The Jordan Report 2000

Accelerated Development of Vaccines

Division of Microbiology and Infectious Diseases
National Institute of Allergy and Infectious Diseases
National Institutes of Health

ABOUT THE COVER

The statue pictured on the cover stands on the grounds of the Pasteur Institute in Paris and commemorates the epochal work of Louis Pasteur on rabies vaccine. Sculpted by Emile-Louis Truffot (1843-1896), it depicts a 16-year-old shepherd boy, Jean-Baptiste Jupille, who in 1885 strangled a rabid wolf that was about to attack a group of six younger shepherds near the village of Villers-Farley in eastern France. Jupille sustained severe wolf bites on both hands and was sent to Paris 6 days later where he became the second patient to receive the complete series of rabies vaccinations developed by Pasteur. Jupille survived and later became the concierge of the Pasteur Institute. He died in 1923.

We are here at the origins of vaccine development. In 1879, Pasteur had been stimulated to a crucial insight by the observation that cultures of the bacteria that cause chicken cholera lost their virulence with aging and that they could serve as a vaccine against challenge with virulent strains. In 1880, he wrote the following words, which express concisely the central idea of live attenuated vaccine development:

> In other words, with a simple change in the way of culturing the parasite, with the fact only of elongating the time between passages, we have a method to obtain progressively decreasing virulence, and finally a true vaccinating agent, which does not kill, which gives a benign illness, and which protects from the mortal disease.

In 1881, Pasteur had succeeded in protecting sheep against anthrax with an attenuated vaccine at the famous public demonstration experiment conducted at Pouilly-le-Fort.

Why did Pasteur choose rabies for his next attempt at vaccine development? Probably because he had been marked by his own experiences as a boy in the Jura region of France, where rabies was enzootic. In addition, rabies was then, as it is now, a virtually 100-percent fatal disease, arousing the same kind of fears as are now aroused by AIDS.

Behind the statue of Jupille, however, there were controversies that engaged Pasteur and that still find their echoes today. When Pasteur vaccinated the boy, he had had experience with a clinical series of three cases: a patient who probably had hysterical symptoms, a patient who had frank rabies and who died despite an incomplete series of vaccination, and Josef Meister, the only true success. Moreover, the preclinical animal experiments were inconclusive. Even today, it is difficult to show protection in animals by postexposure vaccination. Thus, Pasteur was indulging in what at best could be called empiricism when he undertook the clinical trials. Undoubtedly, the last doses of the vaccine contained live rabies virus, and the young Jupille could hardly have given informed consent. In addition, Jupille was not part of a double-blind controlled study, a fact that came back to haunt Pasteur when his critics claimed that his vaccine was causing rabies rather than protecting people from it. Because an understanding of immunology did not yet exist, Pasteur could not measure antibodies to show a correlate of immunity. Even today, although we are able to do those measurements, we still do not have a clear idea of how postexposure protection works.

In the end, however, Pasteur was right. Rabies vaccines are highly effective. The statue of Jupille reminds us of the tremendous value of vaccines and the influence they have had on the lives of millions. In general, vaccinologists cannot identify the particular patients they save from death or disease; for emotional satisfaction they must take comfort in the cold numbers of declining incidence. However, rabies is an exception. This fatal disease occurs in between 15 and 50 percent of exposed but unvaccinated persons, and the vaccinator can easily identify those with high risk who likely would have succumbed to rabies without the vaccine.

Thus, the statue of Jean-Baptiste Jupille is perhaps the only monument in the world to those who remain well as a result of vaccination. It also serves as a reminder that vaccines—for all the good that they do—are, and always will be, controversial.

Stanley A. Plotkin, M.D.

Medical and Scientific Advisor
Aventis Pasteur

Emeritus Professor of Pediatrics
University of Pennsylvania

Recommended Reading:

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PREFACE

The past century will go down in history as one marked by tremendous activity in all aspects of vaccine development and implementation. In 1900 few effective methods existed for preventing infectious diseases. Now, at the close of the century, vaccines are available to protect us against 21 infectious diseases, smallpox has been eradicated worldwide, we are on the verge of eradicating polio, and the number of deaths in the United States from seven other infectious diseases has declined to near zero thanks to universally recommended vaccination.

As we look ahead, the payoffs from basic research will drive much of vaccine development. Just as new tissue-culturing techniques ushered in modern vaccines in the 1950s, and recombinant DNA technology allowed researchers to engineer vaccines in the 1990s, genetic sequencing is expected to result in a new generation of tailor-made vaccines in the next decade. In just the past few years, researchers have published the complete genetic sequences for more than 13 microorganisms. Furthermore, more than 60 other sequencing projects targeting other medically important pathogens are currently underway.

Novel vaccine delivery methods, such as transgenic plant vaccines (potatoes and tomatoes engineered to contain vaccine immunogens), transcutaneous skin patches, and nasal vaccines continue to be developed and clinically tested.

We are not just witnessing an era of new technology in vaccine delivery methods but also a paradigm shift in what we have traditionally considered vaccine-preventable diseases. During the past few years interest has increased in vaccines for nontraditional uses, such as for autoimmune and other chronic diseases. Advances in bioinformatics and microbial array technology may soon give researchers the tools to determine the role of infectious agents in a number of chronic diseases. With this information, vaccines may one day be used to control, prevent, or treat chronic diseases, such as heart disease.

Thanks in large part to the interest and generosity of the Bill and Melinda Gates Foundation, international cooperation to ensure implementation of existing vaccine programs throughout the world is no longer a simple hope but a realistic goal. Globally, donor agencies and researchers have come together to meet the challenge of developing vaccines for diseases such as malaria that are of particular importance to less developed countries.

Nonetheless, this scientific excitement has been tempered by recent concerns about vaccine safety. During the past summer the RotaShield™ vaccine by Wyeth Laboratories, Inc., was removed from the market because of concerns that the vaccine might increase infants' risk for developing bowel obstructions. At roughly the same time, concerns about mercury in routinely administered vaccines led to a temporary revision of the hepatitis B immunization guidelines. Public distrust of vaccines and discomfort with mandatory vaccination programs continue to serve as the focus of numerous reports by the media. We closed the century with congressional inquiries about the safety of routine immunization programs.

Clearly, the challenges and opportunities have never been greater. *The Jordan Report* began as a yearly update submitted to Dr. William Jordan, former Director of the Division of Microbiology and Infectious Diseases, on the progress of vaccine development through the eyes of NIAID staff; it continues to provide that perspective, albeit to a much larger audience. In the first edition of the 21st century, we have tried to present a clear, accurate, and broad perspective of vaccine research supported by the National Institute of Allergy and Infectious Diseases (NIAID).

Carole Heilman, Ph.D.

Director
Division of Microbiology and Infectious Diseases

ACKNOWLEDGMENTS

The Jordan Report for 2000 was prepared under the supervision of the Division of Microbiology and Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health. Appreciation is expressed to the following staff members who assisted in the preparation of the report.

Dr. Carole Heilman Director	Dr. Pamela McInnes Acting Deputy Director	Dr. Michael A. Gerber Editor

Division of Microbiology and Infectious Diseases (DMID)

Ms. Diana Berard
Enteric and Hepatic Diseases Branch

Dr. Dennis Lang
Enteric and Hepatic Diseases Branch

Dr. Pamela McInnes
Respiratory Diseases Branch

Dr. Ann Ginsberg
Respiratory Diseases Branch

Mr. Robert Gulakowski
Respiratory Diseases Branch

Dr. Linda Lambert
Respiratory Diseases Branch

Dr. David Klein
Respiratory Diseases Branch

Dr. Fran Rubin
Respiratory Diseases Branch

Dr. Christopher Taylor
Respiratory Diseases Branch

Dr. Dennis M. Dixon
Bacteriology and Mycology Branch

Dr. Phillip Baker
Bacteriology and Mycology Branch

Dr. B. Fenton Hall
Parasitology and International Programs Branch

Dr. Christopher Beisel
Virology Branch

Dr. James Meegan
Virology Branch

Dr. David Morens
Virology Branch

Division of Acquired Immunodeficiency Syndrome (DAIDS)

Dr. Peggy Johnston
Vaccine and Prevention Research Program

Ms. Blanche O'Neill
Vaccine and Prevention Research Program

Dr. Steven Bende
Preclinical Research Branch

Dr. James Bradac
Preclinical Research Branch

Ms. Mary Allen
Clinical Development Branch

Dr. Carl Dieffenbach
Basic Sciences Program

Division of Allergy, Immunology, and Transplantation (DAIT)

Dr. Charles Hackett
Basic Immunology Branch

SELECTED TOPICS IN VACCINE DEVELOPMENT CONTRIBUTING AUTHORS

Transcutaneous Immunization

Dr. Gregory Glenn
Walter Reed Army Institute of Research
IOMAI Corporation

Dr. David Lang
Enteric and Hepatic Diseases Branch
Division of Microbiology and Infectious Diseases
National Institute of Allergy and Infectious Diseases
National Institutes of Health

Dr. David Taylor
Walter Reed Army Institute of Research

Memories of Pathogens Past—Inborn Antigen Receptors and T Cell Affinity Maturation

Dr. Charles J. Hackett
Chief, Molecular and Structural Immunology Section
Division of Allergy, Immunology, and Transplantation
National Institute of Allergy and Infectious Diseases
National Institutes of Health

Toward a Preventive Vaccine for Type 1 Diabetes

Dr. Charles J. Hackett
Chief, Molecular and Structural Immunology Section
Division of Allergy, Immunology, and Transplantation
National Institute of Allergy and Infectious Diseases
National Institutes of Health

Cytomegalovirus Vaccine Development

Dr. Christopher E. Beisel
Persisting Viral Infections Program Officer
Virology Branch
Division of Microbiology and Infectious Diseases
National Institute of Allergy and Infectious Diseases
National Institutes of Health

Development and Testing of *Streptococcus pneumoniae* Conjugate Vaccines

Dr. David Klein
Bacterial Respiratory Diseases I Program Officer
Respiratory Diseases Branch
Division of Microbiology and Infectious Diseases
National Institute of Allergy and Infectious Diseases
National Institutes of Health

Global Alliance for Vaccines and Immunization—A Millennial Challenge

Dr. G.J.V. Nossal
Department of Pathology
University of Melbourne
Victoria, Australia

In addition to those who wrote feature articles for this report, sincere thanks and appreciation are also expressed to the staff of the Division of Microbiology and Infectious Diseases, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), for updating the material presented in Appendix A; to Ms. Alice Knoben, U.S. Food and Drug Administration, Centers for Biologics Evaluation and Research, Office of Vaccines Research and Review, Division of Vaccines and Related Product Applications, for reviewing and updating the information presented in Appendix B; to Ms. Mary Allen and Ms. Sarah Landry and the staff of the Division of Acquired Immunodeficiency Syndrome, NIAID, NIH, for assembling the information contained in Appendix C. Special thanks are also due to Mr. Abraham Mittelman, Jennifer Noyes, and staff at IQ Solutions, Inc. for their assistance and expertise in preparing this document for publication.

TABLE OF CONTENTS

VACCINE UPDATES

Enteric Infections

Overview

Diarrheal diseases are a major cause of morbidity in developed countries and are a major cause of both morbidity and mortality in developing countries. In the United States, diarrhea is the second most common infectious illness, accounting for one out of every six (16 percent) infectious diseases. In some developing countries, children have more than 12 episodes of diarrhea per year, and diarrheal diseases account for 15 to 34 percent of all deaths. Conservative estimates place the death toll from diarrheal diseases at 4 million to 6 million deaths per year, with most of these occurring in young children. In 1991, an epidemic of cholera appeared in the Western Hemisphere for the first time in 100 years; this, as well as the emergence of a new strain of *Vibrio cholerae* in Asia in 1992, emphasizes the need for both continued surveillance and a research infrastructure capable of rapidly developing vaccines against new or emerging diarrheal infections.

The diversity of bacterial and viral infections that may cause diarrheal disease complicates accurate surveillance and diagnosis, especially in less developed parts of the world where there is little or no access to modern laboratory procedures. Such surveillance is critical to assess the magnitude of the problem, to establish the need for particular vaccines, and to identify and select sites in affected areas suitable for the testing of new candidate vaccines. The value of good diagnostic reagents was made evident this year at a workshop on the caliciviruses. These viruses have been difficult to diagnose due to the lack of specific and sensitive diagnostics. Now that reagents are available, the disease burden of this group of diarrheagenic viruses is becoming increasingly recognized around the world. Despite the continuing difficulty in culturing human strains, surveillance has revealed a large disease burden in both children and adults. This knowledge increases the attractiveness of a calicivirus vaccine strategy as a public health tool. The immunological diversity of viral subtypes and the lack of cross-protection will make this a challenging objective.

Another problem is developing vaccines that will be inexpensive enough to use in developing countries where the largest disease burden occurs. Indeed, in the U.S., industry interest in developing vaccines against bacterial diarrheal disease lags due to the rather small market in this country where they would be used, for the most part, as travelers' vaccines. A recent WHO report (Legros D, et al., WHO Bulletin, submitted) on the problems associated with delivery of an effective cholera vaccine in refugee camp settings illustrates rather dramatically how the cost of vaccines impact on the cost-effectiveness of a preventive strategy. Despite these real barriers, the focus of NIAID's enteric diseases program will continue to be basic research on the pathogenesis of the organisms responsible for diarrheal diseases. This research will help define the protective immune responses to infection and guide efforts to develop new and improved vaccines. Once developed, it will take a real worldwide effort and investment in order to provide these vaccines to the people who can benefit from them the most.

Cholera

More than 100 years after Koch's discovery of the cholera organism, highly effective vaccines remain an elusive goal. The search for a better cholera vaccine is prompted by the results of epidemiological and challenge studies showing that the recovery from natural infection is often followed by solid, long-lasting immunity. The emergence of O139-Bengal in Asia, with its high attack rate in adults, emphasizes the need for continued surveillance and the development of more effective vaccines against this disease. The goal of this effort is to design safe, oral vaccines, composed of either killed or attenuated bacteria, that can provide high levels of protection for at least several years after the administration of one or more doses.

The oral vaccines currently under development are of two types: killed *Vibrio cholerae* bacteria that are combined with purified cholera toxin B subunit (CTB), and strains of *V. cholerae* that are attenuated by virtue of specific gene deletions. Oral vaccination with *whole-cell B subunit* gave adequate levels of protection (about 50 percent) during at least three cholera seasons in field trials sponsored by the World Health Organization and the United States Agency for International Development in Bangladesh. Multiple doses of the vaccine were required over a 4-month period; unfortunately, young children, the major target population for this vaccine, were not well-protected. Since that time, the manufacturer (SBL Vaccin AB) has improved the formulation and added a killed *V. cholerae* O139 component to protect against both O1 and O139 serotypes. A whole-cell vaccine (mixture of four classical and El Tor strains killed by heat or formalin and not containing CTB) has been produced and tested in Vietnam and showed a protective efficacy of greater than 65 percent

against El Tor cholera in both adults and young children. The inactivated vaccines require two doses administered 1 to 2 weeks apart to achieve this level of protection, but they do not require a cold chain.

Under NIAID sponsorship, several live-attenuated strains of *V. cholerae* with known genetic deletions have been constructed and tested in volunteers. A vaccine candidate, CVD 103 HgR, lacking the toxic A subunit but retaining the immunogenic B subunit of cholera toxin, has been derived from the classical Inaba strain of *V. cholerae*. This vaccine has been tested in adults and young children in Indonesia and South America and has been shown to be safe and immunogenic. A large-scale efficacy trial, involving more than 60,000 individuals, was conducted in Jakarta, Indonesia. Unfortunately, the results of this pivotal trial did not show that the vaccine was effective in preventing cholera and a U.S. license was denied by the FDA. A new multisite trial in U.S. volunteers has been conducted with this vaccine, and, based on the results obtained, re-application for U.S. license may be made in the near future by the manufacturer, Swiss Serum Vaccine Inc. (SSVI). CVD 103 HgR has been licensed in some European countries and in Canada.

Live attenuated El Tor vaccines against both O1 and O139 serotypes have been tested in volunteers. These appear promising as vaccines and are being developed by both SSVI and Avant Immunotherapeutics, Inc. Work also is underway by grantees and by intramural scientists of the National Institute of Child Health and Human Development to produce parenteral cholera vaccines consisting of O antigen conjugated to a variety of proteins including cholera toxin. Research is also under way to explore the use of *V. cholerae* as an expression vector as the basis for multivalent vaccines. These conjugate vaccines should be tested for efficacy in a new standardized cholera human challenge model and directly compared with the live oral vaccines.

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Shiga Toxin-Producing *Escherichia coli* (STEC) and Enteropathogenic *E. coli* (EPEC)

Shiga toxin-producing *E. coli*, also referred to as enterohemorrhagic *E. coli*, primarily of the O157:H7 serotype, are responsible for many foodborne illnesses in developed countries. These dangerous strains of *E. coli* express one or both of the Shiga toxins (Stx-I and/or Stx-II). The Centers for Disease Control and Prevention estimates that as many as 20,000 cases per year occur in the United States. Clinical symptoms can include mild diarrhea, severe abdominal cramping, and bloody diarrhea. Children and the elderly or immuno-compromised are at particular risk of developing severe complications including kidney failure due to hemolytic uremic syndrome (HUS). Contaminated food products (undercooked ground beef, unpasteurized apple juice, raw milk, sausages, lettuce, and sprouts) as well as swimming pools and well water have all been identified as sources of infection. Recent outbreaks caused by sorbitol fermenting O157:H- (Germany) and Shiga toxin producing O111 (U.S.) emphasize the need to consider strains other than O157:H7 as potentially dangerous and capable of producing HUS.

Current efforts at vaccine development are focused on animals (cattle and other ruminants) known to asymptomatically carry these organisms and shed them in their feces. Researchers have shown that immunization of pigs with a genetically modified, nontoxic version (E167Q) of SLT-2e prevents the development of disease. Other vaccine approaches target the colonization factor intimin, the protein required for the attaching and effacing lesion characteristic of STEC and EPEC infection in experimental animals. Indeed, if intimin proves to be a good antigen capable of inducing immunity, it would be a useful immunogen against both classes of pathogenic *E. coli*.

Conjugate vaccines targeting the bacterial lipopolysaccharide have been developed by Dr. Robbins' group at the National Institute of Child Health and Human Development. These vaccines have been discussed as being appropriate for both animal and human use. NIAID grantees have expressed the B-subunit of Stx-I in vaccine strains of *V. cholerae* and shown that experimental rabbits accumulated less fluid in ileal loops challenged with Stx-I than did control animals. Another grantee is attempting to express intimin in canola, alfalfa, or other animal feed as an edible animal vaccine. Of course, if this strategy were to work in animals, it could also find use as an edible human vaccine.

The sporadic and relatively rare occurrence of infections due to STEC limits the usefulness of a vaccine for humans. Such a vaccine could be useful during a large community outbreak to prevent secondary spread or in an institutional or childcare environment. If an anti-intimin (or other shared antigens) response could be shown to protect against STEC infection as well as infection with closely related EPEC strains, a stronger case for a vaccine strategy could be made. Such a vaccine could target both groups of pathogens, particularly if EPEC can be shown to be a major contributor to diarrheal disease burden in the U.S.

Therapeutics for treatment of individuals infected with STEC are also under development. Toxoids, if safe and immunogenic in human volunteers, could provide protection against STEC strains and *Shigella dysenteriae*. Antitoxin antibodies could also be purified from donor serum and assessed for their ability to prevent the development of HUS and other serious sequelae in patients presenting with suspected STEC infection. NIAID supported researchers are using recombinant methods to produce "humanized" monoclonal reagents of mouse monoclonals that have been shown to neutralize Stx-I and II. These hybrid antibodies, which contain the specific binding variable regions of the original mouse monoclonals with the constant regions of human antibodies, would also be tested for efficacy in preventing the development of the systemic effects of STEC infection. We hope to initiate phase I trials of this treatment strategy in the next year.

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Enterotoxigenic *Escherichia coli* (ETEC)

A safe and effective vaccine against ETEC would be useful both to young children living in areas of the world where ETEC is endemic and to travelers visiting these areas. ETEC is second only to rotavirus as the cause of severe dehydrating diarrhea in young children throughout the world. Although surveillance data are difficult to obtain, it is estimated that ETEC causes more than 400 million cases of diarrhea per year and more than 700,000 deaths in children less than 5 years of age. ETEC is also the major cause of traveler's diarrhea, which affects at least 8 million citizens of the United States who travel to endemic regions of the world each year; it causes significant financial hardship in developing countries that rely heavily on tourism.

Volunteer studies have shown that infection with ETEC generates protective immunity against rechallenge with the same strain. On the basis of this observation, several attenuated strains have been developed in an effort to mimic the presentation of important ETEC antigens to the immune system, without inducing disease. Protection correlates with levels of intestinal IgA antibody specific for the colonization factor antigens (CFA).

Swedish investigators have produced the vaccine candidate that is currently furthest in development. This vaccine is composed of a mixture of five formalin-inactivated ETEC strains, which together express the major CFAs important in human disease, combined with a recombinant cholera toxin B subunit, which will elicit antibodies against the ETEC labile toxin (LT). Clinical studies in more than 500 volunteers have demonstrated that this vaccine is safe, immunogenic, and capable of generating antibody-secreting cell (ASC) responses equivalent to natural infection in Bangladeshi adults. In studies conducted in Egypt, this vaccine was found to be safe, immunogenic, and to induce both ASC and IgG responses in adults. The presence of measurable IgG responses is important in assessing vaccine response in young children from whom only limited amounts of blood are available. Subsequent trials have been conducted in progressively younger persons, with comparable safety and immunogenicity results. A study is currently being conducted in infants 6 to 18 months of age.

Other investigators have used attenuated strains of *Shigella* and *Salmonella* to express ETEC colonization factor antigens. Animal experiments with the *Shigella* construct have indicated that an immune response to the expressed CFAs is generated following oral or intranasal administration. These approaches wait, however, for the vector strains to become acceptable vaccine candidates in their own right. At such time, vaccine strains may be further engineered as multivalent vaccines by the expression of foreign antigens including ETEC CFAs.

University of Maryland investigators, in cooperation with the Department of Defense, administered colonization factor antigens CS1 and CS3 (CFA/II) that had been encapsulated in biodegradable microspheres to human volunteers. Of 10 volunteers, 5 developed IgA anti-CFA/II ASC following 4 doses of antigen delivered via intestinal tube. Volunteers were challenged with 10^9 colony-forming

units (cfu) of wild-type ETEC; 10 of 10 unvaccinated controls and 7 of 10 vaccinees developed diarrhea (30 percent vaccine efficacy). Additional human trials are being planned that will use this antigen preparation in combination with a nontoxic mutant *E. coli* labile toxin as an adjuvant.

Drs. Charles Arntzen and John Clements have teamed up on a novel edible vaccine approach. Initial success was obtained with the expression of *E. coli* LT-B in tobacco. Since most people are not fond of eating tobacco, another plant vehicle was needed. Potatoes were chosen, and when expression of LT-B was achieved, animal experiments were performed. Mice fed potatoes containing LT-B developed serum IgG and secretory IgA specific for LT-B. Phase I safety and immunogenicity studies in volunteers have been completed at the University of Maryland VTEU. This trial demonstrated that this vaccine was safe and highly immunogenic. Dr. Arntzen's long-term goal is to express antigens in a plant that humans find appetizing, such as tomatoes or bananas. Another group is exploring transgenic corn for use as edible vaccines.

