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Volume 655

Pharmaceutical Biotechnology

Edited by
Carlos A. Guzmán
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DEDICATION

To Michela Morgana and Alessia Federica

PREFACE

Pharmaceutical Biotechnology is a unique compilation of reviews addressing frontiers in biologicals as a rich source for innovative medicines. This book fulfills the needs of a broad community of scientists interested in biologicals from diverse perspectives—basic research, biotechnology, protein engineering, protein delivery, medicines, pharmaceuticals and vaccinology. The diverse topics range from advanced biotechnologies aimed to introduce novel, potent engineered vaccines of unprecedented efficacy and safety for a wide scope of human diseases to natural products, small peptides and polypeptides engineered for discrete prophylaxis and therapeutic purposes. Modern biologicals promise to dramatically expand the scope of preventive medicine beyond the infectious disease arena into broad applications in immune and cancer treatment, as exemplified by anti-EGFR receptors antibodies for the treatment of breast cancer. The exponential growth in biologicals such as engineered proteins and vaccines has been boosted by unprecedented scientific breakthroughs made in the past decades culminating in an in-depth fundamental understanding of the scientific underpinnings of immune mechanisms together with knowledge of protein and peptide scaffolds that can be deliberately manipulated. This has in turn led to new strategies and processes. Deciphering the human, mammalian and numerous pathogens' genomes provides opportunities that never before have been available—identification of discrete antigens (genomes and antigenomes) that lend themselves to considerably improved antigens and monoclonal antibodies, which with more sophisticated engineered adjuvants and agonists of pattern recognition receptors present in immune cells, deliver unprecedented safety and efficacy. Technological development such a nanobiotechnologies (dendrimers, nanobodies and fullerenes), biological particles (viral-like particles and bacterial ghosts) and innovative vectors (replication-competent attenuated, replication-incompetent recombinant and defective helper-dependent vectors) fulfill a broad range of cutting-edge research, drug discovery and delivery applications. Most recent examples of breakthrough biologicals include the human papilloma virus vaccine (HPV, prevention of women genital cancer) and the multivalent Pneumoccocal vaccines, which has virtually eradicated in some populations a most prevalent bacterial ear infection (i.e., otitis media). It is expected that in the years to come similar success will be obtained in the development of vaccines for diseases which still represent major threats for

human health, such as AIDS, as well as for the generation of improved vaccines against diseases like pandemic flu for which vaccines are currently available. Furthermore, advances in comparative immunology and innate immunity revealed opportunities for innovative strategies for ever smaller biologicals and vaccines derived from species such as llama and sharks, which carry tremendous potential for innovative biologicals already in development stages in many pharmaceutical companies. Such recent discoveries and knowledge exploitations hold the promise for breakthrough biologicals, with the coming decade. Finally, this book caters to individuals not directly engaged in the pharmaceutical drug discovery process via a chapter outlining discovery, preclinical development, clinical development and translational medicine issues that are critical the drug development process.

The authors and editors hope that this compilation of reviews will help readers rapidly and completely update knowledge and understanding of the frontiers in pharmaceutical biotechnologies.

Carlos A. Guzmán, MD, PhD
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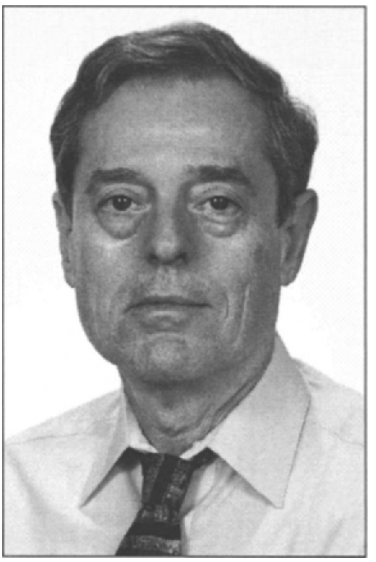

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CONTENTS

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We would also like to express our deep acknowledgement to all contributors, without your engagement this book would have never come to life. We are indebted with you for sharing your knowledge, experience and insights. Finally, we would like to thank the staff of Landes Bioscience for your outstanding support during this enterprise, in particular to Cynthia Conomos, Celeste Carlton and Erin O'Brien; any mistakes are ours—you did a terrific job.

Chapter 1

Translational Medicine—A Paradigm Shift in Modern Drug Discovery and Development:
The Role of Biomarkers

Mark Day, J. Lynn Rutkowski and Giora Z. Feuerstein*

Abstract

The success rate of novel medical entities that are submitted for registration by the regulatory agencies and followed successful marketing has been stagnating for the past decade. Failure in efficacy and safety continue to be the prime hurdles and causes of failure. Translational medicine is a new function within the pharmaceutical industry R&D organization aimed to improve the predictability and success of drug discovery and development. Biomarkers are the essence of the translational medicine strategy focus on disease biomarker, patient selection, pharmacodynamic responses (efficacy and safety) target validation, compound-target interaction). Successful deployment of biomarkers research, validation and implementation is adopted and embraced as key strategy to improved the drug discovery and development towards new medical entities.

Drug Targets—Historical Perspectives

Drugs are natural or designed substances used deliberately to produce pharmacological effects in humans or animals. Drugs have been part of human civilizations for millennia. However, until the very recent modern era, drugs have been introduced to humans by empiricism and largely by serendipitous events such as encounters with natural products in search of food or by avoiding hazardous plants and animal products. The emergence of the scientific era in drug discovery evolved along-side the emergence of physical and chemical sciences at large, first as knowledge to distill, isolate and enrich the desired substance from its natural environment, followed by deliberate attempts to modify natural substances to better serve the human needs and desires.

Scientific evolution throughout the past two centuries enabled identification of biologically active substances in humans (e.g., hormones), which were manipulated chemically to improve (potency, duration of action and exposure), or to mitigate or abrogate undesirable actions. The cumulative knowledge of human, animal and plant biology and chemistry provided the scientific foundation and technical capabilities to purposely alter natural substances in order to improve them. Such evolution marked the era of "forward pharmacology". The era of forward pharmacology is about drug design that emanates from primary knowledge of the action of the biological target that has clear biological action.

The exponential progress in molecular biology since the mid-20th century, culminating in deciphering the complete human genome in the year 2000, brought the dawn of pharmacogenomics

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and the "reverse pharmacology" era. The "reverse pharmacology" era is defined by the need to first clarify the biology and medical perspectives of the target so as to qualify it as a drugable and pharmaceutically exploitable for drug discovery and development scheme. The pharmacogenomic era provides vast opportunities for selection of new molecular targets from a gamut of approximately 30,000 primary genes, over 100,000 proteins and multiples of their translational and metabolomics products. Thus, the permutations in respect to opportunities for pharmacological interventions are unprecedented, vast and most promising for innovative medicines.

The pharmacogenomics era as a source for drug targets also poses unprecedented hurdles in selection, validation and translation into effective and safe drugs. New technologies continue to drive efficiency and robustness of mining the genomic drug discovery opportunities but physiological and integrated biology knowledge is lagging. In this perspective, translational medicine and biomarkers research have taken center stage in validation of the molecular target for pharmaceutical exploitation.

In this chapter we offer a utilitarian approach to biomarkers and target selection and validation that is driven by the translational medicine prospect of the target to become a successful drug target. We hereby offer classification and analytical process aimed to assess risk, innovation, feasibility and predictability of success of translating novel targets into successful drugs. This manuscript provides clear definitions on the type of biomarkers that are core to translational medicine and biomarkers research in modern pharmaceutical companies.

Translational Medicine: Definition

Translational medicine in the pharmaceutical industry is a research discipline aimed to improve the predictability of success of drug discovery and development. Translational medicine research aims to discovery, validate and implement biomarkers in lieue of clinical outcome studies, improve the congruency of preclinical models to clinical reality and establish proof of concept for efficacy and safety based on targeted mechanism of action. In particular, translational medicine aims to establish surrogate biomarkers to aid in early registration and promote personalized medicine for better patients selection for targeted mechanism of action.

Biomarkers—Utilitarian Classification

Biomarkers are the stepping-stones for modern drug discovery and development.[1-4] Biomarkers are defined as biological substances or biophysical parameters that can be monitored objectively and reproducibly and used to predict drug effect or outcome. This broad definition is however, of little utility to the pharmaceutical process since it carries no qualification for the significance and use of the biomarker. The following classes and definitions of biomarkers are therefore offered (see Fig. 1):

I. **Disease Biomarkers**: disease biomarkers are biomarkers that correlate statistically with the disease phenotype (syndrome) for which therapeutics are developed. Correlation of levels (in the circulation, other fluids or tissue) or expression patterns (gene, protein) in peripheral blood cells or tissues should signify disease initiation, progression, regression, remission or relapse. When we apply these criteria to our empirical approach to current strategies to develop drugs for certain diseases, it becomes apparent that our current approaches employed in clinical testing are sub-optimal. One pertinent example is provided by the way industry has approached the development of treatments in schizophrenia.

   a. ***Disease Initiation Misconceptions***: Unfortunately in the past 50 years all marketed therapies have been developed around the dopamine D2 receptor, either in the form of full antagonism or partial agonism. These treatments are only effective on the positive symptoms in around 70% of patients and are associated with treatment resistance and poor side effect profiles. Current clinical practice and drug discovery is based around the concept that the onset of positive symptoms represents the initiation of the disease. Arguably, however, these symptoms arrive late in the chapter of schizophrenia. The focus on the positive symptoms has impeded the development of novel therapeutics

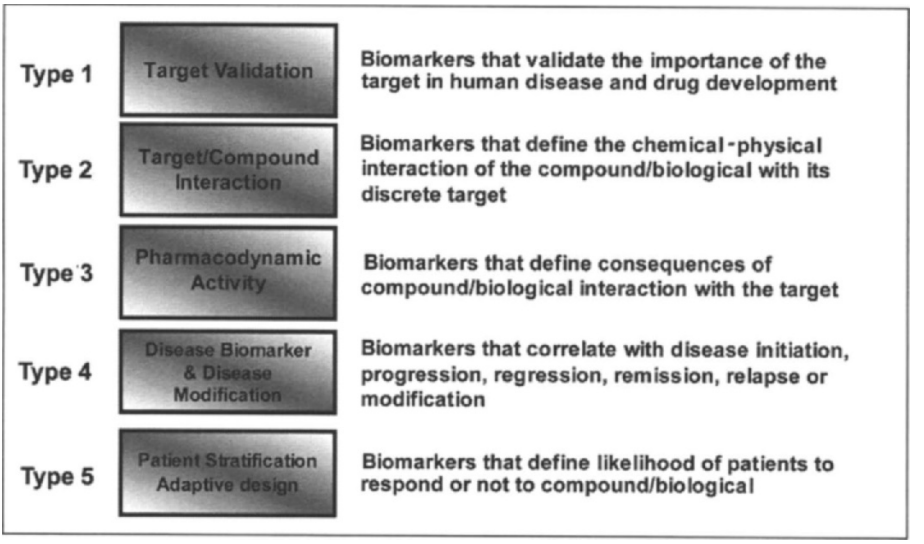

Figure 1. Utilitarian classification of biomarkers types 1-5

driven by an under appreciation of the disease processes. Translational medicine focuses on disease biomarkers and brings new focus and hypotheses to the drug development process. For example, cognitive symptoms manifest prior to positive ones. We now know that individuals who are at risk of becoming schizophrenic manifest, in early adolescence, clear cognitive deficits often associated with low IQ. Some, but not all, of those individuals will go on to manifest positive symptoms (hallucinations, delusions, paranoia).

b. ***Remission***: A second issue is that the treatments that are used to control positive symptoms do not improve functional outcome. In contrast, attenuation of the cognitive deficits do predict functional outcome and in some cases lead to patient rehabilitation into the work place.[6]

c. ***Relapse***: Relapse is associated with thought disorder and cognitive disorganization. As such, cognitive endpoints are seen early on in life and can be seen as an early milestone in the initiation of the disease, worsens when the positive symptoms appear and remission of positive symptoms with improved cognitive function tracks with augmented rehabilitation and functional outcome. Therefore, as cognitive endpoints track more readily with initiation, progression, remission and relapse in schizophrenia it fulfills all the criteria in the disease biomarker definition.

In addition, the duration of aberrantly expressed biomarkers could also be associated with risk for disease even if the level of the biomarker does not change over time. In schizophrenia, this is typified by the fact that these at risk individuals also show sensory gating deficits (e.g., prepulse inhibition) do not "normalize" with the majority of successful treatments of positive symptoms.[7] Since disease biomarkers are defined by their statistical correlation to features of the disease it is imperative that the clinical phenotype is clearly defined. Stratification of all possible phenotypic variables in clearly a prerequisite for accurate assessment of the discrete relationships of the biomarker to the disease. Gender, age, life-style, medications, physiological and biochemical similarities are often not sufficiently inclusive resulting in plethora of disease biomarkers claims that are often confusing and futile.

II. Target Validation: biomarkers that assess the relevance and the potential for a given target to become the subject of manipulation that will modify the disease to provide clear

therapeutic benefits while securing a sufficient therapeutic index of safety and tolerability. This biomarker is intrinsically linked to our understanding of the disease.

a. ***Postmortem Studies as Source of Misleading Target Identification***: Often our understanding or employment of our strategies to a develop drugs is often sub-optimal and may therefore lead to targets being inappropriately or incorrectly identified. However, many of our approaches to target identification are based upon receptor expression from post mortem brain tissues. For example, post mortem brain tissues taken from schizophrenics show heterogeneous neuropathology's ranging from ventricular enlargement, disorganized cell layering (e.g., Layers II and III of the cortex) and reduced dendrite spine count and arborization in regions of the CNS such as the DLPC and hippocampus (e.g., ref 8).

b. ***Animal Models as Target Validation Biomarkers***: However, recent preclinical data has demonstrated that chronic exposure to antipsychotic treatments (e.g., haloperidol and olanzapine) is also associated with significant decreases in total brain weight and volume, gray matter volume, glial cell number.[9] As such, investigation of selective targeted "risk" gene disruption in mice, not only serve as etiologically relevant animal models, but by virtue of modeling the genetic component of the disease can serve as model systems of target validation. A pertinent case in point, several of the emerging "schizophrenia gene" disruptions are showing neuropathology that is seen in post mortem brain tissue as described in section IIa.

III. **Compound-Target Interaction Biomarkers**: biomarkers that define the discrete parameters of the compound (or biological) interaction with the molecular target. Typified by PET and SPECT, such parameters include binding of the compound to the target, its residency time on the target, the specific site of interaction with the target and the physical or chemical consequences to the target induced by the compound (or biological). Industry needs to engage early in the discovery process and develop SAR for TCI biomarkers early on in the process.

IV. **Pharmacodynamic Biomarkers**: biomarkers that predict the consequence(s) of compound (biological) interaction with the target. The pharmacodynamic biomarkers include events that are therapeutically desired or adverse events based on mechanism of action.

a. ***The Concept***: pharmacodynamic biomarkers can best be described by the employment of a new molecular entity (e.g., "compound X"), that has no PET ligand, whose therapeutic benefit is derived from indirect action upon a separate target system (e.g., dopamine) to which there exists a TCI biomarkers (Raclopride "R"). As such we can examine the effects of "X" (e.g., 5-HT2c agonist) for the displacement of dopamine via the use of "R".

b. ***Tracking the Divergence and Convergence of Signaling Pathways***: However, pharmacodynamic biomarkers can be used to report on discrete molecular events that are proximal to the biochemical pathway that is modified by the manipulated target or remote consequences such as in vivo or clinical outcomes (morbidity or mortality). Pharmacodynamic biomarkers are diverse and frequently nonobvious. Advanced and sophisticated bioinformatics tools are required for tracking the divergence and convergence of signaling pathways triggered by compound interaction with the target.

c. ***"Off Target" Effects***: A subset of the pharmacodynamic biomarkers are consequences induced by the compound outside its intended mechanism of action. Such pharmacodynamic effects are often termed "off target" effects, as they are not the direct consequence of the compound interaction with the target. Usually, such pharmacodynamic events are due to unforeseen lack of selectivity or metabolic transformations that yielded metabolites not present (or detected) in the animals used for safety and metabolic studies prior to launch of the compound into humans trails or into human use. These issues will not be dealt with in this chapter.

V. **Patient Selection:** biomarkers that are used for selection of patients for clinical studies, specifically proof-of-concept studies or confirmation Phase 3 clinical trials that are required for drug registration. These biomarkers are important in order to help in the selection of patients likely to respond (or conversely, not respond) to a particular treatment or a drug's specific mechanism of action and potentially predict those patients who may experience adverse effects. Such biomarkers are frequently genetic (single nucleotide polymorphism, haplotypes) or pharmacogenomic biomarkers (gene expression), but could be any of the primary pharmacodynamic biomarkers. Biomarkers for patient selection are now mainstream of exploratory clinical trials in oncology where genotyping of tumors in view of establishing the key oncogenic 'driver(s)' are critical for prediction potential therapeutic benefits of modern treatments with molecular targeting drugs. The success of the new era of molecular oncology (as compared to the cytotoxic era) will largely depend on the ability to define these oncogenic signaling pathways via biomarkers such as phosphorylated oncogenes, or functional state due to mutations that cause gain or loss of function.
   a. Imaging regional cerebral activation whilst patients perform tests of cognitive performance can be used to dissect the discrete neural regions and substrates supporting cognitive performance. In contrast to oncology, it is rare that there are concrete physical matter to quantify based on the heterogeneous nature of neuropathological abnormalities (see section I). However, imaging techniques such as functional MRI (fMRI) are bridging this gap. fMRI has the potential to be a powerful, sensitive and repeatable tool in our armamentarium. This technology affords the potential to dissect patients with cognitive deficits that are driven by, for example, either medial temporal lobe or by frontal lobe dysfunction (e.g., episodic memory vs. executive function deficits) within a clinical trial. Applied in early clinical POC studies we can, in essence, turn our current heterogeneous clinical population into discrete, focused sub groups with which to answer specific and focused hypothesis about the target, patient population and ultimately increase the probability of seeing an effect with our compound whilst improving the potential for differentiation from comparators. This in turn can aid patient selection in larger Phase III confirm studies and can be driven by adaptive trial design.

VI. **Adaptive Trial Design:** The overall objective of adaptive trial design is to enable real time learning. The method is based on computer modeling and simulation to guide clinical drug development. In a first step, decision criteria and assumptions are defined and analyzed strategy and study designs are formulated to test competing hypotheses in one aligned approach.

Once this framework is established, a formal scenario analysis, comparing the fingerprints of alternative designs through simulation is conducted. Designs that appear particularly attractive to the program are further subjected to more extensive simulation. Decision criteria steer away from doses that are either unsafe or nonefficacious and aim to quickly hone in onto the most attractive dose-range. Response-adaptive dose-ranging studies deploy dynamic termination rules, i.e., as soon no effect dose scenario is established and the study is recommended for termination. Bayesian approaches are ideally suited to enable ongoing learning and dynamic decision-making.[5]

The integrator role of "adaptive trials" is particularly strong in establishing links between regulatory accepted "confirm" type endpoints and translational medicine's efforts to develop biomarkers. Search for biomarkers that may enable early decision making need to be read out early to gain higher confidence in basing decisions on them. A biomarker can be of value, even if it only allows a pruning decision.

These considerations highlight the importance of borrowing strength from indirect observations and use mathematical modeling techniques to enhance learning about the research question. For instance, in a dose-ranging study, it is assumed that there should be some relationship between the response of adjacent doses and this assumption can be

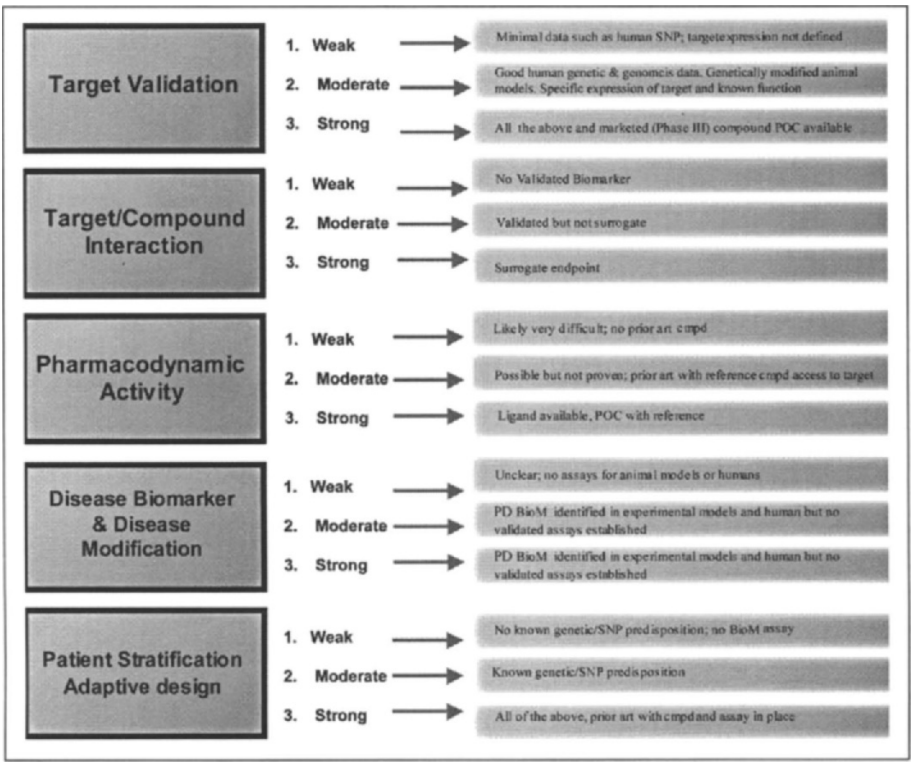

Figure 2. Criterion for biomarker scoring

placed to modeling algorithm. Both safety and efficacy considerations can be built to this model. Ideally, integration of all efforts all the way from disease-modeling in discovery to PK/PD modeling in early clinical development to safety/risk and business case modeling in late development.[4,10-12]

A summary of the biomarker definitions proposed herein is provided in Figure 1. The utility of this system is represented in Figures 2-4, which suggest a semi-quantitative scoring system that helps assess the strength of the program overall and identification of the areas of weaknesses in each of the biomarkers needed along the compound (biological) progression path. Figure 5 illustrates the continuum of biomarkers research, validation and implementation along the complete time-line of drug discovery and development including life cycle management (Phase IV) and new indication (Phase V) when appropriate.

A program for which a 'STRONG' score is established across all 5 biomarker's specifications provides confidence that the likelihood for success from the biological and medical perspectives and is likely to result in a more promising development outcome. Likewise, it would be prudent to voice concerns regarding programs that score 'WEAK', especially if low scores are assigned to target validation, pharmacodynamic and in special cases target-compound interaction (e.g., central nervous system target).

This scoring system is complementary to other definitions of biomarkers based on certain needs. For example, surrogate biomarkers as defined by the Food and Drug Agency (FDA), are markers that can be used for drug registration in lieu of more definitive clinical outcome data. Surrogate biomarkers are few and hard to establish (e.g., blood pressure and cholesterol, Fig. 3).

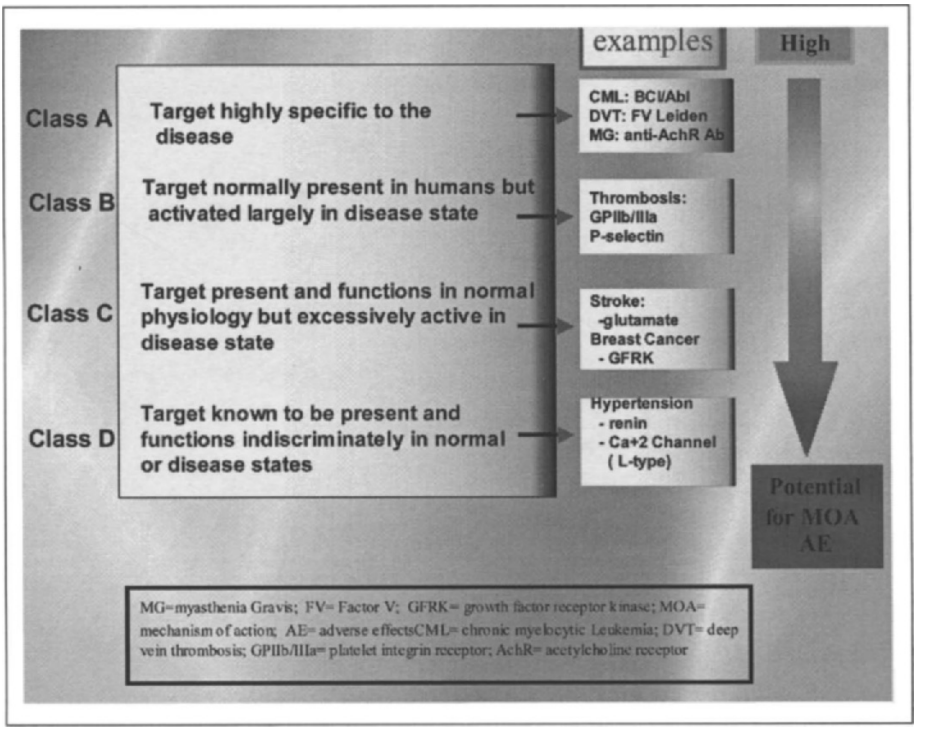

Figure 3. Type 1 biomarkers: target validation translational medicine perspectives

Principles of Target Selection

Two key guiding principles are essential in the early selection process of molecular targets:

A. That modulating the target carries the prospect of unequivocal medical benefit (efficacy) to patients beyond standard of care.

B. That benefit can be garnered while maintaining a sufficient level of safety that can be realized within the attainable compound exposure.

Such a mission is frequently unachievable and hence, establishing an acceptable "Therapeutic Index" is the practical goal for most drug development schemes. Commonly, a therapeutic index is established by calculation of the ratio of the Maximum Tolerated Dose (MTD) and the Minimum Effective Dose (MED) in animal efficacy and safety studies. In this light, targets selected for drug development can be classified with respect to risk assessment based on the following categories (Fig. 3):

Class A: Target only present and contributing to the disease process.

Class B: Target present physiologically but in a nonactive form; but is activated and contributes to the disease.

Class C: Target functions physiologically but at augmented, uncontrolled fashion that contributes to the disease.

Class D: Target functions in normal states and indiscriminately in disease (e.g., no difference in target expression, functions and distribution can be identified in disease as compared to the normal physiological state).

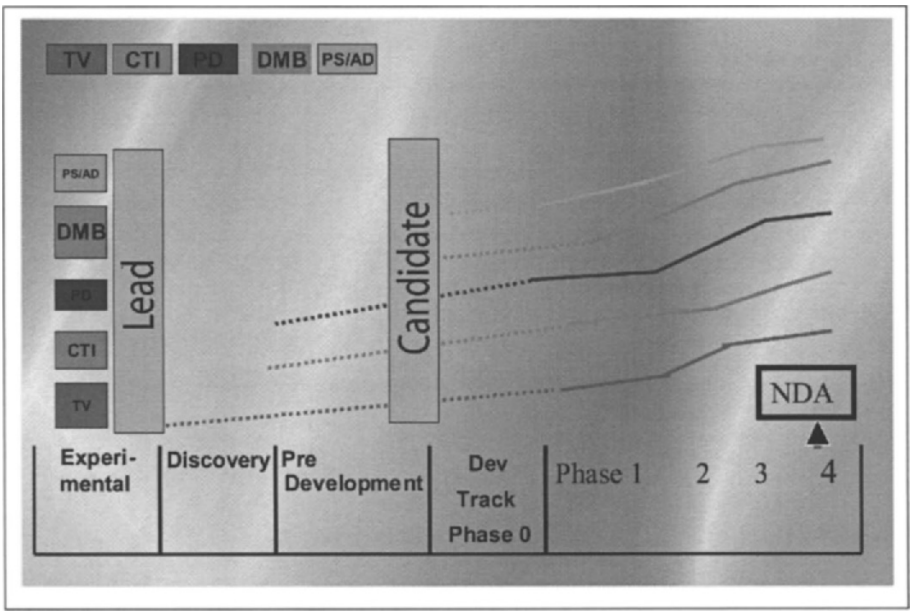

Figure 4. Building translational medicine via biomarkers research

Class A—Disease Specific

A disease specific molecular target should be a molecule that operates only in the disease state and is not participating in physiological (normal) functions. Drug interaction with such targets should provide efficacy with the lowest chance for mechanism-based adverse effects when manipulated by drugs. Examples of such targets are genetic disorders, which result in either over-activity or loss of activity of the target. Such is the case in Chronic Myelogenous Leukemia (CML) that results from aberrant recombination of DNA from chromosome 22 into chromosome 9 (Philadelphia Chromosome), fusing the Bcr and Abl genes into an overacting tyrosine kinase, which drives oncogenic transformation. To cure the disease, potent and selective inhibitors of this aberrant kinase had to be discovered, a task that took over a decade to accomplished.[13] Such targets have the potential for a high safety profile. It is however, important to note that this example may does not necessarily represent the ultimate approach for this disease since the activity of the kinase (Bcr/Abl) is driven by the Abl kinase catalytic site, which is preserved in its physiological format. Thus, inhibitors of this target/kinase by drugs such as Gleevec may still carry the potential for interference with in tissue/cells where the Abl kinase is physiologically active.

Another example that is applicable to this category is a disease such as Myasthenia Gravis where specific antibodies that block the acetylcholine receptors cause progressive muscle weakness. Specific neutralizing agents to these antibodies are likely to provide high efficacy in treating the disease with the likelihood of fewer adverse effects[14] since such antibodies are not physiologically present in human beings. These examples are typical for "Type 1 class A" target validation. The biomarkers that need to be established for this "Type 1 class A" category should focus on validating the specificity of the target to the disease state.

Class B—Target Present Physiologically but in a Non-Active Form, but Is Activated and Contributes to the Disease

This class of targets has little or no discernible physiological activity in the normal states, yet in certain pathophysiological situations, the target is presented, activated and plays a role in a

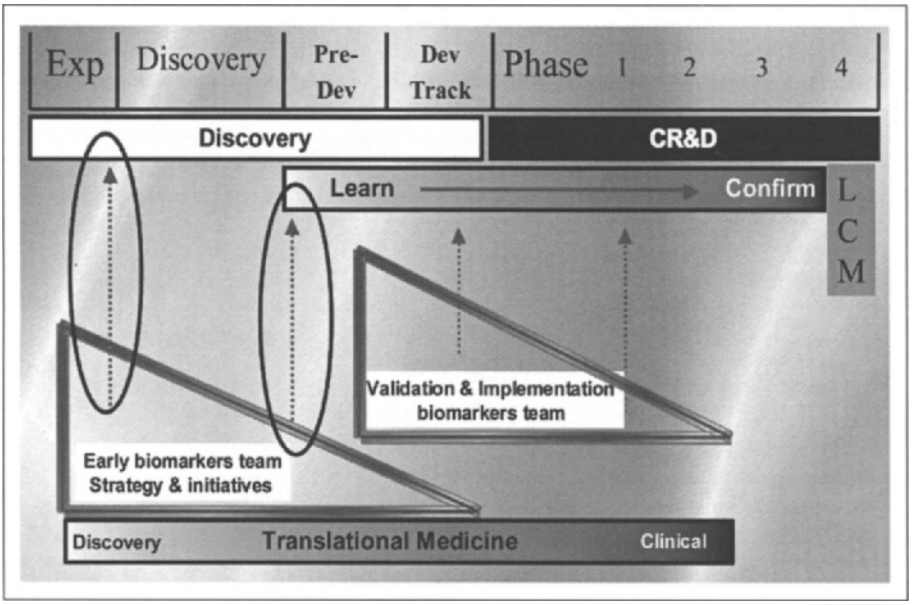

Figure 5. Translational medicine: biomarkers implementation across the pipeline

pathophysiological event. Example of such targets in the "Type 1 class B" category is the P-selectin adhesion molecule. This adhesion molecule is normally cryptic within platelets and endothelial cells. Upon activation of these cells, P-selectin is presented on the surface of the cell and mediates adhesion interaction with its ligand, a mechanism believed to play a role in thrombosis and inflammation. Inhibitors of P-selectin binding to its ligand, the P-Selectin Glycoprotein ligand (PSGL-1), are expected to provide clinical benefit with a lower likelihood of adverse events. To validate this situation, biomarkers that confirm the preferential role of the activated target in a pathophysiological process while maintaining little physiological function is essential.

However, one must be aware of potentially serious limitations to this approach where cryptic targets in the physiological state that are activated in the pathological condition and where inhibition of the target may not only provide for significant therapeutic benefit, but where inhibition of such a target may also expose the patient to some other risk, such as loss of host defense from injury. Such is the case of the platelet adhesion integrin molecule, GPIIb/IIIa, which serves as the final common pathway for platelet aggregation. Interfering with activated GPIIb/IIIa binding to its ligand (e.g., fibrinogen) provides effective and often life-saving therapy in patients with acute risk for thrombosis; however chronic treatment with GPIIB/IIIa antagonists have not been particularly effective in providing benefit due to the relatively high frequency of significant adverse effects due to bleeding since platelet adhesion to matrix protein is essential to seal bleeding sites in trauma and disease conditions. Thus biomarkers for this class must establish the full physiological significance of the target in order to assess the therapeutic index of tolerability (benefit as well as risk).

Class C—Target Functions Physiologically but at Augmented, Uncontrolled Fashion That Contributes to the Disease

This class of targets includes molecules that play an active role in normal physiological processes, some of which may be critical to health. Such is the neurotransmitter glutamate in the central nervous system, which is essential to cognition, memory, thought processes and state of arousal. However, in ischemic stroke or asphyxia, glutamate release is uncontrolled and reaches to high levels over prolonged periods that are believed to be neurotoxic and likely contribute to neuronal death

following a stroke. Inhibitors of glutamate release or antagonists of its action at various receptors are believed to carry the potential for effective treatment for stroke provided the inhibition of excess release of the neurotransmitter can be achieved in a timely manner and only to an extent that preserves the physiological need of this transmitter and over short periods (only that limited period where excess glutamate is neurotoxic). Such targets may be pharmaceutically exploitable when their manipulation is carefully tuned to the pathophysiological context).

Another example of a target in this category includes the Human Epidermal Growth Factor 2 (HER2) inhibitor, Herceptin, which in certain cancers (e.g., breast cancer) is constitutively activated and participates in the oncogenic drive. Inhibition of the HER2, while clearly of therapeutic value in breast cancer, has also been associated with heart failure due to the physiological role of HER2 in the cardiac myocyte survival-signaling pathway.[15] Thus, the biomarker challenge in modulation of class C targets of this nature is in identifying biomarkers that assess the needed 'titration' for inhibition of the target activity only to an extent that is necessary for the maintenance of normal physiological function.

Class D—Target Maintains Physiological Functions in Normal and Disease States

This class of targets encompasses the largest group of molecular targets exploited so far by modern drugs. Many members of this class have yielded highly beneficial therapies. This class consists of molecular targets that are known to have important physiological functions, which cannot be differentiated within a disease context; that is to say the target is not different in its expression levels (gene, protein) or signaling pathway in the normal and disease states. A priori, such targets harbor the greatest risk for mechanism-based adverse effects, as there is no apparent reason to expect that modulation of the target in the disease state will spare the normal physiological function of the target.

Examples of such targets include the coagulation factor inhibitors (e.g., FIX, FXa and thrombin), which are critical to maintain a physiological level of homeostasis; hence, inhibition of these targets carries inherent bleeding liabilities. Likewise, all current anti-arrhythmic drugs (e.g., amiodarone, lidocaine, dofetilide), while effective in treating life-threatening arrhythmias, all carry significant liability for mechanism-based pro-arrhythmic effects and the potential for sudden death. The biomarker challenges for this class are defining the fine balance needed between efficacy in the disease context and expected safety limitations. Biomarkers that define the acceptable therapeutic index are key to the successful utility of drugs that modulate such targets. However, targets in this class do not necessarily exhibit narrow safety margin for clinically meaningful adverse effects. Significant examples are the L-type Ca^{2+} channel blockers. The L-type Ca^{2+} channel is an essential conduit of Ca^{2+} needed for 'beat by beat' Ca^{2+} fluxes that secure precise rhythm and contractility of the heart, skeletal muscle, neuronal excitability and hormone and neurotransmitter release. Yet, L-type Ca^{2+} channel blockers are important and sufficiently safe drugs that are used to treat hypertension, angina and cardiac arrhythmias with undisputable medical benefits. However, inherent to the L-type Ca^{2+} channel blockers in this class of targets are mechanism-based adverse effects associated with rhythm disturbances, hypotension, edema and other liabilities.

Probably the best example for system specificity of physiological targets that provide major medical benefits with a high safety margin is the Renin-Angiotensin-Aldosterone (RAAS) system. The RAAS is an important blood pressure, blood volume and blood flow regulatory system, yet its manipulation by several different pharmacological agents (rennin inhibitors, angiotensin I converting enzyme inhibitors, angiotensin II receptors antagonists) has yielded highly beneficial drugs that reduce risk of morbidity and mortality from hypertension, heart failure and renal failure, despite the fact that the system does not demonstrate significant operational selectivity between normal and disease states (especially hypertension). However, mechanism-based hypotension and electrolyte disturbances can limit the therapeutic benefit of these drugs and elicit significant adverse effects when the RAAS is excessively inhibited.[16] The biomarker challenge for these targets is to define the relative or preferential role of the target in its various physiological

activities, where minor manipulation in one organ might provide sufficient therapeutic potential while providing a low likelihood for adverse effects that result from more substantial inhibition of the same target in other organs.

Conclusion

The analysis and classification offered in this chapter regarding biomarkers in drug discovery and development aim to highlight the need to carefully study and analyze the significance of the target selected for therapeutic intervention as the first cross road for success or failure in the development of effective and safe drugs.[17] The analysis and utility of biomarkers along the process of drug discovery and development has become an integral part of the "learn and confirm" paradigm of drug discovery and development in leading pharmaceutical organizations such as Wyeth research. Such analyses are useful to guide the "learn phase" in search for biomarkers that can better assess the benefits and risks associated with manipulation of the molecular target.

The scope of this paper does not allow for a detailed review of the "learn and confirm" paradigm, for which the readers are directed to recent references.[18-19] Various technological and strategic activities are needed to establish the biomarker strategies for the various targets described. The need to address these issues via biomarkers research, validation and implementation commencing at the very early stages of the drug discovery and development process is emphasized. In the pharmaceutical setting, it means commencing efforts to identify biomarkers for all 5 categories listed above. Such efforts could commence even before a tractable compound (biological) is in hand, a time where target validation is a clear focus of the program. As compound becomes available, compound—target interaction, pharmacodynamic (efficacy and safety) biomarkers and strategies for patient selection and adaptive design needs must be explored. At the onset of the "first in human" studies, all strategies, plans and biomarkers research should be well worked out (as much as possible). We believe that fundamental changes in the structure, function and interfaces of Pharmaceutical R&D is urgently needed to provide for a key role of translational medicine and biomarkers research towards more successful discovery and development of innovative medicines.

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CHAPTER 2

Natural Products in Drug Discovery:
Present Status and Perspectives

Gabriella Molinari*

Abstract

Natural products and their derivatives have been and continue to be rich sources for drug discovery. However, natural products are not drugs. They are produce in nature and through biological assays they are identified as leads, which become candidates for drug development. More than 60% of the drugs that are in the market derive from natural sources. During the last two decades, research aimed at exploiting natural products as a resource has seriously declined. This is in part due to the development of new technologies such as combinatorial chemistry, metagenomics and high-throughput screening. However, the new drug discovery approaches did not fulfilled the initial expectations. This has lead to a renewed interest in natural products, determined by the urgent need for new drugs, in particular to fight against infections caused by multi-resistant pathogens.

Introduction

Historically, chemical substances, derived from animals, plants and microbes have been used to treat diseases. Plants and microorganisms produce unique bioactive substances, providing access to very different types of lead compounds, the natural products.

Natural products have played and continue to play an invaluable role in the drug discovery process, particularly in the areas of cancer and infectious diseases. In fact, more than 60% of the approved drugs are of natural origin. In the modern drug discovery era there are three major sources of new compounds: original natural products, structures derived semi-synthetically from natural products and combinatorial synthetic compounds based on natural products models.[1-3] Bioactive natural products are mostly low-molecular weight organic compounds known as secondary metabolites.[4] The producer organisms can growth without synthesizing these metabolites and produce them in response to environmental cues. These compounds could be produced in nature as "weapons" that organisms use to fight each other.[5,6]

In this chapter will be described the role of natural products in the evolution of drug discovery, giving emphasis to the natural products which provide candidates to be used in infectious disease therapy: the antibiotics. Antibiotics are biologically active molecules with different structures and mode of action made by microorganisms, which are active against other microorganisms at low concentrations. Many antibiotics are made chemically by modification of natural products through a process called semi-synthesis and the effective compound is a semi-synthetic derivative. In Table 1 are summarized different antibiotics from natural origin actually used in the clinic. The majority of antibiotics inhibit targets involved in essential microbial functions: protein synthesis (30S and 50S subunits of the ribosome and RNA polymerase), DNA replication (DNA gyrase) and cell

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Table 1. Microbial secondary metabolites or derivatives used as antibiotics

Antibiotic	Class	Target	Derived From	Produced By
Amikacin	Semisynthetic aminoglycoside	Protein synthesis: binding to the 30S ribosomal subunit	Kanamycin	-
Amoxycillin	Semisynthetic hydroxyampicillin	Cell-wall synthesis: penicillin-binding protein (PBP)	Ampicillin	-
Amphotericin B	Natural polyene macrolide	Fungal membrane	-	*Streptomyces nodosus*
Ampicillin	Semisynthetic aminobenzylpenicillin	Cell-wall synthesis: PBPs	Peniciflin	-
Azithromycin	Semisynthetic 15 membered azalide	Protein synthesis: binding to the 50S ribosomal subunit	Erythromycin	-
Aztreonam	Semisynthetic monocyclic ß-lactam	Cell-wall synthesis: PBP3	SQ-26180	*Chomobacterium violaceum*
Bacitracin	Natural thiazolpeptide	Peptidoglycan synthesis: lipid pyrophosphorylase inhibitor	-	*Bacillus licheniformis*
Caspofungin	Semisynthetic lipopeptide	Fungal wall: glucan synthesis	Pneumocandin B	*Clarea lozoyensis*
Cephalosporin	Natural cephem	Cell-wall synthesis: PBPs	-	*Cephalosporium acremonium*
Chloramphenicol	Natural phenicol	Protein synthesis: binding to the 50S ribosomal subunit	-	*Streptomyces venezuelae*
Clavulanic acid	Natural oxa-1-penem	ß lactamase inhibitor	-	*Streptomyces clavuligerus*
Clindamycin	Semisynthetic thiooctopyranoside	Protein synthesis: binding to the 50S ribosomal subunit	Lincosamine	*Streptomyces lincolnensis*

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Table 1. Continued

Antibiotic	Class	Target	Derived From	Produced By
Dalfopristin-Quinupristin	Semisynthetic streptogramin	Protein synthesis: binding to the 50S ribosomal subunit	Pristinamycin	*Streptomyces pristinaspiralis*
Daptomycin	Semisynthetic lipopeptide	Bacterial membrane	A-21978C	*Streptomyces roseoporus*
Erythromycin	Natural 14-membered macrolide	Protein synthesis: binding to the 50S ribosomal subunit	-	*Saccharopolyspora erythrae* *Streptomyces erythreus*
Fosfomycin	Natural phosphonic acid	Cell wall: synthesis of peptidoglycan precursors	-	*Streptomyces fradiae*
Fusidic acid	Natural fusidane	Protein synthesis: translation stage	-	*Fusidium coccineum*
Gentamycin	Natural aminoglycoside	Protein synthesis: binding to the 30S ribosomal subunit	-	*Micromonospora purpurea*
Griseofulvin	Natural benzohydrofuran	Fungal cell wall synthesis	-	*Penicillium griseofulvum*
Imipenem	Semisynthetic carbapenem	Cell-wall synthesis: PBPs	Thienamycin	-
Josamycin	Natural 16-membered macrolide	Protein synthesis: binding to the 50S ribosomal subunit	-	*Streptomyces narbonensis subsp. josamyceticus*
Kanamycin	Natural aminoglycoside	Protein synthesis: binding to the 30S ribosomal subunit	-	*Streptomyces kanamyceticus*
Methicillin	Semisynthetic penicillin	Cell-wall synthesis: PBPs	Penicillin	-
Mupirocin	Natural pseudomonic acid	Protein synthesis: blocking isoleucin incorporation	-	*Pseudomonas fluorescens*
Netilmicin	Semisynthetic aminoglycoside	Protein synthesis: binding to the 30S ribosomal subunit	Sisomicin	-

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Table 1. Continued

Antibiotic	Class	Target	Derived From	Produced By
Novobiocin	Natural coumarin	DNA synthesis: DNA gyrase	-	*Streptomyces spheroids S. niveus*
Nystatin	Natural polyene macrolide	Fungal membrane	-	*Streptomyces noursei*
Penicillin	Natural ß-lactam	Cell-wall synthesis: PBPs	-	*Penicillium chryseogenum*
Polymyxin	Natural lipopeptide	Bacterial membrane	-	*Bacillus polymyxa*
Rifampin	Semisynthetic ansamycin	RNA transcription: RNA polymerase	Rifamycin	*Nocardia mediterranei*
Sisomicin	Natural aminoglycoside	Protein synthesis: binding to the 30S ribosomal subunit	-	*Micromonospora inyoensis*
Spectinomycin	Natural aminocyclitol	Protein synthesis: binding to the 50S ribosomal subunit	-	*Streptomyces flavopersicus*
Spiramicyn	Natural 16-membered macrolide	Protein synthesis: binding to the 50S ribosomal subunit	-	*Streptomyces ambofaciens*
Streptogramin	Natural macrocylic peptolides	Protein synthesis: binding to the 50S ribosomal subunit		*Streptomyces diastaticus*
Streptomycin	Natural aminoglycoside	Protein synthesis: binding to the 30S ribosomal subunit	-	*Streptomyces griseus*
Teicoplanin	Natural lipoglycopeptide	Cell-wall synthesis: peptidoglycan	-	*Actinoplanes teichomyceticus*
Tetracycline	Natural polyketide	Protein synthesis 16SrRNA	-	*Streptomyces aureofaciens*
Thienamycin	Natural carbapenem	Cell-wall synthesis: PBPs	-	*Streptomyces cattleya*
Vancomycin	Natural glycopeptide	Cell-wall synthesis: peptidoglycan	-	*Streptomyces orientalis*

wall synthesis. Many other cellular functions which are essential for microbes are also present in mammalian cells making them unsuitable targets.

Drug Discovery Evolution

The time frame between the initial discovery of a potential drug candidate and the market launch of a new therapeutic agent usually is 10-14 years (Fig. 1). For this reason it is difficult to measure the real trend in the drug discovery programs during the last two decades. However, the worldwide pharmaceutical natural product patents trend show a decline from 1990 to 1999 and a recent increase of patenting up to 2001.[7] The interest in discover new natural products declined when new technologies became more attractive for the pharmaceutical industry to be implemented in their search for new drug candidates. The evolution of molecular techniques allowed the identification and isolation of purified protein targets. The use of defined molecular targets combined with automatization, modern robotic instruments, sensitive detectors, data processing and control software allowed the development of tests using microvolumes for the screening of libraries of chemical structures: the high-throughput screening (HTS). The definition "hit" and "lead" development were then incorporated to the drug discovery process. Through the HTS millions of tests could be automatically performed, testing compounds for their activity as inhibitors or activators of a specific biological target (i.e., hit). Active structures could be then identify in a short period of time (i.e., leads) and they are the starting point for drug design.[7] Combinatorial chemistry provided the possibility to obtain large collections or libraries by synthesizing combinations of a set of smaller chemical structures.[8] Further development of molecular biology, cellular biology and genomics allowed the discovery of new molecular targets, which were introduced in the HTS screens. This determined that industries concentrated their efforts in the generation and exploitation of synthetic chemical libraries, abandoning the search for new natural products. However, in the target-specific approach only one target can be screened at a time and during target-based discovery preclinical and clinical studies resulted in high failure rates. In addition, despite the speed of synthesis, combinatorial chemistry has not yielded an increased number of leads.[9] It is common knowledge in medicinal chemistry that the removal of chiral centers, introducing additional flexibility into molecules and decreasing its size, generally leads to a less specific and weaker activity.[2] The greater flexibility of combinatorial compounds is likely to have detrimental entropic consequences for the binding of these compounds and could affect also their ability to induce conformational changes in the receptor required for biological function. Finally, commercial trends shifted the interest of industry from the infectious diseases research field to others with more economical success (e.g., drugs to treat autoimmunity and/or chronic cardiovascular or neurodegenerative diseases).

Natural Products Properties

Natural products structures have the characteristic of high chemical diversity, biochemical specificity and high binding affinities to their specific receptor.[10,11] As they are produce in nature, it is easy to suppose that all of them have a function and they have naturally evolved to be more effective. The generation of natural product diversity has occurred within the constraints of available biosynthetic reaction and precursors, but also in the context of biological utility. The synthetic routes for the natural product generation have coevolved with the functional requirements of their ligands.[2] Natural products interact with a wide variety of proteins and other biological targets, acting also as modulators of cellular processes when they inhibit protein-protein interactions.[7]

Different chemical and molecular characteristics from active structures can be investigated to compare diversity between compounds of different origin. Molecular properties such as mass, number of chiral centers, prevalence of aromatic rings, molecular flexibility, distribution of heavy atoms and chemical properties are evaluated. In an analysis performed by Lipinski[12] were estimated the solubility and permeability of compounds by experimental and computational approaches. This work introduced "the rule of five" which defines the properties relevant for the characterization of small molecules for medical use. These properties are: molecular mass (<500

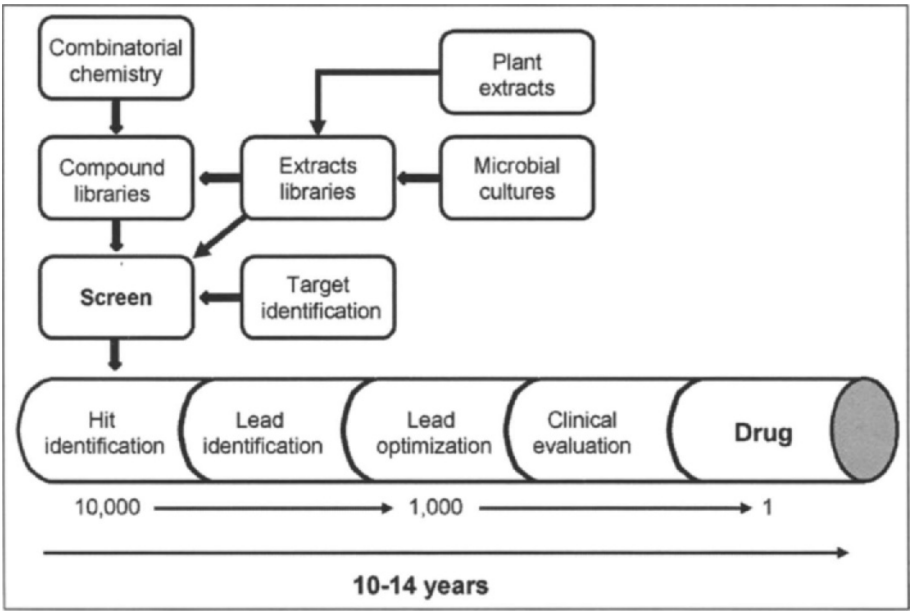

Figure 1. Stages in the drug discovery process.

Da), number of hydrogen-bond donors (<5), number of hydrogen-bond acceptors (<10) and a calculated octanol-water partition coefficient to indicate the ability to cross biological membranes (<5). Other studies characterized the molecular frameworks and substituents,[13,14] the statistical analysis of different drug databases[15] and the introduction of a drug-like index.[16] The work from Feher and Schmidt[2] analyzed the molecular diversity of natural products in comparison to that of compounds obtained from combinatorial chemistry and synthetic drugs derived from natural products. After the analysis of several databases they counted: 10,968 drug molecules, 670,536 combinatorial compounds, 3,287 natural products and 27,338 molecules considered as natural and semi-natural. Many molecular properties, such as number of chiral centers, rotatable bonds, unsaturations, atom types, rings and chains were evaluated and results showed that natural products and combinatorial libraries have different properties, demonstrating that combinatorial compounds are substantially less diverse than either drugs or natural products. Furthermore, the diversity of combinatorial compounds is confined to an area where appears to be little diversity for natural products, thereby raising the question of the real significance of this diversity in the context of biological processes (i.e., why this type of diversity was not positively selected during evolution). In conclusion, products obtained from natural origins show more diversity, contain a greater number of chiral centers and higher steric complexity.

The Urgent Need for New Drugs

Microbial infections are responsible for approximately 26% of deaths worldwide. Thus, there is an urgent need for new drugs, particularly to fight against the multi-resistant infectious agents. Particularly bacteria show an outstanding capacity to develop resistance against common used anti-bacterial drugs through a wide variety of mechanisms. This has lead to a practical exhaustion in the repertoire of active antibiotics, including compounds such as carbapenems, tetracyclines, fluoroquinolones and aminoglycosides. The last resource drugs, as imipenem for gram-negative and vancomycin for gram-positive, are no more effective against the "superbugs". This is the new

term for infectious agents which have developed resistance to almost all commercial drugs. These multiple resistant bacteria are now one of the most challenging problems for modern medicine.

There are limited and rather expensive therapeutic options for infections caused by *Staphylococcus aureus*, particularly for the methicillin-resistant *S. aureus* (MRSA) strains, which show an incidence exceeding 40% in some European countries.[17] The new drugs on the market (e.g., linezolid, quinupristin-dalfopristin, daptomycin) have limitations, such as high rates of side effects or low efficacy in pulmonary infections.[17] Other problematic gram-positive pathogens are the vancomycin-resistant enterococci and penicillin-resistant *Streptococcus pneumoniae*. Another class of superbugs, the opportunistic gram negative pathogens (e.g., *Pseudomonas aeruginosa, Burkholderia cepacia* and *Stenotrophomonas maltophilia*), which are frequently from environmental origin and generally infect compromised patients, are intrinsically resistant to many antibiotics.[18] *Acinetobacter baumanii*, a pathogen which is responsible of 7% of all cases of pneumonia and kills 40% of infected patients, has successfully developed resistance against all common antibiotics including colistin (polymyxin E), the last universally active drug against this pathogen.[19,20] *Mycobacterium tuberculosis*, a pathogen considered under control, is now re-emerging as problematic due to its resistance and persistence.[21] In addition, the emergence of new fungal and viral diseases poses the need of rapid discovery in response to new infectious diseases (i.e., we cannot afford to wait 10-15 years).[22,23]

From Microbial Diversity to Drug Discovery

The battle against microbial resistant pathogens demands new and better drugs, which are more difficult to find after decades of hard work on discovery programs. However, natural products and their derivatives, continue to be rich sources for lead discovery. The question is if classical approaches will succeed in discovering new leads after more than 60 years of natural products search. Classical natural products drug discovery programs are based on extract collections obtained from natural resources, followed by screening, bioassay guided isolation, identification of new compounds, structure elucidation and large scale production (Fig. 2). Microorganisms are capable to carry out a tremendous variety of reactions, adapting to a large array of different environments, allowing a culture to be transplanted from nature to the laboratory flask and later to a fermentor, in order to produce active compounds in large quantities under tightly controlled conditions.

Microbial Sources

Microorganisms, bacteria and fungi were and still are a rich source of natural products. Over 6,000–12,000 compounds of microbial origin with anti-microbial activity have been isolated. However, only five phyla (*Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes* and *Proteobacteria*) include species that produced bioactive molecules which were developed to drugs.[24] In the past, drug discovery programs focused only in group of organisms, as *Actinomycetes* (mostly represented by *Streptomycetes*) and fungi isolated from easily accessible environmental soil samples and cultivated under standard conditions. Approximately 3,500 bioactive compounds have been recognized from the genus *Streptomyces*. In particular, strains from *Streptomyces griseus* and *S. hygroscopicus* produce over 50 and 200 different compounds with anti-bacterial activity, respectively.[4] Other known producers of bioactive compounds are *Bacillus* spp. and *Pseudomonas* spp. Different strains from *Bacillus subtilis* produce over 60 different active compounds. Other novel groups of microorganisms, such as Myxobacteria, resulted to be rich sources of new structures.[25,26] However, despite the huge amount of bioactive compounds characterized each year from microbial sources, only a relatively small number of compounds, approximately 150, have been commercially developed as antibiotics.[27] The most important are listed in Table 1. Most of them are produced by *Streptomyces* raising the question if they are the only microorganisms able to produce active compounds with low toxicity.

Nevertheless, the microbial world represents 90% of all biological diversity and less than 1% has been explored.[28] Mining this microbial diversity is the key for obtaining high compound diversity. A huge source for new natural products remains unexplored and the microbiological efforts are actually concentrated in the study of unexplored niches to access unknown uncultured

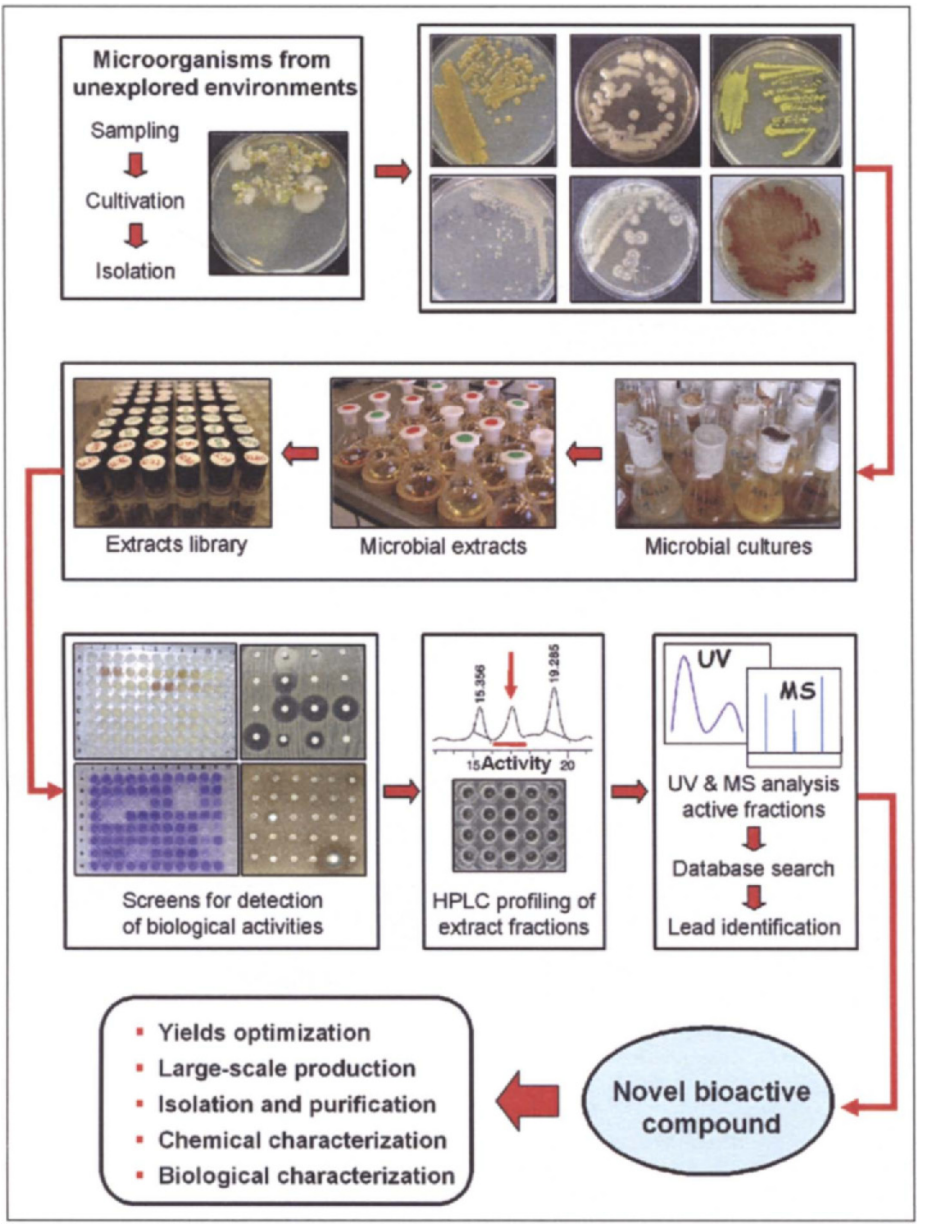

Figure 2. Discovery of natural products of newly isolated microorganisms.

microbial diversity (e.g., marine environments or tropical forests). In addition, the development of new cultivation technologies could let to identify and characterize new forms of biodiversity.[23,24,29] Good examples are the probes obtained from the deep-sea hyper saline anoxic basins of the Mediterranean sea, which harbored uncharacterized biodiversity.[30-32] Novel lineages of microbes have been found and several novel enzymes exhibiting exceptionally catalytic activities of industrial

relevance have been recovered and characterized, indicating that new groups of microbes could exhibit new capacities.

The selection of the environmental niche to explore is matter of continuous search. New companies are able to provide material from exotic environments. However, microbiological expertise is required for the isolation of new microorganisms. New methods, as high-throughput cultivation using microtitre dishes, microencapsulation of single cells and automated processes were recently developed to increase the percentage of strains cultured.[33-35] Once the environmental strains are isolated, antagonist methods could be used for the detection of substances with anti-microbial activity. The cocultivation of a target and a new isolated microorganism in solid media allow a rapid identification of antagonism. However, the observation of antagonism does not imply success in the discovery of the active structure. The antagonist compound could be produced only in presence of the target or in low amounts making difficult the characterization process. Furthermore, this method limits the exploitation of a new isolate to anti-microbial compounds.

Microbial Extracts

Bioactive natural products are secondary metabolites produced and secreted in nature by microorganisms during competition in microbial communities. In the laboratory, during cultivation, microorganisms may also produce many compounds as a result of their secondary metabolism. The extraction of the secondary metabolites produced during growth will let to the generation of a library of extracts, which could be stored and screened in parallel screens using a variety of assays (Fig. 2).

Cultivation is a crucial process since microorganisms could show the capacity to produce bioactive compounds only under specific growth conditions. Few general rules could be applied for the determination of the optimal cultivation conditions for production of bioactivities. First, should be taken into consideration the requirements of the group of microorganisms under study (e.g., the addition of sea salts or the use of sterilized seawater for marine isolates). A wide range of different media have been successfully used for the production of bioactive metabolites. Literature search, once the species of the producer strains are known, could provide useful information for the choice of the appropriate medium and growth conditions (e.g., temperature and aeration). Optimization of production yields should be investigated before shifting to large-scale production. During the initial cultivation of a large number of unknown environmental strains, a general medium in which organisms grown in former studies showed production of bioactive compounds could be applied. However, when strains are selected through the primary screens for their capacity to produce bioactive substances, different cultivation strategies should be further explored. Conditions that influence the yields of secondary metabolites production are: variations of growth substrates (e.g., carbon source and concentration), variation of growth parameters (e.g., effect of time of incubation, temperature and aeration) and the addition of supplements or precursors[36] (e.g., Mn, Mg and DMSO). After cultivation and filtering of the biomass, whole broth extraction is the primary step of the isolation process. In addition to the classical extraction using different solvents (e.g., chloroform, acetone and ethyl acetate), several new approaches were developed over the last years to increase the efficiency of extraction.[37] Alternative, cultivation could be performed in the presence of a resin.[38,39] The resin will adsorb the secondary metabolites produced during the whole incubation time, stabilizing them and reducing the risk of degradation. In addition, this approach facilitates the biomass separation process by collecting the resin on a filter or a sieve. Finally, the adsorbed compounds are eluted (e.g., methanol) and the extracts are concentrated by rotary evaporation.[40]

Crude extracts are complex, containing from 10 to 100 metabolites. The quantity of compounds present in an extract is not know, many of them are present at low concentrations, limiting the possibility to use HTS technology for identification of bioactivities. The risks in performing screens using crude extracts are: (i) the concentration levels of compounds might not be enough for detection of activity and (ii) the presence of other compounds could inhibit the test. Moreover, new HTS assays are based on fluorescence reactions and many crude extracts contain compounds that

could affect the readout of the assay by autofluorescence or absorbtion at the same wave lengths.[7] However, interferences could be overcome using "lifetime discriminated polarization", a technique that reduce potential interferences by rejection of signals from short-lifetime sources[41] or by using red-shifted wavelength dyes.[42] Once available a library of extracts, two main strategies could be pursued for the discovery of new compounds: the biological and the chemical screenings.

Chemical Screening

During the chemical screening each extract is analyzed by high performance liquid chromatography (HPLC). This analysis coupled to ultraviolet (UV) photodiode array detection and to mass spectrometry (MS) using electrospray ionization (ESI) and atmospheric pressure ionization,[43] will provide the mass and UV spectra data of each peak, which will be used to compare with data from databases to recognize new compounds. Liquid chromatography (LC) combined with MS, UV and nuclear magnetic resonance (NMR), using techniques such as LC/UV, LC/MS, LC/MS/MS and LC/MS/NMR quickly provide structure information with minimal amounts of compounds.[44] This enables the differentiation between novel compounds and known compounds present in crude extracts. Isolation of known compounds can be avoided and only substances with novel structures are isolated to generate a natural product library. These libraries contain new molecules that need to be incorporated in biological screens in the search for possible applications. The chemical screening is recommended particularly when extracts obtained from new groups of organisms are analyzed.

Biological Screening

During the biological screening, parallel assays (e.g., detection of anti-bacterial, anti-fungal, anti-yeast and anti-mycobacterial activities; inhibition of enzymatic processes; effects on eukaryotic cells) are performed with the crude extracts libraries. Assay interferences, particularly by the enzymatic tests, could be reduced by screening fractionated samples obtained from crude extracts.[45,46] These samples contain less complex mixtures and the relative concentration of the compounds is increased, thereby increasing the chances of detecting biological activities. However, this strategy requires a first fractionation/purification step (through HPLC) before performing the screening for biological activities and multiplies the number of samples to be screened.

During the primary screens bioactive extracts are selected for further analysis. The compound contained in the crude extract and responsible for the bioactivity should be recognized and identified. One or more rounds of chemical purification and biological assay might be necessary for identifying and isolating the active component in the complex mixture. At this point of the drug discovery process combined expertise from microbiologists and chemists is required. A strategy that allows a rapid identification of the active principle is the HPLC-activity profiling. The active extract is separated on an analytical HPLC and fractions are collected in a microtitre plate. The fractions are tested in the biological assay that allows the identification of the active fraction (Fig. 2). The active peak is further analyzed by LC coupled to UV detection and MS.[47,48] The mass and UV data allow recognition of known compounds through databases[49,50] (e.g., Chapman & Hall Dictionary of Natural Products[51]). When no matches are found, the new molecule should be identified. New activities for known compounds can be discovered by implementing new screens. Then, the active principle need to be isolated for characterization.

Production, Purification and Characterization of a New Natural Product

Larger amount of compounds is required for the isolation and purification. An important and complicated aspect in the drug discovery programs is the transfer of the production process from the small-scale laboratory cultivation or 5 l fermentor to large-scale equipment.[52] Rarely a biological process behaves the same way in large-scale fermentors as in small laboratory cultivation. Mixing and aeration are the main difference between the two growth conditions. Bioengineering expertise is required for fermentation surveillance.[37] The cloning of the complete biosynthesis cluster in a

heterologous host is indicated when the producing organism is difficult to handle, growths slowly or produce low yields of the active compound. The purification of a new molecule from a complex crude extract is a task that limits the speed of the drug discovery process. Different chromatography systems are employed for purification.[7,53] Identification of compounds usually involves a combination of various techniques including NMR, MS, UV and infrared (IR) spectrometry. New advances in NMR and MS spectroscopy allow the structure elucidation with small amount of compound (from 0.5 to 1 mg).[29,43] However, larger quantities of purified principle are required for the chemical and biological characterization, as well as for the study of the mechanism of action and in vivo activity. Structure/activity studies contribute to understand the interaction between the new molecule and its target. As mentioned at the beginning, an active natural product is not a drug. It is a lead which should be further developed to obtain a drug. Active compounds with high toxicity, poor pharmacokinetic properties and questionable in vivo relevance are not further explored or need to be modified in the laboratory. Combinatorial chemistry could yield more useful derivatives through derivatisation or biotransformation.[54] Nature often represents the starting point for the development of semisynthetic compounds with improved stability and/or activity (Table 1). Furthermore, the total chemical synthesis of new compounds could be an alternative method to microbial production or provide insights about the structure/activity relationships of the compound.

Metagenomics for Drug Discovery

Most of the microbial world remains uncultivated; many potentially active compounds are unknown. The number of microorganisms cultured from soil samples represents 1% of the total microbial community.[28] The analyses of 16S rRNA genes amplified from soil discovered novel phyla.[55-57] Marine microbial communities studies using cultivation-independent genomic approaches resulted in the characterization of many new phylogenetic lineages suggesting that the majority of the microbial diversity has to be discovered.[58] Metagenomics, a strategy to access the genetic potential of uncultured microorganisms, could allow the discovery of novel compounds through a culture-independent process.[57,59,60] DNA isolated directly from soil or other samples is cloned into a bacterial artificial chromosome vector to construct genomic libraries, thereby accessing the metagenomes from microbial communities. However, this new technology has limitations for obtaining novel compounds with complex structures. The probability of finding a biosynthetic cluster encoding a new active metabolite in a random DNA library is reduced. Furthermore, *Escherichia coli* is an inappropriate host for functional screening, because genes may be not expressed or the metabolic precursors are not present. However, new suitable hosts (e.g., *Streptomyces*, Actinomycetes and *Pseudomonas*) are actually under characterization. These expression hosts could provide a more appropriate genetic, biochemical and physiological background to produce bioactive molecules from genes that are derived from uncultivated bacteria. However, the cloning and expression of genes from members of different or unknown lineages could fail. The use of metagenomics in drug discovery is a strategy adopted by several companies which are developing high-capacity vectors, suitable transformation protocols and engineered host to accommodate large gene clusters for production of complex secondary metabolites.[60,61]

Natural Products Under Development

There are at least 127 natural products or natural product-derived compounds that are currently under development, of them, only a few have anti-bacterial properties.[62] Leeds et al have reviewed the most important natural products under development with anti-bacterial activity.[29] Seven antibacterial compounds that target lipid II, a membrane-anchored cell-wall precursor, are actually on preclinical development.[63] They are produced by *Lactococcus*, *Actinomycetes*, *Streptomyces*, *Cytophaga* and *Pseudomonas* and show activity against multi-resistant gram-positive bacteria. Because lipid II production is restricted to bacteria, these compounds should have low toxicity in humans.

Other recently described compounds are active against multi-drug-resistant strains of staphylococci and enterococci. They are examples of new compounds found in well-known sources

(e.g., a collection of soil isolates) by using new screens or by sampling previously unexplored environments.

Platensimycin has a novel chemical structure and targets an enzyme involved in fatty-acid synthesis. This compound was discovered by screening 250,000 extracts with a new screen.[64,65] Sampling of unexplored environments from Indonesia also allowed the discover of a new variant of a macrolactin produced by *B. subtilis*, which show activity against MRSA and VRE.[39] Interestingly, this new macrolactin seems to have a novel mechanism of action by targeting the cell division process.

New Strategies in Fighting Infectious Diseases

During drug discovery, extracts showing powerful biological activities in parallel screens are continuously investigated. Moreover, new screens are implemented, according to new therapeutic needs.[66] In addition, the recent availability of complete microbial genomes facilitates structural approaches aimed at the identification of new molecular targets.[67-70] However, natural products are produced in nature in response to competition in microbial communities. Therefore, the microbial producer should be resistant to the action of the compound. This implies that a resistance mechanism pre-exists in nature which is coupled to the production pathway.[71,72] Furthermore, microorganisms can rapidly evolve resistant mechanisms due to their genomics plasticity and the existence of mechanisms for horizontal gene transfer. Thus, the fight against microbial resistance could be a battle lost from the beginning. Therefore, it is necessary to develop new strategies to combat infections agents.

Virulence or pathogenicity refers to the ability of an organism to establish an infection and cause disease in a host.[73] Products that aid the pathogenicity of a bacterium are termed virulence factors.[74] Molecules that inhibit the production of virulence factors, affect the growth, block the spread or interfere during host-pathogen interactions represent alternative therapeutic approaches.[75,76] Rather than kill the pathogen, once the pathogen is weakened, the host immune system could take over and clear infections. Deeper knowledge on host-pathogen interactions and the molecular basis of immunoclearance could yield new targets and new strategies for successful therapies. Another focus of innovation is drug discovery through system biology, a biology-driven approach that involves screening compounds by automated response profiling in disease models based on complex human-cell system.[66,77,78] Infection biology represents the emerging field where the efforts from microbiologists, immunologists, cell biologists, system biologists, chemists, pharmacologists and clinicians are combined to fight infection.

Conclusion

The decline in the discovery of natural products during the last decades was a consequence of the shift of interest when scientists concentrate their efforts in chemical and genetic combinatorial methods, as well as in the development of HTS for testing existing compounds libraries. Nevertheless, nature still contains a treasure of diversity which it is essential to tap, particularly considering the meager outcome from combinatorial approaches. Hopefully, the environment offers us its treasure of unexplored resources, allowing the discovery of new weapons against emerging infectious.

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CHAPTER 3

Protein Pharmaceuticals:
Discovery and Preclinical Development

Davinder S. Gill*

Abstract

Proteins are natural molecules that carry out important cellular functions within our bodies. Their precise role is crucial to the maintenance of good health. Malfunctioning proteins or those not produced optimally result in disease. The foundation of biopharmaceutical drug therapy has therefore been to modulate cellular function by targeting specific proteins expressed on or outside the cell. Because most biopharmaceuticals are natural in origin, they are biologically and chemically very different from conventional medicines. In addition to differences in mechanism of action, biopharmaceuticals differ in the process by which they get manufactured and delivered. Because of their large, complex structure, they must often be produced by culturing cells and then purified from a host of cellular components. This can be time-consuming and costly. Also, most biopharmaceuticals are given by injection under the skin or by infusion into the veins. This creates significant limitations to their utility. Nonetheless, biopharmaceuticals can be very powerful and selective in disease applications such as in rheumatoid arthritis or cancer. This chapter describes methods by which proteins drugs are discovered, optimized and developed. It also covers novel agents and next generation proteins as well as some of the challenges and opportunities in the area.

