization is that you kept your incubation mixture at 37°C. At this temperature, antibodies are released from the cells into the medium and little agglutinates of red cells may occur. These agglutinates may look like rosettes, especially since in the case of complete true rosettes it is often difficult

to see the nucleated lymphoid cell which is all covered by the red cells. One can distinguish the formations better by performing the same preparation in a thin layer between two glass slides, in such a way that the red cells are squeezed away and the nucleated cells appear. Concerning the differences in kinetics between hemolytic plaque formation and ICA, your data are quite consistent with ours, even if your peak response of rosette-forming cells is somewhat lower than ours.

DR. SHEARER: The clusters we recorded looked like berries and were obviously formed by a large bunch of red cells surrounding and covering a spleen cell. They were quite different from the red cell aggregates you caution about which were also seen but were not counted.

DR. BIOZZI: In my personal experience, it is very difficult to distinguish between small round red cell agglutinates and true rosettes in the counting chamber. This is why we carry out all the procedures of ICA in the cold to avoid the release of antibody which will produce hemagglutination.

TABLE III
PLAQUE COUNTS AFTER INCUBATION AT 5°C OR 37°C (PAIRS OF PLATES)[a]

	Incubation Temperature	
Incubation time	5°C	37°C
0	–	44
10 minutes	34, 79	53, 62
1 hour	14, 40	59, 82
5 hours	8, 49	99, 108
1 day	41, 48	Lysed
4 days	102, 283	–
6 days	Lysed	–

[a] 0.1 ml spleen cell suspension and 0.05 ml red cells mixed with 2 ml agar and plated without a base layer. After setting (10–15 seconds), pairs of plates were placed either on ice water at 5°C or incubated at 37°C. After standing for the times indicated, plates kept at 5°C were covered with 1 in 10 guinea pig serum at 0°C, left standing for a further 30 minutes at 5°C, drained and incubated at 37°C for 20 minutes. The plates kept at 37°C were covered with guinea pig serum at room temperature, and incubated at 37°C for 20 minutes.

DR. BERENBAUM: I would like to discuss two points made by Dr. Biozzi. The statement that agglutinating cell counts are more sensitive than counts of hemolysin-forming cells depends on how one defines sensitivity. The true measure of sensitivity in this case is the efficiency of counting, that is, the relation between the number of cells that actually contain antibody and the number that are counted, but to know this, we would have to have in-

dependent methods of detecting antibody-containing cells. Another measure is the sensitivity with which antibody-containing cells can be counted amid a population of other cells that do not contain antibodies. The Jerne technique counts these with a sensitivity of about one in 100 million. You can plate a whole spleen containing, for example, 100 million cells and can pick out the one or two cells containing antibody in a 2–3 minute survey. With the agglutinating-cell technique, suspensions are examined in a counting chamber and you can survey about 3–6 mm^3 of suspension in a few minutes. To do this conveniently, the spleen has to be dispersed in about 10–20 ml of fluid and to survey a whole spleen in this way would take about 100–200 hours which, of course, is not practicable. In practice, therefore, using reasonable survey times, the sensitivity of the agglutinating cell method is about 1 in 10,000 compared with a sensitivity of 1 in 10^8 for Jerne's method. With regard to the point that the cells in the Jerne plate have to be alive, there is, in fact, no good evidence that the cells must be alive although, of course, they may be. First, the whole test (apart from complement lysis) can be carried out in the cold. It takes longer to produce plaques because it takes antibody longer to diffuse out in the cold but plaques are undoubtedly formed (Table III). Second, it has been found by more than one worker that even if you have enough puromycin in the medium to prevent protein synthesis, you still get plaques.Third, although it was shown in the original work with this method that plaque appearance was inhibited by cyanide, this does not prove that the cells have to be alive, as can be shown by a very simple experiment. If you incubate the plate and allow antibody to diffuse out from the cells, but, before adding complement, you add cyanide to the plate and then wash it off, you get no plaques (Table IV). Cyanide splits disulfide bonds and so this could be due to an effect of cyanide on the 19S antibody or perhaps on complement. Certainly there is no evidence here that cyanide is acting on the cells. Of course, no plaques are formed if the cells are damaged before they are incorporated in the agar because the antibody diffuses out of the damaged cells too fast. If cells do not have to be alive

TABLE IV

PLAQUE COUNTS AFTER TREATMENT WITH CYANIDE (PAIRS OF PLATES)[a]

Experiment	Solution added	Period of treatment			
		0 minutes	10 minutes	30 minutes	60 minutes
A	Saline	702, 704	720, 730	462, 594	622, 644
	Saline + KCN, 10^{-2} *M*	–	15, 5	14, 62	7, 19
	Saline + KCN, 10^{-1} *M*	–	4, 6	3,9	0, 4
B	199	3612, 3900	1783, 3458	3055, 3285	2340
	199 + KCN, 10^{-2} *M*	–	1810, 1928	1560, 1995	–
	199 + KCN, 10^{-1} *M*	–	30, 37	42, 41	–

[a] Five spleens taken 3 days after a secondary injection of sheep red cells and suspended in 5 ml 199; 0.1 ml suspension used per plate. Agar for bases and top layers of plates made up in saline in experiment A and in 199 in experiment B. Pairs of plates incubated for 1 hour at 37°C, treated with the solutions specified for 10, 30, or 60 minutes, rinsed in saline or 199 respectively, and treated with 2 ml 1 in 10 guinea pig serum in saline for 30 minutes at 37°C.

Treatment with cyanide after diffusion of antibody out of antibody-forming cells and its attachment to red cells inhibits development of plaques.

to produce plaques, the consequence is that you cannot use this method to study the effects of toxic or immunosuppressive agents by incorporating them in the plate.

DR. BIOZZI: I think that the method of rosettes is more sensitive than that of hemolytic plaque just because it detects a larger number of active cells in the same cell populations from both normal and immunized animals. This means that cells producing a very little amount of antibody are not detected by the plaque method while they are detected by the rosette method. In other words, the threshold of detection is lower for rosette-forming cells than for plaque-forming cells. I do not think that the number of cells inspected is of importance because the number of rosettes is similar in different aliquots of the same preparation. Therefore it is useless to examine the whole cell population of a spleen. I have not studied the requirement for viability of cells producing hemolysis plaque. I know that Ingraham and Bussard [(1964) *J. Exptl. Med.* **119,** 667] using metabolic inhibitors obtained some evidence that active synthesis of antibody is occurring in the formation of hemolysis plaques. I know that everybody does not agree on this point, but I feel that cells must be alive in order to synthesize and perhaps release antibody; or at least some enzymic or surface properties of the living cells must be preserved. On the contrary, for the rosette method, only the presence of the antibody on the surface of the cells is needed. This is one of the reasons for the higher sensitivity of the rosette method.

DR. MITCHELL: Similarly to Dr. Biozzi, we and others have found no stimulation of spleen or lymph node distant from the regional node in rabbits immunized with a soluble antigen, diphtheria toxoid, in the footpad. We could, however, elicit a secondary response by injecting the antigen into the *opposite* footpad 28–31 days later [Mitchell and Calabresi (1964) *Yale J. Biol. Med.* **36,** 421]. This again seemed to be a specific stimulation of the node regional to the second injection; the site injected initially did not respond histologically to the challenge in the opposite paw. Only in the node challenged by the second injection did antibody formation occur, as measured by specific immunofluorescence. The spleen in no instance showed any evidence of antibody-forming cells, so we felt that if distant nodes were "primed," this was probably done not by antigen which had leaked into the circulation, but perhaps by small lymphocytes. According to the scheme that Gowans had proposed, lymphocytes recirculate via the postcapillary venules in the lymph nodes and it seemed that this might be the mechanism by which the distant node became informed of the antigen. Also, there is an observation which may support the notion that the primary response is basically a process of induction, and that any proliferation or antibody formation which occurs at that time may be merely incidental. In one strain of highly inbred hooded rats, used in Gowan's experiments, the primary response to toxoids is almost *nil* with a tanned red cell technique. Yet the secondary response in these same animals is uniformly high, yielding titers of 1 to 80,000 and 1 to 160,000. Whatever its significance, there does not seem to be any correlation between level of titer in the primary response and that after challenge, in that system and animal (unless it is an inverse correlation, as Dr. Šterzl has shown for the hemolysin system).

DR. BIOZZI: The localized response of the regional lymph nodes after antigen stimulation is not a general phenomenon and its occurrence depends upon the nature and the dose of the antigen. The presence of adjuvant in water oil emulsion is also important. In fact, in some experiments we used a large amount of antigen to involve either the spleen or the far located lymph nodes and we found that, if soluble antigen is injected in the footpad in large amounts, it is possible to involve also the far located lymph nodes. In this case, however, the spleen is also involved. This indicates that the antigen spreads throughout the body. On the other hand, the possibility is real that cells migrate from the lymph nodes to other lymphoid districts through the circulation. After immunization involving only the spleen, there is a certain level of rosette-forming cells in the blood and its peak corresponds to that

of rosette-forming cells in the spleen. However, the location of rosette-forming cells in another district of the lymphoid system is very poor, as shown in Table V of our article.

DR. SIGEL: The Variety Children's Research Foundation, Miami. You showed a very high background level of ICA cells with the sheep red blood cells. Does the normal mouse spleen contain a large number of cells which are positive in the immunocytoadherence tests with pigeon red blood cells?

DR. BIOZZI: The results with sheep red cells and pigeon red cells are quite similar. Data obtained with human red cells of the A group are also quite similar [Biozzi *et al.* (1966) *Ann. Inst. Pasteur* **110,** Suppl. 3, 7].

DR. METCALF: The current interest of immunologists, as I understand it, is in the Cole-Playfair-Kennedy techniques which enumerate antigen-responsive cells and these cells are being regarded as the possible precursors of the plaque-forming cells. Now the total number of antigen-responsive cells in an unstimulated mouse spleen is said to be about a thousand, perhaps a little more. Are the cells you described these antigen-responsive cells? I ask this since the antigen-responsive cells are believed to proliferate a little bit earlier than do the plaque-forming cells, just as your cluster-forming cells do. Have you any comment on the interrelationship between these three types of cells?

DR. BIOZZI: The extrapolation from our data (see Fig. 6 of our article) indicating the number of antigen-responsive cells (target cells) in the spleen is in very good agreement with the results reported by Kennedy *et al.* [(1966) *J. Immunol.* **96,** 973]. I have no data from my own experiments which could clarify the question of whether the immune process starting from these antigen-responsive cells, namely, the process leading to the increasing number of antibody-producing cells, is related to cellular multiplication or differentiation or both. The spleen increases very much in cellularity after antigen stimulation. Quantitatively, if one considers that the spleen of the normal mouse contains about 100 million cells, on the third or fourth day after antigenic stimulation the number of splenic cells is almost doubled. So, unless they come from the bloodstream, and I think this is extremely unlikely, they must multiply locally. On the other hand, the doubling time of antibody-producing cells in a secondary reaction is very short, in the order of 6–7 hours or so. It seems to me that this is too short a period to consider that all the process is based upon multiplication, because this would be a very short multiplication time for mammalian cells. So it may be that multiplication and differentiation take place at the same time and that what we see is the summation of the two phenomena.

DR. SHEARER: I would like to comment on the question asked by Dr. Metcalf concerning the relationships of plaque-forming, cluster-forming, and antigen-sensitive cells. We have obtained evidence indicating that the cluster-forming cells and the 19S plaque-forming cells are two distinct populations within the spleen. One piece of evidence is represented by the different kinetics of appearance of the two immune cell types following antigen administration [Shearer, G. M. (1965) *Federation Proc.* **24,** 305]. The second and stronger evidence is given by the following experiment. We incorporated in soft agar a suspension of immune spleen cells and sheep red cells containing specific clusters resulting from previous agglutination. The suspension was plated on hard agar so as to obtain eventually also hemolytic plaques. The number of clusters was counted in a given area before complement addition. After suitable incubation with complement, clusters were recounted and also the newly formed plaques of hemolysis. The latter appeared throughout the plate without lysing the clusters. Since the clusters were not destroyed during the appearance of plaques, one can conclude that cluster-forming cells do not release hemolyic 19S antibody and *vice versa.* It is not known at this time whether these two immune cell types are derived from a single or from different antigen-sensitive cells.

DR. BIOZZI: I do not see why cells synthesizing 19S antibody should not produce rosettes. In fact, these antibodies combine well with the antigen and the phenomenon of rosette formation is based upon the binding of antigen with the antibody located on the surface of the cells. Maybe the 19S antibodies do not remain on the surface of the cells.

DR. LANDY: I am dubious that we will resolve these issues by the study of the progenitors of stem cells in conventionally reared mice, and I would urge that we hear from Dr. Šterzl who for many years has studied immunocompetent cells in newborn germ-free, colostrum-free piglets, perhaps the ideal mammalian model in that it is an immunologically pristine species. In this situation one is really beginning with a clean slate.

DR. ŠTERZL: I would like to mention our experiments with germ-free piglets, and to comment on the question related to the number of cells demonstrating agglutinating antibodies (Zaalberg technique) and hemolyzing antibodies (Jerne technique). Counting clusters, one detects cells producing both 19S and 7S agglutinating antibodies. In contrast, by the plaque technique, one detects predominantly 19S antibody-forming cells. Only by the use of anti-IgG serum can one enhance the production of 7S hemolysis [Šterzl and Riha (1965) *Nature* **208,** 858]. Even more important is the fact that hemolyzing antibodies are directed against other antigenic determinants on red cell stroma than the agglutinating antibodies. Immunizing Forssman positive mice with sheep red blood cells, the effect of Forssman's antigen is excluded. Pretreating the sheep red cells with 0.5% formalin completely destroys the ability of those cells to induce the formation of hemolyzing antibodies without impairing the capacity to induce agglutinating antibodies. Different patterns in the development of hemagglutinins and hemolytic antibodies during primary as well as secondary responses can be seen using various amounts of sheep red cells: large doses given in the primary response enhance the formation of hemagglutinins in the secondary response but inhibit that of hemolysins. These observations imply that not only different antigenic determinants on the sheep red cells, but also different lines of immunologically active cells are involved in these phenomena. With reference to what Dr. Landy mentioned, it is true that probably using normal mice one is starting with cells (about 50 of the 1×10^8 lymphoid cells) which are already producing antibodies. In germ-free, colostrum-free piglets reared on antigen-free diets, we did not detect any background of antibody-producing cells by the plaque-forming technique. Under these conditions, only 1 cell per million lymphoid cells is stimulated to produce antibodies and can be considered as specifically immunologically competent.

DR. BIOZZI: With our method of immunocytoadherence (ICA), we were unable to detect any rosette-forming cells in the spleen and thymus of newborn mice. These cells begin to appear during the development of conventionally reared animals in the absence of antigen injection [Biozzi *et al.* (1966) *Ann. Inst. Pasteur* **110** Suppl 3, 7].

Cellular Differentiation during Immune Responses Studied with the Electron Microscope and Radioautography*

SAM L. CLARK, JR.†
Department of Anatomy, Washington University School of Medicine, St. Louis, Missouri

Cellular differentiation is the process by which cells with a common inheritance grow different under the influences of differing environments. According to present notions, environmental stimuli set off events that culminate in the repression or derepression of some gene, thus selecting which of the many potentialities of the cell will be expressed. For lymphoid cells, antigen is such an environmental influence. The synthesis of RNA, protein, and other substances that result from genetic derepression and that change the structure and function of the cell are the outward signs of an earlier, inward change; by the time they are detected, the critical events of differentiation are already over. In the case of immune responses, differentiation of the genes themselves will need to be considered.

A considerable body of evidence, largely circumstantial, supports the idea that antigen induces proliferation of large, pyroninophilic lymphoid cells that subsequently differentiate into plasma cells responsible for secreting antibody (Nossal, 1962). The application of the electron microscope to this problem (Bessis, 1961; Feldman, 1964; Movat and Fernando, 1965; Clark, 1966b) has opened a new world of structural complexity, and provided circumstantial evidence supporting the old theory, but it has failed to reveal the processes of cellular differentiation, chiefly because cells seen in electron micrographs are dead—no longer differentiating. In the words of Pooh Bah, electron micrographs provide

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"merely corroborative detail to add artistic verisimilitude to an otherwise bald and unconvincing narrative." However, these details do permit more precise definition of cellular types and, coupled with functional indicators such as radioautography, immunohistochemical staining, and other tests for the presence of antibody, can be interpreted in dynamic terms.

In this paper, I will define plasma cells—both mature and immature, blast cells, lymphocytes, and macrophages—both free and fixed—as follows. Mature plasma cells contain abundant rough-surfaced endoplasmic reticulum, a large, centrally placed Golgi complex, and a condensed nucleus without nucleoli. Immature plasma cells are larger, contain less rough-surfaced endoplasmic reticulum, but have numerous free ribosomes in the cytoplasm; the nucleus is large, with dispersed chromatin and a prominent nucleolus. Blast cells are larger still, lack rough-surfaced endoplasmic reticulum, but are especially rich in free ribosomes; the Golgi complex is small but the nucleus is even larger, the chromatin more dispersed and nucleoli more prominent than in immature plasma cells. Large lymphocytes differ from blast cells only in possessing fewer cytoplasmic ribosomes. Small lymphocytes have small, condensed nuclei without nucleoli and the scanty cytoplasm contains few ribosomes and a small Golgi complex. Medium-sized lymphocytes are intermediate in structure. Macrophages, identifiable by the dense remnants of ingested debris in their cytoplasm, vary greatly in appearance. Some possess large immature nuclei and numerous ribosomes, both free and bound to endoplasmic reticulum; others have condensed nuclei and few ribosomes. All have well-developed Golgi complexes and much smooth-surfaced endoplasmic reticulum. Free macrophages are studded with irregular pseudopods and fixed macrophages (phagocytic reticular cells) extend to cover the reticular fibers of lymphoid tissue.

Plasma cells not only contain, but almost certainly manufacture and secrete antibodies. As seen in electron micrographs, they contain ribosomes and polyribosomes. Polyribosomes extracted from myeloma cells can synthesize γ-globulin (Shapiro *et al.*, 1966), and plasma cells actively incorporate radioactive amino acids into protein, as demonstrated by radioautography at both the light and electron microscopic level (Mitchell, 1964b; Clark, 1966b). This newly formed protein is rapidly excreted, and γ-globulin is the only kind of protein released by intact lymphoid cells; therefore it is presumed to represent antibody (Clark, 1966b). In their rough-surfaced endoplasmic reticulum and Golgi complex, plasma cells possess the machinery characteristic of cells that secrete protein, and de Petris and Karlsbad (1965) have detected antibody lying within the cavities of these organelles by immunohistochemical staining and

electron microscopy. Furthermore, sequential analysis of electron microscopic radioautographs provides evidence that protein synthesized in the rough-surfaced endoplasmic reticulum passes through the Golgi complex on its way out of the plasma cell—a sequence of events characteristic of other types of cells that secrete protein (Clark, 1966b).

Thus mature plasma cells appear to be the highly differentiated source of antibody, but they neither reproduce themselves nor remain long in lymphoid tissue. They manufacture no DNA and little RNA (Mäkelä and Nossal, 1962; Nossal, 1962; Mitchell, 1964a). Previously made RNA persists for the life of the cell, and actinomycin D does not inhibit the synthesis of antibody by mature plasma cells (Mitchell, 1964b; Lazda and Starr, 1965)—this has been taken an evidence that the messenger RNA responsible for antibody synthesis has a long life, but in any case it indicates that the highly differentiated plasma cell is no longer receiving nuclear instructions for further differentiation. Furthermore, it seems to lack the capacity for any future differentiation: most plasma cells disappear within a few days of their formation (Mäkelä and Nossal, 1962; Nossal, 1962), and the remaining few will not respond to subsequent antigenic stimulation (Miller, 1964). Their fate is uncertain. Many mature plasma cells found in lymphoid tissue no longer synthesize protein (Schooley, 1961; Clark, 1966b) and in electron micrographs, some appear to be dying (Clark, 1966b). However, Moore *et al.* (1965) reported finding bits of cytoplasm apparently shed from plasma cells and proposed that by this divestment they became small lymphocytes. In any case, new plasma cells must come from the differentiation of some other type of cell.

The "immature plasma cell" is a natural choice as putative precursor for the plasma cell. It combines a well-developed Golgi complex and rough-surfaced endoplasmic reticulum with the vesicular nucleus, prominent nucleolus, and free ribosomes characteristic of differentiating cells. By arbitrary choice of electron micrographs, one can assemble a series of cells ranging in imperceptible transition from mature plasma cells through immature plasma cells back to blast cells that lack rough-surfaced endoplasmic reticulum. These, in turn, cannot be distinguished from the slightly less basophilic large lymphocytes. Thus, from the morphological point of view, a common origin for plasma cells and lymphocytes seems reasonable, but what direct evidence is there for this?

Precursor cells can be expected to proliferate and be active in the synthesis of RNA and protein if they are to continue to produce highly differentiated offspring. Among lymphoid cells, the most active in this regard—as judged by radioautography—are large lymphocytes, blast cells, and immature plasma cells (Nossal and Mäkelä, 1961; Mitchell,

1964a,b; Clark, 1966b). These comprise the large, round pyroninophilic cells of lymphoid tissue and will be referred to collectively as large lymphoid cells.

Furthermore, these cells respond to antigenic stimulation. Cells from spleen and lymph nodes of immunized animals accelerate their rate of proliferation when incubated with antigen *in vitro,* but cells from thymus and bone marrow do not (Chapman *et al.,* 1965). Almost immediately after injection of antigen, a similar burst of mitotic activity can be detected *in vivo.* It reaches a peak within 24 hours and has all but ceased by 48 hours (Nossal *et al.,* 1963; Cohen and Talmage, 1965). Large lymphoid cells are chiefly responsible; their generation time decreases from approximately 30 to 14 hours (Capalbo *et al.,* 1962; Capalbo and Makinodan, 1964). The synthesis of RNA increases simultaneously (Cline and Fudenberg, 1965), and at the height of the response most of the rapidly proliferating cells contain antibody, as demonstrated by immunohistochemical staining (Urso and Gengozian, 1964). As proliferation declines, large lymphoid cells diminish in number, plasma cells become abundant, and antibody appears in the blood. If tritium-labeled thymidine is administered during the proliferative phase, all plasma cells will be labeled by 8 days after administration of antigen, indicating their origin from proliferating cells (Urso and Makinodan, 1963; Urso and Gengozian, 1964). However, if labeled thymidine is administered prior to antigen, the plasma cells that develop will not be labeled, indicating that the cells originally responsive to antigen were not proliferating before exposure to antigen (Cohen and Talmage, 1965). Therefore, the antigen-sensitive cell probably is not a large lymphoid cell.

What, then, is the antigen-sensitive cell—the precursor of the large lymphoid cells that proliferate in response to antigen? There is considerable evidence that small lymphocytes can initiate immune responses, both primary and secondary (Gowans *et al.,* 1962; Gowans and McGregor, 1965; Ford *et al.,* 1966). Thoracic duct lymph, freed of large cells by incubation *in vitro* for 24 hours will restore immunological responsiveness to lethally irradiated animals. At least in the case of primary responses, small lymphocytes seem to play a necessary role. Chronic drainage of thoracic duct lymph depletes the blood and lymphoid tissues of small lymphocytes and impairs primary responsiveness; this deficit is restored by injection of small lymphocytes (McGregor and Gowans, 1963). The specific sensitivity of small lymphocytes to antigen is indicated by the fact that cells from tolerant animals restore competence generally, but do not restore responsiveness to the tolerated antigen. Although small lymphocytes synthesize no DNA and little RNA or protein (Mitchell, 1964a,b; Clark, 1966b), they seem capable of becoming

large lymphocytes. Twenty-four hours after their injection into irradiated animals, large lymphoid cells of donor origin are found proliferating in the host's lymphoid tissue (Ford *et al.*, 1966).

What are the sources of immunologically competent small lymphocytes? If thoracic duct cells are cultured in the closed system of a diffusion chamber and labeled with radioactive thymidine, labeled small lymphocytes eventually appear, apparently derived from proliferating large and medium-sized lymphocytes (Schooley and Berman, 1960). After injection of unlabeled small lymphocytes into an irradiated animal, followed by administration of radioactive thymidine to the host, the small lymphocytes that invade the bed of a homograft during its rejection are labeled, indicating their origin *de novo* from proliferating precursors (Gowans *et al.*, 1962). If labeled large lymphoid cells, taken from an animal at the height of an immune response, are injected into an irradiated animal, labeled small lymphocytes soon appear in the thoracic duct (Ford *et al.*, 1966). Perhaps, then, the small lymphocytes capable of initiating a secondary response arise from the large lymphoid cells that proliferate during an immune response. Makinodan and Peterson (1966b) have reported that the increase in "secondary antibody-forming potential" that occurs during a primary response is due to an increase in the number of potentially responsive cells, but these have not been identified as small lymphocytes. In any case, one important source of small lymphocytes must be the large lymphoid cells of peripheral lymphoid tissue (spleen, lymph nodes, etc.). Another potential source of immunologically competent small lymphocytes is the thymus. Lymphocytes derived from thymic grafts can emigrate to other lymphoid organs and there proliferate in response to antigen (Ford, 1966; Davies *et al.*, 1966), but there is doubt as to whether these proliferating cells participate in synthesis of antibody, and thymic lymphocytes are notoriously poor at immune responses. Whatever the sources of immunologically competent small lymphocytes, they form a pervasive, recirculating population of cells well-suited to encountering and responding to antigens wherever they may turn up in the body.

The basic question of cellular differentiation still remains that of how antigen is recognized and induces the initiation of an immune response. The most obvious form of recognition received by antigens is phagocytosis. Macrophages lining the sinuses of lymph nodes and spleen ingest antigenic and nonantigenic particles without discrimination, but antigens congregate specifically in the phagocytic reticular cells of lymphoid follicles (Ada *et al.*, 1964; Mitchell and Abbot, 1965). This localization has been attributed to the action of opsonins—perhaps natural antibodies—rather than to specific recognition by macrophages. Macrophages have not been found to proliferate in response to antigen (Chapman *et al.*,

1965), but they do form close associations with plasma cells (Thiery, 1962) and transforming lymphocytes (McFarland and Heilman, 1965). After incubation with antigen, macrophages can stimulate lymphoid cells to a primary immune response *in vitro* (Fishman, 1961; Fishman and Adler, 1963). The effectiveness of macrophages does not depend upon previous immunization, but it does require physical contact between macrophages and competent lymphoid cells, and it can be inhibited by exposing antigen-treated macrophages to antibody (Harris, 1965, 1966). Therefore, the role of macrophages may be nonspecific and relatively inactive. Harris suggests that they form a surface to which antigen may attach and become peculiarly accessible to responsive lymphoid cells.

In attempting to analyze the action of antigen further, one must take account of the great diversity of immunological responses. Depending upon the physical state, quantity and route of administration of the antigen and the previous history of the recipient, the result may be the production of antibody or immunological unresponsiveness. Antibodies may be humoral or cellular, 19S, γM or 6.5S, γG immunoglobulins. The response may be primary or secondary, and it may or may not result in immunological memory. Cellular products of proliferating blast cells may be plasma cells or lymphocytes. Such dichotomous choices of behavior suggest genetic alternatives. Cellular differentiation can express only what is in the genetic repertoire of the differentiating cell. Is diversity of response due to genetic differences between lymphoid cells or to selection of genetic alternatives by the environment?

Studies of the sequence of amino acids in immunoglobulins reveal heterogeneity at a few specific sites. It has been concluded that antibody specificity depends upon such variations in amino acid sequence, and that these are genetically determined (Nisonoff and Thorbecke, 1964). Presumably the genetic diversity responsible for these variations arose by mutation, and the question is whether it arose during evolution or develops anew during the life of each individual. In other words, does each lymphoid cell contain the many thousands of allelic genes necessary to respond to any antigen, or does each cell, by somatic mutation, come to contain no more than two alleles—diversity residing among the entire population of lymphoid cells rather than in individual cells? The preservation of genetic diversity through all the generations of germ cells in the higher vertebrate line seems more awkward to account for than somatic mutation. Brenner and Milstein (1966) have proposed a mechanism by which somatic mutation might affect only the variable parts of the immunoglobulin genes, and the rapidly dividing population of lymphocytes in the embryonic thymus would furnish considerable opportunity for the segregation of mutants. The affinity with which antibody binds antigen

is quite variable, even during a single immune response (Eisen and Siskind, 1964); this can be taken as evidence of genetic diversity among the responding cells.

Whether or not lymphoid cells are genetically diverse, they behave as if they were. The number of cells responsive to a primary injection of an antigen is small—approximately one out of a million cells in the spleen (Kennedy *et al.*, 1966). An exception to this rule is the transformation of small lymphocytes into blast cells, produced by incubating together cells from genetically different animals; 1–4% of the small lymphocytes respond. However, it has not yet been established that this response is truly immunological (Johnson and Roberts, 1964; Chapman and Dutton, 1965; Dutton, 1965). Few plasma cells have been found to make antibody of more than one specificity, and none more than two—the theoretical limit for diploid cells (Nossal and Mäkelä, 1961). Colonies of cells making a specific antibody can be located in the spleen by incubating sections of tissue on plates for demonstrating hemolysins; their distribution is not random, and colonies making antibodies to two different antigens do not coincide (Nakano and Braun, 1966). These colonies of cells have been interpreted as clones, each stemming from a single antigen-sensitive cell, and each making only one kind of antibody. If specificity for a particular antigen resides in only one or two allelic genes for immunoglobulin, as would be expected as a result of somatic mutation, then each antibody-forming cell should produce globulins restricted in allotype to one possibility for each locus. In general this has been observed to be the case (Bernier and Cebra, 1964; Weiler, 1965; Curtain and Baumgarten, 1966), but these results do not exclude explanations other than somatic mutation. Thus lymphoid cells responding to antigens behave as clones; whether they are genetically distinct clones remains uncertain.

If immune responses are clonal, are the several responses to a single injection of antigen the work of one or several clones? Approximately 1000 cells in the spleen can respond to a primary injection of antigen (Kennedy *et al.*, 1966); presumably they produce 1000 clones of responding cells. The original antigen-sensitive cells might all have originated from a single, genetically determined precursor, but antibodies of several affinities are produced in a single immune response (Eisen and Siskind, 1964), which can be taken as evidence that several clones take part in the response and that they are genetically diverse. Can the small lymphocytes and plasma cells produced in an immune response stem from a single clone? Apparently small lymphocytes can be the parents of clones that produce plasma cells (Ford *et al.*, 1966), but can such a clone also produce new small lymphocytes? Immunological memory, delayed hypersensitivity, and immunity to grafts have all been attributed to small

lymphocytes, and each of these may develop during an immune response without relation to whether or not antibody is produced by plasma cells (Nossal *et al.*, 1965b). This might indicate that several clones can respond independently to antigen, but it could also be attributed to a single clone responding to different environments within the body. Some cells produce γM antibodies while others produce γG antibodies; there is disagreement as to whether both may be produced simultaneously by a single cell (Nossal *et al.*, 1965a; Moore *et al.*, 1965). γM antibody is characteristic of the early phase of the immune response and is found within relatively immature cells in the splenic sinuses, whereas γG antibody is found later in the maturing plasma cells of the white pulp (Moore *et al.*, 1965). However, immune responses can occur in which only one type of antibody is produced (Nossal *et al.*, 1965b). γM antibodies have been identified in mature as well as immature plasma cells, by electron microscopic examination of plaque-forming cells, but there is disagreement as to whether lymphocytes without visible endoplasmic reticulum also produce macroglobulins (Bussard and Binet, 1965; Fitch *et al.*, 1965; Harris *et al.*, 1965). The heavy-chain portions of γM and γG molecules are different and presumably represent different genetic loci (Nisonoff and Thorbecke, 1964). If, as is suspected, coding for antibody specificity occurs at both loci, then somatic mutation might result in the capacity of a cell to make γM and γG antibodies of different specificities. This would make it difficult for cells producing γM antibodies and cells producing γG antibodies in the same immune response to belong to the same clone, or for a cell to shift from secreting γM antibodies of one specificity to secreting γG antibodies of the same specificity, as Nossal *et al.* (1965b) have suggested may occur. The use of marker chromosomes ought to be useful in settling some of these questions of clonal relationships.

However, understanding the mechanism by which antigen initiates an immune response, produces immunological memory, or induces tolerance will require greater depth of analysis. Committed cells will need to be defined either in terms of multipotent lymphoid cells conditionally committed to a particular antigen, or genetically diverse cells each committed to one or two antigens *a priori* (Burnet, 1958). If lymphoid cells are multipotent, there should be uncommitted cells available to respond to newly encountered antigens. In an environment permeated by antigens, uncommittedness might be difficult to maintain. Perhaps short-lived lymphocytes are uncommitted. They reportedly arise in the thymus (Everett *et al.*, 1964), where antigens penetrate poorly (Clark, 1964). Perhaps the thymus serves as a haven where cells may remain isolated from antigens and thus uncommitted. The putative lymphocytopoietic hormone

of the thymus (Clark, 1966a) might serve to provide a constant supply of uncommitted lymphocytes. On the other hand, if lymphoid cells achieve genetic diversity and precommitment by somatic mutation, the lymphocytopoietic hormone of the thymus might provoke the rapid proliferation necessary to segregate mutants. The ability of animals to maintain immunological competence after removal of the thymus in adult life speaks in favor of the latter alternative. Immunological memory has been attributed to long-lived small lymphocytes (Ford *et al.*, 1966). If lymphocytes are multipotent, maintenance of memory would require some persistent effect from the original stimulus, such as persistence of antigen within the cell. There is much argument concerning the persistence of antigen, but Nossal *et al.* (1965a) reported detecting no antigen in antibody-forming cells, using radioactive antigen of very high specific activity. If the same were true for long-lived lymphocytes, it would favor genetic diversity rather than the persistence of antigen as the basis of immunological memory. It has been reported that small lymphocytes proliferating in response to phytohemagglutinin or secondary contact with antigen demonstrate ephemeral chromosomal abnormalities produced by irradiation given several months previously (Nowell, 1965; Weber and Nowell, 1965). This has been interpreted to indicate that these cells had not divided since the irradiation was given; if true, this technique offers exciting possibilities for detecting long-lived lymphocytes with specific memories. Tolerance is difficult to explain if lymphoid cells are multipotent, because the constant supply of uncommitted cells which would be needed to respond to new antigens should make escape from tolerance easy. But the mechanism by which antigen inhibits the production of antibody is as poorly understood as is the mechanism by which it stimulates antibody production (Mitchison, 1964).

Another characteristic of immune responses that must be explained in order to understand the control of cellular differentiation is the limited nature of these responses. Hardly has cellular proliferation begun in response to the primary injection of an antigen, when it is over—no more than four to seven generations intervening between the original antigen-sensitive cell and mature plasma cells no longer capable of mitosis (Kennedy *et al.*, 1966). Synthesis of antibody continues only as long as plasma cells persist, and these are mostly short-lived (Miller, 1964). If examined for a longer period of time, the primary response may be found to recover after an initial diminution and wax and wane in diminishing waves for most of the life of the animal (Makinodan and Peterson, 1966a). Such behavior suggests some kind of feedback inhibition, and antibody might be the inhibitory agent, but evidence for this is slight (Nossal *et al.*, 1965b).

As our analysis of the problem of cellular differentiation has become increasingly involved in complexity, it has become obvious that present knowledge is not up to making order out of this chaos. However, it can be predicted that future understanding will depend upon continuing to deal with the complexities of the problem. In particular, the potentialities and responses of individual cells will have to be viewed within the complex environment of the whole animal. There are many different ecological niches within the body, but not all seem suited to housing an immune response. When immunologically competent cells are injected intravenously, only 15% take up residence in the spleen, yet that is the only site where they have been found to produce colonies of antibody-forming cells (Kennedy *et al.*, 1966). Within lymphoid organs, antibody-forming cells congregate in specific localities, such as the medullary cords of lymph nodes. In the spleen, cells that produce γM antibodies are found in the walls of sinuses, whereas γG antibody is found in cells in the white pulp (Moore *et al.*, 1965). The ability to produce memory cells capable of initiating a secondary response is reported to be lacking in lower vertebrates in which one specific type of ecological niche is missing—the germinal center (Nossal, 1965).

If clonal selection is a factor in immune responsiveness, then there must be competition among genetically diverse clones for a limited supply of suitable ecological niches. The supply of space in germinal centers does not seem rigidly limited—germinal centers expand during an immune response, as the numbers of cells within them increase (Hanna, 1965); perhaps the limits of space must be sought in the lymphoid system as a whole. As seen in electron micrographs, lymphoid organs contain a wide variety of environments, but they all have one feature in common: the lymphoid cells lie in enclosed spaces, separated from the bloodstream and connective tissue spaces by some cellular barrier (Clark, 1962, 1963a,b, 1964). Competition between immune responses can be demonstrated, and Gorman and Chandler (1964) have explored some of the theoretical implications of such competition.

Perhaps, in the final analysis, the availability of antigen will be seen as the key to the diversity of immunological responses, as Mitchison (1964) has suggested in the case of tolerance. The complex environments of lymphoid tissues may serve chiefly to govern the quantity and character of antigen available, which may, in turn, determine whether an antigen-sensitive cell will proliferate, differentiate into an antibody-producing cell, or become tolerant.

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DISCUSSION

DR. SIGEL: With regard to the possibility that a correlation exists between germinal centers and secondary response, I should like to state that this correlation does not necessarily occur in lower vertebrates such as marine teleosts. Studies in our laboratory have disclosed vigorous secondary responses in at least some of the marine fishes immunized with bovine serum albumin. Yet, so far we have failed to demonstrate anything akin to germinal centers in the spleen of these animals. Moreover, they possess no organized lymph nodes. Preliminary findings indicate that antigenic stimulation provokes an increased DNA synthesis and pyroninophilia in the kidney where germinal centers have not yet been seen. Experiments based on immunofluorescence performed by Mrs. Sheila Helman in our laboratory suggest that the fish thymus plays an important role in antibody synthesis.

DR. THORBECKE: This point was discussed rather extensively at the meeting on germinal centers held in Bern last June. In my recent work I have advanced the theory that cells carrying immunological memory are formed in germinal centers. It became clear at this meeting, and also from my own results, that this is not the only site of formation for cells with immunological memory. In studies with various lower vertebrates, Dr. Good has also found that for synthesis of antibodies in the IgM class a memory can be established in the complete absence of germinal centers. Moreover, definite signs of the presence of a memory for the 19 S antibody response can already be detected very early in the immune response, when germinal centers have not yet formed. In the case of the homograft rejection, Dr. Micklem could suppress germinal center proliferation with radiation given after the first

homograft, and still obtain establishment of a memory for the second set rejection. It seems that the more impressive and long-lasting type of immunological memory, the one for 7 S antibody response, may be the one that is most likely linked to the proliferation of cells in germinal centers.

DR. REIF: We have found that there are three distinct types of small lymphocytes that can be distinguished by immune cytolysis on the basis of cell surface antigens; these three types are thymic lymphocytes, splenic or lymph node lymphocytes, and marrow lymphocytes [Reif, A. E., and Allen, J. M. (1964) *J. Exptl. Med.* **120,** 413]. For each antigen injected, only roughly 3000–6000 out of 100 million cells in the spleen seem to be precursors of antibody-forming cells. Until we can recognize the different types of lymphocyte under the microscope, the electron microscope, or in some other way, we cannot be sure from which type the antibody-producing cells are derived.

DR. CLARK: Certainly there are morphological differences among lymphocytes but these have not yet been described adequately. It has been suggested that thymic lymphocytes contain fewer polyribosomes than peripheral lymphocytes but I have not been able to convince myself of this at the electron microscope.

Concerning the Recognition Reaction and Transfer RNA in Protein Synthesis

PAUL C. ZAMECNIK

The John Collins Warren Laboratories of the Huntington Memorial Hospital of Harvard University at the Massachusetts General Hospital, Boston, Massachusetts

Despite the rapid pace of progress in our understanding of the mechanism of protein synthesis, important deficiencies in our knowledge are still evident. Let me call certain of these to your attention and at the same time weave into the presentation a brief description of work currently in progress in our laboratory.

In the sequence of reactions in which an amino acid participates on its way to becoming part of a completed protein molecule, the most obscure is the nature of the mechanism by which a particular amino acid becomes attached to its own transfer ribonucleic acid (RNA) molecule. This tRNA then transports the esterified amino acid to the correct site for the coding operation with messenger RNA on the ribosomal complex. In the interaction between aminoacyl synthetase and tRNA, it appears requisite that certain nucleotide bases of the tRNA "recognize" one or more side chains of amino acids associated with the aminoacyl synthetase. The fidelity of transcription of the genetic message of DNA depends as much on the accuracy of the interplay between these two different types of macromolecules as it does upon the precision of the base pairing operation between messenger RNA and tRNA during the coding step of protein synthesis.

Let us consider two alternate types of reaction site which may be present on the aminoacyl ligase. To begin with, perhaps the side chain of the amino acid being activated may interact with an area on the tRNA. This possibility appears to have the virtue of simplicity, i.e., the amino acid itself may assure the correct transfer of its identity to the tRNA. It

presents difficulties, however. The "R" group (or side chain) of the amino acid is presumably situated in a closely fitting cavity on the aminoacyl ligase, at the moment when its carboxyl group is interacting with ATP to form the enzyme-bound aminoacyl-AMP. We must now picture this

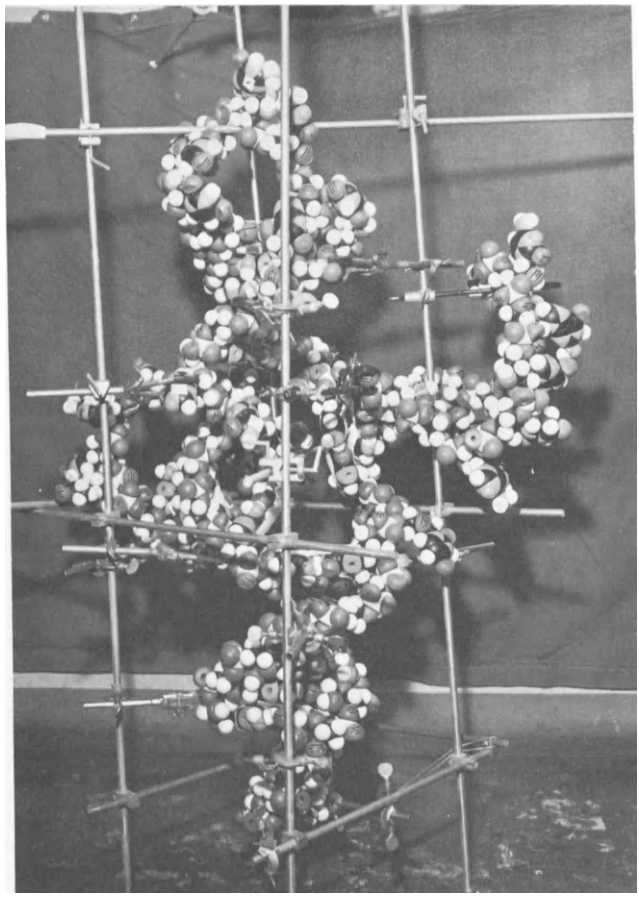

FIG. 1. Front view of a three-dimensional model of $tRNA^{ala}$ (Holley *et al.*, 1965).

aminoacyl side chain as dissociating from its active site on the enzyme and rotating outward from the enzyme surface to serve as a probe for association with the correct tRNA. At this same instant, the AMP portion of the aminoacyl-AMP must remain anchored *in situ* on the enzyme. There is no evidence directly in favor of this aminoacyl probe possibility, and yet no experimental data rule it out. It is difficult to consider that there is enough specificity in the surface of the tRNA to distinguish the

side chain of one aliphatic amino acid from another with the requisite accuracy. A more comfortable possibility is that a group of amino acid side chains in the peptide structure of the aminoacyl ligase constitute a recognition site, which interacts with the tRNA.

We have been involved for some time in efforts to clarify the nature of the recognition interaction. With our colleague Dr. John Haines* we have reacted a mixture of aminoacyl ligases, obtained from a crude protein fraction from *Escherichia coli,* with active site reagents in current use in the study of other enzymic reactions. Following preliminary experiments using cyanate, Woodward's reagent K, and a variety of other reagents, we have evolved assay conditions using the reagent fluorodinitrobenzene, in which arginyl synthetase is inhibited approximately 85% under conditions in which lysyl synthetase is inhibited only of the order of 12%. The use of ^{14}C-labeled fluorodinitrobenzene with semipurified arginyl and lysyl synthetases suggests that arginyl synthetase reacts approximately to the same extent with fluorodinitrobenzene as does the lysyl synthetase. These experiments illustrate a future promising direction for studies of the recognition reaction—namely, a comparison of the effect of protein-active site reagents on purified aminoacyl synthetases. This is a more interesting problem than trying to define the active site of a single enzyme, since the elucidation of an entire transcription code is at stake.

In reference to the aminoacyl synthetases, this family of enzymes is presumably alike in general characteristics, but in a single species must differ one from the other in at least two important ways. Each one of these enzymes is quite specific in its interaction with a single amino acid. This action of the enzyme is usually possible in the absence of added tRNA. Each enzyme is also of course specific in its interaction with a particular tRNA molecule. Several laboratories, including our own, (Marshall *et al.*†) (Makman and Cantoni, 1965; Calendar and Berg, 1966) have obtained molecular weights of purified *E. coli* aminoacyl synthetases of the order of 45,000–95,000. In line with current reasoning, it may therefore be suggested that aminoacyl synthetases of this size may possibly be composed of subunits. If indeed there should be subunits of a single aminoacyl synthetase molecule, the problem would be posed as to whether the amino acid-ATP interaction site occurs on the same subunit as the tRNA recognition site. This would be a point worth establishing, one way or the other. It may be argued that it would be more logical to expect the presence of both the activation site and the recognition site on the same hypothetical subunit. One would otherwise be faced with the

* Haines, J., and Zamecnik, P. C. (1966). Unpublished data.

† Marshall, R. D., Shuster, L., and Zamecnik, P. C. (1966). Unpublished data.

complex situation that a new and precise type of association process between protein subunits would be necessary if the correct amino acid

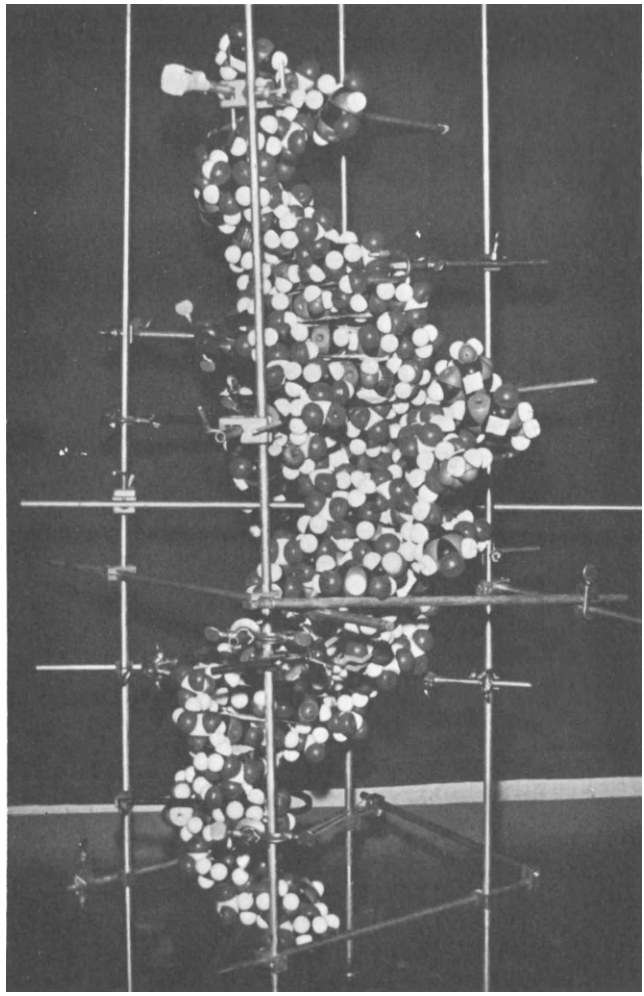

FIG. 2. Lateral view of a three-dimensional model of $tRNA^{ala}$ (Holley *et al.*, 1965).

activation site were to become unerringly part of the same enzyme molecule as the recognition site which should properly go with it.

The recognition reaction may also be examined from the point of view of the tRNA molecule. The elucidation of the complete primary structure of several tRNA molecules has prepared the groundwork for a search for the location of its recognition site. The "clover leaf" type of model

suggested by Holley *et al.* (1965) for RNA^{ala} serves as a good intellectual prop for this type of investigation. The structures of RNA_I^{ser} and RNA_{II}^{ser} (Zachau *et al.*, 1966) and of RNA^{tyr} (Madison *et al.*, 1966) may be arranged similarly, suggesting that the "clover leaf" may bear a resemblence to the correct arrangement of double-stranded regions and single-stranded loop areas common to the tRNA family. In this clover leaf configuration, a T ψ CG and an AGH_2U are features of corresponding loop areas in all four structures, and a Me_2G is located in an identical position, at the transition point between two limbs. My colleagues Drs. John Hershey and Ian Weatherall have constructed a model of yeast RNA^{ala} using Pauling-Corey-Kolter plastic subunits, and front and side views of one form of this model are shown in Figs. 1 and 2. The general compactness of the center of this model is evident. It would appear possible that the hypothetical loop areas might either extend out

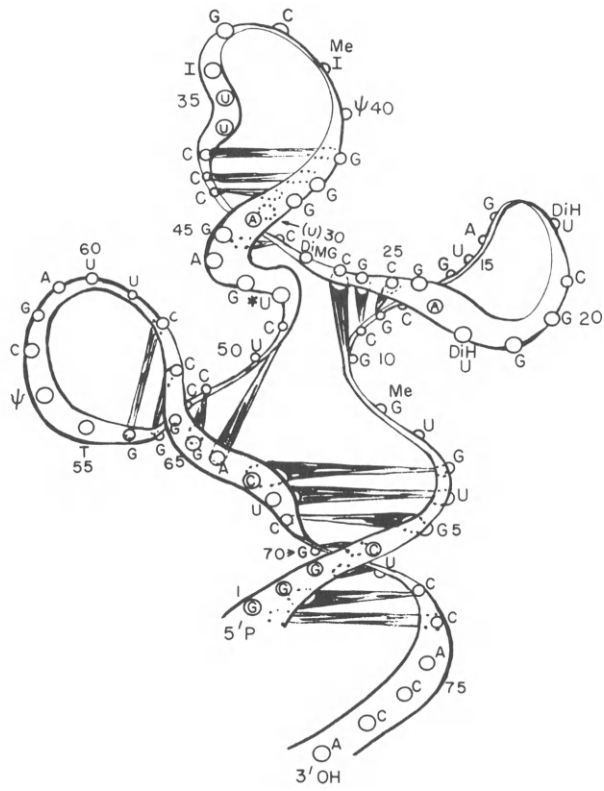

FIG. 3. Schematic conception of Fig. 1, showing in particular the helical rotation involved in the double-stranded areas.

from the central mass of the structure, even permissively approaching the —CCA terminus, or else might rest against the central mass like folded arms, depending on the functional state of the molecule. The lateral dimension of the model (Fig. 2) is more slender than the front view shown in Fig. 1, and would permit close apposition of two tRNA's on adjacent ribosomal binding sites. A schematic representation of the model, in Fig. 3, for which I am indebted to Dr. Weatherall, calls attention to the helical rotation of the double-stranded areas, and to the possibility of sensitivity to alteration of the three-dimensional structure as a consequence of changing the hydrogen bonding or stacking propensities of a single nucleotide base. In this connection, a change in the optical rotatory dispersion (ORD) pattern of amino acid-charged and uncharged tRNA has been observed (Sarin and Zamecnik, 1965). Aqueous bromination of a few nucleotide bases has also been found to evoke a change in the ORD curves (Lamborg *et al.*, 1965) and to impair the acceptor activity of tRNA (Yu and Zamecnik, 1964).

The relationship of the recognition site on tRNA to the anticodon site is still a matter of uncertainty. Earlier evidence on this question, including some from our own laboratory (Yu and Zamecnik, 1964; Weil *et al.*, 1964) suggested that these two sites were distinct from one another. Later evidence has pointed toward the participation of the anticodon nucleotides in the recognition process (Burton *et al.*, 1966; Hayashi and Miura, 1966). At the present moment a reasonable working assumption would be to consider that the anticodon site takes part in the recognition interaction with an aminoacyl synthetase, but does not comprise the entire recognition site. In defense of this assumption, it is well established that the coding reaction in protein synthesis is a rather universal mechanism, and is able to cross the species barrier quite readily in a number of instances. The same is not true for the recognition process, and sharp species specificities have been found in numerous instances. These observations would argue for the presence of distinctive features in the tRNA recognition area which are not present in the anticodon site.

It may be worth while, however, to define more carefully the meaning of the term recognition site, in trying to examine these conflicting lines of evidence. If recognition is achieved by a precise interaction of a few nucleotide bases (for example, three adjacent bases) of the tRNA with a few amino acid residues in appropriate conformation in the aminoacyl synthetase, then a long stem of phosphodiester-linked nucleotides will extend from this site to the —CCA terminus, to which the activated amino acid may become esterified. Alteration of secondary structure of this stem region, induced by chemical modification of the primary structure of the tRNA, may influence the rate and extent of esterification of the

amino acid, without necessarily modifying the recognition site itself. Any study of the recognition site which is based on measurement of esterification of an amino acid to tRNA therefore measures the effect of chemical modification of the stem region (or esterification handle) as well as modification of the recognition site itself. The technique of Hayashi and Miura (1966), Deutscher (1965), and of Letendre *et al.* (1966), in which inhibition of charging of tRNA by oligonucleotides is studied, also involves the possibility of associating with and masking the tertiary structure of both the aminoacyl synthetase and the tRNA in regions other than those of the recognition site. At the moment, therefore, the question of whether the recognition and anticodon sites of tRNA are identical, overlapping, or separate, has not been conclusively answered.

We have for some time been interested in the technique of ORD as a means for investigating the possible individuality of secondary structure of tRNA molecules (Lamborg *et al.*, 1965), and for studying the recognition interaction. It has been gratifying to find that the ORD patterns of purified samples of tRNA's from yeast do indeed differ from each other significantly (Sarin *et al.*, 1966). The differences have been in details of the dispersion patterns in the near and far (Lamborg *et al.*, 1965) ultraviolet regions at a given temperature, and also in the meltout behaviors of the purified samples when the temperature is raised from 20–85°C. These observations are complementary to those which show primary structural differences between, for example, yeast RNA^{ala} and RNA^{ser} (Holly *et al.*, 1965; Zachau *et al.*, 1966). A long-range goal of ours has been to interact purified aminoacyl ligases with their respective purified tRNA's in the spectropolarimeter and to look for evidence of change of secondary or tertiary structure of either the tRNA or the enzyme as a result of their interaction. We have not, however, up to the present time been in a position to carry out such experiments in detail.

As a beginning in this direction, we have observed the ORD pattern of a purified *E. coli* lysyl synthetase and of the same enzyme in the presence of ATP and L-lysine. This latter is of course the situation which occurs when the amino acid is activated. A new Cotton effect was introduced into the ORD pattern of the mixture of lysyl synthetase, lysine, and ATP when these three constituents were incubated together for a short time at 37°C. A search for the chemical explanation of this Cotton effect led to the identification of P^1P^4-di(adenosine-5′)tetraphosphate (AP_4A) and of P^1P^3-di(adenosine-5′)triphosphate as by-products of the amino acid activation reaction (Zamecnik *et al.*, 1966; Randerath *et al.*, 1966). In amplification of this finding, we have observed the formation of an entire family of hybrid dinucleotides of this type which may be formed during the *in vitro* lysine activation reaction (Randerath *et al.*, 1966). Their

formation in this system, therefore, poses the question as to whether they may be compounds which occur within intact cells or simply *in vitro* synthetic artifacts.

During the past few months we have devoted considerable effort to the search for AP_4A and AP_3A in a variety of living cells. Thus far, after a painstaking quest, we have found AP_3A in two separate commercial preparations of crude mixed nucleotides obtained originally from yeast. We have also recently found AP_4A in extracts of rat liver prepared in our own laboratory.*

It is interesting to note that the ORD patterns of AP_4A and of AP_3A differ significantly from the patterns obtained from ApA and ApApA.† Curiously enough, the ORD pattern of P^1P^2-di(adenosine-5′)diphosphate (AP_2A) is entirely unlike that of AP_4A and AP_3A, and resembles that of ApA and ApApA. The inference is that the secondary structures of AP_4A and AP_3A are very different from those of the naturally occurring di- and trinucleotides which have the same base composition. This observation thus poses an interesting conformational problem.

The work of Jacob and Monod (1961) has called attention to the regulation of readout of the genetic message at the level of replication and transcription. It is worth mentioning another point in the pathway from free amino acid to completed protein at which subtle regulation and modification of the initial genetic message may be imposed—namely, at the translation level (cf. also Gorini and Beckwith, 1966). The explanation for the presence of the approximately thirty minor base constituents which one may enumerate as occurring in the family of tRNA molecules is still uncertain. It is clear, however, that methylation or other alteration of amino groups of nucleotide bases which might otherwise participate in hydrogen bonding may alter the secondary structure of a tRNA. Likewise, saturation of the uracil ring in dihydrouracil would have the effect of impairing the π-π bond and hydrophobic stacking which play such an important role in maintenance of the secondary structure of tRNA. Other base modifications may also impair base stacking via steric hindrance. In this way, subtle modifications of tRNA made subsequent to genetic transcription of its primary structure, and during its polynucleotide life, may influence the speed with which a tRNA molecule is able to carry out its function of accepting and transferring amino acid. These minor base modifications may likewise influence the accuracy of performance (i.e., the error level as well as the rate) of either of these dual functions of tRNA and thus modify the final product formed, that is, the protein molecule synthesized.

* Stephenson, M., and Zamecnik, P. C. (1966). Unpublished data.

† Zamecnik, P. C., and Scott, J. F. (1966). Unpublished data.

Let us also take note of the paucity of information available on the mechanism by which subunits of a protein molecule come together to form the biologically active macromolecule. It is possible that such multichain protein molecules are formed on an endoplasmic grid which permits the elongating peptide chains to associate while the incomplete ends are still attached to tRNA molecules on the ribosomes. This step may in particular have relevance to the regulation of antibody formation.

In summary, it may be stated that while major steps in the process of protein synthesis are now understood, the nature of the recognition reaction is obscure; and regulation of the rate of synthesis, the mechanism of initiation and termination of chains, and the mechanics of association of subunits are still matters of current intensive investigation, the outcome of which will be of special interest to immunology.

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DISCUSSION

DR. COUTSOGEORGOPOULOS: I remember that an analogous compound to your AP_4A with 4 phosphates in the middle, which had guanosine instead of adenosine, has been identified in brine shrimp eggs. Such compounds occur then in nature.

DR. ZAMECNIK: That is correct. I should have mentioned that F. J. Finamore and A. H. Warner [(1963) *J. Biol. Chem.* **238,** 344] found GP_4G in brine shrimp eggs 3 years ago and that the same compound has been found recently in *Artemia.* In shrimp eggs, GP_4G is found in large amounts, approximately 1%. Its function is obscure. I do not see any way in which we can get GP_4G from our system at present because the activating enzyme is strictly dependent on having an adenosine in one side chain. I think that GP_4G is another example of a good energy storage compound.

DR. COUTSOGEORGOPOULOS: After the aminoacyl-AMP is formed on the enzyme, can you attack it with nucleoside mono- or di-phosphates in order to make symmetrical compounds with less phosphates in between?