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Helicobacter pylori

It is now well recognized that *Helicobacter pylori* is the main cause of gastric and duodenal ulcers as well as gastritis, and is a contributing factor for the development of cancers of the stomach. In some developing countries, the infection rate approaches 100 percent of the population, while in the United States as much as 40 percent of the adult population is infected with this organism, although not all infected individuals are symptomatic. It disproportionately affects Hispanic and African Americans. Approximately 10 percent of the adult population in the United States is affected by peptic ulcer disease (PUD), and an estimated 25 million Americans have had PUD in their lifetimes. At least 90 percent of PUD cases are caused by *H. pylori* infection, but about 70 percent of the U.S. population is unaware of this association. The NIH Consensus Conference held in 1994 recommended that persons diagnosed with ulcer disease be evaluated for their *H. pylori* status, and, if found to be infected, that they be treated with a recommended antibiotic therapy designed to eradicate the organism. In 1996, the Food and Drug Administration approved diagnostic products and recommended treatment protocols specifically for *H. pylori* disease. In October 1997, the Centers for Disease Control and Prevention launched a national media campaign designed to educate the public and health care providers about the association between *H. pylori* and ulcer disease and to stress the fact that this is an infectious disease that can be cured by antibiotic therapy.

The usefulness of a vaccine strategy to prevent infection with *H. pylori* is worthy of evaluation. The organism has been shown to be extremely heterogeneous at a genetic level; this may make the development of a preventive vaccine difficult. On the other hand, animal experiments have demonstrated that a vaccine composed of purified urease, a known virulence factor of the organism, can be protective. However, for this vaccine to be effective, the co-administration of the potent mucosal adjuvant, cholera toxin, has been required. Clearly this approach cannot be applied to human vaccination because of the severe diarrheal effects of such toxins. However, research is being conducted on the use of nontoxic mutants of either cholera toxin or *E. coli* labile toxin as adjuvants. In addition to urease, other antigens, combinations of antigens, and killed whole cells or cell extracts are being evaluated by a number of investigators and companies. Other approaches include the expression of *H. pylori* antigens in live-attenuated orally delivered vectors. Development of an *H. pylori* challenge model in volunteers is controversial but would facilitate measurement of effectiveness of vaccination in small numbers of humans.

The *H. pylori* DNA sequence has been determined by more than one group, but the Institute for Genomic Research (TIGR) was the first to release the sequence to the scientific community. The availability of the sequence

data will permit a detailed analysis of the genome and, predictably, will identify new genes that, by virtue of their similarity to other known bacterial virulence determinants, will become targets for rational vaccine design.

Companies that are involved in the development of a vaccine against *H. pylori* include OraVax and Astra Research Center, Boston, Massachusetts; Antex Biologics, Rockville, Maryland; IRIS Chiron Biocene, Italy; and Commonwealth Serum Labs, Australia.

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Polio

As worldwide polio eradication efforts accelerate, the number of countries that are free of polio continues to increase. Globally, health officials remain optimistic about eradicating the disease by the end of, or shortly after, the year 2000. In 1998, 6,227 confirmed polio cases were reported worldwide (up from 5,185 in 1997), with 50 countries still having polio. The slight increase may be due, in part, to better surveillance and detection, especially in countries like India. In addition, WHO has identified 14 "priority" nations, all of which have large reservoirs of poliovirus circulation. The Southeast Asian countries were responsible for more than 80 percent of all polio cases in 1998. By WHO regions, the numbers of cases reported in 1998 were African region, 992; Southeast Asia region, 4,673; Western Pacific region, 0; Eastern Mediterranean region, 536; European region, 26; and American region, 0. PAHO documented that the last case of paralytic poliomyelitis associated with a wild-virus isolate in the Western Hemisphere occurred in Peru on August 23, 1991. The successful methods developed during this pioneering regional eradication effort led to a now-standard worldwide eradication strategy of (1) achieving and maintaining high routine vaccine coverage; (2) giving supplemental vaccine doses during "National Immunization Days" (NIDs) to interrupt wild poliovirus transmission; (3) developing sensitive systems for surveillance; and (4) conducting mopping-up immunization campaigns. As of May 1999, only two countries have not conducted NIDs (Sierra Leone and the Congo); in both cases this was the result of ongoing civil wars.

Worldwide immunization is being coordinated by an international coalition of partners including WHO, Rotary International, CDC, the United Nations International Children's Fund, a number of national governments, and many nongovernmental organizations. During 1996 alone, two-thirds of the world's children younger than 5 years of age received oral polio vaccine. Worldwide coverage with three doses of oral polio vaccine among infants younger than 1 year of age has reached 81 percent. In Africa, coverage has increased from 32 percent in 1988 to 60 percent in 1996. A new WHO/partner plan for acceleration of polio eradication emphasizes key priority countries for rounds of NID pulse immunizations in India and sub-NIDs in other key countries. With two regions of the world now polio-free and three other regions close to elimination, global eradication appears to be feasible. Laboratory confirmation of cases is available through a "Global Laboratory Network for Poliomyelitis Eradication," which includes 67 national labs, 14 regional labs, and 6 specialized labs. However, the need for repeated contacts with infants to administer the three doses required to fully immunize and the heat sensitivity of the vaccine remain challenges to the global eradication effort.

The problems of controlling polio in developed countries are different from those of developing countries. Although polio is controlled in developed areas, a small number of cases occur each year, and these appear to be associated with use of the live-attenuated vaccine. The United States currently follows three different polio immunization schedules: all oral polio vaccine (OPV); all inactivated polio vaccine (IPV); or two IPV followed by two OPV. The sequential IPV/OPV vaccination schedule is intended to reduce vaccine-associated paralytic polio (VAPP) while maintaining individual and population immunity. A study of all VAPP cases from 1980 to 1994 in the United States showed that of the 125 cases (annual mean, 8), 76 percent were in immunologically normal vaccines or their contacts. Of the VAPP cases, 97 percent were associated with the first or second dose—1 case per 750,000 children receiving their first dose. Therefore, starting immunization with IPV is hoped to establish solid immunity before the first OPV dose. Recently, the Advisory Committee on Immunization Practices of CDC recommended that an all-IPV schedule be used for routine immunization beginning in the year 2000.

As the world approaches eradication of polio, there have been preliminary meetings to discuss whether there will be a time when all polio immunization could be stopped. This issue is controversial, with some experts recommending continuing OPV, others recommending continuing indefinitely only with IPV, and still others seeing a possibility of stopping all immunization after a period of only IPV. This issue is unresolved and will remain the focus of intense debate.

Another issue for the posteradication era is the safety of performing research on wild poliovirus strains in less than biosafety level 4 containment facilities. After eradication, there is concern that the laboratory or the vaccine manufacturing facility would become a potential source of reintroduction of wild poliovirus into the community. The seed virus for production of IPV is a high-yielding, wild-type poliovirus, and recently, there was a case of accidental transport of the strain from a production facility into the community via an infected but immunized worker. Eventually, if poliovirus immunization is stopped, all poliovirus strains, including vaccine-derived strains, might have to be contained or destroyed. Other unresolved issues about the posteradication era include: (1) Is reintroduction possible from immune-suppressed individuals persistently shedding vaccine strain virus? (2) Could these persistent shedders be controlled with immune globulins or antivirals? (3) Which vaccine would be used if a reemergence occurred? (4) Which vaccine(s) will be needed in the posteradication age? (5) How will these vaccines be produced if all stocks are destroyed or high-containment production facilities are required? (6) Would polio bioterrorism become an important concern?

NIAID currently funds several extramural basic research projects on the virological and immunological aspects of polio. One goal of this work is to apply the knowledge obtained to make better vaccines that will be (1) genetically stable and not revert to a more neurovirulent form and (2) more efficient and efficacious, especially when used in tropical and developing regions of the world.

Several major NIAID-supported discoveries have added greatly to the knowledge of polioviruses, as well as other RNA viruses. Molecular studies have been substantially advanced by the development of quick, reliable nucleic acid sequencing methods and the construction of a cDNA infectious clone of poliovirus. The changes in viral nucleic acid that occur during vaccine reversion to virulence have been defined, and a number of studies are examining the basis of viral virulence and attenuation.

The detailed study of viruses has always been hindered by the fact that viruses must invade a host and replicate within living cells; however, research supported by NIAID shows that it is possible to induce the *de novo* synthesis of infectious poliovirus in a cell-free, test-tube system. This system has provided a number of new research approaches to the study of virus replication.

Another major breakthrough was the ability to insert into mice the human gene responsible for producing the receptor for human poliovirus. Because such “transgenic” mice are able to make the receptor for poliovirus, they become susceptible to infection and develop a paralytic-like disease. These new mice have helped advance research focusing on the pathogenesis of viruses.

These discoveries are of great significance not only for the study of poliovirus but also for research on other viruses. As a model, polio research has led to major breakthroughs, particularly in other RNA viral systems. Nonpolio enteroviruses will remain a problem even after eradication. In a recent study of more than 3,200 cases between 1993 and 1996 in the United States, echoviruses 9, 30, 6, and 11 were commonly isolated, as were coxsackieviruses B5, A9, and B2. Enterovirus 71 has been increasingly linked to neurologic disease, and evidence continues to mount implacating certain enteroviruses in the etiology of diabetes. This group of viruses requires intensified research. The knowledge derived from poliovirus studies will be of great value in the development of new vaccines or antiviral drugs against many other RNA viruses that are now difficult to study.

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Enteric viruses

Rotavirus is the leading cause of severe diarrheal disease of infants in both developed and developing countries. It is particularly satisfying to report that licensure of the first vaccine against rotavirus was granted to Wyeth-Ayerst this past year by the Food and Drug Administration. This vaccine is a tetravalent human-rhesus reassortant vaccine, which was developed by NIAID intramural scientists. However, after receiving reports from the Vaccine Adverse Event Reporting System of intussusception among infants who had received this rotavirus vaccine, in July, 1999, CDC recommended a temporary suspension of the use of this vaccine. In October 1999, the Advisory Committee on Immunization Practices, after a review of scientific data from several sources, concluded that intussusception occurs with significantly increased frequency after immunization with the Wyeth-Ayerst rotavirus vaccine and withdrew its recommendation for routine use of this vaccine. Wyeth-Ayerst subsequently withdrew this vaccine from the market. Another reassortant vaccine, combining bovine and human viruses, has been developed by NIAID grantees and Merck and Company, Inc. This vaccine demonstrates protection in human trials at levels similar to that of the Wyeth vaccine. Data on this vaccine have not yet been submitted for licensing. Both the Wyeth and Merck vaccines contain G serotypes 1-4 that predominate in the United States.

Several additional candidate rotavirus vaccines are being explored. Intramural scientists have developed a cold-adapted human rotavirus candidate vaccine that is currently being evaluated for safety. A virus (89-12) isolated from a naturally infected, asymptomatic child in a nursery is also being evaluated. Avant Immunotherapeutics, Inc., Boston, is developing this virus, which has been further attenuated by growth in tissue culture. It has been in phase II trials in infants. Two additional human viruses isolated from asymptomatic children in India have been evaluated in phase I studies in the U.S. Both strains are safe and immunogenic. Indian manufacturers are considering development of both vaccines. One advantage that these weakened human viruses may have is the lack of vaccine-induced fever, a side effect seen in a small percentage of recipients of the rhesus- or bovine-based reassortant vaccines.

A NIAID grantee has succeeded in assembling virus-like particles from the products of bacculovirus-expressed rotavirus genes. The resultant particles are noninfectious and can be designed to contain structural proteins from multiple serotypes. This recombinant particle vaccine will be given parenterally, and the results obtained thus far in animals have been promising. Human phase I trials will be conducted in NIAID VTEU facilities. NIAID-supported research also is examining the possibility of incorporating rotavirus into microspheres for use as an oral vaccine capable of stimulating a protective mucosal immunity. This technique has been shown to enhance the immune response to either live or killed rotavirus administered either orally or parenterally.

Animal studies performed by a NIAID grantee have indicated that VP6 may be a new vaccine target. IgA monoclonal antibody directed against this protein provides protective immunity against rotavirus in mice. Studies by another grantee have indicated that a nonstructural protein, NSP-4, has secretory activity similar to that of enterotoxins. This is an interesting finding that may help explain the diarrheagenic activity of rotavirus infection. Whether induced immunity to this protein would be a useful vaccine strategy remains to be seen. Another NIAID grantee is testing the possibility of using DNA vaccines to induce protection against rotavirus in animals. The DNA vaccines, administered orally after the DNA was encapsulated in microspheres, were shown to be immunogenic and protective in mice. Studies of this nucleic acid vaccine approach are proceeding in pigs.

Caliciviruses have been shown recently to be significant contributors to diarrheal disease. Norwalk virus surface protein has been expressed in bacculovirus-infected insect cells. When the protein accumulates intracellularly in high concentration, virus-like particles self assemble and can be purified. These Norwalk VLPs, like the rotavirus VLPs, are immunogenic and protective as vaccines in animal studies. Moreover, in the past year, transgenic potatoes containing Norwalk VLPs have been found to be immunogenic in human volunteers. Additional immunogenicity studies in larger numbers of volunteers and vaccine efficacy studies are planned. Measurement of efficacy will require administration of wild Norwalk virus in a challenge protocol.

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Shigella

Shigellosis (bacillary dysentery) is endemic throughout the world. More than 32,000 cases were reported in the United States in 1993; this represents an increase of 35 percent from the number of cases reported in 1992. More than 90 percent of all cases reported in the United States were caused by *Shigella sonnei*. Symptoms of the disease can vary from mild diarrhea to severe dysentery with inflammation and ulcerative lesions of the colon, bloody diarrhea, and hemolytic uremic syndrome; death can result in untreated cases. Although there are 30 serotypes of shigellae, usually only two or three serotypes predominate in a given area. *S. sonnei* predominates in industrialized countries, whereas *S. flexneri* is most commonly found in developing countries; both are associated with endemic disease. *S. dysenteriae* causes epidemic outbreaks of dysentery, as well as significant endemic disease. Therefore, a comprehensive vaccine approach to controlling shigellosis must include components of all three species.

Early studies showed that the O somatic antigens of S*higella* are major immunogens and that the most effective attenuated vaccines were those that transport these immunogens to mucosal tissues where they can generate a local or mucosal immune response. Limited tissue invasion of the vaccine strain would also likely generate a better cell-mediated immune response, thought to be important for protection against invasive pathogens such as *Shigella*.

Attenuated strains of *Shigella* have been created by deleting known virulence factors. Dr. Phillipe Sansonetti at the Pasteur Institute has made a *ics*A, *iuc*A deletion mutant of *S. flexneri* 2a (strain SC602). After a single oral dose of 10^4 cfu, this vaccine candidate provided 100 percent protection against severe shigellosis in seven North American volunteers when they were challenged with *S. flexneri* 2a.

Auxotrophic mutants also appear promising. Animal studies indicate that these mutants may be sufficiently attenuated and still able to induce protective immunity. Researchers at the University of Maryland have created an *aro*A, *ics*A deletion mutant (strain CVD 1203) and a *gua*B-A, *vir*G deletion (CVD 1205) in *S. flexneri* 2a. Two doses of CVD 1203 proved safe at 10^6 cfu and induced IgA-secreting cell responses in 60 percent of volunteers. At higher doses (10^8 and 10^9 cfu) better antibody-secreting cell (ASC) responses resulted but at a price of increased vaccine reactogenicity. Additional deletions of two *S. flexneri* enterotoxins, SHET 1 and 2, from these strains should reduce reactogenicity and should be evaluated in humans in the near future.

Auxotrophic strains are also being developed as vectors for multivalent vaccines. An *S. flexneri aro*D deletion vector expressing *S. dysenteriae* 1 Shiga toxin B subunit has recently been reported. It should be noted, however, that even in persons infected with wild-type *S. dysenteriae*, an anti-Shiga toxin response has not been detected. Therefore, it is difficult to predict the protective efficacy that might be achieved by a vaccine strategy aimed at Shiga toxin. It is assumed that a humoral response directed against this toxin may lessen the chances of developing some of the more serious consequences of infection such as the hemolytic uremic syndrome.

Efforts also are under way in the laboratory of Dr. John Robbins at the National Institute of Child Health and Human Development to develop parenteral vaccines composed of detoxified *Shigella* lipopolysaccharide-protein conjugate. A randomized, double-blind study has been conducted in Israeli military volunteers and demonstrated 74-percent protection. A recent study of O-specific polysaccharides conjugates from *S. sonnei* and *S. flexneri* 2a demonstrated safety and immunogenicity in children 4 to 7 years old.

In studies conducted at the Walter Reed Army Institute of Research, two approaches for the development of *Shigella* vaccines are being examined. In one, subcellular nucleoprotein preparations have provided encouraging results in animal models of *S. sonnei* infection. In the other, a lipopolysaccharide-proteosome preparation specific for *S. sonnei* has also yielded promising animal results. The Department of Defense is pursuing both approaches as well as live attenuated human vaccines.

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Typhoid

Typhoid fever remains a serious public health problem throughout the world, with an estimated 33 million cases and 500,000 deaths annually. It also is a serious threat to travelers visiting endemic areas. In the United States, more than 41,000 cases were reported in 1993. In virtually all endemic areas, the incidence of typhoid fever is highest in children 5 to 19 years of age, which is important since school children can be immunized readily through school-based immunization programs.

Although licensed parenteral whole-cell vaccines are now available for typhoid fever, they are rarely used because they are only marginally effective and they produce adverse reactions in many vaccinees. Oral killed whole-cell preparations, though not reactogenic, are also not protective against *Salmonella typhi*. Therefore, efforts are now directed at the use of purified virulence (Vi) antigens or live orally administered preparations of demonstrable efficacy.

In collaboration with the Pasteur Institute, the National Institute of Child Health and Human Development has developed a nonreactogenic, immunogenic, purified Vi antigen vaccine; the Vi antigen is a linear homopolymer of galacturonic acid. Clinical trials in Nepal and South Africa demonstrated that a single injection of the Vi vaccine has an efficacy of about 72 to 80 percent, in the face of a very high "force of infection." Since the Vi vaccine is effective after only one immunizing dose, it appears to offer some advantages over the Ty21a vaccine, especially for use in developing countries, although it is associated with some minor side effects in some vaccinees. The Vi vaccine has been licensed in France and several countries in Africa; the manufacturer is currently assembling data to apply for a license in the United States. More recently it has been shown that immunogenicity could be increased by covalently conjugating the Vi polysaccharide to protein carriers such as *E. coli* labile toxin. The use of labile toxin or cholera toxin in such a vaccine may also stimulate antibody production to the toxin itself, thereby providing some additional protection against enterotoxigenic *E. coli*, cholera, and diarrheal diseases mediated by these related toxins as well.

An important advance for the control of typhoid fever has been the development of the attenuated *S. typhi* strain Ty21a from strain Ty2. This strain was extensively tested in Egypt and Chile, and although its efficacy may vary widely from site to site and with vaccine formulation, the Ty21a vaccine has been remarkably safe and reasonably immunogenic. It was licensed in the United States in 1991 and is presently being used primarily as a vaccine against traveler's diarrhea. The World Health Organization has recently advocated a head-to-head comparison of the efficacy of Ty21a and Vi to make future recommendations on the use of these two available vaccines in areas severely affected by typhoid.

Several groups of investigators have been developing attenuated deletion mutants as live oral typhoid vaccines. Metabolic pathways and genes critical to virulence expression have been targeted. These include the double *aro* mutants, *aro/pur* mutants, *cya/crp*, and the *pho*P/*pho*Q mutant. Several of these mutants have been used in clinical trials with varying degrees of success. The focus here will be on recent efforts.

The University of Maryland has been pursuing double *aro* mutants derived from wild-type strain Ty2. CVD 908 was shown to be incompletely attenuated because it induced bacteremia in 6 of 12 volunteers at a dose of $5x10^7$ colony-forming units (cfu). The additional deletion of *htr*A made it clinically more acceptable. This strain, designated CVD 908-*htr*A, will soon be undergoing additional clinical studies. These vaccine strains are being developed by Peptide Therapeutics Limited, England.

Another vaccine candidate developed by Dr. Roy Curtiss is the *cya/crp/cdt* triple deletion mutant of Ty2. The *cya/crp* double mutant was found in clinical trials to be incompletely attenuated. Therefore, a portion of the gene adjacent to the *crp* locus was deleted. This gene

was designated *cdt* since its apparent function is to control dissemination of *Salmonella* out of the intestinal tract and *GALT* to visceral organs in animals infected with *S. typhimurium* or *S. cholerasuis*. The strain of *S. typhi* containing equivalent deletions has been named x4073. This strain, or derivatives thereof, containing the balanced lethal plasmid expression vector have been used in two different clinical trials and shown to be well-tolerated and immunogenic. Most of the vaccine studies to date have employed strain Ty2 as the parent. Because this strain has been maintained in the laboratory since 1918 and probably contains a number of unknown mutations, Dr. Curtiss has made identical deletions in a recent clinical isolate in an attempt to define more clearly the genes contributing to attenuation as defined in mice and humans. The goal is to retain enhanced immunogenicity while satisfactorily attenuating the strain. Clinical trials with the first of these attenuated strains began in 1998 at the NIAID-supported St. Louis University Vaccine and Treatment Evaluation Unit. Dr. Curtiss' vaccines are being developed by Megan Health, St. Louis.

The other strain being actively pursued as a vaccine against typhoid is the *pho*P/*pho*Q deletion mutant TY800. This strain has also employed Ty2 as the parent. The *pho*P/*pho*Q virulence regulon is a two-component system composed of a membrane-bound kinase (PhoQ) and a cytoplasmic transcriptional regulator (PhoP). This system regulates a number of genes that contribute to *Salmonella* pathogenesis, and its deletion from Ty2 has created a vaccine candidate that appears to be well-tolerated and highly immunogenic. Of particular interest is the high antibody-secreting cell (ASC) response observed in volunteers to date. NIAID is hopeful that phase II trials with this strain can be conducted in the near future in its VTEU facilities. Avant Immunotherapeutics, Inc. is developing the vaccine.

The recent demonstration of the attenuating effects of a DNA adenine methylase (*dam)* deletion on *S. typhimurium* pathogenesis in a mouse model has identified another virulence factor that should be targeted for deletion in human vaccine strains. This gene, which may be another global regulator, may also be an important contributor to virulence in other bacterial pathogens including other enteric pathogens.

Because *S. typhi* is an invasive organism, it is expected that a significant cell-mediated immune response will be an important component of protection. Additionally, it is still assumed that *Salmonella* vectors can be developed to express foreign antigens and serve as multivalent vaccines capable of protecting against more than one enteric (or other) disease by oral immunization. Although encouraging results have been demonstrated in animals, this concept has yet to be conclusively demonstrated in human trials. Definition of a suitable live-attenuated, orally delivered vaccine against *S. typhi* itself will set the stage for this development.

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Fungal Infections

Overview

Infections caused by systemic fungal pathogens are a significant health problem in both the immunocompetent and the immunocompromised host. Fungi that regularly infect and cause disease in otherwise healthy hosts are termed primary pathogens. These include *Coccidioides immitis, Histoplasma capsulatum, Blastomyces dermatitidis, Paracoccidioides brasiliensis*, and, on occasion, *Cryptococcus neoformans*. Opportunistic fungal pathogens, which more typically require immunosuppression to infect the human host, include *Candida albicans*, which is a normal inhabitant of the human gut, and *Aspergillus fumigatus,* which is ubiquitous in the environment. The primary fungal pathogens each occupy a discrete ecological niche. *C. immitis* is found in the soils of the southwestern United States, Mexico, Central America, and South America. *H. capsulatum* can be found in soils enriched with guano from bats, chickens, and starlings, with a highly endemic focus along the Mississippi River but with documented occurrence throughout the world. *B. dermatitidis* is believed to be present in microfoci of soil worldwide, but primarily in geographic regions of North America that overlap those of *H. capsulatum*. Historically, it has been difficult to isolate *B. dermatitidis* from the environment, but it probably occupies a different niche than does *H. capsulatum*. Recent studies have found *B. dermatitidis* in moist, rich soil at the banks of rivers and waterways in endemic regions. *P. brasiliensis*, the etiologic agent of paracoccidioidomycosis (South American blastomycosis), is restricted to South and Central America. It has an affinity for shady and moist vegetation, particularly near rivers and lakes, with microniches in the armadillo's hole or in the soil rich in organic matter where this animal usually feeds. Virulent strains have been frequently isolated from naturally infected armadillos (*Dasypus novemcinctus*). The increasing incidence of paracoccidioidomycosis in the Amazon region can be associated with recent agricultural settlements, deforestation, and soil churning. Worldwide, roughly 10 million people may be infected with *P. brasiliensism,* and as many as 1 to 2 percent of these people may develop the disease. *C. neoformans* can be found in soils contaminated with pigeon guano and is prevalent worldwide. Infection is initiated by inhalation of microscopic forms of each fungus from a point source in nature.