Introduction

Most biopharmaceuticals today can be broadly classified into one of three major categories—monoclonal antibodies, fusion proteins or native biologics. Monoclonal antibodies are a growing class of Biopharmaceutical products that includes 21 FDA approved drugs to date.[1] The list includes products such as Bevacizumab (Genentech), Panitumumab (Amgen), Rituximab (BiogenIdec/Genentech) and Adalimumab (Abbott). In 2006, this class of drugs generated greater than $19 billion in annual sales worldwide, a figure that is expected to grow at the rate of 30-40% for the next 5 years.[1-2] Fusion proteins are a class of drugs exemplified by Etanercept, the extra cellular domain of the TNFα receptor (p75) fused the Fc domain of immunoglobulin IgG. Since its launch in 1998, Etanercept is used for the treatment of chronic inflammatory diseases such as Rheumatoid arthritis, plaque psoriasis and alkylosing spondylitis. Other examples of this class of drugs include Alefacept (BiogenIdec) for treatment of multiple sclerosis and Abatecept (Bristol-Myers Squibb) also for treatment of rheumatoid arthritis. Fusion proteins are an established class of drugs with medicines such as Etanercept projected to reach $5 billion in global sales by 2010.[3] Native biologics is a class of drugs that are also referred to as secreted proteins or replacement therapies and it represents the earliest category of biotherapeutic drugs approved by the US FDA. It includes erythropoeitins, interferons, insulins, clotting factors, growth hormones and interleukins. By market size, this class of Biopharmaceutical products dominates the global portfolio of protein-based drugs. Erythropoeitins alone drew close to $10 billion in worldwide sales in 2004 and the number is still growing.[4]

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Protein Drug Discovery

Screening

Discovery of Biopharmaceuticals, as with any modern medicine, begins with the identification of a molecular "target", one that is closely associated with disease. The target can be either a missing or an overproduced or a malfunctioning protein or its derivative. A therapeutic hypothesis is formulated around the function of the target protein. Using recombinant DNA techniques, a series of candidate therapeutic proteins are then generated to test the hypothesis. The strategy to screen candidates depends on the type of protein being discovered. For fusion proteins and native biologics, screens tend to be relatively small with evaluation of 10-20 different proteins for lead identification. These candidates generally include variants with small (1-2 amino acid) modifications. In the case of receptor fusion proteins changes are made either in the receptor ectodomain or in the hinge region and in the more rare case in Fc portion of the protein. In the case of native biologics, the variants can be single or double amino acid changes introduce to improve expression or stability either as it relates to product formulation or to pharmacokinetics. In some cases, variants can also include engineering of the carbohydrate composition of the protein for improved pharmacokinetics.[5] The small panel of proteins is then evaluated in a number of in vitro assays to select the most suitable lead protein.

Screens for monoclonal antibodies can be much larger. While monoclonal antibodies have been around now for more than three decades, their development as therapeutic agents is relatively recent however. Screening for monoclonal antibodies has traditionally relied on the hybridoma process developed by Kohler and Milstein in 1975. The process involves somatically fusing rodent B-cells derived from spleen or lymph node with mouse myeloma cells such as Sp2/0. Successfully fused cells can be screened for antibodies that selectively bind target antigen. Typically, primary screens are based on ELISA or a FACS assay. Increasingly however, the trend has been to carry out functional assays upfront where possible. As an example, a reporter gene assay can be used as a primary screen to identify hybridomas producing ligand-neutralizing antibodies or in the rare case a receptor-activating antibody. However since any initial screening is done using pools of typically 500 hybridoma cells per well, cloning by limited dilution must be carried out to isolate single hybridomas from the pool. This is typically done over one or two rounds of subcloning before the monoclonality of the hybridoma is established. Further, not all hybridomas grow at the same rate. This requires constant monitoring of the pool to ensure all possible hits from the fusion screen are isolated and rescued.

Little has changed in the hybridoma technique since the days of Kohler and Milstein. While being overall robust, the hybridoma process is not very efficient. Typically only 1-2% of B-cells in the spleen or lymph node of an immunized rodent are actually antigen specific.[6] The somatic cell fusion technique used to produce hybridomas is blind to the specificity of the B-cell. Thus, a number of irrelevant B-cells fuse along with relevant clones. Moreover, cell fusions conducted using the classic PEG reagent are inefficient with less than 1 in 20,000 B-cells resulting in a viable hybridoma.[7] Techniques such as electric field-induced hybridization techniques have been reported to improve fusion efficiency. Regardless, hybridomas can sometimes be unstable resulting in a loss of antibody producing genes over time.

To overcome these limitations new techniques such as the Selected Lymphocyte Antibody Method (SLAM) have been developed.[8] The aim of these approaches is to bypass the somatic cell fusion by directly sampling B-cells and to rescue antibody-encoding genes from a cells of interest. The SLAM approach consists of two steps. In the first step, biotinylated target antigen is covalently coupled with streptavidin coated sheep red blood cells. Next antibody producing B-cells are mixed with antigen coated sheep blood cells followed by addition of anti-IgG rabbit anti-serum and guinea pig serum as source of complement. Formation of plaques is then visualized under a microscope. In the second step, a desired B-cell, identified from a large pool of lymphocytes by its ability to form hemolytic plaque, is isolated using a micropipette. Antibody producing genes from the single B-cell are then isolated using RT-PCR followed by cloning of the VH and VL

domains. The advantage of the SLAM approach is that it directly samples antibody producing B-cells bypassing the hybridoma fusion step. This allows screening of a much higher number of B-cells (500,000) versus typically 2000-4000 wells in a hybridoma fusion. Rare B-cell clones can therefore be more efficiently isolated from a vast pool of irrelevant clones.

An approach different from the screening of hybridomas is based a completely new technology called phage display that does not require immunization of animals or screening of antibody producing cells. This approach is based on first directly isolating antibody encoding genes from B-cells and then cloning them as repertoires of individual antibodies in the form of binding fragments.[9] These can be either single-domain antibodies, single-chain Fv of Fab fragments. These repertoires can then be displayed on hosts such as filamentous bacteriophage. The advantage of this system is that it not only obviates the need to carry out lengthy immunizations but it also removes any in vivo biases around immunodominant epitopes on target antigens. Further, since bacteriophage can be cultured more easily than antibody producing mammalian cells, the system lends itself to automation that can be integrated into high-throughput screening methodologies.

The recombinant antibody approach begins by mass cloning of antibody genes derived either from an immunized source such as a rodent or even a human exposed to pathogens or infectious agents. With sequencing of the human genome now complete, it is relatively straightforward to design sets of degenerate primers to amplify by PCR genes encoding antibody variable domains. Once the individual gene fragments are cloned the actual format by which they are assembled can vary. The most widely used format is that of the single-chain Fv wherein the heavy and the light chain variable regions of the antibody are linked through a 15-20 amino acid flexible linker. However, several labs have also reported the successful use of the Fab format. The scFv is a less stable molecule and one that is more prone to aggregation than the Fab.[10] However, its single gene construct means it is easier to manipulate. Also, scFv's in general express better than Fabs.[11] Smaller fragments such as single-domain antibodies have also been successfully cloned as recombinant repertoires however bigger fragments particularly those that contain the Fc portion have proved to be challenging.

Cloned ensembles of antibody genes can then be propagated in bacteria or in yeast.[12] By creating a genetic fusion between the antibody binding domain (scFv or Fab) and host surface protein (e.g., phage pIII or yeast Aga2) a link between genotype and phenotype is created. This allows mass screening of antibody repertoire through affinity driven selections. Only those phage or yeast that display binders get selected while the rest get washed away. Selected phage or yeast are then rescued and amplified for successive rounds of selection and amplification. At the end of 3-4 such cycles, phage or yeast are then sampled individually for binding activity. Those phage or yeast that display using binding properties are rescued and antibody genes contained within them isolated for further manipulations.

One of the key advantages of such in vitro screening methods is that the antibody selectivity can be driven based on the desired outcome. For example, if antibodies against a particular epitope or domain of the antigen are desired then counter-selections against undesired regions can be carried out. Or if antibodies with slow off-rates are to be selected then phage or yeast can be incubated in buffer at incubation times before elution. In cancer applications, antibodies that bind surface antigens and get internalized may be of value. In vitro approaches allow direct selection of these types of antibodies.[13] Perhaps the ultimate screening approach is one where antibody repertoires are directly selected in vivo. This has been successfully demonstrated.[14] However, its utility to the drug discovery process remains to be proven.

Optimization

Proteins, like small molecule drugs, frequently require optimization before lead candidates can be identified for further development. Often, the optimization is focused towards improvement of binding affinity or selectivity. But it can also include reduction of potential for immunogenicity or improvement of solubility of stability characteristics. For fusion proteins or native biologics

since the initial screens are small, sometimes optimization in included upfront as in the case of darbopoetin alfa.[5]

Over the past decade several different optimization technologies have been developed. It is not possible to review them exhaustively here. However, it is important to highlight a few key technologies that have had the most impact in the protein optimization process. Most protein optimization technologies have essentially relied on two key steps. The first step has been to generate chemical diversity within the protein through a variety of mutagenesis techniques. Depending on the size of the diversity created, the second step is to use any one of a number of "Display" technologies to sort through the libraries and isolate important variants. There are also semi-rational approaches based on protein structural modeling that generate small and focused chemical diversity that can be screened using standard methodologies.

One of the early approaches used to generate diversity was the use of error-prone PCR.[15] The approach relied on the low fidelity of the polymerases used in the PCR process to randomly create mutations across a stretch of DNA. When the low fidelity of an enzyme like Taq polymerase is combined with certain PCR conditions such as high Mg2+ concentrations, the error rate can be as high as 0.01 mutation/bp/PCR cycle. The advantage of the error-prone PCR process is that it is simple, it introduces mutations across the gene of interest and can therefore be very useful for identify structure/function "hotspots". The disadvantage is that biases with certain polymerases have been reported and newer polymerases have been engineered to overcome the problems but still the overall mutational rate achieved is low. A different approach, targeted mutagenesis, works better when there is knowledge of where mutations need to be introduced as in the case antibody CDR regions.[16] This approach generates far more chemical diversity than error-prone PCR but in a more restricted portion of the sequence. Targeted mutagenesis can involve any number of oligonucleotide-mediated strategies. This can include spiking wild type nucleotides or codons with mutants or replacing stretches of sequences with nucleotides or codons that are completely randomized such that each nucleotide is substituted by every other possibility. Thus if three nucleotides are substituted with four possibilities at each position (G, A, T or C) then this generates 3^4 or 81 possible combinations. For larger sequence stretches the permutations are even larger.

Yet another approach that combines the breadth of error-prone PCR and the depth of targeted mutagenesis is Directed Evolution.[17] Here, codons are designed to introduce all possible mutations at a given position in linear sequence. However, rather than carry this through in a combinatorial fashion all along the sequence, parallel synthesis is conducted to construct small pools of mutagenic sequence. These pools are then assayed using traditional screens to isolate functionally important variants.

Two more mutagenesis approaches are worth mentioning. One involves randomly shuffling DNA to create chemical diversity.[18] The process begins by first digesting DNA into small fragments using a restriction endonuclease such as DNase I. Digested fragments are then randomly combined and amplified using PCR to recreate the full length gene. The diversity of sequences created by this process is sorted for functionally important clones. The process is then repeated until the protein is optimized, for example, until a certain improvement in binding affinity is achieved.

For some of the mutagenesis approaches discussed above there is the limitation that the chemical diversity generated can only be partially sampled. That is because the number of possible permutations rise exponentially with linear sequence sampled whereas the maximum number of variants that can tested is around 10^{10} due to limitations in bacterial transformations. Some of these limitations are alleviated by the use of cell-free systems as described below. But another approach has been to use semi-rational or computational methods to sample protein sequence in silico before any experimental works begins.[19] The process begins with construction of a protein structural model followed by analysis of all possible sequences that permit the fold predicted by the model. Using computational resources a large number of possible sequences are screened and those predicted to retain the protein functional fold given the highest score. Only the most optimal sequences predicted by the algorithm are synthesized and experimentally tested. Thus, sequence space is reduced to fewer functionally relevant sequences.

As discussed above in those cases where very large chemical diversity is generated one needs a selection tool to sort through the enormous collection of variants. Three types of "Display" technologies have been developed and used successfully in the drug discovery process. All three technologies are based on a simple but a powerful principle: a robust link between sequence and function. In the case of phage display, fusing genes of interest with anyone of the genes encoding a phage coat protein creates this link. Several proteins have been used successfully including pIII, pVIII, pVI and pIX although pIII remains the most widely used.[20] The number of different classes of proteins displayed on phage is probably the widest. This includes peptides, single-domain antibodies, scFv's, Fabs, growth factors, cytokines and receptor ectodomains. Thus, as a library selection tool, phage display is probably the most versatile.

Ribosome display is a cell free system in which a gene of interest is transcribed and translated in vitro in a cell free environment using *E. coli*, rabbit reticulocyte or wheat germ extracts.[21] Translated polypeptides are folded in vitro and them made to remain anchored to the ribosome using long polypeptide tethers that are part of the translated sequence. By doing so, the folded protein is linked to the mRNA sequence encoding it. These protein-ribosome-mRNA complexes are then sorted through steps of affinity selection followed by rescue of functionally important complexes. Rescued mRNA is then reverse-transcribed to produce cDNA which can then be amplified by PCR followed by iterative rounds of in vitro translation and selection. The main advantage of ribosome display is that it is cell-free which means sequence diversity larger than phage libraries can be sampled ($\sim 10^{14}$). Further, steps to carry out bacterial transformations and preparation of viral stocks are obviated. Therefore, multiple parallel proteins can be optimized. The disadvantage is that the system is not as robust due to presence of ribosomes and mRNA. Therefore, selection conditions have to be extensively optimized.

A relatively new entrant into the display technology field is yeast display.[22] In this approach a the genetic linkage between structure and function is created by fusing a gene of interest to the Aga2p gene which encodes the adhesion subunit of the yeast agglutinin protein. Unlike phage or ribosomes, yeast is a eukaryote which means that folding and posttranslational modification is different and closer to that in mammalian cells. Further, through the fusion process described above 10,000-100,000 copies of the protein of interest are displayed per cell. This means quantitative screening of variants can be carried out by fluorescence activated cell sorting permitting both equilibrium and kinetic selections. A limitation of yeast display is smaller levels of transformations than permissible in phage or ribosome display thereby limiting the amount of sequence space that can be sampled.

Novel Agents

As mentioned in the introduction, most biopharmaceutical products can be classified as monoclonal antibodies, receptor fusion proteins or native biologics. However, there are some products that do not fall in any of these categories. Two of them are worth mentioning under the category of novel agents.

The first of this novel set of proteins are antibody drug conjugates. These novel agents are part protein and part small molecule drugs. The novelty comes from the role played by each component in producing a safe, pharmacological effect. It is well recognized in fields such as Oncology that if chemotherapeutic drugs could be made safer such that they more selectively kill cancer cells than normal cells, it would greatly improve their safety profile. Antibody drug conjugates do just that. An excellent example of this mechanism is Gemtuzumab Ozogamicin which is approved as monotherapy for treatment of relapsed acute myeloid leukemia.[23] This drug consists of three components: a humanized monoclonal antibody directed against the CD33 antigen expressed on myeloid cells, a hydrolysable bifunctional linker and calicheamicin, a potent cytotoxic drug. By itself, calicheamicin is too toxic and cannot be used without serious side effect. Without the drug the antibody by itself has no effect. However, coupling the two allows the drug to be selectively delivered to target positive cells minimizing nonhematologic toxicity. Upon binding to CD33, the conjugate gets internalized into endosomes whereupon the linker holding the calicheamicin gets

hydrolysed. Calichaemicin gets released resulting in cell death. Since the approval of Gemtuzumab, this type of approach has been used for the development of several conjugates for both hematologic malignancies as well as for solid tumors. In such cases a variety of cell surface antigens such as ErbB2 or CD30 have been targeted using chimeric or humanized monoclonal antibodies conjugated to other cytotoxic drugs such as maytansinoid or aurastatin.[24-25]

Another class of biopharmaceutical drugs that are novel agents is one composed of proteins that exert their pharmacologic effect through the implantation of a device. An example of this class of products is recombinant human bone morphogenetic protein -2 (rhBMP2).[26] rhBMP2 is a member of the TGFβ superfamily that has strong osteogenic properties. This protein is too potent to be delivered systemically. But when administered along with absorbable collagen sponge (ACS) as a matrix, rhBMP2/ACS is effective at inducing de novo bone formation. The drug has been approved by the FDA for three distinct indications in the orthopedic area—interbody spinal fusions, open tibial fractures and for autogenous bone grafts. The choice of the delivery device for rhBMP2 has a strong effect on its clinical activity. It was the development of a suitable carrier that took up a significant time in the clinical development of rhBMP2.[26] Given that several other members of the TGFβ superfamily are candidates as drugs in the muscle, tendon and bone repair areas, lessons learnt from the development of rhBMP2 would help these programs move forward.

Challenges and Opportunities

Perhaps, where protein drug discovery most differs from discovery of conventional drugs is the attention that is paid to maintenance of human composition of the protein drug, to efficient protein synthesis in cultured cells and to the preparation of the drug for delivery by injection. Because conventional drugs are synthetic organic molecules, these considerations normally do not apply. Another difference is that certain tests carried out to verify potential side effects of conventional medicines are not necessary for Biopharmaceuticals. This is because Biopharmaceuticals do not inhibit the function of normal human proteins found in the liver (e.g., cytochyeme p450) or in other critical organs such as the heart (e.g., hERG). Outside of this, criteria to establish safety and efficacy are quite similar between protein and conventional drugs.

The early monoclonals made using the hybridoma process were murine in origin. When tested in clinical trials these proteins were quickly recognized as 'foreign" by the human immune system and eliminated. There was thus the need for approaches to reduce the potential immunogenicity of proteins by reducing their nonhuman content. Early attempts in this regard were oriented towards development of chimeric antibodies. These molecules possessed the minimal binding domains derived from mouse origin with the remainder of the constant domain derived from human IgG. Chimerization reduced the risk associated with immunogenicity and successful product launches were made possible—Rituximab for treatment of non-Hodgkins lymphoma and Cetuximab for head and neck cancer. However, chimeric antibodies did not completely rule out the risk for immunogenicity as there still remained significant amounts of mouse protein in the molecule. Three new technologies arose to fulfill this need and all three have now resulted in successful products.

In a series of advances, the murine content of antibodies was reduced using protein engineering-techniques. The first development was to "humanize" mouse antibodies. In an approach pioneered by Winter and colleagues complementarity-determining regions (CDRs) of murine antibodies were grafted on human framework regions such that the CDRs were the only mouse protein in the antibody.[27] However, a problem with this method was the observation that simply grafting CDRs of mouse antibodies on human antibody framework regions resulted in a loss of binding affinity. This is because the CDRs fold in the form of loops which must be correctly positioned by the frameworks for optimal binding. In a second approach pioneered by Queen, problems associated with CDR grafting were successfully solved.[28] The Queen approach required changes at key framework positions using sequence alignment between the mouse donor sequence and the human acceptor sequence and also by the use of computer models. This approach was vastly successful in overcoming the problems associated with antibody humanization. Indeed, the first

generation of antibodies approved by the FDA was humanized antibodies such as Trastuzumab, Omaluzumab and Natalizumab.

However, the early nineties saw the emergence of two novel technologies that shaped the discovery of the current generation of monoclonal antibodies. The first was the development of phage display technology discussed above. While the early proof-of-concept experiments conducted to validate this technology were based on construction of peptide libraries of single-domain antibodies, the field rapidly expanded to included phage display libraries of scFv and Fab fragments. Construction of large diverse phage repertoires allowed scientists to bypass the hybridoma process and obtain antibodies through completely in vitro approaches. However, antibodies isolated using this approach were not always of the highest affinity as they had not gone through the somatic hypermutation process that antibodies from immunized sources had. Larger repertoires or those derived from autoimmune sources were made to mitigate the problem but the affinity problem was not completely solved.

A second technology that arose was the development of transgenic mouse strains harboring loci of human Ig genes. In this technology, genes encoding endogenous mouse Ig were first inactivated using gene-targeting techniques. This was followed by the systematic introduction of large chunks of the human Ig loci (H, κ and λ) using yeast artificial chromosomes into the mouse germline.[29] Reconstituted mice stably expressed human immunoglobulins at normal levels and had normal B-cell development. Most importantly, when challenged with a human protein as an immunogen, these mice mounted a strong immune response of fully human antibodies which included class switch recombination as well as somatic hypermutation. Thus, creation of such transgenic animals obviated the need to first create a mouse monoclonal antibody and then to carry through humanization. The fact that transgenic mice mounted both a primary and a secondary response meant that high affinity, fully human antibodies of the IgG class could be readily obtained. However, since the process of making such antibodies relied on immunization and screening of hybridomas limitations to the traditional hybridoma approach still applied.

Next Generation Proteins

The nature of biopharmaceutical drug discovery has undergone a fundamental change in the past 5-7 years. Drawing upon the success of first and second-generation protein pharmaceuticals, namely secreted factors such as erythropoetin and receptor fragments such as etarnecept, the industry has increasingly shifted towards the development of monoclonal antibodies as therapeutic agents. Recent product launches by major biotech companies as well as late stage biopharmaceutical pipeline candidates are now mostly monoclonal antibodies. Genentech's Bevacizumab and Wyeth's Bapineuzumab are excellent examples of this trend. However, the industry has also faced several challenges.

While hugely successful, monoclonal antibodies are still very large, complex molecules that require significant engineering at the molecular level to be effective. Enabling technologies to carry out this type of engineering are often held by small biotech companies where access can be restructured. More importantly however, in the recent months, Big Pharma has serially acquired biotech companies that pioneered the development of these technologies.

Another trend that has affected the biopharmaceutical industry has been the rapid advances in protein sequence, structure and function coupled with the commercial need for newer, cost-effective protein therapies. This trend has led to the development of technologies to exploit novel protein scaffolds as therapeutic precursors. Strategies include engineered fragments of monoclonal antibodies as well as nonantibody scaffolds. These strategies have recently been reviewed elsewhere and are referenced here.

Conclusion

The US Food and Drug Administration approved the first protein drug developed using recombinant DNA technology (human insulin) in 1982. Thus, the protein drug industry is relatively young when compared with the traditional drug industry. As discussed in this chapter, significant

new technologies developed in the recent years promise to allow rapid advancement of protien drugs. Biopharmaceuticals have enjoyed tremendous success in the recent years judged by the surge of approvals by the FDA. This success draws upon technological advances made in the field of protein therapeutics but also on a greater realization that there are significant opportunities within pharmaceutical drug development to exploit the power of biopharmaceuticals. Indeed, we are now witnessing the emergence of protein drug development opportunities in areas such as metabolic disorders, Alzheimer's disease and osteoporosis. Traditionally, these areas were reserved for conventional drug discovery. As we move forward into the new millennium, it is hoped that the synergy between biopharmaceuticals and conventional medicines can be further leveraged for safer and more cost-effective treatments. If successful, these therapies will address significant unmet medical needs whose aggregate cost to the healthcare systems worldwide runs into tens of billions of dollars each year.

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CHAPTER 4

The Role of Nanobiotechnology in Drug Discovery

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Abstract

The potential applications of nanotechnology in life sciences, particularly nanobiotechnology, include those for drug discovery. This chapter shows how several of the nanotechnologies including nanoparticles and various nanodevices such as nanobiosensors and nanobiochips are being used to improve drug discovery. Nanoscale assays using nanoliter volumes contribute to cost saving. Some nanosubstances such as fullerenes are drug candidates. There are some safety concerns about the in vivo use of nanoparticles that are being investigated. However, future prospects for applications in healthcare of drugs discovered through nanotechnology and their role in the development of personalized medicine appear to be excellent.

Introduction

Current drug discovery process needs improvement in several areas. Although many targets are being discovered through genomics and proteomics, the efficiency of screening and validation processes need to be improved. This chapter will show how nanotechnology will play a role in improving this process. Nanotechnology is the creation and utilization of materials, devices and systems through the control of matter on the nanometer scale. Given the inherent nanoscale functional components of living cells, it was inevitable that nanotechnology will be applied in life sciences giving rise to the term nanobiotechnology.[1] Technical achievements in nanotechnology are being applied to improve drug discovery, drug delivery and pharmaceutical manufacturing. A product incorporating the NanoCrystal® technology of Elan Drug Delivery Inc (King of Prussia, PA), a solid-dose formulation of the immunosuppressant sirolimus, was approved by the FDA in 2000. Nanotechnologies are already being used in molecular diagnostics.[2] Analyses of signaling pathways by nanobiotechnology techniques may provide new insight into the understanding of disease processes, developing more efficient biomarkers and understanding mechanisms of action of drugs. This will help in designing new approaches to drug discovery. Various nanotechnologies used for drug discovery are listed in Table 1 according to various stages of drug discovery.[3] Selected technologies are described briefly in the following text.

Role of Nanoparticles in Drug Discovery

Older imaging tools such as fluorescent dyes or polymer spheres are either too unstable or too big to effectively perform single-molecule tracking. The role of nanoparticles for drug discovery has been explored but no one type of nanoparticle is suitable for universal application in drug discovery.

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Table 1. Application of nanobiotechnology at various stages of drug discovery

Target identification and validation
Nanoproteomics
Single wall carbon tube nanosensors
High-field asymmetric waveform ion mobility mass spectrometry
Investigating biomolecular interactions with atomic force microscopy
Study of molecular interactions using cantilevers
Study of molecular interactions using nanoprobes
Lead identification
Biosensor
Nanowire devices
Assays based on endocytosis at the nanoscale
Surface plasma resonance
Nanofluidics, nanoarrays and nanobiochips
Nanoflow liquid chromatography
Lead optimization
Nanoparticles
Quantum dots
Gold nanoparticles
Lipoparticles
Use of small molecules attached to nanoparticles
Fluorescence planar wave guide technology
Nanomaterials as drug candidates
Dendrimers
Fullerenes
Nanobodies

Quantum Dots for Drug Discovery

The use of quantum dots (QDs) for drug discovery has been explored.[4] Several QDs are commercially available. Qdot™ conjugates (Quantum Dot Corporation, Hayward, CA) can produce photo resolutions up to eight times more detailed than the older imaging tools. The Qdot™ conjugates are almost "an order of magnitude" brighter than fluorescent dyes and can be observed for as long as 40 minutes compared to about five seconds for the dyes. Length of observation time is critical to studying cellular processes, which change rapidly over a span of several minutes. Since cellular receptors are critical targets for new drug candidates, a more detailed understanding of the behavior of these receptors can open up new treatment options. QDs are ideal for targeting cancer for both diagnosis and therapy. However, some limitations for QD use in drug discovery studies have yet to be resolved, i.e., toxicity, size variation, agglomeration, potential multiple drug attachment to a single QD and blinking.

Gold Nanoparticles

Gold nanoparticles are the most commonly used nanomaterial in diagnostics and have many other uses as well such as a connecting point to build biosensors for detection of disease DNA. Instead of a fluorescent molecule, a gold nanoparticle can be attached to the antibody and other molecules such as DNA, which can be added to the nanoparticle to produce bar codes. Because many copies of the antibodies and DNA can be attached to a single nanoparticle, this approach is much

more sensitive and accurate than the fluorescent-molecule tests used currently. Although they can be used for drug discovery, they need to be combined with another technology for visualization.

Gold nanoparticles have been used to demonstrate multiphoton absorption induced luminescence (MAIL), in which specific tissues or cells are fluorescently-labeled using special stains that enable them to be studied. Gold nanoparticles can emit light so intense that it is readily possible to observe a single nanoparticle at laser intensities lower than those commonly used for MAIL, i.e., sub-100-fs pulses of 790-nm light.[5] Moreover, gold nanoparticles do not blink or burn out, even after hours of observation suggesting that metal nanoparticles are a viable alternative to fluorophores or semiconductor nanoparticles for biological labeling and imaging. Other advantages of the technique are that the gold particles can be prepared easily, have very low toxicity and can readily be attached to molecules of biological interest. In addition, the laser light used to visualize the particles is a wavelength that causes only minimal damage to most biological tissues. This technology could enable tracking of a single molecule of a drug in a cell or other biological samples.

Small Molecules Attached to Nanoparticles

Multivalent attachment of small molecules to nanoparticles can be used to increase specific binding affinity and to reveal new biological properties of such nanomaterials. Gelatin nanoparticles have been used for the attachment of biotinylated anti-CD3 antibodies by avidin-biotin complex formation to provide a carrier system for specific drug targeting to T-lymphocytes.[6] A union between small molecule chemistry and nanotechnology, has the potential of development of a wide range of nanomaterials for biomedical application.[7] One of the applications is in target screening in high throughput drug discovery. This technique can enable small molecule modification to impart desirable biological functions for in vivo visualization of targets and for delivery of therapeutics thus enabling combination of diagnostics and therapeutics, particularly in case of cancer.

Role of Nanoproteomics in Drug Discovery

Proteomics is playing an important role in the target identification and validation phases of the drug discovery process. Application of nanotechnologies in proteomics has been termed nanoproteomics, which is an extension of the scope of proteomics on nanoscale. Most current protocols including protein purification/display and automated identification schemes yield unacceptably low recoveries, thus limiting the overall process with respect to sensitivity and speed and the requirement of greater amounts of starting material. Low abundant proteins and proteins that can only be isolated from limited source material can be subjected to nanoscale protein analysis, nano-capture of specific proteins and complexes and optimization of all subsequent sample handling steps leading to mass analysis of peptide fragments. Some nanotechnologies are refining the application of proteomics for drug discovery and examples are described here briefly.

A technology called magnetism-based interaction capture (MAGIC) identifies molecular targets on the basis of induced movement of superparamagnetic nanoparticles inside living cells.[8] These nanoprobes capture the small molecule's labeled target protein and are translocated in a direction specified by the magnetic field. Use of MAGIC in genome-wide expression screening can identify multiple protein targets of a drug. MAGIC can also be used to monitor signal-dependent modification and multiple interactions of proteins.

Single-Walled Carbon Nanotubes

Single-walled carbon nanotubes as a platform have been used for investigating surface-protein and protein-protein binding and developing highly specific electronic biomolecule detectors. Nonspecific binding on nanotubes, a phenomenon found with a wide range of proteins, is overcome by immobilization of polyethylene oxide chains. A general approach is then advanced to enable the selective recognition and binding of target proteins by conjugation of their specific receptors to polyethylene oxide-functionalized nanotubes. These arrays are attractive because no labeling is required and all aspects of the assay can be carried out in the solution phase. This scheme, combined with the sensitivity of nanotube electronic devices, enables highly specific

electronic sensors for detecting clinically important biomolecules such as antibodies associated with human autoimmune diseases.

Nanoflow Liquid Chromatography

The use of liquid chromatography (LC) in analytical chemistry is well established but the relatively low sensitivity associated with conventional LC makes it unsuitable for the analysis of certain biological samples. Furthermore, standard LC flow rates are frequently not compatible with the use of specific detectors, such as electrospray ionization mass spectrometers. Therefore, due to the analytical demands of biological samples, miniaturized LC techniques were developed to allow for the analysis of samples with greater sensitivity than that afforded by conventional LC. In nanoflow LC (nanoLC), chromatographic separations are performed using flow rates in the range of low nanoliter per minute, which result in high analytical sensitivity due to the large concentration efficiency afforded by this type of chromatography. NanoLC, in combination to tandem mass spectrometry, was first used to analyze peptides and as an alternative to other mass spectrometric methods to identify gel-separated proteins. Gel-free analytical approaches based on LC and nanoLC separations have been developed, which are allowing proteomics to be performed in faster and more comprehensive manner than by using strategies based on the classical 2D gel electrophoresis approaches. Protein identification using nanoflow liquid chromatography-mass spectrometry (MS)-MS (LC-MS-MS) provides reliable sequencing information for low femtomole level of protein digests. However, this task is more challenging for subfemtomole peptide levels.

An ion mobility technology, high-field asymmetric waveform ion mobility mass spectrometry (FAIMS), has been introduced as an online ion selection method compatible with electrospray ionization (ESI). FAIMS uses ion separation to improve the detection limits of peptide ions when used in conjunction with electrospray and nanoelectrospray MS. This facilitates the identification of low-abundance peptide ions often present at ppm levels in complex proteolytic digests and expands the sensitivity and selectivity of nanoLC-MS analyses in global and targeted proteomics approaches. This functionality will probably play an important role in drug discovery and biomarker programs for monitoring disease progression and drug efficacy.

Atomic Force Microscopy for Drug Discovery

Atomic force microscopy (AFM) has become a well-established technique for imaging single biomolecules under physiological conditions. The exceptionally high spatial resolution and signal-to-noise ratio of the AFM enables the substructure of individual molecules to be observed. Used as a sensor, the AFM tip can also probe the charges of biological surfaces immersed in a buffer solution. So far, such approaches have successfully characterized protein interactions but in the future they could be applied to imaging and detecting multiple parameters on a single molecule simultaneously. If a ligand is attached to the end of an AFM probe, one can simulate various physiological conditions and look at the strength of the interaction between the ligand and receptor in a wide range of circumstances. By functionalizing the tip, it can be used to probe biological systems and identify particular chemical entities on the surface of a biological sample. This opens the door to more effective use of AFM in drug discovery.

Role of Nanoscale Biosensors in Drug Discovery

Biosensors are currently used in the areas of target identification, validation, assay development, lead optimization and ADMET, but are best suited for applications related to soluble molecules. Biosensors can overcome many of these limitations of currently used cell-based assays. They are particularly useful in the study of receptors, in that biosensors do not require the removal of the receptor from the lipid membrane of the cell as might be necessary with other assay methods. A primary application of current biosensor technologies is the optimization of limited-scope drug libraries against specific targets.

Optical Biosensors

Optical biosensors capable of exploiting surface plasmon resonance (SPR), waveguides and resonant mirrors have been used widely over the past decade to analyze biomolecular interactions. These sensors allow the determination of the affinity and kinetics of a wide variety of molecular interactions in real time, without the need for a molecular tag or label. Conventional SPR is applied in specialized biosensing instruments. These instruments use expensive sensor chips of limited reuse capacity and require complex chemistry for ligand or protein immobilization. A sensitive technique is being developed for optical detection of gold nanoparticle-labeled molecules on protein microarray by applying the surface plasmon resonance and specific molecular binding using rolling circle amplification.[9]

Cantilevers

Cantilevers transform a chemical reaction into a mechanical motion on the nanometer scale and this motion can be measured directly by deflecting a light beam from the cantilever surface.[10] A state-of-the-art position sensitive detector is employed as detection device. The static mode is used to obtain information regarding the presence of certain target molecules in the sample substance. The surface stress caused by the adsorption of these molecules results in minute deflections of the cantilever. This deflection directly correlates with the concentration of the target substance. The dynamic mode allows quantitative analysis of mass loads in the sub-picogram area. As molecules are adsorbed, minimal shifts in the resonance frequency of an oscillating cantilever can be measured and associated with reference data of the target substance. Both modes can also be operated simultaneously. The controlled deposition of functional layers is the key to converting nanomechanical cantilevers into chemical or biochemical sensors. Inkjet printing is a rapid method to coat cantilever arrays efficiently with various sensor layers. Applications relevant to drug discovery include label-free biochemical assays and investigation of biomolecular interactions as well as multiplexed assays. By attaching specific antibodies to cantilevers the simultaneous imaging of target antigens and identification of antigen-antibody interactions have been demonstrated.

Nanofluidics, Nanoarrays and Nanobiochips

Nanofluidics implies extreme reduction in quantity of fluid analyte in a microchip compared with standard methods. The use of the word "nano" in nanoliter (nl) is in a different dimension than in nanoparticle, which is in nanometer (nm) scale. From one printing run that consumes <1 nanomole of each compound, large combinatorial libraries can be subjected to numerous separation-free homogeneous assays at volumes that are a small fraction of those used in current high-throughput methods.

Nanoarrays are the next stage in the evolution of miniaturization of microarrays. Whereas microarrays are prepared by robotic spotting or optical lithography, limiting the smallest size to several microns, nanoarrays require further developments in lithography strategies such as electron beam lithography, dip-pen nanolithography, scanning probe lithography, finely focused ion beam lithography and nano-imprint lithography. Nanoarrays can measure interactions between individual molecules down to resolutions of as little as one nanometer and can be used in bioaffinity tests for proteins, nucleic acids and receptor-ligand pairs.

Nanomaterials as Drug Candidates

Dendrimers

Dendrimers are a novel class of three-dimensional nanoscale, core-shell structures that can be precisely synthesized for a wide range of applications. Specialized chemistry techniques allow for precise control over the physical and chemical properties of the dendrimers. They are most useful in drug delivery but can also be used for the development of new pharmaceuticals with novel activities. Polyvalent dendrimers interact simultaneously with multiple drug targets. They can be developed into novel targeted cancer therapeutics. Dendrimers can be conjugated to different biofunctional

moieties such as folic acid using complementary DNA oligonucleotides to produce clustered molecules, which target cancer cells that over-express the high affinity folate receptor.[11]

Fullerenes

A key attribute of the fullerene molecules is their numerous points of attachment, allowing for precise grafting of active chemical groups in three-dimensional orientations. This attribute, the hallmark of rational drug design, allows for positional control in matching fullerene compounds to biological targets. In concert with other attributes, namely the size of the fullerene molecules, their redox potential and its relative inertness in biological systems, it is possible to tailor requisite pharmacokinetic characteristics to fullerene-based compounds and optimize their therapeutic effect [25].

Fullerene antioxidants bind and inactivate multiple circulating intracellular free radicals, giving them unusual power to stop free radical injury and to halt the progression of diseases caused by excess free radical production. Fullerenes provide effective defense against all of the principal damaging forms of reactive oxygen species. C-60 fullerene has thirty conjugated carbon-carbon double bonds, all of which can react with a radical species. In addition, the capture of radicals by fullerenes is too fast to measure and is referred to as "diffusion controlled", meaning the fullerene forms a bond with a radical every time it encounters one. Numerous studies demonstrate that fullerene antioxidants work significantly better as therapeutic antioxidants than other natural and synthetic antioxidants, at least for CNS degenerative diseases. In oxidative injury or disease, Fullerene antioxidants can enter cells and modulate free radical levels, thereby substantially reducing or preventing permanent cell injury and cell death. A tris-malonic acid derivative of the fullerene C-sixty molecule (C3) functionally replaces manganese superoxide dismutase and acts as a biologically effective superoxide dismutase mimetic.[12]

Nanobodies

Nanobodies (Ablynx , Ghent, Belgium) are the smallest available intact antigen-binding fragments harboring the full antigen-binding capacity of the naturally occurring heavy-chain antibodies. Nanobodies have the potential of a new generation of antibody-based therapeutics as well as diagnostics for diseases such as cancer.[13] They are extremely stable and bind antigen with nanomolar affinity. They combine the advantages of conventional antibodies with important features of small molecule drugs and can address therapeutic targets not easily recognized by conventional antibodies such as active sites of enzymes.

Nanobiotechnology and Drug Discovery for Personalized Medicine

Personalized medicine simply means the prescription of specific treatments and therapeutics best suited for an individual. It is also referred to as individualized or individual-based therapy. Personalized medicine is based on the idea of using a patient's genotype as a factor in deciding on treatment options but other factors are also taken into consideration. Molecular diagnostics is an important component of personalized medicine and nanobiotechnologies are already being used in molecular diagnostics. Although current efforts using pharmacogenomics and pharmacogenetics include matching the existing drugs to the right patients for optimal efficacy and safety future personalized medicines could be discovered and designed for specific groups of patients using pharmacoproteomics. Nanobiotechnology shows promise of facilitating discovery of personalized medicines apart from facilitating integration of diagnostics and therapeutics.[14]

Conclusion

The examples given in this chapter cover a number of different nanotechnologies. Some of these are already established in research through other well known technologies such as biosensors and biochips. Nanoparticles are still used extensively for developing diagnostics and some of the assays for drug discovery.

With a large number of nanotechnologies and nanomaterials, no generalizations can be made about the overall safety and toxicity. In vitro diagnostic use does not carry any risks to people

but there is a concern for in vivo use of nanoparticles, particularly those that are less than 50 nm, which can enter the cells. There are still many unanswered questions about their fate in the living body. Because of the huge diversity of materials used and the wide range in size of nanoparticles, these effects will vary a lot. It is conceivable that particular sizes of some materials may turn out to have toxic effects. Further investigations will be needed. The FDA approval is essential for clinical applications of nanotechnology and substantial regulatory problems may be encountered in the approval of nanotechnology-based products. Pharmaceuticals, biologicals and devices are all regulated differently by the FDA and it is not yet clear how emerging nanotherapeutics will be evaluated.

Future of Nanotechnology-Based Drug Discovery

An increasing use of nanobiotechnology by the pharmaceutical and biotechnology industries is anticipated. Apart from innovations based on nanoparticles, several other nanotechnologies are in development for application in life sciences. In the near future, it may be possible to fully model an individual cell's structure and function by computers connected to nanobiotechnology systems. Such a detailed virtual representation of how a cell functions might enable scientists to develop novel drugs with unprecedented speed and precision without any experiments in living animals.

Nanotechnology will be applied at all stages of drug development—from formulations for optimal delivery to diagnostic applications in clinical trials. Nanobiotechnology would fit in with the concepts for integration of diagnostics and therapeutics for the development of personalized medicine.

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CHAPTER 5

Conotoxin Venom Peptide Therapeutics

Richard J. Lewis*

Abstract

Venom peptides offer enormous opportunity for the discovery of peptide drug leads. This review focusses on the potential of cone snails that have developed arrays of small peptides as part of highly evolved venoms used for prey capture and defence. Many of these peptides selectively modulate ion channels and transporters, making them a valuable source of new ligands for studying the role these targets play in normal and disease physiology. A number of these conopeptides reduce pain in animals models and several are now in preclinical and clinical development for the treatment of severe pain often associated with diseases such as cancer.

Introduction

Venomous animals have developed rich cocktails of peptides they deliver through specialised envenomation apparatus into the soft tissue of animals. These venom peptides have diverse and selective pharmacologies.[1] Their evolved bioactivity makes venom peptides a unique source of bioactives from which new therapeutic agents and research tools can be developed. To-date, a linear peptide from the saliva of the gila monster lizard (Exendin-4), a disulfide bonded globular peptide from a cone snail (ω-MVIIA or Prialt) and a peptide mimetic (captopril) of a snake angiotensin converting enzyme inhibitor (teprotide) have reach the clinic to change clinical practice for the treatment of diabetes, pain and hypertension.[1] This chapter focusses on the therapeutic potential of the small bioactive peptides conotoxin or (conopeptides) produced by marine molusc of the family Conidae.

Conotoxins are amongst the most interesting of the venomous species. They have evolved hundreds of highly selectivity peptides that help immobilise their prey of either including fish, molluscs or worms. Their small size, relative ease of synthesis, structural stability and target specificity make them ideal pharmacological probes (Table 1). Somewhat surprisingly, many of these classes of conotoxins act on pain targets, allowing the *specific* dissection of key ion channels and receptors underlying pain and providing important new ligands for developing pain therapeutics. It is estimated that in excess of 50,000 conopeptides have evolved for prey immobilisation, with <0.1% characterised pharmacologically. A surprising number are highly selective for a diverse range of mammalian ion channels and receptors associated with pain pathways. In this chapter, we discuss how different classes of venom peptides from marine cone snails (Table 1) can be used to improve our understanding and treatment of pain. Conotoxins act at many of the ion channels and a smaller number of receptors found in pain pathways (Fig. 1). Of particular interest are conotoxins that inhibit pain pathways by blocking calcium and sodium channels, the nicotinic acetylcholine receptor, the norepinephrine transporter, the NMDA receptor and the neurotensin receptor.

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Table 1. Amino acid sequence and pharmacology of analgaesic conopeptide classes

Class[a]	Name	Sequence[b]	Pharmacology
χ	MrIA	NGVCCGYKLCHOC	Norepinephrine transport inhibitor
α	Vc1.1	GCCSDPRCNYDHPEIC*	Neuronal nicotinic AChR inhibitor
μ	PIIIA	RLCCGFOKSCRSRQCKOHRCC*	TTX-sensitive sodium channel inhibitor
μO	MrVIB	ACSKKWEYCIVPIIGFIYCCPGLICGPFVCV	TTX-resistant sodium channel inhibitor
ω	MVIIA CVID	CKGKGAKCSRLMYDCCTGSCRSGKC* CKSKGAKCSKLMYDCCSGSCSGTVGC*	N-type calcium channel inhibitor
Conantokins	Conantokin-G	GEγγLQγNQγLIRγKSN	NMDA antagonist
Contulakins	Contulakin-G	pESEEGGSNAT$_g$KKPYIIL	Neurotensin receptor agonist

[a]Sequences of representative conopeptides of each class are shown. Conopeptides were isolated from fish hunters *C. geographus* (G) *C. magus* (M), *C. catus* (C), *C. purpurascens* (P), or mollusc hunters *C. marmoreus* (Mr) and *C. victoriae* (Vc).
[b]B is 6-bromotryptophan, O is *trans*-4-hydroxyproline, γ is γ-carboxyglutamic acid, T_g is glycosylated threonine and *amidated C-termini. Cysteines involved in disulfide bonds (bolded) that connect is discrete patterns depending on sequence.

Calcium Channel Inhibitors

It has long been established that Ca^{2+} influx into nerve terminals through calcium channels initiates the release of neurotransmitter which allow signalling to other nerves and muscle. In recent years, much has been learned about the nature of these channels which open in response to cell depolarisation. These channels have been classified into six groups according to their electrophysiological and pharmacological properties, termed L-, N-, P-, Q-, T- and R-types. Given this diversity, there is considerable opportunity to develop selective inhibitors of calcium channels playing a key role in pain pathways.

ω-Conotoxins from cone snails are unique tools with which to identify and determine the physiological role of different neuronal calcium channels. Since N-type calcium channels play a key role in pain transmission (see Fig. 1), it is not surprising that ω-conotoxins specific for N-type channels are potent analgesics.[2-4] Extensive structure-activity relationship studies have allowed the development of a pharmacophore model for ω-conotoxins[5] that allows the rational development of further specific N-type inhibitors, including macrocylic peptides and peptidomimmetics.[6] Sub-nanomolar doses of ω-conotoxin MVIIA (ω-MVIIA) or ω-CVID delivered directly to the spinal cord (intrathecally) produce analgesia for up to 24 hr in rats.[11] ω-MVIIA (Prialt, Elan) recently gained FDA approval for the treatment of otherwise unmanageable severe chronic pain. However, development ω-CVID (AM336, AMRAD) has not progressed beyong a Phase I/IIa clinical trial in severe cancer pain sufferers, where is produced clear signs of efficacy. Unfortunately, both ω-conotoxins produced unwanted side effects at therapeutic doses. The discovery of new ω-conotoxins with selectivity profiles that produce fewer side effects may lead to the development of better analgaesics in these classes.

Sodium Channel Inhibitors

Like the structurally related calcium channels, sodium channels play a key role in controlling neuronal excitability, but in this instance they are critical for initiation and transmission of signals along nerve projections. Based on their susceptibility to block by the puffer fish toxin tetrodotoxin (TTX), sodium channels can be divided into TTX-sensitive (TTX-S) and TTX-resistant

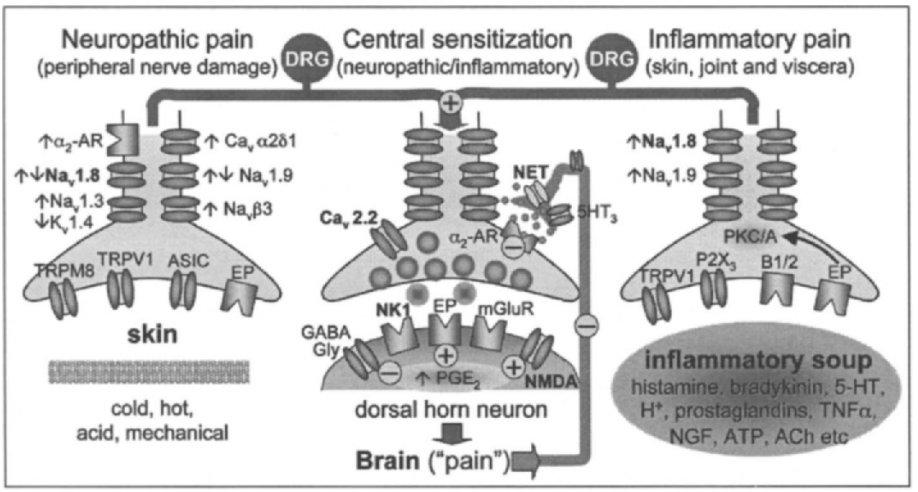

Figure 1. This figure illustrates targets associated with acute and chronic pain states. "Pain" signals arising in the periphery (e.g., the skin, a wound, or in bone cancer) travel through nerves to a key excitatory synapses in the spinal cord. Depending on the strength of this signal and other activating or inhibitory influences, this signal can enter the brain and be perceived as pain of varying intensities. The complexity of the different inflammatory and neuropathic pain types and their associated specific pathways, has frustrated attempts to rationally develop new classes of pain therapeutics. The specificity of conotoxins may circumvent this problem, especially if they can be discovered or developed to act selectively at specific targets expressed by nociceptive primary afferent nerves. Attractive targets include TTX-R channels ($Na_v1.8$), the TTX-S channels ($Na_v1.7$), capsaicin sensitive channels (TRPV1) and the pH sensitive channels (ASICs), as well as neurokinin, neurotensin and glutamate (NMDA) receptors and the descending inhibitory pathway associated with norepinephrine release and reuptake (NET). Bolded targets are inhibited by conotoxins. Several classes of conopeptides (ω-, χ-, α-conopeptides and the contulakins, see Table 1) have entered clinic development as novel analgaesic for chronic pain. ω-MVIIA (Prialt, Elan) is now approved by the FDA to treat otherwise unmanageable severe chronic pain (figure modified from ref. 1).

(TTX-R) classes. A number of these sodium channel subtypes are implicated in clinical states such as pain (see Fig. 1)[7] as well as stroke and epilepsy. Given their critical role in the central and peripheral nervous system, it is not surprising that cone snails have evolved a number of different ways to target this ion channel class. However, despite subtype selective sodium channel inhibitors having considerable therapeutic potential, little progress has been made towards the development of sodium channel inhibitors from cone snails. This may change with the recent discovery that μO-conotoxins MrVIA and MrVIB, selective inhibtors of a key TTX-R sodium channel in pain pathways, is analgesic in animals without affecting mobility.[8]

Antagonists of Nicotinic Acetylcholine Receptors

The nicotinic acetylcholine receptors (nAChRs) are a family of pentameric ligand gated cation channels that play a key signalling role at synapses and neuromuscular junctions. The α-conotoxins are a large and growing class of small peptides from cone snails that competitively inhibit specific subtypes of the nAChR.[9] Muscle selective α-conotoxins (e.g., GI, see Table 1) may represent an alternative to the use of small molecule curare-mimetic muscle relaxants, which are used during surgery but have slower than ideal recovery periods. Interestingly, the novel α-conotoxin, Vc1.1, has been identified as having potential analgesic properties following peripheral administration to rats.[10] This result contrasts the analgaesic effects usually attributed to agonists of the nAChR

acting centrally and appears to be mediated by a specific subtype of the receptor with a previously unrecognised role in pain. Unfortunatley, Metabolic has discontinued clinical trials of Vc1.1 due to an anticipated lack of efficacy in humans (affinity for the human form of the receptor is reportedly much lower than for the rat form).

Norepinephrine Transporter Inhibitors

The norepinephrine transporter plays a key role in reducing levels of neuronally released norepinephrine (also known as noradrenaline). The tricyclic anti-depressants inhibit NET and appear to produce analgaesia by enhancing the descending inhibitory pathway controlled by norepinephrine release. Unfortunately, this class of drugs also have anti-depressant effects and significant off-target pharmacologies that limit there usefulness in the treatment of pain. χ-Conopeptides first isolated from *Conus marmoreus* are highly specific, noncompetitive inhibitors of norepinephrine uptake through the NET[11] that produce potent analgaesia in rats. A synthetic variant of the χ-conotoxins (Xen2174) is currently being developed as a novel analgesic by Xenome Ltd.[12] Xen2174 is currently being evaluated intrathecally in a Phase I/IIa safety trial in cancer patients suffering otherwise unmanageable pain. Initial results are promising both in terms of safety and efficacy. Interestingly, the binding site for χ-conopeptides on the NET partially overlaps the tricyclic anti-depressant binding site but not the NE binding site, providing clues to the development of noncompetitive small molecule inhibitors.[13]

NMDA Receptor Antagonists

Conantokins are specific inhibitors of the *N*-methyl-D-aspartate (NMDA) receptor. These peptides are helical in structure and competitively inhibit glutamate activation of this receptor, especially at NR2B subtype. Malmberg et al[14] showed that intrathecal conantokin G or T also have analgesic activity in pain models of tissue damage, nerve injury and inflammation in mice at doses that were ~20-fold lower than those affecting motor function. Thus, subtype specific inhibitors of the NMDA receptor also have therapeutic potential in the management of pain.

Neurotensin Receptor Agonists

Cone snails produce a glycosylated neurotensin analogue named contulakin-G[15] that is a potent analgesic in a wide range of animal models of pain.[16] Interestingly, contulakin-G is 100-fold less potent that neurotensin for NTR1, but ~100-fold more potent as an analgesic. Based on its potency and wide therapeutic window, contulakin-G (CGX-1160) went into early stage clinical development by Cognetix Inc. for the treatment of pain. Development of the conantokins and contulakins is now on hold with the demise of Cognetix.

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CHAPTER 6

Shark Novel Antigen Receptors—The Next Generation of Biologic Therapeutics?

Caroline Barelle, Davinder S. Gill and Keith Charlton*

Abstract

Over recent decades we have witnessed a revolution in health care as new classes of therapeutics based on natural biological molecules have become available to medical practitioners. These promised to target some of the most serious conditions that had previously evaded traditional small molecule drugs, such as cancers and to alleviate many of the concerns of patients and doctors alike regarding adverse side effects and impaired quality of life that are often associated with chemo-therapeutics. Many early 'biologics' were based on antibodies, Nature's answer to invading pathogens and malignancies, derived from rodents and in many ways failed to live up to expectations. Most of these issues were subsequently negated by technological advances that saw the introduction of human or 'humanized' antibodies and have resulted in a number of commercial 'block-busters'. Today, most of the large pharmaceutical companies have product pipelines that include an increasing proportion of biologic as opposed to small molecule compounds. The limitations of antibodies or other large protein drugs are now being realized however and ever more inventive solutions are being sought to develop equally efficacious but smaller, more soluble, more stable and less costly alternatives to broaden the range of drug-able targets and therapeutic options. The aim of this chapter is to introduce the reader to one such novel approach that seeks to exploit a unique antibody-like protein evolved by ancestral sharks over 450 M years ago and that may lead to a host of new therapeutic opportunities and help us to tackle some of the pressing clinical demands of the 21st century.

Introduction

The world of pharmaceutical development and the treatment of disease were revolutionized in 1982 when the first recombinant protein drug, insulin, was approved for human use. This event was the direct result of previous momentous achievements, notably in 1972 when Paul Berg at Stanford produced the first recombinant DNA (rDNA) and the following year when Herbert Boyer first transformed *E. coli* bacteria with a rDNA plasmid that included a gene encoding a human protein. Boyer went on to establish Genentech, the world's largest Biotechnology Company. The reason that this was possible at all is that the same genetic code is shared by all living organisms, reflecting their common ancestry. Initially clinical developments concentrated on generating recombinant versions of human proteins for the treatment of deficiencies, such as Factors VII, VIII and IX for blood clotting disorders and human growth hormone (hGH). Other popular products were cytokines, eg. interleukins, interferons and

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tumor necrosis factor-α. In the latter cases, it was fortuitous that these proteins exhibited the desired therapeutic effects despite being produced in bacterial expression systems that do not allow for the inclusion of posttranslational modifications, specifically glycosylation. A notable exception to this was erythropoietin (EPO), which initially had poor in vivo efficacy due mainly to its pharmacokinetic profile when not glycosylated. This problem was overcome subsequently by production of EPO in mammalian cell culture.

It was perhaps the emergence of antibodies as therapeutic reagents that have really had the most profound and exciting effect on the development of new treatments for diseases that had, until recently, evaded the best efforts of the medical community.

The Rise and Rise of Antibodies

The history of therapeutic antibodies dates back more than a century, when Emil von Behring first demonstrated that anti-sera could be used to treat conditions from snake bites to diphtheria and tetanus. These treatments were limited however to acute indications and it wasn't until nearly 100 years later when César Milstein's group generated the first monoclonal antibody producing cell line (Hybridoma) that antibody therapies became a real possibility.

Despite the ability now to generate antibodies of known and predetermined specificity in unlimited quantities from these immortal cell lines, other limitations quickly became apparent. The principal problems lay with the murine origin of these antibodies and indeed were the same as those encountered a century before. The immune systems of patients recognized these early antibody therapeutics as being 'foreign' proteins and responded accordingly by generating human anti-mouse antibodies, the so-called HAMA response. This restricted applications to acute indications where a single-dose could be administered. It required the development of several new technologies to gradually overcome these limitations. Without going into details, these technologies provided the means to isolate the antibody-encoding genes, to manipulate and clone the DNA and to gain a greater understanding of structure-function relationships and which elements of the protein sequences define its species origin. The immunogenicity of antibody therapeutics was first tackled by cloning the variable domain genes from desirable rodent antibodies (that part of the antibody that defines its antigen-binding characteristics) onto the constant region genes (defining secondary effector functions) of human origin, resulting in *chimeric* antibodies. Further refinement was introduced by engineering the variable domains, for example cloning the actual antigen-binding loops, or complimentarity determining regions (CDRs) onto a human V-region scaffold that is structurally similar, a process known as CDR-grafting. The success of these approaches is illustrated by the fact that the majority of antibodies approved for therapy currently on the market are of rodent origin.

Whilst additional techniques have been developed since to further humanize, or de-immunize proteins, the drive to generate more precisely human antibodies from the outset progressed significantly in the 1980s and 1990s with the advent of two remarkable advances. The first was the ability to clone entire repertoires of human antibody genes directly from volunteer donors into phage display systems.[1,2] This allowed very large pools (up to 1×10^{11}) of antigen binding sites to be reproduced on the surface of bacteriophage, with one antigen specificity per phage particle, which could then be selected in vitro against any antigen of choice. The second was to introduce sections of human antibody gene loci into mice in which the corresponding loci had been deleted. This resulted in transgenic mouse strains that, upon immunization, would respond by generating human antibodies in vivo.[3] A fundamentally important aspect of both technologies is that they enable scientists to generate human antibodies against human antigens. Again, it is testament to the power of these technologies that the overwhelming majority of antibodies currently undergoing clinical trials (~400) are derived using one or other of these approaches.

Antibodies have now emerged as the major class of biologic therapeutics. An intrinsically high level of specificity for cognate target antigen and amenability to in vitro molecular engineering make them extremely attractive candidates for clinical development. Moreover they are nature's answer amongst the jawed vertebrates to combat infectious agents and malignant cells. However,

there are a number of applications for which the whole antibody was not generally held to be the ideal molecule as they have a number of limitations. IgG antibodies are the most common serum form and the one favored for almost all therapeutic development programs. They are multi-domain, four chain molecules of ~150 kDa with two identical antigen binding sites, each formed by the association of one heavy chain with one light chain. Their size and to a lesser extent their Y-shaped configuration restricts their mobility and targeting to more accessible extra-cellular and cell-surface antigens. This limitation is exemplified by the number of approved antibody drugs and those currently under investigation that target the same narrow range of antigens and clinical indications. The ability to access more cryptic, intra-cellular or recessed epitopes currently intractable to conventional antibodies, will undoubtedly afford the opportunity to exploit a new range of clinically relevant targets.

Accordingly, there is a continual drive within the immuno-therapeutic and diagnostic communities to develop smaller, more stable, highly soluble, high affinity (nM) binding domains. To this end an extensive array of recombinant antibody formulations have been developed, reducing antibodies to minimal binding domains, modulating effector functions, increasing valency and avidity and conjugating to other protein domains to improve pharmacokinetic or in vivo efficacy. An ideal biologic therapeutic will possess all of these attributes. In order to generate promising candidate products, it is also necessary to couple ease of manufacturability i.e., high functional expression yields, low aggregation and consistently homogenous protein production with freedom to operate in a complex intellectual property landscape. One very exciting discovery to have emerged recently and that could have a significant influence on future therapeutic generation is the discovery of a new antigen binding molecule from sharks, the IgNAR. This chapter aims to introduce the reader to this remarkable protein, to describe some of its unique characteristics and to discuss how these might be exploited to lead us into the next generation of biologic therapeutics.

What Are IgNARs?

The immunoglobulin novel (or new) antigen receptor (IgNAR) was first identified in the lab of Martin Flajnik and coworkers in the early nineties. The somewhat serendipitous route of discovery originated from the work of Andrew Greenberg who was studying nurse shark immunoglobulins. He observed cross-reactivity of a nurse shark anti-IgM polyclonal antibody with an unknown band on a Western blot with mobility slightly greater than that of monomeric IgM and which was subsequently shown to be a novel single domain 'heavy chain' previously unknown to science.[4,5] Serum IgNAR exists as a homodimer devoid of light chains, with independent variable domains that exhibit structural flexibility.[6] The levels of circulating nurse shark IgNAR have been measured at approximately 0.1-1.0 mg/ml which is 20-40 x less than IgM.[7,8]

Nurse sharks are members of the order Orectolobiforme. Collectively there are eight orders based on gross anatomical and molecular criteria.[9] Diverging from a common ancestor approximately 450 million years ago, they are the oldest vertebrate taxon to have all the components of an adaptive immune system, namely major histocompatibility complex (MHC), T-cell receptors (TCR), immunoglobulins (Ig), RAG recombinase activity, somatic hypermutation and specialized primary and secondary lymphoid tissue.[10] They belong to the class Chondrichthyes or Cartilaginous fish which can be divided into two subclasses: the elasmobranchs (sharks, rays and skates) and the holocephalans (chimaeras and ratfish). IgNAR has also been identified in other members of the Orectolobiformes, including the spotted wobbegong (*Orectolobus maculatus*) and bamboo sharks (see ref. 11, S. Nuttall personal communication) as well as two species of dogfish, the smooth hound (*Mustelus canis*) and spiny or spurdog (*Squalus acanthias*) (Fig. 1) from the orders Carcharhiniforme and Squaliforme respectively.[12,13] IgNAR sequences from the horn shark—a member of the order Heterodontis, have also been deposited in sequence databases. As spiny dogfish and nurse shark are separated phylogenetically by approximately 200 million years, it is tempting to surmise that IgNAR evolved prior to this split and may be ubiquitous amongst elasmobranchs.

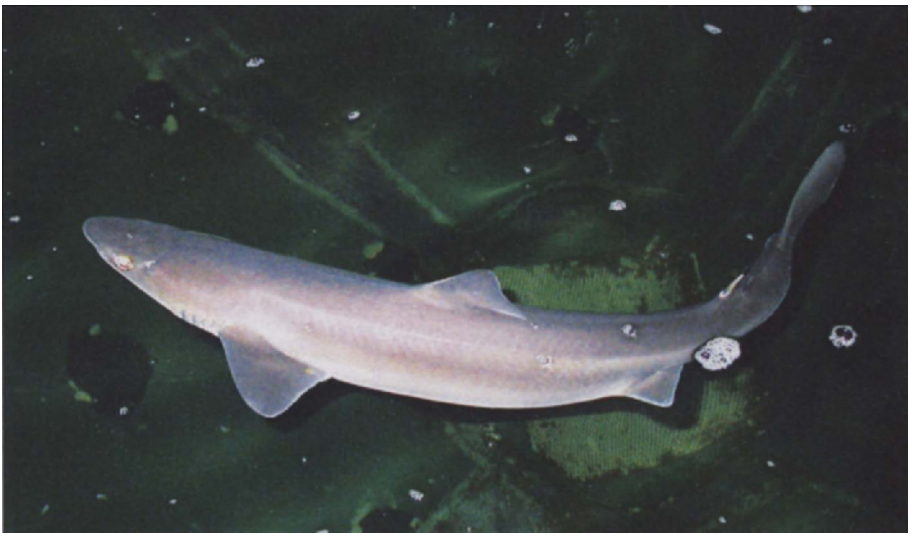

Figure 1. The spiny dogfish *Squalus acanthias*, one of the smaller and easier to keep shark species currently being evaluated as a source of novel IgNAR-based immuno-therapeutics.

The first IgNAR cDNA clones to be sequenced were isolated from a nurse shark spleen library and were shown to consist of a variable domain and five constant domains consistent with immunoglobulin superfamily V and C1-SET domains.[4,5] As with classical B-cell antigen receptors, IgNAR exists as both membrane-bound and soluble forms. In higher vertebrates the former activates lymphocytes while the latter serves to bind serum antigen and provide secondary effector functions. There are two distinct splice-variants of the membrane-bound form, with three and five domains respectively. The soluble form also comprises five domains.[14] Differential amplification of these has shown that the secretory form exhibits a much greater degree of mutation, indicative of antigen-driven selection.[15] What, if any, effector function is provided by the secretory form has yet to be determined. The sequence of IgNAR shows greater homology with IgLk and TCR-V chains than IgH and shows very low levels of sequence homology to human VH.[4,5] From a structural perspective it falls somewhere between cell adhesion molecules, antibodies and T-cell receptors, perhaps providing some insight into its evolutionary origins. It is conceivable therefore that a receptor which evolved independently of antibodies was subsequently adopted by the immune system.[16] The most profound differences between IgNAR and conventional antibodies lie at the hydrophobic interface between VH and VL domains. IgNAR lacks many of these residues and replaces others with hydrophilic ones resulting in a greatly truncated CDR2 region and high solubility in the absence of light chains. The lack of CDR2 makes IgNAR-V the smallest independent antigen binding domain in the animal kingdom with a molecular mass of approximately 12 kDa.

To date, there are three defined types of IgNAR known as I, II and III (Fig. 2). These have been categorized based on the position of noncanonical cysteine residues which are under strong selective pressure and are therefore rarely replaced.[6,17,18]

All three types have the classical Ig canonical cysteines at positions 35 and 107 that stabilize the standard immunoglobulin fold, together with an invariant tryptophan at position 36. There is no defined CDR2 as such, but regions of sequence variation that compare more closely to TCR HV2 and HV4 have been defined in framework 2 and 3 respectively.[19] Type I has germline encoded cysteine residues in framework 2 and framework 4 and an even number of additional cysteines within CDR3. Crystal structure studies of a Type I IgNAR isolated against and in complex with lysozyme[19] enabled the contribution of these cysteine residues to be determined. Both the framework 2 and 4 cysteines form disulphide bridges with those in CDR3 forming a tightly

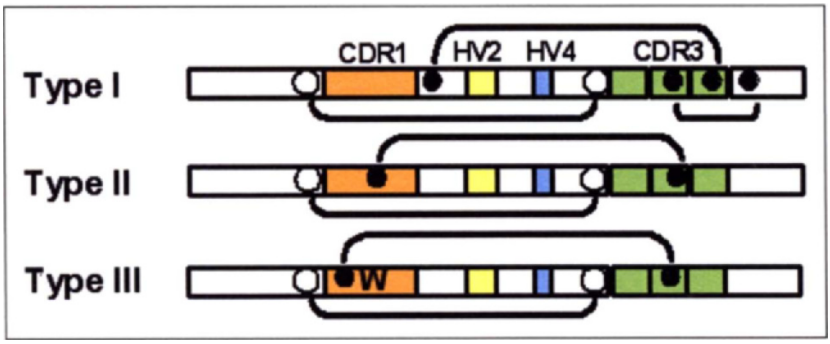

Figure 2. Structure of rearranged IgNAR genes showing positions of canonical (O) and non-canonical (•) cysteine residues, disulphide bonds (connecting lines), conserved tryptophan (**W**) and hyper-variable (CDR/HV) regions.

packed structure within which the CDR3 loop is held tightly down towards the HV2 region.[19] Interestingly, to date Type I IgNARs have only been identified in nurse sharks—all other elasmobranchs, including members of the same order have only Type II or variations of this type.

Type II IgNAR are defined as having a cysteine residue in CDR1 and CDR3 which form intra-molecular disulphide bonds that hold these two regions in close proximity,[19,16] resulting in a protruding CDR3 (Fig. 3) that is conducive to binding pockets or grooves. Type I sequences typically have longer CDR3s than Type II with an average of 21 and 15 residues respectively. This is believed to be due to a strong selective pressure for two or more cysteine residues in Type I CDR3 to associate with their framework 2 and 4 counterparts.[20] Studies into the accumulation of somatic mutations show that there are a greater number of mutations in CDR1 of Type II than Type I, whereas HV2 regions of Type I show greater sequence variation than Type II. This evidence correlates well with the determined positioning of these regions within the antigen binding sites.[20] A third IgNAR type known as Type III has been identified in neonates. This member of the IgNAR family lacks diversity within CDR3 due to the germline fusion of the D1 and D2 regions (which form CDR3) with the V-gene. Almost all known clones have a CDR3 length of 15 residues with

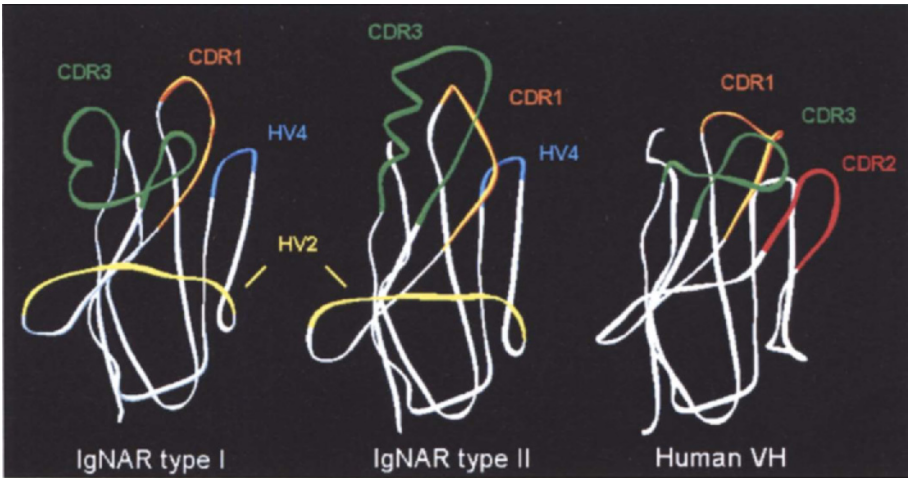

Figure 3. Backbone structures of Type I and Type II anti-lysozyme IgNAR compared with a human VH domain.

little or no sequence diversity. Tissue expression patterns show an initial high level in the spleens of newborn nurse sharks that then declines after 2-4 months, leaving the epigonal organ as the primary tissue of expression throughout adulthood.[20] It is hypothesized that this type of IgNAR may have evolved to protect young pups from early stage exposure to a common pathogen, or to be involved in regulating immune system development.

How Diverse Are IgNARs?

Sharks have three heavy (IgNAR, IgM and IgW) and four light chain (I/NS5, II/NS4, III/NS3 and σ) isotypes identified to date.[8,21] All shark antibody genes are arranged in a "cluster" format, in contrast to the "translocon" organization exhibited in mammalian systems[22] (Fig. 4).

Each cluster comprises one V segment, one or more D segments, one J segment (that can be partially or fully germ-line joined) and a single set of C segments which are rearranged exclusively within individual clusters by the activity of RAG recombinase.[5,24-29] Key to the heterogeneity of the shark naïve repertoire is the junctional diversity achieved through N-additions, trimming and D-region re-arrangement (all three reading frames and possible D-region inversion). As in higher vertebrates, terminal deoxyribonucleotide transferase (TdT) is the enzyme responsible for the nucleotide addition 5' to the D-regions and is highly homologous to that in mammals.[30] Recent work by Malecek and coworkers studying the IgM loci in nurse shark has shown that allelic exclusion does occur in B-lymphocytes but probably not via chromatin condensation, the mechanism adopted in translocon configurations.[31] Currently, there is no evidence to show that recombination events occur between clusters and it is commonly believed that isotype switching does not occur in cartilaginous fish. More probably, isotype is fixed at an early stage of ontogeny.

As IgNARs are devoid of light chain, they lack the combinatorial diversity of convention VH-VL antibodies. This deficiency is compensated for by an increased number of recombination events through the presence of additional D-regions and the introduction of extensive junctional diversity (Fig. 4). IgNAR conforms to the cluster arrangement of V and C domain gene organization and in nurse shark, the gene family is composed of four loci.[5] Diversity of the naïve IgNAR repertoire is achieved by four recombination events (between the V region, three D regions and J region) as well as extensive N and P nucleotide addition which results in considerable CDR3 heterogeneity. Somatic hypermutation, although not responsible for generating the naïve repertoire, is evident in the secretory form of IgNAR. High levels of mutation that share

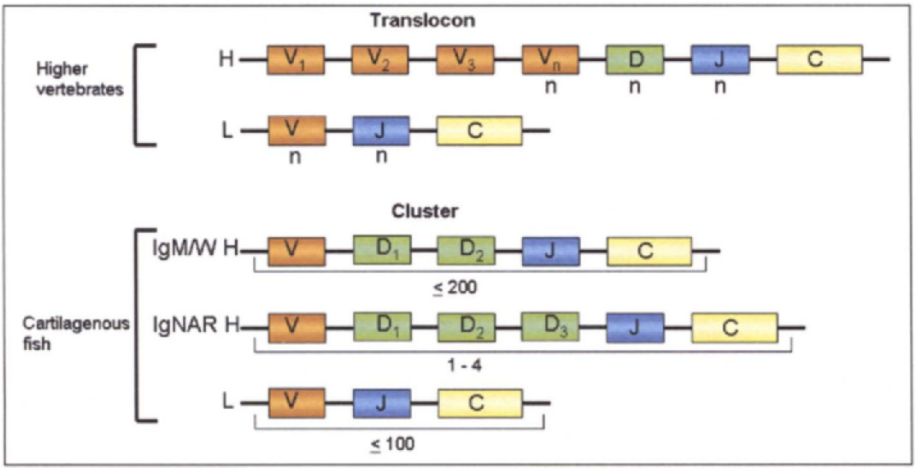

Figure 4. Translocon and cluster arrangements of immunoglobulin genes in higher vertebrates and cartilaginous fish respectively. Adapted from: Flajnik MF. Nature Rev Immunol 2002; 2:688-698;[23] with permission from Nature Publishing Group.

similar mutational patterns and serine codon hotspots (AGC/T) as those seen in mammalian systems have been identified in addition to strand bias and a bias in favor of transitions over transversions. Unusually, base changes occur in tandem (doublets and triplets), particularly in "hotspots" and palindromic repeats.[18]

What Is the Function of IgNAR?