DR. ZAMECNIK: Yes, if one attacks with ADP, one gets AP_3A. So far we have not found AP_2A after treatment with AMP. However, we have not yet tried the reaction using radioactive techniques. J. R. Reiss and J. G. Moffatt [(1965) *J. Org. Chem.* **30,** 3381] found AP_4A, AP_3A, and AP_2A in a chemical synthesis, in which anhydrous pyridine was the solvent. In our enzymic system, it would appear as though pyrophosphate is needed as part of the nucleophilic attacking compound. I should say that we have also wondered whether this sort of mechanism would operate in the case of the luciferin reaction, and Dr. William McElroy has kindly tested this possibility. He tells me that AP_4A is unable to substitute for ATP in the light-forming reaction which involves luciferase and luciferin, at least when AP_4A is used at the 10^{-6} *M* concentration with which we were able to furnish him.

DR. COUTSOGEORGOPOULOS: We have started experiments to see whether symmetrical nucleoside polyphosphates, with at least two phosphates in the middle, such as AP_2A or P^1,P^2dithymidine 5′-pyrophosphate (TP_2T), may serve as precursors for nucleotide incorporation into nucleic acids. In a crude *E. coli* system we have pretty good evidence that we could incorporate thymidine^{14}C into DNA from TP_2T-^{14}C. We have fairly well excluded the possibility that a pyrophosphatase is producing TMP and then a kinase builds up TTP which is then incorporated. AMP could be incorporated by RNA polymerase from AP_2A. We also tested AP_2A in a classical sRNA system and found that AMP was incorporated, presumably at terminal positions.

DR. ZAMECNIK: We have not tried that, but we have tried to link up AP_4dT with Kornberg's deoxyribonucleotide polymerase (in unpublished work done in collaboration with Dr. Morton Swartz), hoping that the labeled TTP formed by the interaction of AP_4dT with lysine and lysyl synthetase would then be used in the presence of three other deoxynucleoside triphosphates and one could have a chain formed in which this thymidine triphosphate would be included. I do not understand the results thus far obtained, but the whole system is inhibited by this compound.

DR. COUTSOGEORGOPOULOS: Once the amino acid has been charged on the sRNA and we are dealing with precharged s-RNA, ribosomes and messenger RNA, how well is it established that only GTP, and not ATP, is needed to make peptide?

DR. ZAMECNIK: I think that this is quite firmly established now from work with the purified system which Lipmann [Nishizuka, Y., and Lipmann, F. (1966) *Proc. Natl. Acad. Sci. U.S.* **55,** 212] has used.

DR. COUTSOGEORGOPOULOS: In other words, you think that this is proved by the fact that not only can one use GTP in the system but also show that GTP acts better than ATP. Also, when one uses more GTP, one gets a better incorporation, but still one does not know whether a transphosphorylase creates ATP from GTP.

DR. ZAMECNIK: We have not actually worked in that area recently. Although the possibility you raise cannot be completely ruled out, it is likely that ATP is not involved. The Lipmann [Nishizuka, Y., and Lipmann, F. (1966) *Proc. Natl. Acad. Sci. U.S.* **55,** 212] and Moldave [Gasior, E., and Moldave, K. (1965) *J. Biol. Chem.* **240,** 3346] systems are well dissected out.

DR. PREHN: You mentioned at several points in your talk various items you thought might have implication for immunology and the connection to me was not entirely obvious. I wonder if you would elaborate on that.

DR. ZAMECNIK: The primary structure of a particular tRNA molecule, for instance RNA^{lys}, is determined by the sequence of DNA bases. The secondary structure of that molecule is also largely, but not completely determined by the primary structure. The evidence is excellent that methylation occurs at the polynucleotide level, when the primary structure of the tRNA molecule is already formed. Presumably also the isopentenyl residue can be attached at this stage. It would appear that the thiol group is incorporated, at least in part, after the chain is formed. There is also evidence that one of the early events occurring after phage infection is an alteration of a tRNA which appears to involve methylation. For each of these alterations of the polynucleotide chain a separate enzyme is necessary which is not present at the initial steps in which DNA plays a role. A subtle control of speed of protein synthesis may be imposed at this point by defining either a better or a worse fit of tRNA onto its activating enzyme, or by possible alteration of the ribosomal association site.

DR. SCHWARTZ: We have two problems in immunology which have a direct bearing on these problems of protein synthesis. One you have already alluded to, that is, "What starts the process in the first place?" But then we have another problem, namely, "What stops it?" We know that antibody formation does not persist indefinitely. Is there any evidence to support the hypothesis that the final product, the finished protein, can modify protein synthesis by a feedback mechanism?

DR. ZAMECNIK: That is a very pertinent question. I do not know of any data on that.

DR. GORINI: A strain of *Sarcina* studied in our laboratory produces a proteinase which is excreted into the medium and this excretion is controlled by different factors. The kinetics of enzyme formation is dual, e.g., one unit per cell and per time unit when it is not excreted, and thousands of units per cell and per time unit when it is excreted. There must be, therefore, an intracellular control that prevents the cell from making thousands of units when the enzyme is not excreted. Since the proteinase is produced as inactive zymogen and the control exists even when the transformation of zymogen is prevented, one has to postulate a control independent from enzymic action. Presumably the zymogen itself controls its own synthesis.

DR. HITCHINGS: It seems to me that there is another possibility related to Dr. Schwartz's question, namely, that a synthesis of the sort that takes place in antibody formation is so intense that somehow it accelerates cell death.

DR. ZAMECNIK: I could not add anything to that, but just to comment on another aspect of Dr. Schwartz's question. It is pretty well established that the primary sequence of amino acids in a single polypeptide chain determines the secondary sequence and the configuration of the molecule to a large extent, but, I would say, not completely. Particularly in proteins which are made up of subunits, one has to face the problem of how the subunits get together after they are formed on the ribosomes, and there are virtually no data on that. It is possible that the completed product, consisting of several subunit chains bound together, might, in sufficient concentration, inhibit the separation of new peptide chains from the ribosome.

DR. PAIGEN: I think that one should add to this collection those cases in which the product of an enzymic reaction controls the synthesis of the enzyme itself. This can occur

either in the negative sense of metabolic repression where the product of a pathway inhibits the formation of the enzymes of that pathway, or in the positive sense of several inducible enzymes which are induced by the product other than the substrate of the pathway. For example, β-galactosidase is not induced by lactose, but only by a transgalactosylation product of lactose metabolism; and glycerol kinase is induced by glycerol phosphate which is the product of its reaction. The same phenomenon is also present in histidine metabolism where the enzymes responsible for histidine breakdown are induced by urocanic acid. I would also like to inject a question. If the AP_4A serves any kind of regulatory function, one might expect that its synthesis would in some way reflect the activity of the lysine-activating enzyme. Is the synthesis of AP_4A sensitive to the presence or absence of lysine? How is it influenced by the availability of the amino acid?

DR. ZAMECNIK: We studied it only *in vitro* using a purified lysine activating enzyme and I do not know whether our observations have general implications for other amino acid activating enzymes. In our system, lysine is indispensable. Both the forward and backward reactions are speeded up by the presence of tRNA. There is evidence, particularly from the work of Loftfield [Loftfield, R. B., and Eigner, E. A. (1965) *J. Biol. Chem.* **240,** P C 1482] and P. Hele [(1964) *Biochim. Biophys. Acta* **87,** 449] that the presence of tRNA may induce a conformational change in the activating enzyme which speeds up both forward and backward reactions.

DR. MANSON: In older experiments with liver microsomes in cell-free systems, it was observed that deoxycholate-treated ribosomes required GTP for peptide formation whereas the crude microsomes did not. Do you know whether this data is still valid and whether the lipoproteins have a role perhaps interchangeable with that of the GTP?

DR. ZAMECNIK: It is quite clear that in crude preparations a number of proteins are associated with and/or adsorbed onto ribosomes. These proteins may already have GTP associated with them. I think the later experiments with ribosomes which are washed in deoxycholate and then with 0.5 *M* sodium chloride or 1 *M* ammonium chloride which show a GTP requirement, are more to be trusted. As to whether lipoprotein plays a role, I do not know.

DR. MARKUS: In connection with the need to switch one sRNA to the position of the other, as has been suggested, could not the following alternate idea solve some of the problems? Instead of having only two grooves on the 50 S particle, you could consider it as a ball lined with grooves all the way around, and instead of having to switch positions you would just turn the ball one notch and put a fresh site into position.

DR. ZAMECNIK: I should have mentioned that possibility, which has been considered and has not been ruled out. The only evidence not in favor of this idea is that, in a well-washed system, one seldom finds more than two tRNA molecules associated with a single ribosome. Of course, this finding does not rule out the idea completely. It was described in detail in a discussion at Cold Spring Harbor by Dr. B. D. Davis [(1963) *Cold Spring Harbor Symp. Quant. Biol.* **28,** 294].

Ambiguity in the Translation of the Genetic Code into Proteins, Induced by Aminoglycoside Antibiotics

LUIGI GORINI

Department of Bacteriology and Immunology, Harvard Medical School, Boston, Massachusetts

The significance of streptomycin (Sm) and related aminoglycoside antibiotics in current investigations of protein synthesis is twofold: (1) Sm is a well-characterized example of an environmental factor which alters the reading of the genetic code without altering the structure of the code itself, and (2) Sm has contributed to our understanding of the mechanism of translation of information from nucleic acid to protein, and, in particular, the role played by the ribosome.

It is well known that the transmission of genetic information from one generation to another is subject to mutations, that is, inheritable changes or errors, which consist of chemical alterations in the structure of the deoxyribonucleic acid (DNA) constituting the genome. However, only recently has it become evident that errors occur as well in the transfer of genetic information to protein within the cell.

It is now known (Yanofsky and St. Lawrence, 1960) that the genetic code is ambiguous since a given codon may be translated differently depending on mutations occurring in the macromolecule tools (activating enzymes and tRNA's) the cell uses in protein synthesis. Some 2 years ago it was discovered furthermore (Gorini and Kataja, 1964a) that the genetic code is also ambiguous in the sense that it can be misinterpreted during the translation process when specific small molecules, like Sm, are present in the cytoplasm (Fig. 1). The effects of a mutation in a translation tool or of the presence of Sm are similar: the genetic script is not altered physically, but it is read differently. There is, however, a basic difference. Altered translation due to an alteration in a translation tool is

inheritable, being due basically to a mutation with an indirect effect. An altered translation due to Sm, however, is strictly phenotypic and lasts only so long as the inducing small molecule is present in the cytoplasm. For instance, a histidine-requiring mutant may grow in the absence of this amino acid provided that, but only as long as, Sm is present in the growth medium. A defective bacteriophage may produce mature particles in a given nonpermissive host, provided that Sm is present during the infection. The phages so produced are still defective and still require Sm for propagation subsequently in the same nonpermissive host.

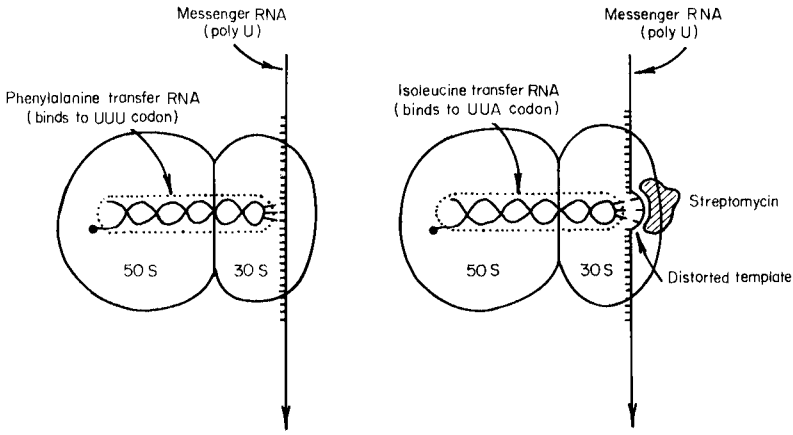

FIG. 1. Action of Streptomycin can be conceived of as an alteration, perhaps some kind of distortion, of the template or of the 30 S subunit of the ribosome such that the codon is "read" incorrectly, binds the wrong transfer RNA, and thus incorporates into the peptide chain an amino acid other than the one the mRNA is coded for. This highly schematic diagram shows how a UUU codon should incorporate phenylalanine (left). Altered by a streptomycin molecule (right), the ribosome causes the UUU to be read as if it were AUU, and thus to incorporate isoleucine.

The first mutant for which this Sm effect was recognized and studied in some detail was an arginine requirer possessing a defective ornithine transcarbamylase (OTC), the enzyme converting ornithine to citrulline, a precursor of arginine (Gorini *et al.*, 1961). Ordinarily, this mutant requires arginine or citrulline for growth and we were very surprised when we unexpectedly discovered that Sm could replace these growth factors. This was in contrast to several other independently isolated OTC-defective mutants which require arginine or citrulline under any growth conditions, with or without Sm. It was easy to demonstrate that growth of this mutant in the absence of arginine was due to small amounts of active OTC produced only in the presence of Sm, and that the enzyme

formed was active and stable also in the absence of the drug. Finally, the possibility that Sm acted on a regulator gene was excluded because the defect in the mutant can be located genetically in the OTC structural gene, and, moreover, this strain is derepressed for the arginine pathway.

We were left with the interpretation that Sm corrects this mutant because it is able to alter the standard meaning of the genetic code at some step in the transmission of genetic information from DNA to protein. Since suppression is the term used in genetics to designate a correction not directly due to a mutation in the gene which is corrected, we called this effect of Sm "phenotypic suppression by Sm"; and since these mutants may be described as dependent upon Sm in the absence of the required growth factor, we designated this new class of mutants "conditionally Sm dependent" (CSD). Such CSD mutants have since been found for a wide variety of bacterial auxotrophs, that is, cells requiring a growth factor, as well as for other types of mutations. Moreover, this type of suppression has been extended to other chemically related aminoglycoside antibiotics.

The relative infrequency of the CSD mutants among auxotrophs obtained from a single parent supported the idea that the similarity of genetic informational suppression and correction by Sm is more than a formal one. It indicated indeed that the reading inaccuracy induced by the antibiotic is selective, like genetic suppression, and it is profitable only to a restricted class of defects in the structural genes. In addition, it was found that the mutation to Sm^R did not always result in cells that were "competent" for Sm-induced suppression of auxotrophy. This observation indicated a linkage between the suppressibility phenomenon and the particular mutation to Sm^R. We suggested (Gorini and Kataja, 1964a), therefore, that suppressibility might be dependent on the structure of the ribosome, because it was already known that the mutation from wild-type Sm^S cells to Sm^R cells involves a change in the structure of the ribosome (Spotts and Stanier, 1961) and more precisely of the 30S ribosomal subunit (Cox *et al.*, 1964; Davies, 1964). This prompted *in vitro* experiments of amino acid incorporation directed by synthetic messengers (Davies *et al.*, 1964) which clearly confirmed our prediction. Misreading is observed in an amino acid incorporating system in which the 30S ribosomal subunits are derived from Sm^S cells and not with those derived from Sm^R mutants. The origin of all other components, transfer RNA, activating enzymes, 50S subunits, is irrelevant. For instance, with polyuridylate, normally coding for phenylalanine, the incorporation of this amino acid is inhibited 50–60% in the presence of Sm and that of isoleucine, serine, and tyrosine is increased from about zero to about 60% of the phenylalanine incorporation (calculated for isoleucine alone).

Thus it was confirmed that misreading is a misrecognition of codon-anticodon through ribosomal intervention altered by the presence of Sm. The same results were obtained (Pestka *et al.*, 1965) by testing the effect of Sm on the binding of amino acid-loaded transfer RNA to messenger in the presence of ribosomes. Finally, we analyzed the amino acid composition of the growing polypeptide attached to the ribosome. With polyuridylic acid as messenger and with no Sm, the polypeptide is a chain of phenylalanine, whereas with Sm added, the polypeptide contains about 40% isoleucine (Old and Gorini, 1965).

It is reasonable to argue that, although misinterpretation of the genetic message by misreading should be chemically possible, evolution would have developed a control against this unwanted possibility. It may be that this control is indeed one of the functions of the ribosome in translation. This is suggested by the following observations obtained in an experiment in which polyuridylic acid (poly U) is used as messenger and the level of isoleucine misincorporation with or without Sm is measured when isoleucine is alone or in association with all other amino acids. It appears that isoleucine is misincorporated in a substantial amount, even without Sm, when it is the only amino acid present and that Sm reverses the corrective effect of the complete amino acid mixture. This result suggests that there is an ambiguity intrinsic to some, or all, codons but that under normal conditions the ribosome and the competition of all loaded transfer RNAs permits only one, or essentially one, unambiguous reading for each codon. Sm alters the structure of the 30 S ribosomal subunit and this alteration permits substantial ambiguous reading of the codon even in the presence of all the amino acids. If the experiment just described is repeated using an amino acid for which misreading with poly U is not observed, for instance, using arginine instead of isoleucine, one does not find ambiguity under any conditions.

Misreading can be induced in a cell-free system in a variety of ways such as by changing the concentration of positive ions, the acidity, or the temperature. The effects of these changes may be laboratory artifacts, however, without biological significance. In the case of the Sm effect, it has been possible to relate misreading in cell-free extracts on the one hand to suppression in living mutant cells on the other. Conditionally Sm-dependent mutants which are sensitive to Sm respond to small, sublethal amounts of Sm in the growth medium and show the misreading in cell-free preparations discussed above. Conditionally Sm-dependent mutants which are resistant to Sm require much more Sm to suppress a genetic defect; these ribosomes show a lower level of misreading, detected only when a highly purified system is used.

As a result of mutation or of the presence of a drug like Sm, the ribo-

some can assume different conformations which permit different types of misreading. Thus some mutations to Sm resistance permit suppression of a particular defect by Sm, while others do not. Even in the absence of drugs, variations in ribosome structure play a role in the accuracy of translation (Gorini *et al.,* 1966). There seems to be a steady low level of misreading, and that level varies with specific ribosomal mutations. Thus ambiguity in translation is inherent in the process of protein synthesis. A cell is capable of a certain frequency of misreading, and drugs or other agents in the environment can increase this frequency by acting on the ribosomes.

Misreading induced by Sm and other aminoglycoside antibiotics has been studied extensively. It has been found that different drugs possess different, characteristic patterns of misreading. With Sm and using poly U or poly C as messenger, we may tentatively define a relatively simple pattern of misreading. For instance, according to the code dictionary compiled to date, it is possible to suggest that misreading affects the meaning of only one base of a triplet at a time. Thus, generally speaking, a given codon is read as if it were one of the triplets connected to it by only one base substitution (Davies *et al.,* 1965). However, only some of the possible connected codons are misread. Moreover, using alternating base copolymers, it has been found (Davies *et al.,* 1966) that the misreading of a given base is controlled by its position. Taken altogether, the evidence *in vitro* indicates that Sm suppresses because it produces ambiguity in base pairing, in contrast to genetic suppressors which are thought to substitute one code meaning for another without altering the conventional base pairing rules defined by Watson and Crick.

The conclusion that the misreading obtained in cell-free preparations does indeed account for the suppression observed in living cells requires unequivocal proof of the occurrence of misreading in such cells. Direct analysis of the amino acid composition of a purified enzyme synthesized in the presence of Sm has not yet been accomplished. However, Sm has been shown (Gartner and Orias, 1966; Gorini *et al.,* 1966) to suppress nonsense mutations, in which the mutated triplet codes for no amino acid and the corresponding protein is therefore missing. Sm misreads the nonsense codon into a "sense" amino acid codon, causing the protein to be synthesized.

Can the *in vitro* results explain the bactericidal action of Sm? Intuitively the answer is yes: if misreading observed *in vitro* with Sm^S but not with Sm^R cells is not an artifact, and if we accept that the only function of the ribosome is to participate in translation, then misreading should be at the origin, close or remote, of all phenomena observed upon addition of Sm to Sm^S cells, first of all the killing. Very suggestive evidence for this

can be seen by comparing resistance to killing and to misreading produced by two aminoglycoside antibiotics, Sm and neomycin (Nm) (Davies *et al.*, 1964). Wild-type *Escherichia coli* is sensitive to both antibiotics and the ribosomes extracted from it are induced to misread with either one of the two drugs. However, ribosomes extracted from mutants Sm^R Nm^S do not misread in the presence of Sm, but they do still misread in the presence of Nm. The converse situation is found when Sm^S Nm^R mutants are tested. In addition, we have checked the ability to induce misreading of certain chemical derivatives of Sm (N′ substituted streptomycylamines) tested for their antibiotic effect some years ago (Treffers and Alexander, 1955–1956). We found that misreading and bactericidal efficiency parallel each other. Moreover, it has been found that the most obvious prediction is verified, namely that, if the killing is due to misreading, a cell is protected against Sm by preventing it from synthesizing proteins by starving it of a required amino acid or by adding chloramphenicol to inhibit protein synthesis. However, in spite of so many strong suggestions, it is hard to see how a flood of bad proteins produced by misreading should irreversibly damage a cell, particularly in the few minutes required for killing. In fact there are prototrophic derivatives of CSD Sm^R strains due to a suppressor mutation in which the cell is filled with faulty protein, inhibited by Sm, but not killed (Gorini and Kataja, 1964b). Thus the elucidation of the antibiotic action remains an open question, especially since a satisfactory model must encompass the genetic evidence described above.

In conclusion, the misreading effect of Sm adds a new dimension, the ribosomal dimension, to the elucidation of the mechanism of protein synthesis. Furthermore, in a meeting predominantly discussing immunological problems, it seems pertinent to underline that suppression by Sm suggests the possibility that in a given genome DNA regions may exist with a high possibility of ambiguous translation and that the presence of molecules extraneous to the DNA and to the translation machinery may specifically direct alternate versions in translations of the same genome.

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DISCUSSION

DR. ZAMECNIK: Is it reasonable to think that streptomycin might attach itself with one end to the messenger, and with the other to a particular sRNA, and that whether it became attached to that particular sRNA or not would depend upon the secondary structure of the sRNA? If the antibiotic could serve in a bidentate way to strengthen the bond between the sRNA and the messenger, then perhaps the hydrogen bonds of two nucleotides rather than the usual three might be sufficient to form a stable combination on the 30 S particle. If such were the case, might not ambiguity in reading be introduced?

DR. GORINI: I have no definite ideas on the mechanism by which streptomycin induces misreading. The only point that is important to realize is that any model one may propose should be reconciled with these three facts: (1) the ribosomal structure controls the type of misreading, (2) the central codon letter is misread as well as the external ones, and (3) a specific pattern of ambiguity characterizes streptomycin and the other antibiotics inducing misreadings.

DR. PRESSMAN: Is not what you call intrinsic ambiguity the same thing as the incorporation of a certain substance which does not bind with as strong a constant but is present in an increased concentration so that it can compete successfully?

DR. GORINI: Whatever the intrinsic ambiguity may be, it is certainly experimentally evident that in the presence of all charged sRNA's there is enough competition to correct any possible intrinsic ambiguity. The fact is, however, that one does observe intrinsic ambiguity for only those amino acids which are misread by streptomycin. For instance, using poly U as the messenger, arginine is not incorporated even when it is used alone, in contrast with isoleucine which is incorporated under the same conditions. When all the amino acids are present, the ambiguity for isoleucine is not seen, but it reappears in the presence of streptomycin. The point to clarify is in which way streptomycin reestablishes this ambiguity. At this time any model can be proposed, provided that it is consistent with the few experimental facts that we have.

DR. TRITSCH: Is the amount of isoleucine incorporated in the presence of streptomycin dependent upon the antibiotic concentration? This could be tested experimentally.

DR. GORINI: One can go down to two or three molecules of streptomycin per ribosome and still obtain the maximum misreading effect.

DR. MUNYON: Does streptomycin show a particularly high affinity for ribosomes?

DR. GORINI: Streptomycin certainly has affinity for RNA and DNA in general. A classical method by S. Cohen uses streptomycin to precipitate nucleic acid. In an experiment done using labeled streptomycin, it was shown that only one molecule of the antibiotic is enough to give the misreading in a Nirenberg system.

DR. SAMUELS: Is it reasonable to conjecture that each antigen might affect the ribosome in a specific way, similarly to what occurs in the case of streptomycin or neomycin? Should this be the case, could different antibodies be produced from the same messenger?

DR. GORINI: Yes, I think this is a reasonable suggestion. There are other cases in which there is evidence for only one cistron controlling the synthesis of more than one protein. For instance, in the case of the alkaline phosphatase of *Escherichia coli*, the genetic indica-

tion is for only one cistron, but electrophoretic studies indicated the existence of more than one protein band. Before the streptomycin misreading effects became known, it was suggested that perhaps there are nonprotein residues that stick onto some of the alkaline phosphatase molecules and make them move differently under electrophoresis even though the actual peptidic chain is the same. Now, of course, the question is open as to whether these bands represent proteins which are different, in the sense that perhaps one of the amino acids in the peptidic chain is different as a result of an ambiguity in translation. It is very difficult to clarify this point. In fact, the problem is to isolate a molecule among hundreds and to prove that it is different by one amino acid and that this difference is not just a question of impurity. One approach is to try to select by mutation a strain of *E. coli* which preferentially makes only one of those enzyme forms. A student of mine obtained a mutant producing an alkaline phosphatase which gave only one electrophoretic band. Three bands were seen again when the mutant was grown in the presence of streptomycin. We are presently studying this system.

DR. BENACERRAF: If an attempt is made to explain the differences in amino acid sequence observed in the variable portion of different Bence-Jones proteins according to a process similar to the one you have discussed, would it be necessary that the same permutation should repeat itself, at least in the variable portion, every time the same codon is translated into its corresponding amino acid in the peptide chain, considering that the synthesis occurs in a cell where the environment is constant? The same ambiguity-generating factor which in your case is streptomycin should also be expected to affect the synthesis of the constant portion of the chain. Also, I would like to point out that in the case of immunoglobulin synthesis, the cell is making a particular chain in very large amounts and is making what appears to be a homogeneous product. If you consider a mechanism which permits different cells to synthesize chains with different amino acid sequences through some form of permutation, it should be one which is working at a very high efficiency. How could you meet such an objection?

DR. GORINI: One has the same perplexity in trying to explain any correction by suppression of missense mutations such as those studied by Yanofsky, for instance. I agree that there is no reason to think that a codon is present only once in any given cistron. It is presently not clear why only one of them is corrected. Khorana showed that some position effect is involved in codon misreading. Moreover, it must be observed that the level of such corrections is generally small. The situation is completely different in the case of a nonsense mutation because a nonsense codon may very well appear only once in a given cistron. I agree therefore that the proposal of ambiguity in the translation as an explanation for a normal biological process such as antibody formation encounters implications very difficult to assess. One is the antigen specificity as ambiguity agents and another is to explain how only a codon, say, in position 10, is corrected with very high efficiency whereas the same codon in another position is not.

DR. BERENBAUM: Using antibody to a bacteriophage produced by rabbit spleen cells, Krueger found that in the presence of streptomycin the antibody lost its neutralizing ability for the phage but retained its complement-fixing ability [Krueger, R. G. (1965) *Proc. Natl. Acad. Sci. U.S.* **54,** 144; (1966) *Proc. Natl. Acad. Sci. U.S.* **55,** 1206]. The antibody cross-reacted with other bacteriophages and it was interesting that its crossreactivity changed. In other words, it reacted with some other phages better than the normal antibody, and less with the phage used for immunization. Since these changes are fairly easily detected by immunological techniques, the majority of antibody molecules must have been changed, not just a small percentage.

DR. GORINI: Yes, I am well aware of Krueger's observations. If their interpretation is

correct, they should indicate that the genome involved in antibody formation can be easily misread by ambiguity.

DR. PRESSMAN: I do not think that there is necessarily a change in type of antibody produced in any particular cell under the influence of streptomycin. Actually, antibodies are produced by many different types of cells giving a particular spectrum of different antibodies. Certain treatments may change the effectiveness of the various cells in producing antibody, and the resulting shift of the spectrum may cause a substantial alteration in the properties of the antibodies formed.

DR. PAIGEN: If one makes a "mistake" theory of antibody formation, one would also require that the theory include some mechanism to provide for a sensible relationship between the product which is formed and the antigen which induced the mistake. Clearly, the problem is not only to produce a new protein, but to produce a new protein which recognizes the antigen. If an antigen came in and caused random mistakes, then very few substances would be antigenic in the sense of being able to induce a specific antibody diverted against themselves.

DR. GORINI: Certainly, one should imply that different antigens are different in causing a mistake and that (like kanamycin, streptomycin, neomycin), each one shows a different pattern of mistake. So many assumptions are required that I would say that the hypothesis is probably incorrect. All I want to stress is that *in vivo* one can show that the same gene may control the synthesis of protein chains which are different in their amino acid composition when it is translated in the presence of drugs like streptomycin. This is certainly true. To prove the hypothesis under discussion, however, one would have to resolve the problem of the efficiency of amino acid permutations, the question of the specificity of antigen-antibody requiring that each antigen induce not only a different type of ambiguity but a type giving the antigen specificity to the resulting antibody. All these prerequisites are difficult to solve, I agree.

DR. REIF: Should we call the streptomycin-induced change in translation of the genetic code into protein an "error," or should we regard it as a preordained but less favored response? Latitude in the reading of the genetic code may constitute a most valuable adaptive mechanism, which may enable bacteria or cells exposed to unusual physiological conditions to survive. An analogy may also be drawn with antibody production. If each cell were able to respond only to the exact antigenic specificity which, according to the clonal selection theory, is preordained for it, then it is very questionable whether antibody production would be as ubiquitous a defense mechanism as it is.

DR. ZAMECNIK: It is difficult to say how faithful or how error-free should be the trinucleotide anticodon which is pairing with a messenger codon. My colleague Loftfield has stated that the difference in the number of H bonds between a trinucleotide which is coded and one which is very similar to it does not represent an enormous energy difference. On that basis one might expect a higher percentage of error than one ordinarily finds in completed protein of a given type. Under ordinary circumstances, during the first step of protein synthesis, in which amino acids are activated and then transferred to sRNA, there is an error level in the order of one part in several thousand.

Macrophage RNA and Antibody Synthesis*

MARVIN FISHMAN and FRANK L. ADLER

Division of Immunology, The Public Health Research Institute
of the City of New York, Inc.
New York, New York

It is generally accepted that the ability of a cell to produce a given protein is determined by its possession of the necessary information, coded in the nuclear DNA, and that several distinct classes of cellular RNA are involved in the transcription of the information, in the transport of amino acids, and in the assembly of the constituent peptide chains. The results of transformation experiments and those of viral infections demonstrate that this rule is not inviolate since nucleic acids foreign to a cell can usurp the function of the cell's own nucleic acids and direct or mediate the synthesis of new proteins. Antibodies, as proteins, nevertheless seem unique in the sense that at least one critical part of their structure, namely the combining group, appears to be formed in specific response to an extraneous agent, the antigen, which may be protein, nucleic acid, polysaccharide, lipid, or a simple chemical coupled to a suitable carrier. Just how such diverse substances can interact with cells so that each evokes antibodies specific for itself is the subject of several conflicting theories of antibody formation and of a large body of contemporary experimental work.

Among the theories, that of "clonal selection" (Burnet, 1959) imposes minimal demands upon the antigen which is required to do no more than to select and stimulate cells which already are endowed with the nucleic acids required in the synthesis of the specific antibody. Entry of the antigen into the cell is not a necessary requirement of this theory, but is an

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essential feature of all other theories. On the experimental level, antigen or antigenic fragments have been found to be present in antibody-producing cells by some (Wellensiek and Coons, 1964) but not by other investigators (Nossal, 1965). Even if this factual contradiction could be resolved, it will be a difficult task indeed to establish experimentally that a causal relationship does or does not exist between the presence of antigen in a cell and the formation of antibody against this antigen.

On the assumption that antigen must enter a competent cell to initiate antibody formation, different functions have been assigned to it by several investigators. It has been suggested that it may combine with nuclear DNA (Schweet and Owen, 1957) where it might cause modifications that are transmitted to a cell's progeny, or where it might act as a derepressor (Szilard, 1960). Combination of antigen or antigenic fragment with RNA has been suggested by others and messenger (Haurowitz, 1965) as well as ribosomal RNA (Garvey and Campbell, 1957) have been suggested as ligating agents. The recently described suppressor RNA (Notani *et al.*, 1965; Weigert and Garen, 1965) offers yet another possible point of attack through which an extraneous agent could modify protein synthesis. Still another suggestion is that of Rinaldini (1965), according to which intracellular antigen stimulates the synthesis of appropriate subunits of immunoglobulin by a feedback mechanism.

With regard to the combination of antigen and cellular RNA, there has accumulated an appreciable body of evidence for the existence of such complexes and their relevance to antibody production. The fundamental work of Garvey and Campbell (1957) provides ample evidence that antigen may persist *in vivo*, presumably in phagocytic cells, for long periods in a highly immunogenic form while complexed with RNA. In the course of our *in vitro* studies on antibody formation, we have extracted from phagocytic cells that had been incubated with antigen two or more RNA fractions, each capable of eliciting specific antibody formation when added to cultures of lymph node fragments from normal animals. It is the purpose of this communication to summarize the evidence that leads us to believe that the RNA in these preparations is essential to immunogenicity, that one of two distinct biologically active fractions contains demonstrable antigen while the other does not, and that this latter fraction contains material which exerts an effect in lymphoid cells that is compatible with the role of messenger RNA.

We have previously reported (Fishman and Adler, 1963a, b; Fishman *et al.*, 1965) some of the aspects of antibody formation to T2 phage *in vitro* which suggested that more than one active principle might be extracted from the antigenically stimulated peritoneal exudate cells (macrophages). Summarized in Table I are data which show that if sufficient

antigen was added to the macrophages, the RNA extracted from such cells gave rise to two waves of antibody formation in lymph node fragments. The first of these two waves was found to consist of IgM antibody, the second mainly of IgG antibody. An early indication that two distinct fractions of macrophage RNA might be involved came from the results of experiments in which it was found that the ability to evoke IgM antibody was more labile to RNAse than that to evoke IgG antibody. The relative resistance of the RNA responsible for the 7 S (IgG) response was thought to indicate the possibility that this RNA might be complexed to either DNA or protein.

TABLE I
THE EFFECT OF VARIATION IN THE AMOUNT OF ANTIGEN ADDED TO A CONSTANT NUMBER OF MACROPHAGES

RNA Macro: T2[a]	Antibody response in tissue culture fluids	
	5 days	9–12 days
1000:1	+	−
1:10	+	−
1:100	+	+
1:1000	+	++

[a] Constant number of macrophages: 1×10^9.

Additional evidence for the existence of two distinct classes of active RNA was obtained in experiments with actinomycin D (Fishman *et al.*, 1965). If the drug was added to the macrophages simultaneously with the antigen, and RNA was extracted after 30 minutes of incubation, such RNA gave rise to the late IgG response only. This activity persisted even when the drug concentration was increased five-fold during the 30 minutes of incubation. However, as seen in the data presented in Table II, if antinomycin D was allowed to react with the macrophages for a total of 2 hours under conditions which, in the absence of the drug, allowed recovery of active material, then neither IgM nor IgG antibody was elicited. If actinomycin D acted in these experiments as a specific inhibitor of DNA-directed RNA synthesis, then the data would suggest that IgM synthesis was mediated by RNA formed after the addition of antigen while preexistent RNA was responsible for the formation of the IgG antibody.

While our early attempts to find T2 antigen in the RNA extracted from stimulated macrophages yielded negative results, subsequent efforts

confirmed the findings of Friedman *et al.* (1965). By incubating RNA from macrophages which had been exposed to relatively high concentrations of T2 phage with potent anti-T2 sera, precipitates were obtained

TABLE II
THE EFFECT OF ACTINOMYCIN D ON BIOLOGICAL ACTIVITY BY RNA EXTRACTED FROM MACROPHAGES INCUBATED WITH T2

Tissue culture fluids (days)	Percent neutralization actinomycin D	
	30 minutes[a]	120 minutes[b]
4	8	0
7	10	8
11	29[c]	0

[a] Macrophages incubated alone for 90 minutes, then actinomycin D and T2 were added and incubation was continued for 30 minutes.
[b] Actinomycin D and macrophages were incubated for 90 minutes, then T2 was added and incubation was continued for 30 minutes.
[c] Significant at 1% level of probability.

(Table III). Extraction of RNA from such precipitates yielded material of high biological activity although the amount recovered was at most 2% of the total RNA. A comparison of the biological activity of RNA recovered from the precipitate, and that reextracted from the supernatant, showed that the former gave rise to a response which was late in onset and mainly IgG, while the supernatant RNA gave rise to the early IgM

TABLE III
PRECIPITATION BY ANTI-T2 SERUM OF RNA-ANTIGEN COMPLEXES FROM MACROPHAGE-T2 RNA

Source of RNA	Antiserum added		
	Anti-T2	Anti-Bacillus *subtilis* phage	NRS
Macrophage-T2	+++	+	+
Macrophage-BSA	+	+	+

response. It seems reasonable to assume that the RNA precipitated by anti-T2 serum is similar to the "super antigen" described by Askonas and Rhodes (1965) and the complexes described by Garvey and Camp-

bell (1957). A more detailed account will be presented in a future publication. The relative role and precise function of the RNA and antigen components of the complex remain unknown.

Our original efforts to purify the active RNA, and, subsequently, to separate the two fractions by physical means, were based on centrifugation in sucrose density gradients. Early data (Fishman and Adler, 1963a) showed that biological activity (IgM antibody formation) resided in a light fraction, presumably RNA of 5–7S. Since resolution of the density gradients was poor, methylated bovine serum albumin columns (Mandell and Hershey, 1960) were used in later experiments. With the aid of such columns, macrophage RNA could be resolved into three fractions of 4, 16, and 23S, respectively. Assays of such fractions showed that IgG antibody was formed in response to the heaviest of these fractions while IgM antibody was evoked by material of smaller size, presumably in the range of 7–10S.

In a most recent series of experiments, we have employed rabbits homozygous at the b locus (light chain of immunoglobulins), using animals of one allotype as macrophage donors and rabbits of the other allotype as lymph node donors. Results of such experiments, shown in Table IV, indicated that the early IgM antibody had the allotypic specificity of the macrophage donor while the late IgG antibody had the allotypic markers of the lymph node donor. These data and other supporting evidence (Adler and Fishman, 1966) once again support the thesis that at least two classes of macrophage RNA may be active in initiating antibody formation in lymphoid cells, and that the fraction responsible for IgM antibody formation may contain messenger RNA activity.

TABLE IV

THE ALLOTYPE OF THE ANTI-T2 GLOBULIN SYNTHESIZED *in Vitro* BY LYMPH NODE FRAGMENTS (b^4b^4) IN RESPONSE TO THE ADDITION OF MACROPHAGE-T2 RNA (b^5b^5)

Tissue culture fluids (days)	Inhibition of neutralization with			Allotype marker
	Anti-4 serum	Anti-5 serum	NRS	
5	+	−	−	macrophages
12	−	+	−	lymphoid cells

In summary, it would appear that macrophages, upon suitable incubation with antigen, yield at least two classes of RNA, each capable of initiating antibody formation in cultures of normal lymph nodes. IgM

antibody is elicited by an RNA fraction of relatively small molecular size which is highly sensitive to RNAse, contains no demonstrable antigen, is formed after the addition of antigen to the macrophage, and is closely associated with RNA that conveys the code for synthesis of the allotypic marker on the light chain. The other class of RNA, responsible for the IgG antibody response, appears to be of high molecular weight, contains demonstrable antigen, is relatively resistant to inactivation by RNAse, and appears to be present in the macrophage prior to the addition of antigen. It seems to function neither as messenger nor does it act as an adjuvant. This possibility was examined with the aid of a model in which physically disrupted phage was assayed for its immunogenicity in the presence or absence of RNA extracted from macrophages.

The relation of these *in vitro* findings to *in vivo* events that follow upon the injection of antigen remains to be established and constitutes part of our present studies and is also under investigation by others (Nossal *et al.*, 1966; Speirs and Speirs, 1964).

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DISCUSSION

DR. GLICK: Have you ever tried incubating other cells besides macrophages with antigen to obtain RNA? Also, have you ever tried incubating other cell types besides lymph node cells with the RNA?

DR. FISHMAN: As I said, our peritoneal exudate is not a pure macrophage preparation. We were not able to get an active RNA from lymph node cells. Discarding the majority of the lymph node cells present in the peritoneal exudate by allowing the macrophages to settle on glass and by removing the cells that do not settle, one can obtain a cell population enriched of macrophages but still containing other cells. This preparation with a decreased number of lymphocytes is still active in giving the RNA response. We are trying to obtain pure preparations by using antilymphocyte serum. We have not tried to put RNA on other types of cells.

DR. GLICK: According to the last experiments you showed, you might be able to predict that another cell type would respond to the macrophage RNA in terms of the first response but not of the second.

DR. FISHMAN: You bring up a point of conflict. What you are asking is whether the lymph node cells are the only cells that are capable of producing antibody. There is evidence from Dr. Holub and from Dr. Bussard that macrophages may also give rise to antibody, and maybe what we are observing is a response which would ordinarily go on in the macrophage population.

DR. SAMUELS: Your results indicated that in a mixture of peritoneal exudate macrophages plus T2 antigen, the addition of actinomycin blocked the early immune response, but not the late one. Since the allotype experiments showed that only the earlier response was derived from the peritoneal exudate cells whereas the lymph node fragments contributed the late response, one would not expect that actinomycin would block the late response because it was not even operating in that reaction mixture. Have you tested the effect of actinomycin in the lymph node system?

DR. FISHMAN: The results obtained with actinomycin D would imply that the allotype RNA was synthesized after the antigen was produced. I have not tried the antibiotic in the lymph node mixture.

DR. SCHLESINGER: What do you think is the general significance of the processing of antigen by macrophages? As you know, macrophages are completely inactive in graft vs. host reactions unless they are derived from a preimmunized donor. In other words, in transplantation experiments it seems that macrophages cannot process the antigen but that macrophages of an immune animal can respond against the antigen.

DR. FISHMAN: The presence of cytophilic antibody around the macrophage may create the desired recognition in the macrophage. In trying to explain macrophage involvement in TB resistance, Fong attempted to transfer the immune status from an immune to a nonimmune macrophage with RNA. Mannik reported that the addition of RNA from immune lymph node cells to nonimmune lymph node cells provided these cells with the ability of eliciting the corresponding homograft response.

DR. UHR: I think Dr. Fishman has properly emphasized the difficult problems of isolating and characterizing RNA and defining the role of such materials in the formation of different types of antibodies. I would like to further comment on one particular problem, the problem of RNAse. Virtually all papers concerned with polyribosome or RNA extractions from lymphoid organs discuss this problem and indicate that measures were taken to reduce RNAse activity, yet most of the published data show extensive degradation by RNAse. Obviously, this is a serious problem in such studies. In trying to determine polyribosome patterns in γ-globulin synthesis. Matthew Scharff and I had to use the unusual maneuver of adding an enormous amount of HeLa cell cytoplasm to prevent messenger RNA breakdown. We presume, but have not proved, that there is an inhibitor present in the cytoplasm. Conventional RNAse inhibitors such as spermidine and bentonite which worked well when judged by conventional *in vitro* assays, were entirely ineffective in preventing messenger RNA breakdown in the polysomal analyses. The reason is that single breaks of individual messenger RNA molecules result in a radical change in polyribosome

profile, but in the conventional RNAse assay, many breaks are necessary to acid-solubilize RNA. Regarding Dr. Fishman's experiments, it is difficult to visualize how an unprotected messenger RNA can get into a new cell, find the right position, and be properly utilized within mammalian cells; it is even more complicated if the messenger has already been chopped down to smaller pieces. I am glad to know that Dr. Fishman is investigating this problem of breakdown with regard to his own system.

DR. FISHMAN: The problem related to the presence of RNAse is complicated by the fact that a phenol extraction of RNA does not eliminate endotoxin, which has been implicated in all phases of antibody formation. Fortunately, we found a way of freeing RNA from endotoxin and to our satisfaction we found that the RNA was still active.

DR. ŠTERZL: I would like to know how much antibody you think is really synthesized in your system. It must be a very small amount compared to that formed in the secondary response evoked by antigen in the *in vitro* system used by Coons and Ambrose. In their experiments, using a hemagglutination technique, titers of 1:600–1200 are obtained. In your system the detection of antibodies by phage inhibition is probably by two or three orders more sensitive. However, you do not detect antibodies in increasing serum dilutions and do not estimate the titer of 50% of phage neutralization, but only the percentage of phage neutralization in one sample compared with controls. Further, I would like to ask you how many cells are involved in antibody synthesis in your experiments. I think that the amount of antibodies which you detect represents the production of only a few cells, calculating that one cell produces $\sim 10^{-6}$ μg of antibody in 24 hours.

DR. FISHMAN: We cannot give the amount of antibody formed in our system. In trying to determine the amount of globulin synthesis by hemagglutination inhibition tests, we have some numbers which may not be meaningful. It is a minute amount, which means that we resort to concentration when we work with gradients. I do not know the exact amount of antibody that is being synthesized nor do I know which cells are involved. We are now trying to see whether a few cells are involved in giving us all the antibodies or whether many cells are involved in giving us a small amount of antibody.

DR. LANDY: Continuing along the line initiated by Dr. Šterzl, I would ask Dr. Fishman two questions. Since all the data you have presented are in the form of percent neutralization by a given quantity of test material rather than being calculated from a series of concentrations, what percent do you regard as a significant value? Dr. Šterzl and I have had considerable experience in analogous assay systems involving the measurement of antibody in terms of the bactericidal action of serum. However, we soon learned that for meaningful quantitation it was essential to obtain dose response data. From the slope of dose response one could then calculate the quantity of serum effecting 50% killing. In contrast, the only data reported here are specific individual values for percent neutralization. Of course, I am very much aware that in your situation, one is working with dilute material and it is not always possible to bring to bear the amount you would use so as to provide the optimal range for quantitative measurement. Would you therefore give us some idea of what can be realistically accomplished with your procedures and what figure for percent neutralization provide maximum reliance?

DR. FISHMAN: This is a problem that we fully realize, Dr. Landy, and therefore we have relied on statistics to determine the significance of our data to the 1% level. It will vary with the sampling errors at the inputting phase. One can actually get a definite number representing the difference between the input and the obtained values which would bring the data to the significant level. This is the only method we can use to determine whether or not our neutralization is significant.

DR. PAIGEN: I gather from your presentation that basically you would like to come to the conclusion that the early response is to a messenger RNA which comes from the macro-

phage, and that the late response is a response to the antigen itself, which is enhanced in some way by the presence of RNA coming from the macrophage.

DR. FISHMAN: Well, this is why I emphasized that unfortunately the marker is on the L chain. We know that the antibody site of globulin is probably on the H chain. Therefore, we still have doubt as to whether or not the coding of allotype I described is significant for antibody synthesis. In the near future we will deal with allotype marker on the H chain as well. We shall see if our ideas are confirmed, and whether the superantigen involves just an increased antigenicity of the fragment or whether the RNA may act as a suppressor RNA bringing about a change in the messenger of a lymph node cell. This would mean that the antigen itself does not have to do it. The whole process can be done by the RNA that is coming along with the antigen, and if this RNA is specific, and this again we do not know, this might be the way in which the globulin synthesis is altered.

DR. CLARK: This is addressed to Dr. Fishman or to anybody else that has had experience enough to give an answer. What are the possibilities that a messenger RNA extracted under your conditions and then put into an *in vitro* system can actually lead to extracellular protein synthesis in a situation where cells are dying and cellular products are present in the medium? How easy is it to induce extracellular protein synthesis within a mass of cells?

DR. FISHMAN: It is very easy for viruses to do it. We cannot be sure that extracellular protein synthesis does not occur under our conditions. We are observing a phenomenon. Our interpretation may have to be modified.

DR. GLICK: I may have some sort of an answer for Dr. Clark's question. We are using a system now where we treat tumor cells with mammalian DNA preparations. [Glick, J. L., and Goldberg, A. R. (1966) *Trans. N.Y. Acad. Sci.* **28,** 741]. We find an increase of RNA synthesis and apparently temporary stimulation of protein synthesis in the DNA-treated cells which are subsequently killed, presumably by the newly formed proteins. This effect is rather specific, depending upon the source of the DNA. Also, indirect experiments indicate that the newly formed RNA seems to be at least in part transcribed by the donor DNA rather than by the host DNA. I wanted to ask Dr. Fishman, though, what is the minimum length of time that the macrophage RNA has to be in contact with the cells? Does the length of RNA incubation depend upon the type of active RNA, that is the RNA itself or the RNA-antigen complex?

DR. FISHMAN: If we added RNAse to our tissue culture medium 6 hours after the RNA was put in, it no longer inactivated the RNA. Therefore all we know is that the process can go on at least within 6 hours. Placing labeled RNA onto lymphocytes, we observed that the process can occur in a few hours.

The Role of Antigen in the Regulation of Antibody Formation*

JONATHAN W. UHR AND KENGO HORIBATA†

Irvington House Institute and Department of Medicine, New York University School of Medicine, New York, New York

The nature and dose of immunogen can markedly affect the rate and duration of formation of particular classes of antibody. For example, 19 S antibody formation of long duration results from immunization of mice with large but not small doses of rat erythrocytes (Borel *et al.*, 1965). Immunization of rats with *Salmonella* flagella stimulates 19 S followed by 7 S antibody formation; immunization with *Salmonella* flagellin stimulates 7 S antibody formation only (Nossal *et al.*, 1964). Such observations have focused attention on the role of antigen in the regulation of the antibody response.

The purpose of this paper is to discuss the possible mechanisms underlying this regulation. Two sets of data that bear on this question will be discussed. The first deals with the kinetics of antibody formation to bacteriophage ϕX 174 (ϕX) in guinea pigs. The second deals with studies of the antibody content of individual antibody-forming cells isolated from rats immunized with *S. paratyphi* B flagella.

I. KINETICS OF ANTIBODY FORMATION TO BACTERIOPHAGE ϕX 174 IN GUINEA PIGS

The effect of dose of phage on the slope of increase of 19 S serum antibody has been studied in guinea pigs over a 10^6 dose range of phage (Uhr

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† Present address: The Salk Institute for Biological Studies, Post Office Box 1809, San Diego, California 92112.

and Finkelstein, 1963). As can be seen in Fig. 1, the slope of increase was constant above a critical dose of phage; with this preparation, just above 10^9 particles. The slope can be conveniently expressed as the time necessary to double the serum antibody level which in this instance is 6–7.5 hours. Below 10^9 particles, and in the above case in two out of five

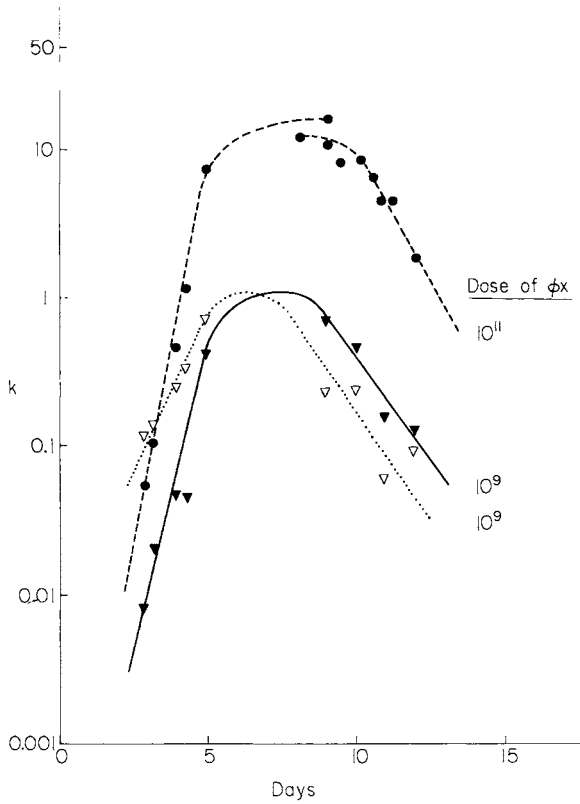

FIG. 1. The primary 19 S antibody response to ϕX in the guinea pig. The responses of representative animals injected with 10^9 or 10^{11} ϕX are shown (Uhr and Finkelstein, 1963).

animals injected with this dose, longer doubling times were obtained. In primary 7 S antibody formation (see Fig. 2) and in secondary 7 S antibody formation, slopes of antibody increase were also dose dependent within a particular range of antigen. Thus, in each of these three antibody responses to ϕX, the dependence of the slope of antibody increase on dose of phage can be demonstrated within particular ranges of antigen.

Results similar to these have been described by Taliaferro and Taliaferro (1963) in the primary hemolysin response to sheep red blood cells

(SRBC) in rabbits. Although the slope of hemolysin response was independent of antigen dose between $10^{6.5}$ and $10^{10.6}$ SRBC, the slope was less steep with doses below $10^{5.2}$. Svehag and Mandel (1964) have shown that the doubling times of serum antibody in the rabbit to a first injection of polio virus decreased from 15 to 6 hours as the dose of virus was increased from $10^{6.4}$ to $10^{9.6}$ plaque-forming particles.

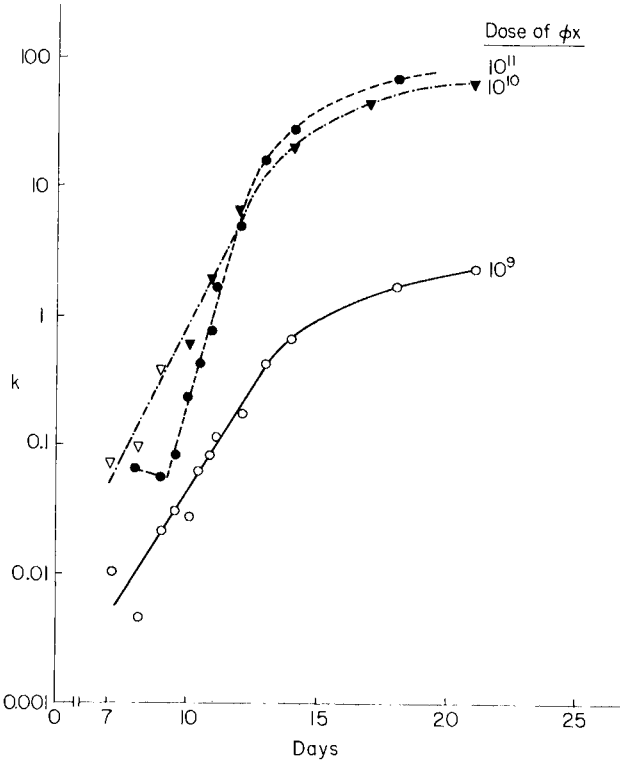

FIG. 2. The primary 7 S antibody response to ϕX in the guinea pig. The responses of representative animals injected with either 10^9, 10^{10}, or 10^{11} ϕX are shown (Uhr and Finkelstein, 1963).

Interpretation of this effect of dose of antigen depends upon an understanding of the cellular and serologic factors that affect the slope of antibody increase. Four possibilities, each of which could theoretically explain a doubling time of approximately 7 hours, are: (1) replication of antibody-forming cells, (2) increase in absolute rate of antibody formation per cell (differentiation), (3) continued entrance of cells into the antibody-forming cell compartment, (4) change in quality of antibody, i.e., increase in efficiency of neutralization per unit weight of antibody.

There is general agreement that replication of antibody-forming cells is an important factor in the secondary response (Coons *et al.*, 1955; Nossal and Mäkelä, 1962a; Urso and Makinodan, 1961; Baney *et al.*, 1962) but no such agreement exists regarding replication in the primary 19 S response. For example, there are three different published interpretations regarding the role of replication in 19 S hemolysin formation in mice: (1) Jerne's interpretation (Jerne *et al.*, 1963) which is that replication of the small number of plaque formers in the spleen before immunization is entirely responsible for the rapid increase of serum antibody concentration after immunization. This conclusion is based on the facts that (a) in adult mice increase in the number of plaque formers in the spleen is precisely similar to the increase in serum hemolysin concentrations. (b) Studies by Koros *et al.* (1966) in which tritiated thymidine incorporation was demonstrated in 55% of plaque formers after a 30-minute *in vitro* pulse. Such short periods of exposure to thymidine-^{3}H only give information on the ratio of *S* period to generation time, however. Therefore, deductions about generation times are only possible if assumptions are made concerning the duration of the *S* period. (2) The conclusion of Šterzl *et al.* (1965) which is that the precursors of the plaque formers are not the "background" plaque formers, but that differentiation of precursors without replication occurs first, followed by their replication. This conclusion was deduced from studies in which thymidine-^{14}C was injected repeatedly into mice during the 24 hours prior to sacrifice in an attempt to label plaque formers. It was observed that 50% of plaque formers were labeled 4 days after immunization but that "shortly after immunization" there was no incorporation into plaque formers. (3) In contrast to these interpretations, Tannenberg (1966) has concluded that 19 S plaque formers are replicating at relatively slow rates based on the results of labeling plaque formers after continuous intravenous infusion of thymidine-^{3}H over a period of 24 hours.

Plaque formers were lightly labeled and a similar percentage of large lymphocytes that were not plaque formers were similarly labeled. Tannenberg assumed that these latter cells are the precursors of the plaque formers, that these precursors are replicating slowly, and that antigen does not change this rate of replication. If one assumes, however, that the small lymphocyte is the precursor of the 19 S antibody-forming cell (Gowans and McGregor, 1965), then Tannenberg has proved that antigen does increase the rate of replication of these cells. Regardless of which of these interpretations is the correct one, it can be appreciated that precise determination of the generation time of antibody-forming cells is difficult to obtain.

The second possibility, an increase in absolute rate of antibody forma-

tion per cell, is suggested by the morphological changes that accompany the differentiation process of plasma cells. Thus, as immature plasma cells change to mature plasma cells, there is an increase in cytoplasmic content of ribosomes as well as endoplasmic reticulum (Bessis, 1961), in other words, the machinery for synthesis and secretion of protein. Kinetics of serum antibody increase and morphological changes have not been systemically studied in parallel in a particular immunization regimen, however, so that the extent of differentiation that takes place during the period of exponential increase is not known.

Little is known about the rate of initial stimulation (i.e., "recruitment") of potential antibody-forming cells. Stimulation of precursors could occur throughout the exponential rise of serum antibody, perhaps at an exponential rate. At the other extreme, stimulation of potential responders may be completed within hours after antigen administration, particularly after secondary immunization. One way of obtaining further information concerning the rate of stimulation may be to study *in vitro* stimulation of immune cells in which the period of stimulation can be better controlled. In addition to the problem of initial stimulation, it is not known whether immunologically competent cells (X cells) (Sercarz and Coons, 1962) differentiate directly to antibody-forming cells (Z cells) after stimulation or whether X cells become "specifically primed" but nonproducing cells (Y cells) first, or both. Thus Z cells may arise from two cell compartments.

Changes in the quality of serum antibody (Taliaferro *et al.*, 1959; Finkelstein and Uhr, 1966; Svehag, 1965) during immunization have been studied, and the results suggest that this is *not* a major factor in the slope of serum antibody increase.

In evaluating the role of these first three possibilities, the apparent exponential character of serum antibody increase should be emphasized. This increase may deviate from true first-order kinetics, however, because the assay for neutralizing antibody is not that precise and the rapid increase in serum antibody is usually observed only over a range of 1000-fold increase. However, since the kinetics look grossly exponential and antibody-forming cells are dividing, the most likely explanation is that proliferation of antibody-forming cells plays a major role in the slope of increase of serum antibody in all antibody responses, whether 7 S or 19 S. Both differentiation and "recruitment" probably also contribute to this slope but there is no data to warrant speculations concerning the extent of these contributions.

The next question to arise is how to interpret the slower relative rates of antibody formation with lower doses of antigen. Lower doses may slow the rates of either of these above three factors regardless of whether replication is the major factor in determining maximal slopes of increase.

For example, "recruitment" of potential responders may be a minor factor responsible for rapid increase in slope but lowering antigen dose might slow "recruitment" and make it rate limiting with respect to increase in slope.