The true incidence of infection by these agents is difficult to assess because the diseases are not reported nationally and can be difficult to diagnose. With the exception of the latex agglutination test for cryptococcal capsular polysaccharide antigen, there are few widely available serologic tests to facilitate rapid laboratory identification of the systemic mycoses. Definitive diagnosis usually depends on culture of the etiologic agent. Recent developments in molecular studies of *C. immitis*, which include cloning and expression of the diagnostic complement fixation (CF) antigen, as well as reports of a sensitive polymerase chain reaction-based method for detecting coccidioidal DNA in patient sputum, provide the basis for new clinical methods of rapid and inexpensive diagnosis of coccidioidomycosis.

It has been estimated, based on the results of skin tests, that there are between 25,000 and 100,000 new infections with *C. immitis* each year. The respiratory disease, known as Valley Fever, can occur in epidemic proportions; 1,500 seroconversions were documented in one county in California in 1991, whereas the number of officially reported cases for the entire State was less than 1,300. This finding emphasizes the problem of underreporting for these diseases. The epidemic in California resulted in more than 3,000 cases occurring in Kern County alone in 1992. It was estimated that the epidemic resulted in more than $45 million in medical costs in Kern County between 1991 and 1993. The California Department of Health sponsored a conference on coccidioidomycosis in 1993. The development of a vaccine was considered to be a promising approach for the prevention of the disease. The Valley Fever Research Foundation, a private foundation incorporated in 1993, commissioned a vaccine feasibility study. The study concluded that a vaccine effort should go forward. Current efforts focused on the development of a vaccine against coccidioidomycosis involve a consortium of seven laboratories funded by research grants from NIAID and the California HealthCare Foundation.

The number of cases of Valley Fever in the Tucson and Phoenix areas increased by 66 percent between 1991 and 1992. A serious complication of the infection is meningitis, a life-threatening disease that is difficult to treat. Primary infections that apparently have resolved spontaneously may leave dormant but persistent fungal elements in lung tissue. Relapse of fungal diseases, such as Valley Fever, is viewed as a potential crisis among immunocompromised patients, such as those with acquired immunodeficiency syndrome (AIDS). One prospective study documented a prevalence of 25 percent in one cohort of HIV-infected patients over a 41-month period in highly endemic areas.

Histoplasmosis also is associated with epidemics in immunocompetent hosts. However, it is becoming an increasingly important infection in immunocompromised

hosts, such as those with AIDS, where the incidence of this fungal disease can be as high as 27 percent. Histoplasmosis can resemble tuberculosis and has been misdiagnosed as such. In one study, 19 percent of the patients with histoplasmosis also had tuberculosis. The disease is geographically widespread, with reports from every continent except Antarctica, and 500,000 new infections are estimated to occur annually in the United States. It is estimated that 99 percent of these infections resolve spontaneously; the remaining 1 percent progress to chronic or disseminated disease. The reasons for this progression in otherwise healthy individuals remain unknown. Clinical disease can be classified as mild, moderate, and severe, with the latter category being the most difficult to treat with available chemotherapy. Given the widespread distribution of disease, the inability to prevent acquisition from a point source in nature, and the remaining problems with antifungal therapy, a vaccine for this disease would have obvious public health benefits.

Blastomycosis occurs mainly as a sporadic infection in immunocompetent hosts, but many cases of opportunistic infection among AIDS patients and other immunocompromised hosts have been described. The true incidence and prevalence of blastomycosis are unknown but appear to be lower than those of the other systemic mycoses described here. A distinguishing feature of blastomycosis is the high proportion of clinically significant disease among infected persons, highlighting the organism's pathogenicity. Another feature of blastomycosis is that it is a common infection among dogs that reside in endemic areas. The severity of most canine infections also is evidence of the potential of *B. dermatitidis* as a primary pathogen.

Although immunosuppressive therapy and infection with HIV are recognized risk factors for the development of severe, progressive coccidioidomycosis and histoplasmosis, they are not prerequisites for human infection with these fungi. Both are primary pathogens. In addition, subclinical infection with these fungi and with *C. neoformans* poses a threat of subsequent reactivation to a progressive form of disease with the advent of immunosuppression. Cryptococcosis (cryptococcal meningitis) is a worldwide problem for immunosuppressed patients. In the United States cryptococcosis is a well-known AIDS-defining illness and occurs in 7 to 11 percent of patients with AIDS. A hospital survey in New York City documented more than 1,200 cases of cryptococcosis in 1991 that were primarily associated with HIV-infected patients, resulting in a yearly prevalence of 6 to 8 percent in this population. Cryptococcal meningitis is also prevalent in HIV-infected individuals in Africa where the costs of antifungal therapy can be prohibitive. Even with the advent of newer antifungal drugs, such as the triazoles, treatment remains suboptimal, and no existing treatment is curative. The situation for coccidioidomycosis and histoplasmosis in patients with AIDS is similar.

Mechanisms of virulence for the pathogenic fungi are poorly defined. The fungi considered above lack toxins that could serve as good targets for a rationally designed vaccine. In addition, they possess a complex, eukaryotic genome that makes elucidation of their molecular biology more difficult than that for either their viral or bacterial counterparts. However, fungi do present numerous effective antigens as demonstrated by the host's response to infection. In general, cell-mediated immunity is thought to be more important in recovery from infection than the antibody response. One possible exception is cryptococcosis, in which antibody specific for the capsular polysaccharide has an opsonizing effect on the encapsulated fungus. With an ever-expanding immunocompromised host population at risk for all of these fungal infections, and with the inability of even new antifungal agents to eradicate fungi from infected patients, serious consideration must be given to the preventive or therapeutic role of antifungal vaccines.

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Blastomycosis

Spores are inhaled into the lungs and converted into budding yeasts, which are large and relatively resistant to phagocytosis and killing by the neutrophils and mononuclear effector cells that constitute the early inflammatory response. Within several weeks after infection of humans and experimental animals, the host develops acquired immunity to *B. dermatitidis* as evidenced by the appearance of delayed-type hypersensitivity, proliferation of lymphocytes *in vitro*, and circulating antibodies in response to antigens of the fungus. In a murine model of blastomycosis, T-lymphocytes but not serum passively transferred from immune to naive animals conferred protection, suggesting that immunity resides chiefly with antigen-specific T cells.

A 120-kD protein, designated WI-1, is displayed on the surface of *B. dermatitidis* yeasts and is an immunodominant antigen during human, canine, and experimental murine infection. Human patients develop strong antibody and T-lymphocyte responses to determinants of WI-1. WI-1 has been cloned and sequenced and shown to contain 30 copies of a repetitive domain of 25 amino acids similar in sequence to a bacterial adhesin, invasin. This so-called tandem repeat mediates binding of the yeast to integrin receptors on human cells, and the expression of WI-1 is altered on genetically related strains of *B. dermatitidis* that differ in virulence for mice, suggesting that WI-1 plays a role in the pathogenesis of blastomycosis. Human, murine, and canine infection is associated with the development of

high antibody titers directed against the tandem repeat. The functional role of monoclonal anti-WI-1 antibodies is under study, and some appear to enhance infection. T-lymphocytes from human patients with blastomycosis respond strongly to WI-1 *in vitro*. At the clonal level, these cells are directed chiefly toward epitopes displayed in a short segment of amino acids at the N-terminus. WI-1 is immunogenic in mice, where protective efficacy has been shown. This supports its vaccine potential, although harmful and beneficial segments of the antigen may need to be separated.

A gene transfer system is available in *B. dermatitidis*, and WI-1 has been disrupted by homologous recombination. WI-1 knockout yeast bind poorly to host tissue and are nonpathogenic in a murine model of pulmonary blastomycosis, emphasizing the role of this adhesin in virulence. Animals that clear knockout yeast can resist a lethal pulmonary challenge with wild-type yeast. Therefore, WI-1 knockout yeast serve as an attenuated vaccine. Antigens responsible for this resistance are under study. The considerable clinical importance of canine blastomycosis in veterinary medicine provides a unique target population of dogs for initial clinical investigation of novel vaccine formulations, such as naked DNA or attenuated strains.

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Candidiasis

Candidiasis is a leading group of opportunistic mycoses caused by any of several species of the genus, *Candida.* Most noteworthy examples include *C. albicans, C. tropicalis,* and *C. krusei.* These and other *Candida* species are normal inhabitants of humans and usually live in harmony with the mammalian host. Factors predisposing to disease include chemical immunosuppression, surgical trauma, and underlying diseases such as diabetes and AIDS. Neutropenia is a major risk factor; patients undergoing immunosuppression to prevent rejection of bone marrow or organ transplantation are particularly vulnerable to infection from either endogenous or exogenous sources.

Novel advances in the identification of protective antibody in models of cryptococcosis described in this report have given hope that analogous situations may pertain to other opportunistic mycoses, including candidiasis. Indeed, a protective antibody has been identified for *C. albicans* in an animal model system. Antigen delivery was key to demonstrating that a mannan adhesin from the fungus could generate immunoprotection. Liposome encapsulation of a mannan adhesin fraction of yeast cells or conjugation of the mannan to a carrier protein have been used to generate protective antibodies that are functional in vaccinated mice and could be passively transferred to protect normal and immunocompromised mice. Both protective and nonprotective antibodies were identified. The latter can be useful in addressing the controversy generated in previous studies where circulating antibodies did not correlate with protection. Two murine monoclonal antibodies, an IgM antibody B6.1 and an IgG3 antibody C3.1, have been demonstrated to be protective in passive transfer experiments, and there is considerable interest in examining the role of immunotherapy as an alternative to chemotherapy in human candidiasis. Because of the newly acknowledged problem of antifungal drug resistance in *Candida*, these findings are of special relevance.

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Coccidioidomycosis

Spores of *C. immitis* are inhaled into the lungs, where they undergo a morphological conversion to a parasitic, spherule form of growth. The spherule enlarges and subdivides into propagative units that are released to repeat the cycle. Patients develop delayed-type hypersensitivity as a consequence of infection. Although complement-fixing and precipitating antibodies are produced during the course of infection, they do not seem to be protective. In

fact, high titers of complement-fixing antibodies are a poor prognostic sign. In experimental infections, immunity is transferred by thymus-derived lymphocytes (T cells) but not by serum.

An experimental vaccine has been prepared from formalin-killed spherules of the fungus grown *in vitro*. After it was demonstrated that the vaccine increased survival in animals after a lethal experimental challenge, a phase III trial was undertaken in human volunteers. The study groups were from Arizona and California and were demonstrated to be skin-test-negative to spherule antigen and to coccidioidin before vaccination. A total of 1,400 subjects received the formalin-killed spherulin vaccine (1.75 mg per injection, with a total of 3 injections), and 1,400 others received placebo. The results of the trial indicate that the vaccine did not prevent clinically apparent coccidioidomycosis. In experimental trials in mice, the vaccine did not prevent infection but did prevent progressive disease and death. Because progressive disease did not occur in either the control or vaccinated human groups, it was not possible to evaluate these potential protective effects. Failure of this trial could have been caused by dose-limiting irritation at the injection site from toxic components of the fungus. That is, the dose used in the human trial was reduced to less that 1/400 of the amount of the spherule vaccine needed to protect mice on a body-weight basis.

Disruption of the whole spherule vaccine and centrifugation of the homogenate at 27,000 x gravity yielded a supernatant preparation (designated 27 K) that was as protective in mice as the killed-spherule vaccine. Cell walls from mechanically disrupted spherules have also shown to produce protection and, when the walls were incubated in phosphate-buffered saline containing 1-percent chloroform as a preservative, a soluble fraction was obtained that induced strong protection against challenge. Alkaline extraction of cell walls has also been reported to yield a soluble fraction (designated C-ASWS), which protects mice against challenge with *C. immitis*. The protective component of the C-ASWS extract was shown to be a glycosylated protein having antigenic identity with the polymeric antigen in coccidioidin that had been designated Antigen 2 (Ag2). In other studies, a 33-kDA peptide was isolated from a chemically deglycosylated lysate of spherules. The 33-kDA peptide expressed T- and B-cell epitopes and, when examined by tandem immunoelectrophoresis, showed complete fusion with the anodal precipitin peak of the Ag2 polymer; hence, its antigenic identity with the protein moiety of Ag2. The gene that encodes Ag2 has been cloned by two groups of investigators and, when expressed in *E. coli*, yielded a proline-rich antigen (PRA) having a molecular size of 19.4 kDA. Immunization of mice with the recombinant Ag2(PRA) protein induced protection against challenge, but a significantly greater level of protection was induced in mice immunized with Ag2(PRA) cDNA. The protective effects of recombinant Ag2(PRA) or the Ag2(PRA) gene vaccine were associated with, and thought to be attributable to, the induction of Th1 responses, evidenced by the acquisition of a delayed footpad hypersensitivity response in mice and increased production of IFN-γ.

Additional vaccine-related research is underway with various fractions of *C. immitis*. A 48 kDA T-cell-reactive protein (TCRP), which is expressed in the cytoplasm of spherules, was shown to stimulate proliferation and IFN-γ production by T cells of spherule-immunized mice. The gene encoding this antigen was cloned and found to have 70 percent homology with mammalian 4-hydroxphynylpyruvate dioxygenase. Mice immunized with the recombinant TCRP protein had approximately 1.5 log lower burden of *C. immitis* in their lungs after intraperitoneal infection. Similar experiments were performed with a recombinant protein expressed by the gene that encodes *C. immitis* heat shock-protein 60 (*HSP60*). The recombinant HSP60 induced proliferation of T cells from HSP60-immunized mice but did not induce protection against challenge. More recently, two additional T-cell-reactive antigens have been isolated and cloned (a spherule outer wall glycoprotein [SOWgp] and urease [URE]); both have been shown to confer immunoprotection in mice against coccidioidal infection.

Although recombinant antigens and gene vaccines have induced protection against challenge with *C. immitis*, none of these vaccines have induced a level of protection comparable to that of vaccines using either the killed spherule or native antigens obtained from *C. immitis* cells or cell walls. The reduced efficacy of the recombinant and gene vaccines could be attributable to inadequate presentation or processing by antigen presenting cells. It is also possible that a multivalent vaccine comprising of several T-cell-reactive molecules expressed during different stages of the parasitic cycle and conserved amongst different isolates of the pathogen will be needed for optimal vaccination against this fungal pathogen.

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Cryptococcosis

Yeast cells of *C. neoformans* are thought to be the infectious form of the fungus. Inhalation of these cells establishes a primary pulmonary infection that is often not apparent. Meningitis is the typical manifestation of disease. Early diagnosis and treatment can arrest but not cure infection in AIDS patients; lifetime suppressive therapy is required.

C. neoformans is delimited by a polysaccharide capsule and, therefore, is unique among the major fungal pathogens of humans. The antibody response to the capsular polysaccharide is minimal in clinically apparent infections. Because most patients with cryptococcal meningoencephalitis have soluble capsular polysaccharide in serum or cerebrospinal fluid, testing for antigen is useful in the diagnosis of this infection. The capsule of *C. neoformans* is a known virulence factor, and attempts have been made to induce a protective immune response against capsular polysaccharide. Injection of mice with capsular polysaccharide alone or with adjuvants does not appear to result in sustained or high-titer antibody response. However, conjugation of cryptococcal capsular polysaccharide to protein carriers may improve the antibody response. Cryptococcal glucuronoxylomannan conjugated to tetanus toxoid has been shown to be immunogenic in mice. Preliminary clinical trials with a glycoconjugate vaccine have been conducted to determine safety and antigenicity; the ultimate goal is to develop a vaccine that will protect patients at high risk of developing cryptococcosis.

Antibody administration has been shown to enhance the efficacy of amphotericin B, fluconazole, and 5-fluorocytosine in mouse models of infection. Studies of antibody efficacy in mice have shown that antibody specificity and isotype are important characteristics for antibody effectiveness. Vaccines that elicit primarily protective antibodies may be effective in preventing infection even if the role of naturally occurring antibody in protection is uncertain.

Confirmation of the protective role of antibody also comes from studies showing that the infusion of monoclonal antibody can prolong life and decrease fungal burden in mice challenged with fungi by the intraperitoneal, intravenous, or intracranial routes. Several protective murine monoclonal antibodies have been used to construct mouse-human chimeric antibodies to the cryptococcal polysaccharide; the goal of clinical studies, in this case, is to determine the efficacy of passive immunization as an adjunct to chemotherapy in cryptococcal meningitis.

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Histoplasmosis

Spores of *H. capsulatum* are inhaled into the lungs and converted into budding yeasts that proliferate within cells of the macrophage lineage. The importance of T cell-mediated immunity in infection is implicit in the emergence of this fungus as a significant pathogen in AIDS. As with coccidioidomycosis, antibodies can be diagnostic but are not thought to play a major protective role. Delayed-type hypersensitivity develops, and immunity can be demonstrated following transfer of T cells in experimental models. These models have shown the expansion of both suppressor and helper cell lines in response to challenge with fungal antigens. The recent development of a transformation system for *H. capsulatum* and an increased knowledge of its molecular biology should facilitate studies on pathogenesis and virulence and provide at least the methodological basis for vaccine development.

HIS-62 is a 62 kD glycoprotein antigen isolated from cell wall and cell membrane extracts of yeast cells of *H. capsulatum*. This antigen induces cell-mediated immune responses in C57BL/6, BALB/c, and CBA/J mice. Vaccination with 80 micrograms of HIS-62 significantly protects all three strains of mice against lethal challenge with viable cells of this fungus. In addition, lymphocytes from humans exposed to *H. capsulatum* respond *in vitro* to this antigen. The gene encoding this antigen has been cloned and sequenced; it has a high homology with the gene that encodes for heat shock protein 60 (HSP60). Recombinant antigen has been generated from *E. coli*, and it stimulates monoclonal populations of antigen-reactive T cells and polyclonal T cells from mice immunized with *H. capsulatum* yeast cells. Vaccination with the recombinant antigen protects mice against pulmonary histoplasmosis. A fragment spanning amino acids 172-443 contained the protective activity of HSP60 although it was not as effective as the full-length protein. Studies are currently underway to determine the mechanisms by which this protein confers protection and to determine the family of T cells engaged by the protein.

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Paracoccidioidomycosis

Natural infection with *Paracoccidioides brasiliensis* is assumed to occur through the respiratory route. Lungs are involved in the majority of patients with paracoccidioidomycosis. Alveolar lesions are exudative or granulomatous. The granulomatous inflammatory response with formation of epithelioid tubercles is the most effective defense against the invading fungus. In the acute lymphatic forms the fungus reaches the lymph nodes by the afferent lymphatics. The earlier and more severe the lymph node involvement, the worse the prognosis. In the chronic progressive forms, dissemination of the fungus to mucocutaneous sites and other organs is accompanied by a vigorous cellular immune response. As the infection becomes more severe, a depression of cellular immunity may occur, leading to the anergic state. This anergy can be reversed with successful treatment. Antibody titers typically rise but do not confer protection in natural infection. Given the similarities between paracoccidioidomycosis and both coccidioidomycosis and blastomycosis, it would be predicted that native antigens exist that can be used to generate a protective immune response. Investigations are underway that support this prediction. Most actively studied is an exocellular 43 kD antigen (gp43) from yeast cell cultures. It represents the major diagnostic antigen and is immunodominant. The gene for gp43 has been cloned and sequenced and the immunodominant T-cell epitope mapped to a 15 aa. peptide (P10). The immune response elicited by either the gp43 or P10 involves T-CD4+, Th1 lymphocytes producing gamma-interferon, which is a key cytokine in the immune protection against *P. brasiliensis*. Mice knockout for gamma-interferon-receptor challenged intratracheally with virulent *P. brasiliensis* are extremely susceptible to the infection, with rapid dissemination and high mortality. Immunization with the gp43 or P10 markedly protects Balb/c mice against the i.t. challenge, with a 200-fold reduction in colony-forming units in the lungs and little or no dissemination to the liver or spleen. Recently, the DNA fragment corresponding to the mature gp43 cDNA and signal peptide was cloned into the VR1012 vector, and Balb/c mice were injected with this plasmid to elicit an immune protection. A type-1 cellular immune response

was obtained that was protective against i.t. *P. brasiliensis* infection.

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Pythiosis

Pythium insidiosum is a filamentous eukaryotic organism, previously classified in the Oomycetes of Kingdom Fungi but recently moved to Kingdom Stramenopila (Protoctista). The organism is aquatic and has a flagellated stage. Cutaneous, subcutaneous, and systemic disease can result in humans, horses, and other animals as a consequence of traumatic implantation. When left untreated, the mortality rate is 100 percent. Choices of chemotherapy are limited, and antifungal drugs are generally not effective. At least two different groups of investigators have generated promising results with therapeutic vaccines consisting of hyphal extracts. Three immunodominant proteins (28, 30, and 32 kD) have been identified. Rates of 53-percent efficacy have been reported following injections of such extracts into infected horses. Refinement of extracts by supplementation with purified protein derivatives has increased efficacy to as much as 70 percent with chronic pythiosis, which is the form least responsive to treatment. This vaccine was effective in curing more than 300 horses with the disease. Three cases of vaccination have been described in Thai humans with pythiosis in their arteries refractory to multiple courses of antifungal and surgical therapy. The infection resolved following vaccination in all cases. Recent studies in experimental rabbits, 35 horses with the infection from Texas, and 2 cases in humans from Thailand have shown that immune modulation from Th2 to Th1 response is behind the curative properties of this vaccine. Investigators have found that IL4, IL5, IgE, IgG isotypes (in study), and eosinophils (all features of Th2 response) are present during pythiosis infections. Although IL2, INFg, IgG isotypes (different from the one detected before vaccination), T cytotoxic lymphocytes, and macrophages (all features of Th1 response) are in place 7 to 20 days after successful vaccination, interestingly, in successfully vaccinated humans and horses, IL4, IL5, IgE, and the eosinophilia of the original immune response had vanished. These data suggest that the modulation of the immune system by curative vaccines is feasible. Similar data from therapeutic vaccines used to treat cancer, allergic diseases, and infections caused by *Leshmania* spp strongly support this idea. Characterization of relevant proteins in *P. insidiosum* in a rabbit model is under investigation.

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Herpesvirus Infections

Overview

The eight human herpesviruses—herpes simplex viruses types 1 and 2 (HSV-1 and -2), Epstein-Barr virus (EBV), human cytomegalovirus (HCMV), varicella-zoster (VZV), and human herpesviruses -6, -7 and -8 (HHV-6, -7 and -8)—are a significant public health problem in the United States. Most of the population has been infected with several of these herpesviruses and, therefore, has lifelong latent infections.

Clinical manifestations

Primary infections are not usually severe or life threatening in healthy persons, but many of the human herpesviruses can produce severe or chronic active infections in certain individuals. While primary infection of young children with most herpesviruses is often unrecognized or mild, primary infection of adults with VZV or EBV can be severe. HSV and HCMV pose a particular threat to newborns whose mothers have had a primary infection during pregnancy.