Higher vertebrates possess an adaptive immune system (AIS) that is mediated primarily by B and T-lymphocytes. In contrast to innate immunity (the first line of defense against invading foreign pathogens) which is based upon restricted germline encoded receptors, the receptors of the adaptive immune system are generated by recombinatorial processes. The levels of hypermutation seen in IgNAR are indicative of a secondary immune response. Tailoring a binding domain through mutation accumulation to increase affinity for target antigen is a classic hallmark of an adaptive immune response. The mechanism by which V(D)J immunoglobulin gene segments are rearranged is such that the repertoire of receptors has sufficient diversity to potentially recognize any pathogen or toxin. Importantly, adaptive immunity also results in memory. Secondary exposure to a pathogen previously encountered results in a much more rapid and aggressive response.

It has been known since the mid-sixties that elasmobranchs are able to respond in an antigen-specific manner when challenged.[32] During this period, more detailed immunization studies were carried out in smooth dogfish (*Mustelus canis*), spotted dogfish (*Scyliorhimus canicula*) lemon shark (*Negaprion brevirostris*) and nurse sharks (*Ginglymostoma cirratum*) characterizing the purified serum fractions responsible for this response and identifying them as 19S and 7S IgM.[7,33-37] Both forms of IgM were present at similar high levels, representing approximately 50% of total serum protein. Upon antigen challenge, there was a temporal response of IgM subtypes with some studies reporting an initial increase in 19S followed by an increase in 7S and another suggesting a secondary enrichment of 19S.[38,39]

Evidence that IgNAR is part of the adaptive immune response in sharks was demonstrated conclusively in the lab of Martin Flajnik by following the antigen specific IgNAR fraction in the sera of immunized animals.[40] Antigen-specific IgNAR and monomeric IgM showed similar expression profiles, plateauing at approximately 4-5 months in response to multiple boosts of hen egg lysozyme (HEL). Increased levels of antigen-specific IgNAR were preceded by an increase in antigen-specific monomeric IgM. Interestingly, sharks are the only known vertebrates to have both pentameric and monomeric forms of IgM. The inter-relationship of the two forms remains to be fully understood, however early studies suggest that the two forms are fully independent and not merely splice variants of the same antibody.[38,41] The current model, though not yet proven, is that the B-cells commit at an early stage to express a specific heavy chain isotype. Class switching, which is observed in higher vertebrates, is not seen in sharks. This is thought to be due to the arrangement of heavy chain genes which is not conducive to recombination between clusters. Based on the immunization studies carried out to date it is believed that the first line of immunological defense in elasmobranchs is low affinity pentameric IgM—with ten binding sites, this large protein benefits from increased avidity enabling it to bind strongly to antigens despite imprecise specificity and poor affinity. There follows a delay before the detection in sera of both monomeric IgM and IgNAR. By ceasing immunization of the animals for an extended period until titers had dropped significantly, Flajnik's group effectively demonstrated that re-immunization with the same antigen elicited a much more rapid response, indicating that sharks generate antigenic-memory.[40]

In higher vertebrates, B-cell maturation takes place in the primary or generative lymphoid organs e.g., bone marrow. Lacking such tissue and indeed a true lymphatic system, elasmobranchs have developed two specialized tissues where lymphopoiesis takes place: the epigonal and Leydig organs. All sharks possess one or both of these primary lymphoid organs, which exhibit similar cellular composition containing large numbers of developing granulocytes, blast cells, plasma cells and lymphocytes. As its name implies the epigonal is attached to the gonads, whereas the Leydig is a mass of lymphomyloid tissue situated in the sub-mucosa of the oesophagus.[42,43] Both organs exhibit expression of RAG recombinases, TdTs and B-cell specific transcription factors

confirming their function as primary lymphoid tissues.[27,44] In adult sharks, many secretory B-cells can be located in the epigonal organ suggesting a role as a reservoir for activated antibody secreting B-cells, similar to the role of bone marrow in mammals.[45]

Spleen and thymus have been identified as the secondary lymphoid organs in sharks. The spleen, which is believed to be the site of antigen driven B-cell activation, changes histologically as sharks mature. Studies in nurse shark pups show the presence of IgM positive but no IgNAR positive cells—it takes approximately five months before the latter are detectable.[45] The spleen is highly vascularized and compartmentalized into red and white pulp zones, although "classical" germinal centers where B-cell activation and antigen-specific affinity maturation occurs in higher vertebrates are not seen. However, the fact that IgNAR does undergo somatic hypermutation and there is white zone compartmentalization of B-cells, T-like cells and dendritic cells, is strongly indicative of the spleen being the site of B-cell activation.[45]

Developing IgNARs as Therapeutics

So far, this chapter has discussed the what, the how and the why of IgNARs in its natural biological context. We now need to explore how all of these physical and biochemical attributes can be exploited in an applied fashion to generate efficacious immunotherapeutics.

Intrinsic Therapeutic Attributes of IgNARs

In the absence of interventive in vitro manipulation, IgNARs V-regions naturally exhibit many of the desirable properties of biologic therapeutics. In its natural context IgNAR is found in the serum of sharks, which contains 350 mM urea (a protein denaturant) as a means of osmo-regulation. It may be the need to retain functionality in this harsh environment that contributes to the extraordinary stability exhibited by IgNAR. In addition to tolerating high concentrations of denaturants, IgNAR have remarkable folding properties. When exposed to extremes of temperature, they 'melt' as expected. However, on return to physiological conditions they refold correctly, restoring full functional binding to most of the protein.[12,46,47] They also exhibit high solubility presumably due to their hydrophilic surface and so produce high functional expression yields in bacterial culture. Whilst interesting in themselves, these features are insufficient to have significant implications on future therapeutic development. There are two additional key characteristics that may confer such a contribution. Firstly, as mentioned earlier, IgNAR are the smallest known naturally occurring antigen binding domain at 12 kDa (Fig. 5). This lends them both to greater mobility and the opportunity to target epitopes otherwise accessible only to small molecules.

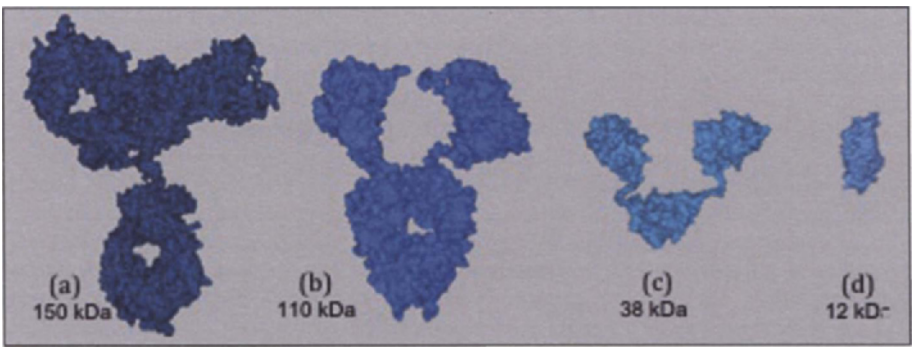

Figure 5. Molecular models illustrating the structures and relative sizes of examples of the many antigen-binding formulations currently being developed for therapeutic applications; (a) whole IgG antibody, (b) scFv-Fc fusion (SMIP®), (c) bi-specific trivalent camelid nanobody and (d) shark IgNAR single domain.

It is the opinion of the authors however, that the unusual topography of the IgNAR binding site, particularly as a result of the lack of a true CDR2 and the lengthy but conformationally constrained CDR3 (Fig. 3), are paramount to its potential. These enable IgNARs to be targeted to recessed epitopes that conventional antibodies cannot reach, even when that part of the antigen is sufficiently exposed. Evidence for this is borne out by the nature of epitopes recognized and targeted by IgNARs, e.g., the active sites of enzymes.[11,46,48-50]

Isolation of Antigen-Specific Clones

IgNAR binding domains are amenable to phage display technology which has been fundamental in revolutionizing recombinant antibody engineering and has driven the development of antibodies and other novel protein scaffolds as therapeutic agents. Phage display couples phenotype to genotype and facilitates the isolation of antigen-specific binding domains and enables subsequent molecular engineering to achieve greater affinity, solubility and stability. A detailed description of this technology and its contribution to drug development is beyond the scope of this chapter, however there are numerous excellent reviews covering the topic.[2,51,52] The first antigen-specific IgNAR that had undergone natural in vivo selection was isolated from a nurse shark immunized with hen egg lysozyme.[46] By comparing the sequences of HEL specific clones isolated from an immunized phage library, the degree of somatic hypermutation and key residue changes that had occurred during the in vivo maturation process to increase affinity were determined.[53] The relationships between progressive mutation accumulation and increased antigen-antibody interactions were identified by comparing the crystal structures of an in vivo matured HEL specific binding domain and its putative germline ancestor in complex with antigen.[54] This study isolated both Type I and Type II IgNAR binding domains with affinities of approximately 20 nM and 1 nM respectively. The isolation of antigen-specific IgNAR binding domains from a phage display library was published by Nuttall et al.[11] This group built a semi-synthetic IgNAR library using natural frameworks isolated from wobbegong sharks. As the diversity of the naïve repertoire is based predominantly on the sequence within CDR3, this group applied random mutagenesis of this region and succeeded in selecting recombinant IgNAR specific for the Gingipain K protease from *Porphyromonas gingivalis*.[11] The study was extended by increasing the library size via the introduction of naturally occurring IgNAR CDR3's from a naive shark repertoire and interestingly resulted in the isolation of two proteins not derived from the synthetic CDR3 library but from natural proteins.[48] A group in the USA were the first to isolate IgNAR binding domains from spiny dogfish[12] using both naïve and semi-synthetic libraries. They isolated binders to SEB, cholera toxin and ricin that had similar affinities (10-300 nM) to those isolated by Nuttal et al from similar wobbegong libraries. This works demonstrates that high affinity antigen-specific IgNAR can be successfully generated from both synthetic and immunized phage display libraries derived from a variety of shark species.

In Vitro Maturation

During the antibody drug development process, isolating a lead binder is just the beginning of a lengthy process. In the majority, if not all cases, there is a need to modify aspects of the protein. This might involve increasing affinity, fine-tuning specificity, increasing functional expression yield/solubility, resolving potential immunogenicity issues or modulating pharmacokinetics. The precise alterations will vary depending on the inherent characteristics of the lead protein and the application for which it is intended. An interesting example is targeting of solid tumors, where experience has shown that improvements in binding affinity do not necessarily equate to improved efficacy. A predominant reason for this in many cases is that antibodies with very high affinities are likely to localize on the tumor surface and not achieve the desired penetration.[55,56] In this instance, a more moderate affinity, combined with reduced molecular weight and associated increases in mobility/diffusion might contribute favorably to efficacy. Indeed it has been demonstrated that a lower affinity diabody (two linked single binding domains) exhibited a greater coverage of tumor targets than the ten fold higher affinity single domain.[57]

Engineering isolated clones to increase affinity and specificity had been achieved by random in vitro maturation,[58] CDR randomized mutagenesis[47,50] and by targeted residue changes based on structural alterations to the CDR3 loop to increase flexibility and antigen cleft binding.[59] The latter example broadened the specificity to encapsulate a greater breadth of species cross-reactivity. As the binding domain in question was isolated against a malarial target, it was hoped that broader specificity would provide an advantage with respect to treating the disease in a variety of states of progression. The ability to readily confer such characteristics to new drugs in development is essential in order to assess efficacy in industry standard (though far from ideal) nonhuman animal models.

Formatting

Lead antibodies are usually isolated initially as single-chain fragments and typically do not have the pharmacokinetic suitable for therapeutic administration. In addition, there is the complication of effector functions which may or may not be desirable, depending on the target and mode of action required. For imaging purposes there is no requirement for effector function, indeed this would be undesirable. Further more, such reagents should ideally be of minimal size, both to maximize tissue penetration and to minimize serum half-life. Antibodies developed for therapeutic purposes often require a much longer half-life to increase efficacy and reduce the need for repeat dosing. Where effector function is desirable, it is typically achieved by engineering the binding site into an IgG-1 format. For certain applications an IgG-4 format is preferred, providing an extended half-life but not recruiting complement activation. Alternative technologies to increase half-life of single domains include conjugation to polyethylene glycol, so-called PEGylation,[60] by targeting human serum albumin (HSA) in a bi-specific format[61,62] or by engineering single-domain-Fc fusion proteins. For many indications, the binding of an antibody is not sufficient to generate the desired cell killing. In such cases, it is often preferred to 'arm' the antibody with a toxin, enzyme or pro-drug. There is little doubt that antigen binding single domains afford a number of potential opportunities that are not found with conventional antibodies or antibody fragments. Chief amongst these is the ability to design and build multi-valent constructs e.g., diabodies, triabodies etc. Not only can such molecules increase apparent affinity due to avidity effects, but can also be multi-specific enabling simultaneous binding to more than one target. Such approaches can be used to modulate pharmacokinetics, increase tissue specificity or to deliver payloads. Our current knowledge on the structure and function of the various domains of antibodies, together with protein engineering advances mean that such modifications are readily achieved. There is currently little published research describing the formatting of IgNAR, one exception being the dimerization to create bivalent IgNARs.[63]

One very important facet of any biologic therapeutic that has been mentioned briefly already is that of host immune responses. It is well known and accepted that whole antibodies from nonhuman species are likely to elicit a degree of anti-protein response in some if not all patients. There are now a number of well established technologies that have been developed to overcome this problem, particularly with respect to antibodies of rodent origin. The past two decades have seen a drive to isolate antibodies from sources that are as human as possible from the outset. There is however growing acceptance that even a 'fully human' antibody can been seen as foreign by a proportion of the patient population. In these cases it is the variable domains that form the binding site that provide immunogenic epitopes, rather than the constant domains which are far more immunogenic when derived from nonhuman origins. This has some very important implications for the bio-pharma industry. On one hand, it is now considered an essential part of the drug development process that any biologic be analyzed for indications of potentially immunogenic epitopes and that appropriate measures are taken to engineer these in such a way as to alleviate the problem without adversely affecting drug function. On the other hand, it also means that the perceived disadvantage of starting with a nonhuman protein is perhaps not such a handicap as was once thought.

Conclusion

Does shark IgNAR represent a suitable platform to produce the next generation of therapeutic biologic molecules? We have witnessed a whole new sector of the pharmaceutical industry emerge around antibody therapeutics over recent decades. There was initially some disappointment that early antibodies derived from rodents did not, on the whole, deliver on the possibly over-ambitious expectations of many commentators that "magic bullets" would lead to a major break-through in the treatment of many diseases, not least cancer. Since those days the industry has matured considerably and our expectations are perhaps more measured, though in many ways no less ambitious. The principal drivers behind the recent resurgence must surely be advances in the generation of 'fully human' antibodies through phage display and transgenic mice and the ability to de-immunize both human and nonhuman proteins. These technologies have had a profound effect on the therapeutic antibody landscape- ten years ago only two monoclonal antibody drugs were FDA approved. Today that number is 21, including 8 'blockbusters', with several hundred at various stages of clinical and preclinical development. Today biologics represent an increasing proportion of pharma companies' pipelines, a trend that is also expected to continue. The time when antibody based therapeutics were considered the answer to our all of our problems is long over however—"one size fits all" simply doesn't work. Greater understanding of human biology and in particular the biology of disease processes has highlighted the many deficiencies of antibodies. We are now entering a new era, where designer biologics will play an increasing role.

Single domain proteins are ideally suited to these new approaches in many respects. The simplicity of a receptor that does not require the correct association and alignment of two separate domains to generate target recognition, e.g., Vh-Vl, has obvious advantages in relation to production of functional material. This is even more so when considering multi-specific molecules where mis-pairing of binding domain partners results in loss of target recognition. The ability to target multiple epitopes or antigens with a single molecule presents numerous advantages, including better cell targeting or tissue specificity in cases where one antigen is over expressed on target cells but is not unique to these cells. It can also be used to bring together different cell types to improve drug efficacy, e.g., having one domain that targets a cell surface antigen and a second that has agonistic binding to T-cell receptors. Other possibilities include targeting HSA to increase serum half-life, or directing an enzyme, radio-label or toxin to a desired tissue so reducing dosage requirements and minimizing off-site toxicity. Of course not all designer bio-therapeutics rely on the antigen recognition element of an antibody. A good example of this Enbrel, where the soluble form of the tumor necrosis factor (TNF) receptor is fused to an antibody Fc region.

Where then does IgNAR fit into the equation? As antigen binding single domains they fulfill all of the attributes and opportunities described above. They are readily expressed at high functional levels in a variety of systems and are amenable to both in vivo and in vitro display systems. The antigen binding loops can accommodate extensive modifications to modulate antigen binding and they can easily be engineered as fusions to additional functional protein domains.

However there a number of other attributes possessed by this unique class of receptors that are generating the real excitement and opening the way for new opportunities to be exploited. At a mere 12 kDa compared to ~25 kDa for the minimal functional binding unit of a conventional antibody, the V-regions of IgNAR are the smallest naturally occurring antigen binding domain. This in itself reduces potential immunogenicity issues and provides for rapid renal filtration and short serum half-life which can be readily extended if required as described earlier. The small size affords the possibility to localize therapies very quickly to target tissues and to improve delivery as a result of increased penetration of solid tumors. They are also able to access antigens that are unavailable to conventional antibodies due to steric considerations. Compared to conventional antibodies, IgNAR exhibit a remarkable degree of stability. They are able to resist high concentrations of denaturants and after exposure to temperatures of 90°C they will refold to their original conformation, so restoring binding function. This property probably results from the adverse environment found in the blood of sharks, which use urea as an osmo-regulator. From a clinical perspective it not only means that IgNAR based therapeutics are likely to have an extended shelf-life, particularly useful

in many areas of the developing world where refrigeration is not always available, but may also open up opportunities for novel routes of administration rather than parenteral. There is a second class of naturally occurring single domain antigen receptors found in camelids and termed VHH.[64] Unlike IgNAR these are clearly evolved from antibody heavy chains and share significant sequence homology. They share many of the properties of IgNAR such as solubility, stability, small size and an extended protruding CDR3 and there are several novel therapeutics in development based on this molecule. It is somewhat remarkable that, despite sharing very little sequence homology and being separated by some 400 million years, shark IgNAR is structurally and functionally incredibly similar to camel VHH—a true case of convergent evolution. VHH share the same noncanonical cysteine as Type II IgNAR. There is however no Type I equivalent in camels and as a result IgNAR has the potential to provide even greater coverage of otherwise unavailable epitopes by virtue of a second distinct binding site topography. We have described earlier the issue of immunogenicity and the impact that this has on the development of biologic therapeutics. By analyzing the structure of crystallized IgNAR proteins we can see that they adopt a conformation that is very similar to that of some human VH domains (Fig. 3). Whilst they appear intrinsically to show very low immunogenicity in mammals (unpublished), it may never the less be possible to engineer them into a more human format by replacing components of the framework regions with those from human antibodies. Conversely, it may be equally plausible to engineer some of the useful characteristics of shark IgNAR into human VH domains by taking the reverse approach.

Overall, there are clearly immense opportunities for future therapeutic development based on this novel protein—harnessing a receptor that predates the age of dinosaurs to tackle some of the urgent clinical challenges of the 21st century.

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CHAPTER 7

Immune Interventions of Human Diseases through Toll-Like Receptors

Cevayir Coban, Ken J. Ishii and Shizuo Akira*

Abstract

Toll-like receptors (TLRs) are the immune sensors for infections, triggering robust innate immune activation followed by protective adaptive immunity against various infectious diseases. Recent evidence, however, has suggested that TLRs are involved in the pathogenesis of many diseases, including not only infectious diseases but also autoimmune diseases, allergy and atherosclerosis. Therefore, prophylactic or therapeutic application of TLR-based immune interventions should be potent, but their safety must be demonstrated using experimental animal models as well as human resources, including analysis of single nucleotide polymorphisms. Here, we focus on recent advances in understanding of the protective and pathogenic roles of TLRs in human diseases.

Introduction

An important role of the innate immune system in the first-line defense against pathogens, and the underlying molecular and cellular mechanism(s) involved not only in infectious diseases but also in cancer, allergy, autoimmune diseases and atherosclerosis, has recently been unveiled. When the Toll pathway in *Drosophila melanogaster* was discovered in the mid-1990s, it was initially thought to be important in embryonic patterning but was then found to be a component of the host defense against fungal and Gram-positive bacterial infections.[1] Subsequently, its mammalian homologues, evolutionarily conserved Toll-like receptors (TLRs) have been discovered.[2]

The mammalian TLRs are a class of pattern recognition receptor (PRR) molecules consisting of at least 11 members that recognize microbial components known as pathogen-associated molecular patterns.[3,4] Similar to the other PRRs, mammalian TLRs are widely distributed on/in the cells of the immune system and are capable of discriminating among a variety of invading pathogens such as protozoans, fungi, bacteria and viruses.[5] Direct interaction of TLRs with the cognate ligand triggers intracellular signaling pathways through multiple adapters, transduction and transcription molecules, leading to robust immune responses characterized by production of inflammatory cytokines, chemokines and immunoglobulins, and up-regulation of costimulatory molecules. In particular, the activation of dendritic cells via TLRs is critically important because of their ability to prime the adaptive immune responses linking innate immune responses to adaptive immunity.[6,7]

In addition to their 'primary function' of fighting invading microbes, TLRs are also involved in the pathogenesis of many diseases. In particular, TLR recognition of self-molecules derived from the host (e.g., nucleic acids) may be linked to autoimmune diseases and possibly to other immunological

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disorders. In human, TLRs and their mutations (e.g., single nucleotide polymorphisms (SNPs)) have recently been linked to susceptibility, not only to infectious diseases, but also to chronic inflammatory diseases, atherosclerosis and asthma (for review, see refs. 8-10).

Such a potential impact of TLR-mediated immune regulation on human diseases has stimulated a wide range of clinical fields. Agonists and antagonists for TLRs, and inhibitors of TLR signaling molecules are presently under development for a variety of therapeutic applications. Choosing a highly 'effective and proper' but 'safer' TLR-agonist/antagonist is critical for the development of improved vaccine adjuvant and immuno-stimulatory agents. TLR antagonists or inhibitors of TLR-signaling molecules, on the other hand, may provide another opportunity for the development of drugs to prevent and/or treat diseases in which TLRs are involved in the etiology or pathogenesis. Effort should also be made to understand their mechanism of action, and to evaluate their safety relying not only on animal models but also on human resources such as SNPs. In this chapter, we discuss recent advances and understanding of the TLR-related research field, in particular the molecular and cellular mechanisms underlying TLR-mediated innate immune responses and their impact on human diseases, in the hope of helping future development of more efficient and safer TLR-based immunotherapies.

Toll-Like Receptors and Their Known Ligands

The mammalian TLR family consists of at least 11 members. TLRs comprise a horseshoe-shaped extracellular domain with dozens of leucine-rich repeat motifs, a transmembrane domain (except for TLR3), and a cytoplasmic Toll/IL-1R (TIR) domain similar to that of interleukin-1 (IL-1) receptors (IL-1Rs).[5] TLR1, 2, 4, 5, 6 and possibly 11 are expressed on the cell surface, while TLR3, 7, 8 and 9 are believed to reside inside cells (e.g., endoplasmic reticulum and/or endosomes). Each TLR is expressed in a variety of immune cells, including macrophages, dendritic cells and B cells, and some stromal cells such as endothelial cells and epithelial cells. TLRs can recognize molecular pattern(s) within lipids, proteins or nucleic acids that are conserved among but unique to microbes that usually do not exist in the host (Table 1). For example, TLR2 alone or together with TLR1 or TLR6 detects bacterial lipoproteins and lipoteicoic acids, which are unique and essential components of the bacterial cell wall; TLR3 recognizes double-stranded RNA, an intermediate generated during viral replication; TLR4 recognizes lipopolysaccharide (LPS), a typical cell wall component of Gram-negative bacteria; TLR5 recognizes flagellin, a protein necessary for flagellated bacteria; TLR7 and TLR8 recognize single-stranded RNA derived from RNA viruses; and TLR9 recognizes unmethylated CpG motifs of DNA observed in certain bacteria and viruses.[11]

New ligands for TLRs are continuously increasing in number and seem to become more diverse and complex than was initially thought (Table 1). Each TLR now has a variety of ligands derived not only from microorganisms, but also from host cells that are particularly damaged or dying for various reasons. For example, TLR9 recognizes a host chromatin-DNA complex as well as hemozoin, a malarial metabolite of the host heme molecule.[12] How such structurally and chemically unrelated ligands can be recognized by the same TLR is not fully understood. While the precise molecular mechanism(s) of TLR ligand recognition thus need further investigation, one such mechanism is that the TLRs are not alone; rather, they orchestrate with other PRRs, thereby enabling the recognition of such a diverse range of molecules.[12,13] Intervention between TLR-mediated protective immune responses and the pathogenesis of a variety of human diseases is therefore a double-edged sword.

Toll-Like Receptor Signaling

Another important key to understanding TLR-mediated immune responses is differential intracellular signaling downstream of the TLR initiated by the TIR domain in its cytoplasmic portion, which is shared with a member of the IL-1R superfamily. Upon stimulation, adaptor molecule(s) that also have a TIR domain are recruited to the TLR-TIR domain. Myeloid differentiation primary response gene 88 (MyD88) is an essential adaptor molecule for most TLRs except TLR3 (Fig. 1). The other adaptor molecule, Toll-IL-1R domain-containing adaptor protein

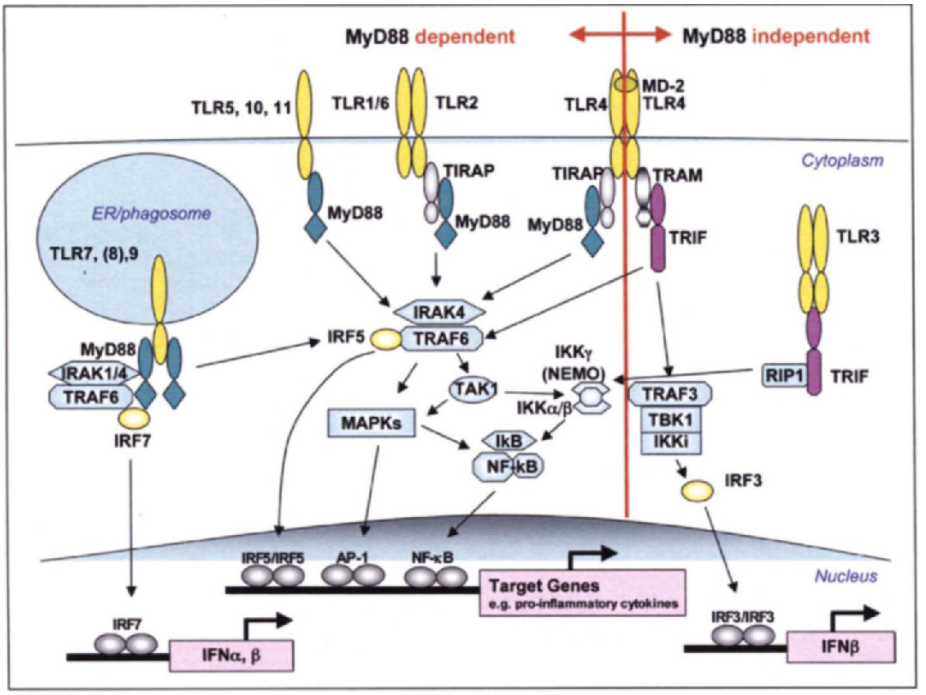

Figure 1. MyD88, Myeloid differentiation primary response gene 88; TIRAP, Toll-IL-1R domain-containing adaptor protein; TRIF, Toll/IL-1R domain-containing adaptor inducing IFN-beta; TRAM, TRIF-related adaptor molecule; TRAF6, Tumor necrosis factor receptor-associated factor 6; TAK1, Transforming growth factor-beta-activated kinase 1; IRAK4, IL-1R-associated kinase 4; TANK/TBK1 , TRIF and TRAF-family-member-associated NF-κB activator/binding kinase 1; IRF3, Interferon regulatory factor 3. (Sightly modified from ref. 12.)

(TIRAP) specifies MyD88-dependent TLR2 and 4 signaling. TLR3 and 4-mediated activations signal through Toll/IL-1R domain-containing adaptor inducing interferon-beta (IFNβ) (TRIF), the so-called MyD88-independent pathway, leading to the induction of IFNβ and IFN-inducible genes. The TRIF-related adaptor molecule (TRAM) further specifies the MyD88-independent, TRIF-dependent signaling pathway of TLR4, acting as a bridging adaptor between TLR4 and TRIF (Fig. 1).

Upon TLR-stimulation, MyD88 as well as TRIF subsequently associate with tumor necrosis factor receptor-associated factor 6 (TRAF6), culminating in the activation of nuclear factor (NF)-κB and/or the mitogen-activated protein kinase (MAP-Kinase) pathway, resulting in the production of pro-inflammatory molecules including cytokines and chemokines (reviewed in ref. 4). TRAF6 forms a complex with ubiquitin-conjugating enzymes (Ub) such as Ubc13 and activates transforming growth factor-beta-activated kinase 1 (TAK1), which in turn activates transcription factors NF-κB and activator protein-1 (AP-1) through the canonical IκB kinase (IKK) complex and MAP-Kinase. The IKK complex is composed of two catalytic subunits, IKKα and IKKβ. NEMO (also known as IKKγ) encodes the regulatory component of the IKK complex, which is responsible for activating the NFκB signaling pathway. IKK phosphorylates IκB and targets it for degradation. The removal of IκB enables NFκB to translocate into the nucleus, where it activates the transcription of various target genes.

In addition to these signaling pathways controlled by IKK complexes, TLR7/9 mediated MyD88-dependent signaling has a distinct signaling pathway for Type I IFN production. MyD88

Table 1. Toll-like receptors and their diverse ligands (based on ref. 12)

TLR	Ligand (Non Host derived = Exogenous)	Ligand (Host Derived = Endogenous)	Source
TLR2	Peptidoglycan		Bacteria
(TLR1+TLR2)	Triacyl lipopeptides		Bacteria
(TLR6+TLR2)	Diacyl lipopeptides		Mycoplasma
	Lipoteichoic acid		Gram-positive bacteria
	Hemagglutinin protein		Measles virus
	GPI		*Trypanosoma cruzi, Plasmodium falciparum,*
	Glycolipid		*Treponema maltophilum*
	Zymosan		Fungi
	Heat-shock protein 60		*Helicobacter pylori*
		Heat-shock protein 70	Host
TLR3	dsRNA		West Nile virus
			Mouse CMV
			Schistosoma mansoni
	siRNA		Synthetic
		mRNA	Host
TLR4	Lipopolysaccharide		Gram-negative bacteria
	Taxol		Plants
	Fusion protein F		Respiratory syncytial virus
	Envelope protein		Mouse mammary tumor virus
	Antrolysin O		*Bacillus anthracis*
	Phosphorylcholine		Filarial nematode
	Glycan		Helminth
	Heat-shock protein 60		*Chlamydia pneumoniae*
			Helicobacter pylori
		Heat-shock protein 60	Host
		Heat-shock protein 70	Host
		β-Defensin 2	Host
		Fibrinogen	Host
		Fibronectin	Host
		Hyaluronic acid	Host
		Heparan sulphate	Host
		Fatty acids	Host
		Tamm-Horsfall glycoprotein	Host
		Surfactant protein A	Host-Lung
		Modified LDL	Host
TLR5	Flagellin		Flagellated bacteria
TLR7	ssRNA		Influenza, HIV-1
			Parechovirus 1
	Imidazoquinoline		Synthetic compounds
		U1snRNP autoantigens	Host

continued on next page

Table 1. Continued

TLR	Ligand (Non Host derived = Exogenous)	Ligand (Host Derived = Endogenous)	Source
TLR8	ssRNA		Viruses Coxsackie B virus Parechovirus 1
	Imidazoquinoline		Synthetic compounds
TLR9	CpG DNA		Bacteria, synthetic ODN DNA viruses
	Hemozoin		*Plasmodium falciparum*
		Chromatin complex	Host
TLR10	Not determined		
TLR11	Profilin-like molecule		*Toxoplasma gondii*

forms a complex with IL-1R-associated kinase 4 (IRAK4) and IRAK1. Depending on the types of cells or stimuli, TLR-MyD88-dependent signaling requires IRAK4 or IRAK1.[14-16] While IRAK4 is necessary for most TLR-mediated pro-inflammatory cytokine production by macrophages and dendritic cells, IRAK1 was shown to be essential only for TLR7- and TLR9-mediated Type I IFN production by plasmacytoid dendritic cells.[17] Recently, TRAF3 and IKKα were found to be indispensable for TLR7/9-induced IFNα induction.[18-20] TLR3 and TLR4 also mediate Type I IFN production via another major adaptor molecule, TRIF and TRAF-family-member-associated NF-κB activator (TANK) binding kinase 1 (TBK1). TBK1 comprises a family with inducible IκB kinase (IKK-i, also known as IKK-ε), and these kinases directly phosphorylate interferon regulatory factor 3 (IRF3) and IRF7.[21,22] TBK1/KK-i-mediated Type I IFN induction is not restricted to TLR3 and 4, but is also involved in TLR-independent virus-, RNA- and DNA-induced Type I IFN production.[23,24]

Transcription factors such as IRF5 and IRF7 were also found to be important mediators for TLR-dependent and -independent signaling pathways. In particular, IRF5 was found to be involved in most TLR-mediated pro-inflammatory cytokine production but not IFNα production, independently of NF-κB or the MAP-Kinase signaling pathway.[25] In contrast, the transcription factor IRF7 was shown to play a critical role in TLR7- and TLR9-mediated IFNα production by direct interaction with MyD88 as well as TLR-independent Type I IFN production induced by viruses.[14,16]

The Role of Toll-Like Receptors in the Human Immune System

In vitro and in vivo experimental models elucidating TLR function and physiological roles in infectious diseases and associated immune disorders have increased our understanding of the importance of TLRs in both protective and pathological immune responses. However, animal studies do not always reflect human physiology. In particular, differences between each mouse and human TLR in terms of ligand specificity, cell types on which the TLR is expressed and the associated diseases (model) may hamper further development of TLR-based preventive and/or therapeutic applications in human. For example, taxol, an anti-cancer drug derived from plants, is a TLR4 ligand in mice, but is not a TLR4 ligand in humans. In contrast, hexa-acylated LPS derived from *Pseudomonas aeruginosa* is a ligand for human TLR4, but not for mouse TLR4.[26] Another example is that CpG DNAs (ligand for TLR9), which stimulate mouse cells, do not stimulate human cells well.[27,28] Murine TLR9 is expressed in wide range of dendritic cells (DC), important antigen-presenting cells including conventional and plasmacytoid DC; however, human TLR9

is expressed in plasmacytoid DC only and not in conventional (myeloid) DC, suggesting that TLR expression patterns in certain immune cells differ between murine and human cells, and that careful human studies should be carried out to determine their potency.

In humans, definitive and suggestive evidence has accumulated by large-scale analyses of SNPs in TLRs and several human diseases such as infectious diseases, asthma, atherosclerosis, and autoimmune diseases (see ref. 10). The idea is that naturally occurring variation in the innate immunity genes has an important role in human susceptibility to a variety of diseases. Identification and functional characterization of polymorphisms in innate immunity-related genes may provide insight into genetically determined susceptibility to disease that might allow us to understand the nature and outcome of the disease, and evaluate diagnostic and therapeutic strategies.[29] Knowing the TLR SNP genotype of a patient suffering a severe infectious disease may allow clinicians to tailor treatment and evaluate the prognosis. In the sections below, we discuss the relevance of TLR SNPs in human diseases and future manipulation tactics.

TLR-Based Immune Intervention in Humans: Promise and Caution

TLR agonists have been used or are under clinical development; the best examples are adjuvants in vaccine formulations. CpG DNA as a TLR9 agonist induces strong T helper 1 (Th1) immune responses, and its efficacy as a vaccine adjuvant has been demonstrated in nonhuman primates and humans.[30,31] Monophosphoryl lipid A (contains lipid A, a component of LPS, a TLR4 agonist), and R848, an agonist of TLR7, are also being developed as vaccine adjuvants.[32-34] Evidence obtained from animal experimental models suggest that TLR agonists are very potent in eliciting an innate immune response, followed by an adaptive, especially cellular, immune response; they are therefore promising candidates as vaccine adjuvants. Accumulating evidence of TLR polymorphism in humans suggest the need for a careful evaluation of genetic as well as functional variation in the target population. The first caution came from the Lyme disease vaccine, which is based on the outer-surface lipoprotein A (OspA) of the pathogen *Borrelia burgdorferi*. Some individuals with very low antibody titers after vaccination were found to have low responsiveness to TLR1 and 2 ligands, and to have lower macrophage expression of TLR1.[35]

The other caution with TLR-based immune interventions is TLR-mediated innate immune activation is so strong that the outcome may be deleterious to the host. For example, with CpG ODN, a promising TLR agonist, it has recently been shown that robust TLR9-mediated innate immune activation can cause multifocal liver necrosis and hemorrhagic ascites.[36] In addition, TLR recognition of self-molecules of the host (e.g., nucleic acids) might be linked to autoimmune diseases and other immunological disorders. For example, the TLR9 ligand CpG ODN has been implicated in triggering autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis.[37,38]

Thus, to develop potent and safe TLR agonists for immune intervention in humans, we need to have knowledge of human TLR functions, human-specific variations such as target TLR-expressing cells and SNPs, and, more importantly, the deleterious effects of TLR(agonist)-mediated immune activation.

TLR2

TLR2 is a major mammalian TLR that can recognize various components of bacteria, viruses, fungi and parasites by forming heterodimers with TLR1 or TLR6.[39,40] Studies in animals have shown that TLR2-deficient mice are more susceptible to infection with Gram-positive bacteria (i.e., *Staphylococcus aureus, Listeria monocytogenes, Streptococcus pneumoniae*), *Mycobacterium tuberculosis* and *B. burgdorferi*.[41] In humans, several SNPs were identified in TLR2 and an Arg753Gly polymorphism was found to be associated with reduced pro-inflammatory responses to *Staphylococcus* infection (Table 2).[42] Another TLR2 polymorphism, Arg677Trp, was associated with susceptibility to certain infectious diseases such as tuberculosis and leprosy, suggesting the importance of TLR2 genetic variants in the response to infections in humans.[42-46]

It is clear that TLR2 plays an essential role in the recognition of several microorganisms, and promotes defense; thus, manipulation of TLR2 function could contribute to the design of new therapeutic strategies for prevention and/or vaccine development against infectious diseases. For example, the TLR2 agonist Malp-2 (TLR2/6 ligand) has already been investigated experimentally in mice as a mucosal adjuvant.[47,48] The TLR2 agonist $Pam_3Cys\text{-}SK_4$ (TLR2/1 ligand) has been examined experimentally to treat established inflammation.[49] On the other hand, TLR2 agonists can result in experimental asthma,[50] but a polymorphism in TLR2 reduced the risk of asthma.[51] A TLR6 polymorphism was also found to reduce the risk of asthma.[52] An additional caution with TLR2 agonists is their link to atherosclerosis. Accumulating evidence suggests the involvement of multiple microorganisms such as *Chlamydia pneumonia, Helicobacter pylori* and cytomegalovirus in the inflammatory etiology of atherosclerosis.[53] Most TLRs, including TLR1, 2, 4 and 5, are expressed in atherosclerotic plaques by several cell types, and can trigger TLR2- or other TLR/MyD88-dependent activation, including the production of pro-inflammatory cytokines and chemokines in atherogenesis.[54,55] However, an Arg753Gln polymorphism of TLR2 in humans was found to be closely related to coronary restenosis,[56] suggesting that the TLR2 pathway may have a dual role in the pathogenesis of atherosclerosis. Nevertheless, the above information should be considered cautiously in the case of using TLR2 agonists/antagonists.

TLR4

TLR4 recognizes LPS, a major component of the Gram-negative bacterial cell wall that plays critical roles in the pathogenesis of Gram-negative bacterial infections such as sepsis.[57-59] It is important to search for new pharmacological interventions, through manipulation of TLR4, due to the increasing antibiotic resistance of Gram-negative and Gram-positive infections, and bacterial product-related complications such as septic shock that can not be treated with antibiotics. By controlling or modifying TLRs and their signaling, the entire septic process may be modified.[60,61]

In a mouse model, TLR4- and MyD88-deficient mice were found to be resistant to LPS challenge.[57,62] A similar phenomenon was observed in humans, in that some people with the TLR4 polymorphisms Asp299Gly and Thr399Ile were hyporesponsive to inhaled LPS.[59] Further studies sought the link between these SNPs and susceptibility to several infections, and revealed that such TLR4 polymorphisms increased susceptibility to respiratory syncytial virus (RSV) infection, brucellosis, severe malaria, and candidal bloodstream infections (refs. 63-66, and see Table 2 for TLR4 SNPs and relation to human diseases). These data are in accordance with previous reports that TLR4 recognizes not only LPS, but also RSV fusion protein,[67] indicating considerable correlation between functional mutation(s) and human infectious diseases caused by a variety of microbes.

TLR4 and several other TLR polymorphisms were found to be related to chronic inflammatory diseases such as Crohn's disease, ulcerative colitis and sarcoidosis. Indeed, TLR4 was considered to trigger the development of Crohn's disease in mice through microbial recognition in the intestine and following inflammatory responses.[68] In humans, TLR4 expression was found to be elevated in patients with ulcerative colitis or Crohn's disease, and the Asp299Gly and Thr399Ile polymorphisms of TLR4 have been linked to the etiology of both of these diseases and the chronic course of sarcoidosis.[69-71] Moreover, TLR4 has been suggested to play an important role in the recognition of *H. pylori* in the gastric mucosa,[72] though TLR4-independent detection is also possible,[73] probably due to TLR2 recognition of atypical *H. pylori* LPS,[74,75] or NOD-dependent recognition of peptide glycan.[76] In addition, *H pylori* HSP60 was demonstrated to activate both TLR2 and TLR4.[77] Further investigation of TLR2 and TLR4 polymorphisms in *H. pylori* infection in humans would be necessary to elucidate the precise molecular and cellular mechanism(s) in *H. pylori*-induced pathogenesis.

In addition, studies have suggested associations of TLR4 polymorphism with chronic obstructive pulmonary disease and severe asthma,[78,79] while other studies have observed no association between TLR4 and TLR signaling molecule polymorphisms and the risk of asthma[80] Clearly, more studies will open new avenues to understanding the role of TLR4 in asthmatic and allergic patients.

Despite the pathogenic roles described above, LPS and its purified products have been used as pharmaceutical agents due to their potency in eliciting innate immune responses. Initially, purified

Table 2. Toll-like receptors and their relation to human diseases

TLR or Signaling Molecule	SNP(s) or Genes	Effect on the Disease	Reference
TLR2	Arg753Gln	Susceptibility to *Staphylococcus* infection	42
		Susceptibility to tuberculosis	44
		Resistance to Lyme disease	137
		Increased risk for coronary restenosis	56
		Associated with severe atopic dermatitis	138
	Arg677Trp	Susceptibility to leprosy	43
		Susceptibility to tuberculosis	45
	GT-repeat polymorphism in intron II	Susceptibility to tuberculosis	46
	-16934	Reduced risk for asthma	51
TLR1,2,6	Nonsynonymous variants	Extension of inflammatory bowel diseases	139
TLR4	Asp299Gly	Hyporesponsiveness to LPS	59
		Susceptibility to meningococcal diseases in infancy	140
		Susceptibility to brucellosis	64
		Susceptibility to osteomyelitis by Gram-negative bacteria	141
		Hyporesponsive to *Porphyromonas gingivalis*	142
		Increased risk for bacterial vaginosis	143
		Association with Crohn's disease	69
		Risk factor for Crohn's disease	144
		Association with ulcerative colitis	69
		Lower incidence of carotid artery stenosis	145
		Lower incidence of acute coronary events	146
		Lower incidence of myocardial infarction	147
		Resistance to chronic obstructive pulmonary disease	78
		Associated with the severity of asthma	79
		Reduces the risk of developing late-onset Alzheimer's disease	148
		Associated with gastric MALT lymphoma	149
	Asp299Gly and Thr399Ile	Susceptibility to septic shock	150
		Susceptibility to severe RSV infection	63
		Resistance to legionnaire's disease	151
		Susceptibility to *Candida* bloodstream infections	66
		Association with chronic sarcoidosis	71
		Decreased susceptibility to RA	152
		Lower incidence of allograft rejection	153
	Thr399Ile	Susceptibility to severe malaria	65
		Association with ulcerative colitis	70
		Increased risk of severe acute graft versus host disease	154

continued on next page

Table 2. Continued

TLR or Signaling Molecule	SNP(s) or Genes	Effect on the Disease	Reference
	C119A	Risk for ischemic stroke	155
	11381G/C	Increased risk of prostate cancer	156
	Variant alleles	Lower risk of prostate cancer	157
	Rare coding variants	Susceptibility to meningococcal diseases	158
TLR5	392STOP	Susceptibility to legionnaire's disease	93
		Association to Crohn's disease	98,100
		Resistance to SLE	94
TLR6	Ser249Pro	Decreased risk for asthma	52
TLR9	T-1237C	Association with Crohn's disease	159
		Increased risk of pouchitis	160
TLR10	c.+1031G>A and c.+2322A>G	Association with asthma	161
	Haplotype GCGTGGC variant	Association with risk for nasopharyngeal carcinoma	132
IRAK4	IRAK 4 deficiency	Susceptibility to pyogenic bacterial infections	135
IKBKG	IKKγ (NEMO) deficiency	Susceptibility to pyogenic bacterial infections and atypical mycobacteria	133
NFKBIA	IkBα-deficiency	Susceptibility to pyogenic bacterial infections and atypical mycobacteria	162
		Association with sarcoidosis	163
		Association with multiple myeloma	164
IRF5	Rs2004640 T	Increased risk of SLE	136
TLR6-1-10 gene cluster and IRAK4	Synergistic effect of several alleles	Increased risk of prostate cancer	165

LPS (contains lipid A) was thought to provide prophylactic protection against subsequent bacterial or viral challenge in animals; however, its extreme toxicity prevented its use.[81] Efforts to eliminate the toxicity of lipid A led to the development of monophosphoryl lipid A (MLA).[81,82] MLA has been in human clinical studies as a new-generation vaccine adjuvant against infectious diseases and seasonal allergic rhinitis, and was proved to be safe and effective.[83,84] Lipid A analogs such as E5531, E5564 (α-D-glucopyranose) and CRX-526[85-87] have been demonstrated to act as LPS antagonists by blocking the effects of endotoxin, and have been in Phase II clinical trial against sepsis and the complications of coronary artery bypass surgery.[84] TAK-242 is another promising compound that acts through blocking TLR4-mediated signaling and has been in Phase III to treat severe sepsis (press release, http://www.takeda.com/press/05072701.htm). Thus, TLR4 ligands are apparently a double-edged sword, requiring attention to safety concerns in order to make use of their potency for therapeutic applications.

TLR5

TLR5 recognizes the bacterial protein flagellin that is found in the flagellar structure of many bacteria.[88] TLR5 is expressed in epithelial cells in the lung and gut, but is also highly expressed in residual dendritic cells, such as in the lamina propria of the intestine.[89] Signaling of flagellin via TLR5 enhances the diversity of the response, probably by engaging MyD88-independent adaptors to activate the interferon pathway, while TLR5-independent recognition machinery was reported

recently.[90] Flagellin is a potent immune activator, stimulating diverse biologic effects that mediate both innate inflammatory responses and the development of adaptive immunity. The protein nature of flagellin is considered an advantage for many immuno-therapeutic applications, mainly due to its ease of manipulation; for example, a DNA vaccine encoding chimeric protein or the protein itself with antigenic protein and flagellin has been studied.[91,92]

Humans with a common stop codon polymorphism at the ligand-binding domain of TLR5 (392STOP) were found to be highly susceptible to legionnaire's disease caused by *Legionella pneumophila*[93] (Table 2). Interestingly, the same stop codon polymorphism was found to protect people from developing SLE, indicating a possible connection of TLR5-mediated immune responses to infectious diseases with the development of autoimmune diseases.[94]

In addition to TLR2 and TLR4, TLR5 has been found to play a critical role against *Salmonella typhimurium* infection of the gastrointestinal system. TLR4 is important in the early detection of *S. typhimurium* infection, whereas TLR2 plays important roles after cellular invasion.[95] More importantly, it appears that TLR5 recognition of *S. typhimurium* flagellin is the major determinant of *S. typhimurium* infection in the gut.[96,97] TLR5-deficient mice were resistant to *S. typhimurium* infection, suggesting that TLR5 may be the sensor, not only for the pro-inflammatory response to flagellin, but also for bacterial invasion of the host via flagellin.[89]

Several studies have shown strong antibody responses to the flagellin of commensal bacteria in Crohn's disease patients but not in ulcerative colitis patients,[98,99] suggesting that flagellin can stimulate both innate and adaptive immune responses, triggered possibly through TLR5, that may promote the pathogenic response in Crohn's disease. Consistently, TLR5 stop codon polymorphism was found to be negatively associated with Crohn's disease.[100] Taking these findings together, use of flagellin or a modified version of it as an immunostimulatory or adjuvant agent in vaccine formulations may be of interest because of its protein nature, though the safety concerns with TLR5 again remain to be elucidated.

TLR7 and TLR8

Single-stranded RNA genome or oligoribonucleotides with sequences derived from HIV or influenza virus, and small synthetic compounds known as imidazoquinolins, are recognized by TLR7 in mice and TLR8 in humans, activating various immune cells that elicit Type I IFNs as well as cellular immune responses.[101-104] Several TLR7 agonists have been approved for clinical use in various viral infections.[105] The TLR7 agonist imiquimod (5% cream) has been shown to be effective for external genital warts, basal cell carcinoma and actinic keratosis,[106-108] and is in a Phase I clinical trial against human papillomavirus.[84] Several other synthetic TLR7 agonist compounds have been in Phase I or Phase II trials against hepatitis B virus, hepatitis C virus, and cancer.[84]

TLR7 also recognizes autoantigens complexed with RNA, such as U1snRNP (nuclear self-antigen) in mice,[109-112] that could play important roles in the pathogenesis of SLE. These recent reports raised safety concerns about the synthetic TLR7 agonists that are already in use in humans, in that they may act in a similar manner to the autoantigens, activating a TLR7-mediated immune cascade and triggering and/or worsening certain autoimmune diseases such as SLE. In turn, TLR7 antagonists could be important pharmaceutical agents for the treatment of SLE. A careful evaluation of dose, formulation and route of administration should be made to overcome these issues.

TLR9

TLR9 recognizes unmethylated CpG (cytosine phosphate guanosine) motifs found in bacterial and viral DNA.[30] Synthetic oligodeoxynucleotides (ODNs) that contain these CpG motifs trigger TLR-mediated, MyD88-dependent signalling of macrophages, dendritic cells and B cells to produce pro-inflammatory cytokines, chemokines and immunoglobulins. The robust innate immune response to CpG ODNs skews the host's immune milieu in favour of Th1 cell responses and pro-inflammatory cytokine production, an effect that underlies their use as immunoprotective agents, vaccine adjuvants and anti-allergens. Preclinical studies provide evidence that CpG ODNs

are effective for each of these uses and can modulate the immune response to co-administered allergens and vaccines.[31] CpG ODNs have had promising results in human use and have entered Phase III clinical trials against several types of cancer, including melanoma, lymphoma, and non-small cell lung cancer, either alone or in combination with chemotherapy.[84] The other promising application of CpG ODN under clinical development is a vaccine composed of allergen antigen conjugated with CpG ODN to either treat or prevent allergic diseases.[113,114] CpG ODN skews the Th2 allergic immune milieu to a protective Th1 immune milieu, whereby allergic symptoms are diminished or reduced.[115]

Recent evidence, however, suggests that CpG ODN should be categorized into several types and is not the only ligand for TLR9. There are at least several types of CpG ODN that activate innate immune responses differentially and that may be used in different applications.[116] Moreover, ODN-based TLR9 agonists that do not require unmethylated CpG motifs have been demonstrated,[117,118] suggesting that TLR9-mediated immunotherapeutic applications can be tailored for respective applications.[119] Although these TLR9 agonists are very potent and promising in eliciting innate and adaptive immune responses, and can be used in many immune-therapeutic applications, there are recent reports suggesting that TLR9 also recognizes other molecules, such as heme metabolites derived from malaria-infected red blood cells,[120] and self-DNA-chromatin complexes.[37,121] These results raised safety concerns for DNA-based TLR9-mediated immune intervention, though the role of TLR9 in the etiology and pathogenesis of SLE is controversial in animal models and human SNP studies.[122-124] It was previously shown that TLR9 ligand-induced arthritis was reduced by guanine-rich ODN (suppressive ODN or inhibitory ODN), an antagonist of TLR9.[125] In addition, the same ODN has been found to reduce the pathogenesis of collagen-induced arthritis, a naturally occurring autoimmune disease,[126,127] suggesting that such TLR9 antagonism may block or reduce the pathogenesis of autoimmune disease and may overcome the deleterious effects of TLR9 ligand therapies.

TLR3, TLR10

TLR3 recognizes double-stranded RNA derived from synthesis (e.g., poly-I:C), the viral genome, or the intermediates generated during viral replication, all of which have been shown to play an important role in anti-viral responses. Poly-I:C was one of the first therapeutic agents used to treat HIV and leukemia patients, but was abandoned due to its toxicity.[128] Several studies have been undertaken to reduce the toxicity of poly-I:C and there are clinical trials against breast cancer and ovarian cancer.[129] To date, there have been few studies investigating the possible involvement of TLR3 in the pathophysiology of human diseases. Pirie et al reported that polymorphisms in the TLR3 gene may be associated with Type 1 diabetes, though studies of a larger population seem to be needed.[130]

TLR10 is found in humans but has no functional homologue in rodents, and to date no ligands have been identified. However, TLR10 shares a common locus with TLR1 and TLR6 that makes hetero/homodimers with TLR2. Thus, it has been speculated that TLR10 could act as a coreceptor with TLR2.[131] Few studies have investigated polymorphisms of the TLR10 gene in relation to human diseases, especially in the lung and nasopharyngeal region, because TLR10 is expressed mostly in the pDC of those regions.[131] Recent studies have found a relationship between various TLR10 polymorphisms and increased risk of asthma and nasopharyngeal carcinoma (Table 2).[29,132]

Other Signaling Molecules

Recent evidence has suggested that primary immunodeficiency diseases of humans could be attributed at least in part to abnormal TLR signaling (for recent review, see ref. 9). In humans, primary immunodeficiency diseases due to natural genetic mutations of genes involved in intracellular signaling for innate immune responses have been reported. Patients with X-linked hypohydrotic ectodermal dysplasia, characterized by increased susceptibility to pyogenic and atypical mycobacterial infections, were reported to possess a mutation in the IKBKG gene that causes defective production of IKKγ (NEMO) protein.[133] This is not surprising, since NEMO encodes

the regulatory component of the IKK complex responsible for activating the NF-κB signaling pathway, which is critical for the TLR-mediated NF-κB activation that regulates immediate transcriptional responses in innate immunity. Similarly, IRAK4 was found to be a key signaling molecule that affects most TLR signaling. Supporting the results obtained from IRAK4-/- mice, which developed defective responses to bacterial infections,[134] patients who have frequent pyrogenic infections were found to have autosomal recessive amorphic mutations in IRAK4.[135] More recently, IRF5, which is important for the transactivation of various pro-inflammatory genes, has polymorphisms in humans that increase the risk of SLE.[136] The intracellular signaling molecules involved in innate immune responses are therefore potential targets for immune intervention, and attention should be paid to their polymorphisms.

Conclusion

Investigations of TLR-mediated innate immune responses have opened new perspectives to the understanding of the pathophysiology of disease. Mouse studies have revealed that TLR agonists or antagonists can be used to prevent or treat immune disorders. In humans, searching for SNPs in TLR genes and their relation to diseases will lead to better understanding and control of disease, and future development of new therapeutic approaches. However, TLR-mediated innate immune activation in response to infection or tissue damage may result in a deleterious outcome such as sepsis, autoimmune disease, or atherosclerosis, depending on the TLR and the signaling pathway. Furthermore, inhibition of TLR signaling may cause innate and/or adaptive immune deficiency, which could be lethal for certain patient populations (i.e., patients with SNPs). Thus, the risks and benefits of the manipulation of TLR-mediated immune responses need to be balanced.

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CHAPTER 8

Genome-Based Vaccine Development:
A Short Cut for the Future

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Abstract

Bacterial infectious diseases remain a major cause of deaths and disabilities in the world. Although conventional vaccinology approaches were successful in conferring protection against several diseases, they failed in providing efficient vaccines against many others. Together to the sequencing of the first genome, a new chapter in the vaccinology history started to be written. Reverse vaccinology changed the way to think about vaccine development, using the information provided by the microorganisms' genome against themselves. Since then, reverse vaccinology has evolved and helped researchers to overcome the limits of the conventional vaccinology approaches and led to the discovery and development of novel vaccines concerning emerging diseases, like *Neisseria meningitidis* B and *Streptococcus agalactiae*. A lot of work must be done, but deciphering the information provided by genome sequences and using it to better understand the host-pathogen interactions has proved to be the key for protection.

Conventional Vaccinology

Vaccine development followed the same basic principles for more than two centuries. When Edward Jenner inoculated James Phipps with a bovine poxvirus to induce protection against the closely related human pathogen smallpox virus in 1796 and then, almost a century later, Pasteur developed a live attenuated vaccine against rabies, the basic principles for vaccine development were established.[1] These approaches served as guidelines for the development of vaccines throughout the twentieth century, allowing the protection against many once lethal infectious diseases. In fact, all existing vaccines were developed using at least one of the following approaches: killed (inactivated), live attenuated or subunit (including the protein-conjugated capsular polysaccharides, toxoids, cell-free extracts, recombinant proteins and stand-alone capsular polysaccharides) vaccines. Because of those basic principles, several infectious diseases can be prevented by vaccines. Table 1 shows the vaccines licensed for immunization and distribution in USA, all of them obtained basically by conventional approaches.[2] Conventional approaches led to great achievements such as the eradication of smallpox and the virtual disappearance of diseases like diphtheria, tetanus, poliomyelitis, pertussis, measles, mumps, rubella and invasive *Haemophilus influenzae* B, increasing the life quality and expectancy.[3] It was also important to provide the basis of vaccinology but showed to be time-consuming, leading to years or even decades of research. Inactivation and attenuation were the first choice for many years, but the difficulty in cultivating some microorganisms in vitro and the fact that even attenuation may result in detrimental or unwanted immune responses showed that these approaches are impractical in some instances.[4] Even the purification of specific antigens failed in many cases in providing a protective vaccine candidate, since the

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Table 1. Vaccines licensed for immunization and distribution in the USA

Vaccine or Target	Type
Anthrax	Subunit
Diphtheria	Subunit
Haemophilus influenzae b	Subunit
Hepatitis A	Inactivated
Hepatitis B	Subunit
Human papillomavirus	Subunit
Influenza virus	Inactivated, live
Japanese encephalitis	Inactivated
Measles	Live
Meningococcus	Subunit
Mumps	Live
Pertussis	Subunit
Plague	Inactivated
Pneumococcus	Subunit
Polio	Inactivated
Rabies	Inactivated
Rotavirus	Live
Rubella	Live
Smallpox	Live
Tetanus	Subunit
Tuberculosis	Live
Typhoid	Live, subunit
Varicella	Attenuated
Yellow fever	Live
Herpes zoster	Live

methods usually used led to the identification of the most abundant but also most variable and less suitable vaccine candidates.[5] Although successful for many pathogens, conventional vaccinology still left many diseases uncontrolled by vaccination. Considering that even new diseases are sure to emerge through evolution by mutation and gene exchange, interspecies transfer or human exposure to novel environments,[6] a faster and more reliable approach must be available to promptly respond to those threats.

Reverse Vaccinology

The sequencing of the first bacterium genome in 1995 led the vaccine development to enter a new era and to open a new chapter in the vaccine development guidebook. Suddenly, all proteins encoded by a microorganism were available and for the first time, after more than two centuries, it was possible to identify vaccine candidates without using the conventional vaccinology principles. This new approach, named reverse vaccinology, gives full access to all the proteins that a microorganism can encode and, by computer analysis, it is possible to identify potential surface-exposed proteins in a reverse manner, starting from the genome rather than the microorganism. Problems related to noncultivable microorganisms and also to antigens that are not expressed in in vitro conditions, conferring the most important obstacles for vaccine development, could be avoided by the reverse vaccinology approach. The feasibility of this approach relies on the availability of a high-throughput system for protective immunity screening and also on good correlates of protection (Fig. 1). The greatest limitation of the reverse vaccinology approach is the inability in identifying nonproteic antigens such as polysaccharides, components of many successful vaccines

Figure 1. The reverse vaccinology equation: the availability of *n* genome sequences, associated to advanced bioinformatic tools and reliable high-throughput systems for protective immune screening have proved to be a balanced equation for vaccine development.

and glycolipids, a new promising group of vaccine candidates.[5] Nevertheless, reverse vaccinology seemed to be a powerful tool that could help researchers to overcome the obstacles of conventional vaccinology and lead to the discovery and development of novel vaccines for the most concerning emerging diseases.

The Classical Reverse Vaccinology Approach

For many years great efforts were concentrated in the development of a vaccine against serogroup B *N. meningitidis* (meningococcus), a bacterium that causes about 50% of meningococcal cases of sepsis and meningitis and has a mortality rate of 5-15% despite the advances in therapeutics. *N. meningitidis* is a Gram-negative capsulated bacterium that can be classified in 13 serogroups on the basis of the chemical composition of the capsule polysaccharide. Among them, only 5 serogroups have been associated with meningococcal meningitis and sepsis: A, B, C, Y and W135. For serogroups A, C, Y and W135 the use of capsular polysaccharides as vaccine components has shown to confer protection in adults and infants, especially when the polysaccharide is conjugated with a carrier protein, eliciting a T-cell dependent immune response and conferring long-term protection. Unfortunately, this approach was not feasible for serogroup B, since its capsular polysaccharide is a polymer of α(2-8)-linked N-acetylneuraminic acid that is also present in mammal tissues and its use in a vaccine could lead to autoimmune responses.[7]

When many other conventional approaches failed to produce an effective vaccine against meningococcus B, the reverse vaccinology approach appeared as a logical and promising alternative to deliver a vaccine. While the *N. meningitidis* genome sequence was still been assembled, computer analysis allowed the prediction of proteins that could be surface-exposed or homologous to known factors associated with virulence and pathogenesis, leading to the selection of 570 potential vaccine candidates. Successful cloning and expression was obtained for 350 proteins in *Escherichia coli*, which were then purified and used to immunize mice.[7,8] Immune sera were then tested in ELISA, FACS and Western Blotting analysis in order to confirm their surface-exposed localization and also in complement-mediated bactericidal assays to test their immunogenicity and protective activity, since this assay correlates with protection in humans. Out of 81 proteins found to be strongly positive in at least one of the mentioned assays, 28 showed to be positive in all of them. To confirm the possibility of using these candidates in a vaccine able to protect against heterologous strains, their presence and conservation were tested in a panel of 31 strains of *N. meningitidis* isolated worldwide and over many years. Out of the 28 proteins tested, five showed to be strikingly conserved in the panel, a result not quite expected since they are surface-exposed proteins. In less then two years, reverse vaccinology achieved what the conventional vaccinology approaches pursuit by decades: surface-exposed proteins in *N. meningitidis* B, able to induce protection and cross-reactivity among distantly related strains and serotypes, suitable to be used in a universal vaccine against this microorganism. Simple by extracting the benefits from genome information and applying them in the development of novel vaccines.

Comparative Genome Analysis: The Second Phase of Reverse Vaccinology

The success obtained with the *N. meningitidis* B experience prompted the application of the reverse vaccinology to other pathogens, such as *Streptococcus pneumoniae, Porphyromonas gingivalis, Chlamydia pneumoniae, Bacillus anthracis, Streptococcus agalactiae, Streptococcus pyogenes,* ExPEC and many others, becoming a routinely classical approach for vaccine development (Table 2). But something unexpected happened when the complete genome of a virulent isolate of *S. agalactiae* (group B streptococcus) was sequenced. *S. agalactiae* is one of the leading causes of bacterial sepsis, pneumonia and meningitis in neonates in the USA and Europe and also an emerging cause of infection in the elderly. Conjugate vaccines based on the five major capsular polysaccharides were currently under development, but they were not able to cover all available serotypes. Since conventional approaches failed in providing a universal and efficient vaccine for the most affected group and since the complete genome sequence of two *S. agalactiae* strains was available in 2002, the classical reverse vaccinology approach sounded logical. But the *S. agalactiae* experience was going to be more challenging. In order to verify the diversity of *S. agalactiae* genome and provide information for a future universal vaccine against this microorganism, comparative genomic hybridization was applied, using the sequenced strain as reference. It was found that approximately 18% of the genes encoded in the sequenced strain was absent in at least one of the other nineteen *S. agalactiae* tested. The problem is that comparative genome hybridization is able to provide information only for the genome sequence that is shared among the strains. Therefore, specific genes in the other strains that are absent in the sequenced genome could not be detected. It could be a problem for a universal vaccine achievement, leading the classical reverse vaccinology to evolve. Sequencing the genome of only one strain could not be enough anymore to provide the information needed for a universal vaccine development, especially when a high variability is observed. To provide the information requested, another additional six genome sequences of *S. agalactiae* were determined. This new information showed that 1806 genes are shared by all strains of *S. agalactiae*, representing the 'core genome' that corresponds to approximately 80% of the average number of genes encoded in each strain. The core genome mainly encodes factors for functions that contribute to the major metabolic pathways, the so called housekeeping genes that usually define the identity of a species. The complementary set of genes, absent in at least one strain, correspond to the 'dispensable genome', probably related to the adaptation of strains in specific environmental conditions by conferring selective advantages. Mathematical extrapolation predicted that, no matter how many strains have been sequenced, each new genome should provide genes that have never been found before. Sequencing additional genomes allowed the estimation

Table 2. Examples of vaccines that have been developed using reverse vaccinology-based approaches

Pathogen	Disease	Status of Vaccine Development	Ref.
Neisseria meningitidis B	Meningitis and septicemia	Phase II	7
Streptococcus agalactiae	Septicemia, pneumonia and meningitis	Preclinical	9,10
Streptococcus pneumoniae	Pneumonia	Preclinical	11
Bacillus anthracis	Anthrax	Preclinical	12
Chlamydia pneumoniae	Pharyngitis, bronchitis and pneumonitis	Preclinical	13
Porphyromonas gingivalis	Periodontitis	Preclinical	14

of *S. agalactiae* pan-genome size (the set of genes that will be observed at least once if an infinite number of different strains would to be sequenced), corresponding to 2713 genes, of which 907 belong to the dispensable genome and also allowed the prediction that the pan-genome is going to grow about 33 new genes every time a new strain is sequenced. This profile is completely different of those observed for other microorganisms, such as *Bacillus anthracis*. Eight genome sequences were determined for this microorganism, but after the fourth it was verified that the number of new genes added to the pan-genome rapidly converged to zero.[15,16]

Comparative genome analysis also provided the information necessary to face the quest of providing a universal vaccine against *S. agalactiae*. Computational algorithms predicted 589 surface-associated proteins, of which 396 belong to the core genome and 193 were absent in at least one strain. Each of this protein was tested for protection and four antigens were able to elicit protective immune responses in the animal model, not only by passive immunization and challenge in newborn mice but also active maternal immunization and challenge of offspring within the first 48 hours of life.[15] None of these protective antigens could be classified as universal, because three of them were absent in a fraction of the tested strains and the fourth, belonging to the core genome, had a deficient surface accessibility in some strains.[17] The cocktail combining the four best candidates conferred 59-100% protection against a panel of 12 *S. agalactiae* isolates, including the major serotypes, as well as two strains from a less common serotype.[15] Without the determination of additional genome sequences, a universal vaccine against *S. agalactiae* would have been compromised. The comparative genome analysis provided new concepts in delivering universal vaccines by the reverse vaccinology approach, even for microorganisms in which a high variability can be observed, opening the pan-genomic reverse vaccinology era.

Although the classical reverse vaccinology approach was efficient in providing protective candidates against serogroup B *N. meningitidis*, subsequent analysis showed that NadA, one of those best candidates, is present in only 50% of strains from patients with meningococcal disease and only 25% of strains carried by healthy people, indicating that also the pan-genome of this microorganism could have the same features of *S. agalactiae* pan-genome.[15] It is possible that the availability of novel genome sequences of *N. meningitidis* could provide even more candidates that could increase the coverage and efficiency already achieved.

Subtractive Genome Analysis: Third Phase of Reverse Vaccinology?

After the sequencing of the first genome in 1995, great efforts have been concentrated in the determination of the genome sequences of other pathogenic bacteria that could lead to the development of novel vaccines based on the reverse vaccinology approach such as the *N. meningitidis* B and *S. agalactiae* experiences. Comparative genome analysis of pathogenic strains (the second generation of reverse vaccinology) have concentrated their efforts in comparing pathogenic strains of a species looking for the identification of antigens that could lead to a maximum coverage in a universal vaccine. But what about nonpathogenic strains? It could sound strange to sequence a nonpathogenic strain genome in order to obtain a vaccine against those pathogenic ones, but a nonpathogenic genome could provide the information necessary for the identification of antigens that really could make the difference in pathogenesis, responsible for the most strictly host-pathogen interactions. In a subtractive comparative genome analysis, genes conserved between pathogenic and nonpathogenic strains of a same or even related species could be discarded, reducing the number of candidates and, consequently, reducing the time for the delivery of a vaccine. Since the ability of causing diseases is frequently related to the integrity of some genes, algorithms must take into account some gene inactivation process such as frameshifting. Of course a whole set of factors are usually responsible for pathogenicity, therefore, also in this case the sequencing of only one nonpathogenic genome could not be enough for a complete understanding of pathogenicity. In some cases, looking for candidates where they do not exist could provide the answers that the comparison between pathogenic strains could not give.

The concept of commensal strains must be also clarified for the success of this approach, since the term 'commensal' is usually associated with nonpathogenic strains or even probiotics.

Table 3. Sequencing genome projects of infectious bacterial species associated with human diseases

Organism Name	Genome Projects
Borrelia afzelii, Clostridium difficile, Fusobacterium nucleatum, Helicobacter pylori, Mycobacterium bovis, Mycoplasma genitalium, Neisseria meningitidis, Rickettsia rickettsii, Shigella dysenteriae, Shigella flexneri, Vibrio parahaemolyticus	3
Acinetobacter baumannii, Borrelia burgdorferi, Burkholderia thailandensis, Salmonella typhimurium, Shewanella putrefacien, Shewanella sp.	4
Burkholderia cenocepacia, Chlamydophila pneumoniae, Legionella pneumophila, Ureaplasma parvum	5
Streptococcus suis	6
Pseudomonas aeruginosa	7
Clostridium perfringens, Mycobacterium tuberculosis, Streptococcus agalactiae, Ureaplasma urealyticum	8
Clostridium botulinum	9
Burkholderia mallei, Coxiella burnetii	10
Bacillus anthracis, Campylobacter jejuni	11
Streptococcus pyogenes	13
Streptococcus pneumoniae	14
Francisella tularensis, Vibrio cholerae	15
Bacillus cereus, Staphylococcus aureus, Yersinia pestis	16
Haemophilus influenzae	19
Burkholderia pseudomallei	21
Listeria monocytogenes, Salmonella enterica	24
Escherichia coli	25

Commensal bacteria are those that usually colonize an individual without causing disease. Within the commensal flora we can find the 'pathogenic commensals' that have the ability to cause disease when the organism is vulnerable. It is important not to confuse those 'pathogenic commensals' (like *S. pneumoniae* and *H. influenzae* B) with the 'nonpathogenic' ones (like some species of *Lactobacillus*).[18]

The structure and composition of the flora is a result of selection at both microbial and host level, leading to a mutual cooperation and stability of this complex system, allowing the flora to form a natural barrier that has numerous protective (pathogen displacement, nutrient competition, receptor competition, etc), structural (barrier fortification, induction of IgA, immune system development, etc) and metabolic effects (metabolization dietary carcinogens, synthesis of vitamins, ion absorption, etc.).[19] Hence the delivering of vaccines against microorganisms that belong to the commensal flora is not an easy task, even for the 'pathogenic commensals'. It has been shown that polysaccharide heptavalent vaccine against *S. pneumoniae* can lead to the replacement with nonvaccine serotypes, but still able to cause infection and that the polysaccharide-conjugated vaccine against *H. influenzae* B led to the increase of serotype F incidence.[20,21] The consequences of a vaccine against commensal flora components are usually unexpected, since reducing the level of competition could promote the emerging of other 'pathogenic commensal'.[19] Considering that

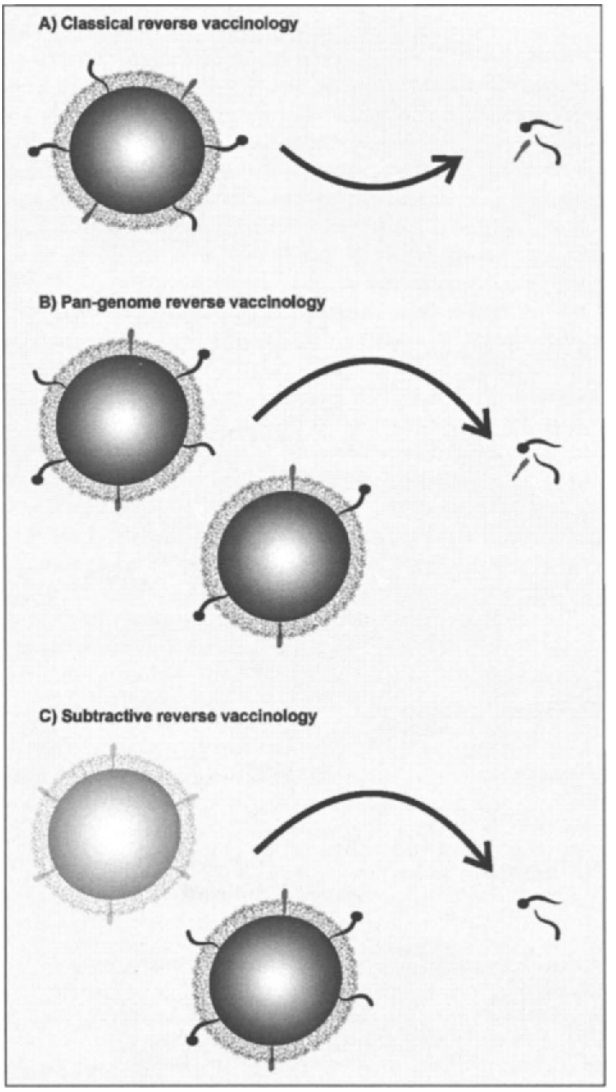

Figure 2. Reverse vaccinology approaches. A) The classical reverse vaccinology approach consists in mining a genome sequence for the identification of putative surface-exposed antigens that could be used as vaccine candidates. B) The pan-genome reverse vaccinology approach compares different genome sequences of different strains to increase the coverage and to avoid the escape of the microorganism by antigen variability. C) The subtractive reverse vaccinology approach consists in comparing pathogenic and nonpathogenic genome sequences in order to select those antigens that could be directly involved in pathogenesis.

virulence, fitness and colonization factors could be overlapping and dependent of the niche and environmental conditions, understanding the 'nonpathogenic' commensals could prevent the development of vaccines with a minimum impact to the commensal flora, especially for microorganisms that possess both phenotypes, such as *E. coli*.