Changes in the kinetics of antibody formation can also be effected by the passive administration of antibody after immunization. For example, ϕX-immunized guinea pigs that received 7 S guinea pig antibody to ϕX 3 days after immunization, showed a diminished peak 19 S response and failed to develop the expected 7 S response (Finkelstein and Uhr, 1964). Generally similar results have been reported with other antigen-antibody systems (Rowley and Fitch, 1964; Möller, 1964; Sahiar and Schwartz, 1964). The specificity of suppression indicates that the injected antibody combines with persisting immunogen and prevents it from further stimulating the immune mechanism. This experiment suggests therefore that the persistence of antigen is important for continued maximum stimulation of the primary 19 S response, possibly because initially stimulated cells need continued stimulation by antigen. Obviously, additional techniques are necessary to obtain further information concerning these possibilities.

II. Immune Functions of Individual Antibody-Forming Cells

Mäkelä and Nossal (1961a,b) have demonstrated the feasibility of studying immunologic and synthetic activities of individual antibody-forming cells. Their means of identification of specific antibody-forming cells is to use flagella from highly motile bacteria as antigen and the capacity of bacteria sharing the same flagella antigens to specifically adhere to antibody-forming plasma cells. Such adherence-positive cells (APC) can then be micromanipulated into microdroplets, their bacteria can be removed by passage in and out of small micropipetes, and the cells can then be washed and deposited in microdroplets for further study.

We have employed the techniques of Nossal and Mäkelä in order to study whether dose of antigen can affect the rate of differentiation of individual antibody-forming cells. We studied such cells following two immunization regimens: (1) injection of flagella alone, (2) injection of flagella followed 24 hours later by injection of homologous specific antiserum. The purpose of the injection of antibody after immunization was to allow initial stimulation of antibody-forming cells which would then subsequently be deprived of continued antigenic stimulation. This type of immunization regimen should increase the possibility of detecting the role of persisting antigenic stimulation. Our objective was to study the rate of antibody synthesis of single cells following these two immunization regimens. Our attempts at cultivating single cells in microdroplets indi-

cated that cells did poorly under the *in vitro* conditions used for single cell studies and that studies of rates of synthesis would be difficult. As a first step toward our objective, therefore, we determined the amounts of antibody contained within single antibody-forming cells by enumerating the number of bacteria that can be immobilized by antibody-forming cells that have been disrupted in microdrops. We reasoned that antibody content of individual cells may be related to rate of antibody synthesis during the phase of rapid increase in the rate of antibody formation.* In other words, as immature antibody-forming cells differentiate to mature antibody forming cells, the cytoplasmic machinery for protein synthesis and secretion increases; hence, there should be more antibody in the process of synthesis and secretion. It was possible to test this assumption partially by determining antibody content of antibody-forming cells removed at different intervals after immunization.

After first determining the conditions for partial suppression by passive antiserum, the antibody content of single cells was then examined at various intervals after such an immunization regimen compared with cells removed from animals immunized with antigen alone (Horibata and Uhr, 1967). As can be seen in Table I, in the immunized group, APC were present 4 days after immunization. At this time none of them contained detectable antibody. By day 5, half the APC contained relatively large amounts of antibody and by day 6 and 7 all the cells showed large amounts of antibody. On days 10 and 14, APC were infrequent and only seven cells were examined of which only one contained antibody; at these times, many of the positive cells had morphological stigmata consistent with loss of viability. It appeared therefore that the peak of the cellular response was over. Consistent with this conclusion were the serologic observations which indicated that like other 19 S antibody responses (Uhr and Finkelstein, 1963; Taliaferro and Taliaferro, 1963; Svehag and Mandel, 1964), peak titer was reached at approximately 1 week and had declined to virtually background levels by 2 weeks.

In the group that received passive antibody 1 day after immunization, APC which did not contain antibody were also observed on day 4. In contrast to the control immunized group, however, none of the cells examined on days 5, 6, and 7 after immunization contained detectable antibody. Only two cells were found on day 10 or 14 after immunization and neither of them contained antibody.

The possibility that APC resulted from passive sensitization by either "free" antibody (Boyden and Sorkin, 1960) or by antigen-antibody com-

* As immunization proceeds and the slope of increase of serum antibody declines, aging plasma cells may begin to store antibody.

TABLE I

EFFECT OF PASSIVELY ADMINISTERED SPECIFIC ANTIBODY ON ANTIBODY CONTENT OF APC FROM IMMUNIZED RATS (HORIBATA AND UHR, 1967)

Passive antibody[a]	Day after immunization[b]	Animal No.	Agglutination titer of serum[c]	No. of APC containing antibody/No. of APC tested	Average No. of bacteria immobilized/APC (range)
0	4	100	256	0/5	< 10
	5	94	256	6/10	400 (80-1,000)
	6	96	4096	10/10	200 (100-300)
	7	86,88	256,1024	7/7	250 (100-500)
	10	90	16	0/1	< 10
	14	92	128	1/6	600
+	4	101	128	0/2	< 10
	5	95	128	0/12	< 10
	6	97	32	0/4	< 10
	7	85,87,89	32,128,16	0/6	< 10
	10	91	16	0/2	< 10
	14	93	8	None observed[d]	

[a] 0.25 ml per rat anti-*S. paratyphi* B injected into each rear footpad 1 day after immunization.

[b] Each rat injected with 5 μg *S. paratyphi* B flagella into each rear footpad.

[c] Serum obtained just prior to sacrifice.

[d] 10^5–10^6 cells scanned.

plexes (Uhr, 1965) was considered. Indeed, during the course of these studies, it was shown that flagella-antibody complexes are capable of sensitizing plasma cells as well as phagocytes *in vitro*. However, the APC which appeared *in vivo* after immunization with flagella or flagella-antiserum combinations did not appear to represent passive sensitization for two reasons: (1) When flagella and antiserum were given at the same time, no APC were detected one week after immunization. (2) Passive sensitization *in vitro* results in preferential sensitization of phagocytes. Phagocytes can frequently be identified under phase contrast microscopy. The phagocytes obtained from the nodes of immunized animals did not appear to be sensitized.

It should be emphasized that the percentage of APC in the immunized group that received antibody was markedly less than the percentage of APC in the conventionally immunized group, varying from 0.01 to 50% in different experiments. There was no obvious difference in appearance under phase contrast microscopy, however, between the positive cells of the two groups. The majority of cells were of a size consistent with plasma cells but smaller cells that could have been lymphocytes were also observed. In addition, there was no observed difference between the numbers of bacteria adherent to the surface of APC from the two groups.

These results suggested a difference in the antiflagella (anti-H antigen) antibody content of cells from immunized animals depending upon whether passive antibody was injected 1 day after immunization. An alternate possibility was that APC detected in the group receiving passive antibody had specificity to the somatic (O) antigen of *S. paratyphi* B and contained antibody which was, therefore, not immobilizing (Nossal and Mäkelä, 1962b). This possibility was definitively tested by doing similar experiments but testing for APC and immobilizing antibody with a strain of *Salmonella* that shares the H but not the O antigen with *S. paratyphi* B, namely *S. atlanta*. In addition, similar experiments were performed in which rats were immunized with *S. adelaide* flagella (an antigen unrelated to *S. paratyphi* B) and were tested with *S. derby* which shares H but not O antigens with *S. adelaide*. The results of this experiment were analogous with those previously reported, that is, all APC on day 6 or 7 in the control immunized groups had detectable immobilizing antibody. In contrast, none of the APC from animals of either of tde immunized groups that received passive specific antibody contained detectable immobilizing antibody on these days.

We recognize that the limitations of the methodology employed above as well as lack of information about the cellular events underlying antibody formation make it impossible to offer a unique interpretation of these experiments. The simplest interpretation is that initially stimulated anti-

body-forming cells have been deprived of antigen by injection of antibody 1 day after immunization and that such cells then differentiate more slowly and perhaps not as fully as they would have. There are two other possibilities that cannot be excluded: (1) potential antibody-forming cells differentiate at later times in the group receiving antibody passively than in the group that is conventionally immunized. This situation could arise through later initial stimulation or later change from the Y to Z compartment as previously discussed. Arguments against this explanation are: (a) both groups were exposed to the same quantity of antigen for the first day of immunization during which time a large proportion of the potential responders is expected to be stimulated. (b) APC were detected in both groups on day 4 and if these cells had differentiated at the same rate in both groups, some antibody containing-APC should also have been detected on day 6 or 7 in the group that received antibody. This was not the case. (c) After 1 week, APC were not detected in significant numbers in the group receiving antibody, i.e., cells entering the Z compartment later than 1 week were not detected. These arguments are weakened by the difficulties of sampling APC which constituted as little as 0.01% in some populations. (2) APC in the group that received antibody are not Z cells but Y cells. This is unlikely because in these animals in which small amounts of antibody were being formed, it would be unlikely *not* to detect the Z cells that are present and only to detect Y cells. Moreover, when antibody formation was completely suppressed by the passive administration of antibody simultaneously with flagella, then no APC were detected.

There are two general mechanisms by which antigen can regulate the immune response: (1) antigen may stimulate cells in an all-or-none fashion such that differentiation and replication at fixed rates occur independent of quantity or quality of initial stimulation or of continued stimulation by antigen. Thus, X cells become either Y or Z cells after stimulation by antigen, and Y cells, in turn, need a second stimulation by antigen to become Z cells. Regulation of the immune response by antigen is achieved mainly by determination of the rate of stimulation of X cells, how many X cells are eventually stimulated, and the rate at which Y cells are stimulated to become Z cells. (2) Antigen can regulate the pathway and the rates of differentiation and replication of individual immune cells. This regulation can arise either through the amount or kind of initial antigenic stimulation or through continued antigenic stimulation or both.

We interpret our results of study of single antibody-forming cells to favor the possibility that antigen can regulate the rate of differentiation of immune cells. In addition, our studies suggest that this regulation arises at least in part from antigenic stimulation after the initial antigenic contact.

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DISCUSSION

DR. BARNES: Did you detect any difference between the two categories of antibody-responding cells you described? Were these cells large or small lymphocytes, or plasma cells?

DR. UHR: Under phase contrast microscopy there was no striking difference in the appearance of the adherence-positive cells from the two groups. First of all, there was no difference in the average numbers of bacteria adherent to cells. Secondly, most of the cells were of a size compatible with plasma cells, i.e., the cells were considerably larger than small lymphocytes. There was a proportion of cells, however, that appeared smaller and that could be considered to fall into the category of lymphocytes.

DR. HOROSZEWICZ: How much antibody did you have to inject simultaneously with antigen to get the suppressive effect and how small an amount of antibody could you inject and still obtain the effect?

DR. UHR: In the system used we were not interested in studying the phenomenon of

antibody suppression per se, and wanted to use antibody-induced suppression only as a tool. In other immunization systems, however, we and others have made the kind of titration you are asking about and the answer depends on the immunization system employed. For example, Dr. Dixon can suppress the rabbit antibody response to hemocyanin by injecting an amount of antiserum which can combine *in vitro* with only 1% of the antigen at equivalence. The injection of antiserum must be given 1 day after antigen; if given simultaneously, much larger amounts of antiserum are required. With ϕX, in the guinea pig, larger amounts of antiserum are required if antiserum is injected after antigen. The reason for the differences observed using different antigens is not known.

DR. BENACERRAF: Since you referred to Dr. Dixon's results I should mention that in his experiments he had to use late antibody to obtain inhibition. From what is known of the maturation of antibody specificity with time, antibodies produced late in immunization are presumed to have high affinity.

DR. SCHLESINGER: You measured the titer of antibody in the serum of rats that received the antigen and the antiserum. Was the test reaction based upon agglutination or immobilization? Is there a different distribution among the different antibodies produced in different cells?

DR. UHR: There was a considerable but incomplete suppression of the primary 19 S response. The results shown were based upon agglutination assays. In addition, we did immobilization assays and the results were analogous except that in this case antibody titers were ten to fiftyfold higher than those obtained by agglutination.

DR. SCHLESINGER: Does this mean that there was dissociation of antibody formation between the APC and the other types of lymphoid cells? Why did not the APC cells contain the immobilizing antibody while the serum did?

DR. UHR: The simplest explanation is that the individual positive cells in the animals that were "suppressed," were forming antibody at absolute rates considerably lower than the positive cells of the conventionally immunized animals.

DR. ŠTERZL: By the use of the specific antiserum, the number of antibody-producing cells may be restricted in the primary response but the number of cells prepared for the secondary response (Y cells) may be increased. One of our students did experiments similar to those you described. There is no doubt that by injecting antiserum the onset of antibody formation is changed, the slope is not as steep as without antiserum, and a smaller number of cells is detected by the hemolytic plaque technique. If, later on, a second dose of antigen is injected, the secondary response is significantly enhanced. I wonder whether the effect of antiserum might not be explained similarly to the results obtained in the case of antigen dose-dependent responses [Šterzl (1966) *Nature* **209,** 416]. It can be suggested that the antiserum bound a certain quantity of antigen otherwise available. The activated cells (Y cells) proliferate with smaller amounts of antigen and only a small number of these Y cells might be turning into Z cells, namely, antibody-forming cells. The secondary response would be prepared, however. Did you test this possibility by eliciting the secondary response?

DR. UHR: No, we did not.

DR. SCHWARTZ: We have done the same experiment as Dr. Šterzl and obtained the same results.

DR. UHR: The effect of passive antibody administration on "priming" for a secondary antibody response appears to depend on the immunization system employed. For example, diphtheria toxoid-rabbit antitoxin complexes, which are very difficult to dissociate, not only do not give a primary response but frequently do not "prime" efficiently for a booster response. If rat antitoxin is used in the complexes instead of rabbit antiserum, then an increase in "priming" efficiency compared to that of "free" toxoid results, in agreement with Drs. Šterzl and Schwartz's findings.

DR. ŠTERZL: I think that this is in very good agreement with our hypothesis. Whether the secondary response is enhanced or suppressed depends on the quantity of antigen available to the cell during primary stimulation.

DR. BIOZZI: I agree with Dr. Uhr that the presence of antigen is certainly a limiting factor in the immune response. In fact, in studies of the kinetics of increase of antibody-producing cells, we have shown that after the fifth day there is no further rise in the number of these cells. This seems to be due to a lack of antigen because if you give a supply of antigen at this time the number of cells continues to rise for 3 or 4 days to about the same extent as during the earlier part of the primary response. This is not a secondary response because the antigen is given during the primary increase in the number of cells. A third supply of antigen has no further effects, however. Therefore, it is possible that there is another limiting mechanism which controls the cell response. In another connection, can memory cells be detected by your technique of adherence? With our method of rosette formation we could not detect memory cells, namely, cells involved in the secondary response which had been initially stimulated by subimmunogenic doses of sheep red cells. Thus, we concluded that cells, which have acquired the specific memory after contact with the first antigen, do not show the adherence phenomenon.

DR. UHR: I think Nossal did not find significant numbers of adherent positive cells in the nodes of immunized rats before secondary immunization. Of course, there is a serious sampling problem, depending upon the number of "memory" cells and the number of cells that can be screened with this technique. Also, many "memory" cells are circulating cells and may not be found by the examination of a particular lymphoid organ.

DR. BARNES: What type of antibody did you use for passive sensitization or blockage? What type of response would you observe if you used, for example, 19 S antibody for tying up the antigen in circulation?

DR. UHR: I have not used 19 S antibody for suppression in this particular system. In ϕX immunization of guinea pigs, the 19 S antibody obtained 1 week after immunization and injected at the same time as antigen can partially suppress the 19 S antibody response. In this case a relatively high titer of neutralizing antibody is needed, however.

DR. BENACERRAF: Can you mimic the type of response you observed in animals which received your standard dose of antigen and the antiserum passively 1 day later by simply decreasing the dose of antigen initially administered? Concerning the interpretation of the data, you choose to consider the suppressive effect of antibody on antibody synthesis as acting on the cell already producing antibodies rather than at a stage previous to differentiation by simply binding antigen. If I understood your interpretation correctly, this would further imply that one would expect a similar phenomenon of inhibition to occur regularly in the cell producing its own antibody.

DR. UHR: I cannot answer the first question. We have not done that experiment. In regard to the second question, I favor the interpretation that the modulating effect of antigen as revealed by the injection of antibody occurs after the initial antigenic stimulation, but I do not want to suggest a particular duration of antigen effect. This duration may be only 1 or 2 days. Certainly, 3 days after the injection of antigen, it is difficult to detect an effect of passively administered antibody. At the other extreme, antigen influence may continue throughout the entire primary antibody response. Other studies of the effect of reinjections of specific antigen on the kinetics of 19 S antibody formation to ϕX support this latter possibility. You also asked why antibody within the cell does not turn off antibody formation. One possibility is that the antigen is outside the antibody-forming cell or, if it is inside the cell, it may be compartmentalized and unavailable to antibody being synthesized within the cell. Secondly, the antibodies produced early in the immune response are less efficient in suppression than the type of antibodies used in our experiments which were obtained

from hyperimmunized animals. The differences in efficiency in suppression between these two types of antibodies may be caused by differences in their binding affinities.

DR. YAGI: In a recent paper on immunoglobulin formation in germ-free piglets, Kim, Uhr, and Horibata [(1966) *J. Immunol.* **97**, 189] suggested that the 19 S antibody seen in the early response is not really IgM, but 19 S IgG, which is quite different from IgM and which is the first immunoglobulin produced in germ-free piglets.

DR. UHR: In 1962 we published a report of the antibody response of newborn humans and also older children to bacteriophage ϕX. We studied two sera from one child, obtained early and late in immunization respectively, by precipitation with rabbit antihuman γM or antihuman γG as well as by sedimentation and mercaptoethanol susceptibility. We were able to precipitate out almost all neutralizing activity from the early antiserum with anti-γM but not with anti-γG antiserum. In the late antiserum, it was anti-γG that precipitated out neutralizing activity and not anti-γM. We therefore thought the results were clear cut. However, only two human antisera were studied in this fashion. Perhaps someone else has further evidence concerning this question.

DR. TOMASI: I would like to emphasize the point that Dr. Yagi brought up, namely, that in defining an antibody as being either IgM or IgG, it is really necessary to do something more than simply to measure its position on a density gradient. Recently we have had the experience of finding a so-called 19 S antibody which actually was of the IgA type, in other words, a polymer of 7 S IgA. In addition, we have been interested more recently in human 7 S antibodies which possess IgM determinants, perhaps similar to certain lower species which have both 7 S and 19 S IgM antibodies. I wonder, in experiments with 6-MP for example, whether the light or 7 S antibodies have really been shown to be IgG. I think it is necessary to do other things such as absorption experiments as suggested by Dr. Uhr, to clarify this fully. This is particularly necessary in view of the enhancing effects of 6-MP on IgM production.

DR. BENACERRAF: I would like to point out that 19 S macroglobulin is not the only immunoglobulin which is apparently synthesized early in immunization and then replaced by 7 S antibodies. The "anaphylactic" type of antibody of two animal species, the rat and the rabbit, that is, the antibody type which causes the release of vasoactive amines from mast cells, is also synthesized very early in the course of immunization. Its serum concentration may peak as early as the seventh to the ninth day. Its synthesis stops by the twentieth day in most cases. Such animals, when reinjected with antigen, no longer make "anaphylactic" antibody. Levels of 7 S and 19 S immunoglobulins continue to be synthesized independently of this immunoglobulin.

DR. NICHOL: You showed a curve representing the kinetics of the response to the phage antigen, Dr. Uhr, namely the rapid increase and leveling off and then a rapid decrease of antibody levels. There is some similarity to the curve that describes the kinetics for the increase and decrease in the activity of certain adaptive enzymes, such as tryptophan pyrrolase and tyrosine transaminase. Since this involves an instance of induction and regulation of the synthesis of a different type of protein, it might have some relevance. The shape of the curve for enzyme activity can be modified by quite a number of factors such as the presence of small molecules which can stabilize the enzyme against proteolytic degradation, the rate of turnover of the messenger RNA, the possiblity that a repressor is formed that shuts off the production of the proteins and the possibility that rapid protein synthesis may deplete the intercellular pool of amino acids required to continue the process. If you were to view the antigen as a drug that initiates the process in some manner, for example, like the initiation of the synthesis of a variety of adaptive enzymes by corticosteroids, would you say that the antigen exerts an effect which is then over and done? Over what period does the antigen exert its effect?

DR. UHR: There is indirect evidence suggesting that in the 19 S antibody response, continued antigenic stimulation is necessary for continued synthesis of 19 S antibodies. This conclusion is based not only upon the effect of passive administration of antibody but also upon the effect of giving a second dose of specific antigen at the time that 19 S serum antibody levels are beginning to fall rapidly. The second dose of antigen renews 19 S antibody formation at rapid rates. Dr. Biozzi showed a similar effect of a second dose of sheep red cells on the increase in number of rosette formers in the mouse spleen. These observations suggest that the 19 S mechanism is running out of antigen. It is probable that 19 S antibody-forming cells that have stopped synthesizing antibody begin to synthesize it again after reinjection of antigen. An alternate possibility cannot be excluded, however. It is that "primed" cells are also being formed at rapid rates during the primary response and that the second injection of antigen is simply restimulating "primed" cells to become antibody-forming cells rather than restimulating the antibody-forming cells themselves.

Alteration of Immunity by Antimetabolites*

ROBERT S. SCHWARTZ

Clinical Immunology Service, New England Medical Center Hospitals,
and the Department of Medicine,
Tufts University, School of Medicine, Boston, Massachusetts

Many cytotoxic drugs having powerful effects on the immune response are now available for experimental and clinical use. These materials include the alkylating agents nitrogen mustard and cyclophosphamide, the antipurines 6-mercaptopurine and azathioprine, pyrimidine antagonists such as 5 fluorouracil, and the folic acid analog amethopterin. When these agents are introduced into simplified *in vitro* systems, they can produce specific metabolic lesions. For example, the purine antagonists cause a block in the interconversion of nucleotides (LePage and Jones, 1961); the pyrimidine antagonists are inhibitors of DNA synthesis (Cohen *et al.*, 1958); amethopterin prevents the utilization of folinic acid (Werkheiser, 1963). However specific these effects may be, all of them ultimately lead to widespread biochemical derangements in organized living cells, since each point of attack involves a central locus of cellular metabolism. Thus, the effects of these cytotoxic drugs may be compared with the collapse of an arch brought about by the removal of its keystone.

In view of what is, in due course, a nonspecific disruption of cellular functions, it is somewhat surprising that highly specific immunological effects may be obtained with these compounds. This presentation will deal with four of them: the development of immunological tolerance, the suppression of 7 S antibody synthesis, the enhancement of antibody formation, and the suppression of antibody-mediated inflammatory lesions. Experiments carried out in laboratory animals will be compared with similar data obtained in man. The literature will not be reviewed compre-

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hensively, since several recent articles have explored the subject of immunosuppressive drugs in considerable detail (Hitchings and Elion, 1963; Schwartz, 1965; Berenbaum, 1965).

Many experiments have established that adult animals can be rendered immunologically tolerant by treatment with antimetabolic drugs such as 6-mercaptopurine, amethopterin, and cyclophosphamide. This form of tolerance resembles in its specificity the tolerant state achieved by injection of newborn animals with antigens: immunologic reactivity to second party antigens remains normal, but responses to the antigen administered during the period of chemotherapy are absent or severely depressed. 6-Mercaptopurine was shown to induce immunological tolerance in adult rabbits (Schwartz and Dameshek, 1959) in the following way: animals were given an injection of bovine serum albumin and simultaneously begun on a 2-week course of the drug, 12 mg/kg daily. Two weeks after the last injection of the antimetabolite they were rechallenged with bovine serum albumin and a new antigen, bovine γ-globulin, was administered. The drug-treated rabbits responded only to the bovine γ-globulin, whereas control rabbits responded to both antigens. It was later shown that at a given dose of 6-mercaptopurine, increases in the dose of antigen resulted in increasingly higher yields of tolerant animals (Schwartz and Dameshek, 1963). These data support the contention that failure of the immune response in the drug-treated animals may in some measure be the result of a state of absolute or relative antigen excess. The induction of immunological tolerance in adult animals with cytoxic drugs thus bears certain similarities to other maneuvers of producing immunological tolerance, such as administration of large amounts of antigen to a newborn animal, the infusion of huge amounts of protein antigen to X-irradiated adult animals, and the administration of large amounts of slowly metabolized polysaccharide antigens to adult mice (Medawar, 1960).

The importance of antigen dosage in the establishment of drug-induced immunological tolerance has also been emphasized by Uphoff (1961). She achieved specific, lasting immunological tolerance of skin allografts in adult mice. The animals were injected with a suspension of spleen cells obtained from the prospective skin-donor strain and they were simultaneously begun on a course of five doses of amethopterin. Two weeks later skin grafts from the strain of mice donating the spleen were applied. These grafts were accepted and showed no sign of breakdown during many months of observation. Very significantly, the recipients rejected third-party skin grafts in a normal manner. Mice receiving only skin allografts and treated with amethopterin retained the grafts for only a short time. Uphoff's important experiments demonstrate the following:

(a) the necessity for a loading dose of antigen, (b) the feasibility of obtaining highly specific immunological tolerance in adult animals by a brief course of chemotherapy, and (c) the importance of proper selection of donor-recipient combinations. In her experiment the donor-recipient pairs differed only in the H_1 histocompatibility locus. Experiments carried out in which the donor and recipient differed in the strong H_2 histocompatibility locus did not result in the acquisition of tolerance. The significance of this latter finding in the field of human transplantation is obvious.

A third example of drug-induced immunological tolerance is the experiment of Salvin and Smith (1964), who produced exquisitely specific deletions of immunological reactivity in guinea pigs with cyclophosphamide. The animals were injected with bovine γ-globulin and treated with eight daily doses of the alkylating agent. They not only failed to form antibodies to the bovine γ-globulin, but also did not respond to a second challenge 2 months later. However, they manifested normal delayed hypersensitivity and humoral antibody formation when challenged at that time with minor variants of the original antigen. For example, guinea pigs rendered immunologically tolerant of BGG by treatment with cyclophosphamide responded in a normal manner to *p*-aminobenzoic acid BGG. As a result of these and other experiments, Salvin and Smith proposed three levels of specificity in the immune response. At the level of drug-induced immunologic tolerance, specificity is oriented toward the whole antigen molecule; at the level of delayed hypersensitivity, specificity is directed to a smaller portion of the antigen (the protein moiety of of the conjugate); and at the level of humoral antibody formation, an even smaller part of the antigen (the hapten moiety of the conjugate) directs the specificity of the antibody molecule.

Two interpretations of the phenomenon of drug-induced immunological tolerance have been offered (Schwartz, 1966). Either a clone of antibody-forming cells has been eliminated by chemotherapy, or uncommitted cells contacted by antigen and thereby induced to the development of a specific immunological function have been suppressed. Operationally, these two possibilities are indistinguishable. The first interpretation implies a genetically enforced restriction of the responsiveness of immunologically competent cells: one antigen, one clone. The second interpretation implies an environmentally guided response of a multipotential presursor cell in the direction of the unipotential antibody-forming cell. A choice between these two alternatives is not possible within the framework of existing data.

A second order of specific immunosuppression is illustrated by the relative sparing of IgM antibody synthesis in animals treated with 6-

mercaptopurine (Sahiar and Schwartz, 1965; Borel and Miescher, 1965), amethopterin (Blinkoff, 1964), or X-irradiation (Svehag and Mandel, 1964). Since each of these agents has important effects on the early stages of antibody synthesis (Schwartz, 1965), it might be anticipated that, under appropriate circumstances, they would affect the orderly development of the immunoglobulin sequence. In studies of this type, a set of conditions in which overall antibody synthesis was not severely depressed was necessary. With 6-mercaptopurine these circumstances were obtained when the antigen was administered in a mildly adjuvant form, when it was injected locally (into a footpad), and when the dosage of 6-mercaptopurine was not excessive (Sahiar and Schwartz, 1965). Under such conditions it was found that the synthesis of IgM antibodies was unaffected, and that the formation of IgG antibodies was severely depressed. Of unusual interest was the finding that 6-mercaptopurine-treated rabbits continued to synthesize IgM antibodies for at least 2 months. In normal rabbits, IgM antibodies elaborated in response to BGG disappeared from the circulation within 3 weeks. It was this prolonged phase of IgM antibody synthesis that led to the finding that IgM antibody synthesis could be terminated by an infusion of immunologically specific IgG antibody (Sahiar and Schwartz, 1964). A mechanism by which IgG antibodies control the synthesis of IgM antibodies thus appeared probable. A similar selective effect on IgG antibody synthesis has also been found in man (Swanson and Schwartz, 1967). Patients with various immunological diseases undergoing treatment with either azathioprine or amethopterin have been immunized with keyhole limpet (*Fissuxella* sp.) hemocyanin at the onset of the chemotherapy. In approximately 25% of these cases it was found that the synthesis of 7 S anti-hemocyanin antibodies was abolished, while the formation of 19 S anti-hemocyanin antibodies remained intact. This finding may have clinical importance, since not all autoantibodies are of a given molecular class. For example, the cold agglutinin responsible for certain types of autoimmune hemolytic anemia is a macroglobulin, and it is of some interest that cold agglutinin disease is generally resistant to therapy with immunosuppressive drugs.

A novel and perhaps important immunological effect of 6-mercaptopurine, the enhancement of antibody formation, has recently been described (Chanmougan and Schwartz, 1966). These experiments were undertaken when it was found that a 1-week course of 6-mercaptopurine given to normal rabbits resulted in hyperplasia of the lymphoid system (Sahiar and Schwartz, 1966). The hyperplasia was discernable within 5 days of the last dose of drug and persisted for at least 3 weeks. Rabbits treated with 6-mercaptopurine and challenged with small doses of a

protein antigen (bovine γ-globulin) at the peak of the hyperplastic phase were found to elaborate large amounts of circulating antibody (Fig. 1).

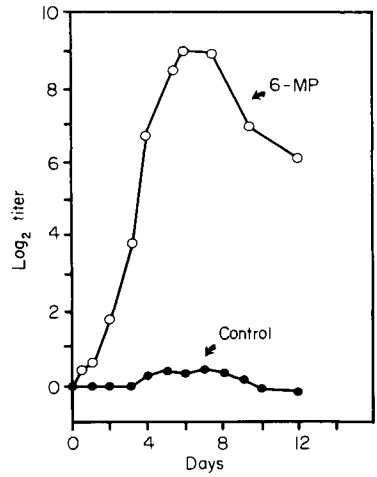

FIG. 1. Enhancement of antibody synthesis by 6-MP. Both groups of rabbits received 20 μg of BGG intravenously. The control animals barely responded to this dose of antigen. Rabbits given the antigen 5 days after the last dose of a 1-week course of 6-MP, 10mg/kg, promptly synthesized high titers of antibody (measured by tanned red cell agglutination).

This response was extremely rapid; the induction period in many animals was 24 hours or less. These serological responses were also accompanied by rapid changes in lymph node cytology. Thirty-six hours after antigenic challenge, antibody containing plasma cells could be found in the lymph nodes (Fig. 2). These were never observed at that time in normal rabbits challenged with the same antigen. It may be of some interest that a similar enhancement of antibody synthesis has also been observed in humans undergoing amethopterin therapy for immunological diseases (Swanson and Schwartz, 1967). In these cases, antibody formation began after a prolonged induction period, but the levels of circulating antibody formed after challenge with keyhole limpet hemocyanin were extremely high. Despite this unusual response, definite amelioration of the clinical condition was observed.

This paradox, beneficial effect of an immunosuppressive drug on an immunological disease in individuals whose antibody responses are actually enhanced, may be related to another important effect of these compounds. There is now substantial evidence that certain antimetabolites have the capacity to block certain types of tissue injury without necessarily inhibiting the immune status of an experimental animal. In other words, the peripheral expression of immunity may be blocked

without a concomitant depression of the antibody-forming system. This phenomenon has been carefully studied by Page and his colleagues (Page *et al.*, 1962) who demonstrated that 6-mercaptopurine can profoundly

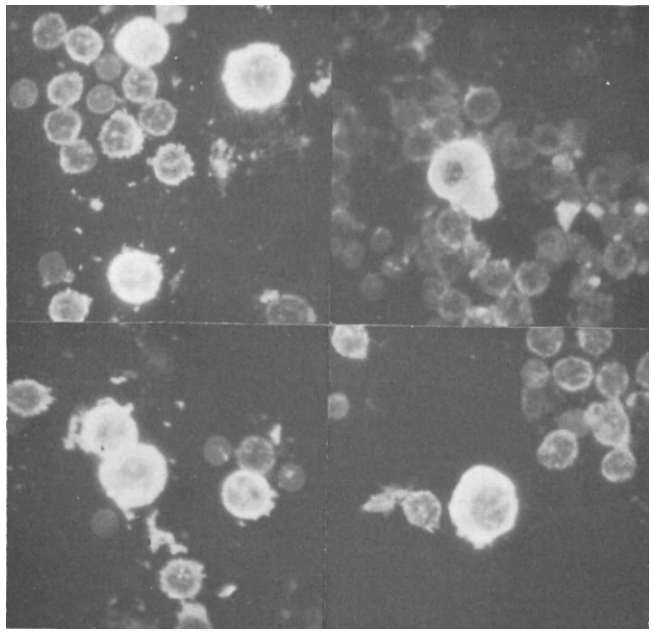

FIG. 2. Specific antibody-containing cells in lymph nodes of rabbits 36 hours after antigenic challenge. These animals were pretreated with 6-MP and challenged with 20 μg of BGG. Such cells are never seen this early after antigenic challenge of normal rabbits.

alter the inflammatory cycle that occurs in rabbit skin following the injection of egg white. Spiegelberg and Miescher (1963) found that 6-mercaptopurine was capable of suppressing experimental allergic thyroiditis in the guinea pig without affecting circulating antibody. Another experiment (Borel and Schwartz, 1964) of this type is shown in Table I. The results recorded there indicate that 6-mercaptopurine can abolish the formation of an active Arthus reaction without affecting levels of circulating antibody or the biological potency of that antibody. Thus, the clinical effects and certain of the experimental findings obtained with these compounds are very complex. The results in experimental animals depend upon the choice of antigen, the dosage employed, its route of administration, and the timing and dosage of drug therapy. The clinical outcome depends not only on these factors, but on the anti-inflammatory properties of these agents as well.

TABLE I
EFFECT OF 6-MP ON ESTABLISHED IMMUNITY[a]

	Experimental day		
	60	74	81
Active arthus	6/6	0/6	6/6
Passive arthus	6/6	6/6	6/6
Hemagglutination titer	>56,000	>56,000	>56,000
Ring test	++++	++++	++++

[a] Inhibition of the Arthus reaction in 6-MP treated rabbits. The animals were injected four times with BGG emulsified in Freund's adjuvant. Following these immunizations, on the sixtieth experimental day, they had the expected immunological reactivity. A 2-week course of 6-MP was then given. At the end of that treatment, biologically active antibody (as indicated by the capacity of the serum to induce a passive Arthus reaction, second line of the table) was still present in large amounts. Nevertheless, the active Arthus reaction was blocked. One week after discontinuation of the 6-MP, full reactivity returned. These experiments show inhibition of the peripheral manifestations of antibody-mediated lesions without central inhibition of immunity. They also demonstrate how such lesions can be blocked in a sensitized individual.

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DISCUSSION

DR. NICHOL: The use of drugs is going to raise some of the questions yet to be answered concerning control and regulation of the immune response. At the outset of our discussion, however, it might be pertinent to make a distinction between what we refer to as the mechanism of biochemical action of some of these drugs and what we sometimes refer to as the mechanism of their chemotherapeutic action. The former really involves some knowledge of the drug receptor and, in the case of most of the drugs that you mentioned, clear identification of the drug receptors is lacking. The mechanism of chemotherapeutic response, however, is related to the innate characteristics of cells of different types, which may explain the selective effects of the drugs on one type of cell and not on another. It is in this area that there is some parallel with the problems related to the effect of drugs on the immune response. Although knowledge about the biochemical action of a drug may give us useful tools with which to approach the problem, actually we know relatively little about either the basis for the chemotherapeutic action or the mechanism of the alteration of the immune response by cytotoxic drugs.

DR. MIHICH: Dr. Schwartz, by showing that one may actually cause an increase in the immunologic response with immunodepressive agents, which happen to be anticancer agents, you are perhaps offering the hope that by proper pharmacological manipulation of some of these drugs, one may obtain an increase of the immunologic reaction which may be useful therapeutically. Some experimental models which we have in cancer chemotherapy lend themselves to testing such possibilities. For instance, using a nonspecific tumor such as sarcoma 180 in Swiss mice, we showed that the curative effect of 6-MP was due to the immunologic response of the host which was not depressed significantly by the drug [Ferrer and Mihich (1967) *Proc. Soc. Exptl. Biol. Med.* (in press)]. In this regard, I would like to mention some of the data which we obtained recently in DBA/2/Ha mice with leukemia L1210, using cytosine arabinoside and 4,4′-diacetyl-diphenylurea-bis(guanyl hydrazone) [Mihich (1966) *Proc. 9th Intern. Cancer Congr.* p. 317]. Both of these agents cause immune depression under particular conditions. Leukemia L1210 is a transplantable tumor which is highly strain specific for the DBA/2 mouse and F_1 hybrids derived from it. Although results obtained with this tumor cannot contribute to the fundamental study of tumor-specific antigens, they are quite pertinent in describing the effects of drugs in a tumor-host system where very weak antigenic differences exist between tumor and host. That L1210 is only weakly immunogenic in DBA/2 mice is indicated by the fact that this tumor kills 100% of the animals with great regularity. When 1×10^6 cells are inoculated intraperitoneally into DBA/2/Ha mice, death occurs in 7–8 days. When the L1210 bearing DBA/2/Ha mice are treated with the two drugs mentioned, 50-day cures are observed in more than 50% of the animals. When these animals are rechallenged with increasing doses of live L1210, they are resistant to transplantation. This state of resistance can be transferred adoptively by transferring spleen cells into untreated mice. Immunity is not just the consequence of immunization with cells killed by the drugs. In fact, if the hosts are X-irradiated, even though the drugs prolong survival of the animals, they do not achieve cures. Therefore, the immunity which developed during treatment was actually participating in determining the therapeutic effect. Then the question arises: is this observation simply due to pharmacological selectivity, namely, the fact that the proliferating tumor is inhibited, whereas the immunologically competent cells are not, or is it due to phenomena similar to that you are describing, namely, an actual enhancement of the immunological response caused by these drugs?

DR. SCHWARTZ: We have been very much intrigued by the possibility that under certain defined conditions these compounds can lead to enhanced immunity. 6-MP is not unique in

this regard. Johnson, of Ann Arbor, has shown a similar effect with several compounds, including cyclophosphamide and a pyrimidine antagonist; under certain circumstances, X-rays will also greatly enhance antibody synthesis. With regard to your specific question, the problem is that we have not yet learned how to use these drugs in such a way that we can get both immune enhancement and antitumor effects.

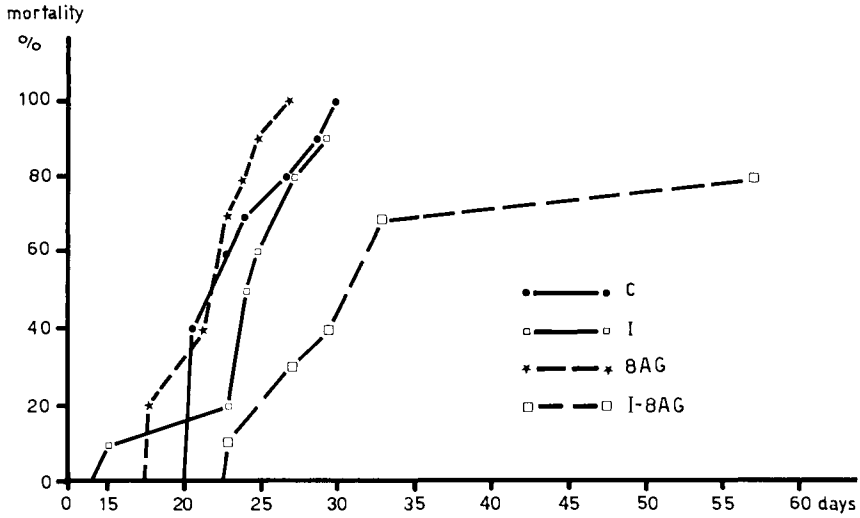

FIG. 1. Synergism between immunity and 8-azaguanine in Swiss mice bearing Ehrlich ascites carcinoma (500,000 tumor cells per mouse were inoculated IP on day 0). C = non-immunized controls; I = immunized controls, injected with frozen and thawed Ehrlich tumor cells in Freund's adjuvant, 2 months before the transplantation of live tumor cells; 8AG = nonimmunized mice, treated intraperitoneally with 8-azaguanine (200 mg/kg), 24 hours after tumor transplantation; I-8AG = immunized mice, treated intraperitoneally with 8-azaguanine (200 mg/kg), 24 hours after tumor transplantation.

DR. KAPLAN: Recently we demonstrated that cytosine arabinoside is a potent suppressor of delayed hypersensitivity during and after its induction, both *in vivo* and *in vitro* [Kaplan and Calabresi (1965) *Clin. Res.* **13,** 543]. We also noted the unusual phenomenon mentioned by Drs. Schwartz and Sterzl in experiments carried out in collaboration with John Northrup, in which the humoral antibody response was measured in rabbits sensitized to tetanus toxoid. Following suppression of the secondary response by treatment with cytosine arabinoside, when we restimulated the rabbits a week later, we found a response much greater than expected or seen in controls [Kaplan and Calabresi (1966) *Clin. Res.* **14,** 333].

DR. BONMASSAR: As Drs. Schwartz and Mihich have already mentioned, in cancer therapy the interactions between immunity and chemotherapy are not confined to the immunosuppressive effects of anticancer drugs. The possibilities of synergism between drugs and the immune response directed against tumors should also be considered. In our laboratory, we have attempted to distinguish between the two effects exerted by antitumor drugs using a quantitative experimental model system based upon the use of Ehrlich carcinoma ascites. Although in this case tumor-specific immunity is not involved because Ehrlich carcinoma is a well established transplantable tumor of the Swiss mouse, the model

is useful to test the possibility that synergistic effects occur between antitumor drugs and immunity. Ehrlich carcinoma cells obtained from mice bearing the tumor in ascitic form were inactivated by freezing and thawing and were injected in two groups of animals. One group was not immunized and served as the control. Twenty-two days after immunization all the animals were inoculated with half a million living tumor cells and both the nonimmunized control animals and one group of immunized animals were treated with chemotherapeutic drugs. The other group of immunized animals remained untreated. The drugs were given 24

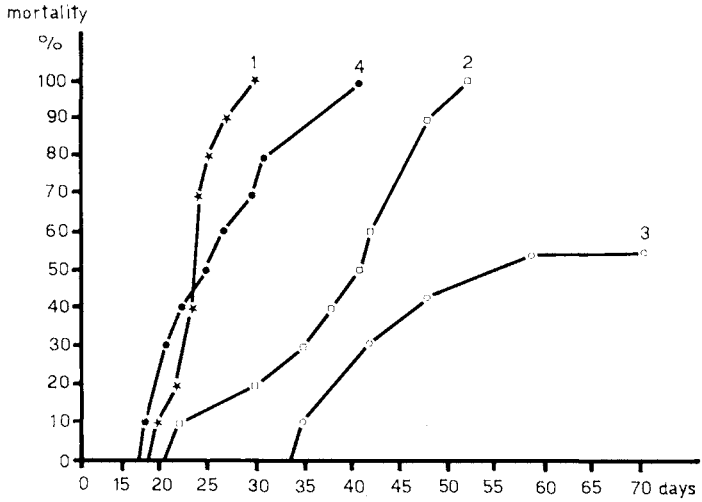

FIG. 2. Inhibitory effect of sarcolysine on the synergism between immunity and sarcolysine in Swiss mice bearing Ehrlich ascites carcinoma (500,000 tumor cells per mouse were inoculated on day 0). 1. nonimmunized controls; 2. nonimmunized mice, treated with a single intraperitoneal injection of sarcolysine (1.5 mg/kg), 24 hours after tumor transplantation; 3. immunized mice, treated with a single intraperitoneal injection of sarcolysine (1.5 mg/kg), 24 hours after tumor transplantation. Immunization was obtained by an intraperitoneal injection of 1×10^7 frozen and thawed tumor cells given 22 days before the inoculum of living tumor cells. 4. immunized mice, treated with sarcolysine (2 mg/kg) once a day for 8 days, starting the day before immunization. The animals were treated again with a single intraperitoneal injection of sarcolysine (1.5 mg/kg), 24 hours after tumor transplantation.

hours after the inoculation of living tumor cells. The antitumor effects were evaluated following tumor growth and mortality rates. The results obtained with drugs such as 8-azaguanine (Fig. 1) indicated that when the chemotherapeutic treatment was given to nonimmunized animals no antitumor effect was seen, whereas when the chemotherapeutic treatment was given to immunized animals, significant antitumor effects were noted. Immunization had no effect in animals not treated with the drug. A number of drugs were studied in this system and many of them were found to synergize with the host defenses directed against Ehrlich carcinoma. Some antitumor agents have a dual effect in the sense that they can also depress the defenses of the host when given prior to the inoculation of the tumor. This immunodepressant action can be evaluated by following the accelerated tumor growth and mortality rates in mice inoculated with a small number of tumor cells. Of

particular interest was the effect of sarcolysin. This drug was synergistic with the host defenses when given to immunized mice. This synergistic effect did not occur, however, in mice which had been treated with the drug prior to the inoculation of the tumor (Fig. 2). This observation indicated that it is possible for an antitumor agent to exert primarily immunodepressant or carcinostatic effects, depending upon the time of administration relative to the host exposure to the tumor. While this observation is consistent with the results of others indicating that immunodepressant drugs are usually not active at a certain time after antigen administration, it also suggests that in the therapy of established tumors the immunodepressant action of therapeutic agents may not be very meaningful.

DR. REIF: I feel that success in chemotherapy awaits not only improvement of the cytotoxic action of therapeutic compounds, but also elimination of unwanted, and reinforcement of desired, effects on the immune response. The regimen of drugs should be optimal not only for cytotoxicity against the tumor cells, but also for minimal interference with the immune response, and, if possible, for reinforcement of this potentially powerful natural defense mechanism.

DR. SIGEL: In connection with the effects of 6-MP on the synthesis of 7 S vs. 19 S antibody in mammals, I would like to mention that in marine teleosts, which lack 7 S γG immunoglobulins. Now, to add a paradox to your list, we were very much surprised to find selective effect you demonstrated occurs only in animals that can make both types of immunoglobulins. Now, to add a paradox to your list, we were very much surprised to find that 5–7-day-old chicks, when radiated with heavy doses of X-ray (850R), developed resistance to induction of tumors by Rous sarcoma virus. The effect is quite specific in the sense that the resistance is to tumor induction by the virus and not to tumor development following transplantation of cells. We thought at first that the effect might be due to destruction of target cells which normally respond to the virus and become transformed. Therefore, we tried the same doses of radiation applied only to the wing into which the virus was going to be inoculated. Such treatment had no effect. It appears, therefore, that this paradoxical resistance requires whole body radiation.

DR. SCHWARTZ: Clearly, 6-MP can totally suppress antibody synthesis in rabbits. In the experiments I described, we wanted to suppress selectively one of the components of the response. In order to have the optimal conditions for this effect we used alum-precipitated antigen and injected it into the footpads. These procedures increased the immunogenicity of the bovine γ-globulin, so that we could "override" the suppressive effects of 6-MP. We could have suppressed both 19 S and 7 S antibody synthesis by either using a less immunogenic form of the antigen, or by increasing the dose of the drug.

DR. SANTOS: If I am correct, this is the first time that a raise in isoagglutinins titer has been shown in man after treatment with 6-MP. The patients that I have seen and those seen at the National Cancer Institute did not have the same life span as your patients and thus we could not measure the delayed response.

DR. SCHWARTZ: The patients I studied did not have cancer, they had immunological diseases. That may be responsible in part for these differences.

DR. SANTOS: There may be two additional reasons why others have not seen the striking enhancement of antibody synthesis that you have shown in humans. The onset of antibody synthesis in some of your cases was quite delayed and may have been missed by other workers. The other possibility is that this enhancement may be related to the peculiarities of the antigen that you have employed.

I would like to comment further regarding the effect of drugs on 19 S and 7 S antibody production. We have recently observed that when relatively low doses of 6-MP are given to patients with solid tumors the 7 S antibody response to Vi antigen may be abolished while the 19 S response may remain relatively intact. On the other hand, Makinodan *et al.* [(1962)

J. Immunol. **88,** 31] have convincingly demonstrated that cells engaged in an early primary response (mainly 19 S antibody) have identical sensitivity to the immunosuppressive action of ionizing radiation, as do cells engaged in a secondary response (predominantly 7 S antibody). These authors employed an adoptive transfer technique that allows a more quantitative approach to the kinetics of antibody formation than can be realized in the intact animal. Recently, utilizing a similar system of adoptive transfer, we have made the observation (unpublished) that cells engaged in a primary agglutinin response have identical sensitivity to the immunosuppressive activity of cyclophosphamide as do cells engaged in a secondary response. These observations have prompted me to wonder if the mechanism that is most sensitive to these agents might be the "switch" mechanism either between populations of cells or within a single cell. I wonder if you might comment on this.

DR. SCHWARTZ: I think your data could be consistent with an effect on some kind of "switch" mechanism. However, I still favor the idea that there are at least two lines of cells involved, one making 19 S antibody and the other making 7 S antibody.

DR. UPHOFF: Some years ago I was attempting to induce tolerance in animals given inoculations of antigen at weekly intervals and amethopterin three times a week over a period of 3 weeks. If the three inoculations of antigen were not given or if the drug treatment was cut short by more than one injection, a typical second set response was observed when these animals were challenged sometime later with a skin allogeneic graft from the same donor strain as that of the antigen. I had failed to induce tolerance and assumed that the animals had been sensitized. Dr. Schwartz's evidence that an increased response may occur after certain types of drug treatment suggests alternative interpretations of this observation.

DR. AMIEL: The enhancing effect of a drug on the immunological reaction in rabbits is seen only when a certain period of time elapses between treatment and antigenic stimulus. In the clinical examples given by Dr. Schwartz, however, the antigenic stimulus was given at the same time as the treatment with methotrexate. We treated some cancer patients with BCG, with the idea of enhancing nonspecifically the immune response. In some of these patients BCG was administered twice weekly after treatment with Imuran given at the dose of 500 mg/m^2. In no instance did we see enhancement of the reaction to BCG. On the contrary, in some cases we observed a complete disappearance of this reaction. Have you any data on the effects of therapy on homograft sensitivity and delayed hypersensitivity in man?

DR. SCHWARTZ: I would like to emphasize that the effects shown in man are not regularly obtained. In some of these patients we gave methotrexate in an interrupted form, once a week or once every 5 days, and this may have been of some importance in producing "rebound" hyperplasia of lymphoid tissue. A possible enhancement of delayed hypersensitivity is very difficult to assess in humans unless the exaggeration is extreme.

DR. UHR: I think it might be a good idea to use a term other than enhancement to define the drug-induced increase in specific antibody formation you have just described, because this term has been used by others for a different kind of immunologic phenomenon. Another point might be taken into consideration regarding the choice of a suitable term for this phenomenon. With poor immunogens, the same drug regimen may cause a decreased antibody response. An alternative explanation of the phenomenon, other than the one you presented, is that the drugs have destroyed a large proportion of the clones of potential antibody-forming cells, allowing overproliferation of the few remaining ones. Thus, good immunogens, like BGG, which may be able to react with many of these remaining clones, may stimulate an exaggerated response whereas poor immunogens may find few or no clones remaining which can react with them.

DR. SCHWARTZ: We were very much interested in the possibility that we might have destroyed many clones by the drug treatment. We thought it might thus be possible to induce tolerance by giving a very large injection of antigen to these "hyperplastic" rabbits. We employed two antigens, BGG and bovine serum albumin and, using saturated concentrations, we were unable to induce tolerance. Your idea of using an antigen "weaker" than BSA is a good one.

DR. HITCHINGS: It is difficult to accept the interpretation that degraded nuclear material provides the stimulus for the hypertrophy of the lymphoid cells. Degradation reactions rapidly remove nuclear materials and convert them to unusable end products. For example, uric acid nephropathy is a common sequella of the radiation or chemotherapeutic treatment of lymphomas. There is nothing to suggest that therapy with 6-MP increases purine turnover, except in patients in whom a high white count is resolved. As a matter of fact Imuran blocks the so-called "shunt pathway" of the gouty hyperexcretor, but neither Imuran nor 6-MP seems to affect the purine turnover in the normal subject.

DR. BERENBAUM: This enhancement or exaggeration of the response is extremely interesting. I want to propose a tentative hypothesis based on two facts. Firstly, you showed that rabbits which were treated with 6-MP before the antigen injection had lymphoid hyperplasia at the time the antigen was given. Secondly, the proliferation obtained in response to antigen seems to be excessive. For example, the total number of cells in the mouse spleen increases from about 80 million to 150 million in a few days after injection of sheep red blood cells. The agglutinin-forming cells increase from 100,000 to 5 million and the hemolysin-forming cells increase from 50 to 50,000. In other words, there is at least ten times as much proliferation as would seem required to account for the increase in detectable antibody-forming cells. I would like to suggest that cell proliferation in response to antigen is a primitive response serving an unknown function and that antibody production has been grafted onto it in the course of evolution. The role of proliferation in antibody production may simply be to provide a population of cells at the right stage to receive, and respond to, an antigen or perhaps the RNA messages that Dr. Fishman was talking about. When you give immunosuppressive agents before the antigen, you destroy or inhibit the activity of lymphoid tissue, and then, when you stop giving the agent, there is a burst of proliferation, giving a partly synchronized population of dividing cells. At this point, the antigen is given. This may explain some of the enhancement, which seems to be produced by a variety of immunosuppressive agents. This hypothesis, however, does not explain why radiation has to be administered after, and not before, the antigen in order to produce enhancement.

Immunosuppressive Agents and the Cellular Kinetics of the Immune Response

M. C. BERENBAUM

Department of Immunology, Institute of Child Health,
University of London, London, England

I. INTRODUCTION

It has been known for many years that immune responses may readily be inhibited by whole-body irradiation if this is administered before the antigen, whereas administration after the antigen is relatively ineffective (Benjamin and Sluka, 1908; Dixon *et al.*, 1952; Taliaferro *et al.*, 1952; Fitch *et al.*, 1956; Gengozian and Makinodan, 1958). The ease with which the time-dependence of the immunosuppressive effects of radiation was established was largely due to the adoption by most investigators of a relatively simple experimental design, using single doses of radiation and of antigen. In contrast, there was considerable delay in establishing the time-dependence of the effects of chemical immunosuppressive agents. This was partly due to the almost uniform adoption of relatively complicated experimental designs, using repeated doses of agent and often of antigen also. The reasons for this are now not clear but investigators at that time may have been more interested in establishing that these agents were in fact immunosuppressive than in studying the secondary issue of time-dependence. It was therefore reasonable to use relatively prolonged courses of administration in order to ensure that immunosuppression would readily be demonstrated. The results obtained, therefore, were those of more or less extended periods of administration, with partial summation of effect, making it difficult to determine the critical period for immunosuppression. In fact, relatively few investigations of timing effects were made for chemical agents since it was

generally believed that they were "radiomimetic" and were not therefore expected to behave very differently in this respect from radiation.

Cortisone was found to behave rather similarly to radiation, being more effective if given before the antigen than afterward (Fagraeus, 1952; Berglund, 1956a,b). Green (1958) found that, in the rabbit, four daily injections of nitrogen mustard were most effective in prolonging the induction period and depressing the peak antibody titer if they were started 2 or 4 days before the antigen was injected, but that the rate at which antibody levels rose was slowed most if the nitrogen mustard course was begun 2 days after the antigen injection. Schwartz *et al.* (1958) gave repeated injections of antigen and 6-mercaptopurine (6-MP) to rabbits and showed that, unlike radiation, this agent could depress antibody production even if its administration was not begun until a week after the first antigen injection, when antibody was already present in the serum.

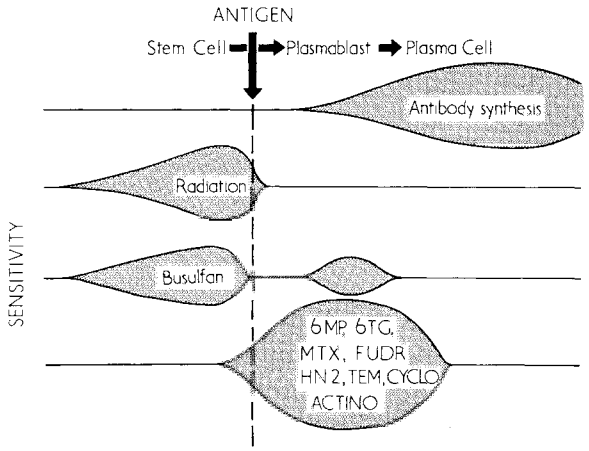

FIG. 1. Stages in the morphological and functional differentiation of antibody-forming cells and in their apparent sensitivity to toxic agents.

In view of the paucity of information on the time-dependent effects of immunosuppressive agents, an investigation of this phenomenon was undertaken using single doses of antigen and agent. The results were as clear cut as they were unexpected. Nitrogen mustard, triethylene melamine, 6-mercaptopurine, 6-thioguanine (6-TG), and methotrexate all suppressed antibody production if they were given after the antigen, usually being most effective on day +2, and were relatively or completely ineffective if given beforehand. The only chemical found to mimic radiation was busulfan, which appeared to inhibit antibody production only if given before the antigen (Berenbaum, 1961, 1962).

It seemed unlikely at that time that the difference between radiation and busulfan (which were termed class 1 agents) on the one hand, and the antimetabolites and other alkylating agents (which were termed class 2 agents) on the other, could be explained by differences in their effects on phagocytosis or on cell division. There was evidence that other types of differentiating cell could vary in sensitivity to different toxic agents in a way that depended on their stage of differentiation and not on their mitotic state (Craig *et al.*, 1958; Glucksmann, 1959; Lucas, 1961; Berenbaum and Calley, 1962). The following hypothesis was therefore put forward. The differentiation of cells mediating the immune response is accompanied by changes in their susceptibility to toxic agents. During early stages of differentiation, before contact with antigen, these cells are sensitive to class 1 agents and insensitive to class 2 agents. During the stages of differentiation following antigenic stimulation, the situation is reversed, the cells losing their sensitivity to class 1 agents and becoming sensitive to class 2 agents. Finally, when fully differentiated and producing antibody, the cells are relatively insensitive to agents of both classes (Fig. 1).

Subsequent work has confirmed these experimental findings and has shown that some other alkylating agents, antimetabolities, and antibiotics behave as class 2 agents. These include uracil mustard, 6-mercaptopurine riboside, azathioprine, 5-fluoro-2′-deoxyuridine and actino-

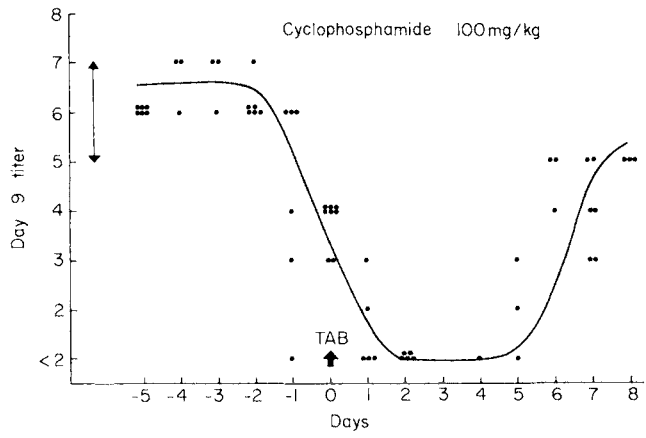

FIG. 2. Time-dependence of the effects of cyclophosphamide on antibody levels. Mice injected with 0.2 ml TAB vaccine i.p. on day 0 and serum taken for titration of H-agglutinins on day 9. Cyclophosphamide, 100 mg/kg, dissolved in saline, injected s.c. at various times on, before, or after day 0. Each point shows the titer (reciprocal of $\log_2$ dilution) of serum pooled from a group of three or four mice. The vertical bar bounded by arrows shows the range of titers in 80% of controls.

mycin C (Frisch and Davies, 1962; Santos and Owens, 1962, 1964; Merritt and Johnson, 1963; Brown, 1964; Buskirk *et al.*, 1965). Cyclophosphamide also suppresses antibody production most effectively if given after the antigen (Fig. 2) although it may be effective if given up to a few days beforehand (Santos and Owens, 1962, 1964; Frisch and Davies, 1965; Buskirk *et al.*, 1965). Melphalan also acts whether given before or after the antigen, being slightly more effective when given 1–3 days beforehand than afterward (Brown and Berenbaum, 1964). Further investigation of busulfan has shown a biphasic action, for it is highly effective if given before the antigen, slightly but significantly effective if given some 2–3 days after the antigen (Fig. 3 and unpublished work), but ineffective if given simultaneously with or shortly after the antigen.