Reactivation-associated disease is often more severe than primary infection. HSV-1, HSV-2, and VZV are associated in some individuals with frequent and/or painful recurrences that manifest themselves as cold sores, genital herpes, and shingles, respectively. Reactivation of herpesviruses in individuals with compromised or waning immunity may result in severe and life-threatening illnesses such as HCMV pneumonia and EBV-associated lymphomas. Therefore, herpesviruses can pose a particular threat to AIDS patients, cancer patients, organ transplant recipients, and the elderly. Induction of immunity that could withstand immunosuppressive regimens would bring significant benefit to these patients. An additional concern with reactivation is that asymptomatic individuals shedding reactivated virus may serve as reservoirs for herpesvirus transmission.

Herpesvirus infection can also have long-term consequences. In certain geographical areas and in certain populations, EBV is associated with nasopharyngeal carcinoma and with Burkitt's lymphoma. More recently, the association of EBV with Hodgkin's lymphoma, T-cell lymphomas, and some gastric carcinomas has been suggested. HHV-8 is a newly recognized herpesvirus associated with Kaposi's sarcoma. There has also been the suggestion of an association between herpesviruses and certain chronic diseases, including HHV-6 and multiple sclerosis, and HCMV and heart disease**.**

Challenges in developing herpesvirus vaccines

Clinically, the goals of immunization against herpesviruses include reducing the severity of disease associated with primary infection; reducing the frequency of reactivation of latent virus; limiting the severity of reactivated disease; and restricting the transmission of virus associated with either primary or reactivated infection. For most human herpesviruses, there is reason to believe that at least some of these goals should be achievable. One effective herpesvirus vaccine, V2V, is already licensed and in use. For other herpesviruses, there is evidence that natural infection can provide at least partial protection against subsequent infection by different viral strains. Further, there are several effective herpesvirus vaccines in use in domestic animals (e.g., pseudorabies virus, Marek's Disease virus, feline herpesvirus, equine herpesvirus, bovine herpesvirus). Experimental vaccination can also provide protection in herpesvirus animal models. Nevertheless, there are several aspects of vaccine research and development that are complicated by unique properties of herpesviruses and their interactions with their hosts.

Immune correlates of protection. Defining the nature of protective immunity for herpesvirus infections is complex because different specificities and types of responses may be needed to prevent primary disease, prevent or limit the establishment of latency, prevent or limit reactivation, control the severity of reactivation disease, and minimize the shedding of infectious virus. In primary infections, the role of antibody is generally limited, with CD8+ T cells and/or CD4+ (TH1) acting foremost in clearing virus. Cellular responses also appear to be essential for limiting the replication and/or spread of reactivated virus. Considerably more work is needed to more precisely delineate the protective responses unique to each of the human herpesviruses. New approaches for measuring specific immune responses, such as flow cytometric assessment of intracellular cytokine production and tetramer analysis, are expected to be valuable in this regard.

Mucosal immunity. Most human herpesviruses infect via mucosal surfaces; reactivated infection may occur at such sites and free virus is typically shed from such sites. Thus, systemic immunity may not provide adequate protection against initial or recurrent infection or virus shedding and transmission; antibody at mucosal surfaces and/or cell-mediated response within mucosal tissues may be required. While the nature of mucosal immune responses is not well understood, it is clear that immunization protocols that successfully induce systemic immunity may not induce adequate humoral and cellular responses at mucosae. Therefore, a major area of interest in herpesvirus vaccine research is the development of strategies for inducing such responses.

Latency. A hallmark of herpesvirus infections, latency presents a dilemma for vaccine development: while it is desirable to prevent latency and thus reactivation disease, latent infection may in some cases be beneficial, if periodic sub-clinical reactivation and immunologic stimulation lead to more durable immunity. In any case, preventing the establishment of latency is likely to be difficult. Few or

no viral proteins are produced during latent infection, eliminating targets for recognition by the immune system. Rapid establishment of latency thus makes it difficult for a herpesvirus vaccine to provide "sterilizing" immunity, although restriction of initial replication may not only mitigate primary disease, but may also reduce the extent of latent infection and thereby the frequency or severity of reactivated replication and disease. If latency is established following vaccination, then a second concern is that the vaccine must induce an immune response of appropriate type and sufficient duration to provide long-term protection against reactivated replication. Durable immunity may depend upon periodic boosting by endogenous (sub-clinically reactivated) virus, as noted above, or by exogenous (wild-type) infection. If wild-type boosting is important for durability it is possible that a vaccination program leading to a significant reduction in circulating virus could actually shorten the duration of immunity and increase the frequency of reactivated infection.

Immune evasion. In addition to avoiding immune recognition through latency, herpesviruses have developed a diverse array of strategies for manipulating and outmaneuvering host immune responses (reviewed in Ploegh, 1998). Specific means include interference with antigen processing, transport, and presentation; negative regulation of cytokine activity; inhibition of CTL-induced apoptosis; interference with NK cell-mediated clearance; and inhibition of complement-mediated antibody attack. The role of these processes in modulating the level of vaccine-induced immunity (for live vaccines) or in blocking the vaccine-induced immune response to a challenge infection is not well understood.

Animal models. Animal models play a critical role in assessing the potential safety, immunogenicity, and efficacy of new human vaccines, but the testing of herpesvirus vaccines in animals is frequently problematic. One major consideration is the host range of the virus. While the alphaherpesviruses (HSV, VZV) have a variable host range and can infect rodents and primates as model hosts, the gammaherpesviruses (EBV, HHV-8) infect only species in the same family or order as the natural host, and the betaherpesviruses (HCMV, HHV-6, and -7) replicate little if at all in species other than their natural hosts. For this latter group, alternative models have included humanized SCID (SCID-hu) mice and the use of related viruses of rodents or primates (e.g., murine and guinea pig cytomegaloviruses). While these systems are useful for some studies of pathogenesis and immune response, they cannot be used for preclinical evaluation of vaccine safety and efficacy. A further concern is the relevance to humans of immunogenicity and protection studies done in animals. For example, the immune responses and efficacy obtained with an experimental vaccine can vary between mouse strains (Manickan et al., 1995), and an HSV subunit vaccine that was very effective in protecting mice was not found to be effective in subsequent human trials.

Vaccination approaches for herpesviruses

Most of the approaches for vaccination available today have been applied to one or more of the human herpesviruses. For each of these approaches, there are both advantages and potential obstacles that derive from the unique nature of herpesviruses and their infections.

Live-attenuated virus. This vaccination approach has enjoyed the greatest success against herpesviruses to date. The live-attenuated Oka strain of VZV used for the prevention of chickenpox is the only human herpesvirus vaccine presently licensed by the FDA. In addition, the USDA licenses effective modified-live vaccines for five different herpesviruses infecting domestic animals. Live vaccines offer a theoretical advantage over other approaches in that the full spectrum of viral proteins is presented in their natural context and abundance. However, by using live vaccines for herpesviruses, latency may be established and there is thus the potential for reactivation-associated or other chronic disease. These concerns are tempered somewhat by the lack of problems seen in long-term followup of healthy and leukemic children who received the VZV vaccine, as well as renal transplant recipients immunized with the attenuated Towne strain of HCMV. In fact, establishment of latency by an attenuated vaccine virus may in some cases be desirable for ensuring durable immunity. A technical problem with traditional attenuation approaches for herpesviruses has been the difficulty of achieving an acceptable reduction in virulence while maintaining adequate immunogenicity. Thus, efforts are underway to engineer new attenuated vaccines for HSV and HCMV by identifying and manipulating regions of the genome or specific viral genes that control latency, reactivation, and virulence.

Disabled virus. One approach that may address some of the problems of live herpesvirus vaccines involves engineering replication-defective strains of virus. Mutations have been introduced into essential genes to prevent the formation of progeny virions (Nguyen et al., 1992; De Costa et al., 1999), or into structural protein genes so that only noninfectious progeny virions are produced (Farrell et al., 1994). This strategy requires a good understanding of the genes controlling a virus's replication and virulence, and has thus far been applied only to HSV, although it is being considered for VZV. Disabled virus vaccines have been able to protect mice against challenge with virulent HSV and appeared to be safe and immunogenic in a phase I trial (Cantab Pharmaceuticals, 1999), suggesting that it may be possible to induce protective immune responses in humans without complete virus replication. An unexpected potential advantage of at least one disabled HSV strain is an apparent inability to establish latency (De Costa et al., 1999).

Vectored subunits. Delivery of one or more herpesvirus proteins via a viral vector (replicating or not) could address concerns with pathogenicity and latency, while delivering adequate quantities of viral antigens and presenting them in a suitable context. The potential of recombinant vaccinia virus has been demonstrated by the successful oral rabies vaccine used for wildlife, and highly attenuated versions of mammalian and avian poxviruses are available for use in humans (reviewed in Paoletti, 1996). Several poxvirus constructs expressing proteins from HCMV, EBV, and HSV have demonstrated immunogenicity or efficacy in experimental animals, but the immune responses observed in human trials of HCMV and EBV recombinants have been relatively modest. Poxvirus recombinants may also be useful for augmenting immune responses through a prime-boost regimen (see Tartaglia et al., 1998, for experience with this strategy for HIV vaccines), as has been described recently for HCMV.

Inactivated virus. The classical strategy of using inactivated virus has a history of yielding safe and effective viral vaccines, but it has several potential limitations for herpesviruses. Viral proteins are not presented in a natural context and only structural proteins are presented, thereby limiting the type and breadth of the immune response obtained. Several vaccines derived from inactivated virions—either complete preparations or partially purified proteins—of HSV and VZV have been evaluated clinically. None of the HSV vaccines has proven effective, and heat-inactivated VZV provides significantly poorer protection against varicella as compared to the live Oka vaccine.

Recombinant subunits. Subunit vaccines containing purified viral proteins are a relatively safe alternative to live vaccines. Most studies have focused on the external viral glycoproteins; however, early viral antigens also have been shown to induce T-cell-mediated immunity. To date, clinical experience with subunit vaccines for herpesviruses has not been encouraging. While those subunits evaluated in phase I and II trials have been safe and immunogenic, a recent phase III trial of an HSV-2 gB+gD subunit vaccine failed to prevent or delay outbreaks in infected individuals (Corey et al., 1999). Approaches for improving subunit immunogenicity, such as novel adjuvants or incorporation of subunits into structures such as viruslike particles (VLPs) or ISCOMs, has received some attention but no clinical evaluation to date.

Peptides. Delivery of specific T-cell epitopes as peptides has the potential to be both safe and exquisitely specific in the immune response induced. Its utility is limited, however, by the need to identify the immunogenic epitopes and by the MHC-specificity of the response. The approach has been tested only to a limited extent *in vitro* and in animals for HSV, HCMV, and EBV; recent results suggest that protection can be achieved with an HSV peptide conjugate (Rosenthal et al., 1999), and an EBV peptide vaccine has been tested in clinical trials.

Purified DNA. The advantages of DNA vaccines for herpesviruses include no risk of disease or latency, presentation of the viral proteins in their native form and context, ability to induce cytotoxic T-cell responses, and the potential for induction of long-lived immunity (see overview by Robinson et al., 1997). Promising results in animal models have been reported for HSV, HCMV, and VZV, and at least one HSV DNA vaccine has moved into phase I trials.

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Cytomegalovirus

Please refer to the section "Cytomegalovirus Vaccine Development" in the chapter titled "Selected Topics in Vaccine Research and Development."

Varicella-Zoster Virus

Background

Primary infection with VZV is manifested as chickenpox (varicella) and results in a lifelong latent infection. Reactivation of the latent virus leads to shingles (zoster).

Varicella. Prior to the introduction of the live-attenuated vaccine, approximately 4 million cases of varicella occurred annually, primarily in young children, with more than 90 percent of the U.S. population becoming seropositive (reviewed in Weller, 1997). Chickenpox was estimated to cost about $400 million each year, much of this representing the cost to parents of lost income from work (Lieu et al., 1994). As the use of the vaccine expands, it will lead to changes in the epidemiology and costs of this childhood illness in the United States.

Varicella can be complicated by a variety of serious conditions, including skin infections that can progress to systemic infections, infections of the brain, and pneumonia (reviewed in Arvin, 1996). Complications of varicella have been responsible for approximately 9,300 hospitalizations and 100 deaths annually. The risk of these complications is highest in adults: while less than 5 percent of varicella cases occur in adults over 20 years of age, 55 percent of the deaths occur in this age group (CDC, 1997).

Zoster. Zoster typically involves large areas of skin that ulcerate and require several weeks to heal. The skin eruption itself is very painful, and it is often followed by post-herpetic neuralgia (PHN), a pain syndrome which may persist for many months or years and which can be very disabling. There is no established prophylaxis or therapy for PHN. The incidence and severity of zoster and its complications increase with increasing age. The incidence among 50-year-olds appears to be between 2 and 4 cases per 1,000 persons per year, and it more than doubles by the age of 80 years. More than one-half of all cases occur in persons 60 years of age and older (Hope-Simpson, 1965). PHN is the major complication of zoster in the immunocompetent host: rare in individuals below 40 years of age, PHN is estimated to occur in from 25 to more than 50 percent of patients with zoster over 59 years of age (Ragozzino et al., 1982).

Current status and key issues in research and development

Both humoral and cellular immune responses are elicited early in primary VZV infections, and their relative contribution to protection from disease is not well understood. The impact of active humoral immunity appears to be limited, but pre-existing antibody has been shown to provide some level of protection. Passively acquired maternal antibody affords some protection to infants, and postexposure administration of VZV immunoglobulin (VZIG) to immunocompromised children reduces disease severity (Brunell et al., 1996). In children receiving the live-attenuated Oka vaccine, the incidence and severity of breakthrough infection are inversely correlated with antibody titer to VZV glycoproteins (White et al., 1992), and possibly with the level of T-cell responses as well (Bergen et al., 1990). Conversely, it is clear that cellular responses play the primary role in preventing disease associated with reactivation of latent VZV. While decreases in humoral immunity are not associated with increased risk of zoster (Webster et al., 1989), the age-related decline in cell-mediated responses to VZV antigens is proportional to the age-related increase in the incidence and severity of zoster (Arvin et al., 1980; Meyers et al., 1980; Ruckdeschel et al., 1977), suggesting that this loss is a causative factor.

The role of viral immune evasion mechanisms in VZV infection is not well defined. For example, VZV is similar to HSV in that its glycoprotein gE forms a complex with gI and can act as an Fc receptor, but it is not known whether the similarity to HSV extends to providing protection from virus-specific antibody (Nagashunmugam et al., 1998). Efforts are currently underway to identify VZV genes that may be associated with evasion of MHC class I- and class 2-mediated immune responses (reviewed in Abendroth and Arvin, 1999)

A live-attenuated varicella vaccine, Oka, was developed in Japan in the early 1970s (Takahashi et al., 1974). In the U.S., this vaccine is produced by Merck & Co. (Varivax), was licensed for use in healthy individuals by the Food and Drug Administration in 1995, and is now recommended for universal use in early childhood by the CDC's Advisory Committee for Immunization Practices (CDC, 1996), the American Academy of Pediatrics (American Academy of Pediatrics, 1995) and the American Academy of Family Physicians. The use of Varivax in the U.S. has been increasing steadily. According to Merck & Co., more than 16 million doses of Varivax have been distributed, and the immunization rate for 1- to 2-year-olds is approaching 70 percent. All 50 U.S. States have ordered the vaccine for use in their immunization programs, and 14 have passed school and/or day-care requirements for varicella vaccination. Post-licensure surveillance in day-care centers indicates that

the vaccine is generally well tolerated and leads to a lower attack rate and protects from severe disease (Izurieta et al., 1997; Buchholz et al., 1999). Long-term monitoring of vaccines to date indicates that immunity persists, and to some extent, is stronger, at 5 years post-vaccination (Zerboni et al., 1998). Further studies will establish whether immunization will provide protection as durable as that from natural infection, or whether boosting will be required to maintain protection through adulthood. The expanding use of this vaccine will undoubtedly alter the epidemiology and costs of varicella in the U.S., and it affords the opportunity to study in greater detail the correlates of protection against infection and disease and the viral functions associated with virulence and attenuation.

It also remains to be demonstrated whether the VZV vaccine will be effective in other populations, such as in the elderly for prevention of zoster, or in immunosuppressed transplant patients. Initial studies of vaccination in the elderly have shown that VZV-specific cell-mediated immunity can be boosted significantly (Hayward et al., 1996; Berger et al., 1998).

In addition to further studies on the live-attenuated virus, there are continuing efforts to evaluate alternate vaccines. Inactivated virus showed some efficacy in protecting bone marrow transplant recipients from shingles (Redman et al., 1997), although this strategy has also been associated with a poorer MHC class I-restricted cytotoxic response (Hayward et al, 1996) and reduced protection from varicella (Varis and Vesikari, 1996) when compared to the live-attenuated vaccine. Other strategies being pursued include disabled virus and plasmid DNA.

Recent accomplishments and developments

The availability of a live-attenuated VZV vaccine that is safe, effective, and FDA-licensed for the prevention of varicella presents an opportunity to determine whether the same vaccination strategy might be effective for preventing zoster in the elderly. In 1994, the Veterans Administration Cooperative Studies Program (VA-CSP) approved a protocol for a multicenter, double-blind, placebo-controlled phase III study to determine whether Varivax can decrease the incidence and/or severity of zoster and its complications in adults age 60 and older. The primary outcome measure for the study is total burden of zoster-associated pain during a first occurrence of HZ. In 1998, the study was initiated as a collaborative effort between VA-CSP, Merck & Co., and NIAID. A total of 21 sites are participating, with a recruitment goal of 37,200. With a 3-year followup period, the study is expected to last approximately 5 years.

Next steps and challenges ahead

The development of either a VZV virus incapable of becoming reactivated or of a subunit vaccine will require much more basic research. Studies of both the antigenic components most important for developing an immune response in humans and novel methods for presenting viral antigens to cells of the immune system are in progress. The results of the phase III study described above will determine whether live-attenuated VZV can help prevent shingles in the elderly. Other populations at risk for severe VZV disease—e.g., pediatric renal transplant recipients—are also candidates for studies evaluating the safety and efficacy of the live-attenuated vaccine.

Cantab Pharmaceuticals (Cambridge, England) has announced a collaboration with Kaketsuken (Japan) to explore the development of a disabled VZV vaccine for both chickenpox and shingles. Vical (San Diego, CA) has a collaboration with Pasteur Mérieux Connaught (Swiftwater, PA) to explore a plasmid DNA vaccine.

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Epstein-Barr Virus

Background

Based on serology, approximately 90 percent of the adult U.S. population has been infected with EBV. Primary childhood infection is often asymptomatic (Henle and Henle, 1970). In most developed countries, 35 to 75 percent of the young adult population remains seronegative. In 25 to 70 percent of such seronegative young adults, EBV infection results in infectious mononucleosis (reviewed in Niederman and Evans, 1997). In limited geographical areas and populations, EBV is associated with nasopharyngeal carcinoma (NPC) and with Burkitt's lymphoma (BL) (reviewed in Rickinson and Kieff, 1996). NPC and BL appear to require environmental, genetic, or chemical co-factors. In immunocompromised individuals, including AIDS patients, EBV is associated with lymphoproliferative diseases and lymphomas. Recent evidence also suggests a possible association with Hodgkin's lymphoma, T-cell lymphomas, and some gastric carcinomas.

Current status and key issues in research and development

The principal target of EBV neutralizing antibodies is the major virus surface glycoprotein gp220/350. A range of cell-mediated responses to EBV infection has also been described, and is likely to be important in controlling persistent infection. CTLs specific for the latent EBV nuclear antigens EBNA-3A, -3B, and -3C are predominant in a large portion of seropositive adults and children (Khanna et al., 1992; Tamaki et al., 1995).

Several vaccine candidates based on this gp350/220 have been developed. For subunit vaccination, this large, heavily glycosylated protein has been prepared from mammalian cell lines (Chinese hamster ovary or mouse C127). Primate studies demonstrate that subunit vaccination can elicit a specific antibody response that is at least partially protective, and suggest that the choice of adjuvant is likely to be important in achieving acceptable efficacy (Finerty et al., 1994). Recently, a phase I clinical study demonstrated that the subunit vaccine is well tolerated in both seropositive and seronegative persons, and that an immune response is induced (Aviron, 1999). Live recombinant vectors have also been used to express and deliver gp220/350. Immunization with vaccinia recombinants provides some protection in primates (Mackett et al., 1996) and in EBV-negative infants (Gu et al., 1995). Clinical trials of a peptide vaccine bearing an EBNA-3A epitope are underway in Australia (Moss et al., 1994).

Recent accomplishments and developments

A phase I clinical trial conducted by SmithKline Beecham Biologicals in collaboration with Aviron has provided initial safety data on a subunit vaccine for EBV. The vaccine under development contains the gp350/220 surface glycoprotein combined with a proprietary adjuvant from SmithKline Beecham. The trial was a randomized, double-blind study to evaluate safety and immunogenicity in 67 healthy young adults. The study showed that the vaccine tested was safe and well tolerated. Laboratory tests showed evidence of immune response in vaccine recipients.

Next steps and challenges ahead

It is not known whether vaccination with gp220/350 alone will be adequate to protect against primary infection, and whether such a protective response would be effective against EBV-associated tumors where the expression of viral gene products is both limited and different. Little has been reported on the use of antigens other than gp220/350 in candidate subunit or recombinant vaccines. Further work is also needed on defining the CTL specificities that a candidate vaccine should target. Following up on their successful phase I trial of a gp220/350 subunit, the next step for SmithKline Beecham Biologicals will be a larger phase II study. Results from the Australian phase I evaluation of peptide vaccination are pending.

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Parasitic and Tropical Infections

Overview

Parasitic diseases continue to plague billions of people in the modern world, killing millions annually and inflicting debilitating injuries such as blindness and disfiguration on additional millions. The World Health Organization (WHO) estimates that 1 person in every 10 is infected with a major tropical disease, and approximately 1 person in 4 harbors parasitic worms. These infections exact an enormous toll on world health and the global economy, particularly in less developed countries, where the diseases are often cited as a major impediment to economic progress. Despite efforts at control, some parasitic diseases are actually becoming more widespread because of drug resistance and changing water and land management policies that have brought humans in closer contact with parasite vectors.

Parasites remain a public health concern in the United States and other developed countries. Many human parasites are widely distributed in this country, but infections remain subclinical because of good nutrition and hygiene practices. In immunologically immature or immunosuppressed populations, however, parasitic infections represent a significant cause of morbidity and mortality. Moreover, symptomatic parasitic infections are becoming more widely observed in the United States as a consequence of the increased number of Americans traveling abroad and of the increased number of immigrants from endemic areas. Recently, isolated endemic foci of some exotic parasitic infections (e.g., malaria and leishmaniasis) have been reported in the United States.

Leprosy

Leprosy, a chronic infectious disease caused by *Mycobacterium leprae (M. leprae)*, has been a scourge of mankind since ancient times. It primarily affects the skin, peripheral nerves, mucosa of the upper respiratory tract, and eyes, often causing substantial disfigurement and disability if untreated.

M. leprae is an acid-fast, rod-shaped bacillus related to the bacterium that causes tuberculosis (*Mycobacterium tuberculosis*). Research on this bacterium has been markedly hampered by a continuing inability to culture it *in vitro* and by its extremely slow doubling time (the slowest known for any prokaryote—approximately 13 days). The bacilli can be propagated in the foot pads of nude mice, but the only established animal model of disseminated disease is the nine-banded armadillo, which poses significant technical challenges of its own.