Conclusion

To date, 1,327 bacterial genomes have been sequenced, of which 520 genome sequences have already been completed. Table 3 shows the number of genome sequencing projects of bacteria that could be associated to human diseases.[22] For most of them, no efficient vaccine is available. In those cases, a comparative genome-based analysis would permit not only the better understanding of strain variability and pan-genome estimation, but also the delivering of a single or combined-antigen universal vaccines in a field where conventional approaches have failed as proved by *N. meningitidis* B and *S. agalactiae* experiences (classical and pan-genome reverse vaccinology). Even for vaccines already available, these approaches could permit the improvement and increment of protection by inclusion of novel antigens, when required. Moreover, the knowledge of antigen gene variability could drive strategies of rational engineering to improve the coverage with proven efficacy. Considering the fact that nonpathogenic strains are present for many genera, sequencing of nonpathogenic genomes could be also important in highlighting the antigens that really could make the difference in pathogenesis. Comparative genome analysis between pathogenic and nonpathogenic genomes would prompt straight to the genes responsible for pathogenesis and, in most cases, responsible for the closest interactions between pathogen and host and, therefore, good vaccine candidates (Fig. 2).

In conclusion, many diseases can not be still controlled by vaccination and more diseases are expected to emerge. After two hundred years of vaccine research and development, genome sequencing provided crucial information required to defeat infectious microorganisms. Nevertheless, we need to learn to decipher the information generated in order to convert it in protective vaccines. Classical reverse vaccinology approach made the first moves, been successful for the development of a vaccine against *N. meningitidis* B. In spite of that, it had to evolve in order to be successful against a more complex microorganism like *S. agalactiae*. And it still needs to evolve, providing novel tools that will avoid the continuing escape of microorganisms from host defense mechanisms.

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CHAPTER 9

The Antigenome:
From Protein Subunit Vaccines to Antibody Treatments of Bacterial Infections?

Carmen Giefing, Eszter Nagy and Alexander von Gabain*

Abstract

New strategies are needed to master infectious diseases. The so-called "passive vaccination", i.e., prevention and treatment with specific antibodies, has a proven record and potential in the management of infections and entered the medical arena more than 100 years ago. Progress in the identification of specific antigens has become the hallmark in the development of novel subunit vaccines that often contain only a single immunogen, frequently proteins, derived from the microbe in order to induce protective immunity. On the other hand, the monoclonal antibody technology has enabled biotechnology to produce antibody species in unlimited quantities and at reasonable costs that are more or less identical to their human counterparts and bind with high affinity to only one specific site of a given antigen. Although, this technology has provided a robust platform for launching novel and successful treatments against a variety of devastating diseases, it is up till now only exceptionally employed in therapy of infectious diseases. Monoclonal antibodies engaged in the treatment of specific cancers seem to work by a dual mode; they mark the cancerous cells for decontamination by the immune system, but also block a function that intervenes with cell growth. The availability of the entire genome sequence of pathogens has strongly facilitated the identification of highly specific protein antigens that are suitable targets for neutralizing antibodies, but also often seem to play an important role in the microbe's life cycle. Thus, the growing repertoire of well-characterized protein antigens will open the perspective to develop monoclonal antibodies against bacterial infections, at least as last resort treatment, when vaccination and antibiotics are no options for prevention or therapy. In the following chapter we describe and compare various technologies regarding the identification of suitable target antigens and the foundation of cognate monoclonal antibodies and discuss their possible applications in the treatment of bacterial infections together with an overview of current efforts.

Introduction

Infectious diseases remain a major threat against human life. Microbial infections are still out of control in many parts of the less developed world where they count for most of the deaths, but also cause an often underestimated toll of death (e.g., community acquired Pneumococcal diseases and *Pseudomonas* infections in patients in intensive care), life-long mutilation (infertility due to *Chlamydia trachomatis*), medical complication due to nosocomial infections caused most often by *Staphylococcus aureus, Enterococcus faecalis, Klebsiella* ssp and fungi. It is estimated that nosocomial infections annually add US$5-10 billion to the cost of the national healthcare system in the United States.[1] Apart from infections caused by viruses and protozoa that only in specific instances can

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be treated with suitable pharmaceuticals, the emergence of antibiotic-resistant strains of nearly all kinds of bacterial pathogens in the community and in hospitals is occurring at an increasingly alarming rate.[2,3] The increase of nosocomial infections, the comeback of bacterial infections in immune suppressed individuals, e.g., TB in AIDS patients,[4] and the lately appeared scenario of bio-terrorism, e.g., in the context of anthrax,[5,6] are reminders that new strategies are needed to master infectious diseases in prophylactic and therapeutic settings.

Vaccination is undeniable the most successful medical intervention in the control of infectious diseases. However, since vaccine-induced immune protection against specific microbes takes more than a couple of weeks to develop and postexposure vaccination is only exceptionally a useful tool, combination with passive immunization is indispensable (e.g., treatment against the rabies virus, reviewed in ref. 7), when instant protection or treatment is required. Therapeutic vaccines are still in the exploratory stage of development and more prone to find their application in the treatment of chronic infectious diseases,[8,9] rather than to become an immediate measure against a sudden infectious threat. On the other hand, most vaccines seem to confer protective immunity to the vaccinated individuals by the means to induce specific antibodies that capture the invading microbe, prior it had an opportunity to colonize in the exposed host. The so-called "passive vaccination", i.e., prevention and treatment with specific antibodies, has a proven record and potential in the management of infections.

Already the pioneers of early microbiology and immunology in the late 19th century, led by their prominent proponents, Emile Roux and Emil von Behring, have realized the concept of "passive vaccination", namely that sheep and horses inoculated with filterable toxin extracts derived from *Corynebacterium diphteriae* cultures were able to mount an "anti-toxin" in their blood. Serum derived from the animals' blood was able to rescue children in the lethal stage of the infection caused by the same pathogen. Revisiting this historical landmark therapy of diphtheria, it was realized that the "anti-toxin" in the serum of inoculated animals is synonymous with a protein species coined today antibodies and the "toxin" with a virulence factor secreted by the pathogen during infection. Thus, the remarkable and groundbreaking therapy concept explored more than 100 years ago, has paved the way to "passive immunization", i.e., all kind of serum-treatments that have found their broad medical applications in prevention of e.g., viral infections or in emergency treatments against e.g., snake venoms.[10,11] Serum antibodies against microbes and even isolated antigens, like the diphtheria toxin, are polyclonal, meaning that they bind—in case of a specific antigen molecule—to a variety of sites or—in case of a microbe—to multiple surface structures.

The advent of the monoclonal antibody technology launched by Georg Kohler and Cesar Milstein nearly 30 years ago, has enabled biotechnology to engineer specific antibody species that bind with high affinity to only one specific site of a given antigen and can be produced in unlimited quantities. Follow-up technologies made it possible to produce monoclonal antibodies that are more or less identical to their human counterparts, employing microbial and tissue culture resources for manufacturing.[12] During the last decade, monoclonal antibodies have infiltrated the therapeutic arena with great success and thereby provided a plethora of novel treatments against a variety of typically devastating diseases including specific cancers, autoimmune diseases and other pathological conditions.[13] The common denominator of all monoclonal antibodies used in therapy is to bind to highly specific sites of typically well characterized protein targets and thereby intervene with biological functions involved in the pathogenic condition; e.g., to growth hormone receptors expressed at the surface of malignant cells.[14,15] Interestingly, monoclonal antibodies engaged in the treatment of specific cancers seem to work by a dual mode; they mark the cancerous cells for decontamination by the immune system, but also block a function that intervenes with cell growth.[16]

Progress in the identification of specific antigens has become the hallmark in the development of novel subunit vaccines that only contain single specific structures derived from the microbe in order to induce protective immunity. The first viral subunit vaccine on the market that has become a great success is directed against Hepatitis B virus and based on recombinant protein

technology. Also pathogen-specific glycosides coupled to carrier proteins are successfully used in so-called conjugated vaccines directed against bacterial infections; an example is "Prevnar" a registered vaccine against *Pneumococcus*.[17] The successful development of subunit vaccines comprising isolated microbial components as antigens has supported the notion that antibodies per se, may suffice to neutralize pathogens in the body even in a setting of "passive vaccination". So far only one anti-infective monoclonal antibody, which is directed against the Respiratory Syncytial Virus (RSV) (Palivizumab), has entered the therapeutic arena.[18] A number of anti-infective antibodies based on specific antigens against bacterial infections are in the stage of clinical and preclinical development (Table 1).

The availability of the entire genome sequence of pathogens and subsequently the application of proteome and genome based technologies have facilitated the identification of highly specific protein antigens suited for the development of novel bacterial subunit vaccines.[19] One of the recently described methods designed to comprehensively mine bacterial genomes for protective antigens, has taken advantage of antibodies derived from humans who have encountered the target pathogen with positive outcome. The sum of all protein antigens that are recognized by cognate antibodies from individuals exposed to the pathogen has been defined as antigenome.[20-22] Typically the antigenome comprises 100 to 200 antigens. Applying a number of selective filters and criteria to the antigenome, in vitro validation makes it possible to reduce the number of best-suitable candidate antigens for vaccine development to about 15 to 30 (unpublished data). Such antigens are presently tested in advanced preclinical and early clinical trials (Kuklin et al[23] and unpublished data). The availability of bacterial protein antigens with promising profiles for vaccine design, but also the identification of specific host targets, have provided novel gates to develop monoclonal antibodies for protection and treatment against specific infectious diseases. In the following chapter we will discuss the impact of discovery and characterization of specific antigens on the development of novel vaccines and antibody treatments.

A New Paradigm in Bacterial Vaccine Development

The capability of the human immune system to identify and eliminate pathogens and pathogen-infected cells is the cornerstone of immunization, the most effective strategy to prevent infectious disease. However, vaccines are still not available against major pathogens including *Meningococcus* serogroup B, *Gonococcus, Helicobacter pylori* and *Shigella*. Traditional vaccines are mainly based on inactivated or attenuated microbes or more recently on polysaccharides of a particular pathogen. Due to the fact that such vaccines cannot prevent numerous diseases, or even worse, induce severe side effects, novel and defined vaccines are being developed to overcome these limitations. Improved vaccines are needed to combat diseases for which current vaccines are inadequate (e.g., tuberculosis) or against pathogens that had not been on the target list for immunization, such as *Staphylococci* and *Enterococci* both with an enormous potential to develop drug resistance.[24,25] The recently emerging threat of bioterrorism boosts the need for new vaccines further.

Most of the new generation vaccines comprise subunits of pathogens (purified protein, toxoid, polysaccharide with or without conjugation) and have made major headways in controlling serious diseases. At present, there are only two vaccines based on recombinant proteins (against Hepatitis B and Lyme disease) that are shown to be effective in preventing human infections. Nevertheless, protein based recombinant vaccines are considered to be the most promising approach to meet the demands of future vaccinology.

In order to design novel subunit vaccines, the proper antigens have to be identified and subsequently evaluated in experimental animal models mimicking human diseases. While vaccine development for obligate pathogens with well-defined virulence mechanisms has progressed well, those bacteria that are in the focus of current vaccine efforts (e.g., opportunistic pathogens and those with multiple serotypes) have more complex pathogenesis.

Vaccinologists are witnessing a remarkable revolution in technologies that now contribute to rapid identification of novel vaccine components against many important human pathogens.

Table 1. Therapeutic antibody approaches against bacterial infections

Drug	Pathogen	Antigen Target	Type of Antibody	Highest Phase	Originator	Indication
Aurograb®	*S. aureus*	ABC transporter	Human-derived single chain variable fragment (scFv) therapeutic antibody	III	NeuTec Pharma	Methicillin-resistant *S. aureus* infections
Altastaph™	*S. aureus*	*S. aureus* Type 5 and Type 8 polysaccharides	Polyclonal (5% Ig prepared from donors immunized with StaphVAX™)	II	Nabi Biopharmaceuticals	Staphylococcal infections
Pagibaximab	*Staphylococcus* spp.	Lipoteichoic acid (LTA) *S. epidermidis*	Chimeric mAb	II	Eli Lilly/Biosynexus	Staphylococcal infections
Aurexis®	*S. aureus*	*S. aureus* ClfA	Humanised mAb	II	Inhibitex	Treatment—in combination with standard-of-care antibiotics—of serious *S. aureus* infections in hospitalized patients
Veronate®	*S. aureus*	Staphylococcal fibrinogen-binding proteins SdrG and ClfA (MSCRAMM)	Human polyclonal	Preregistarion	Inhibitex	Staphylococcal infections in VLBW (very low birth weight) infants: Prevention of hospital-associated infections in premature infants weighing less than 1,250 grams

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Table 1. Continued

Drug	Pathogen	Antigen Target	Type of Antibody	Highest Phase	Originator	Indication
ETI 211	*S. aureus*	*S. aureus* protein A	Antibody conjugate: anti-human complement receptor Type-1 mAb chemically cross-linked with an anti-*S. aureus* protein A mAb	Preclinical	Elusys Therapeutics	Methicillin-resistant *S. aureus* infections
S. aureus mAb	*S. aureus*	IsdB	Fully human mAbs	Preclinical	Merck/Intercell	Staphylococcal infections
SdrG mAb	*S. epidermidis*	SdrG-fibrinogen-binding MSCRAMM protein	Human polyclonal	Preclinical	Inhibitex	*S. epidermidis* infections
Enterococcal mAb	*Enterococcus*	MSCRAMM® proteins	Fully human mAbs	Preclinical	Inhibitex/Dyax	Drug-resistant enterococcal infections
IC 47 therapeutic antibodies	*S. pneumoniae*	Surface proteins	Fully human mAbs	Preclinical	Kirin/Intercell	Pneumococcal infections in immunocompromised patients
KBPA 101	*P. aeruginosa*	Directed against pseudomonal serotypes	Fully human mAbs	II	Kenta Biotech	Pseudomonal infections
Anti-*P. aeruginosa* mAbs	*P. aeruginosa*	Natural human immune response	Fully human mAbs	I	Berna Biotech	Pseudomonal infections
Anti-*P. aeruginosa* mAbs	*P. aeruginosa*	Undisclosed	Fully human mAbs	Preclinical	Millenium Biologix Corporation	Pseudomonal infections

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Table 1. Continued

Drug	Pathogen	Antigen Target	Type of Antibody	Highest Phase	Originator	Indication
MDX 066	*Clostirium difficile*	Toxin A	Fully human mAbs	II	Medarex, University of Massachusetts Medical School	*C. difficile* infections, Diarrhea
MDX 1388	*C. difficile*	Toxin B	Undisclosed	Preclinical	Medarex, MBL	*C. difficile* infections
DiffCAM™	*C. difficile*	*C. difficile* toxins	Bovine immunoglobulins	I	ImmuCell	*C. difficile* infections
Anti-botulism neurotoxin mAbs	*Clostridium botulinum*	Type A-botulinum neurotoxin	mAbs	Preclinical	XOMA	Biological agents used in bioterrorism
Urtoxazumab	Shiga-like toxin-producing *E. coli*	B-subunit of shiga-like toxin II (SLT II)	Recombinant humanised mAbs	II	Teijin Pharma	haemolytic uraemic syndrome
Raxibacumab	*B. anthracis*	*B. anthracis* protective antigen	Fully human mAbs	I	Human Genome Sciences	Anthrax
Valortim™	*B. anthracis*	*B. anthracis* protective antigen	Fully human mAbs	I	Medarex	Anthrax
Anthim™	*B. anthracis*	*B. anthracis* protective antigen	Heteropolymer chemically linked mAb	I	Elusys Therapeutics	Anthrax
Afelimomab	Septic shock	human TNFα	Murine mAbs	III	Abbott GmbH & Co. KG	septic shock
Anti-TNFα	Septic shock (among others)	human TNFα	Sheep polyclonal	II	Protherics	septic shock (among others)

Data were collected from the Adis R&D Insight database and homepages of listed companies in July 2006 using the world wide web. Antibodies were sorted according to antigen target: first, antibodies targeting pathogen surface structures are listed, followed by anti-toxin antibodies and last antibodies against TNFα. Monoclonal Antibodies are abbreviated with mAbs. This is not a complete list.

The availability of complete genome sequences of pathogens has dramatically changed the perspectives for developing improved and novel vaccines by increasing the speed of target identification. Genomics-based technologies have many advantages compared to conventional approaches, which are time-consuming and usually identify only abundant antigens expressible under in vitro culture conditions. Strategies based on genomics have made major contributions to the identification and selection of novel vaccine candidates to combat bacterial infections by exploiting genome sequence information in alliance with adjunct technologies, including in silico prediction (bioinformatics), expression analyses (random mutagenesis, microarrays, in vivo expression technologies), or protein/peptide based selection methods (proteomics and immuno-selection using peptide expression libraries). Although, most technologies can be readily applied to most pathogens, certain strategies are more suitable than others due to distinct advantages and limitations.

The most promising candidate antigens have to be (1) expressed during human disease; (2) accessible (surface bound or secreted) for functional antibodies or effector immune cells; (3) conserved among strains; (4) essential for in vivo survival in order to avoid counter selection; and (5) protective in animal models mimicking the relevant human disease. There is no technology available today that can select antigen candidates fulfilling all five attributes. However, a comprehensive selection procedure meeting the key criteria can be combined with a validation screening that addresses the remaining requirements.

To date, approximately 300 pathogen genome sequences have been determined (http://www.tigr.org/cmr). Genome sequences of bacterial pathogens contain an average of 2700 genes, thus appropriate selection criteria have to be applied to reduce the number of antigen candidates for empirical testing. Bioinformatics has been successfully employed for the prediction of candidate antigens of extracellular pathogens, due to the specific features easing the prediction of cell surface and secreted proteins and/or the identification of genes that show sequence and/or structural homology to known virulence factors.[26] This type of genome-based systematic search for vaccine candidates was termed "reverse vaccinology". The validity of this approach was first confirmed by the identification of protective antigens from *Meningococcus* serogroup B and later from *Pneumococcus* (reviewed in ref. 27). "Reverse vaccinology in silico prediction" typically targets up to 25% of all genome-encoded proteins and, thus, necessitates subsequent high through-put cloning and recombinant protein expression. Inclusion of more restrictive selection criteria became possible through the availability of several genomes for individual pathogenic species. Comparative genomics is another suitable tool to identify genes shared among species of related pathogens or, alternatively, to identify genes present only in pathogenic, but not in attenuated or naturally nonpathogenic strains or species. Such approaches have been successfully applied to Group A *Streptococcus* and *Mycobacterium*.[28-30]

The hallmark of effective vaccine antigens is their ability to induce antibodies and/or to activate immune cells. Regarding this feature, in silico prediction of antigenicity is still in infancy. It is anticipated that with the wealth of knowledge currently being generated, it will be possible to develop prediction algorithms to pinpoint proteins likely to be immunogenic and/or protective.[31] More advanced is the strategy to mine genomic sequence databases of intracellular pathogens for predicted T-cell epitopes and validate them experimentally based on immune recognition.[32,33] Despite all successes, the bioinformatic genome mining approach has limitations due to the inaccuracy of available algorithms, regarding the prediction of (1) open-reading frames that encode proteins; (2) surface and secreted proteins; (3) gene function based on homology searches. Moreover, it is almost impossible to predict the conditions under which candidate antigens are expressed, unless the genes are equipped with well-defined regulatory sequences and promoters.

The availability of complete pathogen genome sequences stimulated the development and wide-spread application of high density DNA-arrays. Comparative microarray analysis identifies genomic diversity and conservation patterns among bacteria. The development of vaccines cross-protective among serotypes and variants of pathogenic species specifically profits from this

analysis, as it was demonstrated by the identification of common genes and protective antigens from major serotypes of *Streptococcus agalactiae*.[34,35]

Profiling of genomic expression with microarrays has revolutionalized the analysis of genes involved in microbial pathogenesis (reviewed in ref. 36). Considering its value in vaccine development, the emphasis is focused on pathogen-host interactions. In several studies novel vaccine candidates were identified, based on requirement for infectious state and dissemination, adhesion or evasion of innate defense mechanisms.[37-40] This approach—that heavily relies on genome annotation and bioinformatics—is most powerful in providing a global view on integrated cellular processes active during infection. Again, it has to be followed by combined application of gene cloning, recombinant protein technology and in vitro functional assays to validate target selection for vaccine development.

Proteome analysis has rapidly developed in the postgenome era and is now widely accepted as a complementary technology to genetic profiling (reviewed in ref. 41). The most direct way of using proteomics technologies for antigen identification is the combination of conventional proteome analysis with serology. There have been a number of recent studies investigating the "immunoproteome" of important human pathogens (for an example see Haas et al[42]). Combining "reverse genomics" and proteomics is especially useful for confirmation of bioinformatic prediction of ORFs and surface location. Moreover, a strong asset of proteomic studies is the identification of surface located proteins that cannot be predicted by bioinformatic means.[43,44] Serological proteome analysis of enriched membrane and cell wall fractions from several pathogens, such as *S. aureus, Bacillus anthracis* and *S. agalactiae* has indeed demonstrated to identify novel surface antigens and protective vaccine candidates without sequence features that could have been recognized by in silico prediction algorithms.[45-47]

The design of proteome-based studies has to be carefully performed, since there is an inherent risk to preferentially detect abundant proteins and to miss those that are expressed only under in vivo conditions and have lower solubility (e.g., membrane and surface proteins). Another need, not necessarily met by proteome analysis, is that protective vaccine components have to be derived from proteins expressed under disease conditions against which prevention is directed. As many virulence factors and antigens are only expressed in vivo, approaches that solely rely on in vitro grown bacteria are likely to miss important protective antigens.

Evaluation of immune responses against any candidate antigen is a crucial validation task and cannot be circumvented. Therefore, techniques using human immunogenicity as their primary screening and selecting parameter on a genome-wide basis seem to be especially valuable for vaccine development. Recently a novel approach combining the advantages of full genome coverage and serological antigen identification was published. The method was first applied to the genome-wide identification of in vivo expressed antigens from *S. aureus* by using antibodies from human serum and comprehensive small-fragment genomic surface display libraries.[22] Subsequently, the technology was extended with an integrated approach for antigen validation as selected clones are directly subjected to generation of epitope-specific immune sera for surface localization and in vitro functional assays. This feature allows the analysis of antigens without the demanding task of high through-put recombinant protein production. This method, named antigenome technology, has been extended to many important human pathogens and validated by the discovery of novel and highly protective antigens, in addition to the identification of the majority of the ones that have been previously described.[20] Since the antigenome technology provides a subset of all genome-encoded proteins, which are expressed by the pathogen in vivo and induce antibodies in humans, the identified antigens fulfill major requirements of vaccine candidate antigens. It is interesting to note that the antigens confined by the antigenome seem often to be involved, as secreted and surface bound proteins, in virulence functions and, thus, being attributed to the "pathosphere" that has been defined as the growing gene pool in which pathogens meet and mingle to cause diseases.[48] It is observed that many of the identified antigens from various pathogens were not or only very weakly expressed under in vitro growth conditions, indicating that a proteomic approach that preferentially selects abundant proteins would likely fail to identify them. As the bioinformatic genome mining approach depends on the accuracy

of available algorithms, potential vaccine candidates can be missed due to a misleading or not existing annotation. Based on the analysis of the antigenomes of fifteen pathogens, approximately 25% of all identified antigenic proteins can only be assigned to hypothetical proteins or proteins with unknown function. Many of the identified antigens would, thus, be not be found by a bioinformatic approach. The cumulative data obtained for the fifteen antigenomes showed that a large fraction of the antigens identified by this method represents cell surface or secreted proteins. Nearly fifty percent of all antigens fell into four cellular role categories: cell wall, cellular processes, transport and binding proteins and determinants of protein fate. In order to pinpoint candidates for vaccine development, a comprehensive and rapid validation strategy to retrieve the most promising antigens from the 100-200 antigens was implemented. Clones selected from peptide display libraries are directly subjected to generation of epitope-specific immune sera used for testing of surface localization and in in vitro bactericidal assays. The human immunogenicity of identified antigens is evaluated with synthetic peptide epitopes. The application of these major selection criteria combined with traditional gene conservation studies reduces the antigenome to a small number of candidate proteins that can be rapidly expressed in recombinant form for subsequent in vivo studies. The re-identification of most of the previously identified protective antigens of *Staphylococci* and *Streptococci*, such as PspA, M1 protein, Sip and ClfA gives further supports the power of the antigenome technology. Most importantly, novel protective proteins yielding animal protection in animal vaccine models, were found in the prioritized groups of antigens derived e.g., from *S. aureus*[23] and *Streptococcus pneumoniae* (unpublished data), respectively. Thus, the utilization of protective antigens—included in subunit vaccines—as targets for monoclonal antibodies, provides an attractive strategy to develop novel treatments against life threatening infections. Such a notion is supported by recent data showing that protection can be conferred to naïve animals, using serum directed against target antigens that have been validated in vaccine models (Nagy et al, personal communication).

The Advent of Monoclonal Antibodies in Disease Treatment

The renaissance of antibody therapy since the mid-1990s was mainly possible through significant improvements in antibody generation and purification (Fig. 1). The first step towards nowadays production technologies was the description of the unlimited generation of monoclonal antibodies by Georges Koehler and Cesar Milstein in 1975,[49] for which they were awarded the Nobel Prize in 1984. They fused mouse myeloma cells with normal antibody-producing splenic B-cells isolated from mice that were immunized with sheep red blood cells as antigen. The resulting hybridoma cells possessed the immortal propagation potential of the myeloma cells and secreted anti-sheep red blood cell antibodies. Selected clones could then be cultured indefinitely and secreted large quantities of monoclonal antibodies.

Despite their success as research tools, mouse monoclonal antibodies as human therapeutics are limited for various reasons. The main problem is the high immunogenicity of these foreign proteins in humans resulting in fast clearance (short half life) and toxicity by human anti-mouse antibodies (HAMAs).[50] Moreover, mouse antibodies have a reduced effect in human recipients due to their nonoptimal interactions with human complement and F_c receptors.[51]

In the early 1980s strategies for chimerization and humanization were ensued to overcome the limitations of mouse monoclonal antibodies. Chimerization demands the joining of the variable regions of mouse antibodies with the constant domains of human immunoglobulins that takes advantage of recombinant DNA techniques resulting in chimeric antibody derived from mouse and human antibody genes.[52] Although being less immunogenic than murine monoclonal antibodies, human antichimeric antibody responses have even been reported for chimeric antibodies.[53] To further reduce the undesirable immune response and confined inactivation, the mouse segment within the humanized monoclonal antibodies has been restricted to the complementarity determining regions (CDR) in CDR-grafted "humanized" antibodies.[54] In order to humanize a mouse monoclonal antibody, the closest matching human immunoglobulin allotype is first identified by structural comparison.[55,56] Then recombinant approaches are used to graft the CDRs from mouse hybridomas to the corresponding selected human immunoglobulin framework. As a result, the

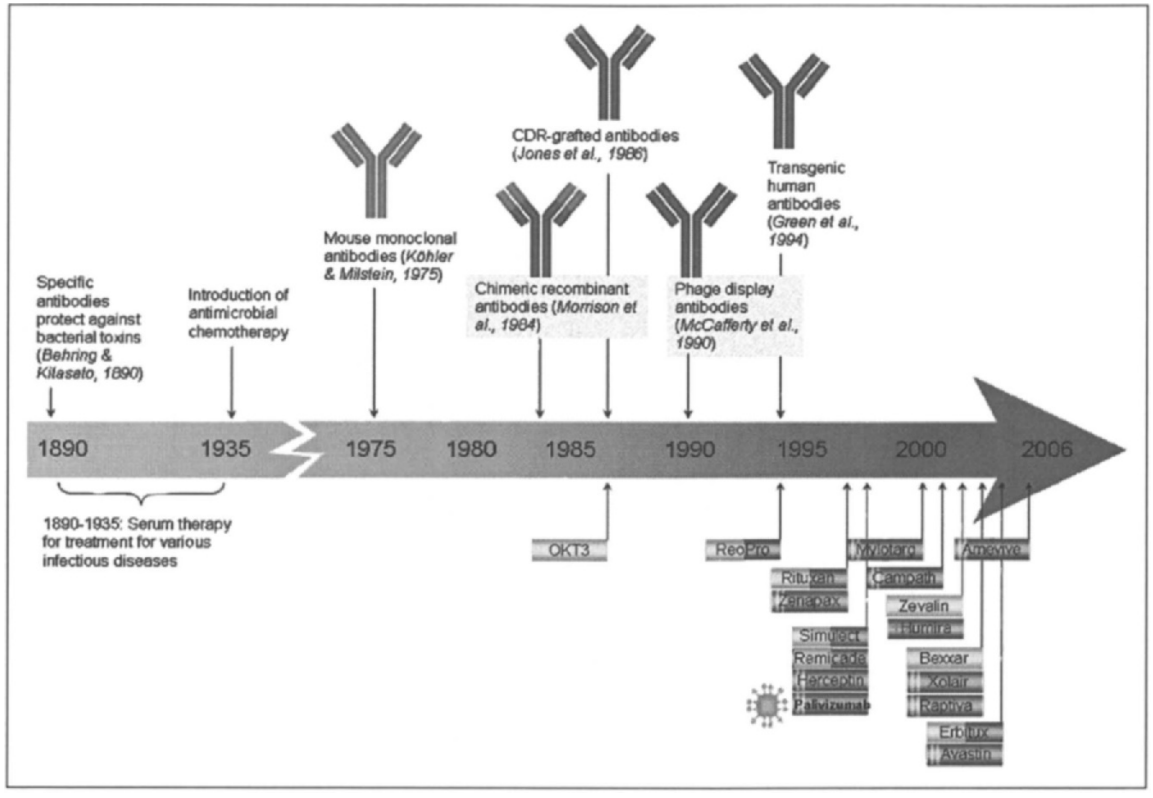


Figure 1. Evolution of antibody therapy and application in the clinic.[49,52,54,61,71] After extensive use of anti serum therapy at the beginning of the last century it was almost abandoned until the advent of monoclonal antibodies. In general new monoclonal antibodies were approved by the FDA 10 years after the development of a new technique was reported in the literature. Palivizumab is the only momodonal antibody currently used in the clinic that targets an infectious disease.

antibody only contains the antigen binding region from mouse origin, while the remainder of the variable and constant regions is derived from a human source.

While routine mouse monoclonal antibody production has been established, human monoclonal antibodies cannot be generated by conventional hybridoma technology, since it was not possible to found human cell lines that secrete constantly high levels of antibodies and, furthermore, humans cannot be challenged with all kind of antigens, due to ethical and safety reasons. Nowadays, phage display technology (reviewed in refs. 57-59) and transgenic mice with a human antibody locus (reviewed in ref. 60) represent established, widespread and robust technologies that allow the generation of potent human antibodies.

Phage display technologies enable in a simple to use and highly versatile procedure for the selection of antibodies against known or novel antigens. The phage display library (first description by McCafferty et al[61]) represents a collection of independent clones carrying a foreign DNA sequence encoding an antibody domain expressed as a fusion with the coat protein of mainly filamentous bacteriophages, as M13 or Fd (reviewed in ref. 62). Monoclonal antibody libraries can be recruited from immune fragments that are already biased towards certain specificities (encoded in the genome of immunized or infected animals or humans), or naïve unbiased fragments that can be derived from nonimmune natural or semi-synthetic sources, bypassing the need for previous immunization. By applying the best suitable selection procedures, those phages that bind to the target antigen with highest affinity are retained. The phages are enriched by selective adsorption to an immobilized antigen ("panning") (reviewed in ref. 12); however various specialized screening techniques exist.[57,63-65] Phage display provides the opportunity to mimic human immune response, also because of the high degree of natural variations found in the replication of the phage genomes.[66] B-cell maturation in vivo requires recombination of germline gene segments accompanied with changes and mutations that can be imitated in vitro by DNA random cloning of VH and VL chain genes.[67] The somatic hypermutation process that naturally contributes to the affinity maturation of antibodies can be achieved artificially by inserting point mutations into gene segments of complementarity determining regions.[68,69]

A method to circumvent the laborious steps of founding humanized and to obtain directly human monoclonal antibodies was developed by engineering transgenic mice with a human immunoglobulin locus as source for antibody producing hybridoma cell lines. (reviewed in ref. 60). Already in 1985 Alt et al proposed to exploit transgenic mice for the generation of therapeutic antibodies,[70] and as soon as 1994 the XenoMouse® (Abgenix, Inc.)[71] and the HuMAb Mouse® (Gen-Pharm-Medarex)[72] were reported to be the first mice carrying both the human VH and VL repertoire created via pronuclear microinjection or yeast protoplast fusion with embryonic stem cells, respectively. For monoclonal antibody generation B-cells are isolated from immunized mice and fused to hybridomas, in a similar manner to the traditional mouse monoclonal antibody production. By employing microcell-mediated chromosome transfer—a technique capable to transfer very large fractions of the human germline—Tomizuka et al generated a chimeric mouse—TransChromo Mouse™ (TC Mouse™) carrying human chromosomes 2 and 14 regions containing the human κ-light-chain and heavy-chain loci.[73,74] In order to increase the low efficiency of hybridoma production due to instability of the Igκ locus, the KM Mouse™ was created by cross-breeding the Kirin TC Mouse™ with the Medarex YAC-transgenic mouse.[75] These mice possess the capability to carry out VDJ recombination, heavy-chain class switching and even somatic hypermutation of human antibody genes in a normal mode to generate high-affinity antibodies with completely human sequences.[76] The resulting antibodies exhibit a half-life similar to natural human antibodies[77] and show only differences in glycosylation patterns, thereby representing a major improvement in hybridoma technology.[12] Although human monoclonal antibodies derived from transgenic mice have not yet paved their way up to FDA approval and registration, so far clinical trials with them have not revealed adverse immunogenic side events in patients,[78-80] in contrast to chimeric, CDR grafted or phage display derived monoclonal antibodies.[81] However there is still a need for confirming these promising data by testing transgenic mouse derived antibodies in larger subject cohorts.

On the other side, the success of phage display technologies in mimicking the in vivo antibody selection process in essence has led to intensive exploration of possible improvements, mainly in the field of new display techniques. All these new selection platforms share four major steps: (1) the creation of genotypic diversity; (2) the linkage between genotype and phenotype; (3) the application of a screening procedure; and (4) the amplification of the selected binding sites.

In the Ribosome and mRNA display method,[82] the antibody and its encoding mRNA are linked by the ribosome which is made to stop without releasing the polypeptide.[83-85] The use of e.g., nonproof-reading polymerases provides additional diversity between generations and therefore represents a very successful technique in the field of antibody affinity maturation.[86]

The attempt in displaying antibodies on the surface of different microbes has only been successful so far, when employing the yeast *Saccharomyces cerevisiae*.[87] Antibodies are displayed via fusion to the α-agglutinin yeast adhesion receptor on the cell wall and selection can be accomplished via flow cytometric cell sorting. Besides yeast display, a lately described *Escherichia coli* based approach is currently under development.[88]

Recently developed antibody platform technologies include retroviral display,[89] protein-DNA display,[90] microbead display by in vitro compartmentalization,[91] in vivo growth selection based on protein fragment complementation[92] and other techniques.[93] However, their advantages over more established systems remain to be demonstrated.

One problem in the application of monoclonal antibodies lies in their restriction to a single specific epitope, limiting their ability in eliminating dynamic and evolving targets and retaining activity in the event of antigen mutation. A new generation of therapeutic antibodies that may overcome the restriction of monoclonal antibodies is the development of a recombinant poly- or oligoclonal antibody technology.[94] For the generation of "Symphobodies"—fully human, antigen-specific recombinant polyclonal antibodies—antibody producing cells are isolated from naturally immune donor blood. cDNA encoding human heavy and light chains are amplified and linked together by Symplex PCR™; pooled PCR products are then inserted into an expression vector and screened for antigen binding. Constructs expressing the selected antibodies are cloned into Chinese hamster ovary cells where they are site-specific integrated into the genome.[94] Thus, such a development of a human antibody repertoire mirrors the human polyclonal immune response against specific antigens.

Besides the fact that the recombinant expression of antibody genes is often difficult because of their large size, the usage of whole immunoglobulins sometimes causes undesired side effects that are mediated by the Fc part of the antibody. To overcome such problems antibody fragments such as Fab, scFv, diabodies and minibodies have been engineered by removing either the entire constant region or the Fc portion.[12] Advantages shared by these antibody fragments include their better clearance from whole body, better tissue/tumor penetration characteristics and their simple and straightforward production in bacteria bypassing mammalian cell based production. The smallest fragments are single chain fragment variables (scFv) formed by tandem arrangement of the VH and VL domains joined by a flexible linker peptide exhibiting a comparable affinity of a Fab.[95,96] Their biological effects can be enhanced through linker length reduction that generates noncovalent scFv dimers "diabodies";[97] by further shortening trimers[98] or even tetramers can be formed.[99] ScFvs have also been modified to deliver toxins and chemotherapeutics to various tumors by binding to cancer-associated antigens, e.g., by coupling the *Pseudomonas aeruginosa* exotoxin A to scFv.[100] Linking of scFvs of different specificity creates bispecific antibodies that bind two different structures on single or different cells.[101] Other truncated antibody variants are Minibodies—homodimers of scFv-CH3 fusion proteins—and Flex minibodies—scFv-IgG1 hinge region fusion proteins.[102]

Whole antibody molecules can be modified as well by coupling with anti-microbial drugs. Antibodies possessing specificity to microbial antigens can be simultaneously linked to toxins, acting as immunotoxins that way. For example, Human Immunodeficiency Virus (HIV) and Cytomegalovirus specific antibodies have been linked to the ricin A chain or the *Pseudomonas* Exotoxin A.[103-105] Unfortunately toxins can elicit immune responses limiting their repeated

therapeutic use. An alternative represents the linking of radionuclides to specific antibodies that do not need to be internalized, like toxins and are unlikely to produce significant immune responses. Radionuclide-labeled antibodies have been tested against *Cryptococcus neoformans* and pneumococcal infections in mice.[106,107] Another development in modifying the antibody molecule was the creation of bispecific antibodies carrying two different Fab fragments and recognizing a microbial epitope for pathogen binding and at the same time a host immune component. This strategy was shown to be successful in animal models for the clearance of bacteriophages[108] and *P. aeruginosa*.[109]

The application of humanized and even fully human antibodies—is associated with low toxicity and high specificity. The benefit of high specificity is that only disease-causing pathogens are targeted and therefore the host flora should not be altered or resistant microorganisms be selected. A caveat is that pathogens with high antigenic variation may require more than one monoclonal antibody for therapy and mutants lacking the antibody determinant could emerge during treatment. Antibody molecules are highly versatile; by binding to a single determinant they can mediate various biological effects including toxin neutralization, microbial opsonization, complement activation and antibody-directed cellular cytotoxicity (Fig. 2). Antibodies can also be used to target host cells and enhance immune functions especially desirable against infectious diseases and tumors or to suppress immune responses by reducing the number of immune cells, neutralizing cytokines or blocking receptors.

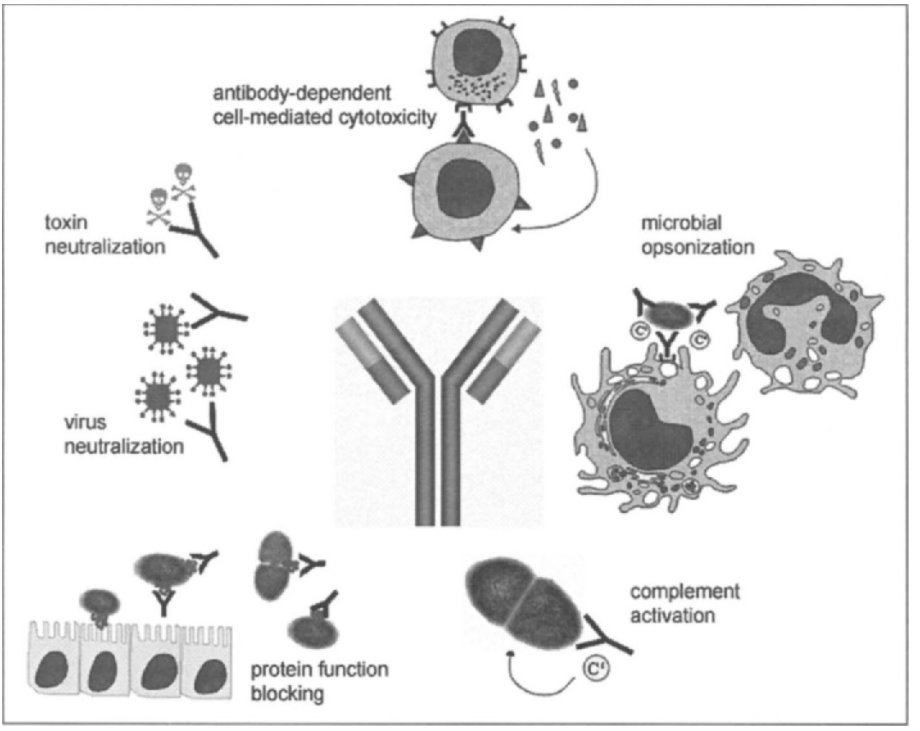


Figure 2. Biological effects of antibodies in infectious disease. Antibodies neutralize viruses and toxins, block protein functions important for microbial adherence or growth, activate complement and microbial opsonization and are a prerequisite for cell-mediated cytotoxicity. All these functions together facilitate the host to combat the invading pathogen (Adapted from Casadevall et al.[8])

The major disadvantages of antibody based therapies are high costs associated with production, storage and administration. Since antibodies have to be produced in live expression systems, the risk of contamination with prions or viruses requires continuous monitoring and testing. Additionally, antibodies have to be administered shortly after infection to be efficient, requiring rapid microbiological diagnosis. Additionally manufacturing of Symphobodies, mimicking polyclonal antibodies in human immune response, may still have to prove that they can be obtained without chance in their composition under stable GMP conditions.

From Serum Treatment to Anti-Infective Monoclonal Antibodies

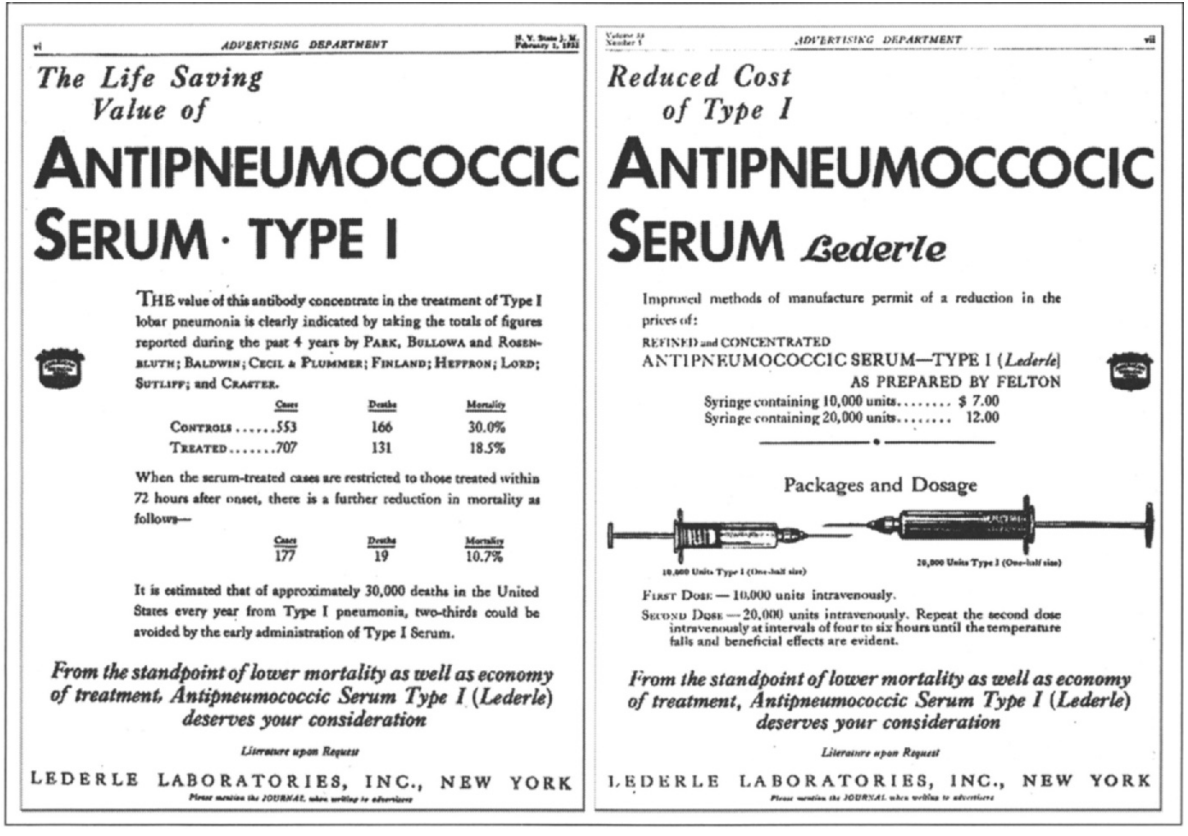
In the late 19th century Behring and Kisato discovered the efficacy of immune sera in treating infectious diseases, such as diphtheria and tetanus.[110] In 1891 the Klemperers already protected rabbits against *S. pneumoniae* with immune sera showing the potential usefulness of passively administered antibodies for the treatment of pneumococcal infections.[111] However reliable anti-pneumococcal therapy was not available until the mid 1920s, since the development of successful serum therapy required the discovery that pneumococci are genetically diverse and only type-specific sera provide protection. Improved vaccination schedules for serum donors to generate good immune responses and advanced antibody purification techniques, as well as the standardization of serum potency were necessary steps in the introduction of serum therapy (reviewed in ref. 112). The high death rate associated with meningococcal meningitis lead to fast developments also in this sector; a significant reduction of the case fatality rates was already achieved with horse sera in the early 20th century.[112,113]

Serum therapy reached its heyday in the 1920s to the mid 1930s when it was standard clinical practice in the treatment of a variety of infectious diseases caused by *S. pneumoniae, C. diphteriae, Neisseria meningitidis, Haemophilus influenzae, Streptococcus pyogenes* and *Clostridium tetani*. The broad application of serum as treatment for pneumococcal disease can be estimated regarding advertisements of that time in medical journals (Fig. 3). However, with the advent of anti-microbial chemotherapy passive immunization with serum was largely abandoned for the treatment of bacterial infections due to major advantages in being less toxic, more effective and cheaper. Serum therapy was often associated with severe side effects including fever, chills and allergic reactions and delayed toxicity called "serum sickness" a syndrome associated with rash, proteinuria and arthralgia. Moreover, for satisfying efficacy precise diagnosis, appropriate and nondelayed dosage was necessary asking for physicians with considerable experience. The production of horse or rabbit therapeutic sera was very expensive because of the need for animal facilities, purification techniques, adequate storage and standardization. Nevertheless lot-to-lot variation could not be fully eliminated (reviewed in ref. 114).

Upon the arrival of the antibiotic era, anti-sera were still used for toxin-mediated disease such as botulism, tetanus and diphtheria in addition to anti-toxin therapy in the treatment of venomous snake bites.[11] The lack of efficient anti-viral treatments also stimulated the use of antibody preparations as postexposure prophylaxis in e.g., rabies or hepatitis B (reviewed in ref. 9).

In spite of the previously experienced shortcomings, long-time neglected antibody based therapies face a renaissance today. The description of hybridoma technology in 1975[49] fired researcher's imagination in developing new therapies against cancer, autoimmune or infectious diseases. As early as at the dawn of the 20th century Paul Ehrlich already dreamed about the use of antibodies as "magic bullet" for the treatment of cancer. Indeed, in the mid' 1980s the first efficient use of a monoclonal antibody for the treatment of refractory lymphoma was reported.[115] The anti-tumor effect was only temporary, since murine monoclonal antibodies have only short in vivo half life and are immunogenic in humans; moreover they don't kill target cells forcefully due to low efficiency in complement activation and antibody dependent cell cytotoxicity. The first FDA approved murine monoclonal antibody for clinical use was OKT3 targeting CD3 in 1986 and was designed for prevention and treatment of organ rejection.[116]

Fortunately, monoclonal antibody techniques underwent continuous and tremendous improvements in reducing the mouse derived portion of the protein and enabling the production of



vi ADVERTISING DEPARTMENT N. Y. State J. M. February 1, 1931

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THE value of this antibody concentrate in the treatment of Type I lobar pneumonia is clearly indicated by taking the totals of figures reported during the past 4 years by PARK, BULLOWA and ROSENBLUTH; BALDWIN; CECIL & PLUMMER; FINLAND; HEFFRON; LORD; SUTLIFF; and CRASTER.

	Cases	Deaths	Mortality
CONTROLS	553	166	30.0%
TREATED	707	131	18.5%

When the serum-treated cases are restricted to those treated within 72 hours after onset, there is a further reduction in mortality as follows—

Cases	Deaths	Mortality
177	19	10.7%

It is estimated that of approximately 30,000 deaths in the United States every year from Type I pneumonia, two-thirds could be avoided by the early administration of Type I Serum.

From the standpoint of lower mortality as well as economy of treatment, Antipneumococcic Serum Type I (Lederle) deserves your consideration

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Reduced Cost of Type I

ANTIPNEUMOCCOCIC SERUM *Lederle*

Improved methods of manufacture permit of a reduction in the prices of:

REFINED and CONCENTRATED
ANTIPNEUMOCOCCIC SERUM—TYPE I (*Lederle*)
AS PREPARED BY FELTON

Syringe containing 10,000 units..... $ 7.00
Syringe containing 20,000 units..... 12.00

Packages and Dosage

10,000 Units Type I (One-half size) 20,000 Units Type I (One-half size)

FIRST DOSE — 10,000 units intravenously.

SECOND DOSE — 20,000 units intravenously. Repeat the second dose intravenously at intervals of four to six hours until the temperature falls and beneficial effects are evident.

From the standpoint of lower mortality as well as economy of treatment, Antipneumococcic Serum Type I (Lederle) deserves your consideration

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Figure 3. Advertisement for serum therapy for Type I *Pneumococcus*. Lederle (now Wyeth) successfully sold pneumococcal treatments already in the early 20th century.

chimeric, humanized and nowadays even fully human antibodies. In the 30 years since the invention of hybridoma technique 21 monoclonal antibody therapies have been approved by the FDA (source: AdisInsight, 07.07.2006); only a single one targets an infectious disease—Palivizumab (Synagis®) against respiratory syncytial virus infections.

In spite of the incredible efforts undertaken to develop novel antibody based treatments with hundreds of monoclonal antibodies being currently under preclinical development or clinical testing, only the minority of these efforts are directed against infectious targets. Among viral infections, AIDS is far the most explored area (Table 2). Due to the extreme variability of neutralizing HIV epitopes, in addition to those combating the virus particle itself,[117,118] many monoclonal antibody approaches target host molecules (such as CTLA-4, CD4, LFA-1, CCR5) to hinder viral entry (reviewed ref. 119). Emerging viral infections caused by the SARS corona virus and West Nile Virus also attracted the attention of monoclonal antibody developers and several preclinical efforts are expected to enter clinical development (Table 2).

Due to the widespread appearance of multi-drug resistant bacterial pathogens and the increasing population of immuno-compromised patients, more and more efforts are focused on antibody-based strategies against pathogenic bacteria and fungi. Especially considerable efforts were and are still undertaken to treat septic shock caused by gram-negative bacteria via neutralization of endotoxin and of TNF-α induced early in the disease, unfortunately with no successful outcome so far.[120-123] The most frequent microbial targets of new developments are opportunistic nosocomial pathogens, such as *S. aureus* and *epidermidis, P. aeruginosa* and Candida species (Table 1 and Table 3). The molecular targets for these monoclonal antibodies are surface structures of these pathogens, including capsular polysaccharides, cell wall glycolipids and surface proteins. The primary aim is to increase opsonophagocytic elimination of the respective organisms with the help of the host's immune cells. Unfortunately, in immuno-compromised patients (under anti-tumor treatment, organ transplantation, old age), the number of effective phagocytic cells is significantly lower than in healthy people and relying only on opsonophagpcytosis may not be sufficient for cure. Monoclonal antibodies that target surface proteins and that also have essential functions in in vivo survival, multiplication (cell division, nutrient acquisition) and pathomechanisms (adhesion, cytotoxicicity, immune evasion), offer another opportunity to reduce bacterial growth and ameliorate infectious damage to the host.[124-127] A single chain anti-fungal antibody that was selected by the hsp90 protein of *Candida albicans* from antibody cDNA libraries of patients who recovered from invasive candidiasis is being developed (Mycograb®). It consists of the antigen-binding variable domains of antibody heavy and light chains linked together to a recombinant protein that is expressed in *E. coli*. Mycograb® is not dependent on recruitment of white blood cells or complement, but simply acts by binding and inhibiting hsp90 of Candida.[128]

Current fear of bioterrorism using biological weapons encourages the development of antibody therapies against anthrax, botulism, ebola or smallpox virus infections and aims to provide immediate immune protection through antibodies that either neutralize the pathogens and toxins themselves, or target the host by blocking corresponding receptors to prevent infection or toxicity. Recently Cohen and coworkers demonstrated the inhibition of the lethal effect of anthrax toxin via blocking of its human coreceptor, LRP6 with LRP6 specific antibodies.[129]

The Next Chapter of the Antibody Success Story: Bacterial Infections

In spite of the historical landmark therapy against diphtheria, antibody therapy against bacterial infections, only exceptionally, has entered the medical arena in the last 70 years. The advent of antibiotics during the forties has certainly discouraged the development of further serum treatments against bacterial pathogens.

Antibiotics have seemingly become a relatively cheep and mostly reliable weapon to control most bacterial infections and epidemics. Alongside with the increase of hygienic standards, the penetrations of mandatory childhood vaccinations and antibiotic treatment, bacterial infections seemed to be a medical problem confined only to less developed parts of the world. The cost-efficient availability, the seemingly ever growing pipeline of novel antibiotics with increasing efficacy

Table 2. Therapeutic antibodies against viral infections

Drug	Pathogen	Antigen Target	Type of Antibody	Highest Phase	Originator	Indication
Palivizumab	RSV	RSV F glycoprotein	Humanised mAb IgG1	Launched	MedImmune	RSV infections
Motavizumab	RSV	RSV F glycoprotein (derived from Palivizumab)	Humanised mAb IgG1	III	Eli Lilly/ MedImmune	RSV infections
2G12, 2F5, 4E10	HIV	HIV gp120, HIV gp41	Human mAb	II	Polymun Scientific	HIV infections treatment & prevention
hNM01	HIV	V(3) region of the HIV-1 envelope protein gp120	Humanised mAb	I	SRD Pharmaceuticals	HIV infections treatment
HIV gp41 mAb	HIV	HIV gp41 surface glycoprotein	Fully human mAb	Preclinical	Medarex/Pfizer	HIV infections treatment
HEBIG™	Hepatitis B	Undisclosed	Pooled plasma of individuals with high titers of antibody to the hepatitis B surface antigen	Launched	Nabi Biopharmaceuticals	Hepatitis B
Civacir™	Hepatitis C	Undisclosed	Plasma of infected patients with high levels of anti-hepatitis C virus (HCV) antibodies	I	Nabi Biopharmaceuticals, Novartis	Hepatitis C
XTL 6865	Hepatitis C	HCV envelope E2 protein	Fully human (derived from human immune cells of convalescent patients)	I	XTL Biopharmaceuticals	Hepatitis C
Anti-SARS mAb	Severe acute respiratory syndrome (SARS)	SARS coronavirus glycoprotein S	Fully human mAb	Preclinical	Medarex/ Massachusetts Biologic Laboratories	Coronavirus infections

continued on next page

Table 2. Continued

Drug	Pathogen	Antigen Target	Type of Antibody	Highest Phase	Originator	Indication
Anti-SARS mAb	SARS	SARS coronavirus glycoprotein S	Fully human mAb	Preclinical	Crucell	Protection against SARS virus infection
Anti-M2 mAb	Influenza virus	Influenza A M2 protein	Fully human mAb	Preclinical	Kirin/Corixa Corporation	Influenza virus infections
WNV mAbs	West Nile Virus (WNV)	Domain II and III of WNV envelope protein	Fully human mAb	Preclinical	Crucell	WNV infections prevention and treatment
Anti-rabies mAbs	Rabies	Rabies virus glycoprotein	Fully human mAb	Preclinical	Crucell/CDC	Rabies
Anti-hMPV mAb	Human metapneumo-virus (hMPV)	Undisclosed	Undisclosed	Preclinical	MedImmune	Metapneumo-virus-infections
Ipilimumab	HIV	CTLA-4	Fully human mAbs	I	Medarex	HIV infections treament, among other applications
TNX 355	HIV	CD4 receptor	Humanized mAb 5A8	II	Biogen Idec	HIV infections treatment
Cytolin®	HIV	4 epitopes of the alpha sub-unit of LFA-1 (CD8)	Murine mAb	II	CytoDyn	HIV infections treatment
PRO 140	HIV	Human CCR5	Humanised mAb	I	Progenics Pharmaceuticals	HIV infections treatment

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Table 2. Continued

Drug	Pathogen	Antigen Target	Type of Antibody	Highest Phase	Originator	Indication
CCR5mAb004	HIV	Human CCR5	Fully human	I	Human Genome Sciences	HIV infections treatment
mAb 1F7	HIV	Idiotype common to anti-HIV and anti-SIV antibodies	Murine mAb IgMκ	Preclinical	Immune Network	HIV infections treatment
mAb B4	HIV	HIV receptor complex	Murine mAb	Preclinical	United Biomedical	HIV postexposure prophylaxis
mAb A3D8	HIV	CD44	Murine mAb	Preclinical	Duke University Medical Center	HIV-1 infections, myeloid leukaemia
CFY 196	Rhinovirus	ICAM-1(receptor for HRV)	Tetravalent humanised Fab fusion protein	Preclinical	Perlan Therapeutics	Rhinovirus infections
Anti- IFN-γ polyclonal antibody	Herples Simplex virus (HSV)	Interferon-γ (IFN-γ)	Polyclonal antibody	I	Advanced Biotherapy	HSV infections treament, among other applications
Bavituximab	Viruses	Phospholipid abnormally exposed on vascular endothelium of tumor blood vessels	Chimeric antibody	I	Peregrine Pharmaceuticals	Cancer, Hepatitis C treatment, Influenza virus infections, Viral infections

Data were collected from the Adis R&D Insight database and homepages of listed companies in July 2006 using the world wide web. Data were grouped into: first, antibodies targeting the virus particle itself and then second, those targeting host structures. Monoclonal Antibodies are abbreviated with mAbs. This is not a complete list.

Table 3. Antibody based therapies against fungal infections

Drug	Pathogen	Antigen Target	Type of Antibody	Highest Phase	Originator	Indication
Mycograb®	*Candida spp. (C. albicans, C. krusei, C. parapsilosis, C. tropicalis)*	Heat shock protein 90 (hsp90)	Human genetically recombinant antibody	Preregistration	NeuTec Pharma	Treatment of systemic candidiasis, Cancer (e.g., Breast cancer)
Candida MAb	*Candida spp.*	MSCRAMM® proteins	Undisclosed	Preclinical	Inhibitex	Treatment of candidiasis
ACE 5033	*Aspergillus fumigatus*	*A. fumigatus* surface protein	Fully human mAb	Preclinical	ACE BioSciences, Genmab	Aspergillosis, Mycoses
Candida mAb	*C. albicans*	Corixa antigens	Fully human mAb	Preclinical	GlaxoSmithKline and Amgen	Fungal sepsis caused by *C. albicans*

Data were collected from the Adis R&D Insight database and homepages of listed companies in July 2006 using the world wide web. Monoclonal Antibodies are abbreviated with mAbs. This is not a complete list.

invading the market has established the attitude in the medical community up into the 1970s of the last century that bacterial diseases may belong to the past. However, the emerging pattern of multidrug-resistant strains of an increasing number of pathogens in hospitals and communities has quickly ended the optimistic belief that the repertoire of anti-bacterial treatments will suffice the challenges in the infectious disease arena (for review see ref. 130). Also the discovery, development and registration of novel antibiotics have not fulfilled the too optimistic expectations that new registrations of treatments may bounce off the threat of untreatable bacterial infections (see commentaries by Clarke[131] and in Biocentury[132]). The infiltration of genomics,[133] intelligent drug design and molecular studies of bacterial host interactions in antibiotic development[134] has rather led to the sobering recognition that the number of suitable targets for new anti-bacterial drugs may be rather limited.[135-137] Furthermore, the often severe side affects, including allergic reactions against specific antibiotics, is restricting their applications, sometimes in critical medical conditions when they are most needed. Last, not least antibiotics often lead to lysis of bacterial cells and thereby freeing endotoxins at high levels, thereby causing overshooting immunity including sepsis.[138]

On the other hand before the advent of the monoclonal antibody technology, treatments of bacterial diseases with antibodies have been out of the reach of economical feasibility. Production of antibodies by immunizing animals as resorts to obtain serum is not a trivial process regarding quality, reproducibility and unwanted contaminations. Also as one has experienced with whole cell bacterial vaccines, immunization with in vitro grown pathogens may not lead to the type of specific antibodies that neutralize them, since they may not display the proper antigens at the surface. Thus, the progress made in defining disease specific antigens for vaccine development has provided novel tools to raise highly specific antibodies that may prevent or block bacterial infections or at least supporting the recovery process.

The skepticism in the medical and scientific community towards the paradigm of anti-infective anti-bacterial monoclonal antibodies is nurtured by multiple lines of thoughts:

1. Existing treatments are sufficient to control bacterial diseases.
2. Monoclonal antibody therapy may not find its way into treatment schedules that would justify the costs.
3. A single monoclonal antibody directed against a specific antigen per se may not be able to counteract the pathogenic course of a bacterial infection.

While all three arguments are widely accepted, a closer look into the paradigm discloses that they are not necessarily substantiated, if one considers the medical need, the progress made in identification of suitable antigenic targets and the positive experience of using monoclonal antibodies against e.g., malignant diseases (reviewed in ref. 139).

The medical need is given, whenever conventional treatment and prophylaxis are not available. *S. aureus* in context with nosocomial infections is equally a target for antibody treatments as *Pneumococcus*, both representing problem germs in intensive care (Table 4).

Moreover, costs for antibody treatments in connection with above described infectious disease outbreaks often missing adequate medical treatments appear to be not too dramatic, if one relates them to the hospital conditions and the underpinning economical efforts spent. Last but not least, the increasingly wide usage of monoclonal antibodies outside of the infections disease area has certainly aided in lowering the costs of development and manufacturing, thereby paving the way to novel treatments.[8]

The question remains what kind of features form the prerequisites for a monoclonal antibody in order to be able to counteract a bacterial infection? The answer to this problem lies—to our opinion—in the selection of the best suitable antigenic targets for the development of monoclonal antibodies. The antigens should be expressed on the pathogen surface during the infectious process; preferred throughout the most important stages of disease manifestation: i.e., during colonization, spreading and invasion. Also the antigens of choice should have a proven record to be a target of antibodies from individuals who have encountered the pathogen with positive or protective outcome. In addition, the selected antigens should be conserved among all clinical strains of the germ causing the underpinning infections.

Table 4. Medical need for novel therapeutics against nosocomial pathogens

Pathogen	Disease	Target Group	Incidence	Medial Need
Nosocomial	Sepsis, bacteremia, pneumonia, wound and surgical site infection, osteomyelitis, urinary tract infections	Hospitalized (esp. elderly, immunocompromized)	Nosocomial infections: 2 million/yr/US; 1 of 20 hospital admissions; 100.000 death/yr/US	High incidence High medical costs (20 Bn$/yr in dev. World) Increasing antibiotic resistance
S. aureus	Sepsis, bacteremia	Premature newborns, <34th pregnancy week	1-2/1000 life birth	Support the premature immune system
S. aureus	Sepsis, bacteremia, pneumonia, wound infection, osteomyelitis	Hospitalized, esp. surgery patients	25% of nosocomial infections	High mortality High incidence High medical costs Multi-drug resistance
S. pneumoniae/ Pneumococcus	Bacteremia Pneumonia Meningitis	Community acquired, elderly	~75.000/yr/US ~500.000/yr/US ~5.000/yr/US	High mortality High incidence High medical costs Antibiotic resistance
Group B Strep/ *S. agalactiae*	Bacteremia, pneumonia, meningitis	Premature newborns, <34th pregnancy week	1-2/1000 life birth	Support the premature immune system, increasing ab resistance
P. aeruginosa *S. aureus*	wound infection, bacteremia, sepsis	Intensive care, burn patients	80% of 2nd and 3rd grade burn patients	High mortality High medical costs Multi-drug resistance
P. aeruginosa	Pneumonia, sepsis	Intensive care, artificially ventilated	15-20% of HAP and 80% of VAP	Multi-drug resistance, high mortality, high medical costs
E. faecalis and faecium	Sepsis, bacteremia, endocarditis	Abdominal surgery patients	10% of nosocomial infections	Mortality, multi-drug resistance (*E. faecium*)
Klebsiella pneumoniae	Nosocomial pneumonia	Hospitalized (esp. elderly, immunocompromized)	10% of nosocomial infections	Multi-drug resistance

Hospital associated pneumonia (HAP) makes up 5-10/1000 hospital admissions and ventilation associated pneumonia (VAP) accounts for 80% of all HAP. Hospitalized burn patients average out 50.000 per year in the US. Of all approximately 9 million life births in the US and Europe per year premature birth before the 36th week accounts for 25% and premature birth before the 34th week for 10%.

All up to here listed features of target antigens may suffice the need to detect the intruder with a monoclonal antibody, particularly if the bound antibody funnels the bacterium into the immunological decontamination program, e.g., opsonization. On the other hand, the lesson learnt from antibiotics is that they have to kill the pathogen or at least disable bacterial growth in the host. In the light of the notion that prevention or treatment of a bacterial infection with monoclonal antibodies may be restricted to a single antibody, one would aim the target antigen also to exert a function needed for bacterial survival in the host. Thus, the antibody will neutralize a virulence factor or an enzyme needed in the infections life cycle of the pathogen. Such a dual mode of action resembles the features of monoclonal antibodies employed in cancers therapy: these antibodies seem to block cancerous cells by marking them for the immunological destruction, but also by blocking their growth. Thus, monoclonal antibodies need to be directed against carefully selected antigenic targets in order to achieve an optimum of interference with bacterial survival in the host.

The recently invented antigen identification procedure that is designed to establish the "antigenome" of pathogens has been instrumental in the development of novel bacterial subunit vaccines.[20-22] Characterization of a *S. aureus* antigen derived from the antigenome—that is presently used in preclinical and clinical programs—has indeed revealed its involvement in virulence and survival function.[23,128,140]

The feasibility of antigens to serve as targets for monoclonal antibody treatments can be pretested in vaccine models where protection of pathogen-challenged animals is accessed.[106,107,109,124,129,141-152] There is no doubt in mind that antigens giving the wanted protection in a vaccine model may not be sufficient when employed for the development of anti-infective antibodies. However, the potency of an antigen in providing protective immunity as vaccine may be a positive and sufficient selective criterion, alongside with all the other features that have been described for antigens selected for subunit vaccine development.

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CHAPTER 10

HSV as a Vector in Vaccine Development and Gene Therapy

Peggy Marconi,* Rafaela Argnani, Alberto L. Epstein and Roberto Manservigi

Abstract

The very deep knowledge acquired on the genetics and molecular biology of herpes simplex virus (HSV), major human pathogen whose lifestyle is based on a long-term dual interaction with the infected host characterized by the existence of lytic and latent infections, has allowed the development of potential vectors for several applications in human healthcare. These include delivery and expression of human genes to cells of the nervous system, selective destruction of cancer cells, prophylaxis against infection with HSV or other infectious diseases and targeted infection of specific tissues or organs. Three different classes of vectors can be derived from HSV-1: replication-competent attenuated vectors, replication-incompetent recombinant vectors and defective helper-dependent vectors known as amplicons. This chapter highlights the current knowledge concerning design, construction and recent applications, as well as the potential and current limitations of the three different classes of HSV-1-based vectors.

Introduction

The human herpesviruses are an important family of viruses, which become established in various tissues for the life of the host.[1,2] Herpes simplex virus (HSV), is a complex human neurotrophic virus that after initial infection and lytic multiplication at the body periphery, generally at oral or genital epithelial cells, enters in sensory nerve endings innervating the site of multiplication and it is retrograde transported to the nucleus of sensory neurons.[3,4]

The genome is a linear double stranded DNA of 152 kb encoding at least 80 gene products. The genome is replicating by a rolling circle mechanism forming head-to-tail concatamers. During the replication, the presence of inverted repeats sequences flanking the two unique segments of the genome (unique long UL and unique short US) are causing the formation of four isomers equally infectious (Fig. 1).[3,5]

The infectious virus particle comprehends an icosahedral capsid, which contains the viral DNA genome in association with core proteins. Around the capsid there is an amorphous layer known as the tegument, containing some 20 different proteins with structural and regulatory roles, surrounded by an external envelope containing different glycoproteins involved in different functions, among which the first steps of binding and entry into the host cell. Once the de-enveloped particle has entered the cytoplasm, it is transported through association with microtubules to the nuclear membrane where the viral DNA is released into the nucleus through the nuclear pores;[6] all the viral replication, from the transcription to the assembly of a new capsid, takes place into

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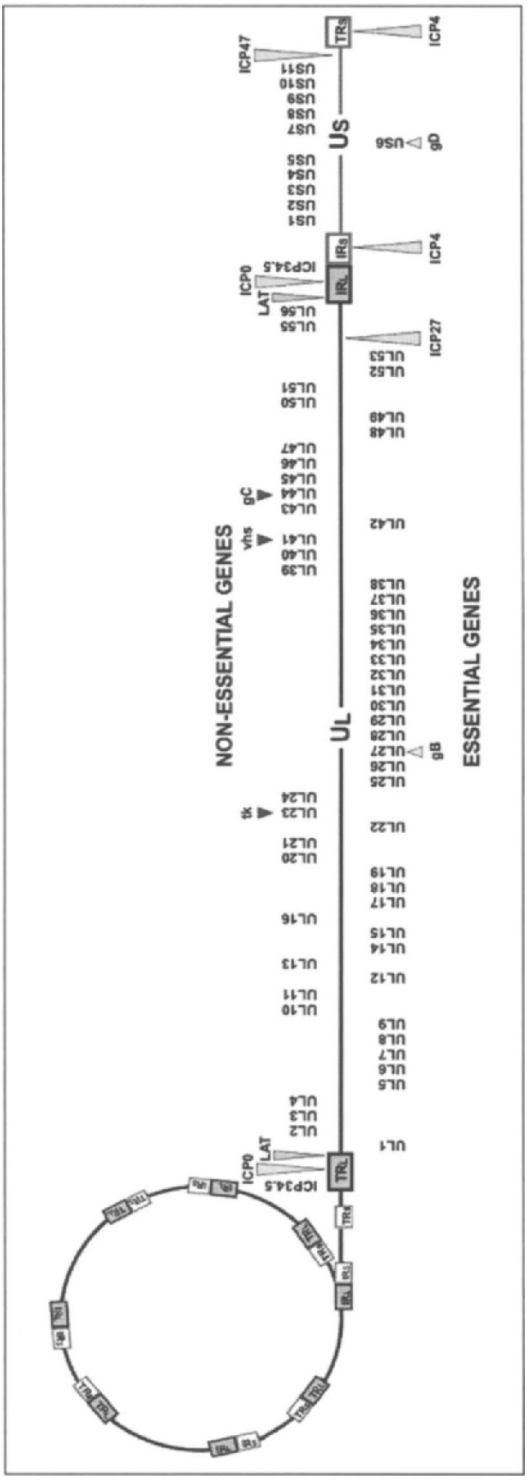

Figure 1. Herpes simplex virus Type 1 genome. The rolling circle replication mechanisms, essential and nonessential accessory genes are depicted.

the nucleus. Following the release of the viral DNA into the nucleus, the viral genome circularizes and a cascade is initiated with the transcription of five immediate early (IE) genes (infected cell protein ICP4, ICP27, ICP0, ICP22, ICP47) through the binding of a viral protein present in the tegument (VP16), in combination with cellular factors, to the enhancer element present in all IE promoters (TAATGARAT).[7] The products of the above genes are responsible of the transactivation of the early genes (E) which are encoding enzymes and DNA binding proteins required for the viral synthesis; the IE and E are followed by expression of late genes (L) which products are principally structural proteins of the capsid, tegument and envelope (primarily viral structural components).[3,5]

Following natural infection the virus is know to be axonal transported from the periphery to the cell body of the sensory ganglion where establishes a lytic or a latent infection.[8] HSV-1 persists in the latent state in the nervous systems of the host for a lifetime where the viral genome persists in an epichromosomal state associated with histones without integrating into the host genome.[9] During a latent infection the virus is in a relative quiescent state where the transcription is limited to a single region of the viral genome and only a group of latency-specific RNAs are detectable in the nuclei of neuronal infected cells. Due to several stimuli, the virus can be reactivated from the latency and usually by anterograde transport gets back to the site of primary infection where it starts a new lytic cycle. Only in few cases the viral particle is retrograde transported to the central nervous system (CNS) and starts a latent or a lytic infection, which evolves in encephalitis.[10,11] The newly replicated virus transported anterograde, usually to a site at or near the portal of entry, may cause a localized cold sore disease lasting 2-10 days with subsequent remission when the cold sores disappear. Over time, periods of remission generally increase in length and the duration of cold sores decrease, until the person rarely has active disease. This process is regulated by specific immunity developed by the patient against the virus.[12] The virus infection is, however, life-long and can be retriggered in some individuals by specific events, such as sunburn, stress or other infections.[13,14]

HSV-1 Genome and HSV-Derived Vectors

The complete knowledge of the HSV sequences and progress of molecular techniques has leaded to the development of HSV as a vector for several potential applications in human health.[15-19] These include (i) delivery and expression of human genes to the nervous system cells,[20,21] (ii) selective destruction of cancer cells,[22,23] (iii) prophylaxis and immunotherapy against tumors [24,25] and (iv) prophylaxis against infections with HSV and other infectious diseases.[26,27]

In the viral genome there are approximately 80 gene products that can be classified as immediate early (IE or α), early (E or β) and late (L or γ) depending on their kinetics of expression during replication. The viral genes can also be categorized according to whether they are essential or non essential for virus replication (Fig. 1). Essential genes are required to produce new infectious viral particles in permissive cell culture infections. Non essential, or accessory, genes encode products that are not absolutely required in cell culture but are important for optimum lytic replication or affect the natural life cycle of the virus in vivo, contributing to host range, pathogenesis, or latency. The viral DNA contains at least 37 essential genes. The US region of the genome contains only one essential gene encoding the glycoprotein D, which offers the opportunity to replace large segments of viral sequences with foreign DNA.[4,28,29] The modified HSV genome should be able to accommodate up to 40-50 kb of exogenous sequences. However, the modification of these viruses to reduce pathogenicity and increase safety often results in the loss of viral activities, which are required for efficient gene delivery and life long association with the host.