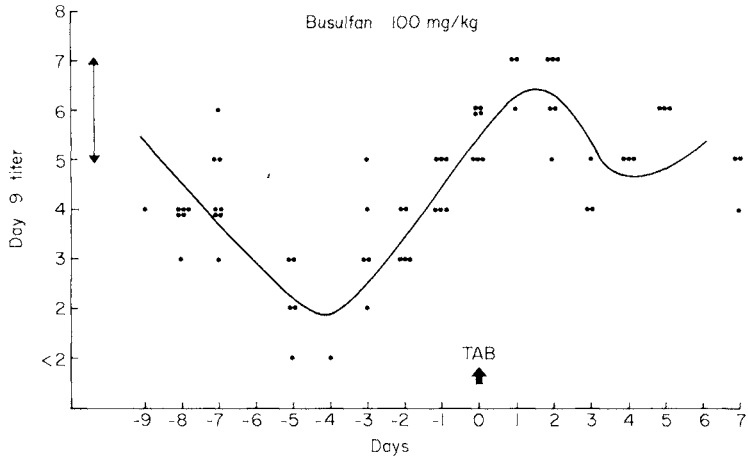

FIG. 3. Time-dependence of the effects of busulfan on antibody levels. Busulfan, 100 mg/kg, suspended in arachis oil, injected s.c. at various times on, before, or after day 0. Remainder of experimental procedure as described in Fig. 2.

These various findings show that the phenomenon of time-dependence is more complex than appeared at first, but they do not invalidate the hypothesis that cell sensitivity varies with differentiation. Indeed, they encourage the belief that it might be possible to dissect the immune response pharmacologically in a way that would throw light both on the mechanisms of the response itself and on the modes of action of the agents used.

However, there are two difficulties in such an analysis. First, changes in serum antibody levels reflect only with considerable delay changes in the population of antibody-producing cells. Second, serum antibody is detectable and measurable only above a certain threshold concentra-

tion and it is not possible, therefore, to use measurements of antibody levels to analyze cellular events in the early and crucial stages of the response, before the threshold has been passed, nor in strongly suppressed responses, when antibody levels have fallen below the threshold.

In view of these difficulties, it was decided to apply the methods recently described (Jerne and Nordin, 1963; Jerne *et al.*, 1963; Zaalberg, 1964) in investigating directly the effects on antibody-producing cells of three immunosuppressive agents, viz., radiation, 6-thioguanine, and busulfan.

II. MATERIALS AND METHODS

1. Mice

Male A2G mice, weighing 18–25 gm at the start of each experiment were used.

2. Immunization

Formolized sheep red cells (Burroughs Wellcome) were washed twice in saline and made up to a 10% (v/v) suspension in saline. Mice were injected with 0.2 ml of the suspension intraperitoneally.

3. Spleen Cells

Mice were killed by cervical dislocation. Each spleen was teased in a disposable petri dish containing about 2.5 ml cold tissue culture medium 199 using a fine-toothed forceps and a scalpel blade. The suspension was transferred to a 64 × 8 mm disposable stoppered test tube, sucked up and down through a Pasteur pipette about twenty-five times to break up cell clumps and, after 3–4 minutes, during which fragments of fibrous tissue settled, the supernatant suspension was transferred to a conical, graduated centrifuge tube and spun at 500 *g* and 4°C for 10 minutes. The supernatant was drained off and the cell deposit resuspended in cold medium 199 to a final volume of 0.4–10 ml, depending on the expected number of antibody-forming cells. In the initial stages of the response or in unstimulated mice, one spleen was usually suspended in a final volume of 0.4 ml; half of this was plated and the rest made up to 1 ml with 199 for use in the agglutinating cell method. At the height of the response, spleens were suspended in 10 ml of 199; 0.05 ml was plated and 1 ml used for the agglutinating cell method.

4. Hemolysin-Producing Cells

Jerne's method was used with minor modifications (Jerne and Nordin, 1963; Jerne *et al.*, 1963).

a. Sheep red cells. Sheep red cells preserved in Alsever's solution (Burroughs Wellcome) were washed three times in saline and a 50% suspension (v/v) made in saline.

b. Absorbed guinea pig serum. Serum obtained by bleeding stock guinea pigs was cooled to 0° C, and one-tenth volume of packed, washed sheep red cells added. The mixture was centrifuged at 1500 *g* and 0° C for 20 minutes and the supernatant serum stored in 1–2 ml aliquots at −40° C.

c. 1.4% Agar plates. Agar-Noble (Difco) sufficient to give a final concentration of 1.4% (w/v) was dissolved over a heated magnetic stirrer in nine-tenths the required volume of deionized water.

Diethylaminoethyl dextran (Pharmacia) sufficient to give a final concentration of 0.4 mg/ml was dissolved in a few milliliters of hot saline and added to the hot, stirred agar solution. One tenth of the final volume of ten-times concentrated medium 199 (Glaxo Laboratories) at room temperature was slowly added. The hot agar was dispensed in 10 ml volumes into 3½ inch diameter disposable petri dishes. When the agar was solid, the plates were placed in plastic bags which were sealed and stored in the refrigerator. They could be used for up to 2 weeks after preparation.

d. 0.7% Agar. Some of the hot 1.4% agar containing 199 (usually about a tenth of the total quantity prepared) was poured in 10-ml aliquots into 150 × 16 mm Pyrex tubes containing 10 ml 199. The tubes were stoppered and their contents rapidly mixed before setting. They were stored in the refrigerator.

e. Plating procedure. Agar plates taken from the refrigerator were shaken to remove exuded moisture and incubated with the lids off at 37° C for about half an hour, or until their surfaces were no longer obviously wet. They were then kept covered in the incubator until used.

Tubes of 0.7% agar were placed in a boiling water bath for about 10 minutes. The melted agar was dispensed in 2-ml aliquots into 64 × 8 mm disposable stoppered test tubes kept in a water bath at 44.5 ±0.5° C. To each of these was added 0.05 ml of the 50% sheep red cell suspension and 0.05–0.2 ml of the spleen cell suspension, depending on the expected number of antibody-forming cells. (The aim was to have about 100–400 such cells per plate.) The tube was then rapidly stoppered and inverted repeatedly to mix the contents which were then poured uniformly over the surface of 1.4% agar plate removed from the incubator immediately beforehand. After the melted agar had set, which took a few minutes at room temperature, the plates were incubated at 37° C for 1 hour. Two

milliliters of 10% absorbed guinea pig serum in saline was added to each plate and they were incubated for a further 30 minutes.

f. Plaque counting. Plates could be examined immediately or after storing in the refrigerator. If it was desired to keep them for more than 1–2 days, the diluted serum was rinsed off with saline and replaced by a few milliliters of 10% formol-saline. Staining with benzidine (Jerne *et al.*, 1963) was considered too dangerous for routine use and, with practice, it was quite easy to count hemolysed plaques in unstained fresh or formalin-fixed gels. The appearance of typical plaques has been adequately described by Jerne and Nordin (1963) and Jerne *et al.* (1963).

Plates were examined over a sheet of white paper illuminated from above, using a 6× hand lens. A 1-cm square grid was placed under the plate to facilitate counting. The total number of plaques per spleen was calculated by multiplying the number of plaques per plate by the reciprocal of the fraction of spleen plated.

TABLE I
EFFECT OF TEMPERATURE AND STANDING TIME ON AGGLUTINATING CELL COUNTS[a]

Time of incubation (hours)	Temperature		
	37° C	22° C	7° C
3	19	369	228
4	49	648	281
6	42	484	582
22	49	188	1082

[a]Spleens from three immunized mice were pooled and suspended in 12 ml medium 199 (see text). The suspension was mixed with 12 ml of 0.25% sheep red cells in 199 and dispensed in 2 ml samples into twelve Pyrex tubes. These stood at the temperatures and for the times specified, their contents were mixed on a rotating blood mixer for 10 minutes, and the number of agglutinating cells per 0.0032 ml counted in a Fuchs-Rosenthal chamber.

5. *Agglutinin-Producing Cells*

The original method of Zaalberg (1964), in which a mixture of suspensions of spleen cells and red cells was examined after 30 minutes' incubation, did not give satisfactory results in our hands. It was found that higher counts were obtained by standing the suspensions for relatively long periods at room temperature or, better still, in the cold (Table I).

a. Sheep red cells. Sheep red cells preserved in Alsever's solution (Burroughs Wellcome) were washed three times in saline and resuspended in medium 199 to a concentration of 0.25% (v/v).

b. Procedure. Both red cell and spleen cell suspensions were cooled on ice before use. One milliliter of each was mixed together in a 150 × 16 mm Pyrex glass tube stoppered with a silicone rubber bung and the tubes stood vertically in the refrigerator (4–7° C) overnight.

c. Agglutinating cell counts. After removal from the refrigerator, the tubes were arranged radially on a Matburn blood mixer and rotated at room temperature at 28 rev/minute for 10 minutes. A drop of the mixture was placed in a Fuchs-Rosenthal counting chamber and, after 2–3 minutes for the cells to settle, the number of agglutinating cells was counted.

Ideally, a typical cell had several (>3) erythrocytes attached to it and was clearly identifiable as a lymphoid cell. Cells obscured by numerous attached red cells were counted as agglutinating cells, unless the red cell mass was so small as to preclude the presence of a lymphoid cell within.

6. Radiation

The mice were irradiated in Perspex boxes in a Maximar 200 machine. The dose was 450 r delivered at 65–75 r/minute (220 kVp, 15 mamp, 0.5 mm copper and 1 mm aluminum added filtration, target distance 34.5 cm).

7. Thioguanine

6-Thioguanine (Lights Ltd.) was ground in a glass homogenizer in 0.25% carboxymethyl cellulose (Hercules Powder Co.) in saline and a 4 mg/ml suspension prepared. Mice were injected subcutaneously with a single dose of 40 mg/kg.

8. Busulfan

This was ground in a pestle and mortar with arachis oil that had previously been heated to 120° C to drive off water and then cooled. The suspension was treated for three periods of 1 minute each in an M.S.E. 60 W ultrasonic disintegrator and was then made up with arachis oil to a concentration of 10 mg/ml. Mice were injected subcutaneously with a single dose of 100 mg/kg.

Experimental procedure. Mice were injected with formolized sheep red

cells (day 0) and different groups irradiated or injected with thioguanine or busulfan on one occasion before or after the antigen injection. Irradiations were carried out on days −7, −1, +1, +3, +6 and +20. Thioguanine injections were given on days −6, −1, 0, +1, +2, +4, +8 and +20. Busulfan injections were given on days −5, −1, 0, +1, +2, +4 and +8. Control mice had antigen only. Groups of mice were killed at intervals after the sheep red cell injection, their spleens removed, and the numbers of hemolysin- and agglutinin-producing cells they contained were determined. In the radiation experiments these determinations were made in separate groups of mice. In the experiments with thioguanine and busulfan both determinations were made on each spleen.

III. RESULTS

A. Controls

In untreated mice there were usually about 50–90 hemolysin-producing cells per spleen. After injection of sheep red cells this number rose rapidly to a peak of 40–80,000 by day +4 and then fell at a rate that varied from one experiment to another, but which generally gave levels of about 10,000 on day +7, 1–6000 on day +14 and 500–2000 on day +21.

The number of agglutinating cells per spleen was usually 25–80,000 in untreated mice (but in some experiments as low as 2–3000). Peak levels of 1–5 million cells per spleen were usually reached after 5–6 days. The subsequent fall was slow, levels of a million agglutinating cells per spleen usually being maintained for at least 3 weeks.

B. Radiation

[These results were briefly reported earlier (Berenbaum, 1966)]. A dose of 450 r caused a rapid fall in the number of antibody-forming cells of both types, followed by a slow climb toward control values, which appeared to be approached after 2–3 weeks in those groups that were followed long enough (Figs. 4, 5). In mice irradiated before injection of antigen, the starting levels were low on day 0 and the climb toward control values very slow. At the time of maximum depression, the levels of lysin-producing cells were about 0.0002 of the corresponding control values in mice irradiated on days −1 or +1, about 0.001–0.003 in mice irradiated on days −7 or +3 and about 0.01–0.025 in mice irradiated on day +6 or day +20. The numbers of agglutinating cells fell to 0.001–0.003 of the corresponding control values in mice irradiated at any time between day −7 and day +6, and the greatest depression after irradiation on day +20 was not much less (0.006 of the controls).

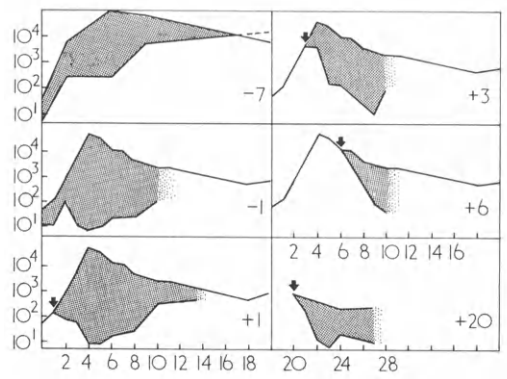

FIG. 4. Effects of radiation on hemolysin-forming cells. Mice injected with sheep red cells on day 0 and irradiated at times indicated. The irradiations on days −1 to +20 were carried out in a single experiment.

The stippled area shows the difference in counts between control mice (top line) and irradiated mice (bottom line) when the former were higher than the latter. When the values in irradiated mice exceeded those in the controls, the former are indicated by a dashed line. Curves are drawn through geometric means of determinations on groups of five to ten mice. The horizontal axis shows the time in days after injection of sheep red cells. The vertical axis shows the number of hemolysin-forming cells per spleen.

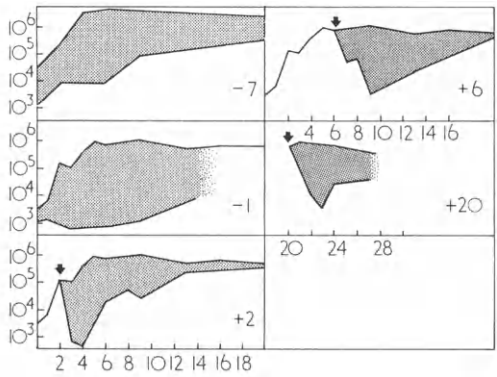

FIG. 5. Effects of radiation on agglutinating cells. Procedure as described in legend to Fig. 4. Curves drawn through geometric means of determinations on groups of five mice. The vertical axis shows the number of agglutinating cells per spleen.

C. THIOGUANINE

Administration on days −6 or −1 had little or no effect on antibody-forming cells of either type (Figs. 6, 7). Administration on days 0, +1 or +2 was highly effective, reducing lysin-forming cells to about 0.003 of the corresponding control values and agglutinating cells to 0.01. Administration on days +4, +8, or +20 was less effective, lysin-forming cells

being depressed to minima of 0.01, 0.06, and 0.25 respectively and agglutinating cells to 0.1–0.15.

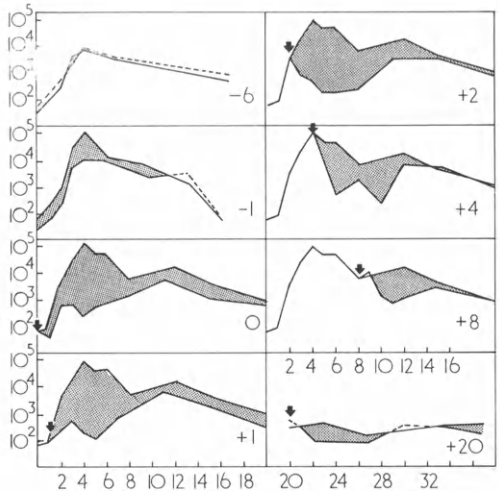

FIG. 6. Effects of 6-thioguanine on hemolysin-forming cells. Curves drawn through geometric means of determinations on groups of eight mice. The thioguanine injections on days −1 to +20 were carried out in a single experiment. Other information as described in legend to Fig. 4.

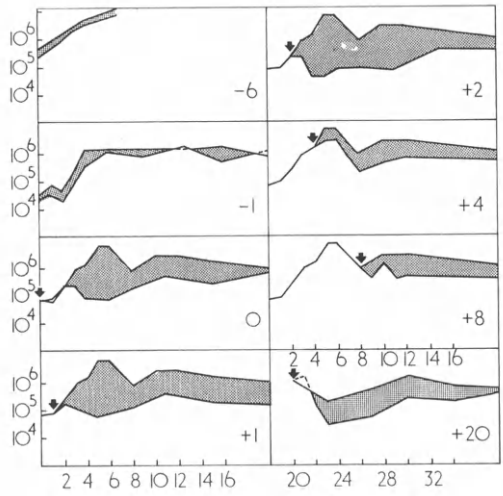

FIG. 7. Effects of 6-thioguanine on agglutinating cells. Curves drawn through geometric means of determinations on groups of eight mice. The same spleens were used for counts of hemolysin-forming cells, shown in Fig. 6.

D. Busulfan

Administration on days −5 or −1 profoundly inhibited the response. Administration on days 0 or +1 was relatively ineffective. Susceptibility returned to a moderate extent on day +2 and was lost again on days +4 or +8 (Figs. 8, 9). The fall of lysin-producing cells following administration on days −5 or −1 was to about 0.0003 of the corresponding control values. Administration on day 0 and +1 gave falls of to 0.06–0.07 of the controls, on day +2 to 0.01 and on days +4 or +8 to 0.25. Agglutinating cells fell to a minimum of 0.01–0.015 of control levels after administration on days −6 or −1. The corresponding fraction for administration on days 0, +1 and +2 was 0.1–0.15, for day +4, 0.2, and for day +8, 0.5.

Administration on days −5 or −1 caused the response to be completely inhibited for the 3-week period of observation, and there was no tendency for recovery of cell numbers as was found after irradiation. Administration to these animals of a second dose of antigen after 3 weeks caused a sharp rise in the number of antibody-forming cells (not shown in these figures), showing that the animals had not been made immunologically tolerant.

IV. DISCUSSION

These experiments were carried out because of the difficulties briefly mentioned in the Introduction, that prevented one from drawing conclusions about the cellular effects of immunosuppressive agents directly from their effects on antibody levels. There were two main problems. In the first place, the rate of decay of antibody already present in the serum is relatively slow compared with the rate of decay of cells damaged by such agents as radiation, corticosteroids, or alkylating agents. The half-life of the former is a matter of days while that of the latter may be measured in hours. Therefore, no matter how rapidly an agent destroyed or inactivated antibody-producing cells, it could not cause the level of serum antibody to fall at a rate faster than that determined by its half-life (unless it also hastened antibody catabolism or excretion). The slower the rate of antibody production at the time of giving an immunosuppressive agent, the longer it would take for a readily measurable difference to appear between serum antibody levels in treated animals and in controls.

In the second place, antibody can be detected and measured only when its concentration is above a threshold level. Morphological studies show that, in the early stage of the antibody response, the fraction of the lymphoid cell population engaged in antibody synthesis increases for 1 or 2 days before the concentration of serum antibody rises to a detectable level (Leduc *et al.*, 1955; Šterzl and Mandel, 1964). Evidently,

measurements of serum antibody cannot help in studying the effects of immunosuppressive agents during this stage, yet it is precisely this stage

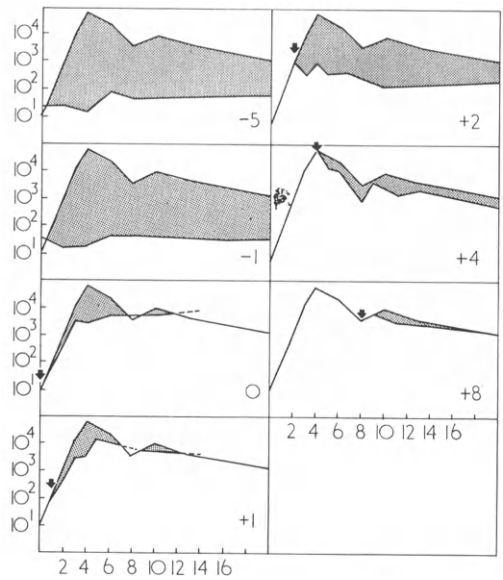

FIG. 8. Effects of busulfan on hemolysin-forming cells. Curves drawn through geometric means of determinations on groups of eight mice. All injections made in a single experiment. Other information as described in legend to Fig. 4.

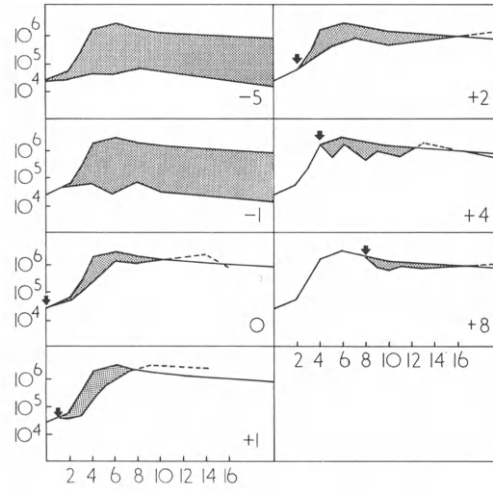

FIG. 9. Effects of busulfan on agglutinating cells. Curves drawn through geometric means of determinations in groups of eight mice. The same spleens were used for counts of hemolysin-forming cells, shown in Fig. 8.

that is of most interest, for it is at about this time that administration of immunosuppressive agents is most effective.

Immunosuppressive agents characteristically cause an apparent delay in the appearance of serum antibody, a reduced rate of rise of antibody levels, and a reduced peak titer. As Fig. 10 shows, the existence of a threshold makes it impossible to distinguish between the various mechanisms that could cause these changes. These include overall slowing of the response, an increased cellular induction period, and destruction of a proportion of the cells. (In the case of the first two mechanisms it would be necessary to postulate also decay of some component of the response so that delay in mounting it would lead to a reduced peak level.)

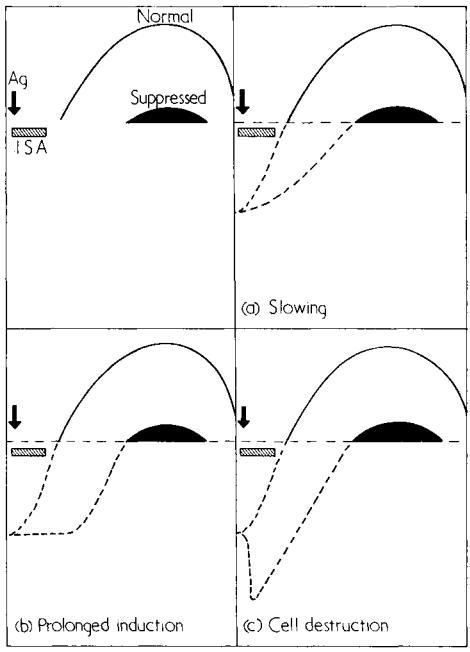

FIG. 10. The delayed appearance of antibody, its slow increase, and reduced peak in the partly suppressed response may be due to different mechanisms that cannot be distinguished by following changes in serum antibody levels because of the threshold below which antibody is undetectable. ↓ = Administration of antigen; hatched bar = administration of immunosuppressive agent; solid line = normal antibody levels; solid area = antibody above the threshold in the partly suppressed response; broken line (below axis) = antibody levels below the threshold.

Such considerations suggested that it might be instructive to devise simple models in which cell sensitivity to toxic agents could be specified, so that the effects of the above interfering factors could be assessed, at

least theoretically. For the purpose of such models, the following simplifying assumptions may be made. The lymphoid cell population includes a small proportion of cells capable of responding to antigen by production of antibody. (Whether or not they produce antibody before antigenic stimulation is immaterial in this context as the proportion of cells is assumed to be so small that any resulting serum antibody levels are well below the threshold of detection.)

After antigenic stimulation, these cells rapidly increase in number and produce antibody, the serum concentration of which eventually rises above the threshold. The time between exposure to antigen and detection of serum antibody is the induction period, as usually defined. Let us assume that effective doses of immunosuppressive agents act by destroying or inactivating a large fraction of these cells. Let it be assumed also that the survivors of such destruction before exposure to antigen proliferate relatively slowly to reconstitute the original number. After antigenic stimulation, the cells multiply with great rapidity and it will be supposed that the survivors of any destructive or inactivating effect of an immunosuppressive agent will simply continue to proliferate at the prevailing rate and produce antibody independently of the fate of the destroyed cells.

A model in which cell sensitivity to a toxic agent remains constant throughout the response is shown in Fig. 11 and has been briefly de-

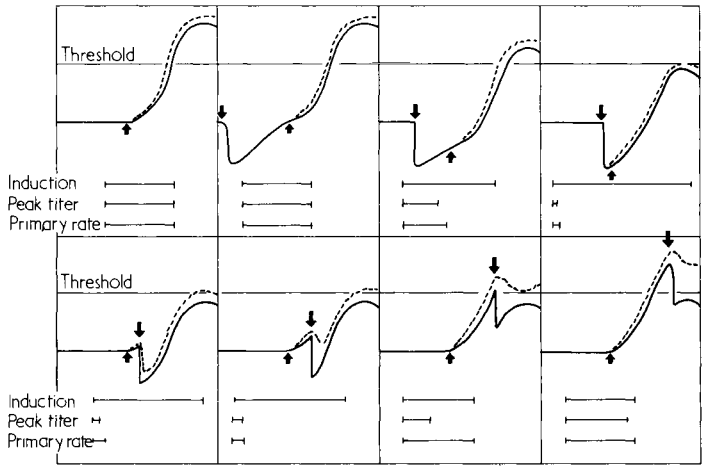

FIG. 11. Model showing the effects of constant cell sensitivity. Solid line = logarithm of number of antibody-forming cells; broken line = serum antibody level; ↑ = administration of antigen; ↓ = administration of immunosuppressive agent. The lengths of the lines indicating induction period, peak titer, and primary rate of rise of antibody indicate the proportionate changes in these in treated animals as compared with controls.

scribed earlier (Berenbaum, 1966). The assumption here is that a particular dose of the agent destroys or inactivates the same fraction of cells whenever it is given in relation to the time of giving the antigen. Administration long enough before the antigen allows time for the original cell number to be restored by the time the antigen is given so that the subsequent response is normal. If the interval between giving the agent and the antigen is shorter, recovery is incomplete. Fewer cells are able to participate in the subsequent immune response, which will be correspondingly reduced. The peak titer will be lowered and, because of the threshold effect, it will appear that the induction period is prolonged and the rate of rise of antibody levels reduced. The shorter the time between administration of agent and antigen, the more marked these effects, and they reach their maximum if the agent and antigen are given simultaneously.

When the agent is given after the antigen, the same proportion of cells is destroyed or inactivated but two new factors affect the result. First, the survivors multiply much faster than before (at least, until about the time that cell numbers usually reach their peak). Second, depending on the time that has passed since giving the antigen, a certain amount of antibody, with a comparatively slow disappearance rate, will be present in the serum.

If the agent is given, say, while antibody-forming cells are multiplying rapidly but before serum antibody has risen above the threshold of detection, the level of antibody will fall with a half-life not materially longer than normal until production by surviving cells and their progeny causes it to rise again. The induction period will be lengthened and the peak titer lowered, as before, but these effects will be partly mitigated by the antibody already present in the serum. The later in the response the agent is given, the greater the accumulation of antibody and the larger its part in counteracting the immunosuppressive effects on the induction period and peak titer.

It appears, therefore, that even if an immunosuppressive agent destroyed or inactivated the same proportion of cells at any stage of the response, there would be marked changes in the induction period, rate of rise of antibody levels and peak titer (and titers at arbitrary times). The period before administration of antigen during which the response could be affected would be a function of the rate at which the cells that were to mediate the response recovered from damage by the agent. The slower their recovery, the longer this period would be. The period after administration of antigen during which the response was susceptible would depend on what was being measured. The induction period and initial rate of rise of antibody levels could be affected only up to the end of the induction period. The peak titer could be depressed only by administration

of the agent before the time when peak titers were normally reached. At later stages, it would become increasingly difficult to show an effect on antibody production, as this would have slowed in any case.

Maximal effects would, according to this model, result from administration of the immunosuppressive agent at the same time as the antigen. This, however, assumes that the cellular effects of the agent are instantaneous. If there were any delay in these effects, the critical period for its administration would be correspondingly earlier.

There are many obvious defects in this extremely oversimplified model. It does not take into consideration homoeostatic mechanisms that might favor extra and complementary proliferation of cells that survive destruction. It ignores the complexity of immune responses as being the result of the integrated activities of several cell types. Nevertheless, there is an indisputable similarity between the effects predicted by the model and those found experimentally for whole-body irradiation in the mouse and rat (Fitch *et al.,* 1956; Gengozian and Makinodan, 1958; Simić *et al.,* 1965). An experimental finding not predicted by the model is that irradiation a few days after the antigen is given may enhance the response. Findings in the rabbit also do not correlate well with the model, for effects on peak titers and primary rates of rise show rapid fluctuations about the time the antigen is given (Taliaferro *et al.,* 1952; Taliaferro and Taliaferro, 1954). The model also fails to account adequately for the fact that the most radiosensitive period is not at the time of giving the antigen but 1–2 days beforehand.

In spite of these defects, consideration of this model suggested that the striking time-dependence of the immunosuppressive effects of radiation might, to some extent, be an experimental artifact and that the radiosensitivity of the cells mediating the response might be much more constant during the response than effects on antibody levels indicated. The experiment described here confirmed this expectation. Although it did not show that the cells were equally radiosensitive throughout the response, it did show that radiosensitivity persisted to a marked degree even at stages of the response generally thought to be radioresistant.

The results of the radiation experiment, however, largely invalidated the original hypothesis, that is, that cells mediating the immune response were highly susceptible to radiation damage only before antigenic stimulation and relatively insusceptible afterward (Fig. 1). To some extent, the characteristic effects of class 1 agents might now be explained by a more or less constant cell sensitivity, the effects of which were distorted by the existence of a threshold of antibody detection and the discrepancy between the decay rate of damaged cells and that of the antibody they produced. In view of this, however, it was possible to predict with confidence

that cells mediating the immune response could not possibly show a constant sensitivity to class 2 agents, i.e., those that produced the greatest effect if given after the antigen. On the contrary, such agents should produce little or no relevant cellular effect when given before the antigen and be highly damaging to the cells when given afterward. A model of one possible state of affairs is shown in Fig. 12.

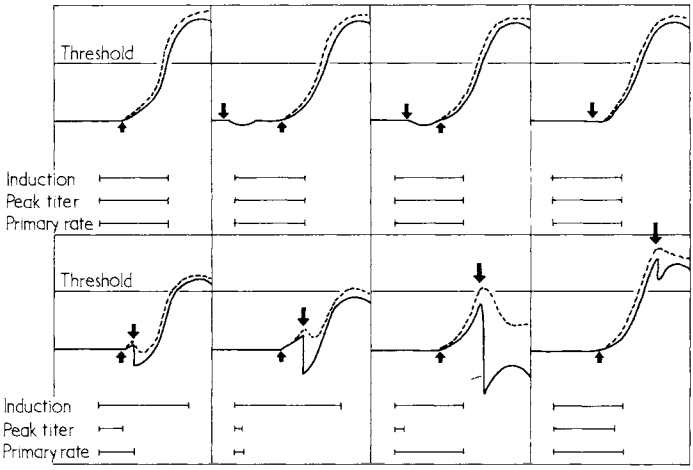

FIG. 12. Model showing the effects of proliferation-dependent cell sensitivity. Solid line = logarithm of number of antibody-forming cells; broken line = serum antibody level; ↑ = administration of antigen; ↓ = administration of immunosuppressive agent. The lengths of the lines indicating induction period, peak titer and primary rate of rise of antibody indicate the proportionate changes in these in treated animals as compared with controls.

Here, cell sensitivity is assumed to be a function of the rate of cell proliferation. (It should be emphasized, however, that cell proliferation is not the only activity that might make cells susceptible to class 2 agents.) Administration of agent before the antigen is seen to be ineffective as it leads only to negligible changes in the number of cells. As the rate of proliferation rises and falls after the antigen is given, the fraction of cells destroyed or inactivated increases and decreases correspondingly. It can be seen that maximum prolongation of the induction period, slowing of the rate of rise of antibody titers, and depression of the peak titer is brought about by giving the immunosuppressive agent at about the time of most rapid cell proliferation.

The cellular effects of 6-thioguanine, a typical class 2 agent, closely resemble the effects predicted by this model, except that cell destruction or inactivation is not instantaneous but takes 2–4 days to reach its maximum.

The experiments with radiation and thioguanine, therefore, suggested the possibility of explaining most immunosuppressive effects in a surprisingly simple way, as follows. The cells mediating the immune response appear to be damaged by class 1 agents to an extent that is independent of the activities or stage of differentiation of these cells and that is more or less constant throughout the response. They are damaged by other agents (of class 2) to an extent that may depend only on their rate of proliferation and that is, again, independent of their other activities or stage of differentiation.

This would have been a rather disappointing conclusion, in spite of its attractive simplicity, for it would have implied that the further study of the effects of immunosuppressive agents could reveal little beyond these two simple mechanisms. The intriguing differences between the effects of the various immunosuppressive agents might be due only to the fact that their different physicochemical properties enabled them to effect these two mechanisms to different extents and at different rates.

These considerations led to the testing of busulfan, which was expected to behave similarly to radiation, reducing cell numbers to a more or less equal extent whenever it was given. However, it did not behave according to expectation. Its effect was profound if given before the antigen, moderate if given 2 days afterward and negligible when given at other times. These effects might possibly be explained by a severely inhibiting effect of busulfan on phagocytosis, together with moderate damage to proliferating cells. However, there is no evidence that busulfan affects uptake of particles by the reticuloendothelial system (Megirian *et al.*, 1959). The possibility of its affecting antigen degradation or recognition mechanisms does not seem to have been investigated. It is also possible that busulfan has selective toxicity for immunologically competent cells before they are exposed to antigen.

In conclusion, it appears that the first hypothesis (Berenbaum, 1962) must be modified with respect to radiation. The position regarding busulfan and class 2 agents has to remain open.

Methods that allow antibody-forming cells to be counted evidently enable a more refined analysis of the effects of immunosuppressive agents than measurements of serum antibody. However, cells are countable only while they contain antibody. Precursor cells cannot be detected, nor can otherwise intact cells in which antibody synthesis has been inhibited. This limitation may explain some of the discrepancies between the effects predicted by the simple models described here and those found experimentally. Indeed, the time taken for the numbers of antibody-forming cells to reach their minima after administration of the agents is at least consistent with effects on precursor cells.

Conclusions drawn from these experiments must therefore be qualified. It is possible that further analysis will require the use of isolated cell populations that can be studied *in toto* and the measurement not only of antibody synthesis but also of other activities of these cells.

V. SUMMARY

In view of the difficulty of analyzing the modes of action of immunosuppressive agents by examining their effects on serum antibody levels, their effects on the numbers of antibody-forming cells was investigated in mice injected with sheep red cells. Although measurements of serum antibody suggest that the immune response is radiosensitive before giving the antigen and relatively radioresistant afterward, it was found that radiation caused a rapid fall in the number of hemolysin- and agglutinin-producing cells in the mouse spleen whenever it was given in relation to the antigen.

6-Thioguanine had little or no effect if it was given before the antigen, was highly effective if given 0–2 days afterward, and less effective if given on the fourth day or later.

Busulfan greatly inhibited the response if given before the antigen, had a moderate effect if given 2 days afterward, and was ineffective at other times.

Simplified models were devised to account for the effects of radiation and thioguanine on antibody levels.

Acknowledgments

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DISCUSSION

DR. PHILLIPS: At the Ontario Cancer Institute Bruce and Meeker performed studies of the effects of chemotherapeutic agents on bone marrow and lymphoma cells [(1967) *J. Natl. Cancer Inst.* (in press)]. The action of these agents could be simply correlated to the proliferative state of the cells at the time of treatment with the drug. For instance, normal bone marrow cells were relatively insensitive to drugs like cyclophosphamide or 5-FU, whereas bone marrow cells regenerating after radiation were as sensitive as lymphoma cells which are always in a state of rapid proliferation.

DR. BERENBAUM: I think it is unwise to come to the conclusion that rapidly proliferating cells are damaged more than slowly proliferating cells simply because they are proliferating. They are carrying out many other processes besides dividing, and it may be one of the other processes that makes them susceptible.

DR. ŠTERZL: You are right in considering the evaluation of serum antibody levels critically. Indeed, this evaluation is limited by the threshold of the methods used. Serum antibodies are detected later than antibodies measured directly at the cellular level by the hemolytic plaque technique. Nevertheless, comparing the relationships between time of administration and action of different immunosuppressive drugs that you reported earlier measuring serum antibodies, with the results presented today using the plaque-forming technique, I do not see any difference. So, what is the basis for your criticism of the methods of detection of antibody in the serum? The second point I would like to mention concerns the interpretation of your results. I think that in 1960 you observed a straight parallelism between the action of drugs which inhibit the proliferation of malignant cells and their inhibitory effect on antibody formation. Also, you reported previously that most drugs,

with the exception of busulfan, are effective when given within 2–3 days after the administration of antigen. This period corresponds to the most rapid proliferation of antibody-forming cells and their precursors during the process of immunization *in vivo*. However, as I stressed in my talk, the effect on proliferation is not the only action of immunosuppressant agents. In order to evaluate the effects of drugs on processes of cellular differentiation, one should use a model system in which proliferation of cells does not play a prevalent role.

DR BERENBAUM: It is true that the overall results I reported today are what one would expect from measuring serum antibody levels, except for those seen in the case of radiation. The determinations of serum antibody levels after radiation show that the response is apparently radioresistant after the antigen is given. But if one measures the plaque-forming activity of cells, one finds that this is not so. The reasons for this discrepancy are two, namely, the threshold of detection of serum antibody and the way in which changes of serum antibody levels lag behind changes in the cells. With agents that affect antibody production if they are given after the antigen, these factors do not operate to the same extent. Regarding the second question, we did not try to correlate inhibition of antibody synthesis with inhibition of cell proliferation, but with effectiveness in treating human tumors. I have never believed that immunodepressant effects are caused simply by inhibiting cell proliferation. I feel that these agents can act in many ways. Arrest of cell proliferation is one way but what may be more important is the destruction of cells, and this may be dependent upon the state of differentiation. This is why I am interested in following the cellular changes caused by each drug in more detail. I do not think that grouping these agents together as antimitotic drugs is very helpful.

DR. UHR: In your theoretical considerations of the mechanism of action of these pharmacologic agents, you discussed only their possible effects on immunologically competent cells. This implied the assumption that there was no effect of these agents on the earlier aspect of the immune response, namely the metabolism of antigen in its broadest sense. Is this assumption justified? Is it not possible that these powerful pharmacologic agents alter the fate of antigen in a manner that is significant to the immune response? Have you any information on this aspect? Perhaps you could study possible drug effects on this early aspect of the immune response by performing cell transfers into recipient animals immunized and treated with drugs at intervals prior to cell transfer.

DR. BERENBAUM: Information on this is rather inconclusive. According to some investigators, radiation inhibits phagocytosis, according to others it does not. Some say it interferes with the breakdown of antigen in the cell, and others say it does not. The same contradiction exists with reference to the corticosteroids. The possible action of the other agents on phagocytosis has not been adequately investigated. Busulfan, for instance, had no effect on phagocytosis under certain experimental circumstances [Megirian, R., Walton, M. S., and Laug, E. P. (1959) *J. Pharmacol. Exptl. Therap.* **127**, 81]. It is not known whether the drug has any effect on the metabolism of antigen.

DR. SCHWARTZ: In connection with Dr. Uhr's question, I would like to mention that we could not find any effect of rather heavy 6-MP treatments on the degradation rate of radiolabeled BGG and radiolabeled BSA in rabbits. In other experiments we could not demonstrate inhibition of phagocytosis by 6-MP.

DR. BENACERRAF: I would like to point out that X-irradiation has no effect on the uptake of phagocytizable material by macrophages. In the liver and spleen, this cell type is extremely resistant to this form of injury. However, after overloading the reticuloendothelial system with particulate material, there occurs a proliferative reaction from cellular precursors to replace the loaded cells. These precursors are very radiosensitive so that X-

irradiation interferes with the restoration of normal phagocytic function after reticuloendothelial system blockade.

DR. HOLLAND: Dr. Berenbaum, none of the drugs that you have used, which were selected because of effects on neoplastic cells, are used in the fashion required to obtain the characteristic biological results in tumor systems. When the drugs are used in a fashion which produces the effects seen on tumors, might one not see different effects on the immunologically competent systems? As a corollary of that, I would point out that busulfan is insoluble and by giving busulfan subcutaneously you may have introduced a repository form of the drug. Thus you might have seen continuing effects of the drug rather than those ascribable to a single dose.

DR. BERENBAUM: To deal with the second point first, this might be a valid criticism, but I do not think it is warranted for two reasons. In the first place, busulfan is not the only insoluble agent we used. Methotrexate, which we used as the base, thioguanine, and 6-mercaptopurine are also highly insoluble. These agents persist in the subcutaneous tissues for days but there is no lag in their effect compared with highly soluble agents such as nitrogen mustard or cyclophosphamide, and maximal suppression is produced by all these agents if given on day +2. In the second place, in work with Dr. G. M. Timmis and Dr. I. N. Brown [(1967) *Immunology* (in press)], we have examined the immunosuppressive effects of a homologous series of sulfonic acid esters, including busulfan. We find that the time of optimum administration for suppressing the immune response varies from day −5 to day 0 in this series and, in general, the greater the chain length of the compound, the shorter the lag period. There is, however, no correlation between the extent of this lag and the water-solubility of the compounds such as would be expected if the lag were due to a delay in absorption from a subcutaneous deposit.

I quite agree with the point about cancer chemotherapy as used clinically. If we had used these agents in the way they are used in the clinic, I do not think anybody could discriminate between them. We would know that they all inhibited antibody production but we would have no information about time-dependent effects. Whether they are used in the correct way clinically, I am not competent to judge. There is evidence presented by Skipper, H. E., Schabel, F. M., Jr., and Wilcox, W. S. [(1966) *Cancer Chemotherapy Rept.* **35** 1], for instance, suggesting that the drugs act by cell destruction and that the repeated doses used clinically simply destroy the same proportion of a proliferating cell population with each dose. The magnitude of the antitumor effect then depends on the proportion of cells kill, the rate of proliferation, and the interval between doses. However, whether clinical drug regimens are correctly applied or not is irrelevant here. The point is that their complexity makes it extremely difficult to analyze the modes of action of these drugs. Since this analysis is our aim, we use simple experimental models instead.

DR. NICHOL: There are some pharmacological factors that bear on this discussion. To group these agents simply as anticancer agents overlooks striking differences in their action. Actually, the alkylating agents represent a group distinctly different from the antimetabolites in that they can affect cells at almost any stage of the cell cycle whereas with a number of the antimetabolites it is necessary to expose cells throughout at least one division cycle in order to achieve any pronounced toxic effect. Some experiments that Dr. Mihich and I carried out several years ago indicated that single doses of some antimetabolites are quite ineffective in altering the viability of tumor cells exposed to the drugs for 4 hours even when these compounds were administered to animals at doses four times the LD_{50} [Mihich *et al.*, (1961) *Cancer Res.* **21**, 323]. A single injection of alkylating agents has distinctly different effects. These considerations may bear on some of the interpretations about inhibiting the proliferation of immunocompetent cells. It is not possible to conclude that an antimetab-

olite is ineffective on cell proliferation simply because it was given as a single dose which might have eliminated only a small fraction of the cell population that happened to be at a certain phase of the cell cycle when exposed to the drug. I think that the pharmacological factors have to be kept in mind in classing the drugs used as active or inactive.

DR. AMIEL: In connection with the comments of Dr. Holland and Dr. Nichol, giving single doses of drugs not only can simplify the problem but, in some cases, it also may give an incomplete idea of the mechanism of action of the immunosuppressive drugs.

DR. BERENBAUM: One is only complicating matters by giving several repeated doses of an agent. In such cases you have to give smaller doses than can be given as a single dose, you are dealing with cumulative effects over a period of time, and you do not really know what the critical time is. You might even introduce a mixture of enhancement and depression. Suppose the course of drug administration started before the antigen is given and finished afterward. You might have partly what Dr. Schwartz described, that is, enhancement from the administration before antigen, and partly depression from the administration afterward. Let us solve the problem using simple experimental designs, and then we can go on to more complicated ones.

DR. SCHWARTZ: I think that we are confusing two issues here. Dr. Berenbaum wants to find a tool which he can use in trying to understand the immune response, and he is attempting to use a number of very interesting compounds to dissect this process. Others of us are interested in a tool which we can use to treat patients. We are both using the same compounds in immunological processes but for entirely different purposes.

DR. SAMUELS: Apropos of Dr. Nichol's comments about the effectiveness of single exposure of cells to alkylating agents, I would like to mention that recently I started a child on a new protocol which initially requires a single intravenous injection of 10 mg/kg of cytoxan in cases of advanced untreated neuroblastoma. One week after the single injection of cytoxan the child was almost totally in remission.

DR. KAPLAN: Applying the principle of using antimetabolites as tools to investigate immune responses, I wonder if you have had a chance yet to use an agent like bromodeoxyuridine which is incorporated into DNA, or cytosine arabinoside which is incorporated into RNA and to a lesser extent into DNA. Such a compound may be incorporated into cells which are not necessarily proliferating but might be differentiating.

DR. BERENBAUM: I have not used them.

DR. SANTOS: The idea of a parallelism between antitumor and antiimmune effects is not valid. These compounds are good cellular poisons. A drug affecting one type of tumor or cell does not necessarily affect another. Therefore the parallelism is merely accidental and incomplete. Second, I wonder whether you have not complicated your system by giving insoluble drugs subcutaneously with saline since they may act as a depot form.

DR. BERENBAUM: I do not think that the concept of cell poison is very helpful. Most agents will act as cell poisons if you give them in sufficient doses. This question was disposed of many years ago by Malmgren *et al.* [(1952) *Proc. Soc. Exptl. Biol. Med.* **79,** 484; (1952) *J. Natl. Cancer Inst.* **12,** 807] and others who gave nonspecific cell poisons to animals during an antibody response. They showed that even though the animals were so poisoned as to die shortly afterward, they still produced normal amounts of antibodies. We did similar work some years ago [Berenbaum (1960) *Nature* **185,** 167; Berenbaum (1962) *Nature* **196,** 384]. We gave a range of substances, some of which were cell poisons, miscel-

laneous antibiotics, boric acid, potassium cyanide, and so on. Some of these were agents used for the treatment of cancer. They were all given in the lethal dose range but only the agents which were useful in the treatment of cancer inhibited immune responses; the other cell poisons did not. Therefore, there is some correlation.

Immunochemical Studies of Mammalian Cell Membranes

WILLIAM BOYLE

Department of Microbiology and Immunology,
Duke University Medical Center,
Durham, North Carolina

I. INTRODUCTION

The study of tissue antigens has increased rapidly in recent years and much of this stimulus has come from areas where there exists an immediate practical application. For example, routine organ transplantation requires the detection and classification of the antigenic differences between individuals; the nature of the immunological specificity of organs and tissues has critical relevance to the understanding and future control of autoimmune disease, while the potential benefits of a clear definition of the antigenic change accompanying carcinogenesis need no repeating.

The accumulation of data in such areas has been massive, as recent reviews testify. Yet because of the comparative newness of the studies and the underlying concept of practical application, presently available information is largely restricted to establishment of specificity, methodology, and phenotypic expression. A significant deficiency is the lack of immunochemical knowledge of tissue antigens. Yet the need exists. A knowledge of the nature of particular antigens in such areas as mentioned above, and the ability to produce the antigen in a stable reproducible form would speed the solution of many practical problems. Beyond this, the development of a comprehensive picture of the fundamental immunochemical organization of cells would help the recognition of new cellular antigens in the future. At present immunochemical investigations of tissue are concerned with isolation and characterization of antigens, and also with attempts to describe their subcellular location.

Knowing this would go a long way in filling the presently abysmal gap in our knowledge of the cellular function of tissue antigens. In view of the specificity of immunological methods and the wide range of tissue and cell types presently under study, not least could be the contribution to the elucidation of the molecular anatomy of cells and tissues.

II. THE IMMUNOCHEMICAL PROBLEM

There are some generalizations that partially explain the present paucity of immunochemical data on tissue antigens. Initially, a period must elapse in which basic immunological assays are developed and to some extent the comparative newness of tissue immunology is a factor here. Much more relevant is the shortage of material for investigation. Especially in areas where isospecificity or organ specificity is being investigated, the inability to obtain bulk materials has been an acute problem, and frequently immunochemical fractionation leading to antigen enrichment in a cell-free fraction has been terminated due to lack of material, when only preliminary results had been achieved.

Two general types of tissue antigen can be distinguished. The first are those which by a simple disruption technique are released from the tissue in a soluble form, or which are found in a soluble form in an associated tissue fluid. For this group it is comparatively easy to determine some of their physical and chemical characteristics (Boyle *et al.*, 1963). This permits correlation of the findings of different investigators, and provides useful markers by which, over a period, cumulative data on such materials can be gathered. Such antigens are amenable to a wide range of gentle purification procedures such as electrophoresis, molecular sieving, ion-exchange chromatography, fractional precipitation, etc. For antigens of this type, if the demands for a quantitative assay and sufficient starting material are met, there is no reason why their complete immunochemical characterization should not be achieved.

The second type of antigen are those which are either part of, or are intimately associated with, the membranous structures of cells and are released with them following tissue homogenization. These membranes are, of course, insoluble in physiological media and, in general, methods are not available which will dissolve or disrupt the membranes, without concurrently damaging the antigenic specificities which they carry. Unfortunately many types of antigen of current interest fall into this class, e.g., transplantation antigens of mice (Davies, 1962; Kandutsch and Stimpfling, 1963) and of man (Rapaport *et al.*, 1965; Bruning *et al.*, 1964; Boyle, 1967) and a number of tissue-specific antigens (Dumonde, 1966). In contrast to the first type of antigen, methods for the purification of this type of antigen are few and arbitrary. The problem is not simply that of

finding a method of solubilization for each antigen. In the absence of more effective methods, any attempt aimed at the description of the subcellular localization of such antigens relies on tissue disruption and organelle separation. The methods of doing this are in many ways an art rather than a science. Invariably, in the absence of well-established protocols, tissues are homogenized in an empirical fashion and separation of organelles is achieved by differential centrifugation. This can be followed by density gradient fractionation, but even in this instance it is usually only in one gradient system. Thus the final products have exploited only two parameters of the organelles and cross-contamination of the products must therefore be frequently expected. Different degrees of aggregation in different homogenization media and the possibility of coaggregation of organelles aggravate the problem. The end result of this can be that correlation of the findings of different investigators is often difficult because the preparations which they employ, though given the same subcellular designation, may be very different in their composition.

This sort of variation has given rise to problems in the interpretation of the immunochemical investigations of tissue antigens and some idea of the extent of this can be shown by a consideration of the studies which have been made on the H-2 antigens of mice. Among all tissue antigens currently being studied, they have probably been the most investigated. These antigens have much in their favor in terms of background information and suitable starting material. They have a well-defined genetic background; they can be assayed *in vivo* by induction of immunity or enhancement of homografts. They can be measured *in vitro* by methods which are as sensitive and reproducible as most immunological assays. Either solid tissues or a wide range of tumors, which exist as populations of almost a single-cell type, are available as a starting source and breeding within most inbred strains presents no great problems so that bulking of the tissue or cell source from many animals is possible. Yet even with these advantages, unique in comparison with other tissue antigen systems, they have been investigated for almost 20 years and as yet there is no absolute data on their chemical nature nor universal agreement on their subcellular localization.

Throughout the years it has been variously claimed that the H-2 antigens were deoxyribonucleic acid (DNA), glycoprotein, not ribonucleic acid (RNA), protein, mucoprotein, and latterly lipoprotein. The "definitive" study showing that they were mineral would have come as a nice completion to the series, but scarcely as a surprise! The consensus of the latest work is that the antigens are part of a lipoprotein complex. The high lipid content precluded their further resolution, and major effort has been concentrated on obtaining an active subfraction with reduced lipid con-

tent. Some success has recently been achieved. Graff and Kandutsch (1966) have reported the preparation of active products with minimal lipid content but they were still insoluble. By enzymic digestion of the complex Nathenson and Davies (1966) have produced a soluble product which contained all the prescribed H-2 antigen specificities and could induce antibody formation. The solubilization method did not release the non-H-2 isoantigenic activity from the complex, which will be a considerable help in immunochemical definition of the H-2 epitopes. But it does show that with membrane-bound antigens any single solubilization method will not release all of them. This raises the question of whether, for every membrane antigen system that will become of interest in the future, we must expect the same tortuous path to solubilization and definition as has been the case with the H-2 system.

In the area of subcellular localization even more confusion arises. In free cell suspensions the presence of the antigens can be shown by direct serological tests which are known to involve reaction at the cell surface. By immunofluorescence on cell sections, the surface location was corroborated but no definite answer was obtained about the intracellular location (Möller, 1961). So most information has had to be achieved by disruption of tissues and determination of the antigen content of the subcellular fractions by antibody absorption or by induction of immunity.

By assay depending on *in vivo* ability to sensitize to homografts, the nucleus of tissues was initially cited as the antigen source (Billingham *et al.*, 1956; Oth and Castermans, 1959). From liver cells a plasma membrane fraction was shown to have activity in serological assays (Herzenberg and Herzenberg, 1961), while from the same tissue most antigenic activity was claimed to occur with mitochondria or lysozomes (Basch and Stetson, 1963). From tumor cells *in vivo* testing showed the antigenic activity to be distributed through most subcellular organelles (Kandutsch, 1960; Dumonde *et al.*, 1963). From studies employing both *in vivo* and *in vitro* testing, it was suggested that the activity found in most subcellular organelles was haptenic, while the complete antigenic activity was limited to the lipoprotein fraction of microsomes (Manson *et al.*, 1963).

Within the past year two very critical studies have been made. By comparison of the absorption of H-2 antibody by intact tumor cells and sonicates of such cells, it appeared that on an average, 87% of the H-2 antigen content of these cells was located on the surface of the cell (Haughton, 1966). In the second study, a number of subcellular fractions were independently prepared from mouse livers, and their degree of purity established by characteristic enzyme assays. Examination of the antigen content of such fractions suggested that the activity was found in

nuclear, lysosomal, plasma, and microsomal membranes, but was absent from the mitochondrial membranes (Heberman and Stetson, 1965). So the confusion remains. Are these differences reflections only of the different tissue source or how much can be explained on the basis of the inadequacy, however carefully done, of the present techniques of subcellular fractionations?

Thus the deficiency of tissue subfractionation hampers the definition of subcellular localization, and gives rise to complexity in comparing studies by different investigators on characterization of the same antigens. This problem is vast and no easy solution can be prescribed. Yet two basic facts stand out. For the H-2 antigens, and for many other membrane-associated antigens, serological tests have demonstrated that the antigens are located on the external surface of the cell. Secondly, in classical tissue fractionation there is no indication of where the surface membrane finally locates among the nuclear, mitochondrial, microsomal, or soluble fractions.

Thus two things seem apparent, namely, that in order to ascribe subcellular distribution to an antigen which is already known to be exposed on the cell surface, account must be made of the contribution of the surface membrane fragments, and secondly, the characterization of such surface antigens might be simplified by the use of plasma membrane preparations for investigation rather than mixed cell membrane sources.

Recently, reports have claimed the preparation of plasma membrane from a number of tissues and the results of these preliminary investigations and the indications they offer for subsequent development are considered below.

III. METHODS FOR THE PREPARATION OF PLASMA MEMBRANES

A. From Solid Tissues

Neville (1960) described a method for the isolation of a plasma membrane fraction from rat liver. The livers were disrupted in weak bicarbonate buffer by gentle treatment in a Dounce homogenizer. After filtration of the homogenate to remove precipitated nucleoprotein and connective tissue, a crude plasma membrane fraction was obtained, by low speed centrifugation, as an overlay on the sediment of nuclei and unbroken cells. Further purification consisted in repeated washing of the plasma membrane layer to eliminate mitochondria, and finally isopycnic banding between two layers of sucrose of density 1.16 and 1.22 to eliminate residual contamination, mainly by nuclear membranes.

The protocol for the purification was established through phase microscopic examination at each stage. The predominant cleavage of the cells

was believed to be at the blood front of the cell, the apposed parts of adjacent cells at the biliary front being sufficiently bound so that their resultant strength was enough to resist breakage. This produced pieces of plasma membrane with characteristic X, Y, and L shapes as seen under phase microscopy. This useful morphological marker was, however, lost as purification proceeded, since the membrane pieces underwent fragmentation. The proof of the plasma membrane origin of the preparation was by electron microscope examination. This showed the presence of double cell profiles similar in size and shape to apposed cells in intact liver. Collections of vesicles were seen lying within a space bound by membranes in a fashion corresponding to the arrangement of bile canaliculi of the intact tissue. No nuclear, mitochondrial, or ribosomal particles were detected by electron microscope examination. The preparations were not examined biochemically or immunologically.

The method of Neville has been slightly modified by Emmelot *et al.* (1964). They improved the final isopycnic banding by introducing a third layer of sucrose of density 1.18. This was claimed to remove more effectively the contamination by nuclear membranes. Their preparations from rat liver showed the same electron microscope morphology as those of Neville. This product received extensive enzymic examination. From these studies it was shown that lysozomal and mitochondrial enzymes were absent. Some enzymes previously detected in endoplasmic reticulum preparations were detected. Electron micrographs had shown some intracellular vesicles near the plasma membrane. These facts together were accepted as an indication of structural continuity between the endoplasmic reticulum and plasma membrane and not as an indication of contamination by endoplasmic reticulum membranes. (The chemical and immunological analysis of these products are described in Section V.) With hepatomas it was found necessary to carry out the initial homogenization in citric acid buffer but subsequent processing could be handled as for normal liver (Emmelot and Bos, 1966).

Neville (1960) chose bicarbonate buffer for his homogenization because other media were found to result in nuclear damage and final contamination with nuclear membranes. Takeuchi and Terayama (1965) have, however, carried out the Neville process in an isotonic sucrose medium augmented with calcium, and have claimed no nuclear membrane contamination in plasma membrane preparations from rat liver.

The method of Emmelot *et al.* (1964) has been applied to intestinal epithelium, liver, and kidney of guinea pigs by Finean *et al.* (1966). Electron micrographs were compatible with the plasma membrane origin of the materials. However, tests for succinic dehydrogenase and glucose-6-phosphatase were applied to determine mitochondrial and endoplasmic

reticulum contamination. The intestinal epithelium product was cleanest but the liver and kidney products showed 15% and 20–30% contamination respectively. All the plasma membrane products were reported to be considerably enriched in 5′-nucleotidase and arylamidase activities and on the basis of these measurements the yield of membrane was thought to be around 5% with respect to protein.

Chemical and immunological analysis of the products described are summarized in Section V.

B. From Free Cells

From Ehrlich ascites cells Rajam and Jackson (1958) described a method which, although probably giving some enrichment of external membrane, was still extensively contaminated with other subcellular organelles even on the basis of morphological criteria.