In the United States there are an estimated 6,500 persons with leprosy, including both those currently undergoing and those off treatment; 112 new cases were reported in 1998. The World Health Organization (WHO), which has led a global leprosy elimination program based on case detection and delivery of effective multidrug therapy (MDT), estimates that in 1997 there were 768,619 registered cases worldwide, with approximately 800,000 new cases detected. These figures represent a dramatic decrease in the prevalence of leprosy over the past few years; however, the number of new cases detected annually has been stable during this same period and recently even appears to be on the increase. The reasons for this discrepancy between the remarkable effect of MDT on prevalence and the lack of noticeable impact on new cases detected are not clear, but one must consider the possibility of previously unknown reservoirs—either environmental or in the form of subclinical human infection. India, Indonesia, and Myanmar currently account for approximately 70 percent of the world's leprosy. Other "hot spots" for this disease continue to exist in Africa, Brazil, Colombia, and parts of central and eastern Europe. Leprosy is still considered endemic in 55 countries.

Dapsone was discovered to be effective against leprosy in the 1940s, but dapsone-resistant *M. leprae* gradually emerged, requiring the recent development of MDT for leprosy. Leprosy patients are classified based on clinical manifestations and skin smear results into paucibacillary (PB) and multibacillary (MB) cases. Standard MDT consists of rifampicin, clofazimine, and dapsone, given in a 6-month regimen for PB disease and in a 2-year regimen for MB leprosy. An United Nations Development Program (UNDP), World Bank, and WHO multicenter trial recently demonstrated that patients with PB disease with a single skin lesion could be cured with a single dose of rifampicin, ofloxacin, and minocycline. WHO has also indicated that it may be possible to adequately treat MB disease with a 12-month rather than 24-month course of standard MDT. These new regimens represent significant practical advances in the effort to control leprosy.

WHO has declared the goal of eliminating leprosy worldwide by the year 2000, defined as reducing case rates to less than 1 per 10,000 population. Although MDT is bringing this ideal closer to reality than had been thought possible even a few years ago, many investigators believe true elimination or eradication of leprosy will not be possible without vaccination.

Major priorities in leprosy research are the development of improved diagnostics (especially a sensitive and specific skin test), furthering our understanding of the basic pathogenesis and epidemiology of the disease (it is not even clear how the disease is transmitted or whether there is a significant nonhuman reservoir), developing alternative treatments, and developing an effective vaccine.

Currently there are only a handful of candidates in the leprosy vaccine development pipeline. One of these

is the antituberculosis vaccine, Bacille Calmette-Guerin (BCG), which has been demonstrated to be effective in preventing leprosy in some settings, but its use remains controversial. The Karonga Prevention Trial Group recently published the results of a double-blind, randomized, controlled trial of single BCG, repeat BCG, or combined BCG, and killed-*M. leprae* vaccine in the prevention of leprosy and tuberculosis (TB) in Malawi. This study demonstrated that a second dose of BCG afforded an additional 50 percent protection against leprosy compared with a single BCG vaccination. In this trial, the addition of killed *M. leprae* did not improve the protection afforded by a primary BCG vaccination. A previous study by the Karonga Prevention Trial Group in the same part of Malawi demonstrated that a single BCG vaccination afforded approximately 50 percent protection against leprosy but none against TB. A recent paper by M.D. Gupte and colleagues in the *Indian Journal of Leprosy* reported on a large leprosy vaccine trial comparing four vaccine candidates to placebo: BCG, BCG plus killed *M. leprae, M.w.,* and "ICRC." The exact nature of the ICRC vaccine has not been made public, but it is reportedly based on a gamma-irradiated non-*M. leprae* mycobacterium. The study enrolled 171,400 subjects and, during a 5-year followup, found overall protective efficacies against leprosy of 65.5 percent for the ICRC vaccine, 64 percent for BCG plus *M. leprae*, 34.1 percent for BCG, and 25.7 percent for *M.w.* These exciting data suggest further analysis and testing of the ICRC and BCG plus killed *M. leprae* vaccines are warranted.

Another approach being pursued in leprosy vaccine development is the identification of major protective antigens and their use as the basis of subunit or recombinant BCG or vaccinia virus vector vaccines. As a recent example of such studies, one such protein, the 35 kilodalton (kD) protein of *M. leprae,* was identified as a major target of the human immune response to this pathogen. The 35 kD protein was expressed in the relatively fast-growing *M. smegmatis* and shown to resemble the native antigen in forming multimeric complexes and in being recognized by monoclonal antibodies and sera from patients with leprosy. The *M. smegmatis*-derived recombinant antigen was recognized by almost all these patients via a T- cell proliferative or immunoglobulin G antibody response, but not by most patients with TB. These findings suggest the *M. leprae* 35 kD protein is a major and relatively specific target of the human immune response to *M. leprae* and that it holds promise as a component of a potential antileprosy subunit, recombinant, or DNA vaccine.

Live atypical mycobacteria, including *M.w* and *M. habana*, are being investigated for their ability to elicit a cross-protective immune response, as are recombinant BCGs expressing other *M. leprae* antigen(s). Clinical testing of all these candidates would be vastly improved by the identification of correlates of human protective immunity.

Sequencing of the *M. leprae* genome is nearing completion and should provide a significant boost to leprosy research, in general, and vaccine development, in particular—even more so than for many other microbial pathogens because of the extraordinary challenges involved in investigating this noncultivatable bacterium.

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Malaria

Malaria is a major health problem in the world's tropical areas, where it is responsible for high rates of morbidity and mortality, especially in children and pregnant women. The annual incidence of malaria is estimated to be approximately 300- to 500-million cases, resulting in 1.5- to 3-million deaths each year. Because the control of malaria is difficult and has been further inhibited by the selection of drug-resistant parasites and insecticide-resistant mosquito vectors, the development of a malaria vaccine has been given high priority. Much work is now being done to determine the immunologic response to infection and to elucidate the protective epitopes that can be used in the construction of a synthetic or recombinant malaria vaccine. Such vaccines would target the infective sporozoite stage, the replicating liver or blood stages, or the sexual stages that are infective for the mosquito vector.

Over the past few years, an increasing number of malaria vaccines have been tested in limited human trials. Trials done in the 1970s with irradiated sporozoites provided good protection in volunteers challenged with infectious parasites. Several years later, an additional study was undertaken to take advantage of improved immunological techniques for the identification of immune correlates of resistance. Four of five vaccinated volunteers were protected, as measured by the absence of, or the delayed onset of, parasitemia following challenge infection. Protected individuals developed antibodies to sporozoites, including the repeat region of the circumsporozoite (CS) protein, as well as to antigens expressed by liver stage parasites. T-cell proliferation, cytotoxicity, and cytokine

production have also been observed in response to recombinant CS protein.

In studies in animal models, CS-based synthetic peptide and recombinant vaccines conferred protection when given with strong adjuvants. Early trials with CS-based vaccines demonstrated enough immunogenicity to warrant challenge studies. When such studies were carried out with adjuvants approved for human use, however, the degree of protection was disappointing. These results were interpreted to mean that better immunogenicity could be achieved if more powerful adjuvants were available for use in humans.

During the 1990s many studies were carried out with various candidate malaria vaccine formulations that included different adjuvants. These studies either failed to demonstrate adequate immunogenicity or failed to demonstrate adequate protection against challenge infection. In early 1997, however, investigators working at the Walter Reed Army Institute of Research reported that a candidate vaccine (RTS,S), based on recombinant fusion proteins of the CS protein and the hepatitis B surface antigen, could provide protection against challenge infection when the vaccine was formulated with an appropriate novel adjuvant. These results are encouraging and validate the importance of incorporating strong adjuvants that elicit appropriate immune responses into vaccine formulations. Unfortunately, subsequent studies indicated that the protection conferred by this vaccine was not long lived. Additional studies are now underway to provide initial assessments of the potential protection conferred by this vaccine under conditions of natural exposure to malaria and to improve the formulation.

An alternative approach that appears promising is to identify specific regions of the CS protein that stimulate immune responses and to then incorporate several copies of those regions into a synthetic structure called a multiple antigenic peptide (MAP). Several research groups are currently examining different MAPs for their potential as candidate vaccines. MAPs based on CS protein structures have been shown to elicit high antibody titers in animal models and are capable of boosting preexisting malaria-specific immune responses. One potential problem associated with evaluation of synthetic peptide-based vaccines such as MAPs is that genetic factors may limit immune responses to the vaccine. This is particularly important because if the responsive individuals are not adequately represented in the initial immunogenicity study, the candidate vaccine might be rejected as nonimmunogenic. To address this issue, collaborating scientists from New York University, the University of Maryland, the U.S. Agency for International Development (USAID), and NIAID developed an innovative design for a recent phase I clinical trial of a CS-based MAP vaccine. Volunteers for this clinical trial were prescreened for presumed immune response genes to ensure that an adequate number of responder individuals were included. In this trial, only the preidentified responder individuals mounted significant immune responses. Further studies will be required to address how candidate vaccines based on this approach could elicit protective immune responses in a less restricted manner.

To address the limitations imposed on such epitope-based vaccines by the genetic restriction elements, investigators from New York University and their collaborators took a novel approach. Peptide epitopes were first synthesized to yield homogeneous products, and these peptide products were then linked to a small core peptide via oxime bonds. These multiple epitope constructs were shown to be immunogenic in mice, and a construct that incorporated a universal T-cell epitope was demonstrated to elicit immune responses in mice of diverse genetic backgrounds. Plans are underway to evaluate this product for further development.

Recently, attention has been directed at the nonrepeat domains of the CS polypeptide. A genetically conserved region within these domains has been implicated in parasite attachment to liver cells. Although shown to be safe and immunogenic in a clinical trial, a vaccine based on a genetically engineered CS-derived polypeptide in which the central repeat region was excised failed to confer protection against experimental challenge in the immunized volunteers.

Although malaria vaccine efforts in the past have focused primarily on the humoral aspects of immunity, increasing attention is being directed to the important role played by T cells. In addition to enhancing antibody responses and conferring immunological memory, T cells also mediate cytotoxic immunity and induce the production of cytokines such as gamma interferon. CS-responsive T cell clones have been established from cells of vaccinees immunized with attenuated parasites; they may prove to be useful in future studies on the development of immune responsiveness. Epitopes of CS polypeptides recognized by helper T cells, as well as by cytotoxic T cells, have been identified and are being incorporated into recombinant vaccine candidates for further testing. To identify new candidate vaccine components, investigators recently have employed a new approach, called reverse immunogenetics. Using this technique, they have identified a peptide component of a liver-stage parasite protein that is efficiently recognized by cytotoxic T cells from individuals who are resistant to severe malaria.

Until recently, obtaining conformationally correct, immunogenic recombinant proteins based on candidate asexual blood-stage vaccines has hampered progress. However, scientists have now established a number of approaches to produce such recombinant proteins. One exciting approach is the use of transgenic technologies. NIAID has recently established a CRADA with Genzyme Transgenics Corporation to evaluate the feasibility of

producing genetically engineered animals capable of secreting a recombinant version of the 42 kilodalton C-terminal fragment of the major merozoite surface protein (MSP-1) in the animals' milk. In addition, recent crystallographic data are providing insights into the structure of the 19 kilodalton C-terminal fragment of MSP-1.

A number of blood-stage vaccine candidates are now entering development. In studies in Aotus monkeys, recombinant protein candidates based on the 42 kilodalton and 19 kilodalton C-terminal fragments of MSP-1 have elicited protection. A phase I clinical trial of the candidate based on the 19 kilodalton fragment of MSP-1 has recently been carried out at the Baylor College of Medicine and demonstrated that the vaccine as formulated was poorly immunogenic and had unacceptable side effects. Additional work will be required before further development and clinical evaluation.

Almost 10 years ago, a blood-stage vaccine (SPf66) developed in Colombia was reported to delay or suppress the onset of disease during trials in that country. In a randomized, double-blind trial conducted in Colombia, the vaccine was reported to have an overall efficacy of 40 percent. Two other clinical trials in South America reported similar results. These studies, however, were carried out in areas of low or seasonal malaria transmission, and thus the utility of this vaccine in areas of high transmission and in other geographic locations has been questioned. To address these issues, randomized, double-blind, controlled clinical trials have recently been completed in Tanzania, Gambia, and Thailand. In the Tanzanian study the estimated efficacy of SPf66 was 30 percent but with wide variability. In both the Gambian and Thai studies, however, no significant efficacy was demonstrated. A later study in Brazil also did not demonstrate any efficacy of SPf66. Still, it is noteworthy that because all these trials addressed only selected clinical features of malaria (i.e., fever and high numbers of parasites in the bloodstream), the potential of SPf66 to reduce either malaria-attributable morbidity or mortality cannot be determined from them.

Antigens of the sexual stages of the malaria parasite that may induce transmission-blocking activity also have been identified. Vaccination with one of them, a 25 kilodalton molecule, when expressed as a recombinant construct in yeast, has shown efficacy in animal models. From these studies, however, it is clear that attaining and maintaining a high titer of transmission-blocking antibody is likely to be important for efficacy. Experiments are currently in progress to identify novel formulations that will address this issue. Phase I clinical testing of this vaccine candidate formulated with alum has been conducted, and results should be available in the near future.

Multicomponent vaccines directed against different antigens and different stages of the parasite life cycle may offer an advantage over single-component vaccines because they may provide multiple levels of protection. Such vaccines may also reduce the spread of vaccine-resistant strains, which can arise when the parasite changes a surface protein to avoid detection by the immune system. Combination polypeptide vaccines have been evaluated but have not generally proven very successful in early stages of testing. An alternative approach has been to use recombinant attenuated viruses because they can incorporate multiple exogenous genes and express the foreign malaria antigens. Vaccinia virus has been used extensively as a smallpox vaccine and has demonstrated a good safety profile in large numbers of individuals. However, disseminated vacciniosis has been a problem in immunocompromised individuals, suggesting that a malaria vaccine based on a recombinant vaccinia virus might not have an appropriate safety profile for use in areas where HIV infection has a high prevalence. This concern is being addressed by the development of attenuated, replication-defective viruses that could be used as a basis for a recombinant vaccine. However, as the virus is attenuated, it becomes less immunogenic; a balance has to be met between safety and vaccine efficacy. An attenuated vaccinia vectored 7-antigen vaccine (NYVAC-Pf7) has been tested in phase I and phase II trials but resulted in poor antibody production and no protection.

Another exciting approach that is being developed for malaria as well as a number of other infectious diseases is a DNA-based vaccine. Such vaccines have the advantage that they may elicit both humoral and cellular arms of the immune response and that they may simplify evaluation of vaccines involving multiple different antigens. Thus, they may find utility at several stages in the vaccine identification and development process. However, because DNA vaccines are so new, experience with them is limited. The issues of safety, immunogenicity, and efficacy, especially in the long term, still need to be addressed. A phase I trial of a CS-based DNA vaccine was conducted at the Naval Medical Research Institute. The vaccine failed to induce antibody responses but did induce cytotoxic T cells. Studies are now underway to elucidate means to enhance the immunogenicity of this candidate vaccine. A number of laboratories have reported that, in experimental systems, giving a primary immunization with a DNA-based vaccine followed by a boosting immunization with a recombinant virus-based or recombinant protein malaria vaccine enhances the immune response. In addition, multivalent DNA vaccines are also under development.

It is clear that before an ideal vaccine can be developed, more information is needed on the immune response to malaria and the factors involved in protection, including the use of immunogenicity-enhancing adjuvants and carrier proteins. Under its Research Plan for Malaria Vaccine Development (see http://www.niaid.nih.gov/dmid/

malvacdv/toc.htm), NIAID has stimulated research in this area with recent initiatives and support activities. Novel vaccine targets, delivery systems, and alternative strategies to prime and boost protective immune responses differentially are being investigated. A resource for the collection of malaria research and reference reagents, named the Malaria Research and Reference Reagent Resource, has been established at the American Type Culture Collection, to provide a central source of quality-controlled, malaria-related reagents and information to the international malaria research community. As part of a consortium, NIAID, along with collaborators from the Wellcome Trust, the Burroughs Wellcome Fund, the Department of Defense, the National Human Genome Research Institute, and Stanford University, is supporting large-scale sequencing of genomes of *Plasmodium* parasites. Such efforts are expected to result in the identification of new targets for potential vaccines and drugs. Finally, efforts are also in progress to expand capabilities to produce candidate malaria vaccines and to accelerate their evaluation domestically and overseas.

As pointed out in a recent report from the Institute of Medicine, "Vaccines Against Malaria: Hope in a Gathering Storm," close coordination and collaboration of malaria vaccine development efforts could accelerate the process. Currently, a number of efforts are in progress to enhance and expand collaborative activities in malaria vaccine research and development. For the past several years, NIAID staff, along with representatives from the Centers for Disease Control and Prevention (CDC), the Department of Defense, and USAID, regularly participate in the Federal Malaria Vaccine Coordinating Committee, an interagency working group that engages in timely exchange of information and collaborative efforts to accelerate malaria vaccine research and development. In an unprecedented meeting of scientists, administrators, and public health officials in January 1997, in Dakar, a number of priorities for malaria control, including malaria vaccine development, were identified. The Multilateral Initiative on Malaria (MIM) was also established. The Wellcome Trust has been the secretariat for MIM for the first 2 years, and the Fogarty International Center will coordinate MIM for the next 3 years. In addition, under other auspices, NIAID actively supports research collaborations with a number of scientists and organizations overseas.

Schistosomiasis

Schistosomiasis is another parasitic disease with a major human health impact. It is estimated that 200 million people worldwide are infected with this helminth, and approximately 600 million people live under conditions in which they are directly exposed to infection. Schistosomiasis is primarily a chronic disease associated with significant morbidity and loss of productivity; nevertheless, the mortality rate is estimated in the hundreds of thousands.

Recent research on schistosomiasis has focused on the identification of candidate vaccine antigens. Several of these candidates have been shown to provide partial protection in a mouse model of infection with the human parasite *Schistosoma mansoni*, a form found in South America and Africa. Many antigens are molecules associated with the invasive larval stage of the parasite; these antigens were initially distinguished by their reactivity with protective monoclonal or polyclonal antibodies. They include the enzymes glutathione-S-transferase and triose phosphate isomerase (TPI), as well as a 38 kD antigen with prominent carbohydrate epitopes that are shared between the larval and egg stages.

Another promising candidate, calpain, was recently identified based on the ability of a T- cell clone to transfer protection against challenge infection in mice. Several other antigens, whose identities have not yet been determined, also have demonstrated partial protective activity. Schistosome paramyosin, a muscle protein, has been shown to induce a protective cell-mediated immune response based on the production of gamma interferon-activated macrophage effector cells. Several vaccine candidates are being tested for efficacy against *S. mansoni* in baboons. One, a 28 kD glutathione-S-transferase of *S. mansoni*, has been shown to reduce worm burden or egg excretion in baboons and cattle. A myosin-like antigen also has shown efficacy against *S. mansoni* in both mice and baboons; MAPs, based on selected regions of TPI and a 23 kD antigen, also have shown promise as candidate vaccines against *S. mansoni* in mice.

Additional investigations on mechanisms to enhance the level of protective immunity achieved with purified native or recombinant-derived antigens are underway; these studies include evaluations of the benefit of combining antigens or of varying the method used to present antigen to cells of the immune system. DNA-based vaccines are also being explored to identify promising routes of administration, combinations of vaccines, and protective immune effector mechanisms. Recent studies carried out in Egypt, Brazil, and Kenya have identified antigen-specific immunologic correlates of resistance to reinfection in populations at risk. Based on these results, plans are now being made for further development of candidate vaccines, including pilot lot production according to good manufacturing practice guidelines and phase I clinical evaluation.

Other Parasitic Diseases

Candidate vaccine antigens have been identified for other parasitic diseases, including leishmaniasis, toxoplasmosis, amoebiasis, and filariasis. Leishmaniasis

is caused by several species of protozoan parasites found in most areas of the world but particularly in the tropics. In its severest forms, this disease can cause serious disfigurement as well as death, and WHO estimates worldwide prevalence to be approximately 12 million cases. Several WHO-supported efficacy trials of vaccines based on a combination of whole, killed leishmania parasites and Bacille Calmette-Geúrin have recently been carried out. In one recently published clinical trial evaluating efficacy against anthroponotic cutaneous leishmaniasis in Iran, no difference was found between the vaccine and the control groups; a subgroup analysis, however, suggested that the vaccine might have a protective effect in boys. This apparent protective effect in boys was unanticipated and may be a chance finding. A second trial in Iran that evaluated protection against zoonotic cutaneous leishmaniasis found no efficacy. An alternative approach involving the development of attenuated leishmania vaccines based on gene replacement in *Leishmania major* is in early stages of preclinical investigation.

Two *Leishmania* surface antigens serve as ligands for the attachment of the parasite to host macrophages, thereby enabling infection to be initiated. They are gp63, a glycoprotein with protease activity, and a glycoconjugate known as lipophosphoglycan. When tested as candidate vaccines, both antigens have been shown to induce protection in a mouse model of leishmaniasis. In addition, a 46 kD promastigote antigen, derived from *Leishmania amazonensis*, has been shown to protect mice when administered as the native molecule admixed with adjuvant or as a recombinant vaccinia construct. Recently, expression cloning has been used to identify a novel parasite antigen, known as LACK, that appears to be related to a family of enzyme receptors. When administered with interleukin (IL)-12, this antigen has also been shown to confer protection against leishmaniasis in susceptible mice.

Over the past several years, NIAID-supported investigators have demonstrated that T lymphocyte-dependent host responses to the leishmania parasites determine whether the disease is progressive or self-limited in experimental animal models. More specifically, when a Th1 lymphocyte response (characterized by the production of cytokines, such as IL-2 or interferon-gamma) is dominant, the disease is self-limited, whereas when a Th2 lymphocyte response (characterized by the production of other cytokines, such as IL-4 and IL-5) is dominant, the disease is progressive. NIAID-supported investigators have demonstrated that incorporation of the cytokine IL-12, a specific stimulator of Th1 responses, into an experimental vaccine against leishmaniasis resulted in complete protection of susceptible mice against progressive disease. Neither IL-12 alone nor the experimental vaccine without IL-12 conferred protection. Other NIAID-supported investigators have extended these findings by demonstrating that immunostimulatory oligodeoxynucleotides given as adjuvants or a recombinant leishmanial antigen, LeIF, are also capable of eliciting IL-12 and Th1 responses and conferring protection.

DNA immunization is also being used to identify and validate candidate vaccine antigens for leishmaniasis. In mice, protection against *L. major* has been demonstrated following immunization with DNA constructs encoding gp63 and LACK antigens.

Toxoplasmosis is primarily a disease of the central nervous system that affects individuals with immature or compromised immune systems. It usually is associated with neurological problems in the developing fetus; however, more recently it has been identified as a major opportunistic infection in AIDS patients. The possibility of effective vaccination against this protozoan parasite was suggested by experiments showing that mice immunized with a temperature-sensitive mutant of *Toxoplasma gondii* were resistant to further infection with a potentially lethal strain. In addition, a major surface antigen of *T. gondii*, called p30, has now been cloned. This antigen has been shown to stimulate CTLs with parasiticidal activity *in vitro*. Purified native p30 recently has been demonstrated to protect mice against parasite challenge *in vivo*.

Amoebiasis, caused by invasion of the intestinal wall and gut-associated organs by the protozoan parasite *Entamoeba histolytica*, has been estimated to result in more than 100,000 deaths per year; the prevalence of infection may be as high as 50 percent in some developing countries. Recent studies have identified a galactose-inhibitable amoebic lectin involved in adherence of the parasite to the colonic mucosa. Gerbils immunized with this lectin showed a significant reduction in development of liver abscesses following infection, suggesting that this molecule might form the basis of a potential vaccine against amoebiasis. Investigators are working to identify the regions of the lectin that elicit protective immunity and to develop genetically engineered and recombinant subunit vaccines based on these regions. In addition, investigators are working to identify new antigens and delivery systems, especially those that would target mucosal immunity.