In recent years, new technologies have allowed researchers to get deeper into these problems.[30] The challenge for many research groups is to develop the tools to render these organisms harmless yet effective for targeted gene transfer and appropriate gene expression. Vectors based on HSV Type 1 are currently (a) amplicon vectors, (b) replication-defective viruses and (c) genetically engineered replication-competent viruses with restricted host range.[19,31]

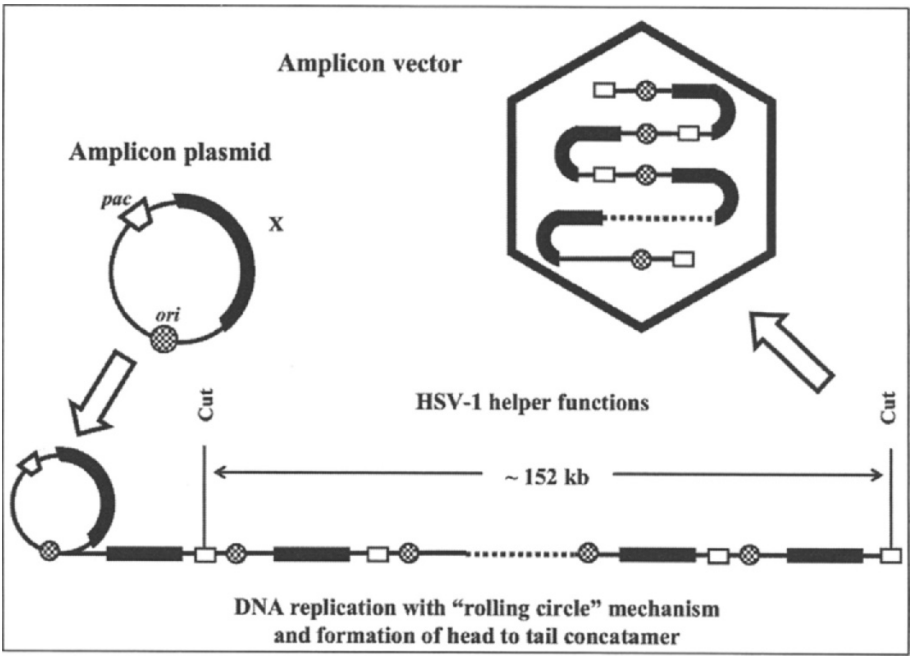

Figure 2. Structure and mechanism of packaging of the amplicon vector.

Amplicon Vectors

Amplicon vectors are HSV-1 particles identical to wild type HSV-1 from the structural, immunological and host-range points of view, but which carry a concatemeric form of a plasmid DNA, named the amplicon plasmid, instead of the viral genome.[32-34] Amplicon vectors possess several advantages as gene delivery vehicles: (a) a large transgene capacity (150 kb); (b) the repetitive character of the genome carried by the amplicon particle ensures the introduction of multiple copies of the transgene per infected cell; (c) the ability to infect a wide variety of cell types, including dendritic cells; (d) the ease of vector construction; (e) the limited toxicity due to the lack of viral coding sequences.

Amplicons are bacterial plasmids that contain one or more transgene cassettes and two non coding viral sequences, an origin of DNA replication (*ori*) and a DNA cleavage/packaging signal (*pac*) and they require a helper system to be produced. In the presence of HSV-1 helper functions, a circular amplicon can be replicated and amplified as head-to-tail concatemers and packaged into HSV-1 particles as approx 152 kb linear DNA (Fig. 2).[35] Classically, amplicon vectors were prepared in cells transfected with the amplicon plasmid and superinfected with helper HSV-1. As the helper virus was generally a replication-defective mutant of HSV-1, the amplicon stocks were produced in transcomplementing cell lines. However, the use of standard HSV-1 as helper resulted in the production of helper-contaminated vector stocks.[36,37] The contaminant helper particles, even if defective, induced significant cytotoxicity and inflammatory responses, preventing their use in gene therapy or vaccination protocols.[30] To overcome these obstacles, different helper systems that produce essentially helper-free vector stocks have been recently developed.[38] The last generation of helper system consists of the entire HSV-1 genome, without pac signals, cloned as a bacterial artificial chromosome (BAC) in *E. coli* supplying the full set of transacting HSV-1 functions.[39,40] Another different helper system recently developed is based on the deletion, by Cre/loxP-based site-specific recombination, of the packaging signals of the helper virus in the cells that are producing the amplicons.[41]

Replication-Defective Vectors

The replication-defective viruses are viral vectors where "essential" genes for in vitro viral replication are either mutated or deleted. Therefore, these mutants cannot grow except in transformed cell lines, where they are complemented in trans. To date, several replication-defective vectors have been constructed in which the IE genes, expressing infected cell proteins (ICP) 0, 4, 22, 27 and 47, have been deleted in various combinations. IE genes are expressed shortly after viral entry into the host cell and are required for initiation of a cascade of E and L viral gene transcription. ICP4 and ICP27 are essential for replication and the deletion of one or both of these genes requires adequate complementing cell lines capable of providing in trans the proteins encoded by deleted viral genes.[42-44] ICP0, 4 and 27 are responsible for E and L gene expression.[45,46] Beside its transcriptional functions, ICP27 also affects the splicing, polyadenylation and stability of mRNAs. ICP0 is a promiscuous transactivator acting on ICP6 gene, which encodes the viral ribonucleotide reductase large subunit and possesses a hybrid promoter, which is activated as an IE and an E function.[47,48] ICP22, the viral product that might be involved in sequestering of cellular DNA polymerase [49] is phosphorylated by two accessory genes UL13 and US3-encoded proteins [50-52] and is required for the optimal expression of the ICP0 protein. Deletion of the ICP22 IE gene can be responsible for an over expression of ICP0.[53] ICP47 inhibits MHC class I antigen presentation contributing to the virus escape from the immune surveillance.[54-56] The "first generation" of replication-defective HSV-1 based vectors consisted of mutants deleted in the single essential IE gene encoding ICP4, namely d120.[45] Although these vectors show reduced pathogenicity and can be used to efficiently transfer and transiently express reporter genes in brain, they are nonetheless cytotoxic for neurons in culture. Cell lines that complement ICP4 and ICP27 have permitted the construction of a "second generation" of highly defective mutants.[43,57-59] To date, several replication-defective vectors have been constructed in which ICP0, ICP4, ICP27, ICP22 and ICP47 genes have been deleted in various combinations.[43,58] Deletion of all five IE genes (ICP 0, 4, 22, 27 and 47) prevents virus toxicity for cells at high multiplicity of infection, allowing the vector gene to persist in cells for long periods [60-62] demonstrating that the residual cytotoxicity of the "first generation" of replication-defective HSV-1 based vectors results from the expression of the other four IE genes.[28] The multiply deleted mutants show an unusually prolonged transgene expression from the ICP0 IE promoter or the HCMV IE promoter in neurons.[63] The advantages of these second generations of replication-defective vectors are characterized by absence of early and late viral gene expression and provide enough space to introduce distinct and independently regulated expression cassettes for different transgenes.[43]

Attenuated HSV Vectors

Deletion of some non essential viral genes results in viruses that retain the ability to replicate in vitro, but are compromised in vivo, in a context dependent manner.[64,65] Among the limitations to the use of HSV is the fact that wt virus is highly pathogenic and cerebral injection causes fatal encephalitis. Toxic and/or pathogenic properties of the virus must, therefore, be disabled prior its use as a gene delivery vector.

Several genes involved in HSV replication, virulence and immune evasion, which are non essential for viral life cycle in vitro, have been identified. These genes are usually involved in multiple interactions with cellular proteins, which optimize the ability of the virus to grow within cells. Understanding such interactions has permitted the deletion/modification of these genes, alone or in combination, to create HSV mutants with a reduced ability to replicate in normal quiescent cells, but that can replicate in tumor or dividing cells. These attenuated viruses harbor further modification so they also serve as therapeutic gene delivery vehicles.[65,66]

Many HSV-1 and HSV-2 genes that are non essential in culture alter virulence in animal models. Among these genes, the ones encoding thymidine kinase (TK), ribonucleotide reductase (RR), the virion-host shut off (Vhs)[67] and the ICP34.5 proteins have been extensively studied.[68] *TK* is involved in optimizing nucleic acid metabolism for virus growth and is necessary for efficient replication in neurons. RR is necessary for the conversion of rNTPs to dNTPs in neurons, which

are otherwise lacking but necessary for the synthesis of new viral DNA during virus replication.[69] The Vhs function of HSV causes rapid destabilization of host RNAs and translational arrest.[70] Vhs also destabilizes viral messages, resulting in over accumulation of IE and E genes during lytic infection.[70-72] The ICP34.5 neurovirulence factor has been found to be essential for HSV pathogenicity.[73] It appears to provide multiple functions to the virus life cycle, one of which is to block the arrest in translation, which usually occurs in virus-infected cells as an anti-viral response preventing virus replication. This effect is mediated through the cellular PKR kinase, which phosphorylates the translation initiation factor eIF2α, thereby stopping translation. ICP34.5 recruits protein phosphatase 1a, to rephosphorylate eIF2α, allowing protein translation and continued virus replication. Tumor cells often display an impaired PKR pathway and/or elevated levels of eIF2α, that allow replication of ICP34.5-deleted viruses, as the inactivation of the PKR response is less critical in this contest.[74,75] Secondly, ICP34.5 seems to be involved in allowing new virions to become packaged and leave infected cells in a cell type-specific fashion. Consequently, in non permissive cells in the absence of ICP34.5 the nucleocapsids are retained in the nucleus and a productive infection cannot ensue.

Use of non replicating viruses or non viral systems as vectors can limit the maximum achievable efficiency of gene transfer. In contrast, use of replicating vectors to allow replication of genes delivered initially to a small number of cells and their subsequent transfer to neighboring cells, as infection spreads, can significantly increase the efficacy of gene delivery.[76-78] Attenuated HSV vectors have been tested as live viral vaccines, as oncolytic viruses and as gene therapy vectors to deliver transgenes to the nervous system.

Engineering Techniques

Alterations of the HSV genome can be achieved in a number of ways. These usually require a two-step process (named: marker transfer/marker rescue) in which portions of the herpes genome, which have been cloned into a plasmid vector, are first modified in vitro. The plasmid DNA is then cotransfected into cultured cells with infectious viral DNA and recombinant viruses are selected. Several methods have been described to insert DNA sequences into the viral genome. Efficient recombination into specific sites within the viral genome has been achieved in vitro using a recombination system derived from phage P1.[79] It is also possible to enhance the frequency of recombination.[59] The initial requirement is the insertion of a reporter gene such as β-galactosidase (*lacZ*) cassette flanked by *Pac*I or *Pme*I restriction enzyme sites not otherwise found in the viral genome. The second phase is the substitution of the reporter gene with other foreign cDNAs by digestion of the vector DNA with *Pac*I or *Pme*I to remove the *lacZ* gene and subsequent repair of the vector genome by homologous recombination with a transgene expression plasmid. Potential recombinant identified by a "clear plaque" phenotype after X-gal staining arose at high frequency (80-100%) (Fig. 3).[59] A different procedure involves transfection of cells with overlapping cosmids containing appropriate insertion or deletions. Expression of genes contained in cosmids leads through recombination to the construction of full-length viral genome.[80,81] To select recombinant vectors it is critical to have a system by which to identify successful recombinants. The viral *TK* gene is particularly useful site for insertion since its inactivation does not affect in vitro the replication of the virus. *TK* mutants can be easily selected in the presence of bromovinyl deoxyuridine or acyclovir.[82,83] Another marker system involves disruption of nonessential viral envelope glycoprotein genes, such as the ones encoding gC or gE. Recombinant viruses are identified by loss of an antigenic determinant of the glycoprotein using specific monoclonal antibodies (black or white plaques staining).

Traditionally, recombinant HSV vectors have been generated through homologous recombination between the HSV genome and a recombination plasmid, which usually requires laborious screening or selection and can take several months. Recent advances in bacterial artificial chromosome (BAC) technology have enabled cloning of the whole HSV genome as a BAC plasmid and subsequent manipulation in *E. coli*. Thus, we sought a method to generate recombinant HSV vectors more easily and quickly using the bacterial recombination machinery.[84,85]

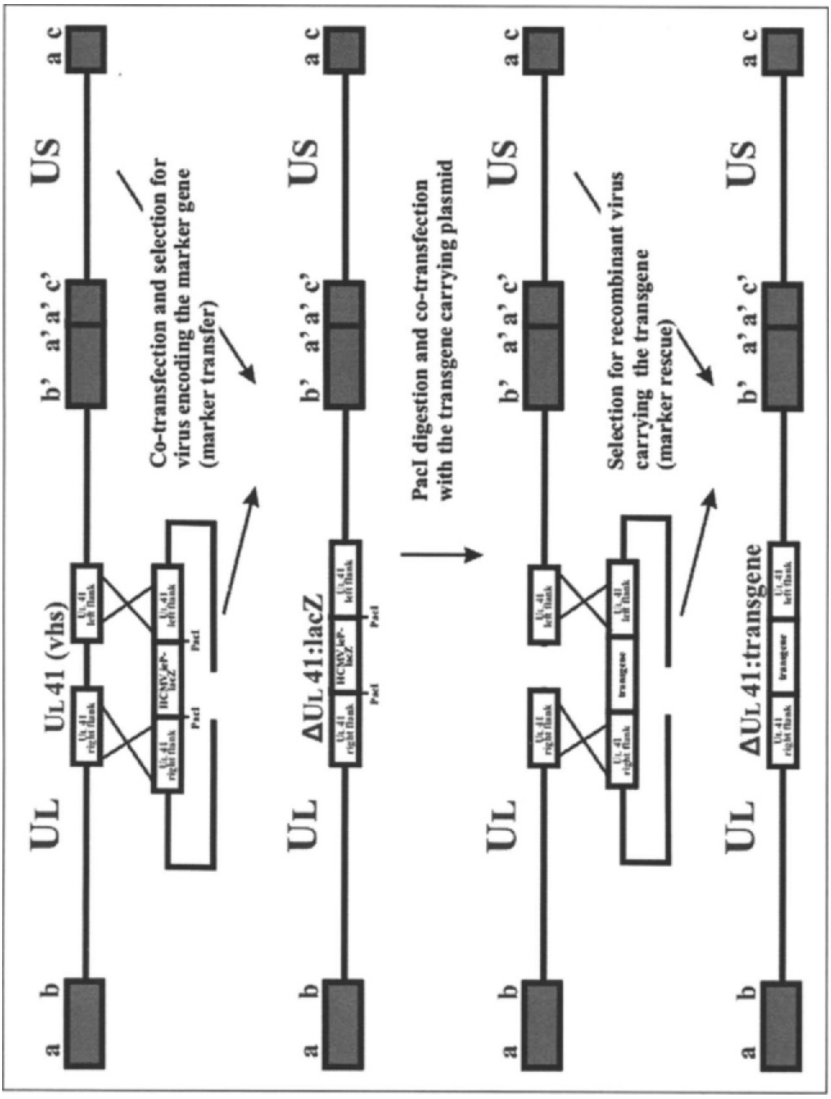

Figure 3. Mechanism of construction of recombinant viruses by classical homologous recombination (marker transfer/marker rescue) and using the "PacI system".

BAC cloning requires insertion of mini F plasmid sequences and antibiotic resistance genes into the viral genome and the length of these BAC backbone sequences is usually greater than 6 kb in total. Insertion of BAC sequences into the wild-type HSV genome (152 kb) will increase the genome length to ~158 kb and there will be no space left for the insertion of additional sequences. To avoid deleterious effects of the BAC sequences, including growth defects and potential transmission between bacteria and man, some herpesvirus BAC clones have been constructed with loxP site-flanked BAC sequences, which can be removed by Cre recombinase.[86-88]

HSV-1 Based Vectors Applications

HSV-1 Based Vectors for Vaccination

Many of the HSV based vectors have been used in gene therapy studies and some of them as experimental vaccines against HSV-1 infection.[26,89,90] However, studies related to the evaluation of the potential of these vectors, as foreign gene or protein delivery systems for immunological studies are very limited. The use of HSV vectors requires the development of mutated viruses that are genetically stable, incapable of replicating in the CNS and of spreading in immunocompromised individuals, not transmissible from immunized individual by contacts and, at the same time, capable of inducing protective immunity against the disease. Recent major breakthroughs in the field of HSV-1 technology authorize and support the use of HSV-1 as vaccine vectors for the delivery of foreign antigens.[89,91-95] In particular, HSV vectors show several advantages for prophylaxis against viral infections. They have been shown: (i) to elicit strong and durable immune responses by various routes of inoculation;[96,97] (ii) the viral DNA persists inside the host's cell nucleus as an episomal element, thus eliminating the safety concerns deriving from the random integration of the viral genome into the host's DNA; (iii) they carry the *tk* gene, that, in case of undesired effects, can be used, in combination with specific anti-viral drugs, to kill the virus-harboring cells.

The efficacy of all of these vectors might potentially be affected by the preexisting immunity to viral antigens in host. The effect of pre-existing immunity on HSV-1 vectors remains controversial, with some studies showing strong immune response in the face of anti-HSV-1 immunity,[96,98] whereas another study showed a reduction in the immune response to a transgene, with the intensity of the reduction depending on the route of inoculation.[99]

Amplicon Vectors

Amplicons were studied as vaccines against HIV[96] or intracellular bacteria.[93] They show unique advantages over other viral vectors.[93] Firstly, amplicon particles are absolutely apathogenic for infected cells since their genome is devoid from HSV-1 genes. Secondly, the repetitive character of the genome carried by the amplicon particle ensures the introduction of multiple copies of the transgene transcription unit per infected cell, likely resulting in strong expression. Lastly, the pantropic properties of HSV-1 particles in experimental systems, which are conserved in amplicons, should allow these vectors to infect a large range of cells, including dendritic cells.

Moreover, amplicons could also allow antigens to be presented by both MHC pathways during the same immunization protocol. This could be achieved (i) by introducing the transgene both in the amplicon genome and in the helper genome or, (ii) by inducing the in vivo production of empty virus-like capsids of selected viruses (e.g., HIV-1, HCV, or HPV-16). Concerning this last property, it has been shown that HSV-1 amplicons encoding Moloney murine leukemia virus gag, pol and env genes can induce the synthesis of retrovirus-like particles in cultured cells. Amplicons have been used to efficiently transduce the full set of proteins of MoMLV retrovirus vectors, thus rescuing integrated retrovirus vectors,[100,101] as well as the nonstructural[102] or structural[103] proteins of HCV.[104] An interesting remark is that amplicon expressing cytokine genes have been found to be a promising strategy for the development of tumor vaccines.[105,106]

Replication-Defective Vectors

Until recently, it was believed that, to be effective, viral vaccines must consist of a live, replication-competent virus or a large dose of inactivated virus. Replication of live virus was believed

to be essential to provide sufficient immunogen to induce a strong immune response. However, several non replicating vaccines, including replication incompetent HSVs, have been shown to induce an immune response.[92,107] These HSV mutants show a reduced cytotoxicity, due to their inability to replicate and to spread in the host, but maintain the capability to infect a wide range of tissues and host species.

HSV replication-defective viruses with mutations in essential genes that fail to form progeny virions and DISC viruses with mutations in structural protein genes that form uninfectious progeny virions have been used as vaccines against HSV infections and as vaccine vectors.[108-110] It has been shown that an HSV-2 double mutant (dl5-29) does not cause any disease in immunodeficient mice indicating that the virus would be safe even in immunocompromised individuals.[111] DISC-HSV-2 has been shown to be an efficient vector for cytokine gene delivery into tumor cells and that the expression of mGM-CSF or hIL-2 enhances the immunogenicity of whole-cell vaccines.[112]

The appealing properties of replication incompetent HSV-1-based vectors inducing strong CTL response, both in murine and in simian models, against foreign genes delivered by viral particles have made them very promising candidates for potential anti-HIV-1 and also other viral or intracellular bacterial pathogens vaccine development.[91,113,114] It has been shown that a mutant HSV-1 virus deleted for the ICP4, ICP22 and ICP27 genes and expressing ovalbumin (OVA) as a model antigen elicited protection in mice against a lethal challenge with a recombinant *Listeria monocytogenes* expressing OVA.[91] A similar vector, expressing HIV-1 Tat protein, has been demonstrated to induce long-term Tat-specific immune responses in the Balb/c murine model.[113] Moreover, vaccination of *Rhesus macaques* with a HSV-1 mutant virus that contains a deletion in ICP27 and expresses SIV Env and Nef antigens showed partial protection against mucosal challenge with the highly pathogenic SIVmac239.[115] In the same animal model, using a prime-boost strategy of vaccination, recombinant HSV-1 vectors deleted for ICP4, ICP22, ICP27 and ICP47 and expressing Gag, Env and a Tat-Rev-Nef fusion protein of SIV, elicited robust anti-Gag and anti-Env cellular responses and induced partial protection against intravenous challenge with SIVmac239.[27,92]

Due to their ability to accept multiple heterologous genes, the IE replication defective vectors could be used for innovative and synergistic strategies of immunization. For example, it is possible to engineer vectors to express specific chemokines and cytokines, together with antigens targeted to MHC-I or II molecules, in order to attract monocytes to the sites of infection, to induce their differentiation into dendritic cells and to favor antigen presentation.

Replication-Competent Vectors

Attenuated live viruses are the most effective to serve as vectors for vaccination. However, a major concern exists about attenuated HSV as a vector. In fact, in addition to the problem of genotypic stability there are other safety issues including questions regarding the potential of vaccine vector to establish latency, reactivate or recombine with virulent wild type strain. To overcome some of these problems, an approach based on defining and eliminating genes involved in neurovirulence, latency or reactivation was developed.[116]

The first and to date the only one, attenuated HSV-1 virus to be constructed and analyzed as a viral vaccine in humans, was the NV1020 (formerly R7020) strain.[117] This virus, based on HSV-1 strain F, has a portion of the unique short region of the viral genome, encoding glycoproteins G, D, I and E, replaced by the homologous region from HSV-2 and possesses only one copy of ICP4. This virus is very strongly attenuated in rodents and primates. In a dose escalation study, local reactions were noted in HSV-1-infected persons. A dose-dependent induction of antibodies occurred in HSV-1 seronegative subjects, but the development of this mutant has been stopped since it resulted too over-attenuated and it was consequently poorly immunogenic.

The goal to construct a safe, less attenuated vaccine candidate, lead to the construction of RAV 9395 mutant.[118] RAV 9395 is based on HSV-2, strain G, which carries deletions in the UL55 and UL56 genes, encoding proteins with unknown functions, the deletion of which causes attenuation and deletion in both copies of the γ34.5 gene (encoding ICP34.5 protein). Concomitant with this deletion, both copies of the open reading frame (ORF) P have also been deleted. The

tk gene was left intact and functional, conferring acyclovir sensitivity to the recombinant virus. When used as a live viral vaccine in a guinea pig model of HSV-1 infection, it was shown to be protective and it was also demonstrated that the immunologic answer depended on the route of administration of the virus.

AD472 is an evolution of RAV 9395, in which deletions in UL43.5 and in the US10-12 region were added to obtain an additional safety level by increasing the genetic and phenotypic stability of the virus.[119] In a guinea pig model, AD472 administered intramuscularly did not prevent infection and viral replication in the vaginal tract, but reduced lesion development and severity in a dose-dependent manner after HSV-2 wt challenge. Moreover, it generally precluded establishment of latency by the challenge virus.

Mutations in TK, especially for HSV-2, do not attenuate the virus sufficiently for human vaccines.[120,121] Other attenuated HSV-1 and HSV-2 viruses with single deletion in vhs or in RR respectively,[122,123] were shown to determine a protective immunity when tested in animal models, but still they are too neurovirulent to be used for human trials.

A further improvement to antigen presentation to the immune system could involve the deletion, from the viral DNA backbone, of the genes that codify for the vhs and the ICP47 proteins. In fact, two mechanisms have been described by which HSV inhibits antigen presentation by MHC class I and class II molecules. The first is related with the ability of the Vhs protein to accelerate the degradation of cellular mRNA molecules[70] and has also been shown to block dendritic cell maturation and thus to inhibit the immune response against the vector-delivered transgene.[67] The elimination of the UL41 locus from the viral genome was reported in the same paper to allow dendritic cell activation and also to stimulate the antigen specific T-cell response in vitro. The second is based on the ability of ICP47, one of the immediate early proteins, to bind to Tap, the transporter associated with antigen processing and to prevent peptide translocation into the endoplasmic reticulum.[54,56]

HSV-1 Based Vectors for Gene Therapy of Nervous System

The Neurotropic Properties of HSV-1

HSV-1 presents several outstanding adaptations to the nerve system and each of them can be rationally exploited in the design of gene therapy vectors with regard to neurological applications. HSV-1 contains genes that control neuroinvasiveness and neurovirulence; this virus can move both in the retrograde and anterograde directions and disseminates transynaptically from neuron to neuron. The virus envelope contains several glycoproteins that mediate entry to neurons due to the recognition of specific receptors (nectins). In most neurons, HSV-1 will establish a latent infection, a situation in which the viral genome will persist as a stable chromatinised episomal element and in which all lytic genes are silenced. Recent studies indicate that, during latency, the viral genome generates a chromatin structure that allows it to behave much like a mammalian minichromosome, with very sophisticated regulation of gene expression. The LATs do not encode proteins and there is increasing evidence suggesting that they can play a major role in the inhibition of the apoptotic response of neurons to virus infection, thereby preventing cell death and favoring eventual reactivation of the virus. The latent virus genome can be reactivated by stress, fever, or immune suppression and, through anterograde traveling along axons, the virus is transported back to the sites of initial epithelial infection, causing recurrent mucocutaneous infections which, in most cases, remain asymptomatic. Recent data indicates that HSV-1 anterograde movement along the axons is dependent upon the interaction of virus proteins with plus-end microtubule motors that move the capsids toward the axon terminals. Many studies indicate that most of these neurotropic features are retained in defective and attenuated HSV-1 vectors, including the abilities to been efficiently transported along axons in both directions and to establish latent infections with prolonged gene expression, both in sensitive and in motor neurons.

Amplicon Vectors

Amplicons have been used to deliver and express transgenes in neurons in vitro and in brain in vivo. They have been used to deliver neurotrophins, like nerve growth factor (NGF)[124,125] or brain-derived neurotrophic factor (BDNF),[124,126] antiapoptotic genes,[127,128] heat-shock proteins [129] or antioxidant enzymes,[130] in attempts to protect neurons against a variety of neurological insults, in many different experimental settings. Amplicons expressing genes affecting neurotransmitter expression or neuroreceptor synthesis have been used to study behavioral features, like learning and memory.[131-134] Amplicons have also been used to deliver tyrosine hydroxylase and other genes to the nigro-striatal system or to cultured striatal cells, in studies aimed to treat Parkinson's disease.[135-139] More recently, amplicons were shown to be able to deliver genes to the retinal pigment epithelial cells of the rat retina, but not to the adjacent photoreceptors.[140] In this study, amplicons allowed rapid and efficient, but transient, gene transfer, following subretinal injection.

The limitation in the amplicons safety profile was the presence of helper virus particles that resulted in some cytotoxicity. This problem has been circumvented recently by using a plasmid-based BAC transfection system to provide the helper functions, although the particle yield is relatively low.[39,40] Gene expression in vivo using amplicons has been reported to persist as long as one month. However, it cannot be excluded that long-term expression from amplicons may be related to persistent low-level replication by contaminating recombinant wild-type virus in the brain. In fact, in contrast with other amplicon preparations, "helper-free" stocks produce only transient expression of reporter transgene in vivo using the same promoter reported previously to remain active long-term. Another limitation of the amplicons was that they cannot accommodate inserts longer than 10 kb. Wade-Martins and coworkers have developed an efficient viral delivery and expression system based on the HSV-1 amplicon vector, termed the iBAC, or infectious BAC that can carry large genomic locus with surrounding sequences.[141-143]

Replication-Defective Vectors

Major advances have recently been made to improve the characteristics of these vectors, in particular to reduce their toxicity, to modulate the greatness and the time-course of transgene expression, to precisely target specific cell populations and to transfer multiple genes.[17,21,144-147] Nonreplicative HSV vectors have been tested in many different gene therapy animal models of various neuropathies, Parkinson's disease,[148-150] Alzheimer's disease,[151] chronic pain[152,153] or lysosomal storage disorders with neurological involvement.[154]

Therapy of lysosomal storage disorders with neurological involvement such as Tay-Sachs (TS) disease requires production and distribution of the missing enzyme into the CNS. Several therapeutic approaches allow restoring the enzymatic activity in many key tissues (kidney, liver, spleen, etc.) but the reduction of the GM2 ganglioside deposits in the CNS is difficult to achieve since CNS, is kept in a privileged environment separated from the blood system by the blood-brain barrier (BBB), which represents an obstacle to therapy.[154] Martino et al have demonstrated that a nonreplicating HSV vector encoding for the hexosaminidase (Hex) A α-subunit (HSV-T0αHex) and delivered into the internal capsule of the TS brain animal model was able to re-established the Hex A activity and removed the GM2 ganglioside storage in both injected and controlateral hemisphere and in the cerebellum one month of treatment. The studies concerning lysosomal storage disorders are particularly important because they represent the first evidence of the distribution of a therapeutic viral vector throughout the entire CNS and suggest that the anatomic structure of the brain may be a useful tool in therapy for genetic neurodegenerative disorders.[154]

Among them, vectors expressing multiple trophic factors seem to be very promising as a side treatment for neurodegenerative diseases. Motor neuron disease (MND) is a group of neurological diseases characterized by degenerative process of the upper and lower motor neuron[155] in different parts of the motor system including the spinal cord, brain stem and motor cortex. One of the major breakthroughs in the field of CNS regeneration is the concept that neurotrophic factors (NTFs), which are endogenous soluble proteins regulating survival, growth, morphological plasticity, or synthesis of proteins for differential functions of neurons, govern the processes involved

in brain and spinal cord repair.[156] Experimental evidence indicates that treatment with multiple neurotrophic factors can significantly increase motor neuron survival in comparison with the delivery of a single factor alone.[157,158] HSV-1 vectors that have been engineered to express multiple neurotrophic factors have been used to deliver these molecules to specific neuron populations.[159] Nonreplicative vectors containing basic fibroblast growth factor (bFGF), ciliary neurotrophic factor (CNTF) and EGFP (as a reporter gene) have already been shown to induce proliferation and differentiation in O-2A oligospheres obtained from newborn rat brains in vitro and also in the rat hippocampus in vivo.[158]

Replication-Competent Vectors

One of the potential target organs of replication competent HSV vector applications, the peripheral nervous system (PNS), seems likely to promise the most successful results. In fact, inoculation of HSV vector by peripheral routes can take advantage of the natural life cycle of the virus, which usually infects axonal nerve terminal at peripheral sites before retrograde transport to neuronal cell bodies where latency is established. It is well known that viral replication is necessary to cross the synapses among neurons and for efficient establishment of latency.[160] In the PNS there are a number of potential applications for HSV replication competent vectors capable of peripheral replication and axonal transport, including the stimulation of regrowth of damaged nerves, the study and treatment of various pain states, the protection of neurons from further degeneration in motor neuron disease, the study and treatment of various neuropathies, the study of neuronal development and the screening of the relevance of genes implicated as being important in any of these processes by a gene delivery approach. Thus, viruses mutated in either gC or TK or RR have been extensively used, which, while being somewhat attenuated compared to wt virus, are also replication-competent. The data obtained to date show the potential of such vectors for gene transfer. Attenuated vectors in fact demonstrated to be highly efficient in driving proenkephalin A (PA) gene expression in dorsal root ganglia (DRG),[161] to deliver genes into monkey eyes[162] and to rodent visual system[163] and to express active nerve growth factor beta subunit (β-NGF) in latently infected DRG .

HSV-1 Based Vectors for Cancer Gene Therapy

HSV vectors have wide-range natural hosts and have been proven to efficiently infect numerous human tumor cell lines in vitro. A number of new therapies have been developed for treatment of cancer and the knowledge of the basic defects that occur in malignant tumors has lead to the conclusion that the association of different therapeutic approaches is the method to eradicate these malignancies. Potential genes should induce a selective anti-tumor response that attacks the primary tumor, inhibits metastasis, prevent recurrence and does not promote drug resistance. Another important feature of HSV-1-based vectors for cancer gene therapy is their capacity to express the autologous *tk* gene, encoding the TK enzyme, a well characterized suicide gene, widely used in gene therapy of different experimental tumors[164-166] and which has already been tested in clinical trials.[167-169] Another advantage of the use of TK/GCV system is that it is capable of killing both vector-transduced and neighboring cells, owing to the effect.[164,170,171]

Recent efforts at modifying the envelope of the HSV-1 virion to target specific receptors, e.g., replacement of the heparan sulfate binding domain in gC in the envelope with a receptor ligand or single chain antibody, indicate that it is possible to selectively increase infectivity of tumor cells bearing corresponding receptors.[172,173] Infection of normally non infectable cells has been achieved using a soluble adapter fusion protein consisting of the HSV-1 envelope gD and a single chain antibody for the epidermal growth factor receptor (EGFR), which is enriched on many tumor cells.[174]

Amplicon Vectors

Most anti-cancer amplicon vectors used to date have employed standard amplicon vectors, which efficiently deliver genes to the cell nucleus but are lost with successive cell division. Therapeutic transgenes used in the context of amplicon vectors have included anti-angiogenic factors, immune enhancing agents, proapoptotic proteins and RNAi.[175]

Stunting of tumor growth can be achieved by inducing hypoxia through inhibition of neovascularization. HSV amplicon vectors have been used to attenuate angiogenesis and thereby inhibit pancreatic tumor growth by expression of a dominant-negative soluble vascular endothelial growth factor (VEGF) receptor, sFlk-1 under control of a promoter induced in hypoxic conditions.[176,177] HSV amplicon vectors have also been evaluated as cancer vaccines by expressing combinations of cytokines and immunomodulatory proteins for treatment of a variety of experimental tumors.[24,175,178-181]

A promising, new approach to cancer is the selective degradation of mRNA by RNA interference (RNAi)[182] or interference with microRNAs that support tumor growth.[183] HSV amplicon vectors expressing siRNAs have been used recently to mediate posttranscriptional silencing of EGFR, which is frequently activated in human glioblastoma cells[184] and to inhibit the expression of BKV T-Ag and tumorigenicity of BKV-transformed cells.[185]

Of the wide range of prodrug activating enzymes tested for cancer therapy,[186,187] only a few have been delivered via HSV amplicon vectors. One of these is HSV TK, which converts nucleoside analogues, such as ganciclovir, into toxic analogues which incorporate into replicating DNA and lead to cell death.[188] A chimeric fusion protein between cytochrome P450 4B1 and GFP in combination with the prodrug 4-ipomeanol was found to confer toxicity to glioma cells with a bystander effect.[189] Amplicon vectors have also been girded with two synergistic pro-drug activating enzymes, TK and cytosine deaminase (CD) for tumor therapy and imaging.[190,191]

The use of a replication-conditional virus to package amplicon vector can improve the efficacy of cancer therapy by combining delivery of therapeutic gene(s) via the amplicon vector with selective viral oncolysis of tumor cells by the replication-conditional virus. It has been demonstrated local and distant immune-mediated control of colon cancer growth with fusogenic membrane glycoproteins in combination with viral oncolysis.[192,193] Moreover, an amplicon vector that expresses an essential viral gene, such as ICP4, can complement ICP4⁻ recombinant viruses to efficiently replicate and cause lysis in prostate cancer cells.[194] Furthermore, it has been shown that the immunostimulatory effects of amplicon vector-mediated cytokine expression enhance direct viral-induced oncolysis in a syngenic squamous cell carcinoma flank model.[105,195,196]

Targeting proliferating tumor cells via the transcriptional control of therapeutic genes can potentially improve the safety and efficacy of cancer gene therapy. It has been shown that transgene expression could be targeted to proliferating cells when cell cycle transcriptional regulatory elements are incorporated into amplicon backbone vectors.[197-199] For example, transcriptional regulation can be rendered specific to human hepatocellular carcinoma cells by inserting the chimeric gene Gal4/NF-YA under the regulation of a HCC-specific hybrid promoter.[200]

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has been shown to induce apoptosis in neoplastic cells. It has been reported the efficacy of amplicon delivered TRAIL and its secreted form (S-TRAIL) in treating tumors in vivo and in monitoring both gene delivery and efficacy of TRAIL-mediated apoptosis by dual-substrate bioluminescence imaging.[201-203]

Replication-Defective Vectors

Multiple immediate early gene-deleted nonreplicative HSV-1 vectors are characterized by high efficiency of transduction of several different host species and cell types, both dividing and non dividing, including various tumor as well as endothelial cells.[23,204-207] Different replication-defective HSV vectors have been produced that deliver anti-cancer transgenes to tumor cells such as melanoma,[43] gliosarcoma,[205,208,209] or glioblastoma.[210] Two or more therapeutic molecules, acting additively or synergistically, can thus be expressed at comparable levels by cells transduced with a combination vector, which is clearly an advantage in comparison with co-administration of two or more vectors encoding a single transgene and also in comparison with co-expression of two molecules, separated by IRES sequences, by a unique vector.

These mutant vectors express, in association with the autologous HSV-1 *tk* gene acting as a suicide gene when accompanied by its pro-drug ganciclovir, further transgenes chosen for their potential to synergize in tumor cell killing and induction of anti-tumor immunity with genes

encoding for soluble human cytokines (IL-2, GM-CSF and IFN-γ), the human B7.1 gene encoding a costimulatory surface antigen (CD80);[43] rat connexin 43 gene improving the HSV-1 TK/GCV killing of glioma cells by increasing the bystander effect [211] or rat connexin and human TNFα.[210,212] Recently, an HSV-1-derived replication-defective vector (T0-IFI16) was developed, [204] which has been shown to efficiently transduce an interferon-inducible gene (IFI16), into primary human umbilical vein endothelial cells (HUVEC), which are usually poorly transfectable. It has also been possible to infect HUVEC cells with similar HSV-1-based vectors expressing anti-angiogenic fusion proteins endostatin::angiostatin and endostatin::kringle 5. The expression of anti-angiogenic proteins by directly infected HUVEC cells has been shown to induce cytostatic effects in proliferation assays in vitro. Also, by addition of gancyclovir to the cell culture media, a major cell killing effect was observed.[206] In vivo, the expression of autologous *tk* gene in association with GCV was shown to be highly efficient in both reducing small tumor masses growth rates and also in inhibiting tumor cell engraftment. The expression by tumor cells of vector-encoded angiostatic proteins was also extremely efficient in inhibiting the tumor establishment, both in presence or in absence of GCV.[206]

The wide spectrum of dividing or nondividing cell types that can be easily infected by non-replicative HSV-1 vectors and among them endothelial and dendritic cells, along with their large exogenous DNA accommodating capacity, makes these vectors very attractive delivery systems. These unique features might be of extreme importance for combined therapeutic strategies requiring the simultaneous expression of high levels of multiple foreign genes, like suicide genes, cytokines or other immunomodulatory molecules, anti-angiogenic proteins, soluble growth factor receptors and so forth. As various types of tumors present different characteristics, the high manageability of large, well characterized HSV-1 genome might permit the combination, in a unique backbone, of the most appropriate exogenous genes for treatment of each particular tumor.

Replication Competent Vectors

Oncolytic viruses, which selectively infect or replicate in cancer cells while sparing normal cells, have been explored as alternative cancer therapies in both preclinical and clinical trials.[77,78] The optimal strategy might be to derive a replicating vector from a highly prevalent but weakly pathogenic human virus,[213] so the reversion to wt would then be of no serious risk to the patient or to the population.

Construction of oncolytic viruses that cannot only target cancer cells, but can also retain their ability to infect, usurp host replication machinery, then release newly made progeny to infect other transformed cells after lysing and killing the host cell, has become a major area of therapeutic cancer research. There are some characteristics that an ideal replication-competent, oncolytic virus should possess above and beyond those viruses that function simply as delivery vectors: (i) be easy to engineer and to produce in large quantities; (ii) selectivity to neoplastic cell alone; (iii) minimal toxicity to normal tissue; (iv) show proliferation within and systematic killing of tumor tissue which itself may be rapidly propagating; (v) ability to disseminate throughout the tumor mass and possibly to sites of invasion distant from the initial inoculation site; (vi) carry low or bearable toxicity; (vii) genomically stable, thus avoiding the generation of toxic, undesirable mutants that could pose a danger; (viii) incorporate a "fail-safe" mechanism for inactivation; (ix) absence of potential spread to the general population; and (x) enduring efficacy despite prospect of encountering a mounting immune response to replicating viruses. Replication-conditional HSV-based vectors have great potential in the treatment of various types of cancers including brain tumors.[64-66,77,214-220]

So far, several oncolytic HSV vectors have been developed. The first generation of these vectors contained mutation in a single gene that restricted their replication to dividing cells. Three such HSV-1 mutants were constructed: (i) *dls*pTk containing a deletion in the *tk* gene;[221] (ii) hrR3 containing an insertion of the *E. coli lac-Z* gene in the early gene UL39, encoding the large subunit of the viral RR (ICP6);[222,223] and (iii) R3616 containing 1 kb deletions in both copies of the γ34.5 gene, encoding the neurovirulence factor ICP34.5.[224-226]

TK mutants are highly neuroattenuated and when used in different mouse models of various nervous system-derived tumor types, showed a slowed tumor growth and prolonged survival. However, clinical trials were not pursued because of (i) undesirable level of toxicity at high titers and (ii) its TK-negative status made it resistant to traditional anti-herpetic treatments, a major disadvantage should any viral toxicity to arise in treated patients.[221,227]

ICP6 mutants have been tested as replicative anti-cancer agents, alone or in combination with acyclovir/gancyclovir, as the mutants retain their sensitivity to such anti-virals. Moreover, the RR$^-$ mutants have been shown to display an increased sensitivity to gancyclovir, compared to the wt virus. These recombinant viruses showed enhanced killing of tumor cells in vitro and showed improved survival of animals. However, like TK mutants, they can cause fatal encephalitis when used at sufficient dose and were not thought to provide a sufficient margin of safety for testing in humans.[223,228]

It has been shown that deletion of ICP34.5, the neurovirulence factor essential for HSV pathogenicity, provides the greatest degree of attenuation of any individual mutation where the virus can still replicate in actively dividing cells. R3616, the prototype HSV-1 deleted in both copies of γ34.5, had demonstrated attenuated neurovirulence but with maintained anti-glioma activity and was found to produce no encephalitis in a nude mouse model.[22,116,229,230] The use of γ34.5 mutated viruses demonstrated considerable anti-tumor efficacy, combined with a good safety profile and different versions of HSV ICP34.5-deleted are currently in human clinical trials.[231]

Following preclinical testing with the above mentioned oncolytic vectors, second generation vectors with multigenic mutations were created. G207 contains deletion in both γ34.5 loci and a *lacZ* gene insertion in the ICP6 gene.[232] These multiple mutations made the reversion to wt highly unlikely and conferred several important safety advantages. Moreover, G207 retains its susceptibility to standard anti-HSV therapies such as acyclovir, since the *tk* gene is intact. After oncolytic activity and safety evaluation studies in the mouse model,[232] G207 neurotoxicity was further evaluated in nonhuman primates.[233] The data obtained in the previous experiment allowed to move into Phase I clinical trials[234] and, presently, enrollment has begun for sequential Phases Ib/II trials employing G207 as an anti-tumor agent for malignant gliomas. Almost simultaneously, HSV1716, derived from the parent wt strain HSV-1 17+ in which both the copies of γ34.5 have been deleted, also underwent clinical trials to evaluate its toxicity in patients with recurrent human glioma,[235,236] after it was demonstrated to be avirulent in mice.[237,238]

Oncolytic herpesvirus have been also studied as an oncolytic anti-tumor therapy against a variety of tumors different than GBM and anaplastic astrocytoma, such as human breast cancer in a brain metastatic model,[25] colorectal cancer and liver metastases,[239] prostate cancer,[240] pancreatic cancer[241,242] and head and neck squamous carcinoma.[243] In many of these studies, the efficacy of G207 has been compared with that of the above mentioned NV1020, which has shown to be too attenuated to work as a live viral vaccine.[117,244] Moreover, NV1020 is currently being investigated in Phase I clinical trials for patients with colon cancer that has metastasized to the liver and has proven recalcitrant to chemotherapy. This is also the first trial to investigate administration via intravascular delivery. In fact, oncolytic viruses can be administered locally, by direct intratumoral inoculation, or systemically, by intravascular administration. However, the route of administration of the virus did influence efficacy, as was observed in the animal model.

Despite the promising results obtained with the engineered HSV-1 based oncolytic vectors described above, it is likely that a multimodal approach to eradicate cancer will be more effective with the final goal to improve safety and efficacy of the system. At this regard, oncolytic HSV vectors have been further modified to augment their anti-tumor efficacy, by incorporation of expression cassettes for the delivery of various transgenes. Moreover, if the therapeutic gene is chosen carefully, this may be synergistic with the anti-tumor effect of virus replication.

Molecules including a number of interleukins and interferons have been tested with oncolytic HSVs. Among these, IL-4,[245] IL-12,[25,246,247] IL-10,[245] GM-CSF,[231,247] and B7.1,[248] which increase tumor immune recognition. This approach also reduces the possibility of toxicity derived from the systemic administration of the cytokine.

Oncolytic HSVs have been tested, which encode different pro-drug-activating systems other than the endogenous TK activity of the virus. Both the 5-fluorocytosine (5-FC) pro-drug/yeast cytosine deaminase (CD) gene system,[249] alone or in combination with the TK/gancyclovir system[186] and the cytocrome P-450/cyclophosphamide (CPA) system,[250,251] were shown to induce beneficial effects.

Nevertheless, ICP34.5 mutants replicate with considerable reduced efficiency in most tumor cells, compared to wt HSV. To improve tumor-selective replication, an ICP34.5 deleted HSV with enhanced growth characteristics was isolated after serial passages on a tumor cell line. This mutant was also found to give improved anti-tumor activity in vivo, without compromising safety.[252] These improved characteristics were found to derive from a second site suppressor mutation in the unique short region, which determines the expression of the US11 gene as an IE rather than a L gene and the deletion of US12, encoding the ICP47 protein, contributing the improvement of anti-tumor immune response.[253] This viral isolate was shown to exhibit enhanced anti-tumor effect.[254]

Most of the oncolytic HSVs analyzed have been based on serially passaged laboratory strains of HSV. These strains have probably lost some of their aggressive properties. It has in fact been recently demonstrated that an oncolytic HSV, JS1/ICP34.5-/ICP47-/GM-CSF, derived from a clinical isolate of HSV-1, possesses a higher ability to kill tumor cells in vitro and in vivo. Moreover, in a model of mouse lymphoma, mice cured with this virus were protected against further tumor challenge.[231]

In early clinical trials, however, treatment with the current generation of oncolytic viruses did not significantly affect tumor growth.[234,235] This suboptimal result may reflect viral gene deletions, which can reduce the replicative potential of viruses in tumor cells. For example, deletion of the γ34.5 gene significantly reduced viral growth even in rapidly dividing cells.[223] A variety of strategies are being pursued to enhance the potency of oncolytic viruses. Overall, the results so far obtained demonstrate that incorporating suicide and/or cytokine transgenes in the viral genome can increase anti-tumor efficacy, especially if used in combination with preexisting anti-cancer treatments such as chemotherapy or radiotherapy.[66,255] Another strategy is to clone therapeutic genes into the viral genome to arm the virus with additional cytotoxic mechanisms that augment the direct lytic functions of the virus. Particularly attractive in this context are cytotoxic mechanisms with potent bystander effect capable of eliminating tumor cells that the virus cannot reach. To this purpose, it has been recently demonstrated that incorporation of cell membrane fusion capability into an oncolytic HSV can significantly increase the anti-tumor potency of the virus.[256,257] These oncolytic HSVs were constructed by three different methods: (i) screening for the syncytial phenotype after random mutation of a well-established oncolytic HSV (to obtain Fu-10); (ii) insertion of the gene encoding the hyperfusogenic membrane glycoprotein of gibbon ape leukemia virus (GALV.fus) into the genome of an oncolytic HSV (to generate Synco-2); and (iii) incorporation of both of these two membrane fusion mechanisms into a single oncolytic HSV (to generate Synco-2D). These vectors have been tested for their anti-tumor activity against liver, breast, ovarian and metastatic prostate cancers showing a significant increase in viral oncolysis; this may lead to an enhanced clinical performance, especially in the late stage cancer patients.

Conclusion

The different kinds of vectors that derive from HSV-1 were conceived to take advantage of the biological properties of this virus. Therefore, recombinant HSV-1 vectors, either attenuated or defective, attempt to exploit different adaptations of HSV-1 to the nerve system, such as latency, the presence of powerful neurospecific promoters, or the occurrence of viral genes controlling neuroinvasiveness or neurovirulence. So far, promising results have been obtained in treatment of several models of PNS and CNS diseases,[154,258,259] in treatment of pain[17,161] and using such vectors as tools for investigation of behavioral traits, like learning and memory[132] and for neuroscience research in general.[146] Although these vectors have been used mainly for gene transfer to neurons or glial cells, they hold a big potential as vector vaccines,[93] both against infectious disease and cancer. In fact, they can efficiently deliver genes to other cell types, including epithelial cells, fibroblasts,

myoblasts, myotubes, embryonic and adult cardiomyocytes and cell lines derived from gliomas, hepatocellular carcinomas, osteosarcomas, epidermoid carcinomas and many other human and murine malignancies. In no case, the vector genomes integrate into host chromosomes, therefore precluding the risk of insertional mutagenesis. The other type of vector, namely the amplicon vectors, attempts to exploit the capacity of the virus capsid to accommodate more than 150 kb of foreign DNA. HSV amplicons possess the unique feature to possibly deliver entire genomic loci including all upstream regulatory elements and downstream introns and to convert them into human artificial chromosomes. One of the major areas of interest in amplicon development regards the possibility to produce still larger amounts of purified vectors than those generated by current procedures. To this purpose, different suggestions regard the improvement of the structure of the amplicon plasmids, of the helper virus systems and of the transcomplementing cell lines where the amplicon vector stocks are being produced. As a second point, there is the possibility of controlling transgenic expression for therapeutical applications and to avoid transgenic silencing. This can be achieved since helper-free amplicons do not express proteins enhancing expression, like ICP4, 27 and 22, or proteins protecting from silencing, like ICP0. As a consequence, transgene expression depends on cell type, multiplicity of infection and cell cycle. It is possible that placing transgenic expression under the control of genuine cellular regulatory sequences will resolve, at least in part, this difficulty.[142,260,261]

Much more work remains to be carried out, especially if we intend to prolong transgene expression and to improve cell targeting. However, although short-term transgene expression represents a great limitation for the use of vectors in the gene therapy of diseases, this is not necessarily the case when considering their use for gene expression that are associated with certain behaviors that are often transient.

Another goal to increase the efficacy of the HSV vectors and to decrease the undesired effects such as infection of healthy cells is to target infection to specific tissues or organs or to restrict transgene expression to predefined subsets of cells. Genetic modifications to the genome of HSV-1 vectors have been generated to preferentially target viral infection and/or replication to tumor cells versus normal cells.[174] Targeting viral infection to particular cells can be obtained by modifying the first steps of the virus life cycle, i.e., adsorption and penetration. Efforts for engineering the HSV-1 envelope to obtain targeted infection are currently in progress.[16,262-264] Altering HSV-1 host range has proved a formidable task because HSV-1 infection is a complex process involving the action of several glycoproteins in cell attachment, entry and cell-to-cell spread.

As a final consideration, although the vectorology area of research is still in continuous development, certainly, more work should be done in order to better understand the vector/host interactions. Anyway, it can be inferred, from what it is known on HSV-1 immune biology, that all the three types of HSV vectors, including amplicons, will induce an anti-viral cellular response, at least in some cell types and will stimulate both the innate and adaptive branches of the immune response in the infected organism. These responses can eventually result in the elimination of the vector or in the silencing of the therapeutic transgenes. Finally, it can be predicted that the large size insert capacity of the amplicon genome, that allow these vectors to express several viral or cellular proteins well-known to down-regulate or to inhibit the anti-viral and immune responses, will be a major advantage of amplicons over other vectors to fight against the silencing cellular forces.

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CHAPTER 11

Virus-Like Particles as a Vaccine Delivery System:
Myths and Facts

Polly Roy* and Rob Noad

Abstract

Vaccines against viral disease have traditionally relied on attenuated virus strains or inactivation of infectious virus. Subunit vaccines based on viral proteins expressed in heterologous systems have been effective for some pathogens, but have often suffered from poor immunogenicity due to incorrect protein folding or modification. In this chapter we focus on a specific class of viral subunit vaccine that mimics the overall structure of virus particles and thus preserves the native antigenic conformation of the immunogenic proteins. These virus-like particles (VLPs) have been produced for a wide range of taxonomically and structurally distinct viruses, and have unique advantages in terms of safety and immunogenicity over previous approaches. With new VLP vaccines for papillomavirus beginning to reach the market place we argue that this technology has now 'come-of-age' and must be considered a viable vaccine strategy.

Introduction

There are many infectious viruses that remain major threats to public health (see Table 1). Where an effective vaccine exists, vaccination is usually the most cost-effective long-term protection against disease and spread for most viruses. The principle of vaccination is to generate sufficient immunity to protect from infectious disease. Thus the vaccine stimulates the body's natural defenses against disease through use of a benign 'decoy' that mimics the virulent pathogen. The more similar a vaccine is to the natural disease, the better the immune response to the pathogen on subsequent exposure. In general, resistance to virus infection depends on the development of an immune response to antigens present on the surface of virions or virus-infected cells. Therefore identification of protective antigens is the first step in the development of effective viral vaccines.

Currently many successful viral vaccines have been developed and are in use. These vaccines are predominantly based on live attenuated or inactivated viruses. The live attenuated vaccines such as measles, mumps, rubella, oral polio, smallpox, varicella and yellow fever are a weakened form of the "wild" viruses. These attenuated virus vaccines rely on limited replication of the virus in the host following vaccination. Immune responses induced are similar to those from natural infections and often these vaccines are effective after a single dose. However, such vaccines may cause severe reactions in some patients, which are often the result of the limited replication of the attenuated virus following vaccination. In contrast to attenuated live virus vaccines, inactivated (or killed) vaccines can not replicate, as their genetic material or overall structure are purposefully destroyed. These vaccines are safer than live vaccines but generally not as effective, requiring 3-5

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Table 1. Viruses that are major health threats

Virus	Disease
HIV	AIDS
RSV	Respiratory Infection
Hepatitis B	Liver Cancer
Hepatitis C	Cirrhosis/Cancer
Epstein Barr Virus	Lymphomas, Nasopharyngeal carcinoma
HPV	Cervical Cancer
Measles	Pneumonia (infants)
Influenza	Pneumonia

Abbreviations: HIV human immunodeficiency virus, RSV rous sarcoma virus, HPV human pappilomavirus.

doses as antibody titer falls over time. They lack the self-boosting qualities of live attenuated vaccines but are safer in the sense that the inherent dangers associated with virus replication are avoided. These vaccines are made as whole cell vaccines (such as Influenza, polio, rabies and hepatitis A) or as fractional or subunit vaccines such as hepatitis B. Subunit vaccines are based on the delivery of only a limited number of viral proteins, often the major protein in the capsid or envelope that is sufficient to confer protective immunity. These vaccines are an incremental step safer than inactivated vaccines because subunit vaccines can be prepared independent to the culture of replicating virus. Indeed, any remaining possibility of incomplete inactivation or batch to batch variation in the safety of the vaccine is eliminated. However, subunit vaccines have traditionally suffered from one important drawback; often single proteins when expressed and purified in the absence of other viral components are less immunogenic than those that are incorporated into infectious virus. This is probably because a proportion of this protein is present in a misfolded conformation relative to the native protein. Thus, more doses with higher amounts of antigen are required to achieve the same level of protection.

A major advance in subunit immunogen production has been assembly of proteins as virus-like particles (VLPs) using protein expression technology in yeast, insect or mammalian cells. VLPs are a highly effective type of subunit vaccines that mimic the overall structure of virus particles without any requirement that they contain infectious genetic material. Indeed, many VLPs lack the DNA or RNA genome of the virus altogether, but have the authentic conformation of viral capsid proteins seen with attenuated virus vaccines, without any of the risks associated with virus replication or inactivation.

VLP preparations are all based on the observation that expression of the capsid proteins of many viruses leads to the spontaneous assembly of particles that are structurally similar to authentic virus.[1-4] In practical terms, the fact that VLPs mimic the structure of virus particles usually means that VLPs should elicit strong humoral response and that lower doses of antigen relative to subunit vaccines are sufficient to elicit similar protective response. In addition to their ability to stimulate B cell mediated immune responses, VLPs have also been demonstrated to be highly effective at stimulating CD4 proliferative and = (CTL) responses.[5-7] This feature of VLP vaccines is likely to be a major contribution to their effectiveness in the field. It is also becoming increasingly clear that precise prime-boost strategies can be important to how effective vaccination is as a strategy to control disease. Therefore, the addition of VLP to the 'arsenal' of vaccine strategies for any disease extends the type of prime-boost regime that can be employed.

To date, VLPs have been produced for many different viruses that infect humans and other animals (see Table 2 and review).[8] One of the most striking features of this group is that it is extremely diverse in terms of the structure of the individual viruses. It includes viruses that have a single capsid protein, multiple capsid proteins and those with and without lipid envelopes. Clearly

Table 2 Baculovirus derived VLPs that have been tested as vaccines

VLP	Family	Proteins Expressed	Vaccine Tested In	VLP Refs.
Papillomavirus,	*Papillomaviridae*	1	Humans (licensed)	10,11,15-20,73
Norwalk and Norwalk-like viruses, Feline calicivirus	Calciviridae	1	Mice, cats, humans (Phase I)	26,27,74-78
Hepatitis E virus	*Hepeviridae*	1	Mice, cynomologous monkeys	28-30
Porcine parvovirus, mink enteritis parvovirus, Canine parvovirus, B19, adeno-associated virus	*Parvoviridae*	1	Pigs, dogs, mink	21-23,79,80,81
Chicken anemia virus, Porcine circovirus	*Circoviridae*	1, 2 (chicken anaemia virus)	Chickens	82-85
SV40, JC virus, murine polyomavirus	*Polyomaviridae*	1	Mice, rabbits (in vitro)	32,86,87
Polio virus	*Picornaviridae*	1 (polyprotein)	-	88
Bluetongue virus, Rotavirus	*Reoviridae*	4 (bluetongue) 2-3 (rota)	Sheep (bluetongue) Mice, pigs (rota)	4,35,42-49,89
Hepatitis C Virus	*Flaviviridae*	3	Mice, baboons	7,51,53
HIV, SIV, FIV, Visna virus, FeLV, BLV, Rous Sarcoma virus	*Retroviridae*	2	Mice, guinea pigs	2,3,50,90-96
Newcastle Disease Virus	*Paramyxoviridae*	1	Chickens	97
SARS Coronavirus	*Coronaviridae*	3	Mice (in vitro)	54
Hantaan virus	*Bunyaviridae*	3	Mice	98
Influenza A virus	*Orthomyxoviridae*	2-4	Mice	52,60,61
Infectious Bursal Disease virus	*Birnaviridae*	1	Chickens	34,52,99,100

Abbreviations: BTV Bluetongue virus, HIV Human immunodeficiency virus, SIV simian immunodeficiency virus, FIV feline immunodeficiency virus, FeLV feline leukemia virus, SV40 simian virus 40, rota rotavirus.

not all of the VLPs that are generated to date are appropriate vaccine targets, some VLPs have been generated to facilitate in fundamental understanding of virus assembly process, morphogenesis or architecture of viruses. However, an important point remains that the structure of the target virion is not limiting to the success of VLP production. Although various expression systems have been employed for VLP production, this chapter will mainly focus on insect cell culture produced VLPs that are being developed as candidate vaccines. The rationale behind this is that among all expression systems, insect cells, together with baculovirus expressing system, appear to be one of the most promising for VLP technology for development of viral vaccines (Fig. 1).

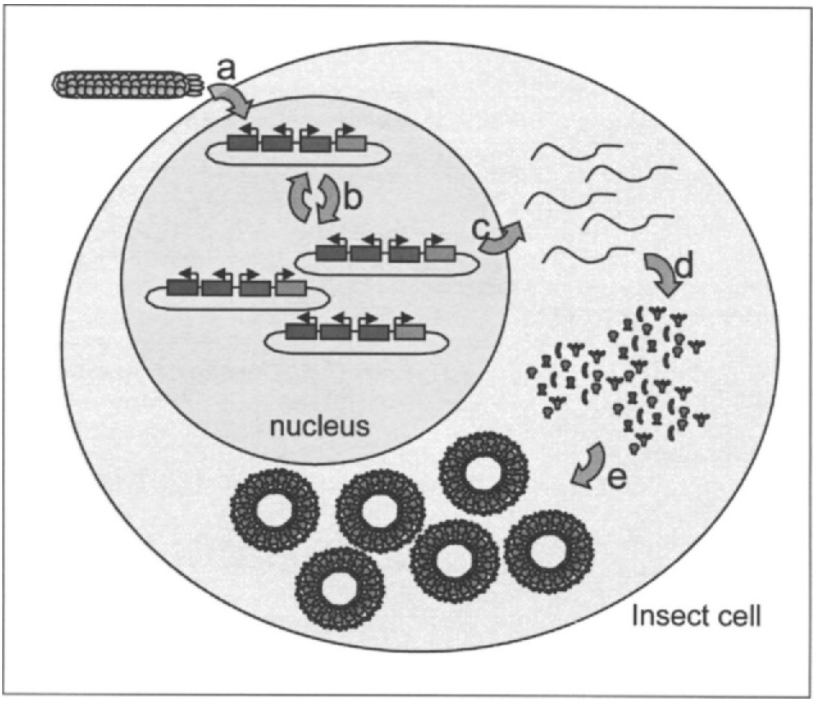


Figure 1. Key stages of intracellular assembly of VLPs using the baculovirus system. a) Baculovirus acts as a vehicle to efficiently deliver DNA, encoding recombinant proteins, to the nucleus of insect cells. b) Viral DNA is uncoated and replicates in the nucleus. c) Recombinant protein expression is driven by strong very-late viral promoters. d) Viral mRNA is used for the synthesis of recombinant proteins. e) VLPs are assembled by the interaction of proteins within the cytoplasm.

Insect Cells and Baculovirus Expression System as Preferred System for VLP Production

As stated above, a variety of protein expression systems are available to express recombinant proteins and particles. However certain criteria for generation of VLPs as prophylactic vaccines, particularly for human viral infection, must be considered. In order for a VLP to be a realistic vaccine candidate, it needs to be produced in a safe expression system that is easy to scale up to large-scale production. Table 2 shows baculovirus expressed/insect cell produced VLPs that have been demonstrated to be highly immunogenic and potential vaccine candidates. This insect cell-based protein production system has many advantages for VLP production. Firstly, extremely large amounts of correctly folded recombinant proteins can be produced in high-density cell-culture conditions in eukaryotic cells. Secondly, baculovirus expression systems have been developed for expression of multiple foreign proteins simultaneously from a single recombinant virus facilitating capsid assembly in each infected cell. Thirdly, as the insect cells that are used for vaccine production can be cultured without the need for mammalian cell derived supplements, the risks of coculture of opportunistic pathogens is minimized. Fourthly, the baculovirus used for recombinant protein expression has a narrow host range that includes only a few species of Lepidoptera and therefore represents no threat to vaccinated individuals. Finally the baculovirus system is amenable to scale-up for large scale vaccine production.[9]

VLPs Produced for Structurally Simple Non-Enveloped Viruses

For a number of nonenveloped viruses viral capsids are formed by only one or two major proteins and thus are relatively easy to manipulate for generation of VLPs by heterologous expression systems. Examples of these are the VLPs formed by the expression of the major capsid protein of Papillomaviruses, Parvoviruses, Calciviruses, Circovirses, Polyomaviruses and Hepatitis E virus (Table 2). All of these viruses are nonenveloped and have a single, virally encoded protein that forms the major structural component of the virion. Papilloma virus VLPs are among the most completely studied of this collection of VLPs and are at the most advanced stage with respect to production of a useful vaccine. VLP of Papilloma viruses are formed from the over expression of the major capsid protein L1.[10-12] These particles are highly immunogenic and are able to stimulate both humoral and cell mediated immune responses.[13-15] Human Papillomavirus (HPV) is the leading cause of cervical cancer. Globally, approximately 70% of all cervical cancer cases are associated with two serotypes of HPV, HPV-16 and HPV-18. VLPs produced in insect cells have been used successfully for Phase I and II human clinical trials in large numbers and were shown to be highly efficacious.[15-19] Moreover, GlaxoSmithKline's cervical cancer vaccine candidate (*Cervarix*™) targeting HPV 16/18 is currently undergoing Phase III clinical trials involving more than 30,000 women worldwide. In this Phase III randomized, double-blinded trial conducted in multiple centres in Denmark, Estonia, Finland, Greece, the Netherlands and the Russian Federation. All vaccinees received the HPV VLPs (HPV-16/18 AS04) as follows: 158 10-14 years old healthy girls and 458 15-25 years old young women received the candidate VLP vaccine according to a 0, 1, 6 month schedule and anti-HPV antibody titers were assessed. At month seven 100 per cent seropositivity was achieved in both groups for HPV 16 and 18 although average antibody titers for both HPV types were at least two-fold higher in 10-14 year-old girls. The vaccine was tolerated by all patients and no vaccine related serious adverse effects were detected. Further, the follow-up study clearly demonstrated the sustained efficacy of HPV-16/18 VLPs up to 4.5 years.[19,20] In conclusion, the bivalent HPV vaccine is highly immunogenic and safe and induces a high degree of protection against HPV-16 and HPV-18 infection and associated cervical lesions.

These studies are not only an important demonstration of the effectiveness of HPV VLP vaccine, and that multi-serotype VLPs are effective, but also highlight the fact that insect cell produced VLPs are a realistic alternative as human vaccines against viral disease. It should also be mentioned at this point that a tetravalent (HPV-6/11/16/18) VLP vaccine, Guardasil™ (Merk), produced in yeast cells was approved by FDA in June 2006 for use in women aged 9-26.

VLP vaccines for various diseases caused by parvovirus infections are also at an advanced stage although as yet none have undergone such large scale trials as those reported for HPV. Synthesis of major structural proteins VP2 of canine parvovirus (CPV) and porcine parvovirus (PPV) led to assembly of VLPs in insect cells.[21,22] Vaccination trials of CPV VLPs in dogs and PPV VLPS in pigs were highly encouraging.[21,23] In one efficacy assay dogs that received as little as or 10 μg or 25 μg of CPV VLP were completely protected from virus infection when challenged with virulent virus. Furthermore a single subcutaneous dose of 3 μg same CPV VLP with 50 μg ISCOM adjuvant was able to protect mink against challenge with the anti-genically similar virus, mink enteritis virus (MEV).[21] Similarly it has been reported recently that a single immunization with 0.7 μg of PPV (porcine starin) VLPs yielded complete protection in targeted animals against infectious PPV strains.[23] Indeed microgram doses of VLPs in gilts were not only highly immunogenic, but were also very efficient in preventing trans-plancental virus transmission and significantly reduced the number of reproductive failures. In addition, the feasibility of safe large-scale production of the porcine parvovirus VLP vaccine has been established complying with the European Pharmacopoeia requirements.[9]

Calicivirus studies have relied heavily on the production of proteins in heterologous systems mainly due to the fact that it is not yet possible to grow the virus in cell culture. Thus, VLP to Norwalk-like viruses have been extremely useful as sources of diagnostic antigen to monitor disease outbreaks. Norwalk virus VLP have also been shown to be effective at stimulating IgG, IgA and humoral responses in mice.[24,25] Preliminary Phase I trials in humans to test the safety and

immunogenicity of insect cell expressed Norwalk virus VLPs has confirmed that they are both safe and effectively stimulate IgG and IgA responses.[26,27]

VLPs for Hepatitis E have been assembled using a truncated form of the virus capsid protein.[28] In immunization studies in mice these VLPs were able to induce systemic and mucosal immune responses following oral administration.[28,29] Furthermore, oral administration of the Hepatitis E VLPs to cynomologous monkeys induced IgM, IgA and IgG responses and was sufficient to protect against infection and disease on challenge with virus.[30] Thus there is clear potential for the application of these VLPs as a vaccine for hepatitis E.

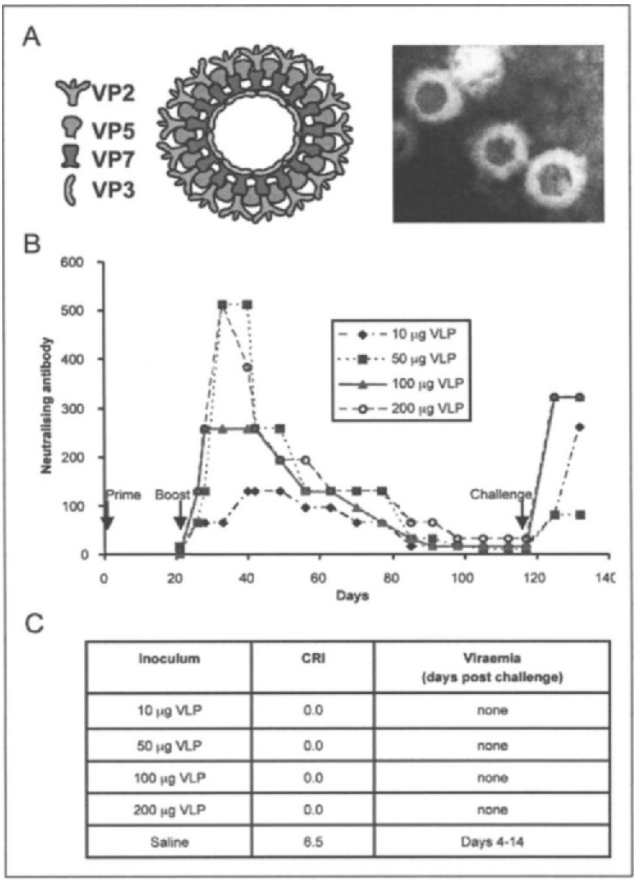
VLP preparations to Circoviruses and Polyoma virus are at a less advanced stage. VLP formation has been reported for Circovirus but as yet no serious attempt has been made at vaccine production. Vaccination of rabbits with VLPs for human JC virus in the presence of adjuvant allowed production of a hyperimmune serum that effectively neutralized infectious virus preparations.[31] However, in the absence of adjuvant there was no response. This pattern of response is unusual for VLPs in general, which often stimulate strong immune responses even in the absence of adjuvant. Indeed, VLPs of murine polyoma virus were able to stimulate a strong immune response in the absence of adjuvant when administered as a single 610 ng dose.[32] Intriguingly, these particles appear to be particularly stable with no alteration of particle morphology or reduction in immunogenicity even after 9 weeks storage at room temperature.[32]

VLPs of Structurally Complex Viral Capsids with Multiple Protein Layers

Viral particles that contain multiple interacting capsid proteins present more of a technical challenge than those that are formed by one or two major capsid proteins. Particularly, it is far more difficult if the assembling proteins of capsids are encoded by multiple discrete mRNAs, but not processed from a single polyprotein as in the case of picornaviruses. This is due the fact that for efficient assembly of a VLP the interacting capsid proteins must be expressed in the vicinity to each other, in other words in the same cell. Assembly of VLPs by processing of polyproteins have been achieved both for poliovirus[33] and for Infectious Bursal disease virus[34] using the baculovirus expression system. More complex assembly of multilayered, multiprotein VLPs have also been efficiently produced for the members of the *Reoviridae.* These viruses have capsids made up of concentric layers of different capsid proteins. Co-expression in insect cells of 2-4 of these capsid proteins, depending on the virus and the particle made, has allowed the production of VLP that are empty of the segmented dsRNA viral genome, but are otherwise indistinguishable from authentic viral particles.[4,35] The first member of the *Reoviridae* for which VLPs were described is Bluetongue virus (BTV), an insect transmitted animal virus. This remains the system in this family for which the largest variety of different VLPs and recombinant single antigen subunit immunogens made by baculovirus expression systems has been tested. In addition, the requirement for efficient co-expression of viral capsid protein in the same insect cell in this system has resulted in the development of baculovirus multigene expression vectors.[36,37] We will focus on this system in some detail as it highlights both the effectiveness of VLP vaccines and some of the technological advances that have been made for the production of VLP with complex architecture.