A method which yields fragments of plasma membrane was elaborated by Kamat and Wallach (1965). This in essence depends on the fact that after disruption of free cells by cavitation in a nitrogen pressure bomb, the plasma membrane and the endoplasmic reticulum are fragmented and these fragments form vesicles with entrapped fluid. Both types of vesicle are sedimentable together in a microsome fraction, but they show different stability. By washing with buffer solutions of decreasing molarity, followed by a period of equilibration in the presence of magnesium ions, the vesicles of the endoplasmic reticulum are preferentially "lysed." The effective step is then to centrifuge the mixture in gradients of a nonionic polymer. As a result, the vesicles will retain within them a fluid which will not equilibrate with the gradient solutions and their effective density will therefore be a function of the density of both the entrapped fluid and the limiting membrane; the lysed vesicles which originated from the endoplasmic reticulum will have a density only of the membrane. Thus a separation is effected in which the plasma membrane fragments are found at a density of less than 1.09 and the heavier endoplasmic reticulum vesicles are found in the region of density 1.16. This method was developed for the Ehrlich ascites cell and the purification protocol was established by the distribution of ATPase and surface antigen markers in a number of density gradient media (Kamat and Wallach, 1965; Wallach and Ullrey, 1964).

The method has been applied to BP8 ascites sarcoma (Boyle, 1967) and the results obtained are described in Section V. It appears likely that the method will be applicable to a number of cell types that can be obtained in suspension. Human normal and leukemic peripheral lymphocytes, mouse and human lymph nodes, and a number of mouse ascites tumor cells have been subjected to this method. In all instances the putative plasma membrane preparation was greatly enriched in the specific iso-

antigens known to occur on the surface of the intact cell (Boyle, unpublished). It should be noted that the period of equilibration in the presence of Mg^{2+} ion during which selective lysis of the endoplasmic reticulum vesicles occurs had to be adjusted for each cell type.

IV. RECOGNITION MARKER FOR PLASMA MEMBRANE

To investigate methods for preparation of plasma membranes, to prove that the final material is in fact derived from the plasma membrane, and to obtain an estimate either of the yield or purity of the preparation, some method of recognition is essential.

A. Morphology

The claim that a preparation is plasma membrane will be more convincing if morphological resemblance can be shown between the final product and the surface of the intact cell. The problem is that, unlike erythrocyte stroma or bacterial cell walls, the plasma membranes of tissue cells are much more fragile and homogenization will always fragment the membrane. The preparations from rat liver which do bear a resemblance to the original tissue were obtained only when very gentle homogenization was applied. The maintenance of the integrity of large parts of the membrane appears to require that the membrane is strengthened by connection with the membrane of adjacent cells. So the success with liver and epithelial tissue may not be reproduced when softer tissues such as spleen are subjected to the same methods. Even with the liver preparations, it should be noted that the characteristic shapes described by Neville for recognition of the plasma membrane fragments by phase microscopy did disappear as purification proceeded.

Electron microscopy will serve as a useful terminal examination but it cannot be expected to be a routine measure for examining the numerous fractions that would be produced during investigations designed to improve yield of membrane. The other insufficiency of the electron miscroscope is that it could not readily be used to give quantitative description. So other recognition markers which could be shown to be distinctive and were easily assayed would be necessary.

B. Enzymes

There exists considerable evidence of the association of Na^{+}-K^{+}-[Mg^{2+}] ATPases with exposed surfaces of a number of cell types. This enzyme activity has been found also in plasma membrane preparations from both solid tissues and free cells. At present it appears to be a distinctive component of plasma membranes (Emmelot *et al.*, 1964; Kamat and Wallach, 1965) and would seem to present the most useful

marker. It has the disadvantage that the ATPase of the surface of the cell may not be distinguishable from that of the endoplasmic reticulum. Further, it is an unstable enzyme even at low temperatures (Wallach and Ullrey, 1964) and where preparation of membranes takes a long time, unless the endoplasmic reticulum and plasma membrane enzymes degenerate at the same rate, its significance as a quantitative marker must be questioned.

The extensive enzymic studies of Emmelot and his colleagues (1964) showed that, apart from ATPase activity, the plasma membrane preparations were more active in 5′-mononucleotidase and NAD pyrophosphatase activities than a corresponding microsomal fraction. El-Aaser *et al.* (1966) have also indicated the use of 5′-mononucleotidase as a plasma membrane marker in their studies on rat liver fractions. Finean *et al.* (1966) report that they employed an arylamidase as a marker in following purification of plasma membrane from a number of tissues but the details of the specificity and quantitation of this enzyme in plasma membrane fractions were not recorded.

C. Chemical Components

Erythrocyte stroma and myelin have often been considered as models for plasma membrane. Both of these have a high sterol content, giving a ratio of sterol to phospholipid in the region of 1:1. Finean and his colleagues (1966) obtained plasma membranes from a number of guinea pig tissues and found a value of 1.17 for intestinal epithelium membrane. The other tissues did not give such a ratio and it was suggested that this probably was due to known contamination of these preparations with other organelles. However, Ashworth and Green (1966) examined plasma membrane preparations prepared by different methods from a number of rat tissues and found that the sterol:phospholipid ratio in these varied from 0.24 to 1.32. So it seems unlikely that this parameter will be useful as a plasma membrane marker.

Many cell types have been shown to have sialic acid exposed on their cell surface by the reduction in their electrophoretic mobility following neuraminidase treatment. As expected, sialic acid has been found in all the plasma membrane preparations examined (Emmelot *et al.*, 1964; Takeuchi and Terayama, 1965; Boyle, 1967). However, there appears to be conflict in whether the sialic acid is also within the cell. Wallach and Eylar (1961) reported that at least 79% of the total sialic acid content of the Ehrlich ascites tumor was exposed on the external surface. Boyle (1967) found that for an ascites sarcoma the endoplasmic reticulum contained about half the content of sialic acid per unit weight as did the plasma membrane. Rat liver plasma membranes prepared by Takeuchi

and Terayama (1965) were reported to contain about 0.3 μmoles of sialic acid per milligram of protein while those prepared by Emmelot *et al.* (1964) contained more than twice this amount. Further studies are obviously required in this area and it would be particularly pertinent to determine if the sialic acids are lipid-bound or protein-bound since this could be a source of confusion.

The minimal amount of chemical characterization which has so far been achieved (Section V, A) has not yet indicated distinctive components in plasma membrane preparations.

D. Immunological Markers

In Section V, B the few immunological studies completed are considered. For the liver plasma membrane preparations of Emmelot *et al.* (1964) the three nonserum antigens released by saline extraction suggest possible markers. However, it will be necessary to examine membrane preparations from the other parts of the cell to establish this. The fact that these antigens can freely diffuse from liver homogenates makes it essential to determine if they exist in a soluble form in the cell and have only been absorbed to the plasma membrane. They failed to readsorb during dialysis against sucrose; a more critical dialysis would have been against hypotonic bicarbonate.

The great enrichment in H-2 isoantigen content found in the plasma membrane fraction from a mouse ascites sarcoma (Boyle, 1967) supports the experiments of Haughton (1966) that the major part of the H-2 antigen content of ascites cells is on the surface. Yet it should be noted that it is unsound to use the antigen content of a putative plasma membrane fraction as a proof that the membrane is of surface origin and the corollary that such antigens are therefore predominantly surface-located. The antigens detected by Boyle (1967) in immunodiffusion tests could not be found when endoplasmic reticulum, nuclear, and mitochondrial fractions were similarly tested. However, the limited quantities of endoplasmic reticulum preparation precluded the necessary control of absorption of the serum with them and until this experiment can be completed, the claim for specific surface membrane location of these antigens can only be tentative.

What does seem a distinct probability is that no single antigenic specificity will be found exclusive to the plasma membrane. However, the quantitation of a number of antigenic specificities of both iso- and species-specificity may reveal a ratio between them which would be distinctive for the plasma membrane. Any future studies of the immunological structure of membrane preparations should examine as many antigenic specificities as possible with this concept in mind.

E. Applied Markers

A suitable marker for recognition of plasma membrane would be a material which could be applied exclusively at the surface, which could be visualized or demonstrated there, and which could easily be assayed in homogenate subfraction. The marker should not exchange between organelles and the assay should be independent of aggregation of the membrane and free from interference by cell products. A useful adjunct to this study would be a second substance, preferably similar in nature to the cell surface marker, which would be known to penetrate the cell.

There is no obvious material which satisfies all these requirements, but some preliminary experiments in this area have been made. A comparison of the subcellular distribution of radioactive chromate and chromic ions has been made for a mouse ascites sarcoma (Boyle *et al.*, 1967). These have the advantage of easy assay without interference but cannot be located by radioautography or by histochemical tests at the levels used. Analysis of the results of these studies suggested that the chromate ion entered the cell, was reduced and combined with intracellular protein, while the chromic ion probably did not enter the cell, in agreement with the results of previous studies on erythrocytes. The plasma membrane preparation obtained from cells labeled with chromic ion, believed to be located only at the surface of the intact cell, contained at best 10% of the total radioactivity of the cell. There was distribution of radioactivity throughout all the subcellular organelles. This did suggest that the percentage yield of plasma membrane was low and that cross-contamination occurred. However, some other preliminary experiments using radioactive iodine bear on this problem. Iodine was covalently linked to intact ascites cells and as a control iodine was allowed to absorb to the cell without covalent linkage (Boyle and Day, 1967). Radioautographs showed that where the iodine was linked to the cell it was limited to the periphery of the cell, and in the control radioactivity was demonstrated throughout the cell. Plasma membrane preparations from cells with the covalently bound iodine contained only 5–8% of the radioactivity of the whole cell. The soluble protein fraction from the cell homogenate contained about 30% of the radioactivity of iodinated cells but only 8% of the radioactivity of cells treated with chromic ion. Thus, labels applied to cell surfaces may react with surface materials which are solubilized by the disruption technique and cross-contamination of subcellular organelles may be due to reassociation of such solubilized materials with other organelle membranes and not due to distribution of complete plasma membrane fragments throughout the subcellular fractions. It appears that continuation of experiments of this sort could yield useful information on

loss of surface materials, coaggregation, and an easy method of investigating methodological improvements. Other markers must be sought. Maddy (1964) described a fluorescent stilbene derivative which attached strongly and specifically to erythrocytes and could be quantitatively recovered in the stroma. While the erythrocyte is a much simpler model and the fluorescent assay of the material could be subject to quenching when examined in nucleated cell homogenates, the use of such compounds should be investigated in nucleated cells.

V. COMPOSITION OF PLASMA MEMBRANE PREPARATIONS

A. Chemical Studies

The rat liver preparations of Emmelot *et al.* (1964) were described as a "phospholipoglycoprotein" core to which were attached proteins which could easily be removed by saline. The whole plasma membrane preparation contained per 100 molecules of phospholipid 40 molecules of cholesterol, 17 of hexose, 16 of hexosamine, and 9 of sialic acid. The hexosamine and 95% of the sialic acid remained insoluble when saline extraction removed 25–30% of the total protein of the preparation. However 40–50% of the sialic acid could be removed by trypsin treatment and 70% by neuraminidase treatment of the whole membranes. The rat liver plasma membranes produced by Takeuchi and Terayama (1965) were composed of 41–54% protein, 26–32% phospholipid, 4–7% cholesterol, RNA 3–4%, hexosamine 1% and sialic acid 0.1%. There are obvious discrepancies between these two sets of analyses for plasma membrane from the same source, but they may reflect a difference in the methods employed to isolate them (see Section III, A). The plasma membranes prepared from guinea pig tissues by Finean and his colleagues (1966) have been examined quantitatively only for sterol : phospholipid ratios (see Section IV, C). By thin-layer chromatography, lecithin, phosphatidylserine, sphingomyelin, and cerebrosides were recorded.

When plasma membrane (PM) fragments have been prepared by the Kamat and Wallach (1965) method from free cells, analyses have so far been concentrated on showing that the fragments differ from the endoplasmic reticulum (ER) and are not just a less aggregated form. For the BP8 ascites sarcoma, the N : P ratio has not been found to differ significantly but the sialic content per unit weight of the PM fraction has been found to be twice that of the ER preparation. Preliminary thin-layer chromatography of the phospholipids of both preparations has revealed only quantitative differences. However, reduction cleavage and alkylation products of both preparations showed on electrophoresis at least

5 common subunits, 2 subunits detected only in the PM and one found only in the ER fraction (Boyle, 1967).

B. Immunological Studies

The preparation of mouse liver cell membrane of Herzenberg and Herzenberg (1961) was shown to carry the strain-specific H-2 antigenic specificities by inhibition of hemagglutination. The plasma membrane fragments prepared from Ehrlich ascites cells showed specific inhibitory activity in the agglutination of Ehrlich ascites cells by a horse antiserum prepared against a whole microsomal preparation of the cells. The activity of the plasma membrane fraction represented 88% of the antigenic activity of the whole microsome fraction and the endoplasmic reticulum fraction 12% (Kamat and Wallach, 1965).

The immunological experiments of Emmelot *et al.* (1964) on the rat liver plasma membranes were carried out using rabbit antisera to their preparations. With a fluorescent-antibody sandwich technique the antisera showed strong reactivity with the plasma membrane preparations. When applied to intact liver sections, the fluorescence was concentrated at the cell surfaces, but some intracellular fluorescence was detected and appeared to be localized to endoplasmic reticulum near the bile spaces and around the nucleus. They found that extraction of the plasma membranes with saline extracted 20–35% of the total protein of the preparation. These saline-soluble membrane proteins gave four precipitin bands on immunoelectrophoresis using antisera to the whole membranes. Similar results were claimed when whole liver homogenate was electrophoresed, and one of the antigens appeared to be present also in rat serum (see Section IV,D).

From BP8 ascites tumor cells the separation of isoantigenic activity determined either by hemagglutination or cytotoxicity inhibition tests was about 65% in favor of the plasma membrane fragment and about 30% in the endoplasmic reticulum in terms of specific antigen content per unit weight of material. On the same fractions, estimates were made of their species antigen content. It had been demonstrated that on the surface of such ascites tumor cells there exist three independently detectable surface agglutinogens (Boyle and Davies, 1966). These were termed T, the dominant agglutinogen of the tumor cell; C, an antigen present on the tumor cell surface and also present on the surface of mouse erythrocytes, and F, a heterophile antigen common to the surface of the mouse tumor cell and the sheep erythrocyte. In Table I it is seen that the plasma membrane fragment showed considerable enrichment of the T and C antigens but that there was a deficiency in the F antigen content. These results

were corroborated by preliminary tests of the immunogenicity of the plasma membrane fragment in rabbits. These preparations induced the formation of antibody with the T and C specificity but there has been no occasion yet where the F antibody activity has been demonstrated in these tests. It is also of interest to note that the endoplasmic reticulum fraction contains T and C antigenic activity. Part of this could be due to contamination with some plasma membrane-derived material but the relative content of the T and C antigens are different from that found for the plasma membrane fragment.

TABLE I
INHIBITION OF AGGLUTINATION OF MOUSE ASCITES TUMOR CELLS (TC), MOUSE ERYTHROCYTES (MRC), AND SHEEP ERYTHROCYTES (SRC) BY PLASMA MEMBRANE AND ENDOPLASMIC RETICULUM PREPARATIONS FROM BP8 CELLS

	Minimum inhibitory concentration[a]		
Inhibitor	TC	MRC	SRC
PM fraction	105	105	>1500
ER fraction	615	2460	>2460

[a] Lowest concentration in micrograms which inhibited agglutination by a standard dilution of rabbit anti-TC serum.

Attempts were made to determine the possible presence of any of the known antigens of mouse ascites tumor cells which had been revealed by immunodiffusion tests (Boyle *et al.*, 1963). In Table II are shown the summary of some such tests. It should be noted that it has so far been impossible to demonstrate any activity between antisera against mouse tumor cells or mouse serum and the plasma membrane fragments.

VI. GENERAL DISCUSSION

The methods which have been described for the isolation of plasma membranes are not perfect and the investigation of the products has not yet been extensive. However, they already have demonstrated their usefulness in three ways.

First, they have shown the variability that can be found in the localization of plasma membrane with the classical homogenate subfractions. The liver preparations were obtained in large pieces following homogenization and sedimented with the "nuclear" fraction, while in the preparations examined from free cells, the fragmentation of the plasma membrane is more extensive and the resultant small particles were found in the

"microsomal" fraction. Had preparations of this type been obtained from tissues or cells with specific antigens on their surfaces, it can easily be seen how for the solid tissue the antigen would have been claimed to be associated with the nucleus, and from the free cell, to have been part of the endoplasmic reticulum. This is probably the basis of the type of confusion that was described in Section II.

TABLE II

SUMMARY OF IMMUNODIFFUSION REACTIONS OF PRODUCTS OF BP8 ASCITES SARCOMA WITH ANTISERA AGAINST PLASMA MEMBRANE AND ENDOPLASMIC RETICULUM AND OTHER MOUSE PRODUCTS

	Antigens[a]						
Antisera against	Ascites cells	Ascitic fluid	Serum	PM	ER	PHC	MCW
Ascites cells	+	+	+	−	−	−	+
Ascitic fluid	+	+	+	−	−	−	−
Serum	+	+	+	−	−	−	±
B	−	−	−	+	−	+	+
D	−	−	−	−	−	−	−
PHC	−	−	−	−	−	−	−
MCW	+	+	−	−	−	−	+

[a] PM = plasma membrane preparation; ER = endoplasmic reticulum preparation; PHC and MCW are phenol and methanol extracts of BP8 cells.

Second, they provide some basis for design of methods to produce better preparations of plasma membrane. For example, it is apparent that had the homogenization of the liver cells been more disruptive, smaller fragments would have been produced. In this case if they were small enough to be sedimented only in a microsomal fraction then they could not have been separated by sucrose density gradient fractionation since their density is of the same order as that of endoplasmic reticulum. This latter problem has been offered a solution by the introduction of the vesicle concept and the use of nonionic polymer gradients by Wallach and his colleagues and this could turn out to be a much-needed concept for the separation of membranes of closely similar density.

Third, the apparent applicability of the methods to tissues or cells other than those for which they were designed offers hope that for many cell types it will be possible to obtain in isolation for detailed study that part of the cell which is predominantly involved in serological reactions and which is thus of prime immunological concern.

Some other areas that need investigation now also become apparent. It is unlikely that methods available give a high percentage yield, but this is only an intuitive guess by the author since we have no accurate method of estimation at present. The problem of markers has been discussed and is mentioned here again only as a reminder of its necessity. To improve methodology we need an easy measurement of success of each method; it is necessary to determine the degree of contamination of other organelles with plasma membrane so that the description of subcellular localization of antigens may be accurate – these are the major needs which require an accurate estimable surface marker. The question of the completeness of the plasma membrane fragment must be investigated, particularly in view of the apparent loss of F-antigen from the ascites sarcoma plasma membrane and the high percentage of protein that could be removed by saline from liver cell plasma membranes. Simpler methods of preparation should also be a goal. The elegant method of obtaining the surface structure of *Amoeba proteus* described by O'Neill (1964) cannot be applied to mammalian cells. This is probably because their membranes are not as strong as the polysaccharide-augmented membrane of the ameba. Yet it does not seem unreasonable to assume that mammalian cell membranes could be strengthened by treatment and then be subject to simpler extraction methods such as that of O'Neill. This also would carry the advantage that for the more complete membrane, morphological criteria could be used to follow isolation and corroborate the origin of the preparation.

Finally, it is to be hoped that when plasma membrane preparations are generally available they will be investigated not just to determine methods of solubilization of particular antigens, but to obtain information on their immunochemical structure as membranes per se. Only in this way will a coherent picture of membrane antigen structure be obtained; only in this way will workers studying each new antigen system be able to draw on experience of previous investigations; and only in this way will the contribution of immunochemistry to structure of cells and recognition of antigen function be fully realized.

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DISCUSSION

DR. GAFFOR: We have tried Wallach's method on human tissues originating from colon carcinoma to study membrane antigens. Our yield was very low. If we started with 10 gm of tissues we could obtain 5–6 mg of protein in fraction B. What was your yield with ascites tumor cells?

DR. BOYLE: With ascites tumors we usually work on batches obtained from 100 mice which probably are equivalent to about 12–14 gm of dry weight material. From this amount we get approximately 5–6 mg total protein of fraction B, which would be equivalent to 8–12 mg in dry weight. The yield is very low. I do not know what the weight of the plasma membrane should be and, as discussed in my paper, I do not know of a foolproof marker that could be helpful in evaluating the yield.

DR. MCCOLLESTER: I would like to mention some of the difficulties we encountered in recovering surface membranes. Work in my laboratory has been directed toward developing methods for isolating surface membranes as ghosts. In the case of L1210 ascites tumor and liver cells we have observed that if the surface membrane ghosts are left in contact with the rest of the cell constituents, even at 0°C, they are very rapidly destroyed, whereas if they are isolated they are quite stable. The question is whether this stability is due to some denaturation, some cross linkage among the subunits which make up the polymer that we

call membrane, or whether it is due to the fact that being separated from the cell homogenate, the membranes are removed from a more hostile environment to one in which they are stable. Hostile conditions, traces of heavy metals and some types of poisons, will cause the membrane to produce numerous blebs [Belkin and Hardy (1961) *J. Biophys. Biochem. Cytol.* **9,** 773]. These blebs can be seen under phase microscopy and often appear to stream from the surface of the cell. The cell, however, may retain identical dimensions and the question arises as to where this material originates. Therefore, when we talk of material isolated from surface membrane, we do not know whether it comes from newly polymerized material from within the cells, or whether it actually represents the surface membranes with which we started. From our experience with skeletal muscle membrane and upon consideration of the dimensions of the surface membrane and of its density, the expected 100% recovery of surface membrane in terms of dry weight would be about one thousandth of the wet weight of the cells.

DR. BOYLE: The bleb formation in cells has interested us. Some years ago it was found that when *Amoeba proteus* was kept for 3 weeks in a medium containing 80% sucrose, initially it became intensely pyknotic but then the membrane reconstituted its original framework whereas the rest of the cell remained unchanged [O'Neill and Wolpert (1961) *Exptl. Cell. Res.* **24,** 592]. With a gentle shearing process an intact membrane "bag" was obtained with essentially no sign of cytoplasmic constituents. Unfortunately, this method does not work with mammalian cell membranes and I think the reason is that the *Amoeba proteus* membrane has a polysaccharide fibrillar coat which is ten times the thickness of the wall and this gives it some rigidity. We tried to toughen up the mammalian cell membrane wall in order to be able to use a similar method but were unsuccessful. Recently we tried to weaken the membrane. Reports by Knight *et al.* [(1966) *Nature* **210,** 1008] suggest that there may be some inhibition of reaggregation of mammalian cells induced by ATP and that there may be an actomyosinlike material on the cell surface which would explain some of its contractile properties. We thought that if we could partially remove the actomyosin, we might be able to change the surface properties. In a medium constituted essentially of 0.6 *M* KCL in bicarbonate and at room temperature, the cells become pycnotic and then, in about 20 minutes, they start to bleb as commonly seen in hypotonic solutions. These blebs will join and eventually one gets a clear vesicle with aggregated material inside the cell. Syneresis alone cannot explain this vesicle since, if the cells were pretreated with rose-bengal, the dye could be seen as a limiting membrane of the vesicle. After gentle homogenization, some of the vesicles are broken and one can see free strands of material. So far we have been unsuccessful in developing this into a preparatory method primarily because the material jells rather rapidly. We are hoping that in time we can collect the structures which actually come floating off this aggregated material.

DR. HOROSZEWICZ: Since you are dealing with a morphologically organized structure, have you tried immunofluorescence staining as a method to confirm the specificity of the isolation procedures followed? If one has antibody against the endoplasmic reticulum or some microsomal fractions, the cell should be more or less uniformly fluorescent, whereas with antibody against the outer membrane one should get a ring. Should this be the case, by fractionation of cells after incubation with the serum which gives only ring fluorescence, it may be possible to detect fluorescence after gradient centrifugation to confirm the fact that one had most of the membranes.

DR. BOYLE: In our hands fluorescent markers were not useful because when we tried to estimate fluorescence throughout homogenate fractions, nonspecific quenching occurred. At present some fluorescent dyes are being synthesized with radiolabel in them to avoid that problem. As far as using an antibody as a marker, theoretically I see many objections to it. Are the two active sites of the antibody going to be saturated by the cell surface? One

does not know what the possible common specificities are within the cell. If only one antibody valence is saturated at the surface, anything that comes from within the cell and has the same specificity would latch on to the other free valence. In terms of specificity, since we do not have a truly purified specific antibody, when we apply antibody to a cell we are putting normal globulin on it as well. In one experiment we iodinated the cell surface directly, thinking that we were probably iodinating the globulin which we know is attached there. When we disrupted these cells, 34% of the iodine was in the cell-free sap. Only 8% of what we had put on the cell was associated with the microsomal sediment.

DR. HOROSZEWICZ: Dr. Pressman showed that antibodies directed against cells do not penetrate the membranes of intact cells. Maybe by incubation of cells with specific antisera, followed by elution, one could obtain a clearer preparation against the outer membrane.

DR. BOYLE: I still do not see how that will solve the problem of differentiating normal globulin from specific antibody globulin. No matter how much one washes the preparation, some normal globulin is going to be present in excess.

DR. PRESSMAN: The experiment that you outlined with iodinated cells may be a very important one because if one iodinates only at a very low level, the few atoms of iodine will react at the cell surface. Since they react rapidly they will not have a chance to get inside. This would be a good marker. Now, if the marker is lost from the surface, and this material is being made soluble, this means that one is not isolating all of the cell surface. In connection with the problems related to the radiolabel antibody attachment and to whether normal globulin as well as antibody would attach, one could use the paired label technique by labeling normal globulin with ^{131}I and antibody with ^{125}I and by determining how much contamination of nonspecific globulin one gets and what part that contamination is playing in the experiment. In connection with your comment about the antibody having a valence of 2 and the possibility of hooking something on with the same specificity, I wonder whether this is such a bad problem.

DR. BOYLE: I think that getting the simplest material possible of only external membrane origin is a necessary goal in trying to establish a degradation pattern that we might be able to relate to species, isogenic, and organ specificities.

DR. MANSON: During the past six years, we have been carrying out a program whose objective is to characterize the isoantigens of the mouse and determine their intracellular localization. In our initial studies we found that the H-2 antigens were associated with the microsomal lipoproteins of lymphocytes and tumor cells [Manson *et al.* (1963) *J. Cellular Comp. Physiol.* **61**, 109–118]. It appeared therefore that the H-2, in addition to its expression on the outer surface of the cell membrane, had an intracellular localization as well. Both liver and kidney microsomal lipoproteins were inactive as transplantation antigens. Recently [Palm and Manson (1965) *In* "Isoantigens and Cell Interactions," Monograph, Vol. 3, pp. 21–33, Wistar Inst. Press, Philadelphia, Pennsylvania; Manson and Palm (1966) *Proc. 9th Intern. Congr. Microbiol., Moscow* Abstr. p. 642] we obtained evidence that liver preparations contain a blocking factor that inhibits the immune response *in vivo*, and this blocking factor was present in our liver microsomal lipoprotein fractions. When these fractions were prepared so that they contained little or no blocking factor, an accelerated homograft response was observed with this material. However, this lipoprotein lacked H-2 antigen activity when tested serologically *in vivo* and *in vitro*. We have thus concluded that in liver and kidney cells, the H-2 antigen may be found only on the cell membrane, whereas in spleen, thymus, and tumor cells it is found on the cell membrane as well as in the endoplasmic reticulum.

Recently we have carried on studies in cooperation with Drs. Leonard Warren and Mary Glick who have devised several methods for obtaining cell-free preparations of the cell membranes of L-cells and L-5178Y lymphoblasts [Warren *et al.* (1966) *J. Cellular Physiol.*

68, 269]. Under phase and electron microscopes membranes prepared by their methods look like isolated, intact cell membranes. Whether the preparations are completely free of intracellular contaminants is not clear. When these preparations were assayed for H-2 isoantigens, both *in vivo* and *in vitro*, some were very active, others were not. Some of the methods used to obtain the intact cell membranes may have inactivated the H-2 isoantigens of the preparations [Manson *et al.* (1967) *Wistar Inst. Symp. Monogr. No. 8* (in press)].

One serious criticism to be discussed is concerned with the methods used to evaluate the isoantigen content of these membranes. As Dr. Boyle said, when cell membranes are disrupted, one almost always finds that the product is a collection of osmotically active vesicular structures. This property has been taken advantage of by Dr. Boyle in isolating the "membrane" fraction. The serological antibody blocking methods used for determining isoantigen content *in vitro* can only detect the antigenic determinants on the outside surface of the vesicle. Antibody could not be expected to penetrate these vesicles. It becomes important therefore to know how the vesicle was formed, if the inside surface of the vesicle is identical with the outside surface, and which of the cellular structures end up on which of the vesicle's surfaces. Because of these considerations, Dr. Palm and I have decided to estimate total isoantigen content of cell-free preparations by *in vivo* methods, hoping that the animal will assay both surfaces of the vesicle for us. Dr. Palm uses an anamnestic response *in vivo* to determine the minimal sensitizing dose of a fraction [Palm and Manson (1966) *J. Cellular Physiol.* **68,** 207]. She has found that a positive antibody response in this test can be elicited by as little as 1 μg of protein per animal of a potent H-2-containing microsomal lipoprotein preparation. At the same time I have determined the minimal sensitizing dose of the fraction in the homograft response—in some strain combinations 10 μg of lymphocyte microsomal lipoprotein per animal are adequate to induce an accelerated homograft response. Some of the membrane preparations are equally active. Thus far, we have not found any preparations to be more active *in vivo* than microsomal lipoprotein preparations. Obviously, the methods of preparation and of characterization of these membranes need to be refined both from the biochemical and immunogenetical points of view before firm conclusions can be drawn in this matter.

DR. BOYLE: I have no comments except that it is good to hear that someone else is having the same type of problems.

DR. DANIELLI: I wonder whether one can distinguish between the plasma membrane and other usually intracellular membranes if an exchange between plasma membrane and internal membranes takes place as suggested by a number of electron microscopists. However, it might be feasible to measure what is actually on the outside of the cell at a particular time. In order to do that one should have on the cell surface something that will not penetrate the cell. This can be done by putting SO_3H residues on a solute, which greatly reduces the rate of permeation. In principle, at least, if one exposes the tissue to a reagent which is capable of forming a covalent bond to the cell surface and also contains these groups which prevent penetration, all one should get labeled is the material that is on the outside of the cell. This would presumably include collagen and other substances but would not include what is literally on the inside unless one used long exposures. I would suspect that to make a full-scale attack on this problem one would have to get the chemist to make a suitable set of reagents which could include azodyes or fluorescence and/or isotope label similar to Maddy's reagent. I cannot see any way of labeling the surface in the way that you want to, without making covalent bonds. I agree with you in being mistrustful of labeling with any component which can come on and off the membrane.

DR. BOYLE: We have been interested in this work of Maddy too [(1964) *Biochim. Biophys. Acta* **88,** 390], and are hoping to synthesize the reagents he used. As far as I know, Maddy did his work only with erythrocytes, and not with nucleated cells, which are quite

different. In terms of your other suggestions, we have been trying very much along the same way of thinking in relation to radiolabel markers. The radioautographs, in the experiments in which we iodinated whole cells, indicated that the label which was covalently bound was only on the outside. We had controls where the iodine was just being adsorbed and not covalently linked, and this was throughout the cell. Upon homogenization, as I told you, 30% of the label went free, suggesting that iodine was marking something which is on the surface but may not be part of the structural membrane. As indicated in my paper, we also tried to use chromium isotopes on the nucleated cells. In red cells, sodium chromate is known to go inside whereas chromic chloride does not. After homogenization, 45% of the isotope applied as chromate is associated with soluble protein whereas only 8% of the isotope applied as chromic chloride is found in this form. This would suggest that the chromic isotope is not labeling the soluble protein from inside the cell. I think this is the type of approach to pursue, but we have not developed a reliable method as yet. Recently it was reported that certain fluorescent dyes localize only on the surface of cells. Since we cannot follow our fractions using fluorescence measurements, we are now synthesizing and iodinating these reagents.

DR. ADA: There are several approaches that would be worthwhile trying. First, I noticed that between the upper B fraction and the lower D fraction there is a twofold difference in sialic acid and I wonder whether this would influence appreciably the charge of these two different types of membranes. It might be worthwhile trying electrophoresis in this connection. If this was unsuccessful or only partially successful, one might block amino groups on the surface membrane, and so accentuate the charge difference. Second, I think it may be worthwhile to see what enzymes like neuraminidase do to the surface membrane. We showed many years ago that the electrophoretic mobility of the membranes of red cells treated with neuraminidase changed from 1.30 to 0.17 μ/sec/volt/cm. This is about a 90% alteration. Perhaps most of the neuraminic acid can be split off from the surface membrane without affecting the properties of the internal membranes. To avoid action of the enzyme on the internal membrane after breakage of the cells one could treat the preparation with the antibody specific to this enzyme. Neuraminidase action against a macromolecular substrate can be inhibited completely in this way. A change in charge should occur as a result, and one might be able to take advantage of this. Finally, there is a possibility that one may be able to change the sedimentation properties of these tumor membranes quite dramatically if one puts something like ferritin onto the outside membrane. As you know, ferritin has an iron core, is very dense, and it should be relatively simple, by means of reagents such as BDB or carbodiimide to attach ferritin to the outside membrane, break the cell, and isolate the modified membrane by differential centrifugation.

DR. WITZ: Crude preparations of microsomes are quite heavily contaminated with plasma proteins. I would like to know in what microsomal subfractions you can find these contaminants, and if they are not found, how you were able to eliminate them so efficiently. Plasma proteins might serve as markers for external cellular membranes.

DR. BOYLE: After one prepares the initial crude microsome suspension there are at least four washings with gradually reduced molarity buffers and a dialysis step. All of these fractions are rich in serum proteins. You may be right that these are important markers. Since the cells we are working with are growing in ascites fluid which is essentially just a 1 to 2 dilution of mouse serum, we do not know which of the serum proteins we detect are truly part of the cell structure and which are just adsorbed. I would like at this time also to thank Dr. Ada for his comments. We are trying electrophoresis, and have not got very far yet, but I am very grateful for the suggestion about ferritin. I think that would be a very good marker and I shall try it.

DR. MILGROM: The distinction that Dr. Boyle made about extractable antigens and

antigens of the cell surface, is quite pertinent in relation to many important biological phenomena. For years the species-specific antigens were studied as soluble cell products such as thyroglobulin, γ-globulin and albumin, and differences were shown among proteins originating from various species. These antigens were not only species specific but also tissue specific. Thyroglobulin is a tissue-specific antigen of the thyroid and one may call γ-globulin a tissue-specific antigen of plasma cells. Mammalian cells contain very strong species-specific antigens that cannot be detected by precipitation tests. We studied these antigens by mixed agglutination in cell cultures. Antisera containing antibody to insoluble antigens were capable of detecting strong species-specific antigens on the cell surface, which were completely devoid of tissue specificity since they could be detected in any cell culture originating from a given species. The second point I would like to make is that antibodies against intracellular antigens can be present in the circulation without damaging the corresponding organs. Antibodies against brain antigen or thyroglobulin can reach very high titers in spite of the existence of the corresponding antigens in the body. No evidence has thus far indicated that they have any damaging effect. In contrast, antibodies against cell surface antigens can hardly be detected in the circulation. In our hands, animals which show high titers of isoantibodies to transplantation antigens lose them after they are grafted with kidney originating from the specific donor. The kidney graft removes the antibodies which are directed against the cell surface. Under autoimmune conditions, it is exceedingly difficult to detect antibodies against surface antigens. Obviously, it is difficult to say whether the animals do not produce antibodies at all or whether antibodies are being constantly removed by the antigens on the cell surface. Available evidence would indicate, however, that a profound abnormality in the immunological mechanisms is necessary in order to achieve the formation of autoantibody against cell surface antigens. This seems to be a teleological phenomenon since antibodies against cell surface antigens are harmful antibodies. Forssman-positive animals can be killed by a minute amount of serum containing Forssman antibodies. In contrast, large amounts of antibodies against, for example, thyroglobulin can be injected into an animal without consequence. Finally, I would like to make a comment about methods which are useful in the study of cell surface antigens. The methods of choice are always based upon agglutination. However, many cells cannot be obtained in a suspension stable enough to be tested by this procedure. Other procedures which were discussed today are based upon immunofluorescence. These procedures suffer from a rather low sensitivity in terms of antibody detection. In our hands mixed agglutination, especially mixed agglutination in cell cultures, seems to be a useful method to detect antigens on the cell surface. For analytical purposes, it would seem that procedures of double diffusion in gel would be best for these studies. The problem would be, therefore, to make cell particles diffuse and react in gel similarly to soluble antigens. This was feasible in our hands and we could indeed analyze particulate antigens by double diffusion in gel.

Tumor Antigens*

RICHMOND T. PREHN

The Institute for Cancer Research and
University of Pennsylvania, Philadelphia, Pennsylvania

The roadway to the understanding of tumor antigens seems to have divided into three rather indistinct and sometimes overlapping trails. One of these trails pursues those tumor antigens which are recognized by their ability to sensitize an animal of a foreign genotype, giving rise to classical antibodies, often precipitins (Blakemore and McKenna, 1964). In general, there is no evidence that this class of antigens is involved in natural tumor immunity. The demonstration of tumor specificity is dependent upon suitable absorptions with normal tissue so that quantitative rather than qualitative differences from normal tissue antigens are difficult to rule out. The appearance of these antigens is often associated with the concomitant loss of normal organ-specific antigens detected by similar means (Guelstein and Khramkova, 1965; Weiler, 1959). Apparently many of the morphologic and enzymic alterations that occur during oncogenesis are detectable by the immunologic apparatus of a different animal species and this fact provides a very useful investigative tool (Gold and Freedman, 1965; Abelev, 1965).

The second trail in tumor immunology concerns the antigens that are known to be directly determined by a viral etiological agent. Some of these give rise to tumor immunity within syngeneic animals while others, apparently nonimmunogenic, are usually detected by complement fixation tests or fluorescent antibody studies (Sjögren, 1965). Many of these antigens appear to be distinct from viral antigens per se, but to be directly determined by viral activity (Sjögren, 1965). Thus, in general, all tumors induced by a particular kind of virus share antigenic specificity but do not cross-react with tumors induced by other viruses.

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The third trail, the one which I am treading and the one which will occupy the remainder of this discussion, concerns those antigens of putatively nonviral tumors which are detected by the immunity they can engender in syngeneic animals or even in the original tumor host (Old and Boyse, 1964). The immunity they produce is strikingly similar in kind to that found in the familiar homograft reaction. This immunity is usually cellular in nature and evidence for the participation of circulating antibodies has been difficult to obtain. However, some recent evidence from diffusion-chamber studies, fluorescent antibody studies, and from studies with chemically altered antigens is highly suggestive of a humoral component (Pilch and Riggins, 1966; Czajkowski *et al.*, 1966; Jeejeebhoy *et al.*, 1966; Ishidate, 1965).

In this discussion, rather than review this entire extensive field, I will limit myself to the following questions concerning the immunogens of presumably nonviral tumors to which recent work in my own laboratory may contribute some insight:

1. What is the relation between the carcinogenic stimulus and the tumor antigens?
2. What is the relationship, if any, between the tumor antigens and neoplastic transformation?
3. Does immunity play a role in controlling these neoplasms?
4. What implications does immunity have for the mechanisms of chemical carcinogenesis?

The first insight concerning the relation of this class of tumor antigens to the carcinogen was obtained as a result of the very early work in this field. Tumor-specific immunity was first described in connection with methylcholanthrene-induced sarcomas of mice. It was immediately evident that the carcinogen was not a part of the tumor antigen, i.e., it did not serve as a hapten complexed with tumor protein (Prehn and Main, 1957). If the carcinogen per se were a part of the antigen, antigenicity could not persist, as it often does, for many tumor transplant generations.

A direct relationship between carcinogen and antigen seems unlikely because of the general lack of cross reactivity between tumors produced by one and the same chemical agent even in one and the same mouse (Globerson and Feldman, 1964). Although occasionally described, detectable cross reactions are indubitably rare. Thus, the relation of carcinogen to antigen seems, at best, very indirect.

The possible direct connection between the chemical carcinogen and the antigen has been rendered even more unlikely by the discovery of the antigenicity of those tumors produced in mice by films and foils of cellophane or other materials (Prehn, 1963a; Klein *et al.*, 1963). In this form of carcinogenesis the chemical nature or reactivity of the material plays

little if any role, the induction of neoplasia being dependent in some mysterious fashion upon the surface properties of the film (Bates and Klein, 1966). Yet these tumors, too, show immunogenicity and probably individual specificity.

Even more pertinent, perhaps, are recent findings with those neoplasms that occur almost inevitably during the cultivation of mouse cells *in vitro*. These tumors appear to occur rapidly regardless of the culture conditions, at least within the range that has been explored, and therefore do not appear to depend upon any carcinogenic chemical in the environment (Sanford, 1965). Rather, it seems to me more likely that neoplasia results when the cells are liberated from *in vivo* homeostatic controls. In any event, recent unpublished work in my laboratory has shown that these tumors are antigenic in the mice of the strain of origin and indeed, often fail to grow unless the recipient animals have undergone thymectomy and X-irradiations to reduce the immune response. Some of these tumors are very immunogenic indeed and are fully comparable to those induced *in vivo* by methylcholanthrene. Whether or not the tumors cross react is presently being determined.

Thus, it appears that there is no direct relationship between the carcinogenic stimulus and the antigenic properties of the presumably nonviral tumors. However, an indirect quantitative relationship involving latent period has been discovered. In general *in vivo,* the earlier arising tumors, and thus those occurring in response to large doses of strong carcinogens, are the more immunogenic (Prehn, 1963a). This relationship, plus the facts of the strong antigenicity of the tissue culture tumors and the immunodepressive effects of the carcinogens, suggests that tumor immunogenicity is inversely correlated with the strength of the immunologic apparatus of the host and the length of time the tumor is exposed to that apparatus.

The hypothesis that the relationship of immunogenicity to latent period is due to the selective pressure of the immune response is supported by the demonstration that tumors tend to lose their immunizing capacity during serial transplantation in immunologically competent mice (Prehn and Main, 1957; Globerson and Feldman, 1964). Furthermore, it has been shown that at any given time, a tumor is composed of numerous clones with differing immunogenic properties (Prehn, 1965). Thus, the natural variability of the neoplastic cells provides a suitable substrate for immunoselection.

The apparently random nature of the specificities and the variability of immunogenic capacities among the tumors must be considered in assessing the possible biologic import of these antigens in the process of neoplastic transformation. Are they to be considered accidents

occurring during tumor progression or are they directly and intimately related to the initial processes of carcinogenesis? No firm answer can be given, but recent work does at least offer some suggestions.

If these immunizing tumor antigens of the nonviral tumors are merely accidental consequences of the inherent variability of tumor cells, one might not expect all tumors to exhibit them. However, it is becoming increasingly evident that, although there are great variations in immunogenic capacity, all tumors, even the so-called spontaneous tumors, probably do contain these antigens to some extent (Baldwin, 1966). Antigenicity may be a universal tumor characteristic and, if so, it seems less likely to be the result of mere chance variation.

If tumor antigenicity is not a chance phenomenon, but is intimately and necessarily concerned with the basic neoplastic alteration, very early tumors or pretumorous lesions might be expected to exhibit antigenicity. We have recently been investigating two systems to determine the possible antigenicity of very early chemically induced lesions. One of my graduate students, Glenn Slemmer, has been studying the hyperplastic nodules induced in the mouse breast by feeding methylcholanthrene. These premalignant lesions can be serially transplanted in "cleared fat pads" following the method of DeOme (DeOme *et al.*, 1959). Although this work is in its infancy, findings thus far suggest that these lesions are antigenic and that they share antigenic specificity with the tumors that arise from them. A very early preliminary observation suggests that sometimes a hyperplastic outgrowth line is not only inhibited in growth but may tend to grow in a more normal and less hyperplastic pattern in immunized animals. In this system, then, antigenicity appears to arise very early in the carcinogenic process.

Another of my graduate students, Marc Lappé, has indirectly approached the same problem with cutaneous tumors induced in mouse skin by methylcholanthrene. He has found that an otherwise subcarcinogenic exposure of mouse skin to the carcinogen is adequate to produce papillomas if the skin is subsequently transplanted to a syngeneic recipient. Apparently the injury of transplantation acts as a sufficient "promoter" to elicit the otherwise dormant papillomas. The interesting point for the present discussion is that transplantation of the "initiated" skin to a sublethally irradiated, and thus immunologically crippled, recipient results in a much larger and more permanent crop of papillomas (Lappé, 1966). This result suggests, but does not rigorously prove, that even the prepapillomatous lesions of the "initiated" mouse skin may be antigenic. Again, the very early appearance of apparent antigenicity, perhaps from the very incipiency of the process argues, I think, against the thesis that antigenicity is a mere chance concomitant

of tumor progression.

If antigenicity is not a chance but a necessary phenomenon in carcinogenesis, what role does it play? To me the most appealing hypothesis, which I have set forth in previous publications, is that the immunogenic tumor antigens may represent nonfunctional heritable alterations in normal growth regulatory sites on the cell surface (Prehn, 1965). Such an hypothesis allows for the wide diversity of specificities which are encountered and is in accord with current ideas concerning some types of cell regulation (Abercrombie and Ambrose, 1962). The fact that there is not a direct correspondence between the degree of apparent immunogenicity and the rate of tumor growth is easily accounted for by secondary alterations during progression, which might block the immunogenicity of cells which actually possess high antigen contents. As an example, Globerson and Feldman have described loss of immunizing capacity without accompanying loss of responsiveness to an already established tumor-specific immunity (Globerson and Feldman, 1964).

Recently acquired data make the hypothesis that the tumor antigens result from true mutational events more unlikely. The syngeneic growth of transplants of two of three sarcomas originally induced independently by methylcholanthrene in C3H female mice seemed to be modified by prior immunization with isogenic mouse embryo tissue. The embryos were 2–3 mm in length and were implanted subcutaneously. Control mice were "immunized" in similar fashion with 2–3 mm fragments of an unrelated and noncross-reacting methylcholanthrene-induced sarcoma. Both the embryomas and the "immunizing" sarcomas were excised after 2–3 weeks of growth and the immunized animals were then challenged by the trocar inoculation of a fragment of the particular test tumor. The growths of these challenge implants are recorded in Table I.

Although much further work is necessary to determine the prevalence of the phenomenon, the above data suggest that at least some of the supposedly tumor-specific transplantation antigens may be found in the normal fetus. It may be that in this system there is no such thing as a truly tumor-specific antigen. It can be presumed that any tumor antigens that cross react with embryo represent a derepression of gene action pathways which normally find expression only transiently during ontogeny and prior to the development of the immune mechanism. Since the tumors themselves seldom cross react, a sizeable number of such antigens is suggested.

Cross reactivity of tumor antigens with embryo has been reported previously in heterologous serum systems (Gold and Freedman, 1965; Abelev *et al.*, 1963). If it is a general phenomenon, the so-called tumor-specific antigens may be merely a manifestation of the "dedifferentiation"

that occurs in cancer. In this event, it seems to me more likely that their appearance is a consequence rather than a cause of the neoplastic process.

TABLE I
GROWTH OF TRANSPLANTS OF METHYLCHOLANTHRENE-INDUCED SARCOMAS IN MICE PREVIOUSLY TREATED WITH EMBRYO TISSUE OR A FRAGMENT OF UNRELATED TUMOR

Tumor	Time after challenge (days)	Immunization with: Embryo — No. of tumors / No. of mice	Embryo — Average size (mm)	Unrelated tumor (control) — No. of tumors / No. of mice	Unrelated tumor (control) — Average size (mm)
513/6 ♀	40	8/29	4.8	13/30	7.8
722/0 ♀	19	11/31	4.8	11/30	5.0
696/4 ♀	43	9/32	3.7	23/29	13.5

In addition to the fact of the general immunogenicity of tumor cells, there is now considerable other experimental evidence to suggest that immunologic mechanisms may normally act to prevent cancer. This seems obvious in the case of viral tumors but is also probably so in the case of the supposedly nonviral neoplasms. The most impressive data come from work showing that newborn thymectomy markedly increases the susceptibility of mice to chemical carcinogenesis (Grant *et al.*, 1966; Grant and Miller, 1965; Maisin, 1964). Also, the newborn animal seems in some cases more susceptible to chemically induced tumor formation than does the adult, a fact perhaps attributable in part to the lack of maturity of the immunologic mechanism in such animals (Toth and Shubik, 1966). The remarkable propensity of rodent cells to become neoplastic in the immunity-free environment of tissue culture is also rather suggestive of a normally inhibitory function of the immune mechanism (Sanford, 1965). However, all of this evidence could be explained on nonimmunologic grounds and it must be admitted that, despite its heuristic appeal, the evidence for the importance of the immune mechanism as a suppressor of incipient neoplasms is still circumstantial. However, the evidence argues strongly against the hypothesis that an immune reaction is necessary to permit carcinogenesis (Green, 1959).

Carcinogens produce a profound interference with normal immunologic reactivity, a phenomenon that seems more than coincidental (Malmgren *et al.*, 1952; Rubin, 1964). Recently, Stjernsward, using the Jerne technique, has shown the impressive correlation between carcinogenic potency and depression of the immunologic response to sheep erythrocytes (Stjernsward, 1966). Methylcholanthrene enhances the growth of antigenic mouse sarcomas in syngeneic mice and appears to do so in relation to the immunogenicity of the tumors (Prehn, 1963b). The facts that the tumors are antigenic and that carcinogens interfere with immunity raises the interesting question as to what role this activity of a carcinogen actually plays in carcinogenesis. Could interference with immunologic activity, so as to allow growth of spontaneously occurring antigenic neoplastic cells, be the necessary and sufficient mechanism of chemical carcinogenesis?

One of my postdoctoral students, Dr. Gerald Bartlett, has approached this problem by studying carcinogenesis within diffusion chambers. An intraperitoneal diffusion chamber can be designed to exclude the immune response sufficiently to permit growth within it of the cells of a highly antigenic allelogeneic tumor (Prehn and Main, 1956). Normal fibrocytes within such a chamber undergo "spontaneous" neoplastic transformation, although the process is much slower than that seen *in vitro* (Shelton, 1963). Our reasoning was that if the chamber largely excluded the immune response, and if the only function of a carcinogen were immunologic depression, then a carcinogen should not act upon the cells grown within the chamber. In other words, we reasoned that the carcinogen should be superfluous in an already immunity-free environment. The result, as yet unpublished, was that methylcholanthrene, placed together with fibrocytes inside an intraperitoneal diffusion chamber, produced neoplastic transformation in those fibrocytes very rapidly. The preliminary conclusion was that methylcholanthrene must have some function in carcinogenesis in addition to immunologic depression. However, this conclusion is dependent upon the virtual exclusion of effective immunity from the interior of the chamber, even in the absence of carcinogen. In view of recent evidence that humoral antibody may be important in a similar tumor-antigen system, this assumption may not be justified, and I think that further work is required before we can definitely conclude that the efficacy of the carcinogen depends upon something more than interference with immunity at the site of tumor formation (Jeejeebhoy *et al.*, 1966; Ishidate, 1965).

Although immunologic depression may or may not be the whole story of chemical carcinogenesis, I feel that there are a number of indications that some form of selection of spontaneous cellular variants may account

for the activity of the hydrocarbon carcinogens in rodents. The random nature of the specificities of the tumor antigens suggests spontaneous variation. The behavior of mouse cells in culture suggests a high rate of presumably spontaneous neoplastic transformation in the absence of any carcinogen (Sanford, 1965). Thus, it is reasonable to suppose that, *in vivo,* carcinogenesis could depend simply upon an interference with normal homeostatic processes, so as to allow spontaneously occurring neoplastic mutants to survive and compete with their normal progenitors. The fact that methylcholanthrene seems to be a poor carcinogen in primates could then be explained by the lack of much spontaneous variation among primate cells (Pfeiffer and Allen, 1948). Primate cells rarely undergo spontaneous transformation in culture, thus suggesting their relative stability (Hayflick and Moorhead, 1961). At least two possible selective mechanisms by the carcinogen could be envisaged as alternatives to, or additions to, immunologic depression.

The first of these other selective mechanisms is differential toxicity. It has been demonstrated that hydrocarbon carcinogens are much more toxic to normal than to neoplastic cells (Alfred *et al.,* 1964; Vasiliev and Guelstein, 1963; Diamond, 1965). This seems to be a general phenomenon unrelated to the etiology of the neoplasm. Perhaps pertinent is the observation that neoplastic cells seem to lack the particular proteins with which the carcinogenic hydrocarbons combine (Abell and Heidelberger, 1962). In any event, it is possible to account for all of the known facts of hydrocarbon carcinogenesis by a theory based upon tumor cell selection by the differential toxicity of the carcinogen (Prehn, 1964).

The second of these other selective mechanisms I shall call the P.T. mechanism or "phenotypic transformation." It is basically an old idea clothed in slightly novel terminology. This speculative mechanism is based upon the idea, for which there is considerable evidence, that surrounding normal cells can suppress or inhibit newly formed tumor cell variants. Perhaps the best evidence for this is the observation that hyperplastic nodules of mouse breast tissue grow in gland-free breast fat pads, but are inhibited by the presence in the fat pad of normal breast tissue (DeOme *et al.,* 1959). Apparently, these incipient tumors respond to growth regulation elaborated by the surrounding normal breast tissue but fail to produce regulatory substances themselves. Somewhat analogous observations have been made in other systems and in tissue culture (Stoker, 1964). Perhaps these examples represent variations on the theme of "allogeneic inhibition" (Hellström *et al.,* 1965).

If a carcinogen combined with and neutralized the regulatory substance elaborated by the normal cells, these cells would be expected to behave and perhaps look like neoplastic cells. They would undergo a

temporary "phenotypic transformation" but the alterations would not be heritable as they are in truly neoplastic cells. In an environment of such "phenotypically transformed" cells, true neoplastic variants, which might arise spontaneously, would not be suppressed. Therefore, clones of such truly neoplastic cells might be able to grow to a large enough size so that some of the cells might be physically isolated from the normal population. Then, even after dissipation of the carcinogen, such cells could continue to grow because regulatory substances from the normal cells could no longer reach them. They would not themselves make regulatory substance because of the heritable defect with which chance variation had endowed them. It is very tempting to equate the hypothetical cell control factor and carcinogen combining factor (the C.C.F.) with the particular macromolecules recently shown by Freed and Sorof to have regulatory properties (Freed and Sorof, 1966; Prehn, 1964). However, such a conclusion, although very attractive, is probably premature.

This hypothetical mechanism of selection by "phenotypic transformation" may account for the otherwise perplexing observation that cells of neoplastic appearance, induced by a carcinogen in organ cultures, seldom seem to grow as tumors when transplanted to the animal of origin (Heidelberger, 1966). Pathologists are painfully aware that it is often difficult to decide on morphologic grounds whether or not a tissue is truly neoplastic. Perhaps environmentally imposed "phenotypic transformation" is a common occurrence in various states of chronic hyperplasia and wound repair.

It should be noted that these three possible selectional mechanisms, depression of immunity, differential toxicity, and "phenotypic transformation," are not mutually exclusive. Any one or all of them may be instrumental in chemical carcinogenesis and perhaps, at times, in other forms of carcinogenesis as well. Much more work will be necessary to define their relative roles and, indeed, to define the role of selection itself as opposed to the role of instruction, in chemical carcinogenesis (Prehn, 1964).

In conclusion, what I have said can be summarized by saying that there appears to be no direct relation between the nonviral carcinogenic agents and the tumor antigens. The random specificity of the antigens suggests a mutationlike process, but if cross reactivity with embryo tissue proves to be a real and general phenomenon, some aberrant form of differentiation is indicated. However, the very early appearance of new antigens in incipient neoplasms and their probably universal occurrence suggest that they may play an important and even necessary role in the neoplastic process. The immune response almost certainly acts as a homeostatic

mechanism in the suppression of neoplasia, but the evidence, while highly suggestive, is not yet conclusive. Interference with the immune response by the carcinogen cannot yet be ruled out as being the necessary and sufficient primary mechanism of hydrocarbon carcinogenesis. Nevertheless, other selective mechanisms, such as differential toxicity and "phenotypic transformation," are equally attractive.

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DISCUSSION

DR. SANFORD: Since you observed an increased carcinogenic effect when methylcholanthrene was used along with millipore filters, I wonder whether the binding of methylcholanthrene to DNA may result in an increase in genetic variation.

DR. PREHN: The chemists tell me that methylcholanthrene binds to DNA, to RNA, and to protein, but which reaction is significant is unknown. Our hypothesis, which we are trying to disprove, is that the action of the hydrocarbon is to interfere with homeostatic mechanisms. You are right that the completely alternative hypothesis, namely that methylcholanthrene instructs changes in the target cells, has not been ruled out. We have experiments under way to attack this basic question. Methylcholanthrene has not shown much capacity as a mutagen, however. My talk was based upon the hypothesis that selective mechanisms play an important role, perhaps the entire role, in the carcinogenic process.

DR. OSOBA: Experiments by Judy Ball and Alec McCarter at the University of Western Ontario bear upon the possibility that the immune response is affected by chemical carcinogens. They use a hydrocarbon which induces thymomas with a latent period of about 8 weeks. After the hydrocarbon is administered to the mice, they test their immune responses at various times before the development of the thymoma and find that these mice, in comparison to controls, have markedly diminished responses to sheep red blood cells. One wonders whether a depression of the immune system produced by the chemical carcinogen may bear upon the mechanism of thymoma induction. Is it possible that methylcholanthrene, when placed within the diffusion chambers as you described, is affecting the immune responses of the animal in which the diffusion chamber is placed?

DR. PREHN: The systemic effects of the methylcholanthrene placed in the diffusion chamber were not tested. The tumors all arose within the diffusion chambers themselves, that is to say, in the tissue that was inside. We were particularly concerned with the strength of the possible immune reactions inside the chambers in the presence and in the absence of methylcholanthrene, but we have experiments under way to evaluate the immunologic status of the animals in greater detail.

DR. WITZ: Why is the induced tumor not antigenic enough in the primary host to be rejected, whereas an isogeneic mouse can be immunized against it? Is it perhaps because the primary host was subjected to the carcinogen, which might exhibit immunosuppressive activity, and as a consequence may not develop an efficient immune response? Was this assumption tested by applying carcinogen to the secondary host which was immunized with the primary tumor?

DR. PREHN: Your own answer is certainly partially right. Since methylcholanthrene can increase the degree of transplantability of the primary tumor into isogeneic mice, I think that in the primary animal the immunodepression caused by the carcinogen is partially responsible for the fact that the tumor is able to grow even though it is antigenic. Probably

another reason is that in the primary mouse the tumor gets a start before there is an immune reaction.

DR. MUNYON: Have you been able to apply carcinogens to tissue cultures and select morphologically altered clones?

DR. PREHN: We have explored the possibility of selecting such clones but our experience has been confined to the mouse. We have obtained so-called spontaneous alteration rather rapidly but we have not been able to accelerate this process with methylcholanthrene. I think that this result may be related to the sensitivity of the system. I am not willing to believe that this is evidence against the possible selection of neoplastic variants by this mean, but I think that the neoplastic variants are occurring very rapidly and frequently in the tissue culture system in the mouse. This may be the reason why we cannot see anything.

DR. MILGROM: Dr. Prehn was generous enough to send me methylcholanthrene-induced tumors from C3H mice and we found that they have hemadsorbing properties. The experiments were performed in what we call closed chamber technique. A cryostat section of methylcholanthrene-induced tumor is placed on a cover slide, which is placed in such a way on a microculture slide with the concavity filled with an erythrocyte suspension that the tissue section is submerged in the suspension. The apparatus is assembled and left upside down to let the erythrocytes settle on the section; then it is reversed and the section is examined microscopically. Methylcholanthrene-induced tumors adsorb erythrocytes of some species, particularly of man. Cell cultures of methylcholanthrene-induced tumors retained this hemadsorbing capacity.