Lymphatic filariasis is endemic in many tropical and subtropical countries, where it is estimated to afflict approximately 90 million people. In its chronic form, this infection causes inflammation and blockage of the lymphatic system, resulting in the condition known as elephantiasis. Immunization with several *Brugia malayi* antigens has been demonstrated to facilitate the clearance of bloodstream forms (microfilariae) of the parasite in animal models. One such antigen is paramyosin, a 60 kD antigen. In addition, filarial collagen has been shown to partially inhibit the development of infective larvae into adult worms.

Vector-Borne and Zoonotic Infections

Anthrax

Background

Anthrax is a potentially life-threatening bacterial disease caused by *Bacillus anthracis*, a gram-positive bacillus that produces heat-resistant spores. Anthrax is rare in humans and occurs in two natural forms (cutaneous and systemic), primarily among those who come in close contact with animals or their products. Cutaneous anthrax is characterized by an inflamed carbuncle covered by a black eschar. If the carbuncle is not adequately treated, the bacilli may spread to regional lymph nodes and then to the bloodstream, resulting in systemic anthrax. Systemic anthrax, which is almost always fatal, also may develop from initial sites of infection in either the lungs (from the inhalation of spores) or the gut (from eating contaminated meat). Death results from massive edema, shock, and pulmonary edema in the case of the inhalation of anthrax.

Natural epidemics of pulmonary anthrax are rare. Because the threat of using anthrax as a bioterrorist agent has increased in recent years, outbreaks of pulmonary anthrax must be suspected as originating from the deliberate release of spores into the atmosphere. This can result in enormous fatalities within a short period of time, well before diagnosis is possible.

Current Status of Research and Development

In response to the potential threat of various bacterial pathogens being used as agents of bioterrorism, NIAID formed a Working Group on Anthrax Vaccines (WGAV) to review the current status of anthrax vaccines. Anthrax Vaccine Adsorbed (AVA), which was licensed for human use in 1970, is the only vaccine currently available for anthrax. It is an alum-adsorbed, killed-cell vaccine, with a shelf life of less than 1 year. It was designed mainly for use by textile workers to protect against cutaneous anthrax (wool sorter's disease) and is administered as six injections over an 18-month period. There are no data to support the efficacy of AVA for pulmonary anthrax in humans.

The results of preclinical studies, conducted by investigators at the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID), have established that the protective antigen (PA) of *B. anthracis* induces significant protective immunity against inhalation spore challenge and that PA is the component of AVA responsible for generating such immunity. The gene for PA has been cloned and inserted into a non-spore-forming, avirulent strain of *B. anthracis*; this enables the production of large amounts of purified recombinant PA (rPA) for use as a vaccine. The administration of two intramuscular injections of rPA (50µg alone or 5µg with alhydrogel as adjuvant) produces 90 to 100 percent protective immunity against inhalation spore challenge in rabbits and monkeys within 3 months after immunization. Levels of serum IgG antibodies against PA, as well as toxin-neutralizing antibodies, in response to inhalation spore challenge parallel the degree of protective immunity generated in response to rPA. On the basis of these and other findings, WGAV recommended that NIAID support joint collaborative studies with USAMRIID to conduct phases I and II clinical trials on the safety and efficacy of the rPA vaccine for humans.

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Dengue

Dengue viruses are the most widespread arthropod-borne viruses (arboviruses). They are members of the *Flaviviridae* family, which includes more than 70 related but distinct viruses, most of which are mosquito borne. Other major pathogens in this family include yellow fever (YF) and Japanese encephalitis (JE) viruses. In 1999, dengue was present on most continents, and more than one-half of all United Nations member-states (discussed below) were threatened by dengue. Epidemics continue to emerge, and this virus causes severe infections in areas where periodic epidemics did not previously occur. The disease will continue to spread as newly urbanized areas become infested with mosquito vectors. In those areas where dengue is endemic, more than 1.5 billion people (including about 600 million children) are at risk. It is estimated that between 35- and 60-million people are infected with dengue and that 2,000 to 5,000 children die from dengue annually. These figures most likely underestimate the scope of this problem.

There are four closely related, but serologically distinct, dengue viruses (types 1 through 4). Because there is no cross-protection between the four types, a

population could experience a dengue-1 epidemic in 1 year, followed by a dengue-2 epidemic the next year. Primary infection with any sterotype often causes a debilitating, but usually nonfatal, form of illness. To date, antiviral drug chemotherapy has not been successful; consequently, most currently used forms of therapy for uncomplicated dengue are supportive in nature.

Some infected patients experience a much more severe, and often fatal, form of the disease, called dengue hemorrhagic fever (DHF), the most severe form of which is referred to as dengue shock syndrome (DSS). Unlike other infectious diseases, the presence of antibodies after recovery from one type of dengue infection is believed, under certain incompletely understood circumstances, to predispose some individuals to the more severe form of disease (DHF/DSS) through immune-enhancement when infected by a different dengue virus serotype. Although all age groups are susceptible to dengue fever, DHF is most common in children.

Dengue viruses are prevalent throughout the tropics, where the urban-dwelling mosquito *Aedes aegypti* is a major vector. Other related mosquitoes, such as *Aedes albopictus*, are also efficient vectors. Although the virus may circulate in endemic cycles, it periodically causes acute, widespread epidemics in which large percentages of the population may be infected. An example was the 1987 epidemic in Thailand, which officially involved 174,285 cases; most were children younger than 15 years of age. Dengue caused 1,007 reported deaths in these children. That year in Thailand, dengue was the third leading cause of illness in children and the leading cause of childhood death. DHF has emerged as an important public health problem in Southeast Asia as new waves of epidemics occur; this appears to be happening in the Western Hemisphere and the Pacific Islands as well. In the Americas, the first epidemic of cases of severe DHF occurred in 1981. The illness was associated with a dengue-2 epidemic in Cuba that followed the dengue-1 epidemic of 1977. During the 1981 outbreak in Cuba, 116,151 hospitalized cases of dengue fever were reported, and 10,312 cases were classified as severe DHF; 158 deaths (many in adults) were reported. More recently, the Caribbean and South and Central America have experienced frequent outbreaks of dengue, with cases of fatal DHF now commonly reported from many countries.

Dengue continues to spread or emerge into areas previously considered not to be endemic but usually is not associated with major outbreaks of the disease. The westward expansion of dengue in Asia was first documented in the late 1980s by the increased epidemics in India and Sri Lanka. Africa and the Middle East also were considered to be areas with a low incidence; however, dengue emerged in these areas in the early 1990s, as demonstrated by the widespread occurrence of dengue infections in U.S. military personnel stationed in Somalia, as well as by reports of dengue in Saudi Arabia.

Because attempts to eradicate mosquito vectors have not been successful in developing countries, the control of dengue will be possible only after an efficient vaccine has been developed. Clearly, the phenomenon of immune-enhancement may be a major problem in developing an effective dengue vaccine. It suggests that instead of a monotypic vaccine, one may have to prepare a multivalent vaccine against all four serotypes of the dengue virus to avoid inducing monotypic-enhancing antibodies that might lead to DHF associated with subsequent natural infections caused by other dengue types. The potential risks of administering a live-attenuated vaccine to a population with preexisting enhancing antibodies are another potential problem that remains to be examined in a systematic manner.

NIAID is now funding several projects that address basic virological and immunological aspects of flavivirus infections in general and dengue infections in particular. WHO is also funding vaccine development programs, and dengue vaccine development programs are in place at a limited number of vaccine manufacturers and small biotechnology companies. The U.S. Army has had a productive, long-term research program aimed at developing a dengue vaccine. However, funds to support this program have been threatened in recent years.

Progress in research on dengue has been slowed, mainly because these viruses grow poorly in cell culture, and there is no acceptable animal model for DHF. NIAID funds several extramural and intramural projects studying basic virological and immunological aspects of flaviviruses such as YF, dengue, and JE virus. Discoveries from these projects cross-fertilize vaccine studies on YF, dengue, and JE. Some of the most promising basic molecular studies that might be applied to the development of an improved dengue vaccine revolve around the development of full-length, infectious dengue cDNAs. Information from studies using this infectious clone has been combined with sequence immunological data to yield new insights into important antigenic regions on the dengue virion. The recent determination of the three-dimensional structure of the E protein of another flavivirus (tick-borne encephalitis virus) has allowed formulation of an even more sophisticated model for understanding antigenicity and pathogenicity of flaviviruses. It is hoped that this research can yield efficient and less costly ways to manufacture safe flavivirus vaccines.

Flavivirus vaccine research has focused on five areas: live-attenuated or inactivated vaccines, infectious clone-derived vaccines, immunogens vectored by various recombinant systems, subunit immunogens, and nucleic acid vaccines.

The most promising set of live-attenuated dengue vaccines has been developed in Thailand with support of WHO. Preliminary trials in adults and children in Thailand were encouraging, with the tetravalent vaccine inducing

broadly cross-reacting antibody in 80 to 90 percent of the subjects. This vaccine has been transitioned to commercial development by agreements with Pasteur Merieux Connaught. Commercial lots have been manufactured, and phase I testing is underway in collaboration with Walter Reed Army Institute for Research (WRAIR).

Because of the success of flavivirus inactivated vaccines against JE in Japan and tick-borne encephalitis in Australia, attempts have been made to develop a killed dengue vaccine. However, because of difficulties in growing high titers of dengue in cell culture, early attempts to make inactivated products were not successful. Recently, WRAIR scientists have utilized certified Vero cells and serum-free media to grow dengue to high titers. A prototype dengue-2 inactivated vaccine purified and concentrated from these cells induces protective levels of antibodies in mice and monkeys. Further testing is planned.

Infectious clones of dengue, JE, and YF are being combined to produce chimeric vaccines, and preliminary mouse studies are encouraging. Scientists at NIH and in Australia have also attempted to alter the genetic structure of the dengue clone to produce live-attenuated vaccine candidates. Mouse and monkey trials have been encouraging, and a number of potential vaccine candidates soon will be tested in phase I trials.

The most advanced studies of flavivirus immunogens delivered by poxvirus vectors have been with JE virus to deliver antigenic JE proteins to humans in phase I trials. Further studies are needed, but these vectors induce both cellular and antibody immunity against JE. Preexisting immunity to the vector attenuated the response. To avoid this problem, vaccinia virus recombinants have also been used to generate subviral particles containing dengue and JE antigens. These particles elicit antibody in mice, but their potential as vaccines is still being explored.

Subunit vaccines for a variety of flaviviruses have been prepared in *E. coli*, baculovirus, yeast, and insect cell systems. The experience with dengue-containing *E. coli* products, and some other expressed products, was not promising. With *E. coli*-dengue products, mice produced good antibody titers, but monkey studies were not as successful. One lesson learned was that flavivirus proteins require extensive processing and folding during maturation. Studies to fine-tune various expression systems to yield more stable flavivirus immunogens are in progress, and baculovirus expressed products and products from drosophila cells appear promising in early mouse testing.

Preliminary studies have been reported on a new nucleic acid vaccine for St. Louis encephalitis, a related flavivirus. PreM and E proteins have been expressed under control of the cytomegalovirus immediate early promoter. Mice immunized with this product developed disappointing levels of antibody but were protected against a live virus challenge. Research by the CDC (Ft. Collins) and the U.S. Navy is attempting to further develop this approach for dengue. In the near future, this exciting area undoubtedly will be a focus of expanded vaccine research efforts.

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Japanese Encephalitis

Japanese encephalitis (JE) is endemic in parts of China, India, Korea, Nepal, Thailand, Vietnam, Kampuchea, Myanmar, the Philippines, Taiwan, Indonesia, Malaysia, Bangladesh, and Sri Lanka and also poses a risk to U.S. travelers and the U.S. military. Infection with this mosquito-borne virus in endemic areas is common; however, clinical disease occurs in only 1 of every 300 to 1,000 infections. These clinical cases have a case fatality rate of up to 40 percent, with severe neurological sequelae occurring in 10 to 30 percent of survivors. Like the closely related yellow fever and dengue viruses, JE virus circulates in endemic cycles, which periodically erupt into major epidemics. Consequently, the incidence of infections caused by JE virus varies substantially and ranges from 10,000 to more than 50,000 cases worldwide per year. Estimates of about 1,000 cases per year have been reported in India, Nepal, and Sri Lanka. An annual morbidity of 6 to 10 cases per 100,000 inhabitants has been reported in heavily endemic areas such as Vietnam and Thailand.

Travelers, military personnel, and others temporarily assigned to endemic areas may require immunization. Exposure to JE virus has increased greatly with rapid economic development of the Pacific Rim countries and the large number of U.S. citizens visiting this region. The treatment of JE is mainly supportive because antiviral drug chemotherapy has not been developed. In developed countries, the control of mosquito vectors or immunization of host reservoirs has limited the spread of virus, but these

public health measures have been difficult to accomplish in developing countries.

An inactivated virus vaccine exists and has been used successfully to reduce the incidence of JE in Japan, Taiwan, and Korea. Currently mass-produced and licensed in Japan, the vaccine has been tested under various experimental protocols. The vaccine is made by Biken, was licensed in the United States in late 1992, and is also distributed by Connaught Laboratories. It consists of partially purified, formalin-inactivated JE virus that is propagated in mouse brain tissue. It requires a series of 3 to 5 injections to stimulate immunity. A different, live-attenuated vaccine (SA 14-14-2) has been developed and tested in China. It appears to be safe and effective in annual Chinese immunization programs involving millions of children. Efforts are underway to reconfirm safety and efficacy in carefully monitored trials in infants and children from 1 to 6 years of age to secure international approval. A recent review of 13,000 vaccinated and control children in Chengdu Province, China, indicated low rates of acute systemic and local side effects, and no central nervous system infections were reported. The vaccine is produced in primary hamster kidney cells. Production issues remain a problem because this is not a widely accepted substrate for the production and licensure of vaccines in some countries, and the vaccine is not currently produced under good manufacturing practice conditions. Further research is also needed to determine the vaccine's thermostability, its ability to revert to a more virulent form of the virus, its efficacy in children with maternal antibody, and its immunogenicity when used in combination with other vaccines.

NIAID currently funds several extramural and intramural projects studying basic virological and immunological aspects of flaviviruses. Some of the most promising molecular studies that might be applied to the development of an improved JE vaccine focus on the development of full-length, infectious JE cDNAs. Information from studies using this infectious clone has been combined with sequence data and immunological data to yield new insights into important antigenic regions on the JE virion.

JE vaccine research has focused on five areas: live-attenuated or inactivated vaccines, infectious clone-derived vaccines, immunogens vectored by various recombinant systems, subunit immunogens, and nucleic acid vaccines.

As mentioned above, further safety and efficacy studies are planned for the live SA 14-14-2. In addition, SA 14-14-2 is being molecularly modified, using infectious clones, to produce a vaccine that is highly stable to reversion. Infectious clones of JE and YF are being combined to produce chimeric vaccines, and preliminary mouse studies are encouraging.

Poxvirus vectors have been employed to deliver antigenic JE proteins to humans in phase I trials. Further studies are needed, but these vectors induce both cellular and antibody immunity against JE. Vaccinia virus recombinants have also been used to generate subviral particles containing JE antigens. These particles elicit antibody in mice, but their potential as a human vaccine is still being explored.

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Lyme Disease

Background

Lyme disease, which is caused by the tick-borne spirochete, *Borrelia burgdorferi*, was first recognized as an infectious disease in 1975. It is the most common tick-borne infection in the United States. In 1998, 14,646 cases were reported to the Centers for Disease Control and Prevention (CDC). This is a 19-percent increase from the 12,289 cases reported in 1997. Increases of 52,16, and 34 percent were reported in major endemic areas such as the New England States, the Mid-Atlantic States, and Upstate New York, respectively.

Ticks infected with *B. burgdorferi* may be coinfected with the agent of human granulocytic ehrlichiosis (HGE) as well as with *Babesia microti,* a parasite that causes babesiosis. Because these are not classified as reportable infectious diseases, precise data on their incidence and morbidity are not available.

Current Status of Research and Development

NIAID has supported an extramural research program on Lyme disease since 1985. The program has grown from 2 research grants in 1985 to more than 48 grants and contracts in 1999. This program supports research on animal models of disease; microbial physiology; mechanisms of pathogenesis; mechanisms involved in the development of protective immunity; vectors, vector

competence, and disease transmission; approaches to the treatment of acute and chronic infection; and the development of rapid, sensitive, and specific diagnostic tests for Lyme disease.

Recent Accomplishments

On December 21,1998, the FDA licensed LYMErix (SmithKline Beecham Biologicals, Inc.), a new vaccine designed to block the transmission of Lyme disease by infected ticks. The major component of this vaccine is highly purified, recombinant OspA, an outer surface protein of *B. burgdorferi*. Immunization with LYMErix stimulates the production of antibodies specific for OspA. When a tick takes a blood meal from an individual vaccinated with LYMErix, it ingests these antibodies, which then bind to the surface of *B. burgdorferi* present in the midgut. As a result, *B. burgdorferi* are prevented from migrating to the salivary glands of ticks where they can then be transferred to a human host and cause disease. Thus, LYMErix is considered to be a transmission-blocking vaccine. After three injections, it has been shown to be about 80 percent effective in preventing Lyme disease in humans between the ages of 15 and 70. Studies are underway to evaluate the safety and efficacy of LYMErix in children younger than 15 years of age. Phase III trials have been completed for another OspA-based recombinant vaccine (ImuLyme; Pasteur Merieux Connaught) that appears to be similar to LYMErix in efficacy. The FDA is currently evaluating ImuLyme for licensure.

Although OspA-based vaccines are effective in blocking the transmission of Lyme disease, NIAID also is supporting basic research to identify other vaccine candidates that, alone or when combined with OspA, will provide even greater efficacy and long-lasting protective immunity. Among the candidates being considered are *Borrelia*-specific virulence-associated antigens or *in vivo*-expressed antigens that, unlike OspA, are capable of boosting the anamnestic immune response soon after infection. The results of recent studies indicate that *B. burgdorferi* decorin binding protein A (DbpA) elicits a sustained serum antibody response that is capable of inducing protective immunity in experimental animals against a wide range of *B. burgdorferi sensu stricto* isolates.

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Rabies

Rabies, an ancient human pathogen, continues to be a significant international health problem. In the United States, the continuing emergence of zoonotic rabies is a problem, especially in the raccoon population along the East Coast. About a dozen human fatalities occur annually in the United States, and the number of postexposure treatments is rapidly increasing, with a substantial associated financial burden. Globally, attention recently has been drawn to potentially significant underreporting of rabies in developing countries. In these areas, a number of vaccines for human use, which vary in quality, are produced nationally and regionally, but postexposure treatment remains costly and often beyond the financial reach of those exposed. Moreover, such treatment must be administered properly, requiring product and delivery infrastructure. The need remains for economic, safe, and effective animal vaccines suitable for mass immunization of domestic animals and wildlife.

Limited studies aimed to improve vaccines are underway with NIAID support. Vaccinia recombinants continue to be studied as an oral vaccine for wild animals, and attempts are in process to develop a nucleic acid vaccine for possible oral administration. To date, large field trials of vaccinia recombinant wildlife vaccine have shown promise, but further studies are needed to better establish efficacy and carefully define safe, optimal application of the vaccine. Research on postexposure prophylaxis focuses on developing a one-shot, easily administered human vaccine and on safe, inexpensive, carefully defined rabies virus-specific immunoglobulins. Although not now available, an antiviral drug against rabies might be useful for postexposure prophylaxis.

Interestingly, in the United States, many recent human victims did not report a bite by a potentially rabid animal. Often the persons reported just “chasing bats” from their houses. In many of these cases, the virus isolated was related to the strain found in silver-haired bats. It is thought this bat could be a significant reservoir for U.S. rabies and that its bite often might go unnoticed. However, some health officials have questioned whether exposure might

have occurred by inhalation of bat excretions. Although the guidelines for commencing postexposure treatment are well established for exposure to domestic animals, updated guidelines will have to be developed for exposure to wildlife, particularly bats. Recently, a bat-associated lyssavirus similar or identical to rabies has been identified in Australia, a previously rabies-free area.

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Yellow Fever

Yellow fever was first distinguished from other tropical febrile diseases during the 1647 to 1649 epidemics in the Americas. Since then, it has caused periodic epidemics in the Americas and Africa. The YF virus is mosquito borne and in humans produces a clinical disease that starts with the sudden onset of acute fever followed by a second phase of hepatorenal dysfunction and hemorrhage. Reported mortality rates vary widely from 20 percent to 80 percent of all cases.

During the latter half of the 20th century, YF circulated in an endemic sylvatic cycle in the Americas, usually infecting up to 500 unvaccinated forest workers per year. In contrast, YF in Africa periodically explodes from its endemic cycle to infect large numbers during major epidemics. The highly successful mosquito eradication campaigns of the early 20th century effectively eliminated urban YF epidemics in South America and limited persistence of the virus to a monkey-mosquito cycle in jungle areas. However, the disease now appears to be slowly reemerging from the forest into those parts of South America where the vector, *Aedes aegypti*, has reinfested urban areas.

In the late 1980s, the total number of cases of YF worldwide (with case fatality rates of about 50 percent) represented the greatest number reported to WHO during any 5-year period since 1948. Numerous studies showed that, in Africa, only a small number of cases of YF are reported. Ironically, 1988 marked the 50th anniversary of the development of the attenuated vaccine for YF—a safe and effective vaccine for this disease.

The 17D YF vaccine was one of the first viral vaccines to be developed. It is a live-attenuated vaccine that is produced in eggs. After one injection, the vaccine induces protective immunity in more than 98 percent of vaccinees for a period of at least 10 years. In fact, protection may be lifelong because neutralizing antibodies have been detected as long as 40 years after immunization. The vaccine is one of the safest viral vaccines ever produced. From 1945 through 1989, only 17 cases (1 fatal in a 3-year-old) of encephalitis associated with YF immunization were reported worldwide. Because all but three of these cases occurred in children immunized at 4 months of age or younger, a review by a panel of experts recommended that the YF vaccine not be given before 6 months of age.

Over the past 40 years, two vaccine-based control strategies have been attempted in Africa. The first consists of routine immunization, whereas the second involves emergency control measures that are implemented after the start of an outbreak. A routine, mandatory YF immunization program was begun in the early 1940s in French West Africa; as a result, the recurring pattern of epidemics in West Africa has been interrupted. However, this strategy was abandoned in 1960, when a postoutbreak, firefighting type of emergency immunization and control strategy was adopted. Since then, there has been a series of epidemics of varying severity. In recent years, with the help of the WHO Expanded Programme of Immunization (EPI), more African countries have apparently, at least partially, incorporated the YF vaccine into their immunization programs. Most give both the YF vaccine and measles vaccine to children at 9 months of age, because the simultaneous administration of both vaccines has been shown to be acceptable. Recently, the Global Advisory Group for WHO-EPI reviewed the status of YF and recommended that all 31 nations in the YF endemic area incorporate the vaccine into their routine immunization programs.

In South America, YF control strategies have been primarily based on reducing mosquito vectors by altering their breeding environment. Extensive studies on the maintenance of YF virus have shown that the virus exists in two cycles: an urban cycle involving humans and *Aedes*

aegypti mosquitoes and a sylvatic or jungle cycle involving forest primates (principally monkeys) and forest canopy mosquitoes, with human infections tangential to the transmission cycle. In the Western Hemisphere, *A. aegypti* mosquitoes were the sole transmitters of urban YF. In 1901, eradication efforts directed toward *A. aegypti* mosquitoes were launched under the direction of Dr. William Gorgas in Havana. These eradication efforts, with concomitant reduction of YF were extended throughout Central and South America in the early 1900s. The eradication program successfully broke the chain of urban *A. aegypti*-transmitted YF. The eradication of the vector, and the subsequent reduction of urban YF cases in the Americas, represents one of the world's most successful public health campaigns against an infectious disease. Unfortunately, *A. aegypti* has now reinfested most of South and Central America and occupies habitats just adjacent to the areas where endemic YF transmission occurs. A major threat is that this species could transmit YF in an urban cycle. The Pan American Health Organization (PAHO) is monitoring the need for incorporation of YF immunization into the EPI programs in South America. Some authorities believe serious consideration should be given to expanding YF immunization in South American EPI programs in an attempt to prevent the reemergence of urban YF.