Bluetongue disease affects mainly sheep and cattle and is classified as an emerging disease in Europe.[38] The disease is caused by bluetongue virus, BTV, which has a multi-layered icosahedral structure formed by nonequimolar amounts of seven viral proteins (VP1-VP7). Three of these structural proteins (VP1, VP4, VP6) are dispensable for the formation of VLPs as they play only an enzymatic role in the virus transcription machinery.[38] The remaining four structural proteins (VP2, VP5, VP3 and VP7) are organised in two capsids. The inner capsid acts as a scaffold for the assembly of outer capsid that is responsible for cell entry and hence contains the major candidate for virus neutralisation.[38]

Expression of all four major structural proteins of BTV was achieved by construcing a baculovirus that simultaneously expressed all four proteins.[37] The advantage of this approach over co-infection with several baculoviruses each expressing a single protein is that equivalent conditions are achieved in all infected cells. Thus assembly of VLP is more efficient as expression



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Inoculum	CRI	Viraemia (days post challenge)
10 µg VLP	0.0	none
50 µg VLP	0.0	none
100 µg VLP	0.0	none
200 µg VLP	0.0	none
Saline	6.5	Days 4-14

Figure 2. Summary of production and testing of VLPs for Bluetongue virus. A) Left, cartoon showing the multi-layered structure of BTV VLPs. Right, electron micrograph of negatively stained BTV VLPs. B) Summary of neutralizing antibody response to VLP vaccination in Merino sheep. Sheep were vaccinated with two doses of VLPs with dose ranging from 10 µg to 200 µg as indicated. Neuralising antibody titre was followed for 117 days, at which point the sheep were challenged with virulent BTV. C) Table showing clinical reaction index (CRI) and length of Viraemia in sheep vaccinated with various doses of VLP and control. No signs of bluetongue disease or viraemia were detected in any of the VLP vaccinated animals.

is controlled at the level of the cell, rather than the level of the culture as is the case with mixed infections. BTV VLPs (Fig. 2) are structurally indistinguishable from virus particles but lack the segmented, double-stranded (ds) RNA virus genome normally present in infectious virus.[38]

Antibodies raised to purified BTV VLPs gave high levels of neutralizing antibodies against the homologous BTV serotype.[4] In subsequent clinical trials 1 year-old Merino sheep were vaccinated with various amounts (10-200 µg) of VLPs for BTV serotype 10. All vaccinated animals developed demonstrable neutralizing antibodies[39,40] and when challenged with virulent virus after four months of vaccination were completely protected from disease. In contrast, unvaccinated control animals developed typical BT clinical symptoms. Even at doses as low as 10 µg VLP was sufficient to protect animals from any signs of disease. Further efficacy tests were performed where VLPs from two different serotypes were combined to vaccinate the same animal. In these animals VLPs vaccination provided complete protection against the two vaccine serotypes and also partial

protection from challenge with related nonvaccine serotypes. The protective efficacy of vaccination in these trials extended over a long (14 month) period.[40] This observation raises the possibility that a broad spectrum vaccine against all 24 BTV serotypes is a possibility by combining VLPs from a relatively small number of serotypes.

The BTV system also demonstrates the efficiency of VLP vaccines relative to immunization with subunit vaccines based on dissociated antigens or unassembled recombinant antigens. In addition the assembled VLPs the two components of the BTV outer capsid, VP2 and VP5, were also prepared and tested in vaccination studies. While 100 μg VP2, the major serotype determining antigen, was only partially protective for a short duration (75 days) against virulent virus challenge, 50 μg of VP2 combined with 25 μg VP5 was protective.[41] In contrast, 10 μg VLPs (containing only 1-2 μg VP2) afforded a better level of protection for a much longer duration.[41] These studies demonstrate that assembly of antigens into VLPs results in a more effective immunogen than delivery of separately isolated proteins.

In addition to BTV, VLP have also been produced for rotavirus, another member of the *Reoviridae*. Intriguingly, VLPs formed from the two inner structural proteins alone of the rotavirus capsid have been shown to be effective immunogens in animal models.[42-48] Indeed in mice even intrarectal immunisation which induces a local mucosal response is sufficient for protection from rotavirus infection.[49] The data from these immunogenicity experiments are encouraging and it is possible rotavirus VLP may provide a viable alternative to the live virus vaccine for rotavirus.

VLPs from Viruses with Lipid Envelopes

Many pathogenic viruses such as Influenza, HIV and Hepatitis C are surrounded by an envelope, a membrane that consists of a lipid bilayer derived from the host cell, inserted with virus glycoprotein spikes. These proteins are the targets of neutralizing antibodies and are essential components of vaccine. Due to the inherent properties of lipid envelope, assembly of VLPs in insect cells for these viruses is a different type of technical challenge to those produced for viruses with multiple capsids. Nevertheless, efficient formation of VLPs of a number of enveloped viruses in insect cells has been reported. For example, VLPs of Hepatitis C virus, several retroviruses, SARS Coronavirus and influenza A have demonstrated correct assembly of the the lipid envelope with the glycoproteins inserted.[50-55] Indeed, for retroviruses, it has been possible to produce hybrid VLPs that contain the gag capsid protein from one virus (SIV) and the envelope protein from another (HIV)[56] in insect cells. Although none of the retrovirus derived VLPs are yet at the stage that they are being used in clinical vaccine trials, initial experiments in animal models are promising.[57,58]

VLPs for SARS Coronavirus as a basis for vaccination were produced rapidly following the SARS outbreak in 2002-2003.[54,55] However the control of SARS Coronavirus by epidemiological measures, continued lack of re-emergence of the virus, and difficulties working directly with the virus have severely limited the development of SARS VLPs as vaccine. Despite this, anti-serum raised in mice against insect cell derived SARS VLPs were able to neutralize a retrovirus pseudotyped with the SARS S protein (Fig. 3).

The Hepatitis C VLPs (Fig. 1) have been tested in mice and baboons and shown to be effective at stimulating both cellular and humoral immune responses.[7,53,59] In one experiment, 6-8 week old female BALB/c mice were immunized intramuscularly three times, at three week intervals with 20 μg insect cell derived HCV VLP, produced by co-expressing HCV coreE1-E2. Because of the lack of a suitable animal model for HCV infections a recombinant vaccinia virus expressing HCV structural proteins (vvHCV.S) was used as a model system. Vaccinated mice were challenged three days after the final immunization with vvHCV.S and then five days later the ovaries of infected mice were harvested and the vaccinia virus titre determined. Five out of seven vaccinated animals had no detectable vaccinia virus in the ovaries at this point. The remaining two animals had five logs lower vaccinia titres compared to control mice.[7] In addition, this study was able to demonstrate that the VLPs efficacy was based largely on its stimulation of $CD4^+$ and $CD8^+$ T-cell responses. A further study in baboons has demonstrated that the VLPs are well tolerated and can stimulate broad and long-lasting HCV targeted immune responses.[53]

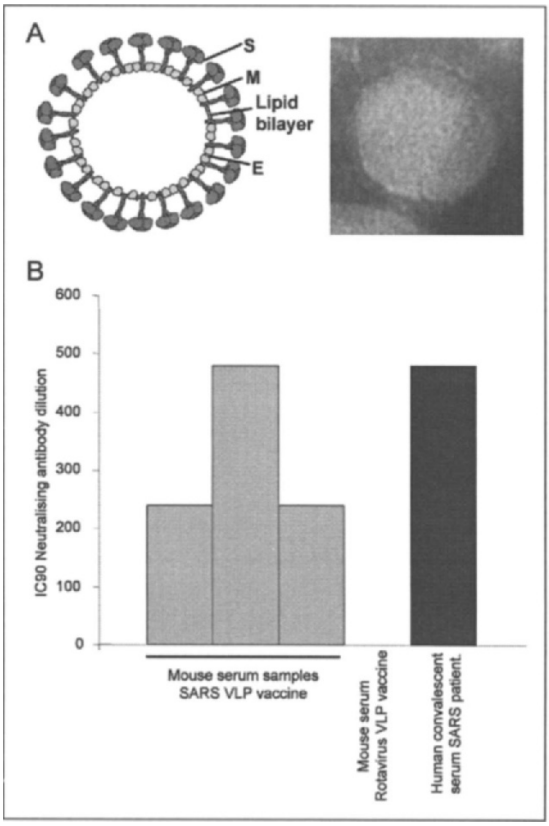

Figure 3. Summary of production and testing of VLPs to SARS coronavirus. A) Left, cartoon and right, electron micrograph of VLPs produced by co-expression of E, M and S proteins of SARS coronavirus. These VLPs were used to raise anti-sera in mice and the ability of these anti-sera to protect against infection with a SARS S protein pseudotyped lentivirus were assessed. B) IC90 neutralising antibody dilution for SARS S pseudotyped lentivirus, using sera from 3 mice immunized with SARS VLP, rotavirus VLP and serum obtained from a SARS convalescent patient.

To date, the most structurally complicated enveloped virus particle that has been used to generate VLP is influenza. VLPs for Influenza A H9N2 and H3N2 have been produced by other groups.[52] These studies have shown that expression of the major structural protein M1 alone is sufficient result in the budding of virus-like vesicles from insect cells.[52] Also, co-expression of M1 with M2, HA and NA leads to the assembly of influenza VLP and M1-HA and M1-HA-NA VLPs confer protection from lethal challenge with the same type influenza A in mice.[60,61] VLP production was also successfully achieved by co-expressing HA, NA, M1 and M2 from influenza virus A/Udorn/72 (H3N2) using a single recombinant baculovirus.[52] To date none of these influenza VLP have been tested in humans. However the potential that HA and NA could be incorporated directly into these VLP from circulating influenza strains without passage in tissue culture has particular advantage for the control of rapidly changing influenza A virus.

Future and Alternative Directions

In addition to the use of VLPs as direct immunogens, the efficiency with which they stimulate cellular and humoral responses has made them prime candidates as carrier molecules for the

delivery of epitopes, DNA and small molecules targeting other diseases. This has been facilitated by the excellent structural information that is often available for virus particles allowing rational design of vaccines where epitopes are exposed on the surface of the VLP. Many of the VLPs that have been developed as vaccines in their own right have also been tested as delivery systems for other molecules. It is not possible here to provide a full account of this approach, as the literature on delivery and display using VLPs is at least as large as that on VLP production for direct immunization (for review see ref. 62). However it is necessary at least to introduce this important area of VLP-based vaccine development. The use of VLPs as carrier molecules for epitopes for other diseases is not limited to those VLPs that are formed from the capsids of economically significant viruses. The reason that many VLPs make excellent carrier molecules for the delivery of epitopes in vaccines is most likely because the particulate VLP structure is readily taken up into antigen presenting cells and thus is able to prime long lasting CTL responses in addition to antibody responses.[6,63,64] Certainly accumulated evidence on VLP vaccines suggests that they are efficient at stimulating both cellular and humoral immune responses.[5-7,64-66] Notable work has been done in this area with both the hepatitis B core particles, human papillomavirus VLPs and parvovirus VLPs displaying T-cell specific epitopes from another protein on their capsid.[5,64-66,67] These studies demonstrate that like bacterial epitope display systems VLPs are efficient stimulators of MHC class I and class II responses.[63] Thus VLPs have great potential as epitope display systems for other diseases. The only major drawback for this approach is that the requirement of the capsid protein to assemble often constrains the size of the foreign sequence that can be tethered to the VLP. One approach that may be of use to overcome this constraint would be to link foreign protein sequences to capsid proteins in such a way that they extend the N or C termini of the protein and extend either inside or outside to particle.[68] Of course, this is only suitable where one or both termini of the protein are exposed on the inside or outside face of the capsid. So far, there are no VLP that we are aware of that have fully exploited the potential of this approach but it has been successfully employed for other protein-based particulate structures that are similar to VLPs in their stimulation of B-cell and T-cell responses and requirement for complex protein-protein interactions for particle assembly.[69-71]

Perspectives: Myths and Facts

Despite the accumulated evidence of the potential of VLPs as potent immunogens for many viral systems that we have discussed, there remains some resistance to the VLP approach as a general vaccination strategy for diseases caused by viruses. In part this is due to some high profile disappointing results for VLP vaccines in the early stages of development, for example an ineffective early vaccine for HIV based on Ty VLPs.[72] This example raises a point of caution for VLP vaccine designers. In general, VLPs stimulate efficient cellular and humoral immune responses but, as with any vaccine, they rely on the long term host response to be effective. VLPs designed to work in immunocompromised individuals need to overcome the same challenges to efficient immune response as any other vaccine approach. The notion that VLPs are ineffective vaccines is clearly a myth that is exploded by the imminent release of two new VLP-based HPV vaccines. Indeed, the accumulated data from the field suggests that VLPs are more effective than many other types of subunit vaccines, because they are more conformationally authentic and are safer than many live virus preparations because they are usually free of viral genetic material. VLP production does not appear to be limited to any one type of virus or virus family, nor is it significantly limited by the complexity of the virus particle.[8]

The use of insect cells as a protein expression system offers exciting opportunities for the synthesis of conformationally authentic VLPs that are formed from the intracellular assembly of multiple proteins expressed in the same cell. The advantage of this system over others used for protein expression is its capacity for industrial scale synthesis of large and multiple proteins and the fact that insect cells are the natural replication reservoir for many pathogenic viruses. Thus the basic cellular machinery that normally processes the infectious form of the virus is present within the expression system and available to produce authentic VLPs.

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Chapter 12

Applications of Bacterial Ghosts in Biomedicine

Petra Lubitz, Ulrike Beate Mayr and Werner Lubitz*

Abstract

Bacterial Ghosts (BG) are empty cell envelopes of Gram-negative bacteria which have been produced by E-mediated lysis. BG are devoid of cytoplasmic content and in combination with the expression of the nuclease SNUC, BG are also devoid of chromosomal and plasmid DNA. Proof of concept and proof of principle studies showed that BG candidate vaccines are highly immunogenic and in many instances induce protective immunity against lethal challenge in animal models. Due to their nature of being bacterial envelope complexes, BG are endowed with intrinsic natural adjuvant activity. BG are able to stimulate the innate and adaptive immune system without any addition of exogenous adjuvants. Although the use of plasmid encoded genetic information is essential for the final make up of BG, BG are not to be considered as genetically manipulated organisms (GMO), as they are nonliving and devoid of genetic information. The latter aspect is of great importance for safety, as no pathogenic islands or antibiotic resistance cassettes can be transferred to other bacteria by horizontal gene transfer. This is an important difference to other chemical-, heat- and pressure- or radiation-inactivated vaccine candidates, which also very often need artificial adjuvants to be added to improve their immunogenicity. The final BG vaccine preparations are freeze dried and are stable for many years at ambient temperature. BG can also be used as carrier and delivery vehicles for drugs or active substances in tumor therapy and due to specific targeting of tumor cells allow a higher specificity of treatment and a reduction of the total amount of drug per application. As carrier of enzymatic activity BG can be used for a new concept of probiotics which can synthesise active compounds from substrates of the environment where they are applied with a certain preference for the gut system. Thus, BG represent a promising technology platform for novel vaccines including combination or DNA vaccines, as drug carriers for therapeutic approaches in tumor treatment and as novel probiotics.

Introduction

The concept of Bacterial Ghosts (BG) has been emerged from basic science studies addressing both the lysis mechanism of bacteriophage PhiX174 after infection of *Escherichia coli* and, the specific mode of action of the cloned lysis gene E of the phage. These investigations resulted in the comprehensive evidence that protein E is able to fuse the inner and outer membranes of Gram-negative bacteria, thereby forming a transmembrane lysis tunnel in the bacterial envelope through which the cytoplasmic content is released. High resolution field emission scanning electron micrographs (FESEM) and transmission electron micrographs (TEM) of the first *E. coli* BG produced by expression of the cloned gene E unambiguously showed the intact structure of the

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bacterial envelope, the fusion (continuity) of the inner and outer membranes at the border of the E-specific transmembrane tunnel and the release of the cytoplasmic content through this hole.[1]

In contrast to other phage lysis systems which lead to a total destruction of the bacterial envelope due to enzymatic degradation of peptidoglycan the latter polymer is not degraded by E-lysis leaving an empty shell of the bacterium with a hole in it which has been called in analogy to the erythrocyte ghost bacterial ghost (BG). For clarity, it should be mentioned that the E-lysis system is restricted to Gram-negative bacteria as only this group of eubacteria has an inner and an outer membrane. In contrast, the large group of Gram-positive bacteria is killed but not lysed by gene E expression.

For medical application such as vaccines, BG of various pathogens and BG as carriers of foreign antigens vaccine candidates have been developed, with a recent focus on vaccines for children against enteric diseases. Among most physicians and health officials there is no doubt that functional vaccines are the most effective medical interventions to safe lives and reduce costs in healthcare. Novel vaccines need to meet the following requirements: (i) to be safe and immunogenic in young children, the adult and the elderly, (ii) include multiple serotypes/species, (iii) be inexpensive, easy to produce, stable without refrigeration and amenable for needle-free administration and, last but not least, (iv) should confirm robust immunity with three or less doses.

As most of the traditionally used vaccines do not meet all listed criteria, there is room for improvement and substitution. In this context, BG are an excellent alternative to vaccines which use chemicals, heat or irradiation to inactivate the pathogen, since all these methods denature essential structural components of the bacteria. As the E-lysis process for BG production is a genetic/biochemical method to open a bacterium from the inside it does not denature any cell component, thereby leading to a superior preservation of their antigenic properties.

Although in the past different routes of immunizations and different animal models have been used for BG vaccine candidates the main focus of our future developmental work for BG vaccines is the mucosal application of BG vaccines in veterinary and human medicine. Other applications in medicine of empty bacterial envelopes are the use of BG as carriers for drugs or other active substances, including DNA or enzymes. The applications of BG are numerous and for this chapter the specific focus is set to their application in medicine as vaccines and drug carriers with a short appendage on advanced applications, such as their use as enzyme reactors for novel probiotics.

Basic Structure of Bacterial Ghosts

On average, the diameter of the E-specific transmembrane tunnel varied between 40 and 80 nm.[1] In figure 1 the E-lysis hole can be seen in a FESEM of BG of *Mannheimia haemolytica*, in comparison to a normal bacterial cell. The variation in size and irregular tunnel structures indicated that the E-specific transmembrane tunnel structure is not a rigid fixed structure. It is dynamically formed by the strong force ejecting the cytoplasmic content through the E-lysis hole due to the osmotic pressure difference between the total solutes of the cytoplasma and the outside growth medium which under normal bacterial growth conditions is more than 1 bar. Due to the integration of protein E in the inner membrane the paracrystaline peptidoglycan net located in the periplasmic space between the inner and outer membrane of the cell envelope structure of Gram-negative bacteria exhibit a higher turn-over rate at potential sites of lysis tunnel formation. As a consequence, the borders of the E-lysis tunnel are determined by the local mash size of the peptidoglycan which is the shape determining rigid structure of the bacteria. BG from rod shaped bacteria such as *E. coli* and other stay rod shaped (Fig. 1), whereas the coma shape of *Vibrio cholera* can also be depicted in the corresponding BG.[2]

Depending of the bacterial species from which BG are derived they exhibit an almost uniform size distribution of 0.5-2 μm length. Their outer surface with all its appendixes, like pili, flagella and lipopolysaccharide, is equivalent to their mother bacteria and their inner surface corresponds to the inside of the cytoplasmic membrane and its associated products which are not released by E-mediated lysis. The space between both membranes is the periplasmic space which by its nature is a gel like environment rich in membrane derived oligosaccharides, specific enzymes, proteins

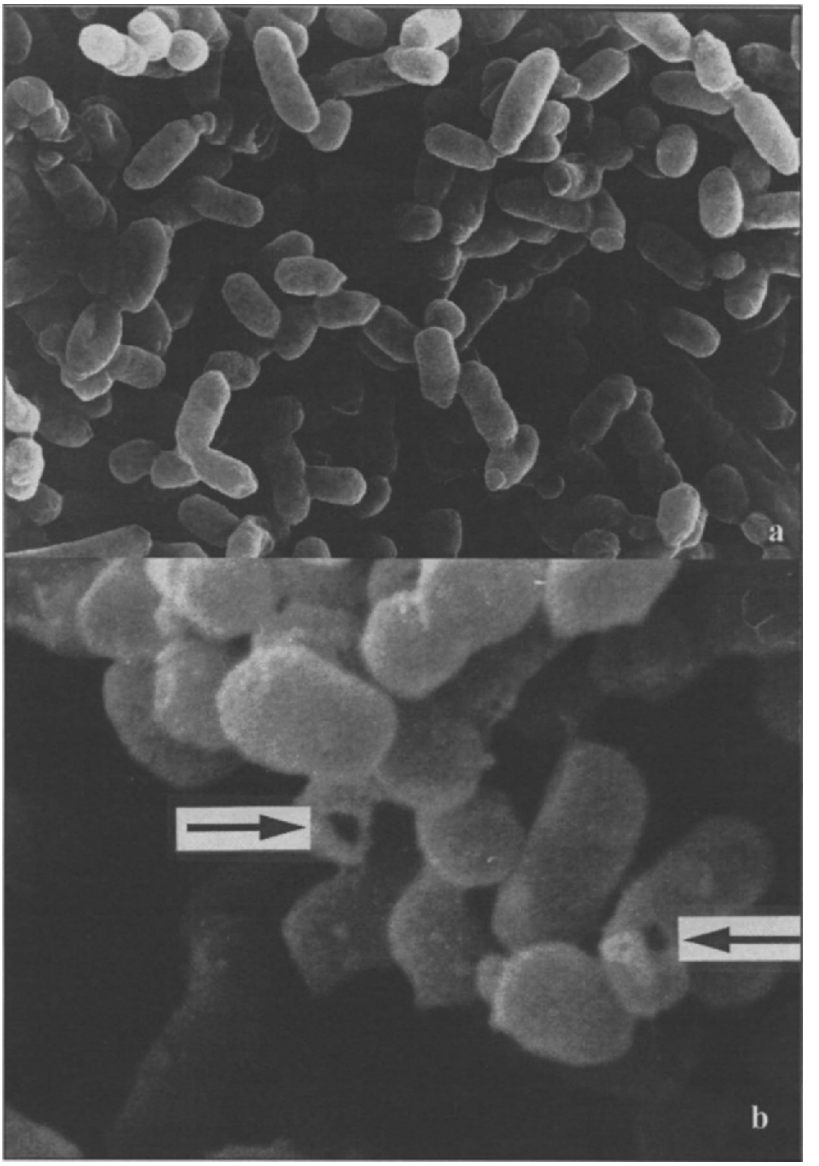

Figure 1. Scanning electron micrographs of (a) viable *Mannheimia hemolytica* bacteria and (b) *M. hemolytica* BG, the arrows indicate E-mediated lysis hole in the cell envelope.

and peptidoglycan. The TEM (Fig. 2) and schematic line drawing of BG envelope (Fig. 3A) does not give details of the complex architecture with numerous single elements and structural units composing the bacterial cell envelope from the inside of the cytoplasmic membrane to the very outermost structure beyond the outer membrane but simplifies the understanding of an empty rod shaped double walled microparticle with a hole.

BG are much more complex and more sophisticated designed by nature for adhesion and eventually uptake by cells or tissues than any engineered liposome, which can be artificially produced. As will

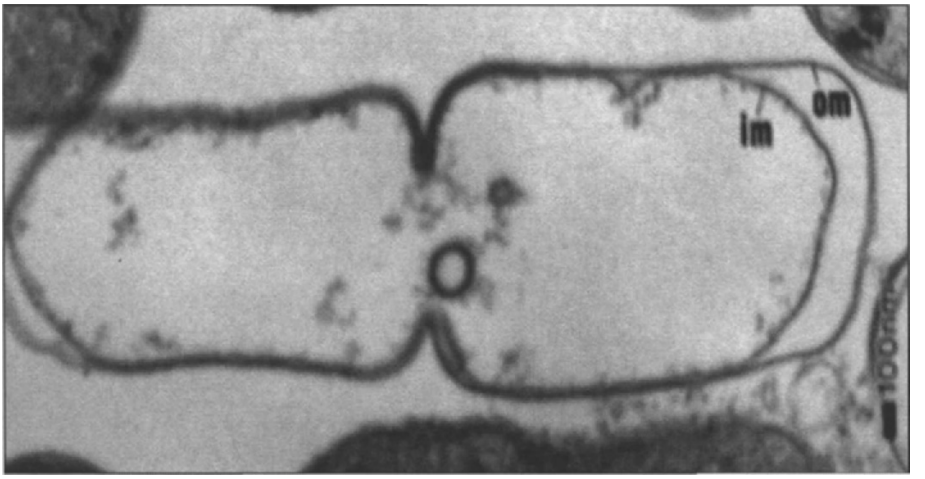

Figure 2. Transmission electron micrograph of an *E. coli* BG, im, inner membrane; om, outer membrane. On the left edge and below the BG parts of full viable bacteria are visible with contrasted cytoplasmic content in contrast to the empty inner cytoplasmic lumen of the BG.

be shown in the following section, although being complex in nature BG provide ample space for modifications and shelter for different additions. BG have been produced from different *E. coli* K12 strains, enterotoxigenic *E. coli* (ETEC) and enterohemorrhagic *E. coli* (EHEC), *Salmonella enterica* serovar *Typhimurium, S. enteritidis, Shigella flexneri, Vibrio cholerae* O1 and O139, *Helicobacter pylori, Neisseria meningitidis, Bordetella bronchiseptica, Actinobacillus pleuropneumoniae, Pasteurella multocida, M. haemolytica and Francisella tularensis* LVS and *Pectobacterium cypripedii.*

BG production requires the transformation of the host bacterium with a plasmid which carries the gene E under an inducible promoter. Since the gene E product is highly lethal for the recipient the transforming plasmiod either brings the corresponding repressor system or use bacteria where the repressor is already preloaded either by chromosomal expression or coded on a plasmid. Thus, the proper establishment of the genetic repression/expression system in a given Gram-negative bacterium determine the success of BG production. A series of plasmids have been developed which carry the gene E under an inducible expression control. The most elaborate systems are derived from the phage Lambda left or right promoter operator system with expression control by the thermosenitive cI857 repressor or derivatives providing the growth of bacteria up to 28, 36 and 39°C and E-mediated lysis at any temperature 2°C above the maximal repression temperature up to 42-44°C for enterobacteriaceae and most other bacteria.[3] Using this system for the control of a second promoter/repressor for gene E expression results in a reversed temperature profile with repression of gene E at high temperature and its expression by temperature downshift.[4] In addition to temperature regulated gene E expression, a chemical induction system such as arabinose, toluolic acid and lactose have also been used.

Bacterial Ghosts as Vaccines

BG production is based on the release of cytoplasm from bacteria by puncturing a hole from the inside to the outside which leaves intact all structural components of the envelope complex of the bacteria used. Killing of bacteria by the break down of the membrane potential occurs slightly before lysis with release of the cytoplasma including chromosomal and plasmid DNA.[5] The loss of nucleic acids minimizes the risk of horizontal gene transfer of pathogenic islands or antibiotic resistance genes by BG. To further diminish the risk of horizontal gene transfer in some of our BG preparations expression and activation of the cloned Staphylococcus aureus nuclease (SNUC)

which degrades DNA and RNA is used.[6] The remaining DNA level of such BG preparations is below the real-time-PCR detection level and sets a new quality criterion of inactivated vaccines.

What are BG vaccines? In the simplest form BG vaccines consist of a freeze dried powder of BG particles without any additions of stabilizers or adjuvant. Such BG preparations are stable at room temperature for many years (oldest samples are now 10 years) and can be used after resuspension in water or buffer for immunization procedures. By their own nature BG have intrinsic adjuvant properties and their particle character makes them attractive for key immune cells, such as dendritic cells, macrophages or monocytes which they address by recognition via toll-like receptors or opsonized antibody facilitated uptake.[7]

Where do we stand with BG as vaccine candidates? BG have been produced from many different bacteria including animal and human pathogens, nonpathogenic *E. coli* and a plant bacterium. Proof of principle for BG production by 20 l fermentation has been established for most of the pathogenic strains and proof of concept for BG vaccines is only missing for *F. tularensis*.

Mucosal vaccine application can be oral, intra-nasal, intra-ocular, intra-vaginal and rectal or aecrogenic. It should be emphasized that aerosol immunization of pigs with *A. pleuropneumoniae* BG induced sterile immunity against bacterial challenge.[8] Pigs are a good model for human diseases and these studies encourage using BG aerosols also in humans either as vaccine or drug carrier. The oral immunization of rabbits with *V. cholerae* BG induced protective immunity determined with the RITARD test and conferred cross protection between classical O1 and the recently emerging O139 strain.[9] Other encouraging highlights are the 100% protection levels against lethal challenge with EHEC after two oral[10] or a single rectal immunization of mice with EHEC BG.[11]

Where to start with a new BG candidate? Minimal amounts of protein E are required to lyse a bacterium.[12] The challenge is to establish the E-lysis system in a given bacterium and to assure the complete repression of gene E. Induction of gene E is not enough to achieve proper E-mediated lysis. There are requirements of the host bacterium which play an essential role for the E-lysis process such as active growth and functional control elements of cell division and of autolytic activity of the bacteria. A reasonable BG production rate of a growing culture is approximately 99.9—99.99% and depends largely on exponential growth of the bacterial culture. Only growing bacteria can be lysed, bacteria entering stationary phase (or mini cells which are not able to divide) are phenotypically resistant to lysis.[13] The molecular trigger events for E-mediated lysis are not completely elucidated and fully understood. Membrane adhesion sites, FtsZ protein in the septosome,[14] cis-trans proline isomerases for conformational change of protein E,[15] chaperones, the strength of the membrane potential,[16] the activity of the autolytic system,[17] the ph and osmotic strength of the medium[18] and other factors influence the E-lysis process. Although it seems to be trivial to E-lyse bacteria a good and efficient E-lysis needs experience and stringent process control.

Bacterial Ghosts as Carrier of Subunit Vaccine

Genetic engineering of the host bacteria which are candidates for BG production can be used to modify their cell envelope to carry foreign protein. Several systems have been developed and adapted to anchor or entrap a foreign protein in the bacterial envelop which after E-mediated lysis remain as constituents of the BG.[19]

Anchoring the foreign protein to the inside of cytoplasmic membrane by fusing it to a N-, C- or N- and C-terminal membrane anchor (Fig. 3B) have been used for E. coli K12 envelopes as carrier of HIV-, ntHi- and HBV-antigens.

Fusion of the target antigens with the maltose binding protein (Fig. 3C) was the method of choice to export zona pellucida protein constructs of ZP2 and ZP3 to the periplasmic space. For the insertion of target antigens on the surface of the outer membrane OmpA-fusion can be used. Also it should be mentioned here that foreign or homologous pili can be inserted in the envelope which can either act as subunit vaccine or to broaden the antigenic repertoire of the host bacterium, e.g., TCP of *V. cholerae*.[20]

The S-layer protein matrices formed by SbsA or SbsB can be modified to carry foreign inserts.[21] As both proteins form sheet like self-assembly structures they are not expelled with the cytoplasma

and remain in the inner cytoplasmic lumen after E-mediated lysis (Fig. 3D). When SbsA or SbsB fusions are exported as maltose-binding protein fusion to the periplasmic space they still retain their self assembly capacity and fill this space with sheets carrying target antigens (Fig. 3E).

A systematic study addressing which of the different possibilities to insert a foreign antigen in the Gram-negative cell envelope is the best has not been performed yet. Therefore it is difficult to decide which antigen presentation is more preferable over the other. There are other practical aspects which also have to be considered. As some of the proteins derived from viral, protozoan or other species or some of the artificial constructs which have been designed to combine different single epitopes can be lethal for the recipient bacterium it is important to preselect the presentation of the construct within the envelope. For instance, it can very well be that the lethal effect of one orientation of membrane anchoring in the cytoplasmic membrane, e.g., N-terminal fusion, can be overcome by the alternative C- and/or N- andC-terminal fusion as it had been the case for HIV reverse transcriptase.[22]

The S-layer protein SbsA seem to have a higher capacity to accept foreign inserts than SbsB and it can be speculated that the p6 self-assembly lattice of SbsA is more robust to tolerate larger inserts than the p2 lattice of SbsB.[23] The periplasmic export of maltose binding protein fusions with target antigens has been used in many cases and it can be stated that most but not all fusion partners were well tolerated. One additional advantage of the maltose binding protein (MalE) domain is that it can be used for quantification of the expression level of the antigen target via the quantitative determination of the MalE part of the construct with MalE-specific antibodies and purified MalE proteins both being commercially available. Quantification of the target antigen is very often not trivial as it needs either the purified antigen and antigen-specific antibodies or purified synthetic peptides which can be used for quantification of tryptic or other digests of the target antigen by MALDI-TOF.[24] This method has also its limitations as not all peptides have the ability to be detected with this method.

If the effort can be justified all different possibilities for envelope insertion can be tested and the one with the best expression is then used for the final BG product. In more general terms it can be stated that the strength of the immune response against a target antigen is correlated to the amount of target antigen presented in the BG carrier. The other message is that multiple antigenic epitopes (proteins) from a specific pathogen inserted in a BG carrier induce a broader and more

Figure 3, viewed on following page. Schematic line drawings of bacterial ghosts and their potential applications. A) Empty BG, inner line corresponds to the inner membrane and outer line to the outer membrane, the space between both lines corresponds to the periplasmic space. B) BG with inner membrane anchored antigens ( ), N-and C- terminal inner membrane anchored foreign protein; N-terminal inner membrane anchored foreign protein;( ), C-terminal inner membrane anchored foreign protein; star, ellipse and heart symbolize different foreign proteins. C) BG as carrier of foreign antigens in the periplasmic space: ( ) periplasmic protein, e.g., MalE protein; ( ), ( ), ( ), different periplasmic fusion proteins. D) BG as carrier of S-layer immobilized foreign antigens in the cytoplasmic lumen: ( ), SbsA, ( ), as carrier of foreign proteins; (Δ), SbsB, ( ), as carrier of foreign proteins. E) BG as carrier of S-layer immobilized foreign antigens in the periplasmic space ( ), SbsA, ( ), as carrier of foreign proteins; (Δ), SbsB, ( ), as carrier of foreign proteins. F) BG as carrier of nucleic acids: ( ),linear double stranded DNA; ( ), circular covalent closed DNA; ( ),inner membrane anchored DNA binding protein, e.g., LacI; ( ), plasmid or minicircle DNA with operator site for membrane bound DNA binding protein. G) BG as carrier of membrane immobilized polymers: ( ), inner membrane anchored streptavidin; ( ), ( ), biotinylated polymere, blue and green line represent different polymers, e.g., dextran and polyhydroxybutyric acid, ( ), drug attached to polymer. H) BG as carrier of water-soluble active substances: star, heart and ellipse are different water soluble compounds. I) BG as carrier of inner membrane attached active substances: ( ), drug, e.g., doxorubicin. J) BG closed with vesicle attached by specific streptavidin—biotin interaction at E—specific transmembrane lysis tunnel: ( ), protein E with in vivo C-terminal biotinylation site; ( ), inner membrane anchored streptavidin on the outside of inside-out membrane vesicles from Gram-negative bacteria.

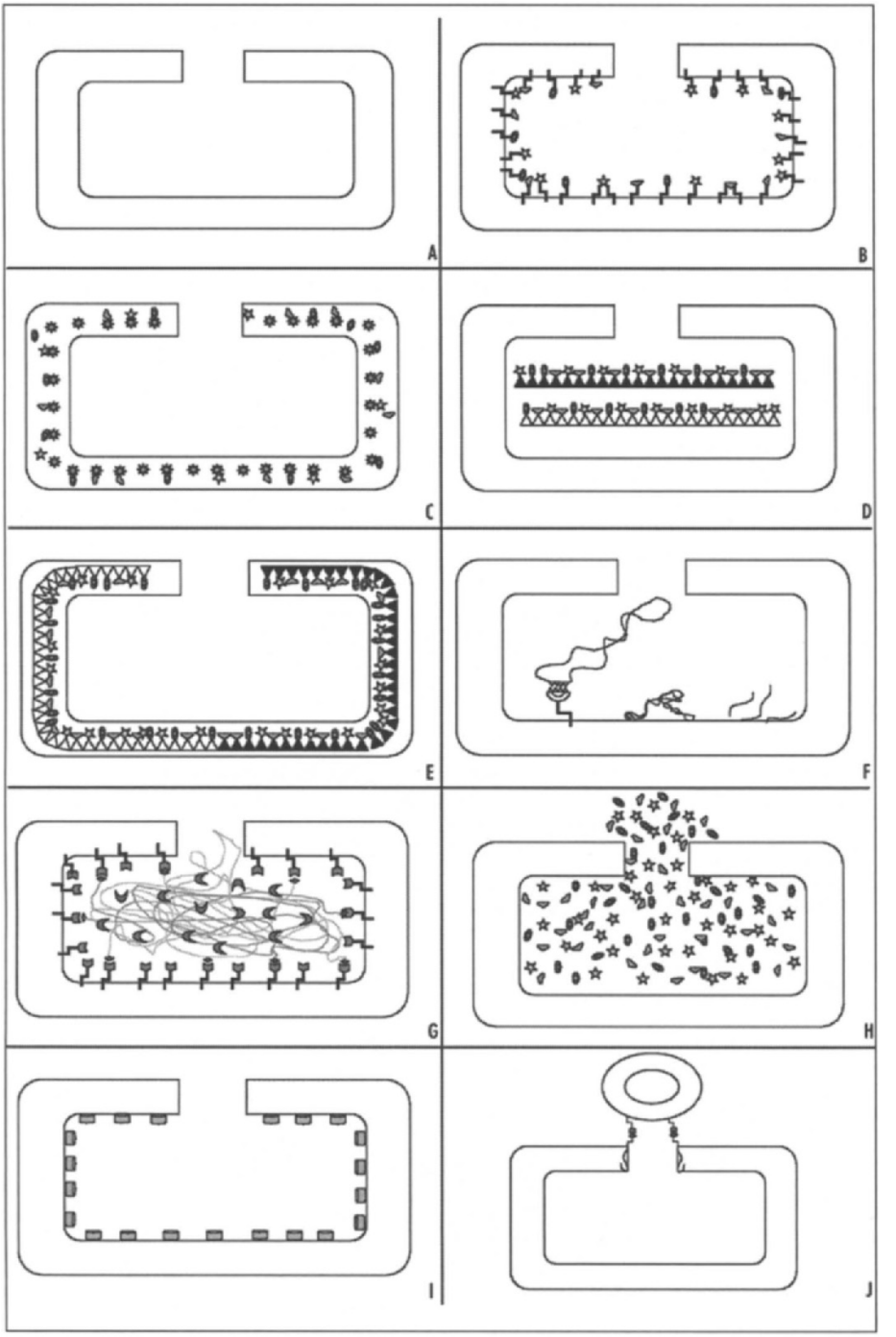

Figure 3. Please see legend on previous page.

robust immune response than a single target protein as it has been seen expressing *Chlamydia trachomatis* outer membrane antigens in *V. cholerae* BG.[25]

Oral vaccination needs roughly 10-times higher doses and intra-nasal or intra-ocular roughly the double dose of a BG vaccine than intra-muscular injections. The immunization regime for mucosal application of BG vaccine candidates has by convention been a primary immunization followed by two boosters. In this context it is of high importance to mention that BG vaccines given orally or by other mucosal routes do not need the addition of adjuvants like heat labile *E. coli* toxin or cholera toxin derivatives to be immunogenic. A recent review about BG as mucosal adjuvants provides detailed information.[7]

Immunization studies with EHEC BG in mice have showed that a single oral dose of EHEC BG was sufficient to protect 75% of the mice against lethal challenge 55 days after the primary immunization with a heterologous EHEC strain and with a single booster 100% protection could be achieved.[10] A single immunization of EHEC BG by rectal route of application achieved already 100% protection against lethal challenge at day 55.[11]

A single oral immunization which provides such robust immunity seems to be rather exceptional and therefore several aspects attributed to the BG should be discussed briefly. EHEC BG envelopes were used as vaccine and thus, the full repertoire of relevant antigens for adhesion was present in the EHEC BG preparation. As adhesion and colonization are the first steps in the pathogenicity of enteric bacterial pathogens it is highly advised to use if possible the whole envelope from such a pathogen. The more efficient rectal versus oral immunization with EHEC BG can be interpreted that the local induced immunity at the site of colonization which is for EHEC the rectal part of the colon could have been of specific importance. These findings correlate well with studies using BG as carrier of recombinant proteins of *C. trachomatis*, were three outer membrane antigens exposed in the same BG carrier gave a better protection rate than a single outer membrane protein. The findings with EHEC BG are encouraging since rectal administration of BG-based formulations is an approach which could be of particular advantage for vaccines aimed at newborns, toddlers and infants.

When we compared the efficiency of the immune response induced of BG envelope inserted antigens with purified target antigens formulated with complete Freund's adjuvant for ZP2/ZP3 constructs it was found that the antigen load of BG which caused the same immunological immunocontraceptive effect was roughly one-third of the dose needed with the purified protein antigen formulation.[11]

Plain BG vaccines as well as BG as carrier of target antigens always induce a humoral and cellular immune response against the target antigen.

Bacterial Ghosts as Carrier of DNA

BG are characterized as empty bacterial envelopes which have lost their cytoplasmic content by E-mediated lysis. It has been observed that DNA either in linear or circular covalent closed form can be filled back to BG (Fig. 3F). The method for filling BG with DNA is rather simple and in its standard version freeze dried BG are resuspended in a DNA solution and after washing off the excess of DNA which is not bound to the inside of the cytoplasmic membrane, the DNA-BG can either be used immediately for gene transfer experiments or can be stored after freeze drying for later applications. Loading and plasmid density within the BG depends on the concentration of the DNA solution used and as can be expected a concentration series can be produced with a given plasmid solution by dilution steps which is then reflected in the amount of DNA loaded per BG. Loading of BG with DNA is very efficient as more than 3,000 copies of a medium sized plasmid can be bound per BG.[27]

DNA transfer of DNA loaded BG to human monocytes derived dentic cells (DC) or mouse macrophages is very efficient with rates of GFP marker gene expression of 75% and 55% respectively.[27,28] It should be mentioned that in contrast to other DNA transfer systems the BG-DNA transfer agent is not harmful to the recipient cells and in the case of immature DC their maturation is efficiently induced as a result of the immune modulatory properties of BG.[28] Therefore, it

is not astonishing that good immune responses were observed when compared to naked DNA in mice DNA vaccination experiments. It was also found that the Th2 (humoral) immune response against the DNA encoded antigen was stronger than the Th1 (cellular) response, which is rather unusual for DNA vaccines and can most likely be attributed to the delivery and BG induced signalling.[29] A more sophisticated version of loading BG with DNA uses the specific interaction of an inner membrane anchored DNA binding protein with the corresponding operator region on target plasmiols (Fig. 3F). This allows a one step DNA loading and BG formation process. In our investigations the self-immobilizing plasmid carries the lac operon which is recognized by the inner membrane bound lac repressor molecule.[30]

For the application of DNA in somatic gene transfer or as DNA vaccine it is desirable to use DNA constructs devoid of antibiotic resistance cassettes or origin of replication and to design the DNA to its minimal size (minicircle) including an eukaryotic promoter the gene of interest with poly A tail and transcription stop sequences. The improved version of our self-immobilisation plasmids encodes the par A resolvase which is a specific DNA recombination enzyme recognizing homologous sequences on the plasmid. After expression of the par A resolvase and the inner membrane anchored lac repressor only minicircle DNA is bound to the inner membrane. By E-mediated lysis BG are produced in a one step production process loaded with minicircle DNA.[31]

BG advantages for use as vaccines are summarized in the following Table 1.

Bacterial Ghosts as Carrier Vehicles for Active Substances in Tumour Therapy

DNA as well as drugs can be used as active substances for tumour treatment and investigations have shown that human tumour cells can be targeted with BG for delivery of DNA or drugs. In a recent study, eight different human melanoma cell lines have been investigated for their capacity to bind and phagocytise BG. Melanoma cells have many functions in common with APC, including their phagocyte activity.[32,33] Bowes cells exhibited roughly 80% expression level of BG delivered marker gene coding for green fluorescent protein (GFP) which was higher than the expression levels obtained with the same amount of DNA and Effectene, as transfection reagent commonly

Table 1. Main properties of BG-based vaccines

Safety profile	Nonliving vaccines which pose no pathogenic threat
	No hazard of horizontal gene transfer
	Do not belong to genetic manipulated organisms (GMOs)
	Do not require addition of adjuvants
Production features	Based on fermentation
	Rapid process
	Low associated costs
Stability	Retain natural surface components
	Recombinant antigens are preserved
	Stable as lyophillized powder
	Cold chain independent
Administration and immunogenicity	Can be administered via various routes
	Amenable for mucosal administration as needle free vaccines
	Natural adjuvants
	Efficient interaction with the host immune system
Versatility	Can be generated from a variety of Gram-negative bacteria
	BG subunit vaccines include protein and/or DNA antigens
	Offer a one step production process for subunit vaccines
	Flexible vaccine platform for incorporation of new antigens

used for DNA transfer.[34] This high ratio of marker gene expression after BG-DNA transfer makes the BG system suitable as vehicle for the transfer of siRNA into tumour and other cells to influence gene expression with therapeutic effects for the host.

The chemotherapeutic drug doxorubicin (DOX) has been also bound to the cytoplasmic compartment of BG (Fig. 3G). Treatment of the human colon cancer cell line CaCo2 with the drug loaded BG showed an effect on proliferation inhibition by two log difference compared to the free drug.[35] As in cancer patients DOX has accumulating side effects on heart functions the tumour therapy often has to be terminated once a critical maximal dose is reached. Thus, by lowering the actual dosis by using DOX loaded BG, the time window for DOX therapy can be considerably extended and can also contribute to an increased quality of life for the patients as side reactions to the drug are also less severe. The results with DOX-loaded BG are encouraging for the concept to specifically target tumour tissue in combination with surgery for colon cancer, head and neck cancer, brain tumours or other, as certain BG have a surface make-up that allow them to bind to and being taken up by the tumour cells. As the binding reaction of BG to the tumour tissue is a fast process only short term rinsing or flushing could be sufficient to deliver the therapeutic cargo.

It is assumed that most of large polycyclic drugs are bound to BG by hydrophobic interactions of organic ring structures with the lipid milieu provided by the membranes of BG (Fig. 3I). Recent investigations of binding polyphenolic compounds to BG confirm this hypothesis and add a group of new compounds to the list of active therapeutic substances for intracellar delivery. On the other hand, polymers such as dextran can also be loaded into BG.[36] Thus, it would be possible to decorate the polymer with drugs (Fig. 3G). The polymer can be retained inside the lumen of BG using the specific interaction of membrane-anchored streptavidin with the biotinylated polymer.

Other Medical Applications for Bacterial Ghost Packaged Active Substances

Supposed the active substance(s) to be carried by BG is highly water-soluble then the compound(s) will leak out the E-lysis tunnel driven by an osmotic gradient if the concentration of solutes in the outside milieu of the targeting region is considerable lower than the solution inside the loaded BG (Fig. 3H). For substances carried in BG in concentration equilibrium with the outside, a rather slow release of solutes out of BG by diffusion is given by the small 40-80 nm orifice of the E-transmembrane tunnel. If binding of BG to a specific surface, e.g., skin is a given property of the BG envelope then the water resistance of substances packaged in the inner lumen of BG is very high. This concept has been tested on plant leaves demonstrating that a high rain resistance of the fungicide carried in the BG envelope could be achieved by this delivery with similar effects on pathogen reduction.[37] The experiments carried out with different plant skins can be translated for human or animal skin applications and open a way for prolonged persistence of substances in the shelter of BG.

Bacterial Ghosts as Enzyme Reactors for Novel Probiotics

The membrane anchoring of proteins, the export of proteins as fusion constructs to the periplasmic space, the presentation as outer membrane fusions as well as integral part of S-layer lattices has been described above for the presentation of foreign antigens in a BG carrier. In the application of BG as enzyme reactors the proteins of interest have enzymatic activities and the whole construct is designed for carrying out metabolic activities. Early examples for such a concept have been described for membrane anchored β-Galactosidase,[38] biotinylated alkaline phosphatase bound to membrane-anchored streptavidin[36] and polyhydroxybutyric acid synthetase fusions with the S-layer protein SbsA.[39] These proof of concept studies can now be extended for the construction of novel BG with metabolic activities causing probiotic effects or more general for health promoting effects.

BG as miniature enzyme reactor can be designed to produce either by metabolic or anabolic enzymatic activities substances from food either as additions to the food itself or as additions during the digestion of food. BG as carrier of β-galactosidase would be able to cleave the milk sugar lactose

in glucose and galactose to avoid lactose intolerance. Another health beneficial effect could come from BG as carrier of polyamine hydrolase to avoid unpleasant allergic or sickness effects from food, or from BG as carrier of alcohol dehydrognease to reduce the adsorption of ethanol after drinking. There is a whole package of other metabolic enzymes that could support digestion or make novel substrates available as nutritive source. Small biosynthetic pathways can also be presented by BG, e.g., for the synthesis inhibitors of pathogens such as reuterin or other substances.

The more speculative aspect of this research field could lead to the design of novel organelles. Some of the requirements for this endeavour have already been solved whereas others are under current investigation. The lysis hole in the BG can be sealed with inside-out vesicles of French press disrupted bacteria (Fig. 3J)[40] and methods to enrich the BG with enzymatic activities have been described above. Restoration of a membrane potential and of transcription/translation machinery are only a few among the challenging open questions on how to revitalize a BG.

Conclusion

A tremendous advantage of BG as vaccines or carriers of active substances is that BG can be produced by fermentation either in a conventional steel fermenter or in disposable devices. Therefore, in a rather short period of time BG production can be scaled-up from small to large batches. Thus, it would be possible to establish local BG production units at low cost with relatively simple infrastructure in developing countries. This would in turn facilitate the establishment of local mass vaccination programs to fight children diseases caused by infectious agents. On the other hand, BG as carriers of active substances and drugs should find their application in tumour therapy. Finally, when custom—engineered for specific enzymatic activities they represent health promoting vehicles which can be used in a concept similar to that of probiotics. Thus, BG represent a promising, cost-efficient and versatile multipurpose technology platform for application in both industrialized and developing countries.

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CHAPTER 13

Immune Modulators with Defined Molecular Targets:
Cornerstone to Optimize Rational Vaccine Design

Thomas Ebensen and Carlos A. Guzmán*

Abstract

Vaccination remains the most valuable tool for preventing infectious diseases. However, the performance of many existing vaccines should be improved and there are diseases for which vaccines are still not available. The use of well-defined antigens for the generation of subunit vaccines has led to products with an improved safety profile. However, purified antigens are usually poorly immunogenic, making essential the use of adjuvants. Despite the fact that adjuvants have been used to increase the immunogenicity of vaccines for more than 70 years, only a handful has been licensed for human use (e.g., aluminium salts, the micro-fluidized squalene-in-water emulsion MF59 and monophosphoryl lipid A). Thus, the development of new adjuvants which are able to promote broad and sustained immune responses at systemic and mucosal levels still remains as a major challenge in vaccinology. Recent advances in our understanding of the immune system have facilitated the identification of new biological targets for screening programs aimed at the discovery of novel immune stimulators. This resulted in the identification of new candidate adjuvants, which made possible the modulation of the immune responses elicited according to specific needs. A number of promising adjuvants which are currently under preclinical or clinical development will be described in this chapter.

Introduction

A key requirement for the immune system is the discrimination between self and nonself, particularly in the context of microbial agents able to cause disease. The recognition of pathogenic micro-organisms is in part performed by the presence of pattern recognition receptors (PRR), such as the Toll-like receptors (TLR), on cells from the innate immune system. This system has also evolved other means to identify potentially dangerous entities, such as the CD1d-mediated recognition of ceramides; the complement system; specialized receptors enabling natural killer cells to sense "nonself", "missing-self" and "induced-self"; and the Nod proteins by which unique microbial motifs are detected (e.g., peptidoglycan), thereby initiating pro-inflammatory signaling cascades.[1,2-5] On the other hand, the recognition of dangerous entities by the innate immune system is a prerequisite for the stimulation of pathogen-specific adaptive immune responses.[6,7] It is now known that adaptive immunity dependency on the innate immune system results from the need for antigen processing and presentation. These functions are displayed by professional antigen presenting cells (APC), such as dendritic cells (DC).[8] The initial uptake and phagocytosis of microbes by APC is facilitated by recognition of pathogen-associated molecular patterns (PAMP) by PRR. This

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leads to the activation of DC maturation, a process entailing up-regulation of major histocompatibility complex (MHC; class I and class II) and costimulatory (e.g., CD40, CD80 and CD86) molecules, together with the production of different cytokines, which in turn results in optimal antigen processing and presentation to naïve CD4+ and CD8+ T-cells. A well orchestrated innate and adaptive immune response usually leads to pathogen eradication and long-lasting immunity against the specific agent (Fig. 1). In contrast, failure to efficiently discriminate between self and nonself can lead to unchecked spread of the pathogen or autoimmunity.

Although highly effective vaccines are currently available for a number of infectious diseases, vaccine formulations can still be improved. The use of well-defined antigens for the formulation has led to less reactogenic subunit vaccines, which unfortunately are less immunogenic than their live or whole cell inactivated counterparts. This has created a major need for efficient adjuvants. On the other hand, conventional vaccines are usually administered by the systemic route. This mainly promotes the elicitation of systemic immunity. However, most pathogens enter the body through mucosal surfaces. Therefore, systemic vaccines are suboptimal, since they fail to induce a local mucosal response able to block the early stages of infection. This roadblock can be eliminated by administering antigens by the mucosal route. Furthermore, mucosal vaccination offers several additional benefits, such as easy administration logistics and high acceptance by the public, as a result of the lack of pain.[9] However, mucosal vaccines have to overcome formidable barriers. Antigens administered by this route can be cleared by non specific mechanisms (e.g., ciliar activity, mucous entrapment, peristaltism), are degraded by enzymes and may be affected by extreme pH, before having the chance of reaching APC. One of the strategies to overcome the poor immunogenicity

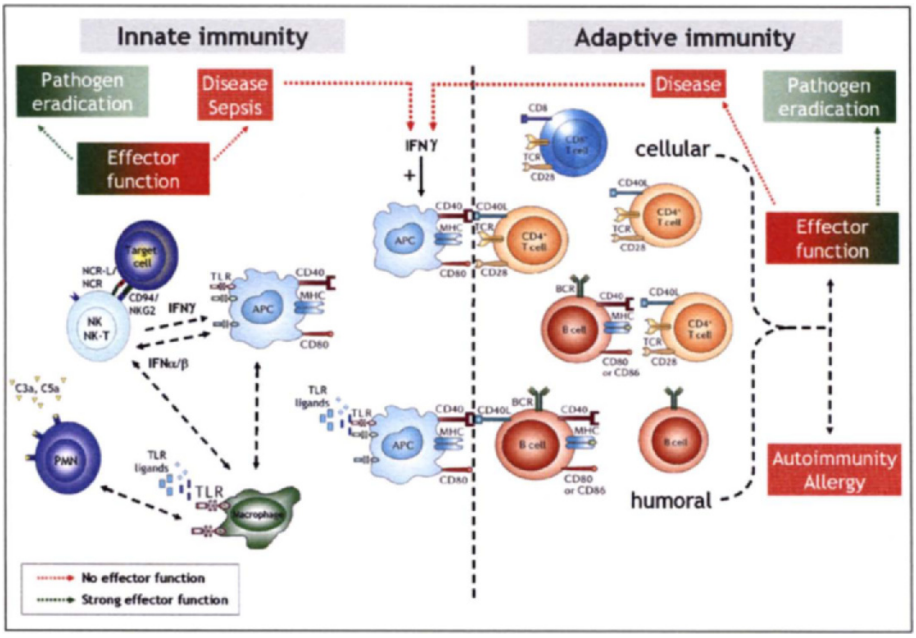

Figure 1. The success or failure of an antigen-specific immune response depends on the interface between innate and adaptive immunity. For a successful removal of a pathogen it is essential that microbes are recognized by components of the innate immune system. The systems for microbial sensing are complementary and they are involved in the development of the ensuing adaptive immune response. Adaptive responses may tend toward tolerance or productive activation of T helper Type 1 (Th1) or Type 2 (Th2) cells. Different responses are required for the elimination of different microbes (e.g., clearance of intracellular pathogens usually requires robust Th1 and CTL responses).

is the co-administration of antigens with mucosal adjuvants.[10] These compounds generate a local microenvironment conductive towards antigen processing and presentation, thereby promoting the elicitation of robust responses at both local and systemic levels.[11]

The chemical nature of the adjuvants and their mode of action are extremely variable. Potential mechanisms by which they exert their biological activity are the "depot" effect, antigen targeting to APC, improvement of antigen processing and presentation and immune activation or modulation through the up-regulated expression of cellular mediators. However, it is also important to consider that their biological activities may not only favor the elicitation of adaptive responses, but also the appearance of side effects. A strong stimulation may result in local or general inflammation and tissue destruction, with the resulting distress for the vaccinees. On the other hand, the stimulation of vigorous responses is insufficient to protect against a specific pathogen. It is essential to promote an adequate type of response. Otherwise, we may even lead to immune pathological reactions or a more severe course of infection. Thus, the identification of adjuvants able to promote predictable responses is a major issue in vaccinology. The lack of adjuvants which are able to stimulate cell-mediated responses represents also a bottleneck. Only a few molecules have been identified exhibiting this property, being most of them unacceptable for human use due to their reactogenicity. Therefore, the choice of a specific adjuvant for a formulation reflects a compromise between the required immune modulatory effect and an acceptable (i.e., low) level of side effects.

Dozens of adjuvants have demonstrated their efficacy in preclinical and clinical studies. Nevertheless, only a few, such aluminum-based salts,[12,13] the squalene-in-water emulsion MF59[14,15] and monophosphoryl lipid A (MPL)[16-19] have been approved for human use (Table 1). In fact, aluminum salts are at the moment the most widely used adjuvants for both human and veterinary

Table 1. Adjuvants included in human and veterinary vaccines

Adjuvant	Administration Route	Side Effects
Aluminium based salts	Systemic	Mild systemic reactions (93%),[27] local side effects (e.g., suprapubic pain and vesical tenesmus)[20,28]
Squalene oil-in-water emulsion	Systemic	Local side effects[29,30]
Monophosphoryl lipid A (MPL)	Systemic and mucosal	Pyrogenic at high doses[31]
Endogenous human cytokines or hormones, (e.g., INFs, IL12, GMCSF)	Systemic	May cause adverse effects
Bacterial toxoids (e.g., derivatives of cholera toxin and E. coli heat labile toxin)	Systemic and mucosal	Mild side effects, may result in retrograde homing to neural tissues when administered by nasal route[32,33]
Muramyldipeptide (MDP), lipid A derivatives	Systemic and mucosal	Currently under study
Lipopeptides, MALP-2	Systemic and mucosal	
Saponins (e.g., QS-21)	Systemic and mucosal	May cause allergic reactions when given at high dosages
Immunostimulating complex (ISCOMs)	Systemic and mucosal	Currently under study
CpG-ODN	Systemic and mucosal	Conflicting data on effect following long term administration, may cause mild damage.[11]

vaccines. Although severe side effects are rare, alum has the potential to cause sterile abscesses, eosinophilia and myofascitis.[20] There are also concern regarding the potential role of aluminum in neurodegenerative diseases.[20] Alum induces strong antibody and Th2 responses. Therefore, adjuvants able to promote Th1 and CTL responses are still needed. Interestingly, the use of alum in combination with IL12 can redirect responses from Th2 towards a Th1-dominant response.[21] On the other hand, MF59 promotes both humoral and cellular immune responses.[14,15,22,23] Squalene or squalane emulsions are efficient adjuvants which can be stabilized by micro-fluidization, so that the emulsions can be frozen or kept for years at room temperature, allowing also their sterilization by terminal filtration.[14] Antigens are added after emulsification, so that conformational epitopes are not lost by denaturation, as well as to facilitate manufacturing.[24,25] Clinical trials of several MF59-adjuvanted vaccines, which were performed in different age groups (from newborns to elderly), have demonstrated their safety and immunogenicity. However, Phase IV studies are mandatory, since the use of oil-based adjuvants may be associated with a higher risk for autoimmunity in experimental models.[26] This seems to be related with the hydrocarbon's ability to induce IL12, IL6 and TNFα. Whether this is of relevance for human vaccination is matter a discussion, since immunotoxicity depends on many factors, such as the species, genetic makeup, route, dose and duration of the administration. In fact, up to now, clinical studies do not seem to support a high risk for autoimmune disease.

Saponins are a chemically heterogeneous group of sterol glycosides and triterpene glycosides, which are common constituents of plants. They are known to cause substantial enhancement of immune responses since the 1920s. Naturally occurring saponins from *Quillaja saponaria* stimulate humoral responses against T-dependent and T-independent antigens and CTL responses.[34] Despite their use in animal vaccines, the development of saponin-based formulations for humans has been impeded by their complexity and concerns about toxicity.[35,36] On the other hand, QuilA, which results from partial purification from crude food-grade extracts, is contained in several veterinary vaccines. Further purification provided concentrated saponin fractions, such as QS-21, which is currently under clinical investigation in humans.[37-39] Interestingly, purified saponins seems to be also effective as adjuvants when delivered by oral route.[37]

Saponins have been combined with cholesterol and other lipids to generate immunostimulating complexes (ISCOMs), which are open cage-like structures with build-in adjuvant activity that promote antibody, T helper and CTL responses. ISCOMs seem to enhance antigen targeting to APC, as well as their subsequent uptake, processing and presentation. The use of ISCOMs also result in the production of pro-inflammatory cytokines, such as IL1, IL6 and IL12.[40] Liposomes represent a related delivery system, which also has build-in adjuvant properties. They are vesicular structures limited by a bilayer membrane composed of phospholipids and cholesterol. Liposomes can carry both membrane associated antigens, as well as water soluble molecules. Their physical properties are highly variable, depending on the composition and the manufacturing method. This allows the optimization of the design for specific tasks (e.g., targeting, co-incorporation of adjuvants). Liposomes have a long history as vehicles for antigen delivery.[41,42] Recently, the so-called virosomes have been developed, by incorporating the hemagglutinin from the influenza virus into liposomes.[43-46] This glycoprotein guides virosomes to APC and promote their fusion with the endosomal membrane at low pH. This in turn leads to the cytoplasmic release of the antigens, thereby providing optimal processing and presentation in the context of MHC class I molecules.

There are other candidate adjuvants in advance stages of preclinical and clinical development, such as a new generation of water-in-oil emulsions (e.g., Montanide, CSA 720), which were demonstrated able to trigger more efficient humoral responses than alum in several animal species and humans.[47-49] A small immunomodulatory peptide, CEL-1000, also promoted the elicitation of protective Th1 responses against infectious agents and tumors in experimental animal models.[50,51] Nevertheless, despite the availability of a number of molecules with adjuvant properties, to optimize rational vaccine design a broader palette of adjuvants able to promote different types of immune response would be necessary. In this context, the availability of immune-modulators with well-defined molecular targets would represent a clear asset. This approach would not only allow

fine-tuning and customizing responses according to the specific needs, but would also facilitate the prediction of potential side effects.

Immune Modulators with Defined Molecular Targets

The advent of whole-genome sequencing of bacterial pathogens has revolutionized the field of vaccinology. This approach not only provides the full array of potential candidate antigens for vaccine design, but has also revealed new PAMP, which might exert immunomodulatory activities by acting on PRR.[52] Adjuvant exhibiting this property deliver a danger signal to the host immune system through the activation of PRR, thereby mimicking infectious agents. This results in the expression of soluble mediators (e.g., cytokines, chemokines) and APC activation. The stimulation of the innate immune system in turn determines and shapes adaptive responses. The exploitation of this knowledge for the identification of new molecular targets is facilitated by recent developments on mass mutagenesis, high throughput screening, gene expression profiling and combinatorial chemistry.[53] However, the intrinsic human genetic variation is a major factor which might lead to differential responses after stimulation with PRR agonists. Therefore, immunogenetics is a critical building block of discovery programs.[54]

TLR Agonists

The mammalian TLR family consists of at least 13 members (10 TLRs (TLR1–10) in human and 12 TLRs (TLR1–9 and TLR11–13) in mice) have been found and each TLR is involved in recognizing a variety of microorganism-derived molecular structures (Table 2).[55,56] TLR comprise a extracellular domain with dozens of leucine-rich repeat motifs, a trans-membrane domain (with the exception of TLR3) and a cytoplasmic Toll/IL1R (TIR) domain similar to that of the IL1 receptors (IL1Rs).[57] The TLR1, TLR2, TLR4, TLR5, TLR6 and possibly TLR11 are expressed on the cellular surface, whereas TLR3, TLR7, TLR8 and TLR9 are believed to reside inside the cells (e.g., endoplasmic reticulum and/or endosomes). Each TLR is expressed in a variety of immune cells, including macrophages, DC and B-cells, as well as in other cell types (e.g., endothelial cells, epithelial cells). TLR can recognize molecular patterns conserved among but unique to microbes, which usually do not exist in the host. Pathogen recognition by TLR provokes a rapid activation of the innate immune system by inducing production of pro-inflammatory cytokines and up-regulation of costimulatory molecules. The activated innate immune system subsequently leads to effective adaptive immunity. Depending on the TLR triggered, specific signaling pathways are activated (Fig. 2). Different TLR can exert distinct, but to some extent also overlapping sets of biological effects.[55] Stimulation of TLR2 and TLR4 failed to increase CTL responses, whereas ligands of TLR3, TLR5 and TLR7 exhibited moderate activity. In contrast, stimulation of TLR9 dramatically increases CTL responses.[58]

Agonists able to either control the over-expression of Th2 cytokines or skew the Th1:Th2 balance towards a Th1 profile would be of clinical relevance for the control of allergic disease.[60] In fact, different TLR ligands were demonstrated to be able to inhibit Th2 cell activation and IgE-dependent release of Th2 cytokines in animal experimental models of allergy.[61,62] TLR are also important for the stimulation of Th2-type responses, since they augment the overall maturity of DC.[63,64] Some of the initial studies performed with native ligands gave conflicting results due to contamination with other moieties. However, the availability of well-defined synthetic derivatives made possible to dissect the signal transduction events triggered by the specific activation of a TLR. Thus, the exploitation of well-defined TLR agonists for the establishment of immune prophylactic or therapeutic interventions seems to be an extremely promising field. However, safety aspects need to be carefully addressed during preclinical and clinical development, since TLR seem to be involved in the pathogenesis of human diseases (e.g., autoimmunity, allergies, asthma and cardiovascular diseases).

In this context, bacterial lipoproteins and their synthetic analogues are strong immune modulators during infection, signaling through TLR2/TLR1 heterodimers. Their synthetic derivatives, such as $Pam_3Cys\text{-}SK_4$, have been exploited as adjuvants and modulators of T-cell responses. Previous

Table 2. Toll-like receptors (TLRs) and their ligands[59]

TLR	Interaction With Other TLR	Ligand (Exogenous)	Ligand (Endogenous)	Source
TLR1 TLR2	TLR 2 TLR1 and TLR6	Peptidoglycan, triacyl lipopeptides, diacyl lipopeptides, lipoteichoic acid, glycolipids	HSP70	Gram-positive bacteria, Measles virus
TLR3	-	dsRNA, siRNA	mRNA	West Nile virus, mouse CMV, *Schistosoma mansoni*, synthetic RNA molecules
TLR4	-	Lipopolysaccharide, taxol, RSV fusion protein, mouse mammary tumor virus envelope protein phosphorylcholine, glycan	HSP70, β-defensin 2, fibrinogen, fibronectin, hyaluronic acid	Gram-negative bacteria, plants, respiratory syncytial virus, mouse mammary tumor virus, *Bacillus anthracis*, helminths
TLR5	-	Flagellin	-	Flagellated bacteria
TLR6	TLR2	Lipopeptides (e.g., MALP-2)	-	Bacteria
TLR7 TLR8	- -	ssRNA Imidazoquinoline, Resiquimod, Imiquimod	U1snRNP (spliceosomal complex) autoantigens -	synthetic compounds viruses (e.g. influenza virus HIV-1)
TLR9	-	CpG DNA, hemozoin	Chromatin complex	Bacteria, synthetic ODN, DNA viruses, *Plasmodium falciparum*
TLR10	-	Not determined	-	-
TLR11	-	Profilin-like molecule	-	*Toxoplasma gondii*, uropathogenic bacteria
TLR12	-	Not determined	-	-
TLR13	-	Not determined	-	-

studies suggested that $Pam_3Cys\text{-}SK_4$ is able to ameliorate established allergic airway inflammation by promoting Th1 response rather than by affecting regulatory T-cells.[65] On the other hand, its co-administration with anti-CD3 antibodies resulted in modulated immune responses, by inducing the proliferation of both regulatory and effector T-cells in the absence of APC. Thus, it seems that $Pam_3Cys\text{-}SK_4$ improves adaptive responses by expansion of effectors and mitigation of suppressive activity of Tregs.[66,67]

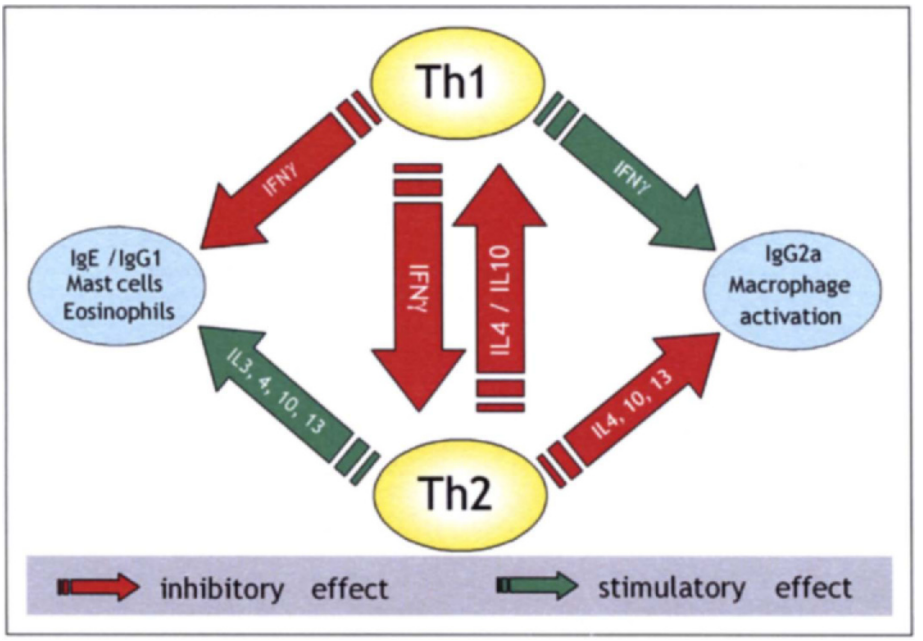

Figure 2. The Th1/Th2 network. IL4 and IFNγ do not promote a direct inhibition of Th1 or Th2 cells differentiation, but rather block the differentiation of these subsets from naive precursors.

Natural and synthetic derivatives of diacylated lipopeptides from *Mycoplasma* spp. and *Francisella tularensis*, such as the macrophage-activating lipopeptide of 2 kD (MALP-2), induce the maturation and activation of APC with release of soluble mediators able to act on bystander cells via activation of the TLR2/TLR6 heterodimer.[68,69] Their co-administration with antigens by either systemic or mucosal route results in the elicitation of strong humoral and cellular responses.[68,70] Additional studies demonstrated that MALP-2 promotes a T-cell-independent activation and maturation of B-cells, increasing also the frequency of Ig-secreting cells. Activated B-cells exhibited increased expression of both activation markers and ligands which are critical for cross-talk with T-cells (e.g., CD19, CD25, CD80, CD86, MHC I, MHC II and CD40). Immunization of mice lacking T-cells also showed that MALP-2-mediated stimulation of TLR2/TLR6 was unable to circumvent the need of T-cell help for efficient antigen-specific B-cell activation. On the other hand, immunization of mice lacking B-cells demonstrated that B-cells are critical for MALP-2-dependent improvement of T-cell responses.[71-73] Thus, B-cell stimulation by PRR seems to be a basic mechanism which can be exploited to improve the immunogenicity of vaccine formulations.

The lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, stimulates adaptive responses. Since the 1970s it was known that the adjuvant effect depends on the integrity of the *lps* locus.[74-77] Then, positional cloning showed that the *lps* locus was identical to *tlr4* locus.[78] However, the toxicity of the LPS prevented its clinical use, making necessary the development of non toxic derivatives. Among them, MPL, a derivative of lipid A from *Salmonella minnesota*, was proven to be safe and effective as a vaccine adjuvant for humans.[79] A new class of fully synthetic lipid A mimetics (e.g., aminoalkyl glucosaminide 4-phosphates, RC-529) have been engineered to target human TLR4 and are showing promise results as vaccine adjuvants and therapeutics against a wide range of infectious agents.[80] Thus, it seems that the potent immune

modulatory properties of TLR4 agonists allow their exploitation as both vaccine adjuvants and stand-alone therapeutics.

Synthetic double stranded polyribonucleotides act at the level of TLR3, inducing the production IFN and other cytokines. This activity can be exploited to increase the effect of anti-viral drugs. For example, the anti-retroviral effect of zidovudine is enhanced by combination with polyribonucleotides.[81] Poly I:C was one of the first therapeutic agents used to treat HIV and leukemia patients, but it was abandoned due to toxicity.[82] Synthetic polyribonucleotides also induce the activation and maturation of DC. In vitro results demonstrated that a novel non toxic analogue of poly I:C (Ampligen) effectively induce in vitro maturation of monocyte derived human DC with sustained production of IL12. DC primed with tumor lysates and matured with synthetic dsRNA may offer a valid alternative for optimizing Th1 anti-tumor responses in cancer patients.[83]

The binding of bacterial flagellin to TLR5 results in DC stimulation and promotion of Th1 responses.[1,84,85] This depends, at least in part, in the up-regulated expression of IL12 resulting from phosphorylation of p38 and c-Jun N-terminal kinase 1/2. Monomeric flagellin acts as local and systemic pro-inflammatory factor via activation of the TLR 5-NF-kappaB axis and IL8 production.[58,86] Co-administration of tetanus toxoid (TT) with flagellin to mice by intranasal route resulted in significantly enhanced TT-specific humoral responses in the mucosal and systemic compartments. Vaccinated mice were completely protected after challenge with a 200-fold minimum lethal dose of tetanus toxin. FlaB colocalized with CD11c as patches in putative DC and a clear increment in the number of TLR5-expressing cells was also observed in cervical lymph nodes.[87,88]

Imiquimod, a synthetic imidazoquinoline which was approved in 1997 for the topical treatment of genital warts, exhibits anti-viral and anti-tumor activities. Imiquimod binds TLR7 and TLR8 on DC leading to the production of pro-inflammatory cytokines, such as IFNα, which in turn stimulates both the innate immune system and the cellular arm of the acquired immune system (e.g., improvement of CTL responses).[89,90] This suggests that imiquimod mimics natural ligands of TLR7 and TLR8 (e.g., ssRNA from viruses). However, recent experimental and clinical data indicate that imiquimod also exerts direct pro-apoptotic activity on tumor cells.[91] Imiquimod is insoluble in water but in most clinical studies its incorporation into an oil-into-water cream emulsion (1-5%) was well-tolerated with mild-to-moderate drug-related side effects, such as itching, burning sensation, pain, erythema, erosion and oedema.[92] An analogue, the so called resiquimod (R848), is able to promote similar Th1-biased responses, but at 10-fold lower dosages than imiquimod.[93] Initial clinical studies suggested that resiquimod reduces the recurrence rate of genital herpes, however, Phase III trials were suspended due to lack of efficacy.[89]

In the course of natural microbial infections, TLR9 recognizes double stranded DNA which, in contrast to mammalian DNA, is characterized by the abundance of unmethylated deoxycytidyl-deoxyguanosine dinucleotides (CpG). The stimulatory activity of microbial DNA can be mimicked by synthetic oligodeoxynucleotides (ODN) containing such motifs (CpG-ODN).[94] The TLR9-mediated stimulation of the vertebrate innate immune system and subsequently, of the adaptive immune system, allows the use of TLR9 agonists as highly effective vaccine adjuvants for infectious diseases, as well as stand-alone or part of combination therapies against cancer.[95,96] TLR9 shows a restricted cellular and sub-cellular pattern of expression, which changes also between animal species. CpG-ODN acts on human B-cells and plasmacytoid DC increasing the production of pro-inflammatory cytokines and promoting APC maturation and Th1 responses.[97,98] These biological activities enable CpG-ODN to act as adjuvants when co-administered with a given antigen by systemic or mucosal route, thereby leading to improved antigen-specific responses. These effects are optimized by maintaining close physical contact between the CpG-DNA and the immunogen. Ongoing clinical studies also indicate that CpG-ODN are well-tolerated in humans.[98,99] During infection, recognition of CpG DNA of intracellular pathogens skews immune response towards a Th1 dominant pattern. This can be also desirable in therapeutic interventions aimed at the prevention of allergic responses.