DR. FJELDE: A few years ago Dr. Turk and I used dimethylbenzanthracene and benzpyrene as carcinogens and dinitrofluorobenzene and oxazalone as skin sensitizers in different strains of mice, some of which were very sensitive to carcinogenesis. On the fourth and fifth day after painting the ears of these mice with the carcinogens, large pyrinophylic cells appeared in the paracortical region of the draining lymph nodes. This always preceded by 1 day the expression of delayed hypersensitivity. This response occurred to a higher degree in the mice more sensitive to carcinogenesis. Recent work with dinotrofluorobenzene suggests that mitogenic and, possibly, mutagenic effects occur which might be related to the genetic constitution of the mouse.

DR. HAUSCHKA: In addition to Dr. Prehn's phenomenon of P.T., phenotypic transformation, there is also G.T., genotypic transformation, normally called mutation. Mutation can occur either on the nucleotide triplet level or on the gross chromosomal level. Everyone who has seen the beautiful cytologic work of Dr. Hellström on chromosome changes in methylcholanthrene-induced mouse sarcomas is aware that karyotypic changes are regularly obtained with this carcinogen and with others as well. Chemically induced tumors exhibit a wide diversity of chromosome patterns. Some of them have clear-cut stem-cell lines, others do not. Now, how is this possibly related to the diversity of the "new" histocompatibility antigens which Dr. Prehn and others have tested for such tumors? There is little or no serologic cross reaction between the carcinogen-induced sarcomas. The normal mouse isoantigens, for example, those of the H-2 system, require a balanced chromosome set for their expression in normal titers. What happens then, if, through the medium of a chemical carcinogen, there is interference with the normal chromosome balance? Let us assume that the stem cells of a given tumor are monosomic for the ninth chromosome which controls the H-2 antigens, and polysomic for another chromosome which does not carry information for a major isoantigen, but, for example, for a tissue-specific antigen. It is possible that there is gene-dosage compensation that would allow some normal, weaker antigens, usually not detected because they do not reside on the cell surface, to come out and suddenly become detectable. In this sense, Dr. Prehn's term "phenotypic transformation" is a good choice, although there may be an underlying karyotypic disorder. The "new"

antigens unveiled in the chemically induced sarcomas may represent exaggerations of normal cell constituents rather than "tumor-specific" attributes.

DR. PREHN: Certainly we do have these two alternate hypotheses, the G.T. and the P.T. I do not deny, of course, that tumor cells represent hereditary changes but I think it is still unsettled whether these changes involve classical mutation or something more peripheral. It is still an open question whether or not the hydrocarbon carcinogen produces either of these events directly. My universe has been severely shaken by the observation that we could immunize against several methylcholanthrene-induced tumors using an early embryo. If this effect is due to embryonic antigens and not to a nonspecific adjuvant type effect, there may be no such thing as a truly tumor-specific antigen. Perhaps if one searches hard enough, one can always find the tumor antigens in some normal tissue or cell. Presumably, the antigens arose in the embryo and disappeared some time prior to the development of the immunologic capacity. The embryo has a lot of structures which arise and disappear and, if these antigens disappear from the embryo early enough, the animals will have no immunological recollection of having ever been exposed to these antigens. This is probably why we can immunize with them when these antigens reappear in the adult tumors. It is also an open question, I think, whether the antigens found in the embryo account for all the antigenicity seen in the tumors.

DR. TRENTIN: With respect to the multiplicity of antigens in chemically induced tumors as opposed to the single antigenicity of virus-induced tumors, what do you think of the possibility that, since methylcholanthrene induces tumors in many cell types, perhaps the particular type of the cell of origin might influence the type of antigenicity? This problem might be resolved by studying the transformation of cell clones in tissue culture. Also, what do you think of the possibility that methylcholanthrene is unmasking a variety of latent tumor viruses?

DR. PREHN: How many viruses can a mouse have? To me it has always been somewhat incredible that by the same procedure one would almost always unmask different viruses. This may be possible, however. I doubt that we are producing tumors in different cell types each time we produce a tumor in a mouse. We have no histologic or any other evidence of this. Many of the tumors appear to be myosarcomas. However, my whole hypothesis is, in fact, that we do have variant cells in a very variant population upon which the carcinogen can act. However, I feel that these variations are of an abnormal type. These are the cells that are already along the road toward malignancy but are normally repressed by homeostatic mechanisms.

DR. SIGEL: I would like to mention some results which may be another illustration of repression and derepression of certain antigens present in the embryo. The serum of hamsters bearing Rous sarcomas contains antibodies to this tumor detectable by the test of complement fixation for avian leukosis. Recently Dougherty and DiStephano [(1966) *Virology* **29,** 586] reported that such serum reacts with normal chick embryo liver antigen which is free of virus. Yet, when these chick embryo cells are grown in tissue culture, after two or more passages, they seem to lose this antigen. This is why in previous work this antigen was not detected in complement fixation tests performed with uninfected chick embryo tissue culture antigens. This antigen becomes manifest again in tissue cultures infected by a virus of the chicken leukosis complex. This is an example of what appears to be a normal constituent of embryonic tissue which is also present in certain specific tumors and which disappears from the tissue upon cultivation *in vitro*. Its reappearance in tissue culture could conceivably be explained either by the virus genome adding the antigen or the virus causing derepression of a cellular gene responsible for this antigen.

DR. WITZ: Dr. Prehn, in most cases you detect no cross reactivity between tumors induced by methylcholanthrene in isogeneic mice, and yet, you have found that immuniza-

tion with embryos was effective against some of those tumors. Would this indicate the presence of a cross-reacting antigen among these tumors, which is also present in the embryonic tissue?

DR. PREHN: The problem is that we do not detect a common antigen. I can conceive of a common antigen masking the detection of individual specificities, but I see no way in which individual specificity would mask a common antigen. The fact that some embryos may share antigenicity with a number of the tumors does not imply that there need be a common antigen among the various tumors themselves. Rather the implication is that the embryo has many transient antigens. The number of tumor types which may ultimately exist should be limited. I cannot conceive of an infinite variety of normal antigenic and transient constituents in the embryo. Thus, the number of types may be limited and the occasional cross reactions among them may be real.

DR. PRESSMAN: With the acetaminofluorene-induced hepatoma in rats and antiserum to it prepared in rabbits, we do get evidence of cross reactions between the various tumors. Of course this cross reactivity may be due to common embryonic components or it may be related to the fact that we are using an heterologous system. How strong is the evidence that carcinogen-induced mouse tumors are all different antigenically? Is there any cross reaction, and if so, what percentage of the tumors do cross react?

DR. PREHN: Both in Klein's laboratory and in ours, the possible cross reactivity of these tumors was investigated and in our laboratory we think we have seen it on several occasions. The techniques used, which are all transplantation techniques, are very cumbersome and imprecise. I do not think that at this time one can quantitate them and say how many tumor pairs one has to investigate before one finds a cross reaction. We have been concerned with the fact that we see a cross reaction so infrequently that this may be an artifact or a mistake. Using tumor pools for immunization, we have some indication that cross reactivity does occur, however.

DR. YOHN: My comments concern the quantitative differences between the amount of "neoantigen" (tumor antigen) found in certain viral-induced and chemically induced tumors. For example, adenovirus-12-induced hamster tumors contain viral-coded tumor antigen in amounts that are readily demonstrable by complement fixation, immunodiffusion, and by immunofluorescent procedures [Pope and Rowe (1964) *J. Exptl. Med.* **120,** 579; Berman and Rowe (1965) *J. Exptl. Med.* **121,** 955]. Recently we demonstrated both the fibrillar morphology of adenovirus-12 tumor antigen by electron microscopy and the serologic specificity of the structure with ferritin-labeled antibody to adenovirus-12 tumor antigen [Kalnins, V. L., Stitch, H. F., and Yohn, D. S. (1966) *Virology* **28,** 751]. The presence of relatively massive amounts of the highly specific antigen in adenovirus-12 induced tumor cells accounts for the serologic cross relationship between all tumors induced by this virus. The question of whether subtle antigenic changes similar to those induced by chemical carcinogens occur, particularly at the cell surface, is almost impossible to clarify by serologic methods. Transplantation studies with adenovirus-12 tumors as well as with polyoma and SV-40 tumors indicate a highly specific antigenic relationship within each virus-induced tumor type. Yet one wonders whether more subtle cellular changes, obscured by viral coded antigenic mass, might be present in viral-induced tumors and, if present, might be more relevant to the malignant properties of the cell than are the viral coded tumor antigens.

DR. MIHICH: Dr. Prehn, could you tell us more about the possible interpretation of your finding that tumors which arise after a longer latency period are less antigenic than those which arise after a shorter latency period?

DR. PREHN: We have done considerable work on the hypothesis that the reason why the earlier tumors are more highly antigenic is a matter of immunoselection. It has been observed that when one transplants serially methylcholanthrene-induced tumors, there is a

tendency toward a loss of antigen expression. This is not to contradict my earlier statement that the antigen can persist indefinitely; it certainly can but it often declines in apparent titer and sometimes virtually disappears. We have done studies to show that in any given methylcholanthrene-induced tumor there are subclones of cells with a very variable expression of the antigenic property. Apparently as long as one studies an early transplant generation, one can always find clones of cells which express antigenicity to a lesser degree than other clones. Consequently there is a very variable cell population and there is every chance for immunoselection. We think then that the reason why tumors with long latent periods are less antigenic is that if these tumors had had high antigenicity they would have been destroyed during their period of slow growth. The only tumors that can have a long latent period are those which, through a process of immune selection, express antigenicity to a lesser degree.

DR. HAUSCHKA: In agreement with your last statement that within the population of a single methylcholanthrene or dibenzanthracene-induced sarcoma there are antigenic subpopulations, we have some supporting evidence obtained using tumors chemically induced in male C57BL mice which were then tested for their capacity to grow in isologous females. The latter should uniformly reject such male tumors. However, they accepted some portions subtransplanted from the same male tumor which had lost the Y chromosome. A cell can survive well without the Y, and this cytologically verified loss is accompanied by loss of the Y antigen [Hauschka and Holdridge (1962) *Ann. N. Y. Acad. Sci.* **101,** 12–22]. Other portions of the same tumor which still had the Y chromosome and the Y antigen were rejected by isologous C57Bl females.

DR. TELLER: The ability of the host to respond to antigens may be modified by age and disease. This was brought out in some of our studies. Sometime ago, we began an investigation of the relation of aging to cancerigenesis, and decided to study changes in homeostatic mechanisms. The first one we tested was the immune reactivity of the host. We found that in the random-bred Swiss HA/ICR female mouse, in which cancer incidence increases directly with age, old mice, compared to young adults, were less capable of rejecting tumor and skin homografts, and tumor heterografts [Teller, Teller, and Robitaille (1964) *J. Natl. Cancer Inst.* **33,** 649]. They also showed a decreased phagocytic activity and produced relatively low titers of hemagglutinating and cytotoxic antibodies [Aoki, Stohr, Curlett, Kubisek, and Curtis (1965) *J. Natl. Cancer Inst.* **34,** 255; Aoki and Teller (1966) *Cancer Res.* **26,** 1648]. The decrease in immune response appeared to be directly related to age. One should keep in mind, therefore, that cellular, as well as humoral, activity may be impaired as a result of aging or disease status.

Expression of Antigens in Normal Mammalian Cells

MICHAEL SCHLESINGER*

Department of Experimental Medicine and Cancer Research
The Hebrew University Hadassah Medical School,
Jerusalem, Israel

I. INTRODUCTION

The expression of any cell characteristic is the result of complex interactions between the genetic information of the cell, the genetic regulation mechanisms, and various environmental factors. A great deal is known about the mode of inheritance of isoantigens (Amos, 1962; Batchelor, 1965; Stimpfling, 1965). Little is known, however, about the mechanisms by which the genetic information for the synthesis of each cellular antigen becomes expressed phenotypically (Schultz, 1959). A deeper understanding of the genetic regulatory mechanism in mammalian cells (Jacob and Monod, 1961) is necessary before the processes underlying the expression of antigens can be comprehended in terms of molecular biology. Some insight into the mechanism of the phenotypic expression of antigens can be gained by studying conditions in which these mechanisms are affected, and the antigenic make-up of the cells is changed. The aim of this paper is to review the information available at present on the dynamics of antigen expression in mammalian cells in different biological systems.

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II. CELLULAR ANTIGENS IN TISSUE CULTURE

A. Phenotypic Expression of Inherited Traits in Cells Grown in Vitro

Tissue culture provides a tool for the analysis at the cellular level of the mechanisms responsible for the expression of genetically determined traits, such as cellular antigens. In older work it was observed regularly that cells in tissue culture progressively lost their tissue-specific traits. Thus pigment cells might not produce pigment when grown *in vitro* (Doljanski, 1930; Whittaker, 1963) and cartilage cells might not produce cartilage (Holtzer *et al.*, 1960). Such "dedifferentiation" of cells was thought to be the rule in tissue culture, and the stability of the differentiated state of the cells was questioned. The recent development of new procedures and culture media made it possible to grow *in vitro* several differentiated cells types as cell clones maintaining their differentiated cellular phenotype. Clones of chick embryo were maintained in culture expressing the morphological and biochemical characteristics of skeletal (Konigsberg, 1963) and cardiac (Cahn, 1964) muscle, of retinal pigment cells (Cahn and Cahn, 1966), and of cartilage cells (Coon, 1966). Under appropriate culture conditions differentiated cellular phenotypes were maintained for many generations in clonal cell culture.

Several factors affect the expression of differentiated traits *in vitro*. Nutritional deficiency may cause poor differentiation. A heat-labile, heavy-molecular weight substance present in embryonic chicken extract may inhibit differentiation *in vitro* (Coon, 1966; Cahn and Cahn, 1966). The expression of differentiated traits in culture may be inhibited by high cell concentrations, while low cell concentrations favor their expression.

Coon (1966) and Cahn and Cahn (1966) demonstrated that pigment and cartilage cells grown under tissue culture conditions in which their tissue-specific traits were not expressed, could regain their differentiated characteristics when grown under suitable conditions. These experiments show that the expression of genetically determined cellular traits may depend on environmental factors, and that the phenotypic loss of a trait may not necessarily imply loss of the genetic information.

B. Manifestation of Antigens in Cells Grown in Vitro

The growth of cells in tissue culture may have different effects on the expression of different cellular antigens. Some antigens may be maintained for prolonged periods of culture, while the expression of other antigens may become lost. Yet other antigens may be acquired by the cells during their growth *in vitro*. It should be realized that similar to other genetically determined cellular traits, the expression of antigens on

cells grown *in vitro* may depend on appropriate environmental conditions. Different culture conditions may be required for the expression of different cellular antigens.

Species-specific antigens persist on cells grown *in vitro* for prolonged periods (Coombs *et al.*, 1961a). Cells of the L culture strain (Earle, 1943) maintained their murine species-specific antigenicity for over 18 years in various laboratories (Coombs *et al.*, 1961b; Defendi *et al.*, 1960; Brand and Syverton, 1962). Similar observations were made with long-term cultures of tissues derived from various species. Indeed, serological tests for species-specific antigens may be relied upon for the identification of the species of origin of cells grown *in vitro* and for the detection of contamination with cells of other species (Defendi *et al.*, 1960; Brand and Syverton, 1962).

Some red blood cell isoantigens (H,MN, and Tj^a) and some leukocyte isoantigens persist on human cells maintained in tissue culture for prolonged periods. Other isoantigens (such as human A and B isoantigens) may cease to be demonstrable on human cells after several passages of tissue culture (Högman, 1960; Coombs, 1963). The H-2 isoantigenicity of mouse cells is fully expressed even after prolonged periods of growth *in vitro* (Defendi *et al.*, 1960; Cann and Herzenberg, 1963). The cells of a subline of the L culture strain retained all the H-2 specificities characteristic of C3H mice, after 25 years of growth *in vitro* (Gangal *et al.*, 1966). Long-term cultures of murine cells may serve as rich sources for the isolation of microsomal lipoproteins carrying H-2 isoantigenic activity (Palm and Manson, 1965). Spencer *et al.* (1964) studied the isoantigenicity of somatic hybrids resulting from *in vitro* fusion of murine cells. A full codominance of the H-2 and non-H-2 isoantigens introduced by the parental cells was found. Spencer *et al.* (1964) suggested that the persistence of these isoantigens over long periods of culture, and the full antigenic codominance in heteroploid hybrids indicates that histocompatibility substances may perform a vital function in cell structure and physiology.

The expression of organ-specific antigens is lost readily in tissue culture, although it seems likely that culture conditions in these experiments may have been unsuitable for the expression of specialized cell phenotypes. Thyroid cells maintained in culture for over 48 hours loose their sensitivity to the cytotoxic antibodies present in thyroiditis patients (Irvine, 1960; Pulvertaft *et al.*, 1961). Jennings and Taylor (1966) showed that the loss of susceptibility of established thyroid cell monolayers to the cytotoxic antibody was correlated with loss of detectable thyroid epithelial cell antigen (Roitt *et al.*, 1964). The addition of various "thyroid stimulators" (including thyroid-stimulating hormone and long-acting

thyroid stimulator) did not increase the antigenicity of thyroid cells grown *in vitro*. Weiler (1959) found that hamster kidney cells lost their organ-specific antigenicity within 72 hours in culture. Similarly, no kidney-specific antigens could be detected in rat kidney cells grown *in vitro* for 6 days (Fogel, 1963). Normal skeletal muscle cells grown in tissue culture shifted from the production of myosin to production of antigenic components of connective tissue (Hiramoto *et al.*, 1961).

Cells grown *in vitro* may possess antigens which they did not show *in vivo*. There are several mechanisms, some of which will be discussed below, whereby cells may acquire new antigens in tissue culture.

Antigenic substances may be adsorbed on cell membranes and attach firmly. Bacterial polysaccharides may attach to the membranes of red blood cells and if the cells are then exposed to antibody against the polysaccharide and complement they will lyse (Adler, 1950; Neter, 1963). Similarly, buccal cells from group O persons or from group A nonsecretor persons readily adsorb the blood group A antigen when incubated in the saliva of a group A secretor person (Swinburne *et al.*, 1961). The artificially acquired A antigen is firmly attached, and mild extraction with alcohol, chloroform, or ether does not dislodge it from the cells. Hamburger *et al.* (1963) found that HeLa cells grown *in vitro* acquired antigenic specificities from the growth medium. Antigenic determinants of horse serum or fetal calf serum were firmly adsorbed on HeLa cells, and antibodies against these heterologous sera had a cytotoxic effect on the modified HeLa cells (Hamburger and Mills, 1965).

Under appropriate culture conditions, cells may be induced to form alien antigens upon addition of the corresponding RNA. Amos *et al.* (1964) grew chicken fibroblast cells under conditions of minimal RNA and protein synthesis, either by the omission of serum from the culture medium or by the addition of actinomycin D. When RNA extracted from either *Escherichia coli* or from mouse myeloma cells was added to the medium, the chicken cells produced proteins resembling antigenically either bacterial or mouse proteins. It seems that in cells in which the endogenous mRNA is exhausted, exogenous RNA may reinitiate the synthesis of those proteins for which it is coded. Fogel and Sachs (1964a) observed that hamster and mouse cells which did not synthesize the Forssman antigen *in vivo*, acquired this antigen upon growth *in vitro*. In primary cultures the antigen appeared gradually within the first 2–3 days after cell plating. It was suggested that synthesis of the F antigen was induced in cells in which originally no antigen was detectable. The synthesis of the F antigen seemed to be affected by the degree of cell organization (Fogel and Sachs, 1964b). While the F antigen was synthesized in cells grown as monolayers or cultured as aggregates by an

organ culture technique, the antigen disappeared in places where histological structures were formed, and in areas of increased cellular contacts. It seems, therefore, that the synthesis of the F antigen is induced *in vitro* in F-negative cells when normal cell contacts are disturbed, and that its synthesis is repressed when the cell contacts are restored. Franks *et al.* (1964) found that cells from F-negative species could pick up the specificity when grown in medium containing sera from F-positive species but became F-negative again after growth in media containing no F substance.

III. EMBRYONIC DIFFERENTIATION OF ANTIGENS

A. Antigenicity of the Fetal Trophoblast

Due to the genetic contribution of the father, the embryo may contain histocompatibility antigens missing in the mother, and may therefore be considered as an allogeneic homograft. The complex immunological problems involved in the maternal-fetal relation have been discussed extensively in several recent reviews (Billingham, 1964; Lanman, 1965; Douglas, 1965; Simmons and Russell, 1966a). The mechanisms that enable embryonic growth and development despite the potential danger of an immune allograft reaction by the mother are still far from clear. However, it now seems that the most important factor for the success of pregnancy is a unique biological phenomenon—the lack of expression of isoantigens on the trophoblastic cells of the placenta.

Medawar in 1953 suggested that the protection of the fetus from an immune reaction by the mother could be due to the following factors: (1) anatomical separation of the fetus from the mother; (2) antigenic immaturity of the fetus; (3) immunological inertness of the mother. Woodruff (1958) demonstrated that pregnant rats and rabbits were capable of immunological response against extrauterine grafts of their own fetuses. While other factors may also contribute to the success of pregnancy, Woodruff's experiments clearly showed that the decisive factor, both necessary and sufficient for the maintenance of pregnancy, is the anatomical separation of the fetus from the mother. The barrier between the mother and the fetus is not achieved by any special properties of the uterine decidua. Schlesinger (1962) and Poppa *et al.* (1964) found that allogeneic intrauterine grafts to nonpregnant, pseudopregnant, or unilaterally pregnant mice and rats were rejected in the same pattern as grafts to other sites. Since the uterus is not a privileged site for allograft growth, it must be assumed that the barrier between the mother and the fetus is formed by the placenta or a part of it. The placenta as a whole possesses paternally determined isoantigens, as shown both by its ability

to induce allograft immunity (Uhr and Anderson, 1962; Hašková, 1963) and by its capacity to absorb isoantibodies (Schlesinger, 1964; Bruning *et al.*, 1964). How does the placenta escape an immune reaction by the mother despite its possession of foreign antigens? It has recently been suggested by Kirby *et al.* (1964) that a layer of fibrinoid forms a protective barrier between the trophoblastic cells of the placenta and the uterine decidua. The possibility that the fibrinoid layer may protect the placenta from an immune reaction of the mother cannot be excluded at present. However, most of the evidence suggests that the privileged fate of the placenta as an allogeneic homograft is the result of the lack of expression of isoantigenicity on the trophoblastic cells which are in direct contact with the maternal decidua. Witebsky and his co-workers suggested many years ago that the chorionic villi of the human placenta were nonantigenic on the basis of their failure to absorb blood group isoantibodies (von Oettingen and Witebsky, 1928; Witebsky and Reich, 1932). These findings have recently been confirmed by studies of Thiede *et al.* (1965) with fluorescent isoantibodies. A deeper insight into the problem of the isoantigenicity of the trophoblastic cells has been possible in studies with trophoblastic cells of the mouse. Grafts of fertilized ova to extrauterine sites, such as beneath the capsule of the kidney or testes or to the spleen, give rise to growths of trophoblastic cells, while the development of embryonic elements is either very limited or completely suppressed (Fawcett, 1950; Kirby, 1960, 1963a,b). Trophoblastic growths develop from fertilized ova grafted to allogeneic hosts as well as from those grafted to isogeneic hosts (Kirby, 1960, 1963a,b; Simmons and Russell, 1966b). Fertilized mouse ova develop into trophoblastic tissue even when grafted to the kidney of rats (Kirby, 1962). The survival of trophoblastic tissue in allogeneic hosts could be attributed to failure of the small inoculum to elicit an effective immune reaction. Simmons and Russell (1962, 1966b) analyzed the growth of trophoblastic tissue in preimmunized allogeneic hosts. The ectoplacental cone (containing trophoblastic cells) of 7½-day-old C3H or (C3H × C57BL)F_1 embryos was grafted beneath the capsule of the kidney of C57BL mice immunized against C3H tissues. Grafts of ectoplacental cones result in vigorous proliferation of the trophoblastic cells in all of the immunized allogeneic recipients. On the other hand, allogeneic grafts of embryonic elements of 7½-day-old embryos were invariably rejected. These studies indicate that the trophoblastic cells do not express histocompatibility antigens. Further evidence that the trophoblastic cells do not possess detectable isoantigenicity was provided by serological techniques. The trophoblastic growths resulting from grafts of fertilized ova to allogeneic hosts failed completely to absorb H-2 isoantibodies (Schlesinger, 1964).

In contrast to the complete resistance of grafts of the ectoplacental cone to immunity of the host, the development of trophoblastic cells from grafts of fertilized mouse ova could be prevented by immunization of the host (Simmons and Russell, 1965, 1966b). The degree of inhibition of trophoblastic growth depended on the degree of immunity induced in the recipients. Animals immunized by two allogeneic skin grafts followed by 8–12 injections of allogeneic spleen cells were more resistant to the development of grafted ova than animals immunized only with one allogeneic skin graft. There also was a gene-dosage effect. Fertilized homozygous ova, from parents of a strain different from the recipient's strain, were more susceptible to inhibition in immune hosts than heterozygous ova in which one of the parents was of the same strain as the recipient and the other parent from a different strain. Grafts of heterozygous 2½-day-old fertilized ova were inhibited to the same extent in immunized recipients when either the father or the mother were of a strain different from that of the host.

It thus seems that early during embryonic development profound changes take place in the expression of isoantigens. Within 2½ days after fertilization both maternally and paternally determined isoantigens become manifest on the fertilized ova. Within the next few days, with the development of trophoblastic cells, a dichotomy appears. The cells of the embryo itself continue to express their isoantigenicity. The trophoblastic cells, specializing as a nonantigenic barrier, cease to express the isoantigens displayed by their immediate cellular precursors.

B. Antigens in the Embryo and Neonate

There is a vast literature on the differentiation of antigens in embryos, and particularly on the differentiation of organ-specific antigens. (See reviews by Langman, 1963; Nace, 1955; Ebert, 1959; Flickinger, 1962.) Embryological differentiation can be looked upon as "a sequence of changes in the patterns of protein synthesis" (Bell, 1964). It is therefore of importance to determine precisely at what developmental stage each protein is synthesized for the first time. There is some controversy about the question of whether specific proteins are synthesized long before morphological differentiation becomes apparent (Ebert, 1959; Wilt, 1962; Bell, 1964) or whether these two processes occur at about the same time (Holtzer, 1961). Ebert (1959) showed that cardiac myosin, detectable by immunological methods, first appears throughout the epiblast of the chicken embryo, and only later becomes confined to the heart-forming regions of the embryo. Ben-Or and Bell (1965) found that feather induction in 5½ to 6-day-old chick embryos was correlated with the appearance of three new antigens, one of which was skin specific. The

formation of adultlike keratin in the developing feather of 13-day-old embryos was paralleled by the appearance of a second skin-specific antigen in the feather. Some recent findings on mouse isoantigens seem to have a bearing on the problem of the time relation between the antigenic differentiation and morphological differentiation. The differentiation of murine isoantigens seems to parallel the structural differentiation of the organs. From the limited data available so far, two generalizations seem to be justified: (1) Each isoantigen may appear at different developmental stages in different organs. (2) In each organ different isoantigens may appear at the same time.

1. Time of Embryonic Appearance of Different Isoantigens

a. H-2 Isoantigens. The full maturation of H-2 antigenicity occurs at different developmental stages in various organs. The H-2 isoantigenicity of the thymus reaches adult levels already around day 17½ of gestation (Fig. 1) (Schlesinger, 1964, 1965a). The H-2 isoantigenicity of the spleen is low in the embryo (Fig. 1) but increases rapidly after birth. There is

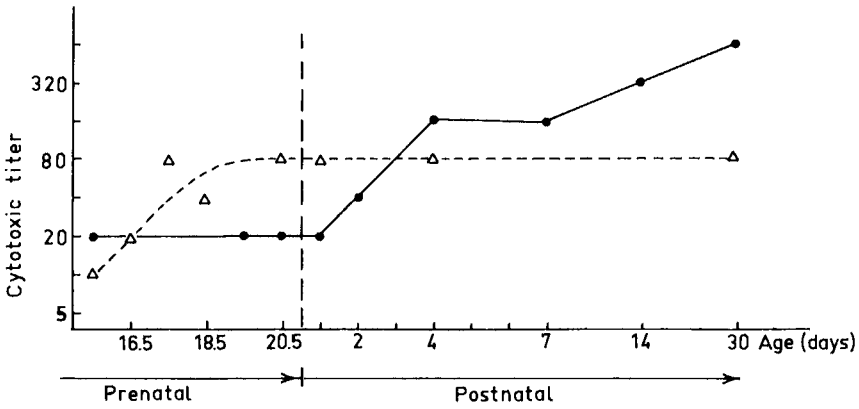

FIG. 1. Differentiation of H-2 isoantigenicity in thymus and spleen cells of C3H mice. The cytotoxic effect of H-2 isoantibodies and complement on spleen and thymus cells was determined in embryonic and neonatal C3H mice of various ages. Solid line = spleen cells; broken line = thymus cells. (Based on data from Schlesinger, 1965a.)

some discrepany between different reports regarding the time at which adult concentrations of H-2 antigens are reached in the spleen. According to Basch and Stetson (1963), 9-day old C57BL/6 mice have only 56% of the adult concentration and adult levels are not attained until the twenty-eighth day of life. On the other hand, according to E. Möller (1963), adult levels are reached already in the spleen of 5-day-old-mice of the

C3H strain. During the first 5 days of life the antigenicity of the liver is higher than that of the spleen, but then, due to the rapid increase of the antigenicity of the spleen, the relation reverses (Basch and Stetson, 1963). The differentiation of the H-2 isoantigenicity parallels closely the structural maturation of each organ. The thymus is the first fully developed lymphoid organ, and the appearance of the H-2 isoantigenicity coincides with the appearance of small lymphoid cells (Ball, 1963). The differentiation of the lymphoid elements in the spleen occurs in the mouse only after birth (East and Parrott, 1964).

b. θ Isoantigens. Reif and Allen (1964) described a non-H-2 isoantigenic system characterized by a high antigen concentration in the thymus and in the brain. While the thymus of the newborn possessed almost adult levels of antigenicity, the antigenicity of the brain reached adult levels only at 5–6 weeks of age (Fig. 2) (Reif and Allen, 1966). The appearance of the θ isoantigens in the brain paralleled the histological (Kobayashi, 1963) and physiological maturation (Uzman and Rumley, 1961) of mouse brain.

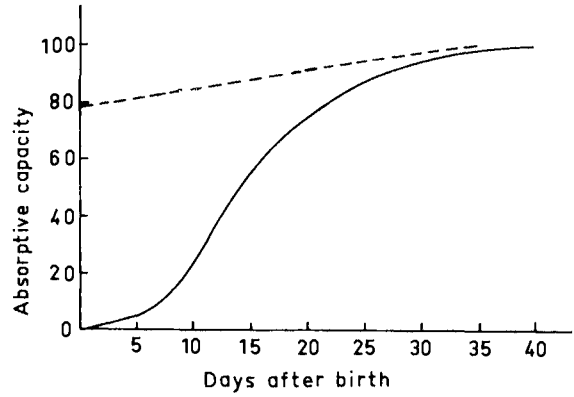

FIG. 2. Differentiation of θ isoantigenicity in the thymus and brain of AKR mice. The thymus and brain of AKR mice of various ages were tested for their capacity to absorb θ isoantibodies. Solid line = brain; broken line = thymus. (Based on data from Reif and Allen, 1964, 1966.)

2. *Embryonic Differentiation of Isoantigens in Different Organs*

The differentiation of isoantigens in the embryonic thymus of the mouse illustrates the concomitant appearance of several isoantigenic systems, which in turn parallels the morphological differentiation of the organ. Between day 15½ and day 17½ of gestation, with the appearance of small lymphoid cells in the thymus, the H-2 isoantigenicity of the thymus rapidly attains adult levels (Fig. 1) (Schlesinger, 1964, 1965a). At

the same developmental stage the TL antigen (Old *et al.*, 1963) differentiates in the thymus of mice belonging to TL-positive strains (Schlesinger, 1966b). The Z isoantigen (Hoecker *et al.*, 1959) has recently been found to have a higher concentration on thymus cells than on spleen cells (Schlesinger *et al.*, 1966b). This antigen also appears in the thymus at the same embryonic stage as the isoantigens mentioned before (Schlesinger and Hurvitz, 1966). It could be argued that the simultaneous appearance of the H-2, TL, and Z isoantigens in the embryonic thymus might be related to linkage of the genes determining these isoantigens, since all belong to genetic linkage group IX (Boyse *et al.*, 1964; Hoecker and Pizarro, 1962). This seems, however, to be an unlikely explanation. The θ isoantigens, whose genetic determination is not linked to that of the H-2 antigens (Boyse and Miyazawa, 1966), are almost fully expressed in the thymus of newborn mice (Fig. 2) (Reif and Allen, 1964). Similarly, the sensitivity of mouse thymus cells to the cytotoxic effect of guinea pig serum appears at the same time as the sensitivity to the cytotoxic effect of isoantibodies and complement (Schlesinger, 1965a). Moreover, recent reinvestigation of the inheritance of the Z isoantigen has shown that it is independent of that of the H-2 locus (Snell *et al.*, 1967, in press).

Much more information will be needed before it will be known whether the pattern of embryonic differentiation of isoantigens observed in the thymus also applies to the differentiation of antigens in other organs. However, the parallelism between the maturation of θ isoantigens in the brain, and of the H-2 isoantigenicity of the spleen with the functional maturation of these organs at least supports such a possibility.

3. Genetic Control of the Embryonic Differentiation of Isoantigens

G. Möller found that the differentiation of the H-2 isoantigenicity of newborn mice of the C57BL and C57L strains ($H\text{-}2^b$) was more complete than that of mice belonging to other strains (G. Möller, 1961, 1963). This was shown by the greater agglutinability of erythrocytes of newborn C57BL mice by isohemagglutinins, and by the higher sensitivity of their spleen cells to cytotoxic isoantibodies. E. Möller (1963) similarly showed that the spleen cells of C57BL mice had a greater capacity to absorb H-2 isohemagglutinins than spleen cells of newborn mice of other strains. In ($H\text{-}2^b \times H\text{-}2^a$) F_1 hybrids, the $H\text{-}2^b$ isoantigenicity reached adult levels before the $H\text{-}2^a$ isoantigenicity (A. Möller, 1963). These experiments suggest that the expression of the H-2 isoantigens might be under control not only of structural H-2 genes, but also of genetic regulation mechanisms.

In contrast to the results of the Möllers, Basch and Stetson (1963) found that the spleen of newborn C57BL mice possesses less than 10%

of the isoantigenicity of the adult organ, and that adult levels are reached only in 28-day-old mice. It is possible however, that the difference in the results may be due to different properties of the sublines used in these studies—C57BL/K1 and C57L/K1 by the Möllers and C57BL/6 by Basch and Stetson. Results of cytotoxic tests with spleen and thymus cells of embryonic and neonatal C57BL/6 mice did not differ from those previously obtained with C3H mice (Schlesinger and Hurvitz, 1966).

Boyse *et al.* (1965) suggested that the occurrence of TL-positive leukemias in mice of TL-negative strains indicates that all mouse strains possess the genetic information for the synthesis of the TL antigen, but that its phenotypic expression is controlled by another gene. Embryos of the TL negative C57BL strain at no stage contained any demonstrable TL antigenicity (Schlesinger, 1966b). This finding may indicate that the gene controlling the expression of the TL antigens operates at least as early as the gene carrying the genetic information for its synthesis.

IV. INDUCTION OF ANTIGENS IN THE THYMUS

A. Thymus-Distincitve Serological Properties

Thymus cells differ in their biological (Billingham and Silvers, 1964) and serological properties from other lymphoid cells.

1. Thymus cells are less sensitive than other types of lymphoid cells to the cytotoxic effect of H-2 isoantibodies and complement (Winn, 1962; Gorer and Boyse, 1959) and are less efficient in the absorption of H-2 isoantibodies (Basch and Stetson, 1962, 1963; Schlesinger, 1964). The yield of H-2 antigenic microsomal lipoproteins per gram of thymus tissue was a little over half of that obtained from spleen (Palm and Manson, 1965).

2. Murine thymus cells show a distinctive sensitivity to the cytotoxic effect of various heterologous sera, notably guinea pig serum (Gorer and Boyse, 1959; Palm, 1961; Reif, 1963; Schlesinger, 1965a). Evidence has been presented that the cytotoxicity of guinea pig serum is due to the presence of naturally occurring antibodies, capable of reacting with mouse thymus cells and complement (Schlesinger, 1965a). It has recently been shown that the toxicity of guinea pig, rat, and rabbit sera for mouse thymus cells could be inhibited by various carbohydrates (Schlesinger *et al.*, 1966a).

3. Old, Boyse and their colleagues found that the thymus cells of some strains of mice possess a thymus-specific TL antigen (Old *et al.*, 1963; Boyse *et al.*, 1965). This antigen is completely absent from any other normal lymphoid cell. Leukemic cells may possess the TL antigen also in strains of mice whose thymus normally does not show this antigen.

4. Reif and Allen (1964) found that isoantisera prepared in C3H/eB/Fe mice against AKR tissues or *vice versa* detected isoantigens whose concentration was higher in thymus cells than in other lymphoid cells.

5. Various isoantibodies were found to have a cytotoxic effect exclusively for thymus cells, although other lymphoid cells could also absorb them (Schlesinger, 1965b; Old *et al.*, 1965; Boyse *et al.*, 1966).

B. Repopulation of the Thymus of Radiation Chimeras and of Thymic Grafts

Popp (1961) and Ford and Micklem (1963) have shown that in mice exposed to lethal whole-body X-irradiation and injected with bone marrow cells, the thymus is repopulated by cells derived from the bone marrow administered. Evidence from parabiotic mice (Harris *et al.*, 1964) and from mice in which only the hindquarters were irradiated prior to bone marrow injection (Ford *et al.*, 1966) indicated that even in non-irradiated animals the thymus may recruit stem cells from the bone marrow. This conclusion is further strengthened by the findings of Metcalf and Wakonig-Vaartaja (1964) that thymus grafts become repopulated by host cells.

The question therefore arises: If the thymus is constantly repopulated by stem cells from the bone marrow, how do the thymus cells maintain their thymus-distinctive serological properties, which so clearly distinguish them from bone marrow cells and from other lymphoid cells?

Mice of the TL-positive A strain (possessing the TL antigen only in their thymus) and of the TL-negative C57BL strain were irradiated lethally and injected intravenously with bone marrow cells from either the same or the reciprocal strain (Schlesinger *et al.*, 1965). The thymus of C57BL mice repopulated by strain A cells showed the TL antigen (Table I), while the thymus of A mice repopulated by C57BL cells was TL-negative. Cells repopulating the spleen and bone marrow were TL-negative in both strain combinations. This experiment clearly showed that the appearance of the TL antigen was determined by the genetic constitution of the repopulating cells rather than by that of the thymus. However, in addition to the genetic information, the expression of the TL antigen also required the thymic environment. In both strain combinations, only the cells repopulating the thymus showed sensitivity to the cytotoxic effect of guinea pig serum.

Similar results were obtained in experiments with thymus and spleen grafts from either strain A or C57BL newborn donors (A × C57BL)F_1 recipients (Schlesinger, 1966a). The thymus grafts were repopulated by host cells and showed TL antigenicity and sensitivity to guinea pig serum,

whereas spleen grafts which were also repopulated by host cells were TL-negative and resistant to guinea pig serum.

TABLE I
REPOPULATION OF THE THYMUS OF RADIATION CHIMERAS[a]

Donor of bone marrow	Recipient	Organ tested	Repopulation by donor cells	TL antigenicity	GPS sensitivity
A	C57BL	Thymus	+	+	+
		Spleen	+	−	−
		Bone marrow	+	−	−
C57BL	A	Thymus	+	−	+
		Spleen	+	−	−
		Bone marrow	+	−	−

[a] Based on data from Schlesinger *et al.*, 1965.

The appearance of TL antigenicity and sensitivity to guinea pig serum in the cells repopulating the thymus of irradiated recipients or thymic grafts could be due to selective accumulation in the thymus of TL-positive GPS-sensitive cells which might be present in the bone marrow in numbers too small to be detectable by ordinary serological methods. This possibility was excluded by exposing A bone marrow to TL antibody and GPS *in vitro* prior to their administration to irradiated C57BL mice (Schlesinger *et al.*, 1965) or by exposing them to TL antibody *in vivo* (Boyse *et al.*, 1965); in no case was the expression of TL antigenicity and GPS sensitivity in the repopulated thymus affected. It therefore seems likely that the appearance of the TL antigen and GPS sensitivity reflects the capacity of the thymus to induce the synthesis of thymus-distinctive structures in stem cells entering it, provided these have the appropriate genetic information.

C. Inductive Phenomena in the Thymus

The thymus plays a central role in the development of the immune capacity of neonatal animals (Miller, 1962; Good *et al.*, 1962) and of adult X-irradiated animals (Miller *et al.*, 1963; Globerson and Feldman, 1964). The precise mechanism by which the thymus confers immunological competence is still under debate. The thymus may serve as a source for the supply of peripheral lymphocytes (Nossal, 1964; Osoba, 1965), particularly during an active immune response (Leuchars *et al.*, 1964). However, not only the quantity of peripheral lymphocytes is re-

duced in thymectomized animals (Waksman *et al.*, 1962) but also their quality (Good *et al.*, 1962; Rieke, 1966). Most of the evidence indicates that the major role of the thymus is the induction of immunological competence in lymphoid precursor cells, which might be formed elsewhere (Miller, 1965; Globerson and Feldman, 1964; Tyan *et al.*, 1966). Immunological competence of thymectomized animals can be restored by the implantation of diffusion chambers containing thymic tissue (Levey *et al.*, 1963; Osoba and Miller, 1964) and by the injection of cell-free extracts prepared from thymic tissue (Trainin, 1966). It thus seems that the thymus secretes a humoral competence-inducing factor (CIF) which may induce immunological competence in potentially competent cells (Miller, 1965).

There is some parallelism between the induction of competence and the induction of thymus-distinctive serological properties by the thymus. For instance, both seem to be impaired in cortisol-treated and in tumor-bearing mice (see Sections V,A and V,B). However, differences between the two processes are apparent. CIF seems to be able to exert its effect in sites distant from the thymus, while antigen induction takes place only in the thymus. Immunological competence is elicited in thymectomized-irradiated hosts after a rather short sojourn of thymic grafts (Cross *et al.*, 1964), while the appearance of thymus-distinctive serological properties occurs only with the repopulation of the thymic grafts during the third week after grafting.

V. MODIFICATION OF THE EXPRESSION OF NORMAL TISSUE ANTIGENS IN VIVO

Little is known about the mechanisms regulating the expression of antigens on normal cells *in vivo,* and about their modification by external influences. As will be discussed below, tumor growth, corticosteroid administration, and hypoxia affect adult animals in such a way that skin grafts obtained from them survive in allogeneic hosts for longer periods than grafts obtained from normal donors. It is possible that under these conditions the skin may have a reduced isoantigenicity, following depletion of its lymphocyte contents. However, it is not yet known whether the skin grafts have a decreased antigencity. It is equally possible that the immunizing capacity of the grafts is decreased, rather than their antigen content. Solid tissue grafts have a greater capacity to induce allograft immunity than cell suspensions (Medawar, 1959; Barnes, 1964). This seems to indicate that the degree of immunity elicited by a graft depends not only upon its antigen content, but also on other properties, possibly on adjuvant-like components located in the connective tissue (Barnes,

1964). It is therefore possible that the "immunogenicity" of the tissues, rather than their antigenicity, is altered in the conditions discussed below.

Another possible mechanism leading to prolonged survival of allogeneic grafts may be their increased resistance to an immune reaction. Eyal *et al.* (1965a) have suggested that the prolonged survival of allogeneic grafts in rabbits treated with various phenothiazine derivatives may be due to stabilization of membranes of the lysosomes, mitochondria, and of the cells of the grafts. *In vitro* treatment of tissues with phenothiazine derivatives prolonged their preservation (Eyal *et al.*, 1965b).

Corticosteroid hormones stabilize lysosomal (Weissmann, 1964) and cell membranes (Jennings, 1966) *in vitro*. It is possible that under stress conditions the increased secretion of corticosteroid hormones may stabilize *in vivo* the lysosome and cell membranes of the skin used for grafting.

A. Tumor Growth

A great deal of information is available on antigenic differences between cancer cells and normal cells (Day, 1965; Old and Boyse, 1964; Hattler and Amos, 1966). However, little is known about alterations in the antigenicity of normal tissues from cancer patients and from tumor-bearing animals. Amos *et al.* (1965) recently found that allogeneic skin grafts from cancer patients had a prolonged survival on normal recipients. Similarly, Robinson *et al.* (1965) observed that in cancer patients the survival of allogeneic skin grafts from donors with malignant disease survived for significantly longer periods than grafts from healthy donors. A similar prolongation of the survival of skin allografts could be demonstrated in mice. Skin grafts obtained from Ehrlich ascites tumor-bearing mice showed a prolonged survival in allogeneic recipients (Ben-Hur *et al.*, 1966; Schlesinger and Golakai, 1966). Several mechanisms have been proposed to explain this phenomenon.

1. Diminished antigenicity of the skin in cancer patients and in tumor-bearing animals. In favor of this hypothesis is the observation of Amos *et al.* (1965) that lymphocytes from cancer patients and from chronically debilitated patients often fail to react in cytotoxicity and agglutination tests with known potent isoantisera.

2. Another hypothesis is that the tissues of cancer patients are coated by autoantibodies which might cause "immunological enhancement" (Kaliss, 1958) of such tissues in allogeneic hosts. In this connection it is of interest that Betts *et al.* (1962) and Anthony and Parsons (1965) found that normal tissue cells of tumor-bearing hosts are covered by globulin. Robinson and Nelken (1963) found that the antiglobulin consumption test of leukocytes and thrombocytes in cancer patients became positive after

radiotherapy and chemotherapy, while it was rarely positive prior to treatment. The destruction of tumors by therapy may release substances that become attached to the surface of normal tissue cells.

3. The increased survival of skin grafts from cancer patients could also be due to an increased resistance to the allograft-rejection mechanism. Schlesinger and Golakai (1966, 1967) studied the isoantigenicity of tissues of Ehrlich ascites tumor-bearing mice of several inbred strains. Two criteria were employed for estimation of the isoantigenic activity of the tissues: (1) capacity of the tissues to absorb H-2 hemagglutinins; (2) sensitivity to the cytotoxic effect of isoantibodies and complement. No significant changes in the H-2 isoantigenicity could be detected in any of the tissues tested (spleen, thymus, liver, and kidney). An interesting phenomenon became apparent when the thymus of tumor-bearing mice was tested for thymus-distinctive serological properties (see Section IV, A). The thymus cells of all strains of mice tested lost their sensitivity to the cytotoxic effect of guinea pig serum within 4 days after the implantation of the tumor (Fig. 3). At the same time the thymus-specific TL antigen disappeared from thymus cells of mice of the A and SJL/J strains. These changes were maintained until the death of the tumor-bearing hosts.

The loss of thymus-distinctive serological properties occurs concomitantly with the involution of the thymus in tumor-bearing animals (Savard, 1948). Part of the thymic involution probably is due to increased

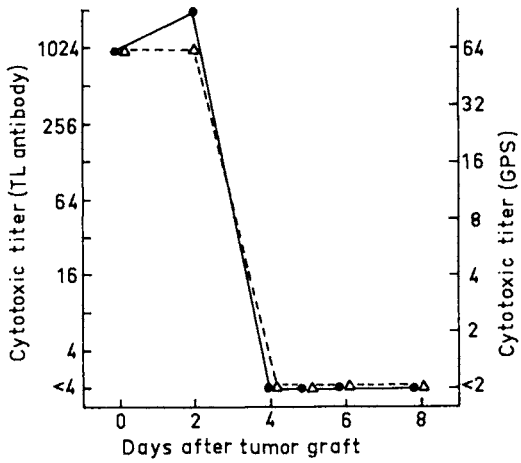

FIG. 3. Effect of growth of the Ehrlich ascites tumor on serological properties of the thymus of the host. The thymus of mice of the A strain was tested for sensitivity to the cytotoxic effect of guinea pig serum (broken line) and TL antibody and complement (solid line) at various intervals after intraperitoneal transfer of the Ehrlich ascites tumor. (Based on data from Schlesinger and Golakai, 1967.)

secretion of adrenal corticosteroid hormones in tumor-bearing animals (McEven and Selye, 1935; Nadel and Burstein, 1956). Indeed, Begg (1953) demonstrated that adrenalectomy restored the thymus toward normal weight in tumor-bearing rats. On the other hand, Savard and Homburger (1949) presented evidence indicating that thymic involution in tumor-bearing animals was independent of the pituitary-adrenal axis. It seems that the involution of the thymus is part of the generalized wasting of tumor-bearing animals, brought about by profound metabolic derangements (cf. Begg, 1958).

B. Effect of Corticosteroid Hormones

Stoerk and his colleagues demonstrated a striking diminution of the immunogenicity of tissues from rats treated with cortisone or other lymphocytolytic agents. This was demonstrated in two fashions:

1. The administration of viable tissues (liver, kidney, or spleen) from rats treated with cortisone failed to elicit in allogeneic rats an effective immunity against allogeneic tumor grafts. Similar immunization with tissues from untreated donors completely prevented the growth of tumor allografts (Stoerk, 1959).

2. Allogeneic skin grafts from donor rats treated with cortisone or nitrogen mustard were rejected much later than grafts obtained from untreated donors (Lambert and Stoerk, 1954).

Schlesinger and Golakai (1966) obtained similar results with allogeneic skin grafts in mice. The administration of 1 mg of cortisol to adult inbred mice significantly prolonged the survival of grafts of their skin to allogeneic recipients. On the other hand, no decrease in the H-2 isoantigenicity of the tissues of treated mice could be demonstrated, neither by absorption studies nor by cytotoxic tests.

Basch and Stetson (1963) found that the daily administration of cortisone to mice resulted in a marked increase in the liver's weight and a corresponding increase in its total H-2 antigenic activity. Since the increase in liver size presumably was not due to cellular proliferation, they inferred that the H-2 isoantigenicity of the liver cells had increased.

The involution of the thymus of cortisol-treated mice was accompanied by loss of thymus-distinctive serological properties similar to that observed in the involuted thymus of tumor-bearing mice. Upon regeneration of the thymus, these serological properties reappeared (Schlesinger and Golakai, 1967). The reactivity of thymus cells of mice of the A and SJL/J strains with TL antibody disappeared within a day after the subcutaneous administration of 1 mg cortisol (Fig. 4). No sensitivity to TL antibody could be detected for 6 days after cortisol administration, whereas afterward the reactivity of the thymus cells promptly returned to

normal. The sensitivity of the thymus cells to the cytotoxic effect of guinea pig serum disappeared for about the same period as the sensitivity to TL antibody. The reactivity of the thymus cells with H-2 antibodies was unaltered or somewhat higher than in normal controls.

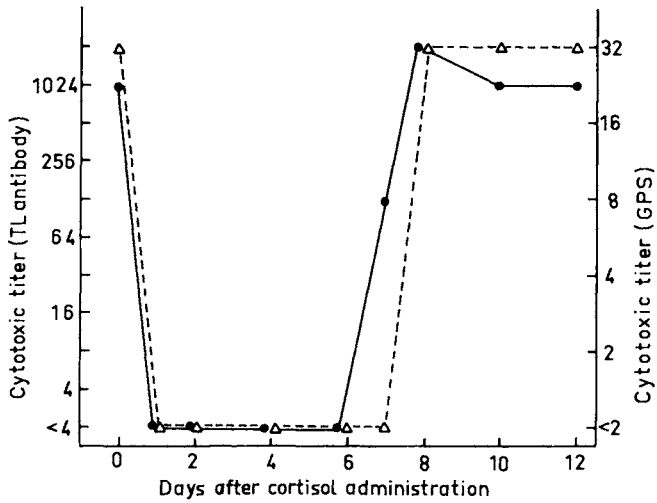

FIG. 4. The effect of cortisol administration on serological properties of the thymus. The thymus of mice of the SJL/J strain was tested for sensitivity to the cytotoxic effect of guinea pig serum (broken line) and TL antibody and complement (solid line) at various intervals after the subcutaneous administration of 1 mg of cortisol. (Based on data from Schlesinger and Golakai, 1967.)

Corticosteroid hormones can stabilize cell and lysosomal membranes *in vitro* (deDuve *et al.*, 1961; Weissmann and Dingle, 1961; Weissmann and Thomas, 1963; Weissmann, 1964). Corticosteroid hormones can inhibit the destruction of cell cultures by sensitized lymphocytes (Rosenau and Moon, 1961) or by antibodies (Fell and Weiss, 1965). High doses of cortisol may inhibit *in vitro* the lysis of red blood cells by antibodies and complement (Jennings, 1966). However, the resistance of the thymus cells of cortisol-treated mice to lysis by guinea pig serum and by TL antibody cannot be explained by stabilization of the membranes; such a nonspecific stabilization should have affected also the sensitivity of thymus cells to H-2 antibodies. Moreover, absorption experiments indicated that the thymus cells which were refractory to the cytotoxic effect of TL antibody also lost their capacity to absorb the antibody.

Several hypotheses may be put forward to explain the antigenic changes observed in the thymus of tumor-bearing and cortisol-treated mice:

1. Tumor growth or cortisol administration may have a selective

detrimental effect on thymus cells possessing thymus-distinctive serological properties, leaving in the thymus only a cell population devoid of such properties.

2. The induction of thymus-distinctive properties in the stem cells entering the thymus may be inhibited. This may either be due to loss of the inductive capacity of the involuting thymus, or due to refractoriness of the stem cells to the inductive capacity of the thymus.

Evidence has recently been presented that cortisol administration may inhibit purine nucleotide and protein synthesis in the thymus and that it may impair the ability of thymic ribosomes to incorporate amino acids (Feigelson, 1965; White, 1965). The disappearance of TL antigenicity and GPS sensitivity of thymus cells may reflect an effect on the genetic regulatory mechanism of thymic cells.

C. The Effect of Hypoxia

Ferguson and Anthony (1965) studied the effect of acclimation of inbred mice to reduced barometic pressure on the rejection of allogeneic skin grafts. Prior acclimation of either the donor or the recipient to reduced barometric pressure prolonged the retention of allogeneic grafts. The finding that grafts from donors acclimated to hypoxia survived on untreated hosts for longer periods than grafts from untreated donors indicates that hypoxia has a profound effect on the skin used for grafts. Ferguson and Anthony suggest that this may be a decrease in the number of lymphocytes in the donor skin, leading to decreased antigenicity of the skin. Hypoxia causes an increased adrenocortical activity associated with thymic involution (Edelman, 1945; Ferguson and Anthony, 1965). It thus seems that the mechanism leading to the prolonged survival of skin grafts from hypoxic donors may probably be similar to that responsible for the prolonged survival of allografts from donors treated with corticosteroid hormones.

VI. DIFFERENTIATION OF IMMUNOGLOBULIN-PRODUCING CELLS

Immunoglobulins differ from the antigens discussed so far in that they do not form an integral part of the cells in which they are produced, and have well-defined functions outside of the cells. The genetic control of the production of immunoglobulins will be discussed, although it may differ in some respects from the genetic control of other cellular antigens.

Immunoglobulins consist of two light (L) and two heavy (H) polypeptide chains (Fahey, 1962; Cohen and Porter, 1964). In man two different types of L chains and four different classes of H chains are

known at present. The localization of these various chains in individual lymphoid cells can be studied by fluorescent antibody techniques. It was found that a given plasma cell in human lymphoid tissues, as a rule, produces only one type of L chain (Pernis and Chiappino, 1964; Bernier and Cebra, 1964). Similarly, only one class of heavy chain is usually produced in an individual plasma cell at a given time (Mellors and Korngold, 1963; Chiappino and Pernis, 1964; Bernier and Cebra, 1965). However, for a limited period they may produce simultaneously 19 S and 7 S antibodies (containing μ and γ classes of H chains respectively) (Nossal *et al.*, 1964). Cells of germinal centers of lymphoid follicles may contain simultaneously both types of L chains (Pernis and Chiappino, 1964), and both 7 S and 19 S antibodies (Chiappino and Pernis, 1964). The different types of immunoglobulin chains in cells of the germinal centers may not be actually produced there, but may be trapped secondarily (Pernis *et al.*, 1965). However, it is possible that these cells are not yet committed to the synthesis of one particular type of immunoglobulin. The production of only one type of immunoglobulin by mature lymphoid cells may constitute a molecular mark of cellular differentiation. During this process different nonallelic genes may be selectively activated or inactivated in different cells. Pernis *et al.* (1965) point out that similar processes may account for differentiation in other organs. Morphologically similar cells in the same organ may be specialized to produce different proteins at a given moment—as for instance, the production of serum albumin and fibrinogen by different liver cells (Hamashima *et al.*, 1964).

Analysis of immunoglobulin allotypes determined by allelic genes of the same locus revealed that single plasma cells of heterozygous animals contain either one or the other allotypic marker (Pernis *et al.*, 1965; Weiler, 1965). Since presumably all the lymphoid cells of a heterozygous individual carry the genetic information for both allelic genes, the presence of only one allotype in a given cell implies a selective inactivation of one of the two allelic genes in each immunoglobulin-producing cell. In other autosomal genes studied so far, both parental alleles are expressed in each cell—as for instance, the genes determining transplantation and blood group antigens or polypeptide chains of hemoglobin. Phenotypic mosaicism similar to that found in the case of immunoglobulin allotypes has previously been shown only in female animals with respect to genes located on the X chromosome (Lyon, 1961).

A phenomenon that might be related to the synthesis of allelic immunoglobulin allotypes by different cells is the selective inhibition of the production of one of the allelic allotypes in heterozygeous newborn rabbits by specific antibodies (Dray, 1962; Mage and Dray, 1965). In-

hibition of allotype synthesis was observed both in rabbits born to immunized mothers, and in rabbits injected neonatally with isoantibodies against one of the allotypes. While the isoantibodies suppressed the formation of the corresponding immunoglobulin allotype, the formation of the allelic immunoglobulin was enhanced so that the total amount of immunoglobulin synthesized remained normal. The effect of the isoantibodies was not simply due to their reaction with the immunoglobulins produced by the recipients since the inhibitory effect persisted long after the administered antibodies disappeared from the sera of the recipients. Moreover, the recovery of the synthesis of the suppressed allotype was insidious, and was not complete throughout life. If the inhibitory effect of the isoantisera had been due to their combination with the allotype formed by the recipient, a sharp recovery would be expected with the formation of enough allotype to neutralize the antibodies. It has been suggested by Mage and Dray (1965) that the antiallotype antibody may either kill or prevent the differentiation of cells which would normally become committed to the synthesis of the suppressed antigen or that it might somehow influence the genetic regulatory mechanism.

The mechanism underlying the inhibition of allotype synthesis may be similar to that involved in the antigenic modulation of the expression of the TL antigen (Boyse *et al.*, 1963, 1966). Specific isoantisera against the TL antigen inhibit the manifestation of the TL antigen in leukemic cells and in normal thymus cells, and increase the concentration of H-2 isoantigens on the cell surfaces. The disappearance of the TL antigenicity was found not to be due to masking of the TL antigen by blocking antibodies. Rather, it seems that the antibody initiates an active metabolic process, which inhibits the expression of the TL antigen. This inhibitory process can be reversed by actinomycin D.

VII. CLOSING COMMENTS

Why during embryonic development do some antigens appear in cells of one organ but not in cells of another organ? Why does the same antigen appear at different times in different organs? How do some cellular antigens disappear in the adult organism? All of these questions, as well as others, constitute part of the general problem of cell differentiation. It has to be assumed that, like the manifestation of other differentiated cell traits, the appearance of cell antigens is brought about by intricate metabolic pathways, involving the genetic regulatory mechanism. Through these processes the genetic information for the synthesis of cellular antigens is expressed phenotypically.