The EPI program provides an excellent way to deliver YF vaccines to a larger population at a reduced cost; however, despite the fact that the current YF vaccine is an excellent public health tool, further studies are needed to better define its role in controlling YF. In the past, the amount of vaccine available has been a limitation at times. The number of surviving infants in the 1990s, in the countries where YF is a potential risk, will be approximately 18 million. Although the vaccine is made in a number of developing countries, including Senegal and Nigeria, only about 6 million doses are produced yearly in Africa. Newer technology, combined with efficient technology transfer, might help solve the problem of availability. The development of a cell culture-produced vaccine might result in increased vaccine production. One recent study of vaccine thermostability showed that further work on stabilizing the YF vaccine is needed because only 5 of 12 manufactured lots met the WHO criteria for vaccine thermostability. More research is needed on the safety of combining this vaccine with other vaccines in a multiple-dose regimen for immunization.

NIAID currently funds several extramural and intramural projects studying basic virological and immunological aspects of flaviviruses. Some of the most promising molecular studies that might be applied to the development of an improved YF vaccine focus on the development of a full-length, infectious YF cDNA. Information from studies using this infectious clone has been combined with sequence data and immunological data to yield new insights into important antigenic regions on the YF virion. It is hoped that this research will yield efficient and less costly ways to manufacture safe flavivirus vaccines. At a minimum, a clone-derived vaccine seed virus might reduce YF vaccine production lot diversity, improve quality control, and reduce the need for vaccine safety testing in primates.

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Respiratory Infections

Overview

Infections of the respiratory tract continue to be the leading cause of acute illness worldwide. Upper respiratory tract infections (URI) such as the common cold, strep throat, sinusitis, and otitis media (OM) are very common, especially in children, but seldom have serious or life-threatening complications. Lower respiratory tract infections (LRI) include more serious illnesses such as influenza, bronchitis, pertussis (whooping cough), pneumonia, and tuberculosis and are the leading contributor to the more than 4 million deaths caused each year by respiratory infections. According to the 1999 World Health Report, acute LRIs and tuberculosis are among the top 10 leading causes of death from an infectious disease, worldwide. In the United States, pneumonia and influenza are the sixth leading cause of death and are responsible for 3.7 percent of all deaths. The populations at greatest risk for developing a fatal respiratory infection include the very young, the elderly, and the immunocompromised. In developing countries, most of the deaths caused by respiratory infections occur in children younger than 5 years of age, and the World Health Organization (WHO) estimates that 30 percent of these deaths are attributable to pneumonia. The most

common etiological agents of pneumonia are *Streptococcus pneumoniae*, *Haemophilus influenzae,* and respiratory syncytial virus. In the elderly, influenza-related pneumonia remains a leading cause of infectious disease-related deaths. Nosocomial or "hospital-acquired" pneumonia is a major infection control problem. Pneumonia is the second most common type of nosocomial infection, accounting for approximately 15 percent of all nosocomial infections with associated mortality rates of 20 to 50 percent. Nosocomial pneumonia can prolong hospital stays by 4 to 9 days, resulting in additional costs of approximately $1.2 billion annually in the United States.

Although generally considered less severe than LRIs, URIs have a major effect on global health. The common cold accounts for approximately 20 percent of all acute illness in the United States, with associated direct costs estimated at more than $500 million annually. OM, which can be caused by a variety of etiologic agents including nontypeable *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Moraxella catarrhalis*, is responsible for substantial morbidity and can have long-term effects on speech and language development in children.

According to data obtained from a 1995 health survey in the United States, there were more than 223 million acute cases of respiratory infections, including more than 13 million cases of bronchitis, 60 million cases of the common cold, 108 million cases of influenza, and 5 million cases of pneumonia. Acute respiratory infections accounted for an estimated 640 million restricted activity days, 152 million bed days, and 134 million days of work lost among employed persons older than 18 years of age.

In addition, respiratory infections were responsible for millions of visits to hospital emergency rooms, outpatient departments, and doctors' offices.

Adequate clinical management of infections depends primarily on the rapid and accurate identification of the causative agent and is essential to avoid the indiscriminate use of antibiotics, which ultimately favors the development of antimicrobial resistance. Treatment of infections caused by antibiotic-resistant pathogens often requires the use of more expensive and potentially more toxic drugs and usually results in longer hospital stays. The difficulty in identifying the causative agent, the rapid global emergence of antibiotic-resistant organisms, and the increased incidence of atypical pathogens as the cause of respiratory infections have complicated the management of LRIs. The burden of respiratory infections is not only the loss of lives but also the substantial effect they have on health resources.

A major goal of the NIAID Respiratory Diseases Branch is to stimulate and support research that may lead to more effective and accepted prophylactic and therapeutic approaches for preventing and controlling respiratory infections. Areas of interest include the development and licensure of vaccines and therapeutic agents for respiratory pathogens; stimulating basic research on the pathogenesis, immunity, and structural biology of respiratory pathogens; developing more accurate and more rapid diagnostic tools; and understanding the long-term health effect of acute respiratory infections in various populations.

Bordetella pertussis

Several acellular pertussis vaccines are now being used throughout the world, as well as new combination vaccines that include acellular pertussis vaccines. Since the licensure of the acellular vaccines in the U.S., research in the area of pertussis has continued along several different paths. This section reviews the types of studies currently supported by NIAID.

Shortly following completion of the pertussis efficacy trial in Sweden in 1996, several vaccines were licensed for use in that country. The decline in the incidence of pertussis was marked, and the disease is currently at a level comparable to that of the late 1960s when the Swedish whole cell vaccine still was efficacious. A similar reduction in the incidence of pertussis disease occurred in Italy following the completion of the NIAID-supported efficacy trial there in 1996 and after the vaccine was marketed on a wide scale.

Efforts continue to emphasize the need for a vigilant pertussis surveillance in Sweden as well as the importance of laboratory confirmation of pertussis. Diphtheria antitoxin levels were shown to decline markedly during the second and third year after the primary vaccination series at 2, 4, and 6 months of age for all studied vaccines. Subsequent data from a Swedish sero-surveillance study, and prebooster data in DTaP5 (Pasteur-Merieux-Connaught) recipients, indicated that the diphtheria antitoxin levels at 5 years of age were lower for children immunized on a 2, 4, and 6 months of age schedule than for those children immunized on the regular Swedish schedule at 3, 5, and 12 months of age. This observation prompted an early booster dose given to children around the ages of 5 to 6 years (normally administered at 10 years of age).

The safety of the acellular vaccines is also being addressed as an extension of the original NIAID efficacy trial contract. The possible long-term effects of hypotonic-hyporesponsiveness episodes (HHE) and convulsions following vaccination in Trial II are being studied using an extensive battery of neuropsychological tests. The first round of the long-term followup of children with HHE started in fall 1998. Thus far 29 children with HHE and 54 control subjects have been evaluated.

Secondary analyses of study infants in households exposed to *B. pertussis* in Trial I showed statistically significant correlations between clinical protection and the levels of serum IgG antibodies against pertactin, fimbriae

2 and 3, and to a lesser extent, against pertussis toxin at the time of exposure. Multi-component pertussis vaccines of proven high efficacy in Trial I and Trial II induced higher antibody levels against pertactin and fimbriae 2 and 3 than less efficacious vaccines. It has been suggested that anti-pertactin, anti-fimbriae 2 and 3, and, to a lesser extent, anti-pertussis toxin could be used as surrogate markers of protection for multicomponent acellular and whole cell pertussis vaccines.

Many important questions about pertussis remain. These include the mechanisms by which the organism adheres to the host and the nature of the responses that it induces within host cells (e.g., binding and release of potent toxins). One interesting approach for examining **host responses to** *B. pertussis* in a unique and more comprehensive fashion involves the use of high-density cDNA microarrays or "DNA chips." Using these chips, investigators have begun to analyze the responses of 16,000 human genes (about 20 percent of all expressed human genes) to *B. pertussis, Bordetella bronchiseptica*, and to mutant strains, each with defects in different virulence factors. One of the most interesting results thus far is that each bacterial species and each of the well-known *Bordetella* toxins appears to induce a characteristic set of responses in human cells (i.e., a "signature") that can be used to both identify and better understand the manner by which the human host recognizes a disease-causing bacterium. One hope is that this approach might lead to a novel means of diagnosing infectious diseases by detecting characteristic response signatures in the host.

The most important bacterial adherence factor associated with *B. pertussis* is a cell-associated and secreted protein known as filamentous hemagglutinin (FHA). It now appears that the FHA protein is also produced by the closely related animal pathogen, *B. bronchiseptica*, but in lesser quantities. Additional studies have characterized the *B. bronchiseptica* FHA protein and the genes responsible for its secretion. This information may help in developing better vaccines against the *B. bronchiseptica*-associated animal diseases. Other studies have shown that FHA itself is a potent pro-inflammatory protein able to elicit "distress signals" by human cells. This is an activity of FHA that was not previously appreciated and might help explain one role played by the secreted form of this protein.

The ability of a microorganism to obtain nutritional iron in the host environment is critical for its success as a pathogen. Certain bacterial pathogens possess multiple iron acquisition systems that are activated in response to iron-limiting growth conditions in the host. Research to determine how bacteria use these iron-gathering mechanisms may lead to strategies that interfere with bacterial colonization of the host and will contribute critical information about iron acquisition of all bacteria, especially microbial pathogens inhabiting specific sites in the host. *B. pertussis* accumulates iron through several different mechanisms. When starved for iron, *B. pertussis* cells produce and excrete a small siderophore molecule named alcaligin that binds iron in the external environment. The iron-alcaligin complex then binds the bacterial cell surface receptor and the iron is subsequently transported into the organism for nutritional use. Experiments have shown that these bacteria actually sense any alcaligin siderophore in the external environment; upon detecting the siderophore, the cell's alcaligin system genes are upregulated further. An important recent discovery was the identification and characterization of the gene encoding the ferric alcaligin receptor that is located on the cell surface. Mutants constructed with defective genes are no longer able to acquire iron from alcaligin. Because this receptor is likely to be involved in the alcaligin "sensing" phenomenon, mutants are now being constructed that no longer sense or detect external alcaligin. Study of these mutants, as well as those that lack the ability to properly regulate their iron acquisition genes, will offer insights into how bacteria retrieve iron in the host and control the expression of their genes in response to environmental host signals.

Although pertussis affects all ages, morbidity and mortality occur primarily in children under 1 year of age. Antibiotics eradicate the bacteria from the nose and throat and limit transmission, but they have no effect on the duration or severity of the illness unless given during the initial phase when pertussis is rarely suspected. Recently, a pertussis immune globulin for intramuscular use showed a modest benefit in children with pertussis. Based on these observations, a high titer pertussis immune globulin for intravenous use (P-IGIV) was developed and shown to be safe for use in young infants and children. A phase III study was initiated with the primary objective of demonstrating the effectiveness of P-IGIV in treating severe pertussis in hospitalized children, and thereby facilitating the licensure of this orphan product. Because the average annual hospitalization rates of infants with pertussis have been less than anticipated, the enrollment period for the trial is expected to continue through September 2001. To date, 22 infants out of a total sample size of 174 with suspected pertussis have been enrolled in the study and have completed infusions and monitoring. If a beneficial effect is demonstrated upon the completion of this study, P-IGIV could become an important new modality for the treatment of pertussis, particularly in the young infants at highest risk.

Physicians and researchers have known for decades that *B. pertussis* destroys the ciliated cells in the epithelial lining of the respiratory tract. The hairlike cilia sweep away mucus, but when they die, coughing provides the only way to clear the airway. Exactly how *B. pertussis* kills these cells has been a mystery. Earlier work had shown that the mediator of this damage is nitric oxide (NO), produced in the respiratory tract in response to

tracheal cytotoxin (TCT). The latter is a toxin thought to be responsible for the actual lung damage that is central to the disease. A significant finding during the past year has been the demonstration that mucus-secreting cells are the source of the nitric oxide in response to TCT and that there is a synergistic requirement for bacterial endotoxin in signalling these cells. Interestingly, the production of nitric oxide does not damage the mucus-secreting cells, but it is devastating to the neighboring ciliated cells that are responsible for mucus transport in the airways. Without the ability to move secreted mucus, coughing occurs. Another interesting discovery was that some substance in addition to TCT was necessary to induce epithelial cells to produce NO and kill neighboring ciliated cells. This substance turned out to be endotoxin, a compound mostly known for causing widespread immune system stimulation that can lead to shock. These findings suggest new possibilities for therapy of pertussis patients, such as interfering with the molecular signals involved in generating lung damage. Current efforts are focused on identifying the TCT receptor in the host.

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Chlamydia pneumoniae

Background

Chlamydia pneumoniae (CP) is recognized as an important cause of acute respiratory tract infections including pharyngitis, sinusitis, and bronchitis; in addition, severe systemic infections, while uncommon, do occur. It is the third or fourth most common cause of pneumonia, accounting for approximately 10 percent of all cases of pneumonia and 5 percent of all cases of bronchitis in the United States. Infection is usually asymptomatic, especially in the younger age groups. Most children become infected between the ages of 5 and 14 years. However, the disease is more severe and has the highest incidences in the elderly; case fatalities of 6 to 23 percent have been reported in this population. Transmission of the disease is person-to-person via respiratory droplets. Although CP has been isolated from the nasopharynx of healthy individuals, the rate of asymptomatic carriage in the normal population is unknown. Epidemics of pneumonia caused by CP have been documented in a number of geographic locations (mostly in northern Europe). In addition, CP has been implicated as a causative agent in chronic obstructive pulmonary disease and has been associated with the exacerbation of asthma. Studies indicate that approximately 40 to 60 percent of the adult population worldwide have antibodies to CP, suggesting that the infection is universal.

Clinical disease manifestations associated with CP extend beyond respiratory illnesses. For example, there has been a recent association of CP with cardiovascular disease. Initially, this association was made on the basis of elevated IgG and IgA antibodies and increased chlamydial lipopolysaccharide (LPS)-containing immune complexes in 50 to 60 percent of patients with coronary heart disease or acute myocardial infarction compared to 7 to 12 percent in control patients. Subsequent to these studies, several other investigators in the United States and other countries have reported similar findings in patients with coronary heart disease and have come to similar conclusions. Recent studies indicate that CP can be identified in post-mortem brain samples of patients with Alzheimer's disease, and in the cerebral spinal fluid of patients with multiple sclerosis. CP has also been associated with Guillain-Barré syndrome and endocarditis. Infections caused by CP can occasionally result in shock and multi-organ dysfunction syndrome and have been associated with acute pulmonary exacerbation in some patients with cystic fibrosis. CP has been isolated from immunosuppressed patients such as those with AIDS; however, its role as an opportunistic pathogen is unclear. Thus, infections attributed to or associated with CP have a substantial impact on the public health in the United States and worldwide. Although conventional antibiotic therapy has been shown to be effective against CP, recurrent infections have been shown to occur following treatment. Consequently, alternative strategies such as vaccine development should be considered.

As a group, Chlamydia cause important infections in animals and humans. Chlamydia are distinguished from other bacteria by having a unique life cycle with an orderly alternation of dimorphic forms that are functionally and morphologically distinct. The infectious form, known as the elementary body (EB), is specialized for invasion into susceptible host cells. Following endocytosis, the EB differentiates into a larger form called the reticulate body (RB). Once inside the cells, the organism resides inside membrane-bound vesicles and can modify the inclusion membrane, resulting in evasion of lysosomal fusion and immune detection. Chlamydia grow only intracellularly and require and use substrate and energy pools of the host cells for growth and as such have been termed "energy parasites." A special property of Chlamydia is their ability to persist in cells, and this property may result in latent

or chronic infections. The chronic state may be related to the ability of the organisms to develop into morphologically aberrant forms that do not divide or differentiate into EBs; this state may favor the development of immune-mediated diseases and the avoidance of host defense strategies. Studies show that these aberrant forms can be induced experimentally by the administration of cytokines such as interferon-gamma and are characterized by the absence of typical inclusions, low-grade infectivity, and altered expression of key membrane surface proteins. There is a lack of understanding about the mechanisms by which Chlamydia cause disease, and very little information is available on factors associated with virulence. The organisms possess two major surface proteins: an outer membrane protein (OMP1) and lipopolysaccharide (LPS). Chlamydial LPS has a low endotoxic activity when compared to the LPS of Enterobacteria; however, the role of LPS or the OMPs in pathogenesis has not been defined. Studies indicate that the aberrant form has an altered expression of the OMP. Chlamydia do not have a peptidoglycan layer but do have penicillin binding proteins on their cell walls. In addition, they express a number of heat shock proteins (hsp).

Certain characteristics distinguish *C. pneumoniae* from two other closely related organisms, *C. trachomatis* and *C. psittaci* such as DNA homology. Thus far, CP has been found to have one immunotype TWAR (derived from the first two strains, TW-183 and AR-39). However, more recent studies indicate that CP strains are antigenically different from each other, suggesting that more than one serovar of CP exist. The organism forms dense round inclusions in tissue culture cells that are more similar to *C. psittaci* than to *C. trachomatis*. In addition, CP has a characteristic pear-shaped EB that is surrounded by a periplasmic space. Ultrastructural studies of the entry of CP organisms into HeLa cells show that the mode of attachment and endocytosis of CP are different from those of *C. trachomatis* and *C. psitta*ci.

Current Status of Research and Development

Very little research has been done on the development of vaccines against diseases caused by CP. At present, most studies are focused on methods of diagnosis, the immunobiology of CP, and the response of the host to infections caused by CP. Recent improvements in isolation techniques have tremendously improved the capacity to detect the organism in clinical specimens. Monoclonal antibodies specific for CP are now commercially available for culture confirmation, and several CP-specific primers have been used in polymerase chain reaction (PCR) detection of organisms. However, efforts are being made to develop a more sensitive multiplex PCR system.

Several studies have been conducted over the years to examine the mechanisms involved in abnormal immune reactions associated with CP. For example, genes encoding hsps associated with immunopathology and those associated with protective responses have been identified in CP. Among these, hsp60, recognized by using sera from individuals infected with CP, is expressed at high levels during periods of stress and is particularly high in the aberrant form of the organisms; for example, high levels are expressed during chronic, persistent Chlamydial infections. In a recent study designed to examine the significance of hsps in the development of atherosclerosis, it was shown that chlamydial hsp60 can induce a variety of proinflammatory cytokines as well as increase the expression of cellular adhesion molecules on immune and vascular cells. In addition, at the molecular level, hsp60 induced the activation of nuclear factor kappa B, which may contribute to the gene expression of these molecules. In another recent study, it was shown that a peptide from heart muscle that has homology with CP outer membrane protein, can induce an autoimmune inflammatory heart disease, suggesting that CP may be linked to heart disease by antigenic mimicry of heart muscle protein.

There is a tremendous gap in the understanding of the host immune responses to infections caused by CP. Cell-mediated immune responses can be demonstrated in individuals infected with CP by blast transformation assays using peripheral blood mononuclear cells and has been demonstrated in experimental studies using *C. pneumoniae* EB antigens. CP infections also induce serum IgM, IgA, and IgG antibody responses. However, the role of cell-mediated or humoral immunity in recovery from infections caused by CP remains to be determined. Studies indicate that immunity to CP may be dependent on the expression of interferon-gamma, a characteristic product of Th-1 type T cells. Recently, a number of species-specific, potentially immunogenic, antigens have been characterized. Two of these, an OMP2 and a heat shock protein, have epitope configurations consistent with the capacity to induce a T cell proliferative response.

Considerable research has been directed at understanding the association of CP with coronary heart disease. Indeed, morphological as well as microbiological evidence indicating the presence of CP in atheromatous plaques has been obtained using electron microscope studies, immunocytochemical staining, and PCR testing of coronary, carotid, and aortic atheroma. In most studies, it is clear that the organisms are more commonly found in diseased than in normal tissue. However, the role of CP infection in the progression to atherosclerosis is unclear. Other studies have focused on elucidating the mechanisms of pathogenesis. The results of these studies suggest that the initial events may be the colonization of CP in alveolar macrophages. Indeed, macrophages or monocytes are likely to play a key role in the infection, serving as a vehicle for dissemination and responsible for the inflammatory response to infection through the elaboration of a variety of inflammatory mediators. Studies

show that CP can grow in blood monocytes, monocyte cell lines, and a variety of vascular cells. CP can also induce the expression of cytokines, including tumor necrosis factor alpha, IL-6, IL-1 beta, and interferon gamma, as well as increase the expression of cellular adhesion molecules. In addition to the release of cytokines from macrophages, activated T cells produce cytokines that cause infiltration of monocytes and lymphocytes from the blood. However, it is not clear how these events lead to the development of atherosclerosis.

It is still not clear whether CP actually causes atherosclerosis or is merely a bystander in the process. Studies in animals show that CP is capable of initiating and accelerating the development of atherosclerosis. For example, a combination of CP infection and small amounts of cholesterol supplementation enhanced the development of atherosclerosis in rabbits; however, antibiotic treatment of rabbits significantly reduced the development of atherosclerotic lesions. Three prospective human studies, conducted to examine whether cardiovascular diseases are amenable to antibiotic treatment, have now been reported. These studies indicate that cardiovascular events are reduced following treatment. However, in light of other studies showing conflicting results, the future of antibiotic therapy is uncertain. Several other treatment trials are underway.

There are currently no licensed vaccines for CP. Recent advances in immunological techniques and molecular genetics now make the development of such a vaccine feasible. Little is known about the microbial components of CP that may serve as vaccine targets. Studies show that the major outer membrane protein (MOMP) of *C. trachomatis* induces the activation of T cells that are protective in infections against *C. trachomatis*. There have been conflicting reports, on the other hand, regarding the immunogenicity of the MOMP of CP. Some studies indicate that this antigen is poorly immunogenic, whereas other studies show a moderate to high level of immunogenicity. Clearly, this area of research needs to be investigated further using purified *C. pneumoniae* MOMP. Recently, two novel genes encoding CP outer membrane proteins have been identified and found to be immunogenic in mice. A major impediment in the development and application of a vaccine against CP is the poor understanding of the host defense mechanisms against this organism. Animal experiments show that a Th1 type immune response to infection promotes protection, whereas animals that are susceptible to infection manifest a Th2 type immune response.

There are three experimental animals available for CP infections: mouse, rabbit, and monkey. Mice have been shown to be the most susceptible to intravenous, subcutaneous, or intracerebral infection. These experimental animal models can be used to examine potential vaccine candidates. For example, although CP is primarily a respiratory pathogen, it is conceivable that vaccine administration may prevent systemic spread to other organs. In an effort to understand latent infection caused by CP, it has recently been reported that CP lung infection in mice can be reactivated by treatment with cortisone; however, the underlying mechanisms remain to be clarified.

Recent Accomplishments and Developments

Although *C. pneumoniae* is a well-known causative agent of respiratory infections, it has also been recently associated with cardiovascular and neurologic disease (including multiple sclerosis, stroke, and Alzeheimer's disease). There is now considerable interest in understanding the mechanisms involved in the process of atherogenesis; there are studies in progress to determine how *C. pneumoniae* organisms colonize and destroy the walls of blood vessels. The earliest lesions seen consist of foam cells (mainly lipid-laden macrophages) and T lymphocytes intermixed with smooth muscle cells. Previous studies using electron microscopy have identified CP within foam cells. In a recent study it was shown that CP lipopolysaccharide, a major bacterial cell wall component, could induce foam cell formation suggesting that CP contributes directly to atherogenesis. In another recent study it was observed that Chlamydial hsp60 induced cellular oxidation of low density lipoprotein; this finding offers a mechanism whereby *C. pneumoniae* may promote the development of atherosclerosis. In addition, a number of treatment trials are ongoing based on the concept that the administration of antimicrobial agents may decrease the risk of cardiovascular disease.