Bacterial Toxins and Their Derivatives

Cholera toxin (CT) and its close relative the heat-labile enterotoxin of *Escherichia coli* (HLT) are A/B moiety toxins. They are composed by an A subunit, which is responsible for the enzymatic activity and a pentamer of B subunits, which mediates the binding of the holotoxin to the receptors present in the membrane of the target cells. CT and LT-I bind to the ganglioside GM_1, LT-IIa binds with high affinity to the ganglioside GD_{1b} and with low avidity to the gangliosides GD_{1a} and GM_1 and LT-IIb binds with high affinity to GD_{1a}.[100,101] These two toxins are powerful adjuvants when co-administered with antigens by either systemic or mucosal route.[102] The molecular mechanisms by which they are able to stimulate the innate and adaptive immune systems are not fully elucidated. However, direct effects on T-cells and APC have been observed, which provide insights into how these molecules may exert their activity. In vitro studies performed with primary B-cells and macrophages showed an increased phosphorylation of different signaling molecules, including Erk1/2 and p38.[103] In this context, the B subunit of CT induces signaling events resulting in cellular activation, expression of surface molecules and production of cytokines, by promoting a transactivation of two transcriptional elements, the cyclic AMP-responsive element and NF-κB.[103] The observed GM1-dependent nuclear translocation of NF-κB in DC demonstrates the critical role played by this receptor in the stimulation of signal transduction cascades.[104] On the other hand, there is a paucity of information regarding the structurally related members of the serogroup II HLT, namely LT-IIa and LT-IIb, which have different binding specificities for ganglioside receptors.

The use of CT and LT in humans was initially hampered by their high toxicity. However, site-directed mutagenesis has permitted the generation of LT and CT derivatives with reduced toxicity, which retain their adjuvanticity.[105] Different mutants, such as LTK63 (LT with a serine-to-lysine mutation at position 63 of the A subunit), have been exploited as systemic or mucosal adjuvants.[106] A preferential induction of Th2-type responses was observed when these molecules were incorporated in the vaccine formulations. Interestingly, their use also prevented or alleviated autoimmune diseases, thereby demonstrating their wide-ranging effects on the immune system. The observation that this improvement is associated with the generation of regulatory T-cells, which in turn inhibit pathogenic Th1 responses, explains in part the two apparently contradictory outcomes after exposure to CT-like enterotoxins. Nevertheless, further investigations are required to unravel the mechanisms leading to either adjuvanticity or tolerance induction to fully exploit their potential in the context of vaccine design and immunotherapies.[107]

CD1d Agonists

NKT-cells represent a unique subset of immune regulatory T-cells, which are able to recognize glycolipids presented by the MHC class I-like molecule CD1d. Because of their biological properties, NKT-cells are attractive targets for the development of immunotherapies. The prototypical NKT-cell ligand αgalactosylceramide (αGalCer), originally isolated from a marine sponge, has potent immune modulatory activities. αGalCer is a ligand of invariant Vα14+ NKT-cells, which is presented by CD1d on APC. Administration of αGalCer to mice results in a rapid activation of NKT-cells, which is characterized by cytokine secretion, surface receptor down-regulation, expansion and secondary activation of a variety of bystander cells from the innate and adaptive immune systems. Different studies have demonstrated the efficacy of αGalCer against metastatic tumors, infections and autoimmune diseases.[108-111] Studies performed with structural analogues of αGalCer also showed that β-anomeric GalCer can induce CD1d-dependent biological activities in mice, albeit at lower potency than α-anomeric GalCer.[112] The response of NKT-cells to distinct GalCer analogues seems to differ both at quantitative and qualitative levels. These findings indicate that specific glycolipids could be exploited to fine-tune NKT-cells responses, thereby shaping the elicited immune responses in vivo. This approach will certainly facilitate the development of effective and safe NKT-cell-based immunotherapies.[24,112-115]

Different studies have also demonstrated that αGalCer could act as an effective adjuvant when co-administered with antigens by either systemic or mucosal route. When αGalCer was

administered with the model antigen ovalbumin by intranasal route to C57BL/6 and BALB/c mice, strong ovalbumin-specific humoral and cellular immune responses were stimulated at systemic and mucosal level, characterized by a mixed Th1/Th2 cytokine profile. Vaccinated mice also showed complete protection against challenge with the ovalbumin-expressing tumor cell line EG7. Furthermore, intranasal vaccination with the hemagglutinin from the influenza virus A/PR/8/34 co-administered with αGalCer also conferred significant protection against viral infection. Interestingly, when αGalCer was given with a replication-deficient adenovirus to mice, significantly enhanced immune responses were detected. The adjuvant effect induced by intranasal co-administration with αGalCer was blocked in CD1d-/- mice, indicating that the immune responses were exclusively dependent on the CD1d molecule present on APC. Experiments performed using CFSE-labeled OT-1 cells which were adoptively transferred into syngenic mice showed that naive T-cells were activated and stimulated to differentiate into functional effector T-cells.[116] The efficacy of αGalCer as adjuvant was also demonstrated in the context of other vaccine relevant antigens (e.g., malaria, influenza, hemagglutinating virus of Japan).[113,117,118] Despite the excellent results obtained using αGalCer, there are major drawbacks preventing the efficient transfer into the clinic, such as its poor solubility. To provide soluble formulations, non organic solvents or detergents are needed, which represent a safety concern and might affect the immunological properties of some antigens. However, a new pegylated derivate, αGalCerMPEG, has been recently described, which is water-soluble and retains both the specificity for the CD1d receptor and the immune stimulatory properties when tested in vitro or in vivo, even at 33-fold lower concentrations of the active moiety.[119]

Cytokines

The process by which mammals meet the dual need of an immediate response to danger and initiation of long-term protection is regulated by pro-inflammatory cytokines, which are primarily produced by cells from the innate immune system. Therefore, cytokines have been widely exploited as natural adjuvants for the design of immunotherapeutic or prophylactic interventions. Many cytokines were demonstrated to be able to enhance protection against infectious agents and tumors, both in preclinical and clinical studies. One of the most exploited ones is IL12, which plays a key role linking the innate and acquired immune systems. IL12 is critical for activation of NK cells, promotion of CTL development, T-cell independent induction of IFNγ, activation of differentiated CD4+ and CD8+ T-cells, development of Th1 responses, provision of complementary immune-regulatory signals to IL2 and defense against intracellular pathogens.[120] Therefore, also other members of the IL12/IL23/IL27 family, which share ligand and receptor subunits and play overlapping roles in innate and adaptive responses are also considered as prime candidates.[121] The use of IL2 also gave promising results. The mechanism of anti-tumor activity of this cytokine is related to its ability to expand and activate the NK and CTL subsets.[122] There is also increasing evidence that local or systemic effector cell dysfunction, which is characteristic of patients with advanced cancer, can be reverted by IL2. Another important cytokine, IL15, is produced by several leukocytes in response to infections. IL15 exhibits many homologies to IL2 and, like IL2, stimulates NK cells.[123] Chemokine-antigen fusions also resulted in enhanced immunogenicity. The rational use of combinations between cytokines and chemokines could promote the targeting of antigens to APC, their subsequent maturation, the attraction of critical bystander cells, the steering of cellular immune responses toward a Th1 response pattern, the improvement of CTL responses and the enhanced production of systemic IgG and secretory IgA. Several review articles have provided additional information about the most commonly used cytokines and chemokines for immunotherapies.[124-130]

Cell Wall Components

Freund's complete adjuvant (FCA) has been the adjuvant of choice for animal experimentation for decades. A water-in-oil emulsion is generated, in which water droplets containing antigen are emulsified in mineral oil containing killed mycobacteria or their cell walls. However, its high

toxicity precludes its use in humans. Nevertheless, subsequent studies allowed the isolation of the active components and a better understanding of the underlying mechanisms of adjuvanticity. This resulted in the identification of molecules exhibiting similar immune modulatory properties and an adequate safety profile. N-acetylmuramyl-L-alanyl-D-isoglutamine (i.e., muramyl dipeptide; MDP) was the first.[131] This is a synthetic derivative of a component present in the bacterial peptidoglycan. The activity of MDP can be attributed to its ability to cause the release of multiple cytokines. Animal studies established that MDP exhibits a broad array of immunological effects, including: (i) enhancement or suppression of antibody levels dependent on the time of administration relative to antigen; (ii) improvement of cell-mediated immunity; (iii) enhancement of nonspecific immunity to bacteria, viruses, fungi and parasites; (iv) stimulation of natural resistance to tumors; (v) promotion of cytokine release and (vi) pyrogenicity. MDP is recognized by NOD2, but not by TLR2 or heterodimers formed by TLR2 with TLR1 or TLR6.[132] In contrast to intact and diacylated MDP, derivatives with a single octanoyl or stearoyl fatty acid chain were found to activate TLR2 and TLR4 (see above) and exert their activities through the MyD88-dependent pathway on APC.[133] Studies performed using a *Mycobacterium tuberculosis* animal experimental model also showed that NOD2 and TLR are two non redundant recognition mechanisms which synergize for the induction of pro-inflammatory cytokines.[134] Interestingly, MDP also exerts pronounced neuropharmacological activities, probably through the interaction with 5-hydroxytryptamine receptors. However, some of these biological activities are undesirable for clinical use.[135] Thus, structurally well-defined synthetic derivatives from the MDP were generated, which exhibit improved pharmacological properties.[73,136,137-139] Among these non toxic derivatives can be mentioned the adamantylamide dipeptide (AdDP), MDP-Lys (L18) and murabutide. The AdDP is a synthetic compound in which the dipeptide was combined with the anti-viral compound amantadine. AdDP exerts its adjuvant properties when administered by either systemic or mucosal route, leading to the elicitation of strong humoral and cellular responses at both systemic and mucosal levels.[140-143]

Co-Stimulatory Molecules

Processed antigenic peptides are presented by APC to T-cells in the context of MHC molecules, which are the only physiological ligands for the T-cell receptor (TCR). However, several additional receptors form part of the immunological synapsis. In fact, ligation of the TCR to the MHC II-peptide complexes (signal 1) leads to various T-cell effector functions, depending on the specific nature of the costimulatory receptors (signal 2). Thus, surface molecules such as CD40 and CD154 are central to the cross-talk between T-cells and antigen-presenting cells. The study of the immune responses against intracellular pathogens showed the relevance of CD40-CD154 interactions in the regulation of IL12 and IFNγ secretion.[144] Furthermore, anti-CD40 antibodies can be effective adjuvants when administered either separately (high dosages) or conjugated (low dosages) to the antigen. In the former case, it is likely that side effects such as splenomegaly would occur, whereas when conjugates are used, side effects may be avoided.[145,146] GM-CSF has been shown to be synergistic with IL12 or CD154 for induction of CTL responses and the triple combination of GM-CSF, IL12 and TNFα appears to induce the most effective protection in some experimental models. The combination of costimulatory molecules with vaccine antigens has also resulted in a synergistic effect.[147] Another potential target molecule is CD134, a member of the TNFR superfamily. CD134 is transiently expressed on T-cells following ligation of the TCR. The CD134 ligand, known as OX40L, is a TNF family member expressed on APC. The OX40/OX40L pathway offers opportunities for both enhancing responses through use of agonists (adjuvants) and abrogating unwanted responses, such as those generated in autoimmune diseases, through the use of blocking reagents and antagonists.[148] Another widely studied costimulatory pathway is the CD28-CD80/CD86. CD28 is constitutively expressed on T-cells and can be seen as analogous in function to CD40 on B-cells. The ligands for CD28 are CD80 and CD86 (also known as B7-1 and B7-2). They are members of the immunoglobulin superfamily, which

are transiently expressed on activated APC. Forced expression of B7 on tumor cells, which do not normally express it, resulted in enhanced T-cell responses.[149-152]

Bis-(3',5')-Cyclic Dimeric Guanosine Monophosphate (cdiGMP)

In many bacteria bis-(3',5')-cyclic dimeric guanosine monophosphate (cdiGMP) signaling determines the timing and amplitude of complex biological processes, such as biofilm formation, virulence or photosynthesis. Besides its role as an intracellular and intercellular signaling molecule in prokaryotes, c-di-GMP also affects eukaryotes.[153,154] This interesting molecule inhibits cancer cells in vitro, by affecting basal and growth factor-induced cell proliferation.[155] Recent studies have also demonstrated that cdiGMP exhibits potent adjuvant properties. Subcutaneous co-administration of antigens with cdiGMP promotes the elicitation of vigorous antigen-specific humoral and cellular immune responses, which were characterized by a balanced Th1/Th2 response pattern.[156] Similar results were obtained when cdiGMP was used as mucosal adjuvant. However, the IgG1/IgG2a ratio of antigen-specific antibodies and the profiles of secreted cytokines suggested that a mixed Th1/Th2 response pattern is promoted when cdiGMP is administered by mucosal route.[157] Intraperitoneal injection of cdiGMP in mice resulted in the local recruitment and activation of monocytes and granulocytes. Human immature DC cultured in the presence of cdiGMP showed increased expression of the costimulatory molecules CD80 and CD86, the maturation marker CD83 and MHC class II molecules. IL12, IFNγ, IL8, MCP-1, IFN-γ-inducible protein 10 and RANTES were also produced by cdiGMP-treated DC, which exhibited enhanced T-cell stimulatory activity.[158] It seems that the activation of the p38 MAPK in human DC and an ERK phosphorylation in human macrophages may be involved in cdiGMP signaling. However, the underlying signaling events need to be further elucidated.[158]

Conclusion

The advent of subunit vaccines rendered obvious the pressing need for developing efficient and safe adjuvants. This is particularly true when mucosal vaccination strategies are coming into consideration, since only a few moieties have been identified so far exhibiting this property. In particular, there is a high demand for adjuvants able to stimulate cellular immunity, which is essential for combating intracellular pathogens and tumors. In this context, it would be helpful to have well-characterized moieties, which are able to exert their biological activity through stimulation of defined cellular targets and/or signaling cascades. This is now possible due to the identification of agonists of PRR present of APC, which are thought to provide a link between the innate and adaptive immune system. An in depth understanding of the underlying mechanisms of action of these moieties, together with the availability of synthetic derivatives with a well-defined structure will certainly reduce the likelihood of undesired side effects. It would be also feasible to choose the most appropriate entity in order to stimulate predictable immune responses, according to the specific clinical needs. Another aspect which requires attention is the minimization of effector functions in untargeted cells. To this end, efforts are being invested to achieve the specific targeting of adjuvants and/or formulations. The targeting encompasses both organ and cell-specific delivery (e.g., local lymph nodes, solid tumors, APC). This is expected to facilitate optimal stimulation with negligible side-effects. Thus, basic research in immunology will continue being the main driving force for innovation in the vaccinology field. Increase predictability for candidates tested in preclinical and clinical studies will facilitate the cost-efficient development of efficient vaccines with an optimal safety profile.

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Chapter 14

Innovative Approaches to Develop Prophylactic and Therapeutic Vaccines against HIV/AIDS

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Abstract

The acquired immunodeficiency syndrome (AIDS) emerged in the human population in the summer of 1981. According to the latest United Nations estimates, worldwide over 33 million people are infected with human immunodeficiency virus (HIV) and the prevalence rates continue to rise globally. To control the alarming spread of HIV, an urgent need exists for developing a safe and effective vaccine that prevents individuals from becoming infected or progressing to disease. To be effective, an HIV/AIDS vaccine should induce broad and long-lasting humoral and cellular immune responses, at both mucosal and systemic level. However, the nature of protective immune responses remains largely elusive and this represents one of the major roadblocks preventing the development of an effective vaccine. Here we summarize our present understanding of the factors responsible for resistance to infection or control of progression to disease in human and monkey that may be relevant to vaccine development and briefly review recent approaches which are currently being tested in clinical trials. Finally, the rationale and the current status of novel strategies based on nonstructural HIV-1 proteins, such as Tat, Nef and Rev, used alone or in combination with modified structural HIV-1 Env proteins are discussed.

Introduction

Epidemiology: Main Global and Regional Trends

Human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) remains one of the most serious threats not only to global health, but also to global development. According to the 2007 AIDS epidemic update by World Health Organization (WHO) and the Joint United Nations Programme on HIV/AIDS (UNAIDS), approximately 33.2 million people were living with HIV in 2007[1] of whom 2.5 million were due to new infection among adults and children (Fig. 1). Today, Sub-Saharan Africa continues to pay the highest toll to the global epidemic, with 68% of new infections, 76% of the estimated 2.1 million deaths in 2007 and 90% of the 2.5 million children living with HIV worldwide. Despite major methodological improvements, which have cut by 16% the former estimates, the statistics still indicate that the number of people living with AIDS increased in 2007, partly due to expanded access to therapy that reduced the number of deaths. This new analysis also shows that the pandemic actually peaked in the late 1990s and

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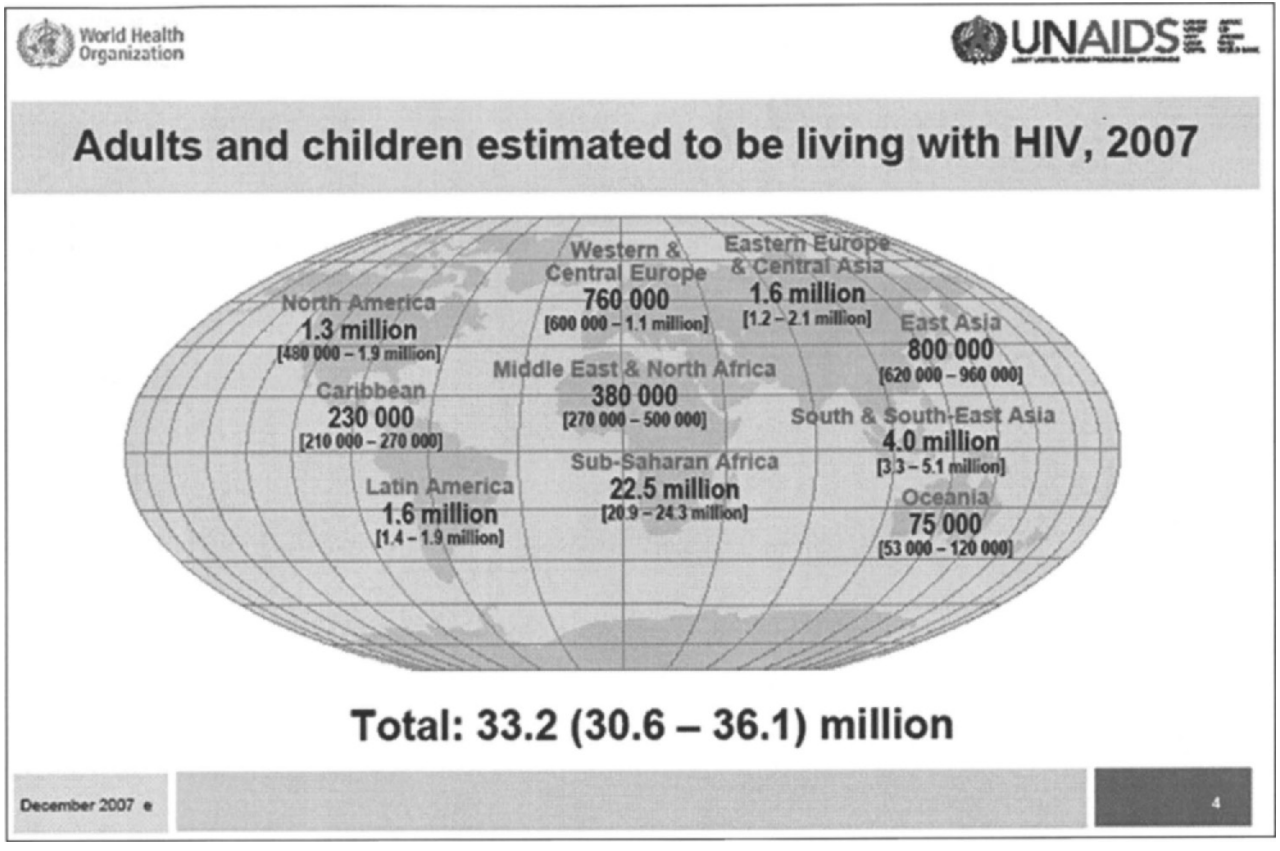

Figure 1. Adults and children estimated to be living with HIV, 2007. Reproduced by kind permission of UNAIDS (2007) (www.unaids.org).

the subsequent decline may at least in part be attributed to the efficacy of prevention campaigns. While this is certainly good news, it has important practical consequences on clinical trials, which may actually result undersized and lack statistical power. As today, longitudinal prospective studies appear the only way to realistically measure the incidence and will have to be included in the preparatory studies for vaccine testing.[2]

Rationale and Roadblocks to HIV Vaccine Development

The increasing social and economical, including therapy, costs of the pandemic make the search for a preventive and therapeutic HIV/AIDS vaccine the highest priority of the world HIV/AIDS agenda.[3] Preventive vaccination is aimed at inducing protective immune responses in individuals naive to HIV, whereas therapeutic vaccination is aimed at increasing the potency and breadth of anti-HIV immune responses in order to avoid or delay anti-retroviral therapy use in HIV-1 infected individuals.[4]

Despite advancements in the understanding of HIV biology and pathogenesis[5] and despite over 20 years of attempts, no vaccine is available to combat the HIV/AIDS epidemic.[6,7] However, the vast majority of vaccines evaluated so far turned out to be safe and immunogenic, with some of them showing some level of protection in preclinical models and therefore advanced to clinical testing.[8,9]

The difficulty in generating an HIV vaccine capable of eradicating the infection depends on many factors. Among the most important, the high genetic variability of HIV (for a review see refs. 10,11), the peculiar properties of the envelope (Env) proteins to avoid and evade immune recognition and neutralization,[12] the lack of identified correlates of immune protection, the complexity of implementing preclinical animal models[1,13,14] and conducting efficacy clinical trials, especially in developing countries. In addition to the elusion of antibody (Ab) neutralization, HIV is able to elude cellular immune responses by multiple mechanisms, including down-regulation of major histocompatibility complex (MHC) class I molecules,[15] emergence of mutants escaping cytotoxic T-lymphocytes (CTLs)[16] and dysregulation of cytokine production.[17-19]

Correlates of Protection

Lessons from the Natural History of HIV-1 and SIV Infection

The comprehension of mechanisms of natural resistance to HIV/AIDS may have implications for the identification of novel anti-viral strategies and in particular for the development of innovative diagnostics, therapeutics and vaccines. It is now clear that both host and viral factors contribute to the outcome of the infection and may explain the higher or lower individual susceptibility to HIV/AIDS (Table 1). In particular, two phenomena of natural resistance to HIV-1 infection or progression to disease have been described: individuals that remain uninfected despite exposure to the virus [multiply exposed uninfected (MEU) individuals, also termed exposed-uninfected (EU), or highly exposed persistently seronegative (HEPS) individuals][20] and individuals that become infected but do not progress to AIDS [long-term nonprogressors (LTNPs)]. Both MEU and LTNPs offer valuable clues to elucidate immune mechanisms involved in the resistance or control of infection, respectively and might thus provide a unique resource to identify correlates of protective immunity to HIV.

MEU include HIV-discordant couples having unprotected sex,[21,22] sex workers[23-25] and health care workers.[26,27] Homozygosis for a mutation in CCR5 gene (the 32 bp deletion, i.e., CCR5-Delta32 allele) is presently considered the most relevant genetic protective factor[28-30] (Table 1). Further, in a cohort of MEU Kenyan sex workers HIV-1 specific CTLs were found both in the blood and in the vaginal mucosa.[31] Interestingly, these CTLs, which had similar specificities, recognized epitopes distinct from those recognised by HIV infected individuals[24] and may contribute to protection from infection with both cytolytic and noncytotoxic anti-viral effector mechanisms.[32,33] In the Nairobi cohort, HIV-1 specific $CD4^+$ T-cells produce interleukin-2 (IL-2) rather than interferon-γ (IFN-γ) and display limited activation and cell death as compared to HIV-1 infected

Table 1. Major host factors associated to resistance or control of HIV infection and relevant to vaccine development

Level		Resistance	Control	Reference
Genetic Polymorphism				
-	CCR5	CCR5 Δ32 homozygosis	CCR5 Δ32 heterozygosis	28,60,61
-	CCR2		CCR2-64I	473
-	CCR5 & CCL3L1		Polymorphisms of these genes affect the capacity to mount immune responses (DTH)	79
-	MHC	A2/6802 supertype	B57, B5801, B27 and additional polymorphisms in B, C and outside B and C loci	62,80,412
-	KIR		Activating (KIR3DS1) and inhibiting (KIR3DL1) NK cell receptors binding HLA-B molecules with isoleucine at position 80 (HLA-Bw4)	66,474
Innate Immune Response				
-	Natural antibodies	Against CCR5	Against CCR5	475,476
-	α and β defensins	Increased in the genital mucosae		36
-	CCL5 (RANTES)	Increased in the genital mucosae		477
-	NK	Increased production of IFN-γ	Conserved lytic activity despite altered phenotype	35,478
Adaptative Immune Response				
-	Antibodies	Neutralizing IgA in the genital mucosae		37,479
-	$CD4^+$ T-cells	Prevalent IL-2 production, limited activation and cell death as compared to infected individuals	Polyfunctional, high per cell cytokine production	480
-	$CD8^+$ T-cells	Polyfunctional, found also in the genital mucosae, with specificities different from those found in infected individuals	Polyfunctional, mostly against Gag, highly cytotoxic	34,63,71

CCR5: chemokine (C-C motif) receptor 5; CCR5 Δ32: a 32-base pair deletion mutant of the CCR5 gene; CCR2: chemokine (C-C motif) receptor 2; CCL3L1: C-C chemokine ligand 3-like 1 (MIP-1α isoform), a high affinity ligand for CCR5; MHC: Major Histocompatibility Complex; KIR: Killer cell Immunoglobulin Receptor; CCL5: Chemokine (C-C motif) ligand 5; NK: Natural Killer cells.

individuals[34] (Table 1). In addition, an increased production of IFN-γ by natural killer (NK) cells was found in these sex workers,[35] which, together with increased levels of chemokines and alpha and beta-defensins (for a review see ref. 36), indicate a substantial role for innate immunity in the observed protection. Abs may also have an important role since in MEU HIV-1-specific IgA but not IgG were detected in the genital mucosae, which displayed neutralizing activity[37] (Table 1).

Taken together, these data suggest that local and to a lesser extent systemic, innate and adaptive immune responses may develop upon sexual intercourses with HIV-1 infected partners, which protect from becoming overtly infected. Intriguingly, quitting sex work resulted in diminished CTL frequency and increased risk of infection, suggesting that long-term memory was not induced and persistent antigenic stimulation was required to maintain protective CTL responses[24] or IgA.[37,38] This short-term immunity may also be consistent with allogeneic responses triggered by sexual intercourses with multiple partners, which have been shown to confer transient immunity,[39,40] an observation that has led to the proposal of alloimmunization for HIV-1 vaccine design.[41]

LTNPs are HIV-1 infected people that remain clinically healthy for over 10 years with low (50-2,000 RNA copies/ml plasma, also defined as "controllers") to undetectable (<50 RNA copies/ml plasma, also defined as "elite controllers") viral load and a minimal loss of $CD4^+$ T-cells in the absence of any anti-retroviral therapy.[42-45] Nonprogression appears to depend on multiple factors. Some LTNPs have mutations in viral genes that influence replication and/or immune control, such as those coding for the regulatory and accessory proteins Nef, Rev, Tat, Vif, Vpr and Vpu.[46-52] The LTNPs harboring viruses with mutations in *nef* are of special interest because of the reported efficacy of vaccination of macaques with an attenuated simian immunodeficiency virus (SIV) carrying a deletion in the *nef* gene (SIVΔnef).[53-55] However, follow-up of the well-characterized Sidney Blood Bank Cohort revealed progression in 3 out of the 8 individuals infected with the very same HIV.[56] Of note, progression was observed also in newborns and a few adult macaques infected with the SIVΔnef.[57] Thus, HIV-1 (and SIV) carrying a mutation in *nef* (and long-terminal repeat) are not safe and, more importantly, the immune responses induced by these attenuated viruses are not effective at efficiently controlling the infection over time. Further attenuations of the SIVΔnef to ameliorate their safety profile reduced the degree of protection from superinfection,[58] indicating that much work needs to be done to implement this kind of vaccine.[59]

As many as 25-30% LTNPs may have specific genetic polymorphisms in the HIV-1 coreceptors CCR5 and CCR2, as well as in the gene encoding the CCR5 ligand macrophage inflammatory protein 1-α, which are associated with an impairment of viral attachment and thus infectivity[60,61] (Table 1).

Certain MHC haplotypes (B57, B5801 and to a lesser extent B27) are over-represented in LTNPs[62] and, together with a lower total magnitude and breadth of HIV-specific CTL responses preferentially targeting Gag, significantly correlate with nonprogression.[63] Of interest, human leukocyte antigen (HLA)-B restricted CTLs to HIV are of low avidity, express low levels of the exhaustion marker PD-1 and accounts for the vast majority of polyfunctional CTLs, confirming the relevance of polyfunctionality (especially IFN-γ and IL-2, see below) and shedding some light on the protective role played by HLA-B alleles.[64] The highest frequency of these low avidity, polyfunctional CTLs was observed in $B57^+$ individuals, which is over-represented in LTNPs. This is somewhat paradoxical in view of the known capability of HIV to downregulate through Nef the expression of MHC-I A and B molecules hampering therefore target recognition especially by low avidity CTLs.[65] However, since B57 is also a ligand for the inhibitory NK receptor termed KIR3DL1, which has recently been shown to strongly associate with nonprogression,[66] it has been proposed that the HIV-driven MHC downregulation may relieve this inhibition, triggering NK cell activity against the virus.[67] It is conceivable that, under these opposing pressures, HIV be forced to limit the degree of MHC downregulation to avoid NK surveillance, thus explaining the persistence of low avidity polyfunctional CTLs, which however keep in check the virus. It will be of interest to determine whether these CTLs are over-represented in LTNPs infected with viruses carrying mutations in Nef that hamper MHC downregulation. Taken together, these findings and

hypotheses suggest that NK cells actively participate with CTLs to the containment of infection and ways to stimulate this arm of the innate immunity should be considered in vaccine design.

Concerning the enhanced and qualitatively different polyfunctional activity [simultaneous production of two or more cytokines (TNF-α, IL-2, IFN-γ, MIP1-β) in conjunction with markers of degranulation, such as CD107a expression] of HIV-specific $CD8^+$ T-cells from LTNPs,[68] the capability of these T-cells to coproduce IL-2 appears the most important feature, possibly related to its ability to sustain proliferation, expanding the number of effector cells.[69,70] Along the same line, virus control has been associated with high frequencies of HIV-specific CTLs with a "nonexhausted" phenotype ($HLA\text{-}DR^{High}$, $CD38^{Low}$), which is consistent with retention of polyfunctionality and a low degree of immunoactivation and a potent ex vivo cytotoxic activity against autologous infected $CD4^+$ T-cells.[71] The importance of polyfunctionality is also suggested by in vitro experiments showing that fully competent autologous monocyte-derived dendritic cells (MDDCs) pulsed with Gag were capable to trigger the expansion of $CD8^+$ T-cells in LTNPs but non in progressors, whose $CD8^+$ T-cells were unable to produce IL-2.[72] These data should be carefully considered when designing therapeutic vaccine trials. In addition to the cytolytic activity, HIV-specific $CD8^+$ T-cells have been reported to secrete unknown anti-viral soluble factor(s) (for a review see ref. 73) that has been associated with nonprogression.[74,75]

$CD4^+$ T-cells are pivotal for induction and maintenance of effective T- and B-cell responses (for a review see ref. 76). Of interest, $CD4^+$ T-cells from individuals that control infection are polyfunctional and secrete higher amount of cytokines (IFN-γ, TNF-α and IL-2) as compared to monofunctional cells.[77] These findings highlight the importance of multi-parametric analysis of HIV-specific immune responses in order to correctly identify the functional properties relevant to protection or control of infection. However, whether these immunological features play a key role in controlling the infection or merely reflect a preserved immune system remains to be elucidated. To gain more insights into host genetic factors impacting control of infection, Consortia are being formed to allow analyses of large cohorts of patients.[78-80] Results from these studies indicate that control of infection is strongly influenced by host genetic factors, of which only some are related to adaptive immunity, underscoring the present need to search and identify novel immunological mechanisms (Table 1).

Despite a broader neutralizing antibodies (NAbs) response was reported in LTNPs as compared to patients with progressive disease,[81,82] no apparent role for NAbs in suppression of HIV replication could be demonstrated in elite controllers as well as in patients responding to HAART.[83] Nevertheless, plasma from LTNPs has been recently screened with random peptide phage libraries in order to identify mimotopes capable of inducing relevant NAbs against conformational epitopes.[84]

Another valuable model to investigate correlates of protection is represented by SIV infection in the natural host in which the virus typically does not induce AIDS despite chronic high levels of virus replication. A better understanding of the mechanisms underlying the lack of disease progression in African green monkeys (AGM)[85] and sooty mangabey monkeys (SM) may provide clues to the pathogenesis of immunodeficiency in HIV-infected humans.[86-88] Recent findings support the idea that in the natural host a strong and rapid immunosuppressive response mediated by regulatory T-cells (Tregs) abrogates immune hyperactivation, resulting in a more benign disease outcome.[89,90] Since control of infection appears to occur early after infection, natural immunity is also believed to be key, either by combating directly the virus, or by instructing an effective adaptive immune response.[91] Strikingly, protection from progression may occur in these animals despite severe loss of $CD4^+$ T-cells both in the blood and in the mucosae, indicating that $CD4^+$ T-cell depletion per se is not sufficient to drive progression and that these species have evolved mechanisms to compensate for the T-helper loss.[92-94] Low levels of T-cell activation and apoptosis, preserved lymph nodes architecture and no sign of immunodeficiency were the hallmarks of nonprogression in these monkeys. Taken together, the findings in human and nonhuman primates suggest that blunting of initial inflammatory response and immune hyperactivation correlate with maintenance of polyfunctional T-cells and lack of phenotypes associated to progression (loss of

polyfunctionality and especially of IL-2 production, upregulation of CD38, PD-1, CTLA-4, downregulation of CD107) and should therefore be the goals of vaccines aimed at containing the virus. As a corollary, immunization strategies resulting in sustained vaccine antigen load and immune hyperactivation may be detrimental. Finally, some concerns about the criteria used to define protection as control of infection. In fact, the above studies in the natural hosts as well as those in vaccinated animals[95,96] question the concept that plasma viremia levels and $CD4^+$ T-cell counts are always reliable indicators of vaccine efficacy. Extended follow-up of "bona fide" protected animals is therefore recommended.

What We Have Learned from Vaccination Studies

Although the correlates of protective immunity are still elusive,[97,98] there is substantial evidence that preventive and therapeutic vaccine approaches elicit both humoral (broadly NAbs)[99] and cell-mediated (T-helper and -cytotoxic) responses[100] (Table 2). NAbs are believed not to play a pivotal role in early HIV-1 clearance in the natural infection, since they appear when substantial destruction of the gut associated lymphoid tissue (GALT) has already occurred and the virus has colonized virtually every tissue.[101,102] Conversely, it is conceivable that the induction of a robust Ab response by vaccinating prior to exposure to the virus may protect from infection.

In order to prevent infection by a diverse range of HIV-1 isolates, a vaccine must elicit Abs that are broadly neutralizing. The HIV Env glycoproteins gp120 and gp41 mediate binding and entry into target cells and are the main viral targets for NAbs. However, the domains of gp120 and gp41 which are critical to cell attachment and entry are hidden from the attacking Abs, thus enabling HIV to evade neutralization.[103]

Nevertheless, a few monoclonal Abs (mAbs) with broad neutralizing activity have been identified in HIV-1 positive patient sera and well characterized (2F5, 4E10, 2G12 and b12).[104-106] Passive transfer of these mAbs protected neonatal macaques from subsequent challenge with SIV[107] and delay viral rebound after anti-viral treatment interruption in acutely and chronically infected patients.[108] However, polyspecific reactivity with autoantigens, reported for 2F5 and 4E10, the two NAbs targeting the membrane proximal external region of gp41, suggests that their production might be regulated by tolerance mechanisms.[109] This raises doubts about the feasibility and safety of inducing in healthy individuals Abs with these specificities.[110]

Although NAbs do not appear to contribute to the control of viremia in acute HIV-1 infection, Abs to the Env can be detected at the time of reduction of plasma viremia and other effector functions of Abs may play a role in viral clearance.[111] In fact, significant reduction of set-point viral loads and preservation of central memory $CD4^+$ T-lymphocyte counts were observed in rhesus macaques challenged with a pathogenic SIV (SIV_{mac239}) and passively immunized 7 days later with SIV_{mac239} specific NAbs, despite the rapid disappearance of the NAbs from the plasma.[112] Further, recent data indicate that HIV-1 infected primary cells and in particular macrophages and dendritic cells (DCs), are more susceptible than cell lines to Ab effector functions, including neutralization,[113] suggesting that the effectiveness of the Ab responses elicited so far by vaccination might have been underestimated. Moreover, there is evidence that immunization may induce memory B-cell responses that can last for several years and that can be boosted upon recall vaccination.[114,115]

Finally, antibody-dependent cell-mediated cytotoxicity (ADCC) has recently been reconsidered as an important Ab effector function that may contribute to HIV control (for a review see ref. 116). ADCC is an immune mechanism in which Abs bind target cells, making them vulnerable to the attack by immune cells carrying receptors for the Fc portion of the Ab (FcR) such as NK cells, γδ T-cells, neutrophils and macrophages. Although ADCC against HIV and SIV has been reported since many years,[117-123] only recently it was shown to correlate with protection against SIV in a prime/boost AIDS vaccine approach in rhesus macaques.[124] More recently, the protection afforded by passive immunization with the broadly neutralizing mAb b12 against an intravaginal challenge with the R5-tropic $SHIV_{SF162P3}$ was strongly diminished in monkeys immunized with a b12 variant devoid of FeR binding activity,[125] underscoring the relevance of additional Ab effector functions even in the context of neutralization. This is of importance for several reasons: (i) Abs

Table 2. Key scientific issues to successfully develop an Env-based vaccine according to a Working Group convened by the Global HIV Vaccine Enterprise[†]

Structure-Assisted Immunogen Design	- To improve understanding of the structural basis of antibody binding to the HIV-1 Env glycoprotein - To stabilize gp120 into more immunogenic forms or to scaffold conserved neutralization epitopes into foreign proteins - To identify epitopes capable of inducing neutralizing antibodies or antibodies contributing to neutralization (additive or synergistic effect)
To Assess the Role of Fc Receptors and Complement	- To standardize assays that measure these anti-viral activities - To assess their biologic relevance in passive protection experiments in animal models using antibodies that exhibit the different effector functions in vitro
Assay Standardization and Validation	- To standardize and compare neutralizing antibody assays in order to identify the most reliable assay or combination of assays to measure neutralization - To use more than one assay to assure that all neutralizing antibodies are detected - To prioritize standardization of the PBMC assay because it is the only one partially validated in passive antibody experiments in animal models - To validate neutralization assays based on new technologies in passive antibody experiments in animal models - To generate new SHIVs from nonclade B viruses to address the above issues
Immunoregulation of B-cell Responses	- Better understanding of B-cell responses regulation (and dysregulation) is needed in order to identify the best way to induce long-lasting neutralizing antibodies - To identify genes that are associated with the wide variation in neutralizing antibody responses in HIV-1-infected individuals and in vaccine recipients - To study in nonhuman primate models the potential functional contributions of B-cells to HIV infections - To establish a research consortium to study fundamental B-cell biology as it relates to HIV-1 vaccines

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do not need to be neutralizing, (ii) effector cells belong to native immunity and are therefore ready to go, (iii) ADCC does not attack the virus, but clears infected cells displaying viral antigens, a mechanism reminescent of T-cell immunity. However, unlike CTLs, ADCC does not distinguish infected cells from uninfected ones on which viral particles or antigens are absorbed (for example, soluble gp120 on membrane CD4). Despite this limitation, it is conceivable that a preventive vaccine inducing high titers of Abs mediating ADCC should be effective at curbing viral replication at the very beginning, tipping the balance in the virus—host dynamics in favor of the host.

Although these recent advancements have spurred new interest in the feasibility of inducing protecting Ab responses, many vaccine approaches, such as those based on DNA and viral vectors, have recently focused on the induction of cellular immune responses.[6,126-128] The rationale is that a cellular immune response against HIV, although unable to provide sterilizing immunity, should hopefully enable vaccines to control virus replication following infection, contain viral load, slow down progression toward disease and reduce the probability of secondary infections.[129]

Long-lived memory virus-specific T-cell responses have been shown to be critical to the control of viral replication in many chronic infections including cytomegalovirus, Epstein-Barr virus, human papilloma virus, hepatitis C virus and also HIV (for a review see ref. 29). Despite a large body of evidence suggesting that CTLs,[130,131] T-helper cells[130,131] and Tregs[91] play an important role in controlling infection, direct proof is still lacking. Further, in contrast to Ab responses, T-cell responses are technically more difficult to investigate. In fact, in most vaccine studies IFN-γ ELISpot is used to evaluate CTLs because of its ease, robustness, reproducibility and sensitivity.[132-134] However, while this assay is suitable to measure vaccine immunogenicity, IFN-γ production by CTLs alone did not correlate with control of infection[135,136] (for a review see ref. 137). Similarly, multimer staining technology has provided powerfully insights into dynamics of epitope specific CTLs and their pressure to select escape viral variants. However, this technique requires knowledge of MHC haplotypes and at present it can be applied to a limited number of specificities, making it unsuitable to monitoring of the global T-cell response.

More recent advancements in the measurement of T-cell responses demonstrated that polyfunctional $CD4^+$ and $CD8^+$ T-cell responses are a much better correlate of control of infection or survival. Prolonged (4 years) control of SHIV-89.6P infection in macaques primed with DNA and boosted with modified vaccinia virus Ankara (MVA), both encoding Gag, Pol and Env of SHIV-89.6P, correlated with low breadth and frequency of polyfunctional (IFN-γ and IL-2) $CD4^+$and $CD8^+$ T-cells.[138] The low breadth and frequency of these responses might reflect the limited antigenic stimulation due to the strong suppression of virus replication. However, it might also be due to containment of the detrimental immunoactivation observed in pathogenic HIV and SIV infections, since naturally SIV-infected sooty mangabeys, which have a high viral load but limited immune activation, develop a similar pattern of $CD8^+$ T-cell responses.[139] In a SIV model, the prolonged survival of rhesus macaques vaccinated with plasmid DNA followed by boosting with replication defective adenoviral vectors encoding SIV Gag, Pol and Env and challenged with SIVmac251 correlated with the magnitude and preservation of Gag-specific polyfunctional central memory $CD4^+$ and $CD8^+$ T-cells, while set-point viral load was not predictive.[96,98] In a similar study, preservation of polyfunctional $CD4^+$ T-cells during the first two weeks of infection was a strong predictor of prolonged survival and it was associated to the rapid appearance of Abs neutralizing the challenge virus, which may have contributed to the significant reduction of set point viral load observed in vaccinated animals.[140] Intriguingly, this prolonged survival occurred despite vaccinated animals underwent a substantial, although not massive, $CD4^+$ T-cell depletion in the gut, suggesting that CD4 loss in the gut is not invariably associated to progression, as indicated by the studies in natural hosts.[92-94] Taken together these data suggest that preservation of virus-specific polyfunctional T-cells is an important predictor of control or prolonged survival. The differences observed in these studies may be due to the different vaccine strategies, which may have different correlates of protection, as well as to the virus type and dose chosen for the challenge (for a review see ref. 14). The relevance of the challenge virus is suggested by the monovalent Ad5-gag vaccine developed by Merck (MRKAd5), which was shown to elicit a potent CTL response in rhesus

monkeys and protect from disease progression upon intravenous challenge with the pathogenic SHIV-89.6P,[141,142] but to a much lesser extent against an intrarectal challenge with SIVmac239.[143] Notably, the limited protection upon SIVmac239 occurred in the presence of T-cell responses that correlated with protection in the former study.[144] Thus, the recent failure (no reduced transmission or viral load in vaccinees without pre-existing immunity to the vector) of the Phase II clinical trial based on the MRKAd5 trivalent vaccine would suggest SIV as a more rigorous challenge virus and better predictor of vaccine efficacy in human.[145,146] An additional factor complicating the identification of correlates of protections is the possibility that T-cell responses effective at controlling progression in LTNPs, when induced by vaccination prior to infection may actually favour the selection of escape mutants and accelerate progression.[147] Such an acceleration may also occur because of the expansion of HIV-specific $CD4^+$ T-cells induced by vaccination, which actually increases the number of susceptible target cells to the incoming virus.[148] Thus, despite major advancements in the field, solid correlates of protection are still lacking. The recent introduction in the HIV vaccine field of midsize Phase IIb test of concept (TOC) trials basically acknowledges this weakness and is aimed at obtaining valuable information about vaccine efficacy before moving to much larger, longer and costly Phase III efficacy trials.

General Strategies Adopted to Induce Protective Immunity

Vaccines Aimed at Inducing Neutralizing Abs: Vaccines Based on HIV-1 Env Protein

Former subunit vaccine candidates were mainly focused on the use of the structural protein Env as the immunogen aimed at blocking virus adsorption to the target cells by inducing broadly NAbs.[99,149] However, vaccination with the monomeric HIV Env subunit (gp120), elicited Abs that were able to neutralize lab-adapted but not primary virus isolates[150] and homologous but not heterologous viruses in preclinical challenge models.[151,152] Thus, despite numerous attempts, these approaches have failed so far in eliciting durable, cross-clade NAbs needed to achieve sterilising immunity, as recently soberly confirmed by the failure of the first two Phase III clinical trials of gp120 envelope subunits, AIDSVAX B/B and AIDSVAX B/E by VaxGen, tested in over 5,000 at-risk volunteers in the United States and in Thailand, respectively.[153-156] The reason for this failure was likely related to the complex structure of Env and its high variability.[157-159] Further, heavy glycosylation of the gp120 molecule creates a glycan shield, protecting the protein from incoming NAbs, a phenomenon unknown at the time of the first Env immunizations.[160] In fact, Env hides its Ab binding sites under the protein loops and the heavily glycosylated sites on its surface, hampering recognition of relevant, mostly conformational, epitopes by NAbs.[161-163] Despite the tremendous effort and the sobering results, HIV-1 Env remains a key target for new vaccine strategies (Table 2). In the recent years much effort has been devoted to the construction of oligomeric (trimeric) forms of Env which closely mimic the structure of the native protein present on the viral envelope[164,165] and have been shown to be superior at inducing Abs directed towards conformational epitopes[166,167] and capable of neutralizing both T-cell-line adapted (X4) and selected (R5 and X4) primary isolates of HIV-1,[168,169] as compared to the monomeric gp120.[170] However, Env trimers are extremely unstable and several approaches have been undertaken to stabilize them. In one approach, the gp120-gp41 cleavage site was disrupted by mutagenesis, generating an uncleaved form of gp140 ($gp140_{UNC}$).[171-176] Some of these uncleaved forms of the Env proteins were moderately superior to monomeric gp120 for induction of NAbs in small animal models.[167,177-180]

In another approach, an intermolecular disulfide bond between gp120 and gp41 (SOS gp140) was introduced, with an expectation to induce both neutralizing and fusion-blocking Abs.[181-183] Priming with DNA encoding a membrane-bound form of the SOS gp140 protein followed by repeated immunization with the soluble trimers resulted in high titer Abs that neutralized neutralization sensitive lab strains and, to a much lesser extent, primary heterologous HIV-1 strains.[184] Since the gp41-gp41 interactions in SOS gp140 were too weak to maintain the protein in a trimeric configuration,[183] a single residue change, I559P, within gp41 was introduced.[182] This variant of

SOS gp140, designated SOSIP gp140, appear to be fully cleaved, to be predominantly trimeric and to have favourable antigenic properties.[165] In a recent study, comparison of the immunogenicity of SOSIP gp140 trimers with uncleaved gp140 trimers and monomeric gp120 using a DNA prime-protein boost immunization regimen in rabbits, indicated that SOSIP gp140 trimers were superior to $gp140_{UNC}$ and gp120 proteins at inducing NAbs.[170,184] and SOSIP gp140 trimers with Env from other clades have been generated, which have comparable or better stability and capability to induce NAbs as compared to a prototypic strain (JR-FL, subtype B).[164,185,186]

In a third approach, heterologous trimerization domains at the terminus of the gp41-ectodomain[187,188] were introduced to stabilize the molecule and 30 aminoacids in the second hypervariable region (V2) were deleted to expose neutralizing epitopes shielded by V2.[189,190] This particular variant, termed ΔV2 Env, has been developed and tested in preclinical models including rabbits and monkeys and is currently being evaluated in a gag + env DNA/PLG prime-ΔV2 Env protein boost preventive Phase I trial.[168,169,177,189-191]

Since critical neutralizing epitopes are displayed very transiently upon binding to CD4, another strategy developed by Merck and by the University of Maryland[192] is based on covalently linked monomeric gp120 or oligomeric gp140 to soluble CD4 or to synthetic mimetics of the CD4 receptor in order to induce the conformational changes that take place upon binding of the virus to CD4 prior to virus entry, thus revealing critical neutralizing epitopes, such as those involved in coreceptor binding. This approach was tested in macaques and shown to elicit broadly cross-reactive NAbs.[193] Finally, broadly NAbs are presently being exploited to select peptides from phage-display libraries that mimics the Env neutralizing epitopes (mimotopes) with the goal of identifying immunogens capable to elicit broadly NAbs (for a review see ref. 105).

Vaccines Aimed at Inducing Cellular Immunity: Vaccines Based on Gag, Pol or Nonstructural HIV-1 Proteins: Rev, Tat and Nef

Due to the obstacles encountered in the preparation of an anti-Env vaccine providing sterilising immunity and the protective role of cellular immunity (see correlates of immunity section), a second generation of vaccines based on the structural protein Gag has been developed, with the concept of inducing strong and broad T-helper and CTL responses, which, would contain virus replication, thereby protecting from disease progression and reducing virus transmission to healthy individuals. In fact, Gag protein seems to be the most potent of all HIV-1 antigens in eliciting CTLs, it is more conserved in its immunodominant epitopes than Env and, most importantly, the breadth of CTLs to Gag but not to other HIV proteins appears to correlate with nonprogression in a large cohort study conducted in South Africa on untreated individuals.[63] Ongoing trials with the Gag antigen show promising induction of cellular immunity in primates and humans (for a review see ref. 194).

A Gag-Pol DNA vaccine has been recently tested and resulted safe and well tolerated. However, no HIV-specific Ab responses and only low-magnitude HIV-specific T-cell responses were detected.[195] Further, evidence from preclinical testing of a Gag/Env DNA vaccine in monkeys indicate that initial control (i.e., undetectable plasma viremia level) against challenge with the pathogenic virus SHIV89.6P was overcome over time by the appearance of escape mutants despite apparently preserved anti-viral humoral and cellular responses.[196,197] Alternative strategies have been recently considered, based on the new concept of "reverse vaccinology" with the aim of blocking virus replication and disease onset by targeting nonstructural HIV regulatory genes such as Tat, Rev and Nef, which are essential for replication and infectivity.[153]

In particular, these proteins share desirable features for the generation of a promising vaccine since they exert key functions in the early virus life cycle, contribute importantly to infectivity and pathogenicity, induce a broad immunity and they are highly conserved in their immunogenic domains across HIV-1 clades[198,199] (Table 3). In fact, these proteins are produced very early after infection, Tat and Nef even before HIV integration in quiescent T-cells in which they promote cellular activation and viral replication.[200] Emerging data indicate that, despite their small size, regulatory and accessory proteins are targeted by cellular immune responses very early in the course

Table 3. Rationale to use Rev, Tat, and Nef for an HIV/AIDS vaccine

Evidence	Description	Reference
Pathogenetic	Rev, Tat and Nef are expressed very early and strongly dysregulate the immune system contributing importantly to the establishment of infection and to disease progression	15,200,205,241,251
Epidemiological	In asymptomatic individuals responses to these nonstructural proteins significantly correlated with non-progression to disease	74,201,225,229,481
Immunological	Rev, Tat and Nef are conserved in their functional and immunogenic regions (both B- and T-cell epitopes). Further, Tat and Nef display immunomodulatory effects on APCs exerting adjuvant effects and determining the type of immune response elicited	198,212,215,216,482
Preclinical	Tat, Rev and Nef, either alone or in combination, have demonstrated in preclinical models to be safe and to elicit broad and specific immune responses and, more importantly, to control viral replication and to block disease progression	For a review, see refs. 240,483
Clinical	Tat, Rev and Nef either alone or in combination have demonstrated in Phase I trials to be safe and to elicit broad and specific immune responses	For a review, see ref. 240

of natural HIV-1 infection and contribute importantly to the total HIV-1-specific $CD8^+$ T-cell responses, since multiple CTL epitopes have been identified in functionally important regions of these proteins.[201-204] Furthermore, they have immuno(dys)regulatory effects aimed at facilitating target cell recruitment and activation, further promoting HIV replication and spreading.[205-207] Of note, Tat and Nef are also found extracellularly and in this form they exert effects on different cell types, including chemotactic activity for HIV target cells.[208]

In particular, extracellular Tat can enter both infected and uninfected cells, where it promotes HIV replication or modulates the expression of cellular genes, respectively (for a review see ref. 209). Among others, extracellular Tat upregulates the expression of chemokine receptors and HIV coreceptors, CCR5 and CXCR4.[210,211] Extracellular Tat has also important effects on immunoregulatory functions (for a review see ref. 209) (Table 3). In particular, bioactive soluble Tat selectively binds and enters both immature and mature DCs (iDCs and mDCs, respectively), drives iDCs maturation and activation toward a T-helper 1 (Th-1) inducing phenotype,[212] gains access to the MHC class I pathway of presentation,[213,214] and modulates the proteasome catalytic subunit composition, modifying the hierarchy of the CTL epitopes presented in favor of subdominant and cryptic epitopes.[215,216] This latter activity might be of relevance since one way used by HIV-1 to escape CTL recognition is to mutate residues in the epitope that prevent or impair processing and presentation.[217,218] Accordingly, in the majority of the multiply exposed uninfected sex workers of the Nairobi cohort, CTLs recognize epitopes that are either subdominant or not recognized in infected women.[24] It remains to be seen whether Tat contributes to it in the course of natural infection, whether targeting Tat impacts on this type of immune evasion, or whether this property of Tat may be exploited to induce broader T-cell responses by including it in HIV/AIDS vaccines targeting other antigens. Indeed, preliminary data indicate that in mice co-immunization with Gag and Tat induces CTL responses against 11 different T-cell epitopes, as compared to mice vaccinated with Gag alone, which only responded to 6 epitopes.[219] Both cellular and humoral Tat-specific immunity may contribute to the control of infection and/or disease progression. Because HIV-infected cells express Tat very early after infection, vaccine-induced anti-Tat CTLs may eliminate infected cells and block HIV infection at an early stage.[220] In fact, rapid induction of anti-Tat CTLs has been reported in naïve rhesus macaques acutely infected with the pathogenic SIVmac239 molecular clone, leading to the selection of apparently less aggressive virus variants.[221] Notably, anti-Tat CTLs were found more effective than anti-Gag CTLs at suppressing virus replication in Mamu-A*01 rhesus macaques.[222] Of outmost importance, these data have been recently confirmed in patients enrolled prior to seroconversion and in which a strong temporal correlation between anti-Tat CTLs appearance (as early as 8 days postinfection), viral load decline and $CD4^+$ T-cells recovery was found.[74]

Consistent with this hypothesis, the presence of Tat-specific CTL responses correlates with nonprogression to AIDS both in SIV-infected monkeys and in HIV-positive individuals.[223-226] Furthermore, anti-Tat Abs can sequester the extracellular protein, thus preventing the extracellular Tat-driven enhancement of infection and immune dysregulation associated with them. Strikingly, anti-Tat Abs, which are found only in a minority (15-20%) of HIV-1 infected individuals, are almost exclusively present during the asymptomatic phase of infection and correlate with nonprogression to AIDS.[227-231] Whether this indicates that neutralization of extracellular Tat may impact on disease progression or is merely a reflection of an underlying effective and broad immune response is currently under investigation. Tat is also highly conserved in its immunodominant domains, as suggested by the observation that sera from Ugandan and South African individuals infected with nonclade B HIV-1 strains cross-react with the Tat protein of an HIV-1 clade B strain.[198]

Vaccines based on HIV-1 Tat (both protein and DNA) have proven to be safe and immunogenic in mice and protective in monkeys.[232-236] However, these results have not been confirmed by other investigators utilizing different Tat formulations and vaccination strategies.[237-239] Whether these apparently conflicting results are due to the nature of the vaccine antigen (DNA, native versus inactivated Tat protein, vectored antigen), the monkey species, the route of the administration,

the antigen dose and schedule of immunization, the adjuvant used, or the virus challenge dose, still remains to be elucidated.[240]

Rev is also absolutely required for HIV replication since it facilitates the nuclear export of intron containing viral mRNAs allowing the transition from the early to the late phase of gene expression and proviruses lacking Rev do not produce virions.[241] While it is presently unknown whether Rev is released extracellularly and exerts effects on neighbouring cells, Rev, like Tat and Nef, is often targeted by CTLs in HIV-positive individuals[201] and is broadly conserved among different HIV-1 clades in its functionally constrained and immunodominant domains at the N terminus.[242] However, spontaneously occurring mutations in Rev reduce HIV-1 structural gene expression to levels undetectable by CTLs and may represent a mechanism to escape immune recognition.[205] Of importance, HIV-1 Tat and Rev are the dominant viral proteins produced before Nef down-regulates MHC class I molecules on the cell surface, hampering recognition of infected cells by CTLs[65] (Table 3). Vaccine based on Rev and Tat has proven protective in monkeys.[243] Rev alone or in association with Nef and Tat has been used for therapeutic vaccination and resulted to be safe and immunogenic in HIV-1 infected individuals.[244-246]

HIV-1 Nef protein is a myristoylated, membrane-associated cytoplasmic protein abundantly expressed in the early phase of HIV-1 replication and released in the extracellular milieu.[247,248] Nef protein serves multiple functions and is likely to contribute to viral pathogenesis by downregulating CD4 and MHC class I on the surface of infected cells[249] (Table 3). Among its biological activities, Nef mediates receptor down-regulation, T-cell activation and cytoskeleton rearrangement.[250,251] The Nef protein also enters B-cells and suppresses immunoglobulin class-switch contributing to the evasion of protective T-cell-dependent IgG and IgA responses.[252] In addition, Nef interferes with the ability of CTLs to kill infected T-cells by decreasing the surface expression of MHC class I on HIV-1-infected T-cells[65] and favours spreading of HIV to T-cells by increasing the expression of DC-SIGN on DCs, which traps infectious HIV particles[253] and by promoting DC maturation.[254] Further, differently from Tat, Nef induces FasL upregulation and apoptosis in bystander cells both in vitro and in vivo,[255] whereas it selectively spares infected cells,[256] contributing to immune evasion and pathogenesis. Finally, infection of MDDCs with a wild type but not with a nef defective HIV-1 induces the release of soluble factors recruiting and activating lymphocytes, which consequently become targets for productive HIV infection.[257]Taken together these data strongly support the view of Nef as an important vaccine candidate alone or in association with other HIV antigens (for a review see ref. 240). Immunization with plasmid DNA or a MVA vector expressing Nef was demonstrated to be safe and immunogenic in preclinical and clinical studies.[258-260]

Vaccines Combining Structural and Nonstructural HIV-1 Gene Products

Experimental evidence on the role played by regulatory and structural HIV gene products in HIV infection and pathogenesis represents the rationale to develop new vaccination strategies based on the combination of the two classes of proteins (Table 4). These strategies range from a "minimalistic" approach in which only two antigens, one regulatory (Tat or Nef) and one structural (ΔV2-Env) HIV protein are combined, to a "maximalistic" one, which is aimed at imitating a live attenuated vaccine and therefore combines many HIV structural and nonstructural genes. Several of these new generation vaccines are currently under evaluation in preclinical and clinical trials in the context of the European AIDS Vaccine Integrated Project (AVIP) (http://www.avip-eu.org).[261-266] Such combined vaccines should be able to generate immune responses to both viral products, which are expressed early (regulatory proteins) or late (structural proteins) during the viral life cycle, thus maximizing immune targeting of viral infection. The criteria for an advancement of any of these combined vaccines towards Phase II/III trials in Developing Countries are safety and the demonstration of stronger and broader immune responses against each antigen, compared to those elicited upon immunization with each antigen separately.

In particular, the combination of the regulatory HIV-1 protein Tat with the structural protein ΔV2-Env, represents one of the most recently developed HIV vaccine candidates.[261] Preclinical studies in mice and monkeys have shown that the Tat/ΔV2-Env vaccine is safe, immunogenic and

Table 4. Combined vaccine candidates to be tested in Phase I trials within the AVIP Consortium: previous work and development stage of single components

Combined Vaccine Candidates	Single Components	Mice Immunogenicity	Mice Efficacy[1]	Monkey Efficacy	GMP Development	Approval for Human Use	Clinical Trials (Performed or Ongoing)[2]
Tat + ΔV2Env[3] (clade B)	Tat	+	-	+	+	+	Completed (P + T)
	ΔV2Env (clade B)	+	-	+	+	Pending	To be started (P)
Nef + ΔV2Env (clade B)	MVA[4]-Nef	+	+	+	+	+	Completed (T)
	ΔV2Env (clade B)	+	-	+	+	Pending	To be started (P)
Nef, Rev, Tat, Gag, RT, Env	Nef, Rev, Tat	+	+	+	+	+	Completed (T)
	Gag, RT, Env	+	+	-	+	+	To be started
Multi HIV B-clade Ags and epitopes[5]	Multigene[6]	+	+	-	+	+	Completed (P + T)
Multi HIV A-clade Ags and epitopes[5]	Multigene	+	+	-	+	Pending	To be started
Multi HIV C-clade Ags and epitopes[5]	Multigene	+	+	-	+	Pending	To be started
Multi HIV FGH-clade Ags and epitopes[5]	Multigene	+	+	-	+	Pending	To be started

[1]Mice efficacy is evaluated as the capability of HLA-A2 transgenic C57Bl/6 mice to eliminate the engraftment of HIV-1/MuLV-infected syngeneic splenocytes injected intraperitonally;[484] [2]P: Preventive; T: Therapeutic; [3]ΔV2Env: Env deleted in V2 (see text for further details); [4]MVA: Modified Vaccinia Ankara virus; [5]Multi HIV, also termed MultiHIV DNA vaccine is a plasmid expressing an antigenic fusion protein composed of the regulatory HIV-1 proteins Rev, Nef and Tat, Gag p17/p24 and a stretch of 11 cytotoxic T-lymphocyte (CTL) epitope clusters from Pol and Env, which was cloned into a novel DNA vector named the Gene Transport Unit (GTU). Four different plasmids expressing the same immunogens but originating from subtypes A, B, C consensus, or FGH ancestral sequences, are currently under evaluation; [6]Multigene is a cocktail of seven plasmids encoding clade B Nef, Rev, Tat, RT, clade A and B Gag and clade A, B and C Env proteins.

protects monkeys against an intrarectal challenge with $SHIV_{SF162p4}$ (ref. 267 and Ensoli B, unpublished data) or an intravenous challenge with SHIV89.6P.[268] Based on these promising results, a Phase I clinical trial will start in 2008. The minimum criterium of success for Env-specific responses will be the induction of NAbs against the vaccine strain (i.e., homologous neutralization) in at least 50% of the trial participants.

The other minimalistic approach developed within the AVIP consortium is based on the combination of Nef and ΔV2-Env[264] (Table 4). This vaccine is composed of the *nef* gene inserted into the MVA (see below) in combination with the same ΔV2-Env protein mentioned before. As for the Tat/ΔV2-Env approach, the Nef/ΔV2-Env vaccine will be administered in HIV-negative volunteers, seeking to induce mostly anti-Nef cellular immunity and anti-Env humoral immunity, in particular NAbs, to prevent (or reduce) virus entry and to control virus replication. As for the Tat/ΔV2 Env vaccine, criteria for advancement of the Nef/ΔV2 Env vaccine beyond Phase I will be proven safety and broader and more potent immune responses against the components of the combined vaccine, as compared to those obtained upon vaccination with the single antigens. Phase I trials with Nef or ΔV2-Env alone have been completed[189,259] preclinical testing in macaques of the Nef/ΔV2-Env combined vaccine will be carried out in 2008 and preventive Phase I studies will follow shortly.

The multigene strategy represents another attractive vaccine approach based on the design of a cocktail of genetic immunogens (DNA constructs) encoding several viral components from various subtypes of HIV-1, including structural and regulatory proteins as well as viral enzymes.[266] Two multigene approaches are being evaluated in the AVIP Consortium, one containing a cocktail of seven plasmids encoding Nef, Rev, Tat, Gag, RT and Env antigens, termed HIV multigene;[266] the second one, based on the genes coding for Rev, Tat, Nef, Gag, p17, p24 full length antigens, also includes over 20 T-cell epitopes from Pol, Protease and Env antigens and is therefore termed Multi-HIV antigens/epitopes.[265] A prophylactic Phase I trial with the HIV multigene was recently conducted in Sweden and shown to be safe and highly immunogenic. More than 90% of the 38 volunteers mounted T-cell responses (proliferative and IFN-γ ELISpot responses) against the vaccine antigens upon administration with a needle-free device (Biojector) of DNA encoding Env (clade A, B, C), Rev (B), Gag (p17/p24, A and B), RT (mutated, B) followed by a boost with MVA expressing Env, Gag and Pol of CRF01A_E.[269] Based on these encouraging results showing that this administration strategy and the use of GM-CSF as adjuvant increases the immunogenicity of a DNA prime followed by MVA boosting in human, a new preventive Phase I/II study started in 2006 in Tanzania.[270] As part of the AVIP program, a therapeutic Phase I-II trial has recently started in UK and Sweden with the aim of comparing in individuals infected with HIV clade B the immunogenicity of the multigene vaccine based on clade B antigens with that of the clade A-C multigene vaccine. Of note, both vaccines include a newly developed plasmid encoding Tat/Nef as a single fusion protein.[266] The Multi-HIV antigens/epitopes approach exploits a novel delivery system termed gene transport unit (GTU), a proprietary technology of FIT BIOTECH, which increases the immunogenicity of DNA vaccines thereby avoiding the need for an heterologous boosting.[265] Phase I studies carried out in Finland have proven that GTU-MultiHIV (B clade) is safe and immunogenic in healthy and HIV-1 infected individuals and it is currently being tested in a therapeutic Phase IIa clinical trial in South Africa.[265] A second generation vector, called Auxo-GTU, was more recently prepared and a constructs expressing multiple antigens and epitopes from several clades (A, B, C, FGH) was made and it is going to be evaluated in a Phase I/II trial in 2008.

To address the issue of viral diversity, a novel strategy to broaden cross-clade T-cell responses has been recently proposed. According to this strategy, polyvalent vaccines can be made that comprise a mosaic of several naturally occurring sequences computationally optimized to include the maximum number of potential T-cell epitopes from relatively conserved and immunologically relevant HIV-1 proteins.[271] In vivo testing will verify the validity of this approach.

Key Issues Relevant to HIV Vaccine Development: How to Get the Right Responses in the Right Places

Mucosal Vaccines

Since most of the HIV-1 infections are caused by mucosal transmission both horizontally (sexual intercourse) and vertically (child delivery and breast-feeding), an AIDS vaccine must primarily elicit a robust immune response at the mucosal surfaces.[110] Most of the AIDS vaccine candidates that are currently in clinical trials around the world are delivered by intramuscular or intradermal injection. These routes of administration induce Ab and cellular immune responses in peripheral lymphoid tissues (systemic immunity), although evidence in human of mucosal responses upon intramuscular vaccination with a recombinant canarypox HIV-1 vaccine have also been reported.[272] Furthermore, studies of SIV infection in macaques indicated that, regardless of the route of infection, the gastrointestinal and vaginal mucosae represent the major site of virus replication and amplification and the initial sites of $CD4^+$ T-cell depletion.[273-275] In particular a rapid depletion of $CD4^+$ T-cells has been observed in the vagina of SIV-infected macaques, particularly among the $CCR5^+$ $CD4^+$ subset that is the preferential target for elimination by SIV infection.[276]

Several studies indicated that secretory IgA inhibit virus assembly and intracellular release and play an important role in inhibiting HIV transmission via the mucosal route, in multiply exposed females.[277,278] Further, multiple rectal exposures to low doses of SIV induced MHC class I-restricted cytotoxic responses that protected against a mucosal challenge with an heterologous virus.[279] Similarly, studies in the macaque model provided insights on the protective role of high-avidity mucosal CTL responses generated upon intrarectal vaccination.[280,281] Taken together these data suggest an important protective role for IgA and CTLs at the portal of virus entry. In this regard, mucosal immunisation appears to be more effective than systemic vaccination at eliciting humoral (IgA and IgG) and cellular ($CD8^+$ CTLs) immune responses.[282,283] However, not all the mucosal routes of vaccine administration are equally good at inducing immune responses at the different mucosal surfaces in the body. For instance, oral vaccines are effective at preventing infections that primarily target intestinal tissues, but are not very efficient at inducing IgA in the vagina, one of the main ports of entry of HIV-1.[284] A few studies have suggested that local administration of protein vaccines to the mucosa of the genitourinary tract induces a weak to modest local immunity at the site of immunization.[283,285] In contrast, intranasal (IN) immunization has been shown to induce local immunity not only in the nasal-associated lymphoid tissue and lung, but also in the female genital tract in rodents,[286,287] human[288] and nonhuman primates.[289] Of note, it has been recently reported in the mouse that the nasal associated lymphoid tissue is also an inductive site.[290] Taken together, these data make this type of immunization, which is more practical than vaginal vaccination, appealing to AIDS vaccine researchers. In this regard, immunization of HIV-1 seronegative women with an Env subunit vaccine administered either intranasally or intravaginally, together with a mucosal adjuvant shown to be effective in mice, failed to induce detectable IgA or IgG.[291] These negative results should not stop the attemps to use the IN route. In fact, various protein-, DNA- and RNA-based immunopotentiating adjuvants/delivery systems as well as of live bacterial and viral vectors, which may differ in their ability to induce a specific type of immune response (e.g., CTLs versus antibody responses) at the desired site,[292] are available for IN immunisation and should be evaluated. However, since the nose is a well-known port of entry for neurotropic viruses, there are safety issues that will need to be fully addressed before testing IN vaccines in clinical trials. Of note, tonsillar immunization with an attenuated SIV (SIVΔNef) induced systemic immune responses and conferred protection upon intrarectal challenge of rhesus macaques with a pathogenic SIV.[293] It will be of interest to determine whether safer vaccine approaches will afford similar protection. Recent reports have suggested that combinations of mucosal and systemic immunizations may enhance both mucosal and systemic immune responses.[294-297]

Mucosal tissues are rich in antigen presenting cells (APCs), specialised immune cells that are involved in the induction and regulation of anti-viral immunity. DCs represent the most potent APCs for naïve T-lymphocytes and they are among the first cells in the body to come in contact

with HIV.[298] These cells are critical in the early phases of infection, working as sentinels, alerting the immune system and controlling its early decisions. Furthermore, they exert a crucial role in the induction and regulation of adaptive immune responses.[299] Therefore, it is critical to design vaccine formulations capable of properly targeting and stimulating DCs to induce strong immune responses at mucosal sites. It is likely that the stimuli received by DCs in the peripheral compartments affect their ability to activate T-cells and/or B-cells as well as the type of T-cell response elicited.[300] Of interest, in the mouse, a single intracolorectal administration of a replication defective adenoviral vector expressing OVA or Herpes simplex virus (HSV)-2 glycoprotein B antigen targeted mucosal DCs, which migrated to the draining lymph nodes and induced adaptive immune responses at the rectal and vaginal level that protected the animals against a challenge (by either route) with vaccinia expressing OVA or a lethal dose of HSV-2, respectively.[301] Similar protection was not afforded by IN, intravaginal, or subcute vaccination, suggesting that adenoviral vectors, which naturally target DCs, may be particularly suitable to induce protective humoral and cellular immune responses at sites that represent the major portals of entry of HIV. It remains to be seen whether these promising results are confirmed in human. In fact, intramuscular immunization with replication-defective Ad5 vectors expressing HIV-1 gag, pol, or nef failed to reduce transmission or lower viral load in high risk individuals that became infected.[146] In this regard, manipulation of antigen presenting cells to elicit virus-specific cellular responses is a promising tool to control persistant viral infections.[302-309] In fact, studies in monkey[310] and human[311] indicate that inactivated whole virus-pulsed DC vaccines may be an effective strategy for treating people with chronic HIV-1 infection.

Delivery Systems

Subunit (proteins or peptides) vaccines are generally very safe, with well-defined components. However, these antigens are often poorly immunogenic and adjuvants are required to induce a measurable and supposedly adequate immunity. Thus, a vast array of delivery systems (e.g., micro/nanoparticles, emulsions, ISCOMS, liposomes, virosomes and virus-like particles), immunomodulators (cytokines, chemokines or costimulatory molecules) and, as mentioned above, even autologous DCs pulsed with viral antigens have been proposed and are presently being used to increase the efficiency of vaccines against HIV/AIDS (Table 5). Furthermore, several highly attenuated replicating and nonreplicating vectors have been or are being tested in a number of preclinical and clinical trials (ref. 240 and Table 6)

These strategies, reviewed elsewhere,[240] have proven effective in controlling viremia and progression to AIDS in nonhuman primates, but observations in early phase clinical trials in humans have not been promising. In fact, some of the trials had to be stopped at various stages due to adverse reactions to the delivering vector[312] or the inability of the expressed immunogen to cover genetically diverse isolates prevalent in the geographical areas.[313] Nevertheless, the outcome of several ongoing clinical trials is expected to deliver good news about safe vaccine delivery vectors and, if possible, an effective vaccine against a particular strain of HIV-1[9] (Table 6). Here we briefly review the different approaches utilized to deliver vaccines that are currently being evaluated in clinical trials (Table 5).