As should be clear from the present paper, much is yet to be explored in this field. It is to be expected that in the future more will be learned about the effect of a variety of factors on the expression of many types of antigens in different organs. More will be known about the effect on the expression of cellular antigens of various physiological and pathological conditions influencing the homeostasis of the organism. Above all, it is to be expected that it will be possible to obtain some insight into the role of regulator and structural genes, and the transcription and translation of genetic messages in the appearance of cellular antigens.

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DISCUSSION

DR. AMOS: This presentation was extremely interesting to me, particularly in relation to the transplantation experiments, performed in cancerous animals, showing the significance of thymic activity in the recipient. In some of our studies in humans, two normal subjects received a small skin graft from a cancerous patient. We were concerned that we would by chance pick two recipients who had impaired immunological activity, so we cross-grafted the two individuals about 30 days after they had rejected the skin from the original donors. Both grafts were rejected as classic white grafts. We were interested because normally white graft reactivity is lost about 10–12 days after the transplant. In more recent experiments we transplanted skin from C3H mice bearing methylcholanthrene-induced tumor, at various times after the induction of tumor to normal DBA-1 mice. As a control, on some of these animals, we also included a skin graft from a normal C3H animal. The results were not always consistent, but where we had prolongation of the survival of skin graft from the cancerous animal we very frequently had prolongation of survival of graft from the normal animal. We could not understand this. I think that now we might try to follow thymic changes in this strain of mice.

DR. SCHLESINGER: The appearance of white grafts in normal skin grafts applied after a rejection of a skin graft from a tumor-bearing individual would indicate that the first graft from the tumor patient had induced a state of immunity. In some preliminary experiments Dr. Robinson and I got similar results in the mouse. If one first applies a skin graft from a tumor-bearing animal, and later a graft from a normal mouse, one sees prompt rejection. But if one transplants the skin graft from the normal animal first, followed by a skin graft from a tumor-bearing mouse, these grafts have a prolonged survival. Skin grafts obtained from tumor-bearing animals seem to show increased resistance. This could in part be correlated with the increased excretion of corticosteroids by tumor-bearing animals. Corticosteroid hormones might stabilize cell membranes in a nonspecific way. Adrenalectomy of tumor-bearing animals did not seem to reduce the prolongation of survival of skin grafts derived from these donors, however.

DR. PREHN: We have been much interested in the fact that the cancer bearer is a good donor of skin grafts. Dr. David Steinmüller, working in my laboratory, has investigated this problem, confirming most of what others have found. He has observed, however, that the results were quite poor in animals bearing early passage methylcholanthrene-induced sarcomas. We found that the phenomenon was largely restricted to certain tumors, particularly the Ehrlich carcinoma. As you indicated, mice kept in an anoxic state for 4 or 5 days serve as preferential donors for skin grafting. It is conceivable that this is the common

mechanism underlying the phenomenon, and that, when an animal develops ascites from the Ehrlich tumor, it becomes anoxic and the anoxia produces the effect? Dr. Steinmüller has found that the subcutaneously growing Ehrlich tumor also influences the resistance of the skin grafts. You have shown how the thymus changed in response to the growth of the Ehrlich tumor, but I did not hear what your hypothesis was as to how these changes related to the fact that the animals were preferential donors for skin grafting. Is there a direct connection?

DR. SCHLESINGER: As indicated in my paper, Ferguson and Anthony reported that animals kept under hypoxic conditions are better donors of allogeneic skin graft and that hypoxia caused dramatic changes in the thymus indicative of increased adrenal cortical steroid excretion. With regard to your second question, it may well be that in parallel with the changes in the thymus some antigens of the skin, possibly other than H-2 isoantigens, might be lost.

DR. SANFORD: Dr. Prehn questioned whether prolonged survival of skin grafts from animals bearing tumors might be limited to mice with Ehrlich carcinoma and, possibly, a few other exceptional tumors. We have found that skin grafts from A mice carrying the TA3 tumor survive longer on C3H hosts than grafts from normal A mice. It may be of interest that TA3 tumor cells are characterized by a heavy sialomucin coating at the cell surface and that there is some evidence that this substance is produced by the tumor cells.

DR. WITZ: Concerning the cytotoxic substance present in the guinea pig serum, I wonder what criterion you have to call it antibody. Is it a globulin? What is its specificity? You indicated it could be absorbed by other tissues; what are they? I would suggest including this "antibody" in the group of cytotoxic factors against various tissues as those reported, for instance, by Dr. Landy. The second question is whether you think it is possible that the thymus cells of tumor-bearing mice were coated by certain plasma proteins and thus were protected from the cytotoxic action of the guinea pig serum?

DR. SCHLESINGER: As to the first question, I think that all or at least most of these natural cytotoxic factors, unless otherwise shown, are probably natural antibodies cross-reacting with the cells that are tested. In our laboratory Professor Gross and Dr. Laskow are studying the natural cytotoxic factor present in normal human serum which reacts against HeLa cells and other tumor cells. They have almost completed the isolation of that factor showing that it is a 19 S molecule with γ-globulin properties. The guinea pig serum factor can be absorbed by various murine tissues, is complement dependent, and has a broad specificity directed toward very simple sugar components. The cytotoxic effect of guinea pig serum can be inhibited by several simple sugars. This might indicate that the difference between the cell surface of the thymus and the cell surface of other lymphoid cells is somehow correlated with carbohydrate determinants, which may be different either in quantity or configuration. The possibility that cells are coated by breakdown products, of either tumor or normal cell, would require investigation. Should this possibility be real, one would have to assume that cortisone causes an increased breakdown of some tissue components.

DR. PALM: Several years ago we noticed in a large series of experiments that 10–25% of the cells of thymus suspensions were consistently resistant to the guinea pig serum even when the serum was in considerable excess. Do you find this resistant cell population, and if so, do you think it may be just the type of resistant cell population one frequently encounters in lytic tests, or do you think it may indicate two serologically distinct cell populations within the thymus?

DR. SCHLESINGER: I agree with you that one never gets 100% cytotoxicity with guinea pig serum. It might well be that the resistant cells are not small lymphoid cells but rather epithelial cells, or even myoid cells which are present in the thymus.

DR. METCALF: I think that the decrease in the percentage of susceptible cells in the tumor-bearing animal and in normal animals after cortisone treatment can be explained on the basis of the compound nature of the thymus. In this organ not only are there non-lymphoid cells like epithelial and reticulum cells, but there are also lymphoid cells, and the presumption is that the sensitive cells are the lymphoid cells. But within the lymphoid population there are two subgroups of cells, those which reside in the medulla and those which reside in the cortex. By all parameters applied so far to the medullary lymphoid cells, they appear to be a different cell population. They are similar in size to lymph node cells, following tritiated thymidine labeling they have the kinetics of lymph node population rather than that of thymic cortical cells, and they are relatively resistant to the effects of irradiation and cortisone. The cortical cells outnumber the medullary lymphoid cells at least by 20 to 1 and are more readily obtained on teasing out the thymus than the medullary cells are. The tumor-bearing animals are subjected to stress and they should exhibit thymic atrophy. Following cortisone, as we and others have described [Ishidate and Metcalf (1963) *Australian J. Exptl. Biol. Med.* **41**, 637], there is a very rapid depopulation of the thymus cortex, and for a period of up to 7 days there are almost no lymphoid cells in the cortex. If one postulates that only the cortical lymphoid cells are sensitive to both the lytic effects of guinea pig serum and anti-TL antibody, then your results are exactly what one would expect following a deletion or reduction of the population of cortical lymphoid cells. It may be that what is happening is not a qualitative change within individual cells but rather a deletion of the susceptible population.

DR. SCHLESINGER: This is of course a very important question. I agree with you that there is thymic atrophy following tumor growth. It has been shown that this effect is not corticosteroid-mediated, since it is not inhibited by adrenalectomy. We are still trying to find out whether the effects described are related only to a change in the population of sensitive cells. The reappearance of TL antigen and guinea pig serum sensitivity cannot be due to the entrance into the thymus of cells that have these properties. Whether the very rapid disappearance of these properties from the thymus is due to the very rapid disappearance of particular cells or to the disappearance of the capacity to produce this antigen, is not known at the present. Some experiments done recently by Boyse, Old, and Stockert bear on this question. In newborn and adult animals which received anti-TL antibodies from the mother or by injection, the expression of the TL antigen disappears very rapidly and, as far as I know, this disappearance is not accompanied by any visible atrophy. In an *in vitro* test, where the addition of the anti-TL antibody induces a disappearance of the TL antigen, Boyse, Old and Stockert showed that the addition of actinomycin D inhibits this disappearance. A possible interpretation of these findings is that with anti-TL antibody one activates a messenger RNA-dependent system which inhibits the phenotypic expression of the TL antigen.

DR. BERENBAUM: You showed that grafting tumor cells causes a rapid loss of antigenicity in thymus cells. I wonder whether you tried grafting normal tissues, or using simple protein antigens. It has been claimed recently by Black that administration of antigens causes a very rapid change in the histones of the thymus. It may be that the response you detect is to antigens in general and is not specifically related to the cancerous nature of the graft.

DR. SCHLESINGER: We have done this control experiment but we did not find any change in the thymus.

DR. BARNES: In relation to the issue of the expression of antigens in normal mammalian cells, we have some evidence indicating that, under hormonal influence, the antigenic characteristics of prostatic secretions are modified in parallel with the histological characteristics of the gland. In human subjects and rats, specific soluble antigens of vesiculor-prostatic

origin can be detected by agar gel diffusion in urines from adult males, after concentration, by antiserum to prostatic fluid or extracts [Barnes *et al.* (1965) *J. Lab. Clin. Med.* **66,** 741; Barnes (1966) *Federation Proc.* **25,** 355]. Similar antigens are not usually detected in urines from adult females or prepuberty males. Our interpretation is that the adult prostate, as a normal function, is continually secreting specific proteins into the urinary tract. In urines from castrated rats, treated with 1 mg of testosterone per day, a particular antigen, which is not present before treatment, begins to appear at day 8, and is fully expressed by day 12. This antigen is present in urines and prostate extracts from intact males. In the castrated, testosterone-treated rats there is a correlation between the appearance of this antigen and the histologic changes observed in the vesiculo-prostatic complex.

DR. REIF: There seems to be a connection between Dr. Prehn's observations that the most antigenic newly derived tumors grow fastest, and Dr. Schlesinger's thesis that tissues develop stronger antigenicity as they differentiate. Both sets of data raise the question as to why tissues develop antigens, and why they do it in parallel with cell differentiation. In neither case do we know whether repression or derepression of genes, or both, is involved. However, in both situations the data suggest that those cells which biochemically, morphologically, and functionally have differentiated or dedifferentiated most from the cells of origin also show the greatest change in antigenicity.

DR. SCHLESINGER: I should mention that some antigens may be lost in differentiating cells as, for instance, in the case of the trophoblastic cells of the placenta. On the other hand, trophoblastic cells acquire new antigens [Hulka (1963) *Nature* **198,** 501] which are organ specific. Differentiation may lead to an increase of some antigens and a decrease of others. Actually differentiation is the acquisition of some properties and the loss of others.

Mechanism of Complement Action and Means of Complement Depletion*

HANS J. MÜLLER-EBERHARD

Department of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, California

I. PHYSIOGENIC AND PATHOGENIC ROLE OF COMPLEMENT

Complement is a complex effector system which has been implicated both in physiological and pathological processes. It is thought to constitute an essential part of the mechanism of resistance to infections. On the other hand, it is considered capable of mediating tissue damage and of producing histamine-dependent toxic effects.

In vitro experiments have established that complement action may manifest itself in one of the following ways. Together with specific antibody to cell surface antigens, it causes ultrastructural membrane lesions which lead to cell leakage and subsequent cell death. Upon activation by antigen-antibody complexes it becomes able to direct the migration of polymorphonuclear leukocytes. It can coat cell surfaces to render them palatable to phagocytes, and it is capable of attaching antigen-antibody complexes to the surface of cells. It can produce a split product which is active in the release of histamine from mast cells.

Its participation in mechanisms of resistance to infections can be reduced to the following basic functions. Together with specific antibody to invading microorganisms, complement may damage the bacterial cell to an extent where cell death occurs. Presumably more prevalent *in vivo* is the effect of complement on phagocytosis. In the course of a com-

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plement reaction the fifth, sixth, and seventh components of complement become activated and, in their associated form, they constitute the chemotactic principle which attracts polymorphonuclear leukocytes to the site of antigen-antibody reactions (Ward *et al.,* 1966). In the case of larger particles, coating of the surface with the third component appears to be required for ingestion by phagocytes (Nelson, 1962).

Its role in pathological processes is based essentially upon the same principal activities that render the complement system physiogenic under normal circumstances. When autoantibodies to cell surface antigens arise, or when circulating antigen-antibody complexes attach themselves to membranes, a subsequent complement reaction may lead to membrane damage. Damage to host tissue by autologous complement may be caused in one of two ways. Complement may act directly on membrane sites leading to typical ultrastructural lesions measuring 80–100 Å in diameter which have been interpreted to represent holes (Humphrey and Dourmashkin, 1965). Or it may cause damage indirectly by attracting polymorphonuclear leukocytes and rendering the cell surface susceptible to attack by the enzymes of the white cells. In addition, complement may mark certain cells such as circulating erythrocytes to be withdrawn and sequestered by the reticuloendothelial cells of the liver. Toxic effects of complement may result primarily from the production of a histamine-releasing substance called anaphylatoxin. This substance is derived from the third component of complement from which it is released by the action of C'3 convertase (Dias Da Silva and Lepow, 1966). The potentially harmful role of complement is exemplified by its requirement for the production of certain experimental diseases and phenomena such as nephrotoxic nephritis (Unanue and Dixon, 1964) and immune vasculitis (Arthus phenomenon) (Cochrane and Ward, 1966). Its role in human disease mechanisms is indicated by the fact that complement components are bound *in vivo* at the site of the tissue lesions in glomerulonephritis, lupus erythematosus (Lachmann *et al.,* 1961) as well as in the joints of patients with rheumatoid arthritis (Rodman *et al.,* 1966).

No definitive information is available on whether or not complement is required for the rejection of homografts. The only observations that are suggestive of a participation of complement in the rejection mechanism are the following. Patients who are in the process of rejecting kidney homografts exhibit decreased serum levels of the second component (Austen and Cohn, 1963). Rabbits with an inherited deficiency in the sixth component of complement will accept skin homografts with a certain frequency, while heterozygous members of the same strain with normal complement levels reject homografts without failure (Volk *et al.,* 1964).

In view of the diverse functions of complement, it may become desirable for the clinical investigator and eventually for the physician to obtain means by which the complement system can be manipulated *in vivo*. Depending on a given situation, a physician might either desire to enhance the activity of the complement system or to reduce it. In order to provide the basis for such *in vivo* manipulations it will be necessary to understand completely the anatomy and the reaction mechanism of the complement system and to study means of its enhancement and inactivation *in vitro*.

II. MECHANISM OF ACTION OF HUMAN COMPLEMENT

The human complement system is composed of nine components (Müller-Eberhard *et al.*, 1966a). Listed according to their sequence of action, they are designated C'1, C'4, C'2, C'3, C'5, C'6, C'7, C'8 and C'9. C'1 is composed of three subunits called C'1q, C'1r, C'1s. These three subunits form a calcium-dependent macromolecular complex which has a sedimentation rate of 18 S and which is capable of attaching itself to antigen-bound antibody (Lepow *et al.*, 1963). In immune cytolysis C'1 bound to an antibody molecule on the cell surface catalyzes the attachment to the cell surface of C'2 and C'4. C'4 becomes directly attached to the cell membrane and functions as the acceptor for C'2. Linked together, they form a complex enzyme designated C'3 convertase or in the systematic language of the complement field, C'4,2a (Müller-Eberhard *et al.*, 1966). This enzyme catalyzes the uptake to the cell surface of C'3. Once this is accomplished, C'4,2a and C'3 both act on subsequent components. These are C'5, C'6, and C'7 which interact in the fluid phase to form a reversible complex and which after "activation" seem to impart their effect on the cell membrane as one functional unit (Nilsson and Müller-Eberhard, 1967). After their action, the cell membrane has become susceptible to C'8 and C'9; only after C'9 has acted do ultrastructural lesions appear. However, the lesions are not directly caused by C'9, as it is possible to prevent cell lysis by 0.09 *M* ethylenediaminetetraacetic acid (EDTA) subsequent to C'9 action (Hadding *et al.*, 1966). It appears that complement sets in motion as-yet-unknown processes within the membrane which in turn cause the appearance of the membrane lesions.

The multiple component nature of complement and the complex reaction mechanism provide the possibility for interference with the reaction at many different points. Probably most, if not all, components of complement are endowed with enzyme activity. Accordingly, it should be possible to find ways of specifically increasing or inhibiting the activity of one or the other component.

III. ENHANCEMENT OF COMPLEMENT

It is possible to enhance the hemolytic activity of human C'2 approximately tenfold by treatment with iodine (Polley and Müller-Eberhard 1966). Enhancement does not seem to be caused by substitution of a tyrosine residue with iodine as in the case of carboxypeptidase (Vallee, 1964). Instead, treatment with iodine appears to result in the oxidation of a critical group, probably a sulfhydryl group. The nature of the group was inferred from experiments showing that iodine-treated C'2 is not affected by *p*-chloromercuribenzoate (*p*-CMB), whereas untreated C'2 is virtually inactivated by *p*-CMB. The effect of iodine is reversed by reducing agents such as sodium dithionite. Iodine-treated C'2 is therefore referred to as ^{oxy}C'2.

Oxidation of C'2 not only leads to an enhancement of its activity, but also to a much increased stability of C'3 convertase. This enzyme is labile, its half-life being 12 minutes at 32° C. However, when the enzyme is generated with ^{oxy}C'2, the half-life at 32° C increases to 150–200 minutes (Polley and Müller-Eberhard, 1966). The *in vivo* effects of a greatly increased activity and stability of C'3 convertase have not been investigated as yet.

IV. INHIBITION OF COMPLEMENT

As the catalytic activity of the first component of complement depends on its esterase activity, it is possible to inhibit it with organic phosphonates such as diisopropylfluorophosphate (Becker, 1956). The *in vivo* application of this type of inhibitor will only become possible if a compound will be found which is highly specific for C'1 esterase and does not interfere with other physiologically essential enzymes.

Peptides of certain aromatic amino acids are capable of inhibiting the third component of complement. The mode of inhibition is probably competitive as inhibitory peptides undergo cleavage (Cooper and Becker, 1967). In addition, salicylaldoxime (Basch, 1965) and phlorizin (Müller-Eberhard *et al.*, 1966b) also inhibit C'3.

The chemotactically active C'5,6,7 complex was found to be inhibited by glutamyltyrosine. In this case the inhibitory effect appears to be due to an allosteric effect on the complex. When the active complex was examined by density gradient ultracentrifugation in the presence of inhibitory amounts of the dipeptide, the complex was found to be completely dissociated (Ward *et al.*, 1966).

V. INACTIVATION OF C'3 BY COBRA FACTOR

It has been known since the beginning of this century that certain snake venoms are capable of inactivating complement. This effect has

been shown to be due to the selective inactivation of C'3 (Klein and Wellensiek, 1965). Injection of the partially purified active principle of cobra venom into experimental animals was found to be well tolerated (Nelson, 1966).

The active principle was isolated and obtained in highly purified form by preparative electrophoresis and Sephadex filtration (Müller-Eberhard, 1967). It was found to be a protein with a sedimentation rate of 7 S and an electrophoretic mobility similar to that of a β-globulin. Its approximate molecular weight is 140,000 and its concentration in lyophilized crude venom 0.5% w/w. When purified C'3 was treated with purified cobra factor, no inactivation resulted. By contrast, when cobra factor was added to whole serum, C'3 was selectively inactivated and its physicochemical properties were changed in the process. This pointed out the requirement of at least one serum factor for the inactivation of C'3 by cobra factor. This serum factor was localized in the β-globulin fraction of human serum. It was found to have a sedimentation rate of 5 S, to be heat labile, and to have an approximate concentration in human serum of 60–80 μg/ml. This factor is distinct from any complement component.

Further analysis of the mode of action of cobra factor revealed that it combines with the 5 S serum protein to form a stable 9 S complex. It is in this snake protein-human protein complex that C'3 inactivating activity resides. Formation of the complex is inhibited by EDTA. Action of the C'3 inactivator on C'3 results in the release of a fragment from C'3 which has an approximate sedimentation rate of 1 S. This fragment was found to have anaphylatoxin activity (Cochrane and Müller-Eberhard, 1967).

Injection of a suitable amount of purified cobra factor into rabbits and guinea pigs (approximately 1 mg/kg) was followed by loss of overall complement activity. After a single injection, complement depletion lasted for more than 100 hours. Immunochemical analysis of the serum of C'3-depleted animals revealed total absence of the C'3 protein. Preliminary results indicate that animals with selective C'3 depletion are incapable of producing an Arthus phenomenon and develop a much milder form of nephrotoxic nephritis than the controls (Cochrane and Müller-Eberhard, 1967). C'3-depleted animals are now being used in this laboratory for a variety of immunological studies.

It is hoped that studies on selectively complement-depleted animals and on animals with inherited complement defects will delineate the role of complement in life processes and will provide the basis for a safe iatrogenic regulation of the complement system to the benefit of the patient.

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DISCUSSION

DR. METCALF: Has the tissue origin of any of these complement factors been established perhaps by tissue culture of cells of various types?

DR. MÜLLER-EBERHARD: Dr. Thorbecke has found that macrophages obtained from mice can actually synthesize C'3 factor. Lately she found that macrophages are also able to synthesize the C'1q and possibly C'6.

DR. SIGEL: Dr. Nelson uses different designations to indicate the various complement factors. I believe the sequence of the components is c b e f a d. How do your designations correlate with his?

DR. MÜLLER-EBERHARD: The designations C'3 c b e f a d correspond directly to our terms C'3,5,6,7,8, and 9. I do not think that there is any principal disagreement on the number of components or their sequence of action.

DR. BARNES: To the list of diseases in which complement possibly plays a role one could add experimental hypertension in animals and essential hypertension in man. Complement components are localized in the vascular lesions that are formed in some types of hypertension in man [Paronetto, F. (1965) *Am. J. Pathol.* **46,** 901]. Do you feel that any particular component of complement has a role in the etiology of vascular diseases that are claimed to be immunological in nature?

DR. MÜLLER-EBERHARD: I certainly cannot make any definite statement on this question. The evidence of an involvement of complement in vascular human diseases is clearly circumstantial and indirect. In experimental models such as the Arthus phenomenon or nephrotoxic nephritis, we can definitely show the dependence of the pathological lesion upon the presence of complement. We may extrapolate from there and say that similar phenomena, as they relate to human disease, may also be mediated by the same mechanisms in man. Findings by various investigators indicate that in some human diseases complement components may be fixed *in vivo* at the site of the tissue lesion. It is somewhat premature to draw the conclusion that this is a causal connection but I think it is a good guess to assume that complement is involved in some immunological diseases of man.

DR. MILGROM: I may add amyloid to the list of diseases in which complement is probably involved. In cooperation with Dr. Calkins, Dr. Schultz in my laboratory showed that amyloid contains complement of the B1C/1A complex.

DR. MAO: Complement is more easily inactivated by exposure to ultraviolet light than to heat. After 10 minutes of exposure, the hemolytic activity of the U.V. treated samples is completely lost whereas that of the same serum samples incubated at 56° C for the same period of time is still present. In studies of sensitivity of the components of complement it was found C'1 is the component most sensitive to U.V. whereas C'3 and C'4 are relatively resistant. As Lepow and his co-workers have first shown, C'1 is a complex which consists of three subunits; namely, C'1q, C'1r, and C'1s. All these subunits are required for the hemolytic reaction but only C'1s is responsible for enzymic activity. It was felt that possible differences of sensitivity to U.V. light among these three units may be exploited as a tool to isolate a kind of functionally pure complement fraction(s) of C'1. Recent studies performed in cooperation with A. Strampp at Rutgers State University indicated that the hemolytic activity of the C'1 component is much more sensitive to U.V. denaturation than its enzymic activity.

DR. GLICK: I would like to ask whether the complex of complement to antibody must take place only when antibody is bound to the antigen. If not, can complement bind to the heavy chain of antibody alone? Also, can complement bind to antigen when antibody is no longer bound to antigen?

DR. MÜLLER-EBERHARD: Using analytical ultracentrifugation, we have recently studied the interaction between C'1q and native γ-globulin. We isolated γ-globulin by electrophoresis and allowed it to react with C'1q. We could see complex formation which was dependent upon the γ-globulin concentration in the cell and also upon the type of γ-globulin used. In collaboration with Dr. Howard Gray we studied various myeloma proteins that had been typed with respect to their heavy chain subgroups, and it appears that there are considerable differences in C'1q-γG interaction dependent on the subgroup studied.

DR. TOMASI: I wonder whether you have any information on the number of molecules of C'1q that attached to a molecule of γ-globulin? Do you get one on each H chain?

DR. MÜLLER-EBERHARD: We do not know the exact molecular weight of the C'1q as yet, but I think that it is fair to assume that it has a molecular weight of approximately 450–500,000. Measuring the area of the complex at full saturation, we find that the C'1q-γG ratio is 1 : 1. This would mean that one molecule of C'1q could interact with three molecules of γ-globulin.

Immunogenicity of Synthetic Polymers of Amino Acids; Role of Carrier and Genetic Background*

PAUL H. MAURER and PAUL PINCHUCK
Department of Biochemistry, Jefferson Medical College,
Philadelphia, Pennsylvania

I. INTRODUCTION

The contributions that biochemistry on the one hand and cytology on the other are making to our understanding of the molecular and cellular aspects of the immune response have been well reviewed in this symposium. What more can be said? What we should like to contribute are several areas of investigation, mainly from our laboratory, employing synthetic polymers of amino acids as antigens. Perhaps by the use of these simple and well-defined molecules, we may learn more about some of the parameters responsible for (1) the induction of the formation of humoral antibody or sensitized cells and (2) the induction or selection of the specificity of antibody. The areas to be covered are:

1. The immunogenicity of linear polymers of α-D-amino acids and homopolymers of α-L-amino acids.

2. The role and requirements of a "carrier" for enhancing the immune response to the above nonimmunogenic polymers.

3. The genetic control of (a) the recognition of a given material as an antigen (immunogenicity); (b) the specificity of the antibody formed; and (c) the amount of antibody formed. There may well be other factors under genetic control, such as the minimal amount of antigen needed to

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trigger a standard level of the immune response or to induce tolerance which may vary among different inbred strains.

II. IMMUNOGENICITY OF LINEAR POLYMERS OF α-L- AND α-D-AMINO ACIDS*

In recent years, considerable work elucidating the factors affecting the antigenicity of proteins has been reported using synthetic polypeptides (branched or straight chain) of α-L-amino acids as models of proteins (see reviews by Maurer, 1964; Sela, 1966). Most of our own studies have employed linear, random polymers composed of known ratios of α-L-amino acids, and having molecular weights of the order of proteins (10,000–100,000). In a number of studies with rabbits (Maurer, 1957; Maurer *et al.*, 1959), guinea pigs (Maurer, 1957), mice (Pinchuck and Maurer, 1965a), and man (Maurer, 1957), polymers of only a single amino acid (homopolymers) have been shown to be nonimmunogenic by themselves. Copolymers containing two or more amino acids of varying proportions are usually immunogenic, but the pattern of response is different, depending upon the species studied (see reviews by Maurer,

TABLE I
RESPONSE OF VARIOUS SPECIES (NONINBRED) TO COPOLYMERS

Copolymer[a]	Response[b]		
	Rabbit	Guinea pig	Man
$G_{60}L_{40}$	8/17(3)	68/181(10)	0/15
$G_{60}A_{40}$	60/101(5)	6/21(2)	–
$G_{90}T_{10}$	5/12	0/18	–
$G_{60}A_{30}T_{10}$	30/41(4)	3/7	0/4
$G_{57}L_{38}A_5$	7/11	10/22(2)	2/6
$G_{58}L_{38}T_4$	9/12	7/24(2)	3/5
$G_{42}L_{28}A_{30}$	51/52(7)	12/24(3)	6/8
$G_{36}L_{24}A_{40}$	4/6	3/5	11/12
$G_{36}L_{24}A_{35}T_5$	53/59(4)	8/8	16/20(2)

[a]Subscript refers to mole percent of amino acid in copolymer. G = glutamic acid; L = lysine; A = alanine; T = tyrosine; etc.

[b]Number responding/number tested. Values in parentheses indicate results of the number of experiments.

*This presentation will deal mainly with the work from our laboratory. For extensive discussions of the work of others see review by Maurer (1964), Sela (1966), and the articles by Benacerraf (1966) and Pinchuck and Maurer (1966) in Symposium on Regulation of the Antibody Response, Toronto, Canada, Jan. 1966. (To be published by Charles C. Thomas.)

1964; Sela, 1966). Generally, the immune response displays the characteristics of the response to conventional antigens, i.e., both delayed and immediate hypersensitivity may be present. It has been found in immunizing the above random-bred animals that only a portion of the animals responded. With inbred animals, the response is usually an all or none affair (Kantor *et al.*, 1963; Levine *et al.*, 1963; Pinchuck and Maurer, 1965b, 1966). The more complicated the polymer and the more amino acids present in the polymer, the greater is the percent of animals reacting in all species studied. Tables I and II present some typical findings. Polymers containing two α-L-amino acids were immunogenic in rabbits, guinea pigs, and sheep, but not in mice, rats or men. Terpolymers

TABLE II
RESPONSE OF SWISS MICE TO POLYMERS

Polymer injected	Number responding / Number tested
$G_{90}T_{10}$	0/20
$G_{60}A_{40}$	0/38
$G_{60}L_{40}$	0/58
GAL_{10}	11/16
GAT	20/20
GLT	20/20
GLPhe	20/20
GLAT	9/9
GLA_5	17/36
GLA_{10}	23/25
GLA_{20}	28/33
GLA_{30}	6/6
GLA_{40}	10/10
GLA_{60}	10/10

were immunogenic in all species studied, depending upon the amino acid composition of the polymer. These latter findings are related to the recognition of foreign structures and the genetic control of this recognition system as discussed in Section III.

Entirely different findings were observed when the random polymers consisted only of α-D-amino acids. None of the polymers containing 2, 4, α-D-amino acids (Table III) were immunogenic in rabbit (Maurer 1963, 1965a), guinea pig (Maurer, 1963, 1965a), mice (Pinchuck and Maurer, unpublished), man (Maurer, 1963), and sheep (Maurer, 1966b). Not only was no antibody detectable by the techniques of passive cutaneous anaphylaxis (PCA), hemagglutination (HA), or precipitin

(Ppt.) reaction, but also no type of immediate or delayed reactions were observed in guinea pig or man.

TABLE III
POLYMERS OF α-D-AMINO ACIDS

Polymer	Average molecular weight
$G_{60}A_{40}$	47,000
$G_{60}L_{40}$	105,000
$G_{90}T_{10}$	85,000
$G_{42}L_{28}A_{30}$	70,000
$G_{56}L_{38}T_{6}$	21,000
$G_{60}A_{30}T_{10}$	33,000
$G_{36}L_{24}A_{35}T_{5}$	15,000

Another sensitive method for determining whether synthetic polymers have any immunogenicity is to incorporate haptens of the classical type into the molecule and see whether the polymer can act as a carrier for this hapten specificity. The α-D-amino acid polymers, conjugated with haptens, which were negative in both guinea pigs and rabbits are listed in Table IV (Maurer and Benacerraf, 1966). There was no evidence of any immune response either against the polymer or hapten as

TABLE IV
NONIMMUNOGENIC POLYMER (α-D-AMINO ACIDS)-HAPTEN CONJUGATES

Polymer	Hapten conjugated to polymer[a]	Species studied
L	DNP	Guinea pig
GL	DNP	Guinea pig, rabbit
GAT	Ars	Guinea pig
GLT	DNP	Guinea pig, rabbit
GLT	DNP, Ars	Guinea pig, rabbit
GLA	DNP	Guinea pig

[a]Ars = arsanilate conjugate; DNP = dinitrophenyl conjugate.

determined by PCA, HA, systemic, or cutaneous anaphylaxis. A typical protocol in guinea pigs using the arsanilic acid conjugate of GAT of α-D-amino acids (Benacerraf *et al.*, 1963) is presented in Table V. In contrast to these negative findings, the homopolymers and copolymers of α-L-amino acids could act as immunological carriers for various haptens (Kantor *et al.*, 1963; Levine *et al.*, 1963). A summary of some

TABLE V

IMMUNE RESPONSE OF GUINEA PIGS TO ARSANILIC ACID CONJUGATE OF D-GAT[a]

No. of guinea pigs	Immunizing antigen	24-hour skin reactivity at 14 days[b]		Anti-AS-Ab		Anaphylactic reactivity to	
		D-GAT	ASD-GAT	Ppt.	PCA	ASD-GAT, 200 μg	ASGPA,[c] 250–500 μg
	ASD-GAT						
10[d]	16 arsanil groups/mole 100 μg	0/10	0/10	–	–	0/10	–
7	7 arsanil groups/mole 100 μg	–	0/7	–	–	–	0/7
10	7 arsanil groups/mole 0.01 μg	–	0/10	–	–	–	0/10
13[e]	13 arsanil groups/mole 100 μg	0/13	0/13[f]	0/13	–	–	0/13

[a] From Benacerraf *et al.* (1963).

[b] All skin tests: 10 μg of antigens.

[c] ASGPA refers to arsanil conjugated guinea pig serum albumin.

[d] Eight of the animals were immunized 7 days later with 100 μg ASL-AT, 11 groups/mole. All guinea pigs developed skin sensitivity and anaphylactic reactivity to this antigen at 2 weeks.

[e] The sera of these animals were tested for γ2 complement-fixing antiarsanilic antibodies by passive lysis of tanned SRBC coated with AS bovine fibrinogen; no lysis was observed at a dilution of 1/10.

[f] These guinea pigs were also negative when tested intradermally with 50 μg of ASD-GAT.

TABLE VI

IMMUNE RESPONSE OF GUINEA PIGS TO ARSANILIC ACID CONJUGATES OF L-GAT[a]

No. of guinea pigs	Immunizing antigen	24-hour skin reactivity at 14 days[b]		Anti-AS-Ab		Anaphylactic reactivity to		
		L-GAT	ASL-GAT	Ppt.	PCA	ASD-GAT, 200 μg	ASL-GAT, 200 μg	ASGPA,[c] 250–500 μg
	ASL-GAT							
9	11 arsanil groups/mole 100 μg	8/9	9/9	5/9	4/4	–	–	–
9	10 arsanil groups/mole 100 μg	4/9	9/9	3/9	6/6	–	–	–
9	10 arsanil groups/mole 100 μg	5/9	8/9	–	–	5/5 dead	4/4 dead	–
10	5 arsanil groups/mole 100 μg	–	10/10	–	–	–	–	9/10 dead 1 severe shock
8	8 arsanil groups/mole 50 μg	–	8/8	0/6	6/6	–	–	–

14[a]	8 arsanil groups/ mole 100 μg	8/14	14/14	–	–	–	–	12/14 dead 2 severe shock

[a]From Benacerraf *et al.* (1963).

[b]All skin tests: 10 μg of antigens.

[c]ASGPA refers to arsanil conjugated guinea pig serum albumin.

[d]The sera of 13 of these animals were tested for $\gamma 2$ complement-fixing antiarsanilic antibodies by passive lysis of tanned SRBC coated with AS bovine fibrinogen, 5 of these sera contained lytic antibodies in a titer up to 1/80.

of the findings in guinea pigs with Ars-GAT of α-L-amino acids (Table VI) indicates that responses were obtained both against the polymer (carrier) and hapten (Benacerraf *et al.*, 1963).

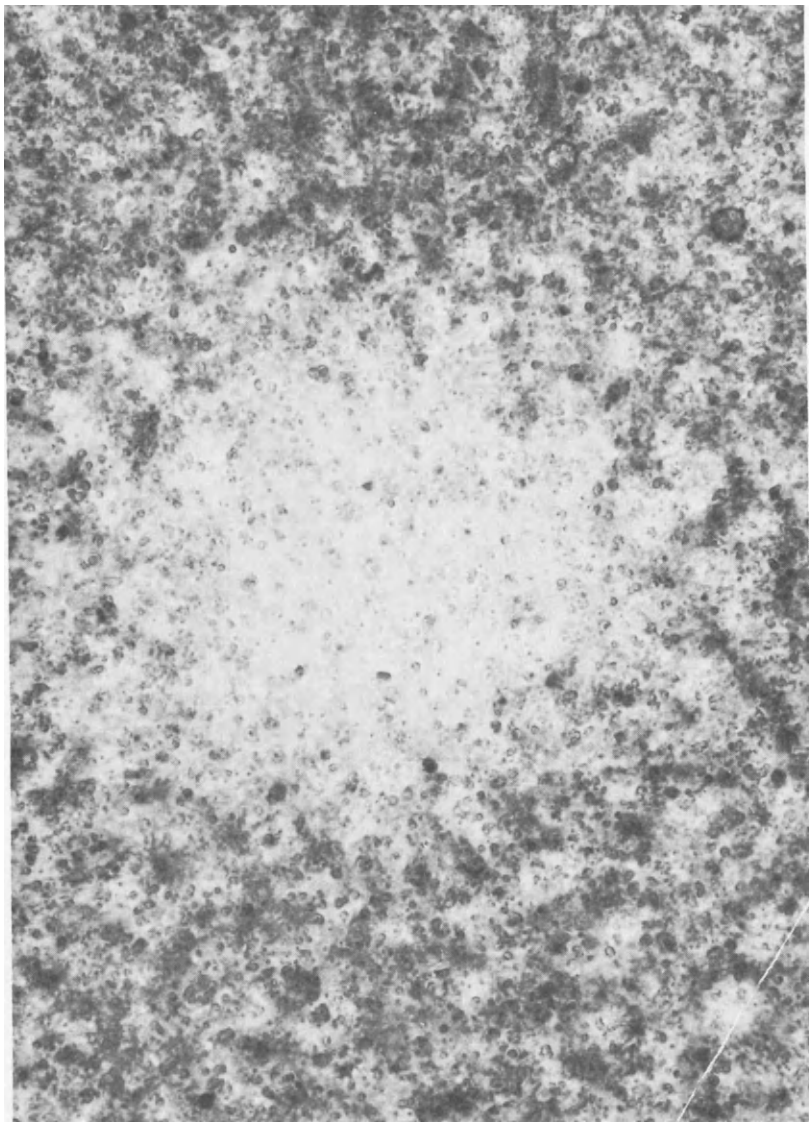

FIG. 1. Typical hemolytic zone observed when rabbit lymph node tissue sensitized against the synthetic polymer GLA_{30} was reacted in agar plates containing GLA coated sheep erythrocytes (150 × magnification).

Recently, Dr. Walsh and Miss Egan in our laboratory have been successful in adapting the Jerne-Nordin local hemolysis plaque method for detecting responses against synthetic polymers of α-L-amino acids, employing tannic acid-treated sheep erythrocytes coated with the polymers containing lysine (Maurer *et al.*, 1963) and draining popliteal lymph node suspensions from immunized rabbits. Typical hemolytic plaques obtained are shown in Fig. 1, and some data in Table VII. It has been possible to detect antibody-forming cells 4–6 days after the first injection of the polymer, even in the absence of positive serological tests for antibody. The specificity of the response was checked by noting the inhibitory effects of the incorporation in the agar-erythrocyte suspension of either sheep antirabbit γ-globulin serum or specific polymer (Walsh *et al.*, 1966). Again, with this technique, no response could be detected against polymers made solely of α-D-amino acids. However, as will be

TABLE VII
PLAQUE-FORMING CELL (P.F.C.) RESPONSE IN LYMPH NODE TISSUE OF RABBITS IMMUNIZED WITH $G_{42}L_{28}A_{30}$-CELLEX GE RESIN[a]

Rabbit no.	Days after injection	P.F.C./10^6 Lymph node cells in presence of		
		Complement only	Normal sheep serum	Sheep antirabbit-globulin sera
First injection				
199	5	0.3	0.2	0.4
201[b]	5	107	85	0.6
203	5	6	2	0.1
204	5	60	57	0.4
215	5	0.6	1	0.5
225	6	35	32	0.1
216	10	2	1	4
217	10	2	0.9	25
219	10	0.6	1	15
Second injection				
194	4	25	20	0.8
195	4	102	70	1.3
202[b]	4	0.4	0.4	0.2
207	4	75	86	0.5
208	4	47	—	1.0
209	10	30	21	0.4
210	10	25	21	0.3
197	10	4	1	2

[a]Test system GLA_{30} coated sheep erythrocytes.
[b]Control rabbits.

seen later, responses could be detected if the D-amino acid polymer was incorporated as a hapten with an immunogenic carrier.

These few lines of evidence indicated to our satisfaction that the polymers of α-D-amino acids alone were not immunogenic. Also, as we have shown previously, the substitution of a D-amino acid for an L-amino acid in copolymers of terpolymers reduces the response or abolished completely the immunogenicity of the polymer containing both L and D amino acids (Table VIII) (Maurer, 1965a, 1966a). We and others have, therefore, postulated that "metabolism or degradation of the polymer is a necessary but not sufficient criterion for immunogenicity" (Maurer, 1964, 1965b). The findings by others of RNA complexed with fragments of antigen have also implicated proteolytic digestion of the antigen as being important for immunogenicity (Campbell and Garvey, 1963;

TABLE VIII

SUMMARY OF IMMUNE RESPONSES IN SEVERAL SPECIES AGAINST SYNTHETIC POLYMERS CONSISTING OF L AND D α-AMINO ACIDS[a]

Polymer	Species injected	Response[b]
$G_{60}A_{40}$ (L,D)	Rabbit	0/10
	Guinea pig	0/10
$G_{60}A_{40}$ (D,L)	Rabbit	0/10
	Guinea pig	0/10
$G_{42}L_{28}A_{30}$ (L,D,L)	Rabbit	5/10[c]
	Guinea pig	7/12[c]
	Sheep	2/2[c]
	Man	0/4
$G_{42}L_{28}A_{30}$ (L,L,D)	Rabbit	2/6[c]
	Guinea pig	5/10[c]
	Sheep	1/2[c]
	Man	0/3
$G_{42}L_{28}A_{30}$ (D,L,L)	Rabbit	2/6[c]
	Guinea pig	2/17[c]
	Sheep	2/3[c]
	Man	0/2
GAT (L,L,D)	Rabbit	3/5[c]
	Sheep	2/2[c]

[a] Letters in parentheses indicate configuration of amino acid, i.e., $G_{60}A_{40}$ (L,D) α-L-glutamic acid and α-D-lysine, etc.

[b] $\frac{\text{Number responding}}{\text{Number tested}}$

[c] Includes very weak positive reactions.

Askonas and Rhodes, 1965; Friedman *et al.*, 1965). Exactly where this digestion occurred, or in what step of the complex recognition scheme of antibody formation this was important, was not, and still is not, too explicit.

III. ROLE OF CARRIER IN INFLUENCING THE IMMUNE RESPONSE

It is a known concept in immunology that there is a sharp difference between the ability of a polymer, macromolecule, or simple chemical to be immunogenic per se and the ability of the same molecules to act as haptens when coupled either to immunogenic or nonimmunogenic but metabolizable backbone structures. Some of the most potent haptens employed in immunological studies such as dinitrophenyl or arsanilic acid are not immunogenic, yet immune responses with considerable specificity against these simple molecules can be elicited if the hapten is conjugated to the proper protein carrier. The following published findings from our laboratory prompted us to reinvestigate the immunogenicity of some homopolymers and copolymers of α-L and α-D-amino acids.

1. Although polyglutamic acid (α-L) was not immunogenic, a large degree of specificity of the antibody produced in rabbits and guinea pigs against the $G_{60}A_{40}$ polymer (α-L) was directed against polyglutamic acid. Polyglutamic acid did not precipitate the antibody but did react with it as determined by complement fixation and PCA techniques (Table IX) (Maurer, 1963; Maurer *et al.*, 1964). The cross reaction of

TABLE IX
CROSS REACTIONS WITH RABBIT ANTI $G_{60}A_{40}$ SERA (POOL 3)

Polymer	Complement fixation (%)	Ppt. (%)	PCA titer
$G_{60}A_{40}$ (L,L)	100	100	1000
$G_{60}A_{40}$ (D,D)	6	0	200
$G_{90}A_{10}$ (L,L)	65	28	–
G_{100} (L)	61	0	200
G_{100} (D)	0	0	100

the α-D-amino acid polymers G_{100} and $G_{60}A_{40}$ with this antiserum is most unusual as this is the only instance of such cross reactions among optical isomers. This may reflect the stereochemical similarity between L and D glutamic acid, as shown by the similar unusual activity of gluta-

mine synthetase with these amino acids (Kagan and Meister, 1966). Employing oligopeptides of glutamic acid, the size of the antibody site was also shown to be complementary to about five to six glutamyl residues (Maurer, 1964).

2. Neonatal injections into rabbits of polyglutamic acid (α-L) could produce tolerance against subsequent immunizations with $G_{60}A_{40}$ polymer (Table X) (Maurer *et al.*, 1965).

The reinvestigation was done initially employing the technique described by Plescia *et al.* (1964). The method involves the use, as the immunogenic material, of an aggregated precipitate formed by the reaction of polyglutamic acid (or negatively charged polymers) and the methylated bovine serum albumin (MBSA) (positively charged carrier). When dealing with a positively charged polymer or copolymer such as polylysine, negatively charged proteins or carriers [acetylated (Ac BSA) or phosphorylated BSA (PBSA)] are used to form the insoluble aggregate. In all of the studies with rabbits, guinea pigs, and mice, immunization was with complete Freund's adjuvant. Antibody was tested for by PCA, systemic anaphylaxis, HA, and passive lysis. Table XI summarizes results obtained with homopolymers and copolymers of α-L-amino acids. Some of the more pertinent observations were: (1) The aggregate of MBSA + polyglutamic acid was immunogenic for rabbits and guinea pigs. The molecular weight of the polyglutamic was an important factor, as the low molecular weight polymer (5000) was not effective in this system. (2) MBSA was not an effective carrier in rabbits and guinea pigs for the polyaspartic acid response or for SIII in guinea pigs. Yet, this latter system (SIII) works well in rabbits (Plescia *et al.*, 1964). (3) The aggregate formed with poly-L-lysine and poly-L-glutamic acid was not immunogenic for either component. (4) Neither acetylated BSA nor phosphorylated BSA aided in eliciting a significant response in rabbits or guinea pigs against poly-L-lysine.* In spite of the above negative findings, techniques are available for producing antibody with poly-L-lysine, SIII, or aspartic acid specificities. The use of polypeptidyl proteins has been most effective in eliciting responses against many different oligopeptides, (Sela, 1966) and oligosaccharides (Borek *et al.*, 1963). Therefore, the nature of the carrier and the method of formation of the hapten-carrier complex, and the method of degradation of the complex may be very important in determining whether a response will ensue against the haptenic component.

In all of these studies, it should be remembered that, in addition to these weakly immunogenic polymers, we are presenting the host with a

*These carriers yield positive responses with poly-D-lysine (Green *et al.*, 1966; Van Vunakis *et al.*, 1966; Maurer, 1966b).

TABLE X

EFFECT OF NEONATAL INJECTIONS OF POLYGLUTAMIC ACID IN RABBITS ON SUBSEQUENT RESPONSE TO $G_{60}A_{40}$ POLYMER

Polymer injected			Immune response					
Neonatally[a]	Adult		First course			Second course		
	i.v.	Adjuvant[b]	No. of reactors[c]	PCA titers[d]	Antibody per ml serum[e] (μgN)	No. of reactors[c]	PCA titers[d]	Antibody per ml serum[e] (μgN)
G_{100} (25)	G_{100}	$G_{60}A_{40}$	3/5	75	8.0	3/5	1500	98
G_{100} (50)	G_{100}	$G_{60}A_{40}$	0/3	–	–	0/1	–	–
G_{100} (100)	G_{100}	$G_{60}A_{40}$	0/2	–	–	0/2	–	–
G_{100} (2×50)	G_{100}	$G_{60}A_{40}$	0/3	–	–	1/2	500	40
	G_{100}	$G_{60}A_{40}$	3/3	150	50	3/3	2000	127
		$G_{60}A_{40}$	5/6	150	40	5/5	2000	170
G_{100} (100)	G_{100}	$G_{42}L_{28}A_{30}$	5/5	750	67	5/5	2000	170

[a]Values in parentheses indicate milligrams polyglutamic acid injected neonatally.

[b]Adjuvant injected in footpads on day 90.

[c]Number of reactors based on PCA reactions in guinea pigs.

[d]Average includes only rabbits having antibody detectable by PCA.

[e]Polymer injected intravenously on day 75.

TABLE XI
RESPONSE TO IMMUNIZATION WITH
POLYMER (α-L-AMINO ACID)-AGGREGATE COMPLEXES

Polymer	Carrier[a]	Species injected[b]	
		Rabbit	Guinea pigs
G_{100} (M.W. 50,000)	MBSA	13/17	41/55
G_{100} (M.W. 50,000)	P-LL	0/6	0/27
G_{100} (M.W. 50,000)	MRSA	3/5	
G_{100} (M.W. 5,000)	MBSA	1/6	0/9
Asp	MBSA	0/4	0/18
PLL	AcBSA	0/12	0/6
PLL	PBSA	0/6	0/6
GT	MBSA	9/12	13/21
GT	L_{100}	–	0/15
SIII[c]	MBSA	–	0/10

[a]MBSA = methylated BSA; AcBSA = acetylated BSA; PBSA = phosphorylated BSA.
[b]Number responding/number tested.
[c]Pneumococcal type III capsular polysaccharide.

number of highly immunogenic materials (carrier protein and mycobacterial antigens). The phenomenon of competition among the different antigens (Adler, 1964) as well as between immunodominant and immunorecessive determinants of a single antigen or carrier-hapten complex can influence the response in which multipotential cells may participate and the nature of the antibody formed. Additional observations, employing the aggregate technique and copolymers support some of the above ideas. The responses obtained by immunization of guinea pigs with the aggregates of α-L-amino acid copolymers and MBSA were much poorer than with the polymer alone (Table XII). Also, immunization of guinea pigs with complete adjuvants alone containing H37Ra mycobacteria or with MBSA and adjuvant before subsequent immunization with an ordinarily immunogenic aggregate has led to a significant depression in the response against the polymer. A similar effect can be produced by introducing a very potent hapten such as DNP into weakly immunogenic polymers. In mouse, rabbit, and guinea pig, after immunization with the polymers GLA5 or GLT, the response is predominantly against GL. However, when DNP, which is a strong immunogenic determinant, is introduced into the above polymers, the major response is now directed against the haptenic groups. This is an example of "internal antigenic competition" among determinants for "triggering" the proliferation of committed stem cells.

TABLE XII
GUINEA PIG RESPONSE TO IMMUNIZATION WITH α-L-AMINO ACID POLYMER OR POLYMER-AGGREGATE COMPLEX

Polymer injected	Response to[a]	
	Polymer aggregate	Polymer alone
GL	0/5	68/181
GAT	4/21	3/7
GLT	1/4	16/34
GLA_{30}	3/31	12/24

[a]Number animals responding/number animals tested.

Extension of some of the ideas referred to above led to an investigation of the immunogenicity of aggregates formed with polymers of α-D-amino acids. Data obtained with guinea pigs and mice (Table XIII) indicate that with this technique, immune responses could be produced against D-amino acid polymers. The hemolytic plaque assay of Jerne and Nordin was also positive when rabbits were immunized with the aggregate of GLA_{30} polymer (D-amino acids) and MBSA.

TABLE XIII
RESPONSE TO D-AMINO ACID POLYMER MBSA AGGREGATES

Polymer injected	Response[a] (guinea pig)	Polymer injected	Response[a]	
			Guinea pig	Mouse
G_{100} (α,D)	26/29	GL	11/12	4/8
G_{100} (γ,D)	13/13	GLT	40/48	5/9
GA (D,D)	24/30	GLA_{30}	24/31	4/9
GA (L,D)	0/13	GAT	29/33	–
GA (D,L)	5/6	–	–	–

[a]Number animals responding/number animals tested.

About 8–14 plaque-forming cells/10^6 lymph node cells were detectable with sheep red blood cells (RBC) coated with the GLA_{30} (D,D,D) polymer. The question could be raised whether the MBSA was acting only as a nonspecific adjuvant because of the formation of a particulate aggregate. To answer this question, different kinds of "aggregating carriers" were employed as noted in Table XIV. The nonimmunogenic

TABLE XIV
EFFECT OF DIFFERENT CARRIERS ON IMMUNOGENICITY IN GUINEA PIGS OF SYNTHETIC POLYMERS

Carrier[a]	Polymer[b]			
	GAT		GLT	
	α-D	α-L	α-D	α-L
Cellex-D	0/7	6/7	0/7	4/7
PVA	0/6	5/6	–	–
PLL	0/5	0/6	–	–
$L_{30}A_{70}$	3/10	4/6	2/10	5/5
$G_{30}L_{70}$	1/6	1/6	0/5	0/4
G.P. red blood cells	–	–	2/7[c]	–
Sheep red blood cells	–	–	7/14[c]	–
MGPSA	3/5	–	0/5	–
MBSA	10/10	3/10	17/19	0/5

[a]PLL = poly-L-lysine; PVA = polyvinylamine; Cellex-D = DEAE cellulose.
[b]Number animals responding/number animals tested.
[c]Data obtained with $G_{42}L_{25}A_{30}$ (D,D,D).

materials such as DEAE cellulose, polyvinylamine, and the non- or poorly immunogenic polymers of α-L-amino acids were not effective carriers for enhancing the response against D-amino acid polymers in guinea pigs. Methylated guinea pig serum albumin (MGPSA) and guinea pig erythrocytes were not as effective carriers as were the more foreign and immunogenic materials such as MBSA and sheep RBC. Similar findings have been reported by Green *et al.* (1966) with the DNP-PLL system in guinea pigs (Table XV). Only immunogenic carriers were

TABLE XV
CLASSIFICATION OF CARRIERS FOR DNP-POLY-L-LYSINE RESPONSE IN GUINEA PIGS[a]

Effective	Noneffective
AcBSA,[b] BSA	Dextran sulfate, heparin
HSA, Ea[c]	Polystyrene sulfonate, CM cellulose, kaolin, DNA, hyaluronic acid
Ac GPA, Ars GPA	GPA

[a]From Green *et al.* (1966).
[b]Also with DNP-PDL.
[c]Ea = ovalbumin.

effective in enhancing the response against ordinarily nonimmunogenic polymers or haptens coupled to nonimmunogenic polymers. All of the above data with homopolymers of α-L or D-amino acids and copolymers of α-D-amino acids, etc., agree with what is known about the nature of the haptenic response. In all of these studies, it appears that the systems necessary for the recognition of the foreign structures are present in these animals, but important metabolic steps associated with handling or degradation of an immunogenic carrier are necessary for triggering this recognition machinery. Experiments in our laboratory have indicated that in contrast to the α-L-amino acid polymers, those consisting of α-D-amino acids are not attacked by any of known proteolytic enzymes. Moreover, the latter polymers can actually act as inhibitors of the enzymes (Liu and Maurer, 1966). The reported data on *in vivo* metabolism and localization in the kidney of externally labeled (^{131}I and ^{59}Fe) polymers of α-D-amino acids and excretion of radiolabel in the urine do not support the concept of proteolytic degradation of D-amino acid polymers (Gill *et al.*, 1964, 1965; Gill and Mann, 1966). The carrying out of the degradation steps may well be one of the functions of the macrophage, i.e., to process the antigen to an appropriate piece (RNA-antigen fragment) which can now be handled by lymphoid stem cells already committed (Levine and Benacerraf, 1964).

The carrier is also involved in the specificity of the antibody as shown by Green *et al.* (1966) in studies with MBSA + DNP-PLL aggregates, i.e., specificity is directed against more than the DNP-PLL determinants. As previously postulated, two possible explanations may be given for the need for degradation in the above scheme: (1) to allow the formation of a "specific inducer," (2) to allow a degraded piece to react with recognition factor present on or in stem cells which then proliferate. Whether or not cells are committed to participate in the above reactions is related to the area of the genetic control of recognition. The data to be presented in the next section will show that this recognition mechanism, or the presence of immunological competence, is under genetic control.

IV. GENETIC CONTROL OF IMMUNE RESPONSE

The results we have presented thus far indicate at least two major steps in an immune response; a metabolic step, part of which seems to be proteolytic, which we may refer to as the carrier phase, and a selection of a specific portion of the molecule as a determinant group, the hapten phase. Both of these appear to be under direct control of the genome. The differences in the immune response of various species to the same materials, both natural and synthetic, as well as the differences between individuals of a species, are best interpreted in this way. With man and

the rabbit, the response has been most heterogeneous. Because genetic studies here are more difficult to perform, most of the discussion will center on findings with guinea pigs and mice.*

The response of guinea pigs to hapten-poly-L-lysine conjugates has been well studied by Benacerraf and co-workers. Only a portion of the Hartley (i.e., noninbred) animals can respond to this material in the absence of an aggregating agent such as AcBSA or PBSA (Kantor *et al.*, 1963; Schlossman *et al.*, 1966). When these animals are selected on the basis of responders vs. nonresponders, the ability to respond is transmitted as a codominant, Mendelian autosomal trait (Levine *et al.*, 1963; Levine and Benacerraf, 1965). Similarly, all guinea pigs of inbred strain 2 are responders, while those of strain 13 are nonresponders. The F_1 and backcross generations show the same pattern of transmission as was found with Hartley animals. When the Hartley guinea pigs are selected on the basis of their response to DNP-PLL, for example, it is found that their ability to respond to other hapten-PLL conjugates such as penicilloyl-PLL, and to such high lysine copolymers as $G_{60}L_{40}$, $L_{70}A_{30}$, GLA_5, and GLT is also determined by what appears to be the same factor. When the amino groups of the hapten-PLL conjugate are succinylated, the factor is apparently unable to handle the modified carrier, and there is no antibody response (Levine, 1964). It should be emphasized that these same nonresponders can produce hapten-oriented antibody if immunized with DNP-BSA or with the MBSA aggregate·DNP-poly D-lysine (Green *et al.*, 1966). Thus, recognition is at the level of the carrier function, and is apparently concerned with some metabolic phase beyond the level of proteolytic digestion. This latter statement is based on data showing that nonresponders degrade PLL in much the same way that responders do (Levine and Benacerraf, 1964).

As mentioned in Section I, mice lack the ability to respond to homopolymers as well as copolymers of amino acids. However, the introduction of small amounts of a third amino acid in the copolymer leads to the formation of a good immunogen. In the case of GLA_5, about 50% of Swiss mice (random-bred) respond to this terpolymer, and the ability to respond is transmitted as a codominant, autosomal Mendelian trait (Table XVI) (Pinchuck and Maurer, 1965b). Furthermore, when six inbred strains were immunized with GLA_5, four proved to be 100% responders while the others were completely nonresponders (Table XVII). Interestingly, if a polymer with a higher alanine content is used (i.e., GLA_{10}), these nonresponders do respond. Quantitative immunochemical

*In preliminary collaborative experiments with Dr. Carl Cohen, employing pedigreed rabbits, evidence has been obtained that the ability to respond to $G_{60}A_{40}$ differs between families.

TABLE XVI
IMMUNE REPONSE TO GLA_5 AMONG PROGENY OF RESPONDER AND NONRESPONDER SWISS MICE

Parental phenotype	Generation	No. tested	Percent responders
Nonresponder × Nonresponder	F_1	19	0
Responder × Responder	F_1	18	100
Responder × Responder	F_1	9	67
Nonresponder Progeny of F_1	F_2	9	0
Nonresponder Progeny of F_2	F_3	7	0

TABLE XVII
RESPONSE OF INBRED MOUSE STRAINS AND SPECIFIC F_1 HYBRIDS TO GLA_5 AND GLA_{10}

Strain	Percent Responders to	
	GLA_5	GLA_{10}
C3H/HeJ	100	100
C57BL/6J	0	100
C3B6F1[a]	100	Not done
BALB/cJ	100	100
A/J	0	100
CAF[b]	100	Not done

[a]Hybrid of C3H × C57Bl/65.
[b]Hybrid of BALB/c × A/J.

studies have shown that the antibody to GLT, GLPhe, GLA_5 in responders and antibody to GLA_{10} in both responders and nonresponders to GLA_5 is predominantly directed against GL, which has been consistently nonimmunogenic (Table XVIII). Antibody against GLA_5 could be detected by passive hemagglutination.

These results have been interpreted as indicating that in mice the presence of some third group, almost regardless of its nature, is required to cause formation of an active "triggering product," and that this is dependent upon the presence of a sufficient concentration of groupings containing three different residues. If the concentration of one of the

TABLE XVIII
GL SPECIFICITY OF INDIVIDUAL TERPOLYMER MOUSE ANTISERA

Immunizing antigens	Homologous reaction Ab ppt'd/ml serum (μg N)	Cross reaction with $G_{60}L_{40}$ (%)
GLPhe (Serum 1)	408	100
GLPhe (Serum X)	1190	80
GLT	67	69
GLA_{10}	125	100

amino acid residues in a terpolymer is low, it may be concentrated in a relatively small portion of the molecule due to differences in the rate at which the different amino acid *N*-carboxy anhydrides polymerize (Bamford *et al.*, 1956). In such a case, one gene at the locus involved may not be able to detect the presence of these triresidue groupings (nonresponders) whereas the allele would.

To test this hypothesis, we have taken advantage of (1) the ease of detection of anti-DNP antibodies, and (2) the fact that in a polymer containing a large amount of lysine, coupling small amounts of DNFB should introduce DNP groupings scattered randomly throughout the molecule. The results obtained with lightly coupled (2–3% substitution of lysine residues) polymers of L-amino acids are shown in Table XIX. As predicted, anti-DNP antibodies were produced by all of the animals immunized with conjugates containing three different groups, i.e., the copolymers GL and LA and all of the terpolymers. On the other hand, none of the animals immunized with DNP-PLL produced antibody.

Several points about the results presented deserve to be emphasized. (1) Even where there was only a small amount of lysine in the polymer, i.e., $G_{90}L_{10}$ and GAL_{10}, there was an anti-DNP response, although in the former case the response was slow in developing. (2) The response to the hapten was independent of the response to the polymer itself, there being no antibody produced to the GL, but the expected response to GLA_5, GLA_{40}, and GAL_{10} (Pinchuck and Maurer, 1965a). (3) The response to the polymer portion seemed to be weaker and to appear more slowly when the hapten-polymer conjugate was used than when the polymer alone was used for immunization, i.e., "internal antigenic competition." (4) Exactly the same pattern of response appeared with another hapten, the pipsyl group. (5) Two points are apparent with regard to the animals; (a) all of the random-bred animals responded, and

TABLE XIX
RESPONSE OF SWISS MICE TO DNP CONJUGATES

Immunizing conjugate	Response[a] to	
	DNP	Polymer
DNP-PLL	0/17	Not done
DNP-$L_{70}A_{30}$	4/4	Not done
DNP-$G_{60}L_{40}$	33/33	0/33
DNP-$G_{90}L_{10}$	8/8	Not done
DNP-GLA_5	18/18	8/18
DNP-GLA_{40}	28/28	23/28
DNP-GAL_{10}	9/9	4/9
DNP-$G_{60}L_{40}$ (G57Bl/6J mice)	8/8	0/8

[a]Number responding/number tested.

(b) an inbred strain, C57BL/6J which did not respond to GLA_5, also gave a positive response.

Several different preparations of DNP-PLL were prepared and tested, and these, as well as DNP-PDL were nonimmunogenic. However, the aggregates of DNP-PLL and PBSA, DNP-PDL and PBSA, or DNP-Succ PLL and MBSA were all immunogenic for DNP in Swiss mice (Table XX). Acetylated BSA was not a satisfactory carrier molecule, paralleling the results of VanVunakis *et al.* (1966) in rabbits. These results show that the nonimmunogenicity in mice of PLL conjugates is not related to inability to detect the antibody formed (antihapten) or to a difference in the linkage, etc., of the hapten to the homopolymer, but rather to some aspect of the initiation of the response.

TABLE XX
RESPONSE OF SWISS MICE TO DNP-POLYLYSINE AGGREGATES

Conjugate	Aggregating agent (carrier)	Response to DNP[a]
DNP-PLL	Phosphorylated BSA	10/10
DNP-PDL	Phosphorylated BSA	10/10
DNP-Succ PLL	Methylated BSA	10/10

[a]Number animals responding/number animals tested.

Because of the findings in guinea pigs, where a moderate lysine content seemed to enhance the immunogenicity of polymers, a further series of conjugates was studied. Swiss mice were immunized with DNP-PLL, DNP $G_{60}L_{40}$, and DNP $L_{70}A_{30}$ (Table XXI). Portions of these conjugates were further modified by exhaustive deamination (DNP-Deam-PLL, DNP-Deam GL, DNP-Deam LA); or succinylation (DNP-Succ PLL, DNP-Succ GL, DNP-Succ LA). The expected responses were obtained; 0% responders to DNP-PLL, DNP-Deam PLL and DNP-Succ PLL, and 100% response to the above-modified copolymer-conjugates.

TABLE XXI
RESPONSE OF SWISS MICE TO CHEMICALLY MODIFIED DNP-POLYMER CONJUGATES

Polymer	Response to DNP[a]
DNP-PLL	0/10
DNP-Deam PLL	0/20
DNP-Succ PLL	0/21
DNP-LA	10/10
DNP-Deam LA	20/20
DNP-Succ LA	18/18
DNP-GL	10/10
DNP-Deam GL	19/19
DNP-Succ GL	15/15

[a]Number animals responding/number animals tested.

However, when the DNP-PLL was only partially modified by deamination (i.e., the formation of "triresidue groupings"), effective hapten carriers were formed (Table XXII). A possible explanation for the effectiveness of the "triresidue groupings" may reside in the needed associa-

TABLE XXII
RESPONSE OF SWISS MICE TO PARTIALLY DEAMINATED DNP-PLL

ϵ-NH_2 Groups Removed (%)	Response to DNP[a]
0	0/10
5	14/15
12	12/14

[a]Number of animals responding/number of animals immunized.

tion of a minimal concentration of determinant with a committed cell to initiate proliferation. Possibly the presence of the third amino acid increases the association or binding constant of the polymer-RNA piece with the recognition factor present on or in the cell.

Implicit in the concept of antigenic competition, as discussed above, is the assumption of a hierarchy of antigens. That is, immunogenic molecules consist of determinants of unequal potency in ability to stimulate the formation of antibodies. There are various aspects of this hierarchy; thus, Silverstein found that fetal sheep could respond to some antigens, such as ϕX bacteriophage and ferritin, long before they could respond to others such as ovalbumin and diphtheria toxoid (Silverstein *et al.,* 1963). Even in an immunologically mature animal, there are differences in the strength of determinants, both inter- and intra-molecularly. Some of our results indicate that the selection of the immunodominant determinant (Luderitz *et al.,* 1966) out of the total range of possible groupings on an antigen is determined by genetic factors, and it is these which we now wish to discuss.

One of the systems we have most intensively studied in mice has been the response to $G_{60}A_{30}T_{10}$ (Maurer *et al.,* 1964). Previously, we had found this polymer to be a good immunogen in rabbits, where it closely resembled $G_{60}A_{40}$ in its response. GAT cross-reacted very well with GA antiserum (Table IX). However, with the rabbit anti-GAT serum, about 20% of the antibody would not react with GA, possibly because sequences which include tyrosine as well as glutamic acid and/or alanine are involved.

When a number of individual Swiss mouse ascites fluids produced following intraperitoneal immunization with GAT in complete adjuvant were studied, a completely different pattern of response was found (Table XXIII). None of the animals produced antibody which cross-

TABLE XXIII
SWISS MOUSE ANTI-GAT SERA PRECIPITIN CROSS REACTIONS

Serum No.	Anti GAT, Precipitated/ml (μg N)	Cross reaction	
		GA (%)	GT (%)
7/20	405	4	95
A3	232	6	108
B2	136	0	107
B6	324	5	110
8/3	435	35	79
B4	392	29	88

reacted strongly with GA and some antisera failed to show any cross reaction with this copolymer. However, all of the sera did show an excellent cross reaction with $G_{90}T_{10}$. Indeed, more antibody was precipitated by GT than by GAT from those sera which showed no GA cross reaction. This anomoly can be explained if one assumes that large amounts of soluble complexes are formed even in antibody excess, as we had, indeed, found previously with rabbit-anti-GAT (Maurer *et al.*, 1964). The response of some inbred mouse strains to GAT is seen in Table XXIV. The pattern of reactivity found is similar in that all of the sera reacted well with GT, while showing much poorer cross reactions with GA. The greater than homologous precipitation of antibody by GT was especially marked with some of these strains (CBA, BALB/C).*

TABLE XXIV
INBRED MOUSE STRAINS—ANTI-GAT PRECIPITIN CROSS REACTIONS

Strain	Anti GAT, Precipitated/ml (μg N)	Cross reaction	
		GA (%)	GT (%)
C57BL/6J	0	–	–
CBA/J	102	0	123
BALB/cJ	160	14	134
DBA/2J	249	16	82
C3H/HeJ	421	23	123
129/J	802	40	95

One of the strains, C57BL/6J, is noted as producing no precipitable antibody (Table XXIV). In "hyperimmune" animals, there is no antibody to GAT detectable by either hemagglutination or precipitin tests. After only two courses of adjuvant immunization this strain produces a very low titer of hemagglutinating antibody, which disappears rapidly. Similarly, CBA/J mice, after producing a fair antibody response (100 μg AbN/ml), respond to further boosting with reduced levels of antibody, the level falling first to 60 and then to 30 μg AbN/ml.

When the C57BL animals were immunized with a solution of GAT plus pertussis organisms (1 mg polymer and 10^9 dried, killed bacteria), and challenged as indicated with 1 mg of a solution of GAT intra-

*In some respects, there are differences between the Swiss and inbred strains, as in the C3H animals where there was both a cross reaction with GA and greater amount of antibody precipitated by GT than by GA. More detailed studies now under way, including studies with specific F_1 hybrids will, it is hoped, increase our knowledge of the details of these differences.

venously, they responded within 15 minutes as shown in Table XXV. This response is short lived, is induced only when pertussis is used as the adjuvant, and has no apparent correlation with hemagglutinating antibody. It thus seems to be similar to the "mast cell sensitizing" antibody, or reagin, which has been described in rats under similar circumstances (Mota, 1963; Binaghi and Benacerraf, 1964). This points up yet another aspect of the immune response, namely that different classes of antibodies may be differentially induced. Other strains, such as C3H, also form this "reagin," but, in addition, form antibody detectable by precipitation and other techniques.

TABLE XXV
PRODUCTION OF "REAGIN" TYPE ANTIBODY BY C57BL/6J MICE

Time of challenge[a] (days)	Anaphylactic reactions[b]	No. dead[c] / No. challenged
9	3/8	0/8
15	7/7	4/7
19	4/5	3/5
28	–	0/4

[a]Animals challenged with 1 mg solution GAT.
[b]Results during 15 minutes observation.
[c]Includes deaths occurring with 12 hours.

The response of mice to the terpolymer GLA_{40} provides some excellent examples of the genetic control of several aspects of the immune response. Figure 2 shows the passive hemagglutination titers of hyperimmune sera of individual mice of three inbred strains. Although all the sera agglutinated sheep cells coated with the homologous polymer, the sera from the three strains differed considerably in the mean titers. This is an expression of genetic control of the amount of antibody produced (Sang and Sobey, 1954; McDevitt and Sela, 1965). When these same sera were tested with sheep erythrocytes coated with GL, all of the C3H mice showed a cross reaction, but none of the C57BL or 129 strain animals did, even though the 129 strain produced more antibody than did the C3H mice.

Table XXVI shows the numerical values for the homologous and heterologous HA reactions of these strains, and of the specific F_1 hybrids. As expected, the latter mice all show the cross reaction with GL. The quantitative precipitin reactions also show the difference in antibody levels. More detailed studies of the specificity of the anti-GLA_{40} are presented in Table XXVII. It can be seen that the C3H mice react

quite well with polymers containing only small amounts of alanine (GLA_5, GLA_{10}) and essentially react completely with GLA_{20} and GLA_{30}. On the other hand, 129 and C57Bl/6 mice do not show a complete cross reaction even with GLA_{30}. Thus, we have the picture of different genotypes selecting similar, but different determinants in the same polymer.

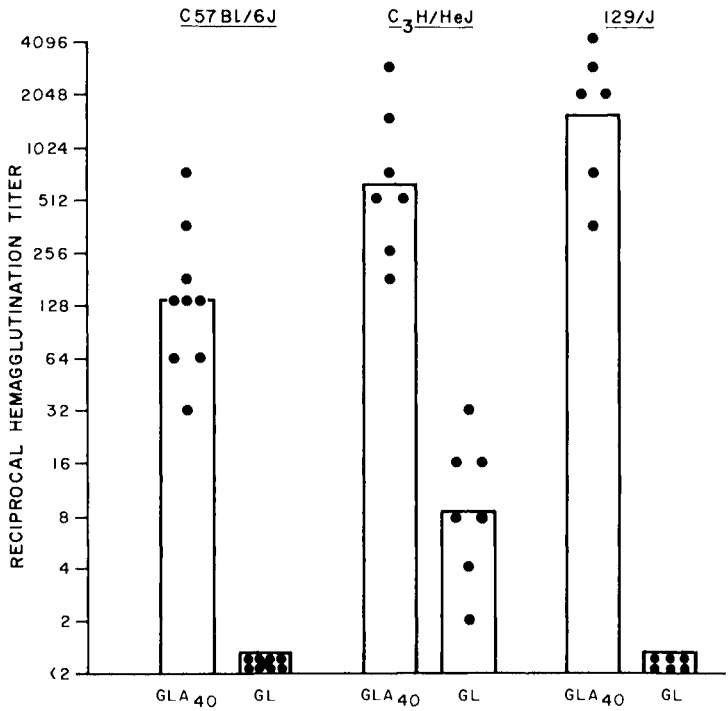

FIG. 2. Reciprocal titers of hyperimmune sera of inbred mouse strains immunized with GLA_{40}. Tanned sheep erythrocytes coated with GLA_{40} or $G_{60}L_{40}$. Height of bar represents mean titer, ● represents individual animal response.

Some of the questions which now remain to be answered in our studies include the possible role of differences in the nature of the antigenic fragments produced by the various strains, the nature of the differences in the antibody-combining sites, and the way in which certain of the antibody specificities the organism may make are selected from the considerable number of antibodies which can be made. This latter point has two aspects. First, the question of how different primary structures, i.e., amino acid sequences determined by DNA base sequences, are selected by antigen, and secondly, the relation of different antibody-binding sites to the different immunoglobulin classes.

TABLE XXVI
PASSIVE HEMAGGLUTININ REACTIONS OF INBRED MOUSE SERA TO GLA_{40}

Strain	Passive HA Titer[a]; Cells Coated With	
	GLA_{40}	GL
A. Parental		
C3H/HeJ	7/7 (640)	7/7 (10)
C57BL/6J	9/9 (128)	0/9
129/J	6/6 (2100)	0/6
B. Specific hybrids		
$(C3B6)F_1$	16/16 (48)	16/16 (9)
$(C3129)F_1$	8/8 (64)	8/8 (6)

[a]Number responding/Number tested. Values in parentheses refer to mean passive hemagglutination titer.

TABLE XXVII
CROSS REACTIONS OF INBRED MOUSE STRAIN ANTIBODY TO GLA_{40} WITH RELATED POLYMERS

Polymer	*Cross Reactions Strain*		
	C57Bl/6J (%)	C3H/HeJ (%)	129/J (%)
GL	0	6.8	0
GLA_5	17	28	17
GLA_{10}	49	69	40
GLA_{20}	71	91	65
GLA_{30}	80	95	77
GLA_{40}	100 (460)[a]	100 (1273)	100 (1510)
GLA_{60}	110	98	94

[a]Values in parentheses indicate micrograms antibody N per milliter serum precipitated by homologous antigens.

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DISCUSSION

DR. BENACERRAF: First I will consider some of the data you presented and the differences between what you observed in mice and what we observed in guinea pigs following immunization with hapten conjugates of poly-L-amino acid. In contrast with mice, guinea pigs were not able to make an immune response to the hapten unless they were able also to react to the carrier polymer. This has been observed in the case of DNP-poly-L-lysine, DNP-GL and DNP-LA. Another difference is that in contrast to the observation that homopolymers are generally nonantigenic, there is evidence that poly-L-lysine is recognized as an antigen by guinea pigs that possess the dominant gene which classifies them as responders. These are small differences and on the whole our observations are very similar. I would like to discuss also the significance of some of the results of the genetic studies made with polyamino acid conjugates. The crucial observation concerning this type of genetics is that it seems to govern the recognition of the carrier rather than that of the hapten. In order to explain these findings, two possibilities must be considered. According to the first one the carrier molecule would be recognized by some step previous to the recognition of the actual and total antigenic specificity of the determinant. This would involve, for instance, the formation of some essential inducer bearing the antigenic determinant through a metabolic step. This interpretation could also explain why nonantigenic homopolymers such as poly-L-glutamic acid or poly-D-amino acids can induce an immune response when they are combined with foreign proteins. The other possibility to be considered is to postulate that a portion of the carrier molecule is an essential part of the total antigenic determinant and is indeed involved in an important portion of the specificity of the antibody produced. More work is necessary to allow a choice to be made between these two hypotheses.

Another question that has to be raised at this time concerning the genetic observations which have been made on antibody synthesis is related to the exact significance of these findings. It has been clearly demonstrated with synthetic antigens possessing rather simple structures that some process of antigen recognition is clearly under genetic control and in some cases under the control of single dominant genes. This, however, does not allow us to state how this recognition is affected nor to conclude that this type of genetics controls also the sequence and structure of the immunoglobulins which are synthesized in response to these antigens. In order to demonstrate that the structure and synthesis of antibodies are indeed under strict genetic control, one would have to study the antibody population made by a given animal or a given strain in response to a well-defined haptenic determinant. An attempt must be made to explore whether in spite of the heterogeneity of the antibody population, definite patterns can be observed in the specificities of these antibodies with respect to their cross reactivity with related structures, which are distinctive of the individual or of the strain and to some extent inheritable. This is at present under investigation.

DR. PINCHUCK: Basically, I agree with you, but there are a few points I would like to make. First of all, when I said that GL does not induce an immune response, obviously I meant to say that it does not produce a certain detectable response. As shown in our paper, when an animal responds to a polymer like GLA_5 or GL tyrosine (GLT), almost all the antibody cross-reacts with GL, which itself is nonimmunogenic or only minimally so. Possibly these polymers cannot initiate a triggering process or processes because the association constant or other physicochemical characteristics of these compounds are not high enough to put in motion the physiological processes triggering an immune response. The

presence of a third group may carry the stimulus over a "threshold." The other point I would like to make concerns the differences in specificity which we have found in the GAT system and in other systems as well. Different genotypes reproducibly determine different patterns of specificity. We hope to learn whether this means that different genotypes have different base sequences specifying the amino acid sequence in the immunoglobulin combining site. Possibly the control of specificity does not necessarily exist on the basis of inheriting certain specific amino acid sequences. With some systems, especially GLA_{40}, there is a wide spread of complexity, and yet one can pick out definite differences between different inbred strains.

DR. GARSA: Recent studies [Pernis, Chiappino, Kelus, and Gell (1965) *J. Exptl. Med.* **122,** 853; Gell and Kelus (1966) *Nature* **211,** 766] seem to indicate the existence of a correlation between the type of allotype and the capability of producing antibody against a given antigen. Have you tried to find a similar correlation?

DR. PINCHUCK: In the GLA_{40} system, which is presented in detail in the paper, F_1 hybrids of responder and nonresponder strains respond to the antigen. Since you imply that the nonresponder strains have a tissue protein which has the same determinants as the antigen to which they do not respond, one would have to consider the fact that the F_1 hybrids would have protein of the parental type. I certainly think that this kind of factor could have an effect leading to tolerance. I do not think that this can be used to explain the facts observed. A rare example of cross reaction between a synthetic polypeptide and a natural substance is the reaction of rabbit anti-GLA_{30} lymph nodes with normal sheep erythrocytes which was observed in our laboratory by Dr. Walsh.

DR. FJELDE: I wonder whether you have looked at the lymphocytes in animals which are responders. Dr. Sorkin and I did some work with DNFB in Swiss mice, and about 10% of the animals became sensitized and had characteristic lymph node and skin changes. In lymphocytes from the peripheral blood of these mice we saw a mitogenic, and possibly also a mutogenic, response upon challenge in tissue culture with DNP-lysine (DNFB conjugated with lysine). There were abnormalities in the mitotic figures, some of them apparently arising from faulty spindle formation.

DR. PINCHUCK: We have not done any studies of that kind but I would no more expect responses to occur to these materials than to any other naturally occurring protein antigens.

DR. AMOS: Have you followed the survival of the substances in the circulation of these animals to make sure that they are not making an antibody which can combine but not precipitate? Have you followed the split products of these compounds, looking specifically for the presence of enzymes that may split them?

DR. PINCHUCK: We have not done immunoelimination studies. Drs. Benacerraff and Levine showed that there is no gross difference in the effects of spleen extracts from responder and nonresponder guinea pigs on poly-L-lysine. Some observations on the proteolytic degradation of these polymers are discussed in the paper.

DR. ADA: Have you carried out a dose response study using different doses of the antigen? This may be of particular interest in regard to the D-amino acid polymers in view of the discrepancy in the literature concerning their immunogenicity.

DR. PINCHUCK: We have not done these studies. Most of the materials used are relatively poor antigens and those that are reasonably good antigens, such as GLAT, are very complex antigenically.

DR. BENACERRAF: With the hapten poly-L-lysine system with which we have been working we can answer most of the questions of Dr. Ada and Dr. Amos. Responder animals which possess the gene are capable of responding to doses of DNP-PLL ranging from 1 μg to 1 mg, a thousandfold range, while nonresponding animals will not respond in this

dose range. Concerning the level of detections in this system, we used the sensitive technique of equilibrium dialysis to measure the antihapten antibodies formed. The level of binding of hapten by the globulin fraction of nonresponding animals was in the same range as that observed in the case of unimmunized control animals.

DR. PINCHUCK: The response to GLA_5 was similar in that the serum from nonresponder animals did not exhibit any more antigen binding capacity than normal serum.

Studies on Transplantation*

T. E. STARZL, T. L. MARCHIORO, Y. IWASAKI, N. KASHIWAGI

Department of Surgery, University of Colorado School of Medicine
and the Denver Veterans Administration Hospital,
Denver, Colorado

There is now ample evidence that homotransplantation of whole vital organs will be a useful and increasingly important method of therapy in the coming years. The attitude of gloom with which both physicians and basic scientists viewed this possibility only recently has been replaced by a sometimes unbridled optimism.

The principal reasons for this change have been the demonstrations that many uremic patients can be materially benefited by homotransplantation of the kidney, that rejection is an inherently and often easily reversible phenomenon, that the need for stringent immunosuppressive therapy tends to decrease with the passage of time, and that the patient with a really good result can live an essentially normal life.

I. PAST EFFORTS

The facts mentioned above became evident soon after the now widely used combination of immunosuppressive drugs was evolved. Using most importantly azathioprine and prednisone, and in some cases actinomycin C and local homograft irradiation, 64 patients were treated in Denver from November 1962 to March 1964, employing homografts from volunteer living donors. Today, now 2½ to almost 4 years later, exactly half of these recipients are still alive by virtue of their still chronically functioning kidneys. The principal mortality was during the first few postoperative months (Fig. 1), after which the chance of continuing survival was relatively good.

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There was a striking difference in the results, depending upon the source of the homograft. Forty-six of the foregoing recipients received kidneys from consanguineous donors; 29 (63%) are still alive. In contrast, only 3 of 18 patients who received nonrelated kidneys survive to date (Fig. 2).

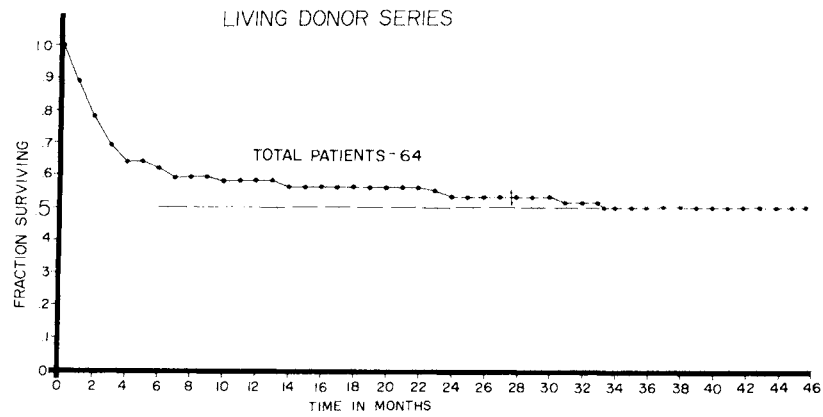

FIG. 1. Life survival curve of the first sixty-four patients who received renal homotransplants from November 1962 to March 1964. All donors were living volunteers. The recipients now have been followed for 2½ to almost 4 years. Exactly half of the patients were still alive on September 26, 1966.

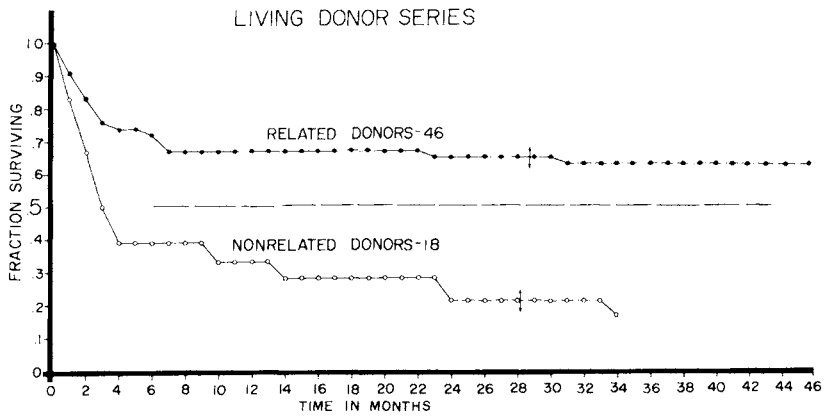

FIG. 2. A breakdown of the results shown in Fig. 1 according to the source of the homograft. Note that 63% of the patients who had consanguineous donors are still alive compared to only 3 of 18 who received nonrelated kidneys.

This experience was accumulated at a time when the clinical problems which were to be encountered were either understood poorly or not at all.

Nevertheless, the substantial salvage rate obtained has proved in principle the feasibility of the undertaking. Furthermore, these, as well as similar results from other institutions (Hamburger *et al.*, 1965; Hume *et al.*, 1966; Murray *et al.*, 1965; Straffon *et al.*, 1966), have defined areas into which intensive effort has already been invested.

II. CAUSES OF FAILURE

The vast majority of deaths after renal homotransplantation are more or less directly caused by the immunosuppressive agents used whether this occurs early, at an intermediate time, or late. In turn, the toxicity of the various agents is related to the difficulty with which rejection is controlled.

A pertinent example is shown in Fig. 3. The patient, who received a

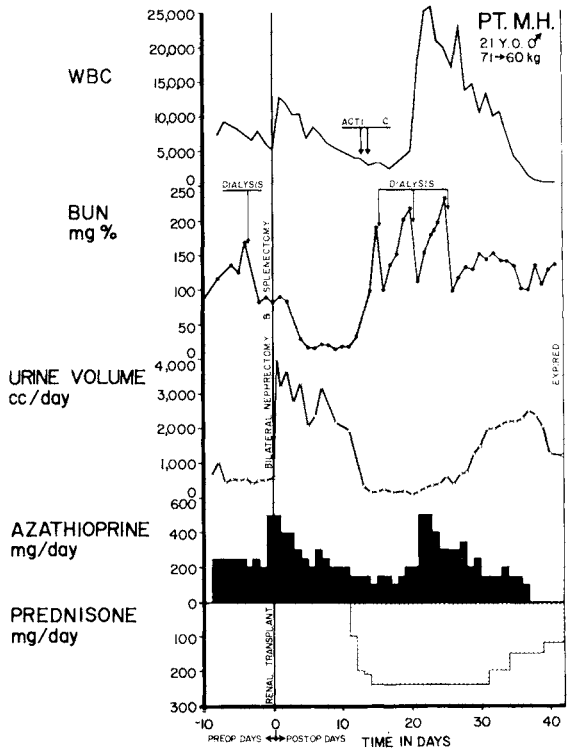

FIG. 3. Typical unsuccessfully treated case. The donor and recipient were brothers, both of A+ blood type. A violent rejection crisis followed good early function, and anuria developed which lasted 2 weeks. Although the rejection was reversed and a secondary diuresis began, the patient died from drug toxicity, leukopenia, and septicemia. Acti C, each arrow is 200 μg intravenous actinomycin C. (By permission of *Surgery* **56,** 296, 1964.)

kidney from his brother, had good renal function for more than week. Severe rejection then ensued, causing temporary renal shutdown, but with maximum immunosuppression, urinary output resumed. In spite of this temporary improvement, the patient died shortly afterward of fungal and bacterial sepsis which were primarily the consequence of bone marrow depression. The responsible drug was azathioprine. It has since been appreciated that the dose of this agent must be reduced during periods of depressed urine excretion inasmuch as the drug has an important renal pathway of detoxification. Dose control is extremely difficult under these circumstances.

With increased experience, such deaths from acute bone marrow depression have become rare, and it seems probable that azathioprine is the safest of the immunosuppressive drugs being used for chronic therapy. When azathioprine can be used as the sole therapeutic agent, infectious complications or other morbidity are negligible. Unfortunately, this is frequently not possible. It is often found that continued homograft function can be maintained only with the additional use of substantial quantities of prednisone. It is the need for high dose steroid therapy which most severely limits the life expectancy of any individual patient.

The tragic consequences which may ensue during steroid therapy, either early or late, are illustrated in Fig. 4. This patient, who had a severe late rejection almost a year after operation, needed large doses of prednisone to prevent further deterioration of homograft function. As a consequence, he developed typical cosmetic deformities, bone demineralization, a duodenal ulcer, pancreatitis, and parenchymal liver disease. Until the time of death, his renal function was adequate to sustain life in a normal person. At autopsy, he had pneumonitis and infestation of several organ systems with cytomegalic inclusion virus. Such patients often become infected with fungi or bacteria which ordinarily have a low grade pathogenicity.

III. HISTOCOMPATIBILITY MATCHING

Since toxicity from immunosuppression accounts for a heavy mortality, usually because large doses are mandatory, intensive efforts are being made in several centers to sharpen the criteria by which a donor is decided upon for any given recipient. With proper selection, it would be expected that the need for potentially lethal immunosuppressive measures could be at least partially eliminated. This approach has been evaluated in collaboration with Dr. Paul Terasaki of Los Angeles, whose method of lymphocyte analysis appears to measure something which is at least related to histocompatibility antigens (Porter *et al.,* 1966; Terasaki *et al.,* 1966).

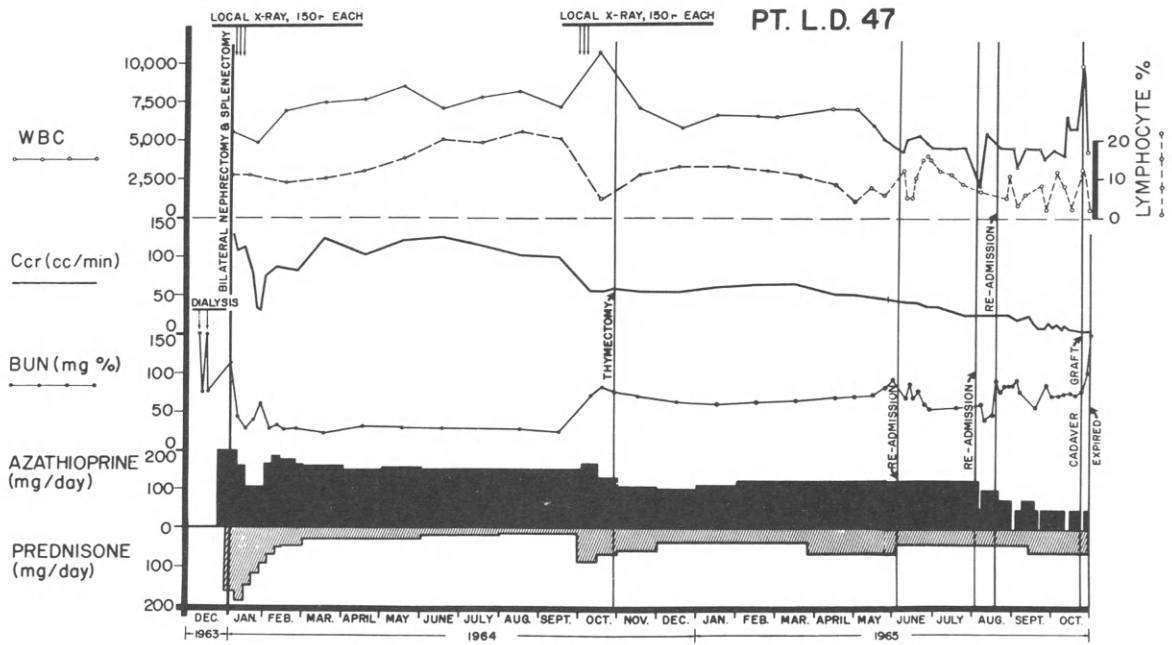

FIG. 4. Course of a 37-year-old man who received a kidney from his younger brother. Both were A+ blood type. Note the severe late rejection after 9 months and the subsequent slow deterioration of renal function. The late thymectomy did not induce either lymphopenia or make easier the subsequent management; the postthymectomy changes in lymphocyte counts were related to adjustments in steroid dosage. (From Terasaki *et al.*, 1966.)

The fate of the first 26 patients for whom donors were chosen by Terasaki on the basis of the best available antigen match is shown in Fig. 5. The transplantations were between blood relatives in half the cases, and between nonrelated pairs in the other half. After a follow-up of 12 to 22 months, the results were exactly the same in the two subgroups. The upgrading was expected in the unrelated subgroup if the test had any discriminatory capability. Failure to increase survival in recipients of related kidneys was a disappointment. The drug toxicity described earlier was still the chief cause of death in both groups. It became clear that the high mortality could not be eliminated even with effective typing procedures without the evolution of better immunosuppressive treatment.

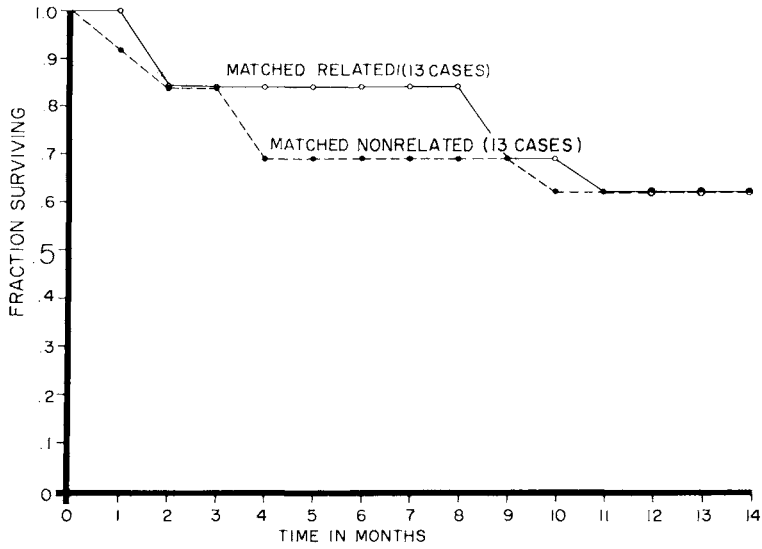

FIG. 5. Results of renal transplantation in a more recent series of patients whose donors were selected by Terasaki's antigen matching method. Note that survival after 13–22 months was exactly the same in both the related and nonrelated cases. Compare these results with those shown in Fig. 2.

IV. IMPROVED IMMUNOSUPPRESSION

The most promising new treatment we have used is antilymphoid serum, raised in a heterologous host by immunization with lymphoid tissue from the species to be eventually treated. Such a product was described by Metchnikoff in 1899 and by numerous others in the ensuing 60 years. Its use to potentiate homograft survival was first suggested by Woodruff (1960; Woodruff and Anderson, 1964) and Waksman *et al.*

(1961). The value of antilymphoid serum for this purpose has been established in a number of subsequent studies (Gray *et al.*, 1964; Jeejeebhoy, 1965; Levey and Medawar, 1966a,b; Monaco *et al.*, 1965, 1966; Nagaya and Sieker, 1965).

In our laboratories, antilymphoid derivatives have been developed for use in both dogs and man (Iwasaki *et al.*, 1967). Horses were subcutaneously immunized with lymphocytes from the spleen, lymph nodes, or thymus of the appropriate species. The equine leukoagglutinin and lymphoagglutinin titers rose to as high as 1:16,000 (Fig. 6). The horse serum which was toxic in its raw state was absorbed with pooled red cells and serum of the species which provided the immunizing tissue.

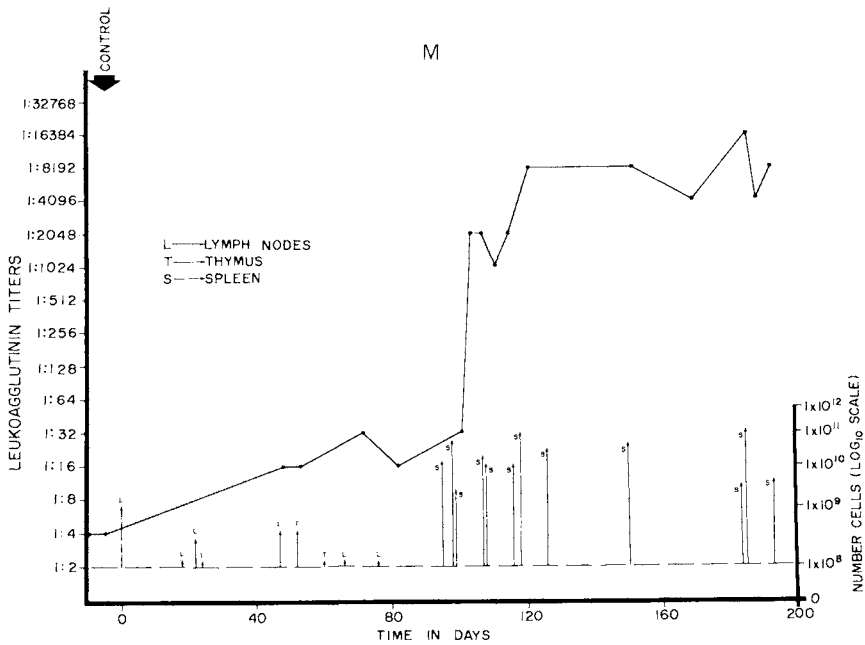

FIG. 6. Effect of immunizing dose upon the leukoagglutinin titer of a horse inoculated with cadaveric human lymphoid tissue. Note that the rise in titer was very modest during the first 3 months, during which time small doses of cells were used. When the quantity of antigen was increased by the use of spleen cells, abrupt increases in titer were observed within a few days. (From Iwasaki *et al.*, 1967.)

Next, studies were conducted to localize the desired antibody (Iwasaki *et al.*, 1967). By separation with the DEAE cellulose column, and by determination of the leukoagglutinating titers and electrophoretic characteristics of the various eluates, it was found that the antiwhite cell antibody was chiefly in γ-globulin with lesser activity in β-globulin (Fig.

7). These constituents could easily be removed by double or quadruple precipitation with 0.4 saturation ammonium sulfate (Fig. 8).

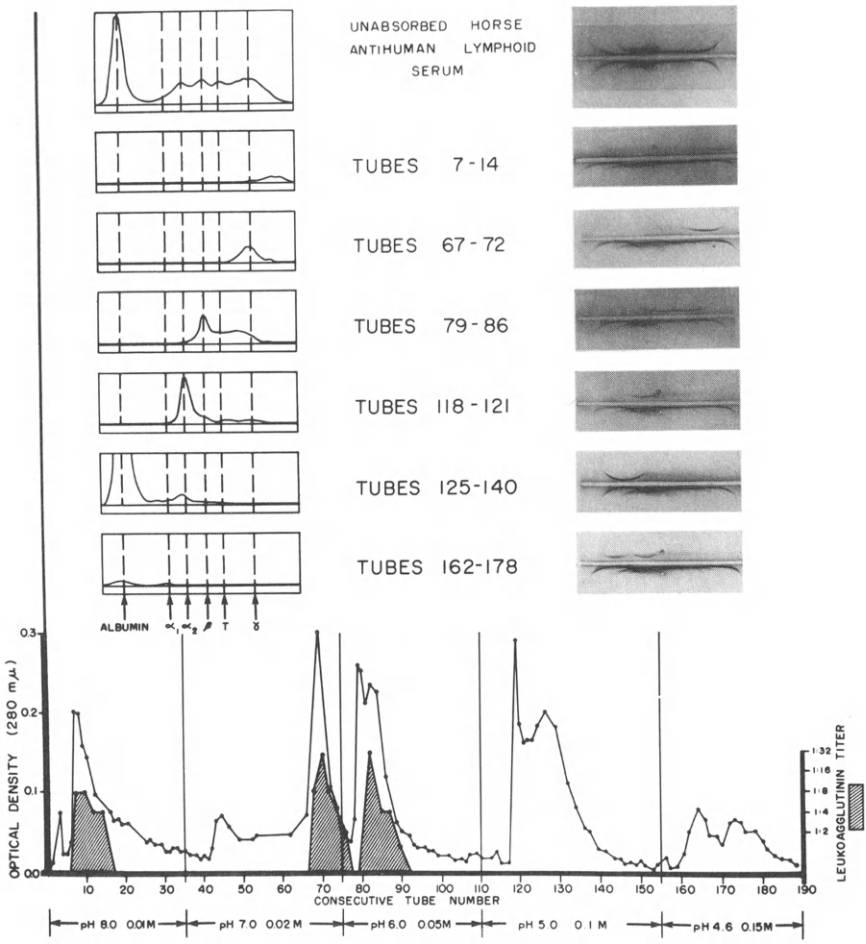

FIG. 7. Studies of the leukoagglutinin-containing fractions in antihuman-lymphoid serum employing column chromatography, electrophoresis, and immunoelectrophoresis. The various eluates from the DEAE cellulose column were analyzed spectrophotometrically for protein content (expressed as optical density), and the presence or absence of leukoagglutinins determined for each collection tube. The electrophoresis and immunoelectrophoresis permitted relatively complete classification of the active immunoglobulins. (From Iwasaki *et al.*, 1967.)

Ammonium sulfate-precipated antidog-lymphoid globulin has been extensively studied in the laboratory (Iwasaki *et al.*, 1967; Starzl *et al.*, 1967). When administered subcutaneously as the sole therapy, it pro-

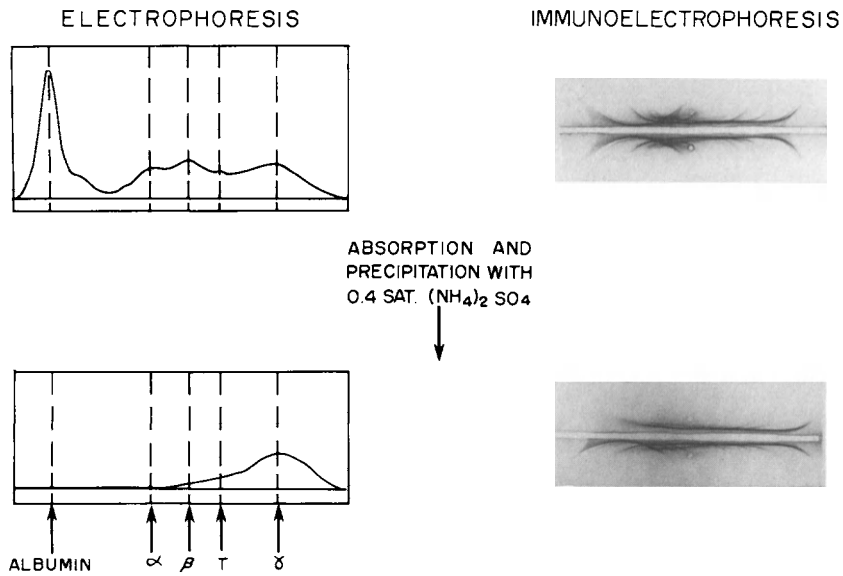

FIG. 8. Electrophoresis and immunoelectrophoresis of absorbed antihuman-lymphoid serum and the protein obtained from it by four precipitations with 0.4 saturated ammonium sulfate, four dialyses and lyophilization. The final product, which was used clinically, consists almost entirely of γ G globulin. (From Iwasaki *et al.*, 1967.)

longed the survival of either kidney or liver homografts (Fig. 9) only slightly less than azathioprine (Starzl *et al.*, 1967). There was little overt toxicity although a substantial fraction of either control or transplanted kidneys developed electron-dense bodies in the glomeruli (Iwasaki *et al.*, 1967) which did not cause a demonstrable reduction in function. The studies indicated that the immune globulin was relatively safe, at least for short-term therapy, but that it was not potent enough to warrant use as the sole method of immunosuppression.

For clinical trial, antihuman-lymphoid globulin was therefore combined with the older standard drugs, azathioprine and prednisone (Starzl *et al.*, 1967). As in the past, a conscious effort was made to use as little prednisone as possible. Eight patients were treated from 9 weeks to 3 months ago. Their renal function, drug doses, and peripheral hematologic patterns during the first 9 postoperative weeks were then compared to those of three groups of previously treated recipients in which the homograft sources had been comparable.

The results are shown in Fig. 10. During the period in question, only 46% as much prednisone was required as for any of the preceding series. The renal function was comparable. The peripheral lymphocytes were

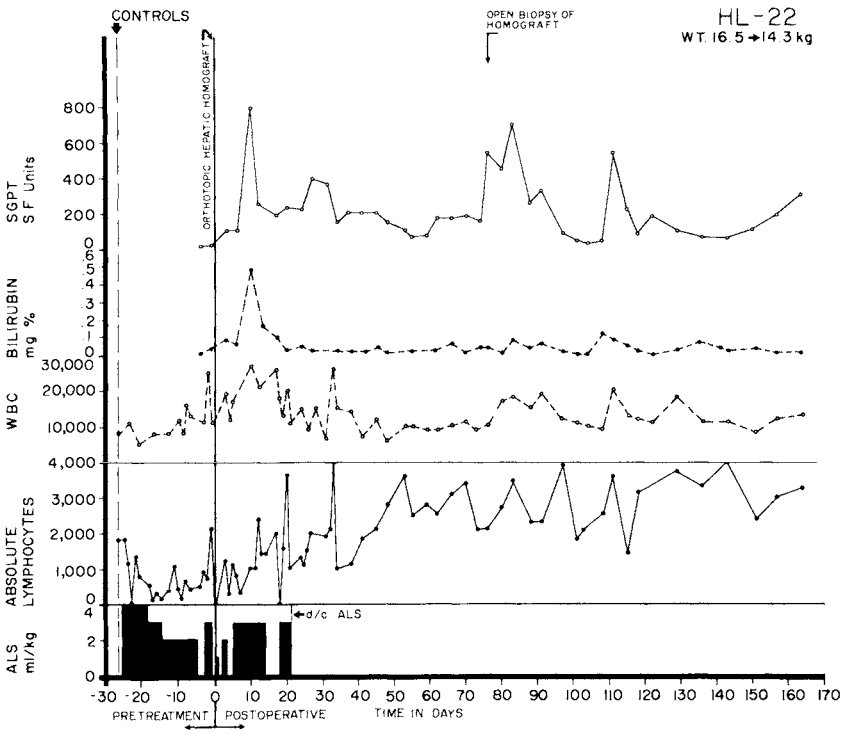

FIG. 9. Course of a dog after orthotopic liver transplantation. The donor and recipient were nonrelated mongrel animals. The only immunosuppression used was horse antilymphoid serum given intraperitoneally. This treatment was stopped 3 weeks postoperatively and the animal has lived for more than 5 subsequent months during all of which time the peripheral lymphocyte count has been high. (From Starzl *et al.*, 1967.)

little changed, a finding which is consonant with the brilliant studies of Levey and Medawar (1966a,b) who have shown that the immunosuppressive effect is not dependent upon either lymphoid depletion or lymphopenia. All eight patients are clinically well.

The aforementioned studies are purely investigational. Whether long-term toxicity, including foreign protein injury to the homograft itself, will occur, can only be determined with longer follow-up. These data do indicate, however, that the possibility of better immunosuppression is not just a mirage.

V. OTHER ORGANS

Clinically, only the kidney has been transplanted successfully to date. It seems inevitable that other organs will follow. In our laboratories, dogs

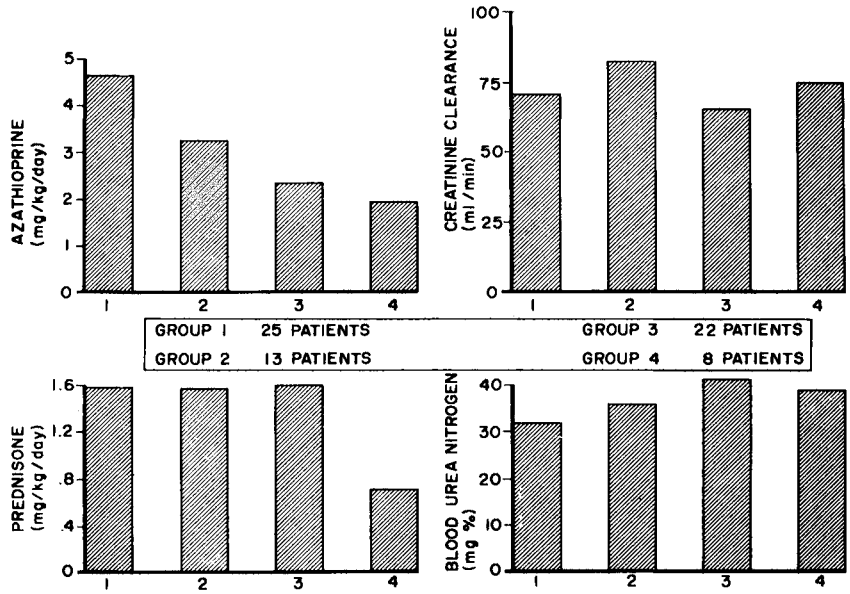

FIG. 10. Variations in immunosuppression and renal function during the first 63 post-operative days in four successive groups of patients who received kidneys from blood relatives.. Since the blood urea nitrogen and creatinine clearance were not determined each day, these were compiled on a weekly basis. Those in series 4 received adjuvant therapy with antilymphoid globulin. Note the drastic reduction in average prednisone dose which was achieved in these patients without significant loss of renal function. (From Starzl *et al.*, 1967.)

are alive as long as 2½ years after complete hepatectomy and replacement with a homograft from a nonrelated mongrel donor (Starzl *et al.*, 1965). In our experience, this kind of chronic survival can be achieved more often with hepatic than with renal homografts. Other workers have had long-term success with cardiac and pulmonary homotransplantation.

VI. SUMMARY

Results with clinical renal homotransplantation have been reviewed. The patterns of mortality have been cited as these relate to research in histocompatibility typing and in the search for better immunosuppressive regimens.

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DISCUSSION

DR. BERENBAUM: Have you any information on the susceptibility to infections of dogs or patients treated with antilymphocyte sera as compared with conventionally treated cases?

DR. STARZL: We do not have a precise quantitative evaluation in this respect as we do in patients with Imuran and prednisone. We have had no infections in patients treated with immune globulin and, in animals, infections have not been a prominent cause of failure. The patients are extraordinarily well off after treatment with antilymphoid globulin given in combination with azathioprine and small doses of steroids.

DR. AMOS: What is really known about the physiological adaptation to immunosuppressive drugs? The patients starting off on azathioprine are often in a very unstable position from the beginning. As you pointed out, in dogs one can stop drug treatment at a certain time after grafting, and an adaptation seems to occur between the transplant and the host. There may be also an adaptation of the host to the drug, and if we knew exactly what the individual drug requirements of a patient were, I think this might help in reducing initial mortality.

DR. STARZL: Yes, it would help very much, because there is a great individual difference. Some patients can tolerate only 0.5 mg/kg and some 4 or 5 mg/kg.

DR. BACH: It was encouraging to see the survival of kidneys from unrelated donors matched by leukocyte typing as compared to the survival of those from related donors that

you observed. I wonder how easy it was to find the unrelated donors who matched to the degree that they were matched.

DR. STARZL: The results obtained earlier with randomly selected related donors were quite good and were not significantly improved by matching according to leukocyte typing. Very often, within the related group, there is not a large number of people from whom to select and therefore the quality of the match cannot be significantly improved by leukocyte typing. Also, within a family, the number of really bad matches is relatively small. In a nonrelated population, however, the chances of getting a bad match are exceedingly high. These may be the reasons why in nonrelated groups, but not in related groups, a significant improvement in the matching could be achieved following the criteria of leukocyte typing.

DR. AMIEL: I would like to comment on the possibility of successfully selecting a donor to match the prospective recipient by phenotyping leukocyte antigens. Experiments in mice performed in our laboratory showed that, when bone marrow cells from more than one donor are transplanted into one recipient, those from the most compatible donor prevail and can be recovered some weeks after grafting. In a clinical case, four members of the family of a prospective recipient (father, mother, and two brothers of the mother) and two unrelated donors chosen from a list of about fifty volunteers on the basis of leukocyte typing (agglutination and cytotoxicity) served as bone marrow donors. Two months later, it was found that the successful graft originated from one of the two unrelated donors. This observation indicates the validity of matching by leukocyte typing. As a second point I would like to mention that sometimes rejection crises of the kidney can be completely overcome in the absence of treatment.

DR. STARZL: I appreciate very much hearing about the clinical case you mentioned. Concerning your second point, I think that one would not often see spontaneous reversability of rejection crises if it were not for the basic therapy with immunosuppressants. Recently, we did orthotopic liver transplantations in about 100 dogs using azathioprine as the sole method of therapy, and we found that one half of the animals went through a crisis in which serum bilirubin values went up and then came down spontaneously without the addition of further therapy. It would seem that rejection of transplanted organs is characterized by spontaneous remissions and exacerbations.

DR. WITZ: Dr. Starzl, I wonder whether immunosuppression is the only possible mechanism for the effects of antilymphoid antisera on kidney transplantation. Is it possible that a phenomenon similar to passive immunological enhancement, as described by Kaliss, might occur in your system?

DR. STARZL: I do not know the mechanism of the antilymphoid serum. Lymphopenia was not seen in a dog bearing a long-living liver graft. In some experiments antilymphoid globulin was given deliberately at such low levels that it had no lymphopenic effect, and yet definite therapeutic results were obtained. In most animals treated with the serum, the host spleen and lymph nodes were actually hyperplastic. Therefore the notion that the antilymphoid serum must cause lymphopenia and lymphoid atrophy to be effective requires revision.

DR. AMOS: The same sort of paradox is represented by the lack of correlation between lymphopenia and drug-induced immunosuppression.

DR. HOLLAND: Dr. Starzl, renal donation is not without hazard to the donor. Has any technique of genetic matchings been helpful in improving cadaver kidney usefulness?

DR. STARZL: A dialysis center could be established at which a large number of patients could be typed, who could then be matched if a cadaveric donor became available. This is just a converse of what most centers are doing at present in which a recipient is matched against a panel of donors. There are patients who have a great number of white cell antigens

and who are comparable, in the context of blood transfusion, to patients of AB blood type. They are "universal tissue recipients." We have done several cadaveric transplants starting 6 to 12 months ago into patients who are "universal recipients." Of these, only one has died.

DR. REGELSON: In view of the fact that lymphohyperplasia occurs despite the immunosuppressive treatments applied, have agents which produce lymphocytosis, for example, pertussis vaccine or thyrotropic hormone or thyroid hormones been tried? These could be tried in association with immunosuppressive drugs in attempts to see whether the lymphoid cells are still potentially functional or whether an increased immunosuppressive effect can be obtained.

DR. STARZL: It has been suggested by Dr. Medawar that the mechanism of antilymphoid globulin may be similar to that of phytohemagglutinin, and that what the globulin does is to send the lymphoid tissue off "on a wild goose chase" with a lot of activity and no specificity.

DR. TURK: Have you seen any signs of serum sickness in patients treated with horse antiserum? Did any of these patients develop eosinophilia?

DR. STARZL: Some of the patients developed eosinophilia. We studied quantitatively the response of patients to horse proteins by determining the antihorse protein precipitin titers which rose from 1:2 to 1:16, which was the highest level seen. On a few occasions the antibody titer returned to normal levels during treatment. Hemagglutinin titers against sheep red cells rose to levels as high as 1:512 in patients who had no other sign of toxicity. The clinical toxicity seen included a local reaction at the site of serum injection which tended to be much more severe on the first than on subsequent injections. Out of twelve patients, one developed hives once, another became acutely hypertensive for about 30 minutes, and a third one became hypotensive for about 10 or 15 minutes and developed air hunger. All these patients have subsequently received globulin on a number of occasions without recurrence of these effects.

DR. TURK: The rise of antibody against sheep red cells observed by Dr. Starzl in patients treated with horse antisera might be due to Forssman antigen which may be present in horse globulin as well as on sheep cells.

DR. MILGROM: Some explanation may be offered for the graft disease that Dr. Starzl described, namely, the lesions within the grafted, nonrejected kidney. I think that immunosuppressive drugs save the graft from rejection but do not suppress significantly the formation of humoral antibodies. These humoral antibodies, directed against transplantation antigens, cannot be detected in the circulation since they are removed by the kidney. This was quite clearly demonstrated in dogs in which the humoral antibody was completely removed within hours after they were regrafted with the kidney from the original donor. Also, in humans we could not observe humoral antibodies as long as the graft was present, but we detected them after surgical removal of the graft. It seems quite plausible that the humoral antibodies directed against transplantation antigens act as Masugi's antibody in combining with antigen within the grafted kidney and inducing pathologic changes. Another point I would like to make is in connection with the use of heteroimmune sera which contain antibodies against human antigens. As Dr. Starzl demonstrated, these antibodies are not lymphocyte specific. They are just directed against man, as a species, and they combine with antigens on the cell surface. This type of antibody, from what is known, is a rather dangerous tool. I am far from criticizing Dr. Starzl's attempts, because without having anything better, he seems to be justified in trying this tool. I wonder whether this antilymphocytic serum does anything besides causing a depletion of lymphocytes. One could achieve a depletion of cannulation of lymphatics without running into the risks deriving from the use of sera which may be quite toxic for the recipient.

DR. STARZL: I do not think one could achieve with lymphocyte depletion the same effect as that seen with the antilymphoid serum. I believe that the remarks you made on another

occasion deserve repeating, namely, that if the antiserum has any degree of specificity at all it may be not because of immunologic specificity but because the lymphocyte represents a spectacular target. In another connection I would solicit Dr. Milgrom's comments about the humoral antibodies directed against the graft which have been found in almost all our patients after renal homotransplantation.

DR. MILGROM: I am aware of Dr. Starzl's attempts to find humoral antibodies which are primarily being detected by using sheep erythrocytes in antiglobulin consumption techniques. Most likely, differences in procedures account for different experimental results.

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