Plasmid DNA

Genetic vaccines (naked-DNA vaccines), employ DNA plasmids as "Trojan horse" vectors to deliver genes that code for HIV epitopes (for a review see ref. 314). These expression vectors remain in their episomal form into the host cell where they produce peptides that induce cellular immunity. Compared to viral and bacterial vectors, DNA plasmids focus the immune response on more narrowly on HIV insert sequences, do not induce (and are not affected by pre-existing) immunity to the vector, are cheap and have several regulatory, safety, handling advantages.[314] Immunization with DNA plasmids containing HIV inserts has been demonstrated to elicit substantial cellular response in mice and nonhuman primates,[315,316] but not in humans.[317]

Therefore, many strategies have been undertaken to enhance the immunogenicity of genetic vaccines, which include delivery systems, modifications of the vaccine construct, formulation with immunostimulatory molecules.[314] In particular promoter modification and inclusion of genes

Table 5. Major HIV-vaccine approaches

Types of HIV Vaccine	Advantages	Disadvantages	Type of Response Elicited
Whole HIV Viruses			
- Killed/Inactivated Viruses	Simple to prepare; they might present HIV surface proteins in a relatively native conformation depending on the inactivation procedure; no mutation or reversion	Little efficacy in nonhuman primates; safety concerns (inactivation efficiency)	Few NAbs, no CTL response
- Live Attenuated Viruses	Mimic natural infection; high levels of protection in animal models	Safety concerns: mutation; potential reversion to virulence	Long-lasting cell-mediated and humoral immunity
Recombinant Viral Proteins (Subunit Vaccines)	Safe; simple and inexpensive to prepare; defined composition (mostly structural proteins)	Immunogenic response is restricted to selected antigens; responses are not durable; no protection in two efficacy trials; adjuvant required	Target humoral immunity, no CTL response
Peptides	These vaccines use small pieces of HIV proteins as an immunogen; safe, inexpensive, potentially useful for broad antigenic diversity	Poorly immunogenic in human trials; stability issue	Poorly immunogenic in human trials; adjuvant required
Naked DNA	Safe; stable; no cold chain required; inexpensive; potential to encode multiple antigens; immunogenic in animals; prolonged immunity; more effective in heterologous prime-boost strategies	Poorly immunogenic in humans; concerns about DNA integration into human cells	Cellular immune responses; heterologous prime-boost strategies needed to induce humoral responses in primates
Viral Vectors	The vaccine is a weakened virus, unrelated to HIV, into which HIV genes are inserted; high-level production of protein antigens directly within cells of the immunized host, potential adjuvant effects of the viral delivery system itself; delivery of antigen directly to components of the immune system, such as antigen-presenting cells	Complicated to prepare; viral escape mutants; potential immunodysregulatory effect of the vector proteins; pre-existing immunity	Depending on the vector, systemic and mucosal humoral and cell-mediated immune responses

continued on next page

Table 5. Continued

Types of HIV Vaccine	Advantages	Disadvantages	Type of Response Elicited
- Poxviruses	Highly immunogenic, they grow to high titers, are very stable when lyophilized and are capable of accepting large transgene sequences; potential to be administered by different routes	Safety concerns	Mucosal and systemic antibody and T-cell responses
- MVA ***- NYVAC***	Safe because they do not replicate in mammalian cells; possibility of introducing large amounts of DNA; effective in nonhuman primate models	Limited immunogenicity and durability of immune responses in humans	Both CTL and Ab responses at both systemic and mucosal sites, depending on the route of immunization
- Canarypox (CPV) ex. ALVAC ***- Fowlpox (FPV)***	Safe because they do not replicate in mammalian cells; no concerns about pre-existing immunity against these vectors in humans	Limited immunogenicity	T-cell responses
- Adenoviruses (Ad)	Safe (replication incompetent Ad), stable; highly immunogenic; wide tropism; high efficiency of cellular uptake; can be administered at mucosal sites	Responses limited by pre-existing immunity (especially to Ad5)	Robust mucosal and systemic humoral and cellular immune responses, especially when replication-competent Ad vectors are used
- Adeno-Associated Viruses (AAV)	They establish a persistent infection in the host	Poorly immunogenic in human; in mice they induce T-cells with an altered phenotype and functionally impaired	Humoral and cellular immune responses
- Alphaviruses: ***- Sindbis (SIN),*** ***- Venezuelan equine encephalitis (VEE),*** ***- Semliki Forest (SFV) virus***	They can infect a large number of animal cell types and transiently express high amounts of viral and heterologous proteins; safe because their replication occurs exclusively within the host-cell cytoplasm; lack of preexisting immunity; ability to induce apoptosis of transduced cells, favoring DC cross-priming	Anti-vector immune response	Humoral and cellular immune responses

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Table 5. Continued

Types of HIV Vaccine	Advantages	Disadvantages	Type of Response Elicited
- **Herperviruses (HSV)**	Durable immunity; ability to activate the innate immune system; ability to accommodate large inserts of DNA	Safety concerns in immunocompromised individuals; pre-existing immunity	Humoral and cellular immune responses
- **Rhabdoviruses (VSV)**	Rapid growth; relatively safe; mucosal efficiency	Potential safety concerns in immunocompromised	HIV specific CTL response
- **Polioviruses**	They grow to high titers; easy to prepare and to deliver orally; stable in the intestinal tract where they infect the mucosal M-cells responsible for antigenic presentation; immunogenic in nonhuman primates	Pre-existing immunity	Mucosal humoral and cellular immune responses
Bacterial Vectors	Simple and inexpensive to prepare; probably safe; they can be administered at mucosal site	Stability issue	Humoral and cellular responses; mucosal immunity
- **Mycobacterium (BCG)**	Safe in humans; easily administered and affordable; able to induce long-lasting immunity; mucosal delivery of HIV antigens; ability to accommodate large inserts; adjuvant activity	Safety issues especially in children and immuno-compromised hosts	Mucosal and systemic humoral and cellular iimune responses
- **Listeria**	Stimulates innate and adaptive immune responses	Safety issues especially in children and immuno-compromised hosts	$CD4^+$ and $CD8^+$ T-cell responses
- **Salmonella**	Safe; inexpensive; offers the benefit of oral delivery; mucosal targeting	Safety issues especially in children and immuno-compromised hosts; poor immunogenicity in humans	Mucosal and systemic humoral and cellular immune responses
VLPs	Safe; mimic the virus particle, displaying HIV proteins in a relatively native conformation	Difficult to prepare	Good cellular and humoral immune responses at systemic and mucosal level
Microparticles	Enhance the bioavalaibility of the antigen, reducing the number of doses in the immunization schedule; targeting of DCs	Instability and manufacturing difficulties	CTL response

encoding cytokines that increase the expansion of antigen-specific T-cells (IL-2 and IL-15), or attract and induce the maturation of APCs (GM-CSF and B-chemokines) have been evaluated (for a review see refs. 318, 319). Furthermore, novel formulations are being pursued to increase the in vivo expression of DNA vaccines and to protect them from rapid degradation, involving adjuvants or carriers such as liposomes, bacterial endotoxin, macroglobulins, CpG oligodeoxinucleotides, peptides and polymers (described below).[320-322] Nevertheless, DNA vaccines recently tested in clinical trials displayed limited immunogenicity[195,323-325] and most DNA vaccines are presently delivered as a prime in heterologous prime-boosts strategies (see below).

Bacterial Vectors

Among bacterial vectors, live attenuated recombinant *Mycobacterium* spp. and enteric bacteria such as *Salmonella* spp. are microorganisms that can be administered at a mucosal surface and should be able to specifically induce mucosal cellular and humoral immune responses.[326] Bacterial DNA vaccine delivery demonstrated in vivo efficacy in several experimental animal models of infectious diseases.[327] Attenuated strains of *Salmonella* spp. have been developed as potential vectors for stimulating immune responses in the gastrointestinal mucosa.[328] An advantage of these vectors is the possibility to exploit the Type III secretion system, a multicomponent system that allow delivery of antigens directly into the cytoplasm, favouring MHC class I antigen-processing and presentation.[329]

A *Salmonella* in which the SIV gag transgene had been fused to a Type III-secreted bacterial protein was used in a Salmonella-prime/MVA-boost regimen to stimulate SIV Gag-specific CTL responses in the gastrointestinal tract of rhesus macaques.[330] Although low levels of CTLs were detected after the priming, upon MVA boosting strong CTL responses were detected in the blood and in the colonic mucosa. However, no protection against intrarectal challenge with SIV_{mac239} was observed.[330] In a Phase I dose escalation trial oral delivery of *Salmonella* expressing HIV Gag resulted safe and induced strong immune responses to *Salmonella* antigens, but modest immune responses to Gag.[331,332]

Mycobacterium bovis Bacillus Calmette—Guérin (BCG) is another promising vector. BCG has a long record of safety in humans and is able to induce long-lasting immunity.[333] However, despite extensive testing in small animals (mice and guinea pigs), evidence in nonhuman primates of promising immunogenicity[334,335] and efficacy against an homologous challenge after a single inoculation of rBCG expressing the $HIV_{MN}V3$ loop,[336] recommendations by WHO and UNAIDS to further explore the use of rBCG as a potential vectored vaccine for HIV,[337] no clinical trial has started yet with this vaccine approach. While pre-existing immunity to BCG does not seem to be a problem, use of this vector in developing countries where *Mycobacterium tubercolosis* (and tubercolosis) is highly prevalent and even BCG vaccination may be fatal in immunodeficient children,[338] raise some concerns on the feasibility of large scale vaccination with this platform.

Another interesting vector is represented by *Listeria monocytogenes* (*Lm*), a facultative intracellular bacterium that enters the cell by phagocytosis and colonizes the cytosol of the host cell.[339] Several properties make *Lm* an attractive HIV vaccine vector. First, this bacterium is a good agent to stimulate innate as well as adaptive immune responses since it specifically infects and induces maturation of DCs. Second, foreign antigens encoded by *Lm* are efficiently processed and presented by both MHC class I and MHC class II molecules, thus activating both $CD8^+$ and $CD4^+$ antigen-specific T-cells.[340-342] Third, *Listeria*-derived vaccine vectors may be given orally.[343] In animal models, oral or parenteral immunization with *Lm* engineered to express a number of HIV/SIV antigens induced strong cell-mediated immune responses, but demonstrated little efficacy against a SIVmac239 challenge in macaques vaccinated against Env and Gag in DNA prime-r*Lm* boost regimen.[339,344,345] However, as *Lm* can cause serious infections in neonates, pregnant women and immunocompromised hosts, different attenuation strategies are being undertaken to overcome safety issues associated with the use of live *Lm* as vaccine vector in humans. In particular, a live attenuated *Lm*[346] and a killed but metabolically active *Lm*[347] have been recently developed and shown preserved immunogenicity and efficacy in tumor mouse models.[346-349] In nonhuman primates,

vaccination with an attenuated strain termed *Lmdd* expressing HIV-1 *gag* induced Gag-specific T-cell responses upon oral administration, whereas combined oral/intramuscular administration induced strong Gag-specific systemic and mucosal Ab responses. This difference was also evident for anti-vector Ab, indicating that the route of administration strongly influences the type of response elicited. Of note, a very late boost failed to induce a robust increase of anti-Gag Ab titers, suggesting that anti-vector Ab may severely limit the vaccine immunogenicity.[350] In this regard, an heterologous DNA prime-oral *Lm* boost strategy appears a more promising approach, in that it induced in rhesus macaques mucosal SIV-Gag-specific $CD8^+$ T-cells expressing the $\alpha4\beta7$ integrin gut-homing receptor.[351]

Viral Vectors

Safety concerns about live attenuated viruses and inactivated vaccines[352,353] have led scientists to look for better and safer ways of making an AIDS vaccine. Most of the more promising AIDS vaccine candidates currently being developed and tested use viral vectors, viruses that are not harmful and act as the delivery system to carry HIV antigens to the immune system.[354] The potential advantage of the viral vector strategy is to mimic as closely as possible the efficacy of live-attenuated vaccines, while at the same time offering much greater safety. Since several of such vectors are replication competent, the emergence of viral escape mutants may represent a concern. Further, although used as vectors, these are actually viruses that have developed multiple and sophisticated ways to modulate and evade the defense system,[355] which may affect the immunogenicity of the transgene, both qualitatively and quantitatively. Also, some of the proteins of the vector may be highly immunogenic, thus hampering the immunogenicity of the transgene. Thus, a better knowledge of these aspects is critical when designing vaccination approaches based on viral vectors. A number of different viruses have been developed as vectors for vaccines. The different vectors all have their own advantages and disadvantages. Among viral vectors, poxviruses and adenoviruses have received the most attention in the design of HIV vaccine.

Poxvirus Vectors

Several poxviruses, relatives of vaccinia (the smallpox vaccine), are attractive vectors since their large genome allows for the inclusion of multiple heterologous genes, including those encoding antigens, costimulatory molecules and cytokines. Moreover, poxvirus vectors may be used for mucosal immunization.[356] Attenuated vaccinia strains such as MVA and NYVAC (derived from the Copenhagen strain by further deletion of 18 open reading frames encoding molecules implicated in pathogenicity and host-range regulatory functions) are the most frequently used poxvirus vectors. These vectors have been shown to be safe in immunocompromised macaques and in human Phase I/II clinical trials.[357-360] Pre-existing immunity to vaccinia is of a limited concern since its use for smallpox vaccination has ended more than twenty years ago. Most of the recombinant HIV vaccine using poxviruses are effective in nonhuman primate models,[361-364] however, they have much less immunogenicity and less durable immune responses in humans.

Vaccination with rMVA alone has failed to show sufficient immunogenicity in preventive and therapeutic Phase I clinical trials[365,366] and because of the inherent high immunogenicity of the vector MVA is currently used as boost for DNA vaccines (see below). However, therapeutic vaccination with MVA-nef was safe and induced novel immune responses in the majority of the 14 volunteers.[259,367]

Other poxvirus vectors presently being tested include canarypox[368] and fowlpox (for a review see ref. 369). Despite promising results in monkeys.[370] Canarypox vectors expressing different HIV genes have shown limited immunogenicity in humans even at high doses, which were associated to high reactogenicity.[371,372] Results from a Phase II trial recently conducted using the recombinant canarypox ALVAC vCP1452A administered alone or together with rgp120 failed to demonstrate sufficient immunogenicity to grant advancement to Phase III trials.[373] Based on composite data from Phase I and Phase II trials, Aventis and the Thai Ministry of Health, together with the US National Institutes of Health (NIH) and the US Military HIV Research Program, have launched a

Table 6. Preventive and therapeutic ongoing HIV-vaccine clinical trials

Vaccines Based on HIV Structural Gene Products

Vaccine Type	Trial N°	Organizer, Producer	Vaccine Product (Clade)	Phase (I,II,III) Start Date
DNA	HVTN 070	Univ. Pennsylvania	DNA gag, pol, env (B) ± IL-12 or IL-15 DNA	I (preventive) Sep-07
	Env DNA	St. Jude, NIAID	DNA env (A, B, C, D, E)	I (preventive) May-05
Protein	C86P1	SGUL, Richmond Pharmacology, Novartis Vaccines	Prime: HIV-gp140—LT-K63 Boost: HIV-gp140—MF59	I (preventive) Sep-06
	HVRF-380-131004	Moscow Institute Immunology	Env, Gag (B)	I (preventive) Mar-06
Vectored Antigen				
- Poxviruses	HPTN 027	NIAID, Sanofi	ALVAC-HIV vCP1521 env (B, E)	I (preventive) Oct-06
	RV138/VR811	USMHRP	ALVAC- HIV vCP205 env gag, pol (B)	I (preventive) Mar-06
	HIVIS 02	Karolinska Institute, SMI, USMHRP	MVA env (E), gag (A), pol (E)	I (preventive) Jan-06
	RV 158/WR 1143	USMHRP, WRAIR	MVA gp160, gag, pol (A, E)	I (preventive) Jul-05
- Adenoviruses	VRC012	NIAID-VRC	Ad35 env (A) Ad5 env (A)	I (preventive) May-07
	HVTN 054	NIH-VRC	Ad gag, pol (B), env (A, B, C)	I (preventive) Jul-05
Heterologous Prime Boost	HVTN 072	NIAID	Prime: DNA env (A) Boost: Ad5 or Ad35 env (A)	I (preventive) May-07
	HVTN 049	NIAID, Chiron	Prime: DNA/PLG microparticles gag, env (B) Boost: oligomeric V2-deleted gp140 (B)—MF59	I (preventive) Jan-05

continued on next page

Table 6. Continued

Vaccine Type	Trial N°	Organizer, Producer	Vaccine Product (Clade)	Phase (I,II,III) Start Date
Vaccines Based on HIV Structural Gene Products				
	HVTN 042/ANRS VAC019	NIAID, ANRS	Prime: ALVAC-HIV vCP1452 env, gag, pol + CTL epitopes from nef/pol (B) Boost: LIPO-5 or ALVAC (CTL epitopes) from Gag, Pol, Env (B)	I/II (preventive) Apr-04
	RV144	DoD,Thailand MOPH,NIAID TAVEG,Sanofi,VaxGen	Prime: ALVAC env (B, E) Boost: Gag (B), Pol (B), Env (B, E) proteins	III Oct-03
	PO1 AI47490	University of Maryland	Prime: Salmonella Typhi env (B) Boost: Env Protein(B)	I (preventive) Jun-03
	NO1 AI05394	NIAID	Prime: DNA env, gag (A, B, C, E) Boost: Env, Gag protein (A, B, C, E)	I (preventive) May-03
Vaccines based on combined HIV structural and nonstructural gene products				
DNA	N/A	Guangxi CDC	Multiclade DNA plasmids (B, C)	I (preventive) Mar-05
	HIVIS 01	Karolinska Institute, SMI, Vecura	DNA env (A, B, C), gag (A, B), RT (B), rev (B)	I (preventive) Feb-05
	040254; 04-I-0254	NIAID	Multiclade DNA plasmids: gag, pol, nef (B), env (A,B,C)	I (preventive) Aug-04
Protein	108706	GlaxoSmithKline	Gag, Pol, Nef	I/II (preventive) Feb-07
	HVTN 064	DAIDS, Pharmexa-Epimmune	Protein epitopes Env, Gag, Pol, Vpu (B) and/or DNA gag, pol, vpr, nef (A, B, C, D, F, G)	I (preventive) Jan-06

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Table 6. Continued

Vaccines Based on Combined HIV Structural and Nonstructural Gene Products

Vaccine Type	Trial N°	Organizer, Producer	Vaccine Product (Clade)	Phase (I,II,III), Start Date
Vectored Antigen				
- Poxviruses	IAVI C003	ADARC, IAVI, Rockefeller	MVA env/gag-pol, nef-tat (C)	I (preventive) Nov-06
	HIV-POL-001	Bavarian Nordic	MVA HIV polytope vaccine	I (preventive) Oct-06
	IAVI D001	IAVI, Therion	TBC-M4 MVA env, gag, tat, rev,nef ΔRT (C)	I (preventive) Dec-05
	IAVI C002	IAVI-ADARC	MVA env/gag-pol, nef-tat (C)	I (preventive) Jan-05
- Adenovirus (Ad)	AIN504-A5218	NIAID, Merck, HVTN	Ad5 gag, pol, nef (B)	II (therapeutic) Sep-05
	HVTN 057	NIAID	Ad gag, pol (B), env (A, B, C)	I (preventive) Nov-04
- Adeno-associated viruses (AAV)	IAVI A002	IAVI, Targeted Genetics	AAV gag, PR, ΔRT (C)	II (preventive) Nov-05
Heterologous Prime Boost	HIV NAT 064	The National Centre in HIV Epidemiology and Clinical Research, The University of South Wales	Prime: DNA gag, pol, tat/rev, env (A, E) Boost: rFPV gag, pol, tat/rev, env (A, E)	I (preventive) May-07
	HVTN 067	NIAID, Pharmexa-Epimmune	DNA vaccine and MVA (alone or in prime-boost regimen) env, gag, pol, vpu (B); gag, pol, vpr, nef (A, B, C, D, E, G)	I (preventive) Apr-07
	HVTN 069	NIAID	Prime: DNA gag, pol, nef (B), env (A, B, C); Boost: Ad gag, pol (B), env (A, B, C)	I (preventive) Nov-06

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Table 6. Continued

Vaccines Based on Combined HIV Structural and Nonstructural Gene Products

Vaccine Type	Trial N°	Organizer, Producer	Vaccine Product (Clade)	Phase (I,II,III), Start Date
	VRC-011	NIAID, VRC	Prime: DNA gag, pol, nef (B), env (A, B, C) or Ad gag,pol (B),env (A, B, C) Boost: Ad gag, pol (B) env(A, B, C)	I (preventive) May-06
	HVTN 065	NIAID, Geovax	Prime: DNA gag, pro, RT, env, tat, rev, vpu (B); Boost: MVA gag, pol, env (B)	I (preventive) Apr-06
	HVTN 068	DAIDS, NIAID, VRC	Prime: Non replicating Ad gag-pol (B), env (A, B, C) or DNA gag, pol, nef (B), env (A, B, C); Boost: Ad gag-pol (B), env (A, B, C)	I (preventive) Mar-06
	EuroVacc 02	EuroVacc Foundation	Prime: DNA env, gag, pol, nef (C) Boost: NYVAC env, gag, pol, nef (C)	I (preventive) Feb-06
	IAVI V001	IAVI, NIAID	Prime: DNA gag, pol, nef (B), env (A, B, C); Boost: Ad gag, pol (B), env (A, B, C)	I (preventive) Nov-05
	HTVN 063	NIAID, Wyeth	Prime: DNA gag (B) ± IL-15 Boost: DNA gag (B) ± IL-15 or DNA gag (B) ± IL-12	I (preventive) Sep-05
	HTVN 060	NIAID, Wyeth	Prime: DNA gag (B) ± IL-12 Boost: DNA or peptide gag (B) or gag, env, nef (B) + GM-CSF	I (preventive) Aug-05
	HIVIS 03	MUCHS, Karolinska Institute, SMI, Vecura, USMHRP	Prime: DNA env (A, B, C), gag (A, B), RT (B), rev (B); Boost: MVA env (E), gag (A), pol (E)	I/II (preventive) Dec-06

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Table 6. Continued

Vaccines Based on Combined HIV Structural and Nonstructural Gene Products

Vaccine Type	Trial N°	Organizer, Producer	Vaccine Product (Clade)	Phase (I,II,III), Start Date
	RV 172/WR 1218	USMHRP, NIAID	Prime: DNA gag, pol, nef (B), env (A, B, C); Boost: Ad gag, pol (B); env (A, B, C)	I/II (preventive) May-06
	HVTN 204	NIAID, Vical, GenVac	Prime: DNA gag, pol, nef (B), env (A, B, C); Boost: Ad gag, pol (B), env (A, B, C)	II (preventive) Sep-05
	HVTN 055	NIAID, Therion	Prime: MVA env, gag (B) + tat, rev, nef, pol (B); Boost: FPV env, gag (B) + tat, rev, nef, pol (B)	I (preventive) Sep-04
Whole HIV	DCV-02	Hospital Clinic Barcelona	DCs pulsed with inactivated autologous HIV	I/II(therapeutic) Nov-06
	5P01AI57127-2	NIAID	HIV immunogen (whole killed gp120-depleted HIV inactivated)	I/II (therapeutic) Oct-05
	2000456-01H	Ottawa Health Research Institute	Remune (vaccine) and ALVAC (vaccine)	I/II (therapeutic) Sep-05

ADARC: Aaron Diamond AIDS Research Center; **ANRS:** Agence Nationale de Recherches sur le SIDA (France); **DAIDS:** Division of AIDS; **DoD:** US Department of Defense; **EuroVacc:** European Vaccine Effort Against HIV/AIDS; **Guangxi CDC:** Guangxi Centre for Disease Control and Prevention; **HPTN:** HIV Prevention Trials Network; **HVTN:** HIV Vaccine Trials Network; **IAVI:** International AIDS Vaccine Initiative; **MoPH:** Ministry of Public Health; **MUCHS:** Muhimbill University College of Health Science; **NIAID:** US National Institute Allergy and Infectious Diseases; **NIH:** US National Institutes of Health; **SGUL:** St George's, University of London; **SMI:** Swedish Institute for Infectious Disease Control; **St. Jude:** St. Jude Children's Research Hospital; **TAVEG:** Thai AIDS Vaccine Evaluation Group; **USMHRP:** US Military HIV Research Program; **VRC:** Vaccine Research Center; **WRAIR:** Walter Reed Army Institute of Research; **MVA:** Modified Vaccinia Ankara; **FPV;** fowlpoxvirus; **vCP:** viral Canarypox; **LT-K63:** nontoxic mutant of *Escherichia coli* heat labile enterotoxin (LT); **PLG:** Polylactide- coglycolide; **GM-CFS:** Granulocyte-macrophage colony stimulating factor.

Phase III trial with a HIV-1 Env-based vaccine in which a canarypox prime is followed by boosting with the VaxGen gp120 protein (Table 6).

Similarly, despite promising results in the monkey model,[374] a Phase I therapeutic trial with a fowlpox vector expressing Gag and Pol failed to display any immunogenicity of the transgenes, although it induced anti-vector Abs.[375] Immunogenicity was demonstrated in a more complex therapeutic trial in which immunization with fowlpox expressing several HIV antigens was combined with HIV lipopeptides (synthetic fragments of HIV proteins associated with lipids that facilitate the induction of a cellular immune response) and followed by administration of IL-2, confirming the intrinsic weak immunogenicity of this vector.[376] Fowlpox vectored vaccines performed poorly also in heterologous DNA prime-fowlpox boost approaches both in monkeys and Phase I preventive or therapeutic trials.[375,377,378] Subsequent studies in the monkey model suggest that the poor immunogenicity observed in human as compared to monkeys might be due to the vaccine dose used, which might be insufficient to trigger adequate responses.[379] However, higher production costs, multiple inoculations and reactogenicity are serious obstacles to scaling up vaccine dosing and might hamper further development of vaccines of this type.

Adeno and Adeno-Associated Vectors

Adenoviral vectors have a broad host range and infect both proliferating and quiescent cells. The tropism of adenoviruses for mucosal epithelium makes them extremely attractive as vectors for HIV vaccine development since they can been delivered orally or intranasally and induce mucosal immune responses.[380] Recombinant adenovirus vectors can accommodate larger inserts, mediate transient but high levels of protein expression and can be easily produced at high titers. Furthermore, adenoviruses targets DCs in which they up-regulate costimulatory molecules and MHC class II expression and induce production of Th-1 and pro-inflammatory cytokines.[381,382] Of note, Adenovirus-based vaccine candidates have produced the most impressive cellular immune responses seen so far.[383] Both replication-competent and—incompetent vectors are being developed as vaccine against HIV. However, while replication-competent adenovirus vectors induce stronger and more persistent humoral and cellular immune responses compared to the nonreplicating vectors, there are safety concerns about their use in clinical trials.[383-385]

The replication-incompetent recombinant adenovirus Type 5 (rAd5) is a modified form of Ad5, the virus that causes some forms of the common cold. It is replication-defective to enhance safety and represents one of the most promising viral vectors for HIV vaccines. However, prior exposure to Ad5 may boost anti Ad-5 antibody response blunting the expression of the transgene and the percentage of volunteers responding to the vaccine. This anti-vector immunity may represent a major problem in the developing world, where the prevalence of prior exposure to Ad5 is greatest.[386] This has prompted the development by Merck, Crucell and Transgene, in collaboration with IAVI, of candidate vaccines based on less prevalent human Adenovirus serotypes (Ad6, Ad35, Ad11, or Ad 24) to replace the Ad5 vector in fusion HIV trials.[387-390] Another approach to circumvent pre-existing immunity to Ad5 has been to modify the vector by substituting key neutralizing epitopes on the surface of viral capsid proteins with those from the less prevalent serotype Ad48. Such chimeric vector is called Ad5HVR48.[391]

Finally, pre-existing immunity to adenovirus may be overcome by heterologous prime-boost strategies, including DNA priming followed by adenovirus vector boosting,[96,392] or the use of different adenovirus serotypes, including the above mentioned Ad5HVR48, for priming and boosting.[393,394]

Upon extensive testing in nonhuman primates Merck found out that intramuscular vaccination with SIV Gag delivered by Ad5 (E1- deleted) was superior to DNA or MVA at inducing CTL responses and at protecting against disease following pathogenic intravenous challenged with SHIV-89.6P,[141,142] but not against an intrarectal challenge with SIVmac239.[143] Noteworthy, the limited protection upon SIVmac239 occurred in the presence of T-cell responses that correlated with protection in the former study.[144] Co-immunization with Ad5 carrying Gag and Env was less effective than Gag alone at controlling infection in rhesus macaques challenged intravenously with

SHIV-89.6P[238] confirming the detrimental role of CTL responses to Env observed in the natural infection.[63] In order to increase the breadth of response against HIV-1 and to improve the vaccine efficacy, replication defective Ad5 vectors carrying pol and nef were constructed and a Phase IIb trial started in 2004 in which 3,000 high risk individuals were immunized intramuscularly 3 times with replication defective Ad5 vectors expressing gag, pol and nef (Ad5MRKAd5 trivalent). This trivalent vaccine was generally safe and well tolerated at all doses studied and immunogenic eliciting responses against the 3 antigens included in the vaccine. However, preliminary data indicate that vaccination did not protect from infection or lowered viral loads. Further, there was an apparent higher susceptibility to infection in vaccinees with pre-existing immunity to the vector.[146,395] Reduced expression and immunogenicity of the transgenes, as suggested by the much lower proportion of vaccinees responding to all the three HIV antigens (Gag, Pol and Nef) in the group with pre-existing immunity to Ad5 as compared to vaccinees with no pre-existing immunity, immune activation generated by the response to the vector and/or an increase of HIV-specific target T-cells induced by the vaccine[148] are some of the hypotheses that have been proposed to explain these findings. Thus, although very preliminary, overall preclinical and clinical data may suggest that SIV is a more rigorous challenge virus and a better predictor of vaccine efficacy in human and that boosting of pre-existing immunity to the vector may actually enhance the susceptibility to infection as compared to placebo or vaccinees with no pre-existing immunity. Another nonreplicative adenoviral vector (deleted of the genes coding for E1 and E3 proteins) has been developed by NIH Vaccine Research Center (VRC), together with a DNA-based vaccine. These are multicomponent vaccines, which express the Env glycoprotein from clades A, B and C and the Gag, Pol and Nef proteins from clade B and are designed for use in a DNA prime-Ad5 boost regimen strategy.[396] Despite differences in the vaccine design, initiation of Phase II trials has been postponed to late 2008, when a better understanding of the reasons of the Merck's vaccine failure will clarify whether vaccination with the VRC candidate would pose the same risks.

Replication-competent adenoviral vectors have also been developed as vehicles for AIDS vaccines.[397] Studies in both chimpanzee and rhesus macaque models have demonstrated that priming with replicating Ad recombinants encoding HIV or SIV genes followed by boosting with viral protein subunits elicits potent humoral, cellular and mucosal immune responses.[385,398-405] Of note, vaccination of Rhesus macaques with Ad vectors expressing HIV-1 Tat and Env conferred a strong protection against a challenge with the pathogenic SHIV 89.6P, which was superior to that provided by a larger vaccine formulation including SIV Gag and Nef in addition to HIV-1 Tat and Env.[268] This underscores the importance of properly selecting the antigens to combine together and provides one of the strongest evidence in favor of the Tat + Env vaccine.

Other viral vectors used as AIDS vaccines in clinical trials include Adeno-associated viruses (AAV) which are not adenoviruses but are often found in adenovirus infections.[406,407] These vectors are currently used in Phase I and Phase II clinical trials.[9] However, the weak immunogenicity recorded in a multicentric Phase I study[408] has led to halting the initiation of Phase II trials in India and spurred a debate on the ethics of conducting the ongoing Phase II trials in Africa in the face of such disappointing Phase I results. The slightly better immunogenicity recorded at the highest dose tested in the Phase I trials may suggest that a dose increase could solve this problem. However, recent data indicate that, in mice, vaccination with high doses of AAV expressing Gag induced Gag-specific effector $CD8^+$ CTLs that were weak producers of IFN-γ, expressed exhaustion markers and failed to become memory cells. Transition to the memory phenotype and restoration of full functionality was achieved upon adoptive transfer, suggesting that chronic exposure to the trangene might have been the cause of the CTL dysfunction.[409]

Other Viral Vectors

Other viral vectors have been tested as vaccine vectors for HIV-1 and have shown various degrees of success (for a review see ref. 410). Among them, the alphaviruses include weakened forms of three viruses named Venezuelan Equine Encephalitis (VEE), Sindbis (SIN) and Semliki Forest Virus (SFV). The first alphavirus vector candidate, AlphaVax's VEE, is designed as a replicon

particles containing self-replicating RNA encoding the VEE replicase proteins and expressing a gene of interest in place of the viral structural protein genes. An appealing feature of alphaviruses is their known ability to induce apoptosis of transduced cells, favouring DC cross-priming.[411] These replicon particles have shown protection against other viruses and have elicited significant cell-mediated and antibody immune responses with SIV antigens, perhaps due to the propensity of the vector to target antigen-presenting cells.[412] The VEE vector is currently being tested in clinical trials.[413]

Viruses belonging to the rhabdovirus family and in particular the vesicular stomatitis virus (VSV) are also being used. These vectors offer the advantage to be highly flexible, easy to manipulate and able to express large and multiple foreign genes.[414] Intramuscular vaccination of mice with a single-cycle vector expressing HIV Env elicited strong Env-specific humoral and cellular responses.[415] Furthermore, immunization of macaques with recombinant VSVs (rVSVs) expressing SIV Gag and HIV Env has been reported to protect from pathogenic SHIV89.6P.[416,417] These promising results have led to the development of rVSV for use in humans.[418] However, since the prototypic rVSV vector was found to be insufficiently attenuated for clinical evaluation, novel highly attenuated vectors have been designed, which are less neurovirulent and more immunogenic than the prototypic rVSV vector.[419]

Other potentially powerful vaccine delivery systems are represented by Polioviruses.[420] Both replication-competent and replication-deficient recombinants have been shown to be immunogenic in nonhuman primates when used through various routes of immunization, including mucosal delivery.[421] However, restrictions to the use of these vectors include the stability and size of heterologous gene inserts[422] and the presence of high levels of pre-existing immunity to polio vectors in the general population.

Replication-competent and replication-defective herpesviruses (HSVs), including HSV-1, represent suitable vaccine vectors against AIDS. Important advantages include broad host cell range, high infectivity and easy of production of high-titer stocks of viruses, long-term expression of foreign antigens and stimulation of both humoral and cellular arms of the immune system. Vaccination with replication-competent or replication-defective HSVs vaccine vectors expressing SIV Env and Nef, protected macaques against a challenge with SIVmac239.[423] However, the overall toxicity and the pre-existing immunity against the vector may represent a safety issue for their use in humans and current strategies focus on the development of replication-incompetent viruses used in prime-boost regimen with DNA.[424]

New Particulate Delivery Systems

Microparticles have been effectively used for many years as particulate delivery systems for drugs, therapeutic proteins and various types of vaccines including recombinant proteins, plasmid DNA, peptides and other vaccine components (e.g., immune potentiators).[425,426] Among antigen-loaded microspheres, injectable, biodegradable polymeric particles prepared with poly(d,l-lactide-co-glycolide) (PLG) or poly(d,l-lactide) (PL) polymers represent a successful method for in vivo delivery of peptide, protein or DNA antigens.[427] Both particles have been shown to be effective, especially for oral delivery.[428] Antigen instability and manufacturing difficulties have been overcome by the recent findings that adsorption rather than microencapsulation of the antigen onto PLGA is easier, cheaper and ensures better antigen stability.[429] In comparison to standard aluminum-based adjuvants, these microspheres have many desirable features, including the ability to enhance the bioavalaibility of the antigen, allowing pulsating antigen release and to reduce the number of doses in the immunization schedule, mimicking the conventional prime-boost regimen. Furthermore, for adjuvanting vaccines against intracellular pathogens and cancer, selective targeting of PLGA microparticles to DCs has been achieved and induction of CTLs has been attained in both small animals and nonhuman primates.[430] In particular PLGs have been demonstrated to enhance the immunogenicity of DNA vaccines to HIV-Gag and HIV-Env in rhesus macaques.[431] PLG particles are currently being evaluated in a gag + env DNA/PLG prime-ΔV2 Env protein boost preventive Phase I trial.[189]

Two novel classes of biocompatible core-shell anionic microspheres have been used as an efficient delivery system for vaccination with the Tat protein.[432] These microspheres, synthesized by dispersion polymerization, are characterized by an increased shelf-life and the capability of reversibly adsorbing native proteins at their surface. In particular, these microparticles consist of negatively charged microspheres, made of either poly(styrene) or poly(methyl methacrylate) and in which hemisuccinated poly(vinyl alcohol) or Eudragit L100/55 were used, respectively, as steric stabilizers.[432] These microspheres prevented Tat from oxidation, maintaining the native and biologically active conformation required for vaccine efficacy and efficiently delivered Tat intracellularly. In the mouse model, delivery of Tat by these microspheres was safe and immunogenic.[433,434]

VLPs

Virus-like particles (VLPs) are self-assembling, nonreplicating, nonpathogenic particles that are similar in size and conformation to intact virions.[435,436] VLPs offer a number of advantages over conventional protein immunogens and have been therefore considered as an ideal HIV vaccine candidate.[437] In fact, these particles can be easily produced in large amount in heterologous expression systems (baculovirus, vaccinia virus) and easily purified. In addition, since VLPs lack regulatory proteins as well as infectious genetic material, they are both replication- and infection-incompetent, making VLPs safer than live-attenuated viruses. Further, VLPs express viral proteins in their native conformation and generally induce more effective humoral and cellular immune response than their soluble counterparts, in both the systemic and mucosal immune compartments.[437-439]

However, due to their nonreplicating properties, VLPs are less effective at inducing cellular immune responses as compared to live-attenuated viruses or replicating viral vector vaccines. For this reason, novel approaches are being developed in order to increase their immunogenicity, including DC targeting.[440] The mucosal administration of VLP vaccines has also emerged as a promising strategy to elicit mucosal and systemic anti-HIV humoral and cellular immune responses.[441]

To date, numerous types of VLPs have been produced utilising the ability of capsid and envelope proteins to self-assemble into highly organised particulate structures. In particular, the Gag protein is required for their assembly, budding and release from host cell. VLPs, based on HIV-1 p55gag, presenting the entire gp120 molecule from an Ugandan clade A HIV-1 isolate, have been shown to induce strong systemic and mucosal humoral and cellular immune responses in mice.[442,443] More recently, IN administration in a mouse model of these VLPs together with the Eurocine L3 mucosal adjuvant (a monoglycerides/fatty acid lipid suspensions)[444] in a heterologous (DNA + VLPs) prime-boost strategy induced higher titers of NAbs and stronger anti-Env T-cell responses as compared to vaccination with adjuvanted VLPs only.[445] Further, a combined multiepitope VLP-based HIV vaccine (Combi HIVvac) carrying both B- and T-cell epitopes (from HIV-1 Env, Gag, Pol and Nef proteins) resulted safe and highly immunogenic in mice.[446] Of interest, vaccination of rhesus macaques with p55gag VLPs in the absence of adjuvant induced broad, durable anti-Gag CTLs.[447] However, therapeutic vaccination with HIV-1 p17/p24:Ty virus-like particles, which contain part of the HIV-1_{IIIB} Gag sequence and are produced by expressing a *TYA:p17/p24* fusion gene in yeast[448] did not appear to slower HIV-1 disease progression,[449] or to impact $CD4^+$ T-cell decline in patients with advanced HIV infection.[450]

Prime Boost Strategies

Many of the vaccine studies combine various approaches in a prime-boost fashion to optimize the immune responses elicited. A heterologous prime boost strategy is the administration of one type of vaccine (the primer is usually DNA) followed by the administration of another form of the vaccine (the booster is usually recombinant proteins or attenuated viral vectors). The goal of this approach is to complement the priming by a different stimulation of the immune system to enhance the body's overall immune response to HIV, a result that may not be achieved with a single type of vaccine. For example, while DNA or microparticles are optimal for inducing T-cell responses, they are poor inducers of Ab, which, however, are readily induced upon boosting with protein or a recombinant vector. Another advantage of this strategy is that it circumvents the

relatively common and detrimental immunodominance of the vector that may result in a reduced immunogenicity of the transgene and the impossibility to use the same vector twice because immunity to the vector strongly reduces or prevents the transgene expression. To overcome this latter problem sequential immunizations with different viral vectors have been used as an alternative prime-boost approach, as reported in nonhuman primate models against SHIV[451] and SIV[143,144] challenges. DNA prime-viral vector boost approaches may also be exploited to target mucosal site either because of the intrinsic tropism of the vector or because they can be applied mucosally. A variety of protocols using alternative viral vectors for both priming and boosting have also been reported, both alone and in combination with DNA and have been successful at limiting disease progression, but not at offering protection against infection. For example, DNA priming followed by a recombinant MVA expressing multiple HIV proteins did not prevent but effectively controlled infection upon challenge with pathogenic SHIV89.6P in rhesus macaques.[361,452] Based on the promising results in monkeys, GEOVAX is currently testing in 4 different Phase I trials a DNA prime-MVA boost approach in which priming with DNA encoding Tat, Rev, Vpu and Gag is followed by boosting with MVA expressing Env, Gag, Protease and RT. Preliminary data indicate good safety and CTL responses in over 50% of the vaccinees[453] and a Phase II trial is planned for 2008. Similarly, McMichael and coworkers at the Oxford University have shown in Phase I studies that DNA prime-MVA boost HIV vaccines are well-tolerated and immunogenic, but the percentage of volunteers responding to the vector and the durability of $CD8^+$ cell-mediated responses have not matched so far the responses observed with the rAd5 vector.[132,366,454] However, the lack of solid correlates of protection and the large body of evidence showing that natural control of infection is not necessarily associated with strong immune responses should not impede advancement of these type of vaccines to Phase II trials.

International Networking to Ease and Accelerate HIV/AIDS Vaccine Development

The first Phase I trial of an HIV vaccine was conducted in the USA in 1987. Since then, more than 50 candidate vaccines have been tested in about 100 Phase I/II clinical trials, involving more than 35,000 healthy human volunteers. Two Phase III trials have been completed and a third one is in progress. The vast majority of these vaccine candidates, including those tested in Phase III trials, were based on structural HIV-1 proteins and primarily aimed at inducing NAbs. Most of the efforts to develop and evaluate HIV vaccines is borne by the NIH, CDC and WRAIR in the USA and by ANRS in France, with strong help from the International AIDS Vaccine Initiative (IAVI) in New York (http://www.iavi.org), the European Union (EU), initiatives in WHO (http://www.who.int/en) and UNAIDS (http://www.unaids.it) and the recent commitment of the Bill and Melinda Gates Foundation for a Global Enterprise (http://www.gatesfoundation.org/GlobalHealth/Pri_Diseases/HIVAIDS).

The HIV Vaccine Trial Network (HVTN) established by NIAID in 2000, with 25 clinical sites in four continents, represents a major resource for clinical HIV vaccine research (http://www3.niaid.nih.gov/about/organization/daids). The EU has also established a comprehensive program aimed at strengthening integration of science among countries of the EU and promoting, among the others, vaccine development against poverty diseases (i.e., HIV/AIDS, TB, Malaria). The AIDS Vaccine Integrated Project (AVIP) (http://www.avip-eu.org), Mucosal Vaccines for Poverty Related Diseases (MUVAPRED) (http://www.mucosalimmunity.org/muvapred/index.asp) and the European Vaccine effort against HIV/AIDS (EUROVAC) (http://www.eurovac.net), are among the most important projects recently cofunded by the EU. In addition, the European and Developing Countries Clinical Trials Partnership (EDCTP) has been created with the aim of helping developing countries to build up their capacity in testing the efficacy of new drugs, microbicides and vaccines.

Conclusion

Several advancements have been made over the past few years to improve vaccine strategies aimed at inducing protection against HIV. Ideally, the aim of an effective vaccine would be to produce sterilizing immunity in all recipients. However, also a vaccine able to control rather than prevent the infection might have important benefits, reducing HIV levels in the body, delaying progression to AIDS and initiation of anti-retroviral therapy and reducing the chance of HIV transmission.

The current knowledge suggests that an effective HIV candidate should induce both humoral and cellular immune responses, to ensure durable immunological memory and to boost both the adaptative and innate immune system. The latter one is particularly important at mucosal sites of HIV transmission.[455] One of the major impediments to the development of an HIV-1 vaccine is the lack of knowledge of the immune correlates of protection. Although studies of MEU and LTNPs continue to provide valuable information on mechanisms of natural protection, which can then be applied to vaccine design, it should be kept in mind that immune responses in LTNPs may represent a correlate of preservation of immune competence in a host containing infection rather than the actual factors controlling the virus. Natural resistance to infection has been attributed to a combination of genetic, innate and acquired immune system-mediated mechanisms.[20] Therefore, a novel approach for treatment and/or prevention of HIV infection might be represented by the manipulation of these restriction factors in order to improve and broaden their activities.[456] The early containment of HIV-1 and SIV replication in acutely infected individuals and monkeys is temporally associated with the emergence of a virus-specific CTL response and high levels of circulating CTLs are associated with good clinical status in chronically infected individuals[457,458] and acutely infected monkeys.[459] Importantly, experimental in vivo depletion of $CD8^+$ T-cells in monkeys, abrogated control of SIV replication during primary infection and the animals died after a rapidly progressive disease course.[460-462] While this has generally been interpreted as the definitive proof of the key role of $CD8^+$ T-cells in the containment of infection, it should be reminded that also NK cells express the CD8 molecule and their experimental depletion may contribute to the loss of virus control.

Interestingly, loss of control of infection has been reported also upon B-cell depletion during primary SIVmac infection of rhesus monkeys,[460,463,464] suggesting that either Abs are indeed crucial for containing the virus even at the very beginning of the infection or, more in general, that severe disturbance of a component of the immune system disrupts the proper function of system as a whole, underscoring the integrated nature of the defense system[360,463,465-469] and the contribution of multiple arms to an effective control of infection (for a review see ref. 470).

Because of safety concerns, traditional immunization approaches, including those based on live attenuated and inactivated viruses, have been almost abandoned. Vaccine candidates based on purified or synthetic proteins are mainly developed to induce NAbs, whereas recent advances in molecular biology and genetic engineering have led to the development of a new generation of vaccines, which includes DNA- and microorganism-vectored vaccines, which are primarily aimed at inducing T-cell responses. In this regard, vaccinia viruses, canarypox constructs, replication-competent and replication-defective adenovirus vectors are the main live vectors currently being evaluated. The success of these vectors is believed to depend also on their capability to trigger innate immune responses, which would induce proper adaptive immunity. Although replication-competent adenoviruses have the advantage of persistently infecting the host and stimulating the immune system,[383] safety issues need to be fully addressed before their advancement to clinical trials. The recent failure of the Merck trial clearly indicates that even replication-defective adenoviral vectors may be harmful in the presence of pre-existing immunity to the vector. Thus, DNA vaccines with increased immunogenicity and microbial vectors that circumvent pre-existing immunity to the vector are needed. In this regard, optimization and further exploration of new adjuvants for DNA and protein antigens are currently being heavily pursued.[471] VLPs have also been employed as multi-epitope vaccine since they offer the advantages of (i) mimicking the virion without having the safety concerns of live-attenuated viruses, (ii) inducing both mucosal and systemic immune

responses, (iii) activating both endogenous and exogenous antigen presentation pathways (MHC class I and II, respectively) and (iv) maintaining the antigens in their native conformation.

Effective vaccination may ultimately require two or more vaccines used in conjunction (heterologous prime-boost strategies), an approach to vaccine development that differs from traditional vaccine design and is presently the preferred strategy for many vaccine candidates against HIV/AIDS (Table 6). In this regard, there is a general agreement that when exploited combined, the vaccine components used for the prime and the boost are expected to stimulate a broader and more diversified immune response than using any of them repeatedly. In addition, the single use of a viral or bacterial vector will avoid the interference, on a second administration, of pre-existing immunity to the vector.

Effective anti-HIV/AIDS vaccines may require targeting of several HIV-1 antigens. Among these multi-component vaccines novel minimalistic vaccination strategies, combining structural (ΔV2-Env) and nonstructural (Tat or Nef) proteins have been rationally designed to induce NAbs and T-cell responses against key early and late HIV antigens. In particular, preclinical testing of the Tat/ΔV2-Env combination in macaques has shown efficacy (ref. 268 and Ensoli B, in preparation) and clinical trials with this vaccine candidate will start in 2008.

However, HIV vaccine development still faces significant challenges. The availability of an effective HIV vaccine requires scientific and public-health efforts and the establishment of Consortia such as the "European Consortia for HIV vaccine development" (including AVIP, MUVAPRED, VIAV), the "Neutralizing Antibody Consortium"; the "HIV Global Enterprise", an international Consortium of nongovernamental and governamental organizations.[472] Clinical trials must also be performed with appropriate ethical rules, especially in developing countries, avoiding duplication of efforts, using standardized genetic inserts as immunogens and implementing immunological assays for preclinical and clinical testing to compare candidate vaccines. This is important because the laboratory assays used to assess immune responses may not be comparable, severely hampering decisions about which candidates to pursue for further testing. In addition, new knowledge about the immune response to HIV is raising concerns that current assays overlook important aspects of those immune responses.

Open questions remain to be answered, such as how to induce high titers of NAbs; whether any of the vaccines being currently developed will elicit cellular immune responses that will correlate with protection from infection or disease progression; the type (poly- or mono-functional) of $CD4^+$ and $CD8^+$ T-cell responses elicited by the vaccines currently being developed; the magnitude, breath and durability of the vaccine-induced $CD4^+$ and $CD8^+$ T-cell responses; the best combination of vaccines that in the prime-boost immunization strategies will stimulate an immune response similar to that thought to confer protection from disease progression.

The challenges the scientific community still faces are formidable. However, looking back, enormous progresses have been made in each aspect of vaccine development, from basic science to clinical testing, that let us be optimistic about the eventual generation of effective preventive and therapeutic vaccines against HIV/AIDS. While vaccines able to slow disease progression and decrease transmission rate should be at reach in the medium term, recent advancements in the generation of Env-based immunogens, their association to key regulatory or accessory HIV-1 proteins and present reconsideration of the several Ab effector functions, make us hoping that even a sterilizing vaccine may be not too far distant.

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Chapter 15

New Strategies to Overcome the Drawbacks of Currently Available Flu Vaccines

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Abstract

Vaccination represents the most efficient tool to control morbidity and mortality resulting from influenza infections in humans. The currently licensed influenza vaccines provide good protection levels in healthy adults, whereas lower protection is generally achieved in ageing individuals who are at a higher risk of developing severe clinical manifestations. Future improvements in influenza vaccines should address the needs of high risk groups including the elderly, small children and chronic patients. Recently, due to the increased incidence of avian influenza pandemic outbreaks, the prevention of a potential human influenza pandemic turned into another crucial issue in the influenza vaccination field. The development and validation of manufacturing processes for efficient and safe pandemic vaccines became one of the top priorities of health, regulatory and funding agencies all over the world. In the pandemic context, the development of novel vaccines administered via the mucosal route may play a significant role by reducing virus shedding from infected individuals. This chapter provides insights in the limitations of existing manufacturing processes, new approaches to overcome limitation in vaccine production, mechanisms of action of current vaccines and discuss potential strategies to improve the immunogenicity and efficacy of influenza vaccines.

Introduction

Influenza viruses A, B and C are enveloped viruses with a segmented, negative single-stranded RNA genome. They are members of the *Orthomyxoviridae* family together with the Thogotovirus and Isavirus. Within each influenzavirus genus no separate species has been recognized, but there are clusters of strains that can genetically reassort with each other.[1,2] Studies on the ecology of the influenza viruses led the hypothesis that all mammalian influenza viruses derive from an avian influenza reservoir. Thus, after long periods of evolution, human viruses originate from reassorted influenza viruses of avian/mammalian origin. The level of nonhuman virus adaptation to the human host has a considerable impact on the severity of human influenza disease. Human influenza is mainly caused by Influenza virus A and, to a less extent by Influenza virus B.[3] Based on the antigenicity of the surface glycoproteins hemagglutinin (HA) and neuraminidase (N) the influenza virus A is further classified in different subtypes. Actually, 16 different HA and 9 different N have been identified. In the past, human influenza virus A infection has been caused by 3 different HA

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(H1, H2 and H3) and 2 different N (N1 and N2). However, new avian restricted strains (H5N1, H7N2, H7N3, H7N7, H9N2 and H10N7) have recently been reported to infect humans.[4,5]

Although associated with a significant morbidity and mortality on groups at risk, such as the elderly, small children and patients suffering of chronic diseases (e.g.,, chronic heart, lung and kidney diseases, diabetes or immunosuppressive conditions), annual epidemics usually resulting from infections with circulating influenza A strains represent reduced threats, as compared to a pandemic situation. Indeed, pandemic avian/mammalian influenza viruses which were able to cross a species barrier and infect humans as a result of genetic re-assortments are associated with severe complications and high mortality rates.[6]

Human influenza epidemics are caused by circulating viruses and usually begin in November-December in the northern hemisphere and in May-June in the southern hemisphere, favored by lower temperature and lower relative humidity,[7] in spite of the availability of prophylactic vaccines with proven epidemiological and clinical benefits. One reason for this is the natural high genetic instability of influenza A viruses. Repeatedly occurring point mutations in antigenically important regions of the genes encoding both the viral HA and N without affecting replication capacity, leads to significant alterations in the immunogenicity of these surface glycoproteins (so-called antigenic drift). The new so originating virus strain is able to escape pre-existing host immunity allowing the new strain to spread.[6] The frequency of antigenic drifts represents one of the most crucial hurdles in the manufacturing of effective interpandemic vaccines. To overcome this obstacle, according to the current WHO recommendations, all manufacturers worldwide produce yearly trivalent vaccines containing antigens from two different subtypes of influenza A strains (currently H1N1 and H3N2) and one strain of influenza Type B. The specific strain selection is based on surveillance data from the worldwide network of national influenza centers and WHO collaborating centers. Because vaccines have to be manufactured before the actual epidemic strains are known, a failure to anticipate emergence of a strain relative to the vaccine will result in a substantial reduction or abrogation of vaccine-mediated protection

Human pandemic influenza, against which various vaccines are still under development, results of the combination of several factors in a stepwise manner.[6] Historical influenza pandemics emerged through an adaptation process of avian or mammalian influenza viruses to the human host. The crucial steps in such adapting process are the development of hybrid virus, termed reassortants, harboring genetic information from both human and animal viruses. Indeed, due to the fact that the segmented genome of influenza viruses consists of eight individual RNA segments, human-avian virus RNA reassortants can be produced by cells co-infected with both viruses.[6] Thereby completely novel subtypes of Influenza A virus can emerge in a process called antigenic shift, which is mechanistically different from the much more frequent genetic drifts resulting of point mutations. Thus, genome segmentation is a characteristic feature of influenza A viruses enabling a rapid and drastic evolution of the virus resulting in the escape of immunosurveillance in newly infected immunologically naive humans. Currently, the establishment of efficient vaccines to prevent pandemic influenza represents one of the most crucial global problems to be solved by scientists and the vaccine industry.

Manufacturing of Influenza Vaccines

The influenza virus was for the first time isolated in 1933 by Smith, Andrewes and Laidlaw.[8] In the 1940s the virus could be propagated in embryonated hen's eggs. The first vaccines against influenza were formaldehyde inactivated whole virus vaccines. They have been developed by the US armed services and made commercially available in 1945 in USA. However, these pioneering vaccines were highly pyrogenic due to the suboptimal virus purification process and exhibited low efficacy. An improvement in the purification process was achieved after introduction of zonal ultracentrifugation in the 1960s.[9] Nowadays the purification of virus from infected chicken embryos using zonal ultracentrifugation is still the manufacturing process of choice for all marketed influenza. Currently, several manufacturers provide worldwide interpandemic influenza vaccines mainly in the form of inactivated split or surface antigen (subunit) vaccines (see Table 1).[10] Split vaccines are prepared from sucrose density gradient purified whole viruses by fragmenting viral

particles into smaller pieces using detergents. Subunit vaccines contain further purified antigenic proteins. The conventional products comprise both major surface glycoproteins HA and N and are generally applied intramuscularly.[6] FluMist, a cold adapted influenza virus vaccine is administered intranasally; it is commercially available in the USA since 2003 for active immunization of people between 2 and 49 years of age.[11] This vaccine is a live attenuated whole virus vaccine characterized by a satisfying protective efficacy in adult. However the vaccine faces some issues related to the biosafety profile of live attenuated influenza viruses.

The manufacturing of current seasonal influenza vaccines is associated with several hurdles that may limit vaccine production capacity. Natural isolates of new epidemic influenza strains to be included in the recommended vaccine composition have to be genetically modified to create high-yielding strains suitable for antigen production. This is achieved using classical methods of virus reassortment using embryonated chicken eggs as a grow substrate. Reassortants are generated by co-infection of eggs with the current epidemic strain and a high yield donor strain. Next, the selected strain is used as a seed virus stock to grow and manufacture the desired virus type. Seed virus strain bearing the recommended HA and N are provided to vaccine manufacturers by one of the Control Agencies, such as the Center for Biologics Evaluation and Research (CBER) in the US, or the National Institute for Biological Standards and Control (NIBSC) in the UK. In order to maximize virus yield from eggs, reassortants of influenza A strains are often utilized. These possess the surface proteins from the desired wild-type H1N1 and H3N2 strains, while the other viral proteins are from a high-yielding strain, such as A/Puerto Rico/8/34. For logistic reasons new virus production substrates that would avoid the dependence on henn's eggs would be a key improvement of the manufacturing process for influenza vaccines.[6] Indeed, the limited availability of eggs as a growing substrate may sometimes be critical, especially in an avian flu pandemic situation. Further disadvantages linked to egg production concern the risk of the presence of residual

Table 1. Currently marketed interpandemic influenza vaccines

Subunit Vaccines	Split Vaccines	Whole Virus	Virosomes
Aggripal *Chiron/Novartis, I*	Afluria *CSL Limited,AU*	FluMist (live attenuated, intranasal) *Medimmune, USA*	Inflexal V *Berna Biotech/Crucell, CH*
Fluad (MF-59 adjuvanted) *Chiron/Novartis, I*	Begrivac *Chiron/Novartis,D*		Invivac *Solvay, NL*
Fluvirin *Evans/Chiron/ Novartis, UK*	Fluarix *GSK, UK*		
Influvac *Solvay, NL*	FluLaval *ID Biomedicals, CA*		
	Fluvax *CSL, Au*		
	Fluviral S/F *ID Biomedicals, CA*		
	Fluzone *Sanofi Pasteur, F*		
	TIV *Medimmune, USA*		
	Vaxigrip *Sanofi Pasteur, F*		

allergenic egg's proteins in the final product; the presence of microbial contaminants that might interrupt the antigen supply considering the removal of thimerosal from the production process and finally the egg waste disposal. Currently, 90% of the worldwide influenza vaccine production capacity is concentrated in the USA and Europe, which in turn represents only 10% of the world's population. The current global manufacturing capacity (~300 million doses per year of the seasonal trivalent vaccine, containing 15 ug/dose of each antigen) would be inadequate to meet global needs during a pandemic, especially as it is expected that higher antigen dosage and a two dose regime would be required for protection against a pandemic strain.[5] Novel approaches, including application routes other than intramuscular, the use of novel adjuvants, alternative cell-based substrates for antigen production, new vaccine formulation (naked DNA immunization, viral vector based vaccines) could pave the way to greater vaccine accessibility with associated public health benefits.

Strong efforts to replace embryonated hen's eggs as the growing substrate in the manufacturing process have been explored after the introduction of reverse genetics into the influenza vaccine field. The reverse genetics technology is an egg independent method allowing to generate influenza vaccine strains with selected gene combinations, while avoiding the time-consuming selection of appropriate reassortants.[6] Several manufacturers are actively working towards the establishment of influenza antigen production in mammalian cell cultures or even in plants. First experiences with the cell culture system were promising, however the first cell line (293T) used to express antigens could not be licensed due to regulatory restrictions. The improvement was achieved by the implementation of MDCK,[12] Vero[13] or PER.C6®[14] cells into manufacturing process. According to public available information, Vero (Chiron, D; Baxter Au), MDCK (Solvay, NL) and PER.C6® (Sanofi Pasteur) cell culture based influenza vaccines are currently under clinical evaluation[15] (Sanofi Pasteur press releases 26 Sept 2006). In 2007 EMEA has issued the marketing authorization valid throught the European Union for the Optaflu (Novartis Vaccine and Diagnostics GmbH & Co. KG), an influenza vaccine based on purified Ha and N, derived from viruses propagated in MDCK cells. Moreover, the need for faster and more flexible production systems leads to the tentative design of novel recombinant vaccines. Naked plasmid DNA or immunization with recombinant vectors encoding HA were proven to be efficient in animal models,[16] but were less successful in humans.[17] Purified HA and N vaccine antigens produced with the baculovirus expression system have been shown to be effective in a mouse model.[18] Recent studies have shown that adenovirus-vectored influenza vaccines are able to elicit robust human immune responses if delivered intranasally.[19] Using an adenoviral vector system, a new HA strain can be constructed within one month. A feature in the development of vectored vaccines seems to be the establishment of molecular strategies enabling for viral tropism modulation. However, due to selection pressure, such kind of modifications might lead to expanded tropism or abolishment of the adenoviral native tropism, which can in turn lead to unintended spread to new and undesired cell types, as well as to horizontal transmission of the vector. However, all these approaches are still far from commercialization.

Strategies to Improve the Immunogenicity and Efficacy of Current Influenza Vaccines

The immunogenicity of influenza vaccines is currently measured by their capacity to induce functional neutralizing HA-specific antibodies in serum,[20] which have been proved to provide acceptable protection against disease. However, the level of vaccine's protective capacity differs depending on age and health status of population groups. In case of a good antigenic match between vaccine and circulating influenza strains healthy adults, who usually undergo only mild symptoms of disease and recover within time period up to two weeks, show 70-90% protection against proven influenza illness upon conventional immunization. As stressed above, individuals at the highest risks of severe seasonal influenza infections are elderly, as well as adults or children suffering of chronic health conditions, such as cancer, immunosuppression or immunodeficiency, cardiac and pulmonary disorders, diabetes and other metabolic diseases, or renal disease, who require regular medical follow-up or hospital care. Only 50-70% individuals belonging to these

population groups are protected by conventional vaccines.[21] Lower efficacy of vaccination in elderly might be related to decreased function of cellular components of immune system.[22] Also, the immune system of small children differs from the one of adults who have usually been facing a long history of contacts with flu viruses. Therefore, current influenza vaccines generate more efficient immune responses in healthy adults than in infants. Moreover, due to the fact that vaccination is recommended in children, elderly and immunocompromised individuals, the safety profile of the vaccine should be very carefully considered. Live attenuated influenza vaccine FluMist exhibits high efficacy in protection against influenza in healthy adults. However, due to the risk of residual replication of virus in the respiratory tract, it is not recommended to be used in high risk population groups.[23] Taken all these aspects together, the challenges for future interpandemic influenza vaccine manufacturers is clearly concerned with the improvement of immunization strategies for individuals belonging to high risk population groups.

The most evident immunogenic effect of conventional influenza vaccines is the generation of systemic antibodies specific to the surface glycoproteins of influenza virus. New vaccination strategies are focused on the induction of more balanced and broadened immune responses. The improvement of the immunogenicity of vaccines can be achieved by: (i) immunopotentiating the immune responses via the addition of an adjuvant; (ii) the induction of a mucosal immunity in addition to the systemic immunity; and (iii) broadening the immune responses by including more conserved antigenic epitopes, mainly derived from internal proteins of influenza viruses.[24]

Immunopotentiating of Immune Responses by Adjuvants

Currently marketed inactivated split- or subunit-vaccines provide superior safety over traditional whole-virus vaccines. However, the immunogenicity of these vaccines leaves space for improvement. Immunogenic properties of subunit vaccines can be improved by the addition of immunostimulating molecules that target signaling pathways of defense immunity. An appropriate antigen-adjuvant combination could overcome this obstacle.

In case of a pandemic flu vaccine, the requirement for a higher antigen dosage seems to be an important issue. It seems that a conventional vaccine formulated with an adjuvant can allow for antigen sparing. Currently, three adjuvants are approved for human use: Aluminium precipitate,[25,26] liposome (virosome)[27] and MF59 water/oil suspension.[28,29] Virosomal formulations of influenza surface antigens have been shown to possess significant adjuvant effect due to the repetitive arrangement of presented surface antigens as well as the maintenance of viral HA in its native and biologically active conformation.[30] In search for optimal adjuvant candidates highly attractive targets become TLR-ligands that activate appropriate signaling pathways in a highly natural and specific way.[31-33] Some adjuvant candidates currently tested to improve efficacy of influenza vaccines are listed in Table 2.

The general safety requirements for any vaccine are extremely high, driven by the fact that vaccines are generally administrated to healthy individuals. Particular risk/benefit ratio consider-

Table 2. Adjuvant vaccine candidates under evaluation for influenza vaccines

Proteosomes[34]	OMP of *Neisseria meningitis* (TLR-2/1 ligand)	Human
LTK63 /Biovector[35]	*Escherichia coli* LT mutants	Human
CTA-1 DD[36,37]	CT derivative (A1 subunit of CT + Ig-binding element of *Staphylococus aureus*	Mouse model
ISCOMATRIX[38-40]	Quil-A-based	In vitro human model mouse model
MALP-2[41]	TLR "2/6 agonist	*Mouse model*

ations are required if immunocompromised individuals are concerned. Regarding the selection of adjuvant molecules the following characteristics should be considered: (i) preferentially, adjuvant molecules should be produced synthetically rather then biologically, (ii) the size and structure of molecules should be precisely defined, (iii) adjuvant signaling and functional pathways must be deciphered; and (iv) a specific way of action will be preferred.

Improvement of Immune Responses Using Alternative Application Routes

The intramuscular application route is routinely used for vaccines administration. However, the implementation of alternative routes, e.g., intranasally, could result in the triggering of additional immune pathways to those activated by intramuscular immunization.

The pathogens causing respiratory diseases invade their host via the nasal/tracheal mucosa. On the other hand, parenteral immunization is not appropriate to induce a local immune response in the respiratory tract.[42] Therefore, the early viral colonization of upper respiratory area during infection and the postinfection process of virus shedding cannot be prevented by parenteral vaccines.[7,43] Conversely, pre-existing mucosal immunity is able to reduce or prevent both colonization and virus shedding. Thus, the intranasal route appears most attractive to prevent influenza infection.

For several years, live-attenuated influenza vaccines for nasal application have been used successfully in the Russian Federation. The current live Russian vaccine is based cold-adapted variants of an H2N2 strain which is reassorted with epidemic H1N1 and H3N2 strains and combined with a cold-adapted reassortant of influenza B virus.[5]

FluMist, is a live-attenuated intranasal influenza vaccine, has been shown to be safe and effective in healthy adults, but its role in the general prevention of influenza is yet to be defined. The vaccine induces IgA antibodies in mucosal secretions, IgG antibodies in serum, as well as CTL cellular responses, leading to an increased efficacy and crossprotective potential between several subtypes of viruses.

As stressed above, the live virus vaccine approach ensures an acceptable immunogenicity but includes inherent safety concerns (genetic stability of the vaccine strains, potential reassortments with circulating viral strains, shedding in immunocompromised vaccinees). In addition, the intranasal application of vaccine is often associated with mucosal discomfort, inflammatory reactions causing sore throat, as well as febrile reactions in vaccines.[44]

The efficacy of a proteosomal influenza vaccine containing outer membrane proteins from *Neisseria meningitidis* (FluInsure, GSK) and a LTK63/Biovector adjuvanted influenza vaccine are currently tested in clinical studies.[5]

Inclusion of Conserved Epitopes Derived from Influenza Proteins in Vaccine Formulations

The inclusion of new conserved immunoepitopes is expected to strengthen cellular responses against influenza, facilitating the direct elimination of infected cells from organism. Moreover, due to the conserved character of epitopes, the generated immunocompetent cells might be cross-reactive and may have a significant impact on cross-protection between different virus subtypes. This could be of help in the control of pandemic influenza outbreaks.[45] Some internal components of influenza virus might have impact on the generation of immune responses against influenza. The outer envelope of the virus is built up by a phospholipidic membrane with intercalated, outside projected influenza surface proteins HA and N. The inner side of the membrane is lined by the matrix protein (M1). The integral protein (M2) is forming ion channels within the membrane. The internal protein nucleocapsid is surrounded by the viral envelope. The nucleocapsid of Influenza A virus consists of 8 genome segments, packaged into the core. Each segment is formed by helically organized nucleoprotein (NP) binding a negative-strand ss RNA molecule. Three polymerase polypeptidic subunits (PA, PB1, PB2) are associated with the NP-RNA structure. Nonstructural proteins NS-1 and NS-2 are located at the inner site of nucleocapsid[1]. The functional characteristics of individual proteins are summarized in Table 3.

Currently available influenza vaccines which are parenterally administered are known to stimulate effectively MHCII restricted immune responses involving activation of B-cells and CD4 T-cells

specific for influenza surface glycoproteins, mainly the genetically highly variable HA. This process seems to be sufficient to achieve good levels of protection against the homologous subtype of influenza virus in healthy adults, however, it is suboptimal in high risk populations, e.g.,, the elderly. Since the surface glycoprotein NA was shown to undergo genetic mutations to a lesser extent as compared to HA, the molecule have attracted attention and studies performed so far have confirmed the potential of N-based vaccines to induce efficient immune responses and protection against

Table 3. The protein-components of influenza virus and their properties

Protein	Segment	Biological Properties	Anti-Viral or Immunological Properties
HA	4	- Binding to sialic acid receptors on cell surface	Induction of B-cell responses, CD4 T-cells responses, antibody production
		- Fusion of virus with endosomal membrane inside of endosomes	
NA	6	Release of newly synthetized viruses from infected cells	Induction of B-cell responses, antibody production?
NP	5	RNA binding, nuclear/cytoplasmic transport of viral RNA	Induction of CD8 T-cell responses
M1	7	Matrix protein forming capsid	Induction of CD8 T-cell responses
M2	7	Ion channel through membrane, important in the uncoating of viruses in endosomes	Induction of B-cell responses, antibody production
			Target molecule of anti-virals amantadine and rimantadine
PA	3	Transcriptase	Unknown
PB1	2	Transcriptase	Induction of CD8 T-cell responses
PB2	1	Transcriptase	Induction of CD8 T-cell responses
NS1	8	Effects on cellular RNA transport, splicing, translation, anti-IFN protein	Induction of CD8 T-cell responses
NS2	8	Unknown	Unknown

drifted viruses.[46,47] Enhancing the NA in HA based vaccines could be of help to control influenza via the induction of more balanced and broadened influenza specific humoral responses

The stimulation of cytotoxic $CD8^+$ T-cells responses specifically targeted to the conserved antigenic determinants derived mostly from internal proteins seems to be a promising approach to improve efficacy of interpandemic vaccines, as well as to develop efficient pandemic influenza vaccines. Following MHCI-restricted epitope presentation, cytotoxic lymphocytes induce apoptosis of infected cells. An approach including conserved internal CTL epitopes would have an advantage over conventional HA-based strategies by potentially conferring an heterologous cross-reactive immunity between different subtypes.[48,49] Although not sterilizing, cellular immunity was shown to prevent illness and deaths in animal models.

Due to HA antigenic drift, HA-based stockpiled vaccines might only provide limited protection against an emerging pandemic influenza strain. The monitoring of pandemic strains derived from the same viral subtype allows to identify the most conserved human epitopes within the internal proteins of influenza virus.[50] New strategies are mostly focused on the inclusion of nucleoprotein (NP), or M1, M2 proteins into vaccines. The efficient induction of heterologous crossprotective responses after immunization with immunodominant NP epitopes have been demonstrated in mouse models.[51,52] A conjugated M2 peptide based vaccines (M2 coupled to KLH or OMP from *Neisseria meningitidis*) were tested in mouse, ferret and rhesus monkeys. The vaccines have been shown to be highly immunogenic and to confer protection against lethal challenge with H1N1 and H3N2 strains in all species. Monkey antisera tested for reactivity with different strains of human influenza A were crossreactive, however, they failed to react with M2 peptides derived from highly pathogenic H5N1 strain.[53] In contrast, if the M2 peptide was coupled to a hepatitis B core particle carrier, the conjugate failed to generate protective responses even against interpandemic strains.[54] Another approach made use of an adjuvanted plasmid vaccine encoding the M protein encoding gene applied topically on the skin. The vaccine induced cytotoxic and humoral responses and provided cross-reactive protection in mice.[55] A new generation of live attenuated vaccines against influenza is based on NS1 protein mutants. The nonstructural protein 1 has been shown to inhibit Type I interferons mediated responses. Such cytokines have a high impact on the regulation of pathogenic effects induced by influenza infection. The attenuation and immunogenicity of NS1 truncated mutants was confirmed in vivo in mouse model, in which was able to confer protection.[56]

Thus, a variety of approaches have been tested in animal, mainly mouse models so far. The proof-of-concept in humans still has to be established. The general immunoregulatory factors that need to be considered in the development of vaccines targeting cellular immune responses are still unclear. Up to now we have learned, mostly from mouse studies, about the immune mechanisms triggered by such vaccines. Antigen availability, antigen processing, epitope stability and individual T-cell repertoires seem to be critical elements to be taken in account during the vaccine development process. In addition, the formulation of individual components needs to be optimized and an alternative way of administration of the vaccine should be evaluated as well.

Conclusion

Influenza vaccines available today are in use since over 50 years. During this time they underwent only small improvements. The efficacy of vaccines is acceptable in healthy adults but suboptimal in at risk population groups, in which the infection can progress to very severe, complicated disease and even to a fatal end. Furthermore, there is currently no efficient tool to efficiently fight the predicted next influenza pandemy. The avian H9N2, H7N2 or H5N1 influenza strains have been already shown to cause human infections, although these avain strains did not acquire the capability of human to human transmission yet. It is essential to improve our understanding of disease mechanisms to facilitate the development of better measures to control influenza. In order to protect humans against pandemic influenza threats, as well as to improve existing interpandemic vaccines for high risk population groups, new vaccine approaches must urgently be identified and developed. That will imply major scientific and industrial investments in order to convert promising improved candidate vaccines into marketable products.

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