Future Steps and Challenges

Efforts should be made to obtain more accurate and more rapid diagnostic methods to ensure timely detection of CP. Studies should be done with more sensitive assays to obtain a better understanding of the epidemiology of diseases caused by CP. Important risk groups should be defined because immunization recommendations will depend on who is at risk. Studies should be conducted to obtain information on the cell biology and molecular genetics of the organism, to characterize CP-specific proteins, and to identify microbial components that may serve as vaccine targets. Molecular mechanisms associated with attachment and invasion should be defined, and the host defense mechanisms, strategies for immune evasion, as well as the underlying mechanisms of protection should be elucidated. Major efforts should be made to develop vaccines against infections caused by CP. It is also necessary to develop appropriate animal models that could be useful in investigating chronic or latent CP infections. Specifically, basic research studies should be conducted to determine which factors contribute to the development of atherosclerosis as well as other cardiovascular and neurological diseases. Further, experiments should be done to evaluate the impact of

antibiotic treatment on CP-associated coronary heart disease as well as the impact of such treatment on the mortality associated with CP infections.

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Group A Streptococci (GAS)

Group A streptococci cause an estimated 25 million infections each year in the United States. These infections occur primarily in school-aged children and present as upper respiratory tract or skin infections. Antibiotic therapy is usually effective in eliminating the infection. However, these bacteria have the potential to initiate life-threatening infections such as rheumatic fever, rheumatic heart disease, or acute glomerulonephritis. Acute rheumatic fever occurs in all parts of the world and is the major cause of heart disease in children in developing countries. Rates of rheumatic heart disease range from 0.2 to 0.5 per 100,000 in affluent, developed communities to 125 to 960 per 100,000 in segregated, low socio-economic populations.

Although the incidence of rheumatic heart disease in the United States and other developed countries declined markedly during the late 20th century, the 1980s witnessed a resurgence in serious GAS infections, often with complications that included severe soft-tissue invasion and toxic shock-like syndromes among otherwise healthy individuals.

Current research studies are focused on elucidating the mechanism of invasion, the host factors that may determine severity of GAS disease, and virulence factors involved in the pathogenesis of GAS infections. In addition, studies on the use and efficacy of antimicrobial agents for controlling GAS infections have become focused on macrolide antibiotics because erythromycin-resistant GAS have been isolated in several parts of the world. This is a major concern because erythromycin has been the drug of choice for treating streptococcal infections in patients allergic to penicillin.

The most significant obstacle to developing a vaccine against GAS is circumventing an autoimmune response due to the immunological cross-reactivity between epitopes on the streptococcal cell and human tissues (heart, kidney, brain, and joints). All clinical isolates of GAS produce immunogenic surface proteins that have a role in the ability of the bacteria to colonize the host and cause disease. Streptococcal M protein is one of the surface molecules that was first described as being important in pathogenesis; it is responsible for resistance of GAS to phagocytosis and is a major virulence factor.

There is a family of M proteins that have several shared structural properties among GAS. Although the amino terminus of the M protein is hypervariable, containing type-specific nucleic acid sequences, the carboxyl terminus is conserved among the different M proteins. More than 100 different types of M proteins have been identified. Although antibodies against M protein have been shown to mediate type-specific protective immunity, specific M protein epitopes have been shown to cross-react with proteins of the host (e.g., myosin, laminin). It may be possible to eliminate this cross-reactivity by constructing vaccine candidates with only specific epitopes of M protein that are thought to be protective.

Currently, NIAID is supporting research aimed at identifying protective epitopes of M proteins from strains of GAS. Animal models are being used to assess the production of opsonizing and non-cross-reacting antibodies elicited after exposure to vaccines under development. An immunofluorescence assay using human heart tissue is one of the methodologies employed to test for heart cross-reactive antibodies. In addition, a number of different approaches to vaccine development are being funded that are based on other streptococcal proteins associated with virulence of GAS (i.e., C5a peptidase, cysteine protease, and other streptococcal pyrogenic exotoxins).

Strategies for vaccine development have included immunization at parenteral and intranasal sites. The parenteral route has been used because it is well

recognized that protective immunity to GAS infection can be achieved with serum-derived IgG directed to type-specific immunodeterminants of the M protein. The intranasal route has been used because early studies indicated the importance of local immune factors.

Pharyngeal challenge studies have been performed following immunization with a highly purified, acid-extracted M protein. Individuals immunized at the intranasal site had lower rates of nasopharyngeal colonization and clinical illness as compared to individuals immunized subcutaneously who exhibited a decrease in clinical illness only and had no change in the level of nasopharyngeal colonization.

Several vaccine candidates have been developed using synthetic or recombinant peptide fragments derived from type-specific sequences of the M protein. A number of recombinant, multivalent M protein vaccines have been constructed by placing protective peptide fragments of various M genes in tandem. Research studies indicated that the response to specific epitopes in hybrid M proteins depends on the size and location of the subunits, which were important considerations for constructing these vaccine candidates. When tested in rabbits using a parenteral route, a hexavalent M protein vaccine evoked a broadly protective immune response as measured by opsonic antibodies with bactericidal activity against all six serotypes and none of the antisera reacted with human heart tissue. The hexavalent vaccine was as immunogenic in alum as it was in complete Freund's adjuvant.

An issue to be addressed is the multiplicity of M serotypes expressed by GAS. Thus, an important consideration is the selection of M serotypes to be included in a vaccine. Epidemiologic studies will guide this selection and will focus on those serotypes that are associated with rheumatic fever, cause serious disease, or are frequent causes of uncomplicated infections. It may be necessary to develop several multivalent proteins that contain protective epitopes that could be mixed according to geographic differences in serotype prevalence.

Because human tissue cross-reactive epitopes identified to date have been located in the nonconserved regions of the M proteins, an alternative approach has been to use the highly conserved region of the carboxyl terminus of the M protein as a target for protective antibodies. In addition, most adults living in areas where exposure to streptococci is common have antibodies to peptides in the conserved region. A live vector was recently developed to evoke a GAS-specific mucosal immune response to the conserved region of the M protein. Although live-vector antigen delivery approaches have been used to develop vaccines, most vectors have been pathogens that require attenuation before cloning heterologous nucleic acid sequences. The use of a nonpathogenic commensal organism circumvents many of the problems involving appropriate attenuation while retaining invasive properities. The conserved region of the M6 protein was cloned and expressed on the surface of *Streptococcus gordonii*, a commensal organism of the oral cavity. Both IgA and IgG antibodies were produced in mice following oral and intranasal administration of this vaccine candidate. Similar studies in rabbits using the same routes of administration evoked M-specific IgG and IgA. In addition, there was no reactivity to human heart tissue when serum antibodies from rabbits colonized for 11 weeks were tested. An issue still to be addressed is the unknown long-term effects of colonization with an engineered oral commensal expressing a conserved epitope of GAS M protein. Colonization followed by eradication of the vaccine strain would be an appropriate cautious approach for initial safety and immunogenicity studies.

Another GAS vaccine candidate based on the conserved region of the M protein is being developed. Overlapping synthetic peptides spanning the conserved region of the M5 protein were tested with serum from adult Australian Aborigines and Thais living in areas where exposure to streptococci is very common. Because high levels of antibody specific for peptide 145 were detected in these individuals, this peptide was chosen for further evaluation. In preliminary studies mice immunized by the subcutaneous route with peptide 145 emulsified in complete Freund's adjuvant produced antibodies that opsonized streptococci of different M types. In a recent study in Australian Aboriginal communities, opsonic activity was demonstrated with affinity purified human sIgA and IgG specific for peptide 145 and acquisition of p145-specific sIgA was shown to increase with age. These studies suggest that this conserved region peptide has an important role in human immunity to GAS.

Although initial efforts to develop a vaccine involved the M protein, the potential for M protein epitopes to trigger autoimmune sequelae has led investigators to develop vaccine candidates based on other GAS proteins. Recent studies have focused on C5a peptidase, a ubiquitous protein among GAS. This highly specific surface–bound endopeptidase expressed by GAS (SCPA) is positioned on the bacterial surface to eliminate the complement chemotaxin C5a. In addition to GAS, group B and G streptococci of human origin also produce nearly identical endopeptidases. In mouse models, SCPA retards the influx of phagocytic cells at subdermal sites of GAS infections as well as the clearance of streptococci from oral-nasal mucosal infections. Intranasal immunization with SCPA was shown to speed clearance of GAS following intranasal challenge. Because SPCA from all serotypes is more than 95 percent identical, only a single protein should initiate protection against all present and new serotypes that circulate in human populations. A potential additional benefit of this vaccine candidate is protection against group C and G streptococci, which can also cause

pharyngitis as well as more serious infections. Vaccine development has focused on the use of a truncated form of the peptidase with mutations in the catalytic site. Parenteral administration of this active enzyme with adjuvant induces neutralizing antibodies and enhances clearance of streptococci from intranasally challenged mice. Experiments are ongoing to confirm the lack of toxicity and the potential to induce a tissue reactive immune response.

Streptococcal pyrogenic exotoxins (SPE) belong to a large family of proteins secreted by GAS and *Staphylococcus aureus* that can cause toxic shock syndrome in the antibody deficient host. These toxins are classified as superantigens based on their unique mechanism of stimulation of T lymphocyte proliferation. They cause serious human disease through massive cytokine release from both T lymphocytes and macrophages and may produce hypotension and shock as a consequence of cytokine effects on the vascular endothelium. SPE A and C have been shown to cause toxic shock syndrome in rabbits (and in two physicians who injected themselves) whether administered as purified exotoxins or when toxin producing GAS were injected subcutaneously. Antibodies against the toxins provided protection to the animals. Finally, toxoids of both SPEs A and C have been prepared through use of PCR mutagenesis of the respective genes. These purified toxoids lack detectable toxicity in either rabbits or mice, and do not stimulate proliferation of rabbit, mouse, or human T cells. The toxoids protect rabbits from lethal challenge with homologous SPEs and have potential use as vaccine candidates.

Studies with extracellular cysteine protease encoded by the *speB* gene in GAS have been focused on developing a vaccine candidate for prevention of severe invasive disease. Previous studies using a murine intraperitoneal model of lethal infection demonstrated that inactivation of the *speB* gene had different effects in several GAS strains, ranging from mild to extensive attenuation of virulence. When mutant and wild type isogenic serotype M3 strains were compared, results indicated that the genetic inactivation of the cysteine protease decreased the resistance of the mutant to phagocytosis and impaired its subsequent dissemination to organs. Recent studies in a mouse model of invasive skin infections demonstrated cysteine protease expression in the infected tissue by immungold electron microscopy. In addition, the wild type strain produced extensive cutaneous necrosis, bacteremia, and death while the *speB* mutant produced discrete abscesses that gradually regressed over time. Because cysteine protease is conserved among all GAS isolates and recent studies support the role of this enzyme in the production of cutaneous and invasive disease, a vaccine based on this protein holds great promise.

A protective immune response against *S. pyogenes* in mice has been demonstrated after intranasal vaccination with the fibronectin-binding protein SfbI. This protein is a major streptococcal adhesin involved in bacterial attachment and allows GAS to evade phagocytosis by polymorphonuclear leukocytes. SfbI specific humoral (IgG) and lung mucosal (14 percent of total IgA) responses were induced in mice following intranasal administration of SfbI alone or coupled with cholera toxin B subunit (CTB). Another study demonstrated that SfbI immunized mice showed 80 percent and 90 percent protection against homologous and heterologous challenge, respectively, as compared to 90 to 100 percent lethality after challenge with *S. pyogenes* among mice immunized with only CTB.

A number of different approaches have led to the development of several promising GAS vaccine candidates. Depending on disease manifestations and geographic location, a combination of vaccines may be needed for control and prevention of GAS diseases and their sequelae (i.e., rheumatic heart disease). A GAS vaccine for prevention of streptococcal pharyngitis would need to evoke a mucosal immune response aimed at preventing colonization of GAS. However, a vaccine for prevention of rheumatic heart disease, streptococcal toxic shock, and necrotizing fasciitis would need to evoke circulating opsonizing antibodies. Safety issues involving human heart cross-reactive antibodies can now be addressed by using recombinant DNA techniques to select potentially protective epitopes to be included in vaccine candidates and thereby exclude epitopes associated with cross-reactivity.

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Group B Streptococci (GBS)

Group B streptococci (GBS) are the leading cause of morbidity and mortality from bacterial infections in newborn infants as well as a frequent cause of infections in peripartum women and in adults with chronic medical conditions. In the past 5 years, significant progress has been made in the development and clinical testing of vaccines to prevent GBS infections in newborn infants. Different types of GBS have been identified by the variations in the composition and structure of their capsular polysaccharide (PS). GBS types Ia, Ib, II, III, and V can all cause disease. Antibodies generated against the capsular PS have been shown to be protective.

In the United States, neonatal disease prevention strategies are currently focused on the identification of vaginal and rectal GBS colonization in pregnant women and the use of antibiotics during labor and delivery in those women who are colonized or present with other risk factors. There is early evidence to suggest that, while this strategy is effective, it has not been able to totally eliminate GBS disease and encourages the widespread use of antibiotics. It has been estimated that 30 percent of women in labor and delivery are receiving intravenous ampicillin or penicillin. Active immunization of women during the third trimester of pregnancy to induce antibodies and passively protect their newborns has great potential for the prevention of both maternal and infant disease and does not rely on detection of colonization. It is also possible that the immunization of these women may affect their subsequent colonization with GBS.

In the 1980s, a type III GBS polysaccharide vaccine was evaluated in a small group of third-trimester pregnant women. The vaccine was safe but not highly immunogenic, with only 63 percent of nonimmune women responding to the vaccine. To improve immunogenicity, GBS polysaccharides were conjugated with protein components as had been done earlier with *Haemophilus influenzae* type b polysaccharide. Several type Ia, Ib, II, III, and V polysaccharide-protein conjugate vaccines have been prepared and evaluated in a series of phase I/II clinical trials. All of the conjugate vaccines have been found to be safe, nonreactogenic, and immunogenic. For example, the GBS type III-tetanus toxoid conjugate vaccine was evaluated in phase I and II clinical trials in women of child-bearing age. The vaccine was found to be safe and significantly more immunogenic than the unconjugated PS vaccine. Antibodies evoked by the conjugated vaccine recognized a conformationally dependent epitope of the type III capsular PS and promoted opsonophagocytosis and killing of GBS. In a mouse model, maternal immunization with the conjugated vaccine protected the neonatal offspring from lethal challenge with type III GBS.

The National Institute of Allergy and Infectious Diseases has supported the production of a larger lot of GBS type III-tetanus toxoid conjugate vaccine for clinical evaluation as a potential maternal immunogen. A third-trimester maternal immunization trial with this vaccine has just been initiated. In addition, ongoing research activities are focused on the use of alternative protein carriers and on the use of alum-based adjuvants to enhance immunogenicity with decreasing concentrations of the polysaccharide and protein components.

C5a peptidase expressed by GBS is being evaluated as a protein carrier for use in a GBS capsular PS conjugate vaccine. C5a peptidases produced by group A streptococci (SCPA) and by group B streptococci (SCPB) are 95 to 98 percent identical in sequence. Because SCPA is recognized as a GAS virulence factor, SCPB may play a role in GBS virulence, and immunization with SCPB could generate protection against GBS. In recent studies SCPB-type III carbohydrate conjugates induced antibodies that were bactericidal for other serotypes when presented to macrophages. In addition, SCPB vaccine produced antibodies that neutralized peptidase activity when administered to rabbits. These studies suggest that in addition to functioning as a carrier protein, when conjugated to GBS capsular PS, SCPB may provide additional protection against GBS.

The widespread use of a successful multivalent GBS conjugate vaccine could significantly reduce the morbidity and mortality associated with this major neonatal pathogen. Consequently, one of the goals of the NIAID GBS program is a phase III vaccine efficacy trial for the prevention of GBS disease. Before this trial can be initiated, considerable work needs to be done on the epidemiology and pathogenesis of GBS disease, the basic biology of GBS, and the further development of immunogenic vaccine constructs. To address these and other relevant issues, NIAID awarded a research and development contract, in 1992, to the Brigham and Women's Hospital. The contract provided support for collaborative as well as multifaceted clinical and basic research efforts on GBS disease. This contract was recompeted in 1997, and a new award was made to the Brigham and Women's Hospital. The contract workscope now includes a focus on the natural history of GBS colonization of women and on the role of GBS as a pathogen in adults with underlying chronic disease.

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***Haemophilus influenzae* Type B**

Research in this field continues to define the genetics and function of the human antibody repertoire of the *H. influenzae* type b capsular polysaccharide (Hib PS). Using the Hib PS as a model, efforts have been made to delineate the rules governing the expression of antipolysaccharide antibody specificities in humans and elucidate the structural determinants of protective immunity to this encapsulated bacteria. Studies have demonstrated the importance of avidity in determining protective efficacy of antibodies to Hib, and recently, this observation has been extended to the pneumococcal system. Several molecular mechanisms have also been identified that can account for variation in anti-PS antibody avidity. These mechanisms include differential variable (V) region gene utilization, subtle alterations in V region sequence acquired during the process of antibody gene assembly, and extent of somatic hypermutation. Molecular analysis of the infant Hib PS repertoire has shown that infant antibodies contain amino acid polymorphisms not previously observed in adult antibodies. This structural variation among infant antibodies provides a molecular explanation for the differential functional efficacy of antibodies elicited in infants by Hib-PS protein conjugate vaccines. Researchers have shown that closely related germline V gene homologs are not equivalent in their potential to form high affinity anti-Hib PS antibodies; therefore, inherited differences in the V repertoire can, in principle, affect the ability to generate protective polysaccharide immunity. Recent efforts involve studying the development of the Hib PS antibody repertoire from fetal life to old age. The results indicate that certain V region genes encoding Hib PS antibodies are assembled as early as the beginning of the second trimester. This pattern of V gene usage in the Hib PS repertoire is maintained throughout adult life and into advanced age. Additional studies are identifying inherited and somatically acquired polymorphisms involved in generating antibody diversity in man. This knowledge will help in understanding the cellular and molecular bases of protective immunity and may allow for the design of more effective vaccines against encapsulated bacterial pathogens.

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Nontypeable *Haemophilus influenzae*

Nontypeable *H. influenzae* (NTHi) is one of the most common causes of otitis media in children and lower respiratory tract infection in adults with chronic lung disease. Otitis media is the most common reason for children to visit pediatricians, accounting for 25 million office visits annually in the United States. Furthermore, ear infections are the most common reason for children to receive antibiotics in this country. Children who have recurrent ear infections may suffer from hearing loss at a time when hearing is critical for language development. As a result, these children may experience a delay in speech and language development. The annual direct health care costs for otitis media is estimated to be $3 billion. Preventing otitis media by vaccination would relieve an enormous amount of suffering and disability in addition to saving billions of dollars in health care costs.

A puzzling question about children who experience recurrent otitis media has been why children who appear

to have perfectly normal immune systems suffer from recurrent infections. Recent efforts have led to a critical observation that helps explain this apparent paradox. Infection by NHTi results in an immune response that is effective exclusively against the specific strain of NHTi causing the infection. Studies on the molecular structure of the outer membrane proteins revealed that the human immune response is directed at highly selected portions of the major outer membrane protein of NTHi. This results in an immune response that is protective against the strain causing infection but leaves the child susceptible to infection by many other strains of NHTi.

Chronic obstructive pulmonary disease (COPD), which includes chronic bronchitis and emphysema, is the fourth leading cause of death in the United States. COPD accounts for 10 million office visits and 2 million hospitalizations annually in the United States, with a large proportion of these the result of exacerbations caused by infections. NHTi is one of the most common bacterial causes of respiratory tract infections in patients with COPD. Infections are the most common single identifiable cause of death in patients with COPD. Preventing such infections could reduce morbidity and mortality, improve quality of life, and reduce health care costs. Adults with COPD represent another population that would experience a benefit from vaccines to prevent infections caused by NTHi.

Understanding the specificity of the human immune response is critical in guiding vaccine design. Other investigations have led to the identification and characterization of a protein that is a potential vaccine antigen. Considerable evidence indicates that immunization with outer membrane protein P6 will induce an immune response that will protect from infection. Phase I trials with P6 in human volunteers have been initiated recently.

Studies have also examined the interaction of NTHi with primary human airway cells in tissue culture. These studies have shown that NTHi binds to a subset of human airway epithelial cells that constitute about 10 percent of the total population of cells. Once NTHi binds to the surface of these cells, there is an active process of airway cell cytoskeletal protein rearrangement. This results in protrusions of the airway cell membrane, which extend like sheets from the surface and engulf the attached bacteria. The bacteria are then taken into the airway cell. At the present time, their fate in this cell is unknown (i.e., whether they survive and replicate or whether they are degraded by the airway cell). Additional studies have identified intracellular NTHi from bronchial biopsies of patients with chronic bronchitis indicating that invasion of airway cells by NTHi can occur in diseased patients.

Another area of interest is the role that NTHi lipooligosaccharide (LOS) might play in attachment and adhesion of NTHi to airway epithelial cell surfaces. These studies have shown that LOS can act as an adhesin. Additional efforts have shown that binding of LOS-coated beads can be blocked by LOS in solution and that the sugar responsible for the binding is the phosphocholine residue present on the LOS of NTHi. Invasion studies using organisms expressing LOS with phosphocholine and other organisms whose LOS lacks phosphocholine show a highly significant increase in invasion if the phosphocholine residue is present on the LOS. Studies are in progress to identify the receptor on airway epithelial cells to which the phosphocholine binds.

Investigators have also successfully cloned and determined the nucleotide sequence of three genes (i.e., *hgp*) that encode hemoglobin/hemoglobin-haptoglobin binding protein in *H. influenzae*. These newly discovered proteins have been named HgpA, B, and C. Studies of the cloned genes indicate that they are similar to each other and that they all contain a series of tetra-nucleotide repeats (CCAA) in the protein-coding region. Using a genetically engineered gene reporter fusion, it has been demonstrated that this region of repeating units allows the expression of the genes to vary randomly and that the change from an expressed form of the gene to an unexpressed form occurs at a rate of approximately 1 percent. To elucidate the role of phase variation of these genes in the pathogenesis of NTHi infections, multiple mutations of the individual genes were constructed. Single, double, and a complete triple mutant of the host strain, HI689, were constructed. Growth studies, using the mutants and the wild-type strain, indicated that expression of any single *hgp* gene was sufficient to allow normal growth *in vitro*. The complete triple mutant, lacking all the identified *hgp* genes, had a markedly reduced ability to grow using the hemoglobin-haptoglobin complex as a sole heme source. These studies also showed that incubation of a double mutant, in which the remaining intact *hgp* gene was in an unexpressed state with either hemoglobin or hemoglobin-haptoglobin complex, leads to a population in which all the bacteria express that gene product. These results are consistent with a proposed hypothesis of immune avoidance. It has been suggested that the acquisition of heme from hemoglobin and hemoglobin-haptoglobin is a vital step in pathogenesis. These data demonstrate that an initial step in the acquisition of heme is cell surface binding of the carrier proteins, indicating that the binding proteins are surface expressed and potential targets for a host immune response.

Studies are now underway to better understand the structure and function of NTHi pili, which are complex bacterial surface proteins that mediate NTHi adherence to human respiratory epithelial cell. Efforts have focused on a single protein, HifE, which is located at the tip of a pilus and contains the adhesive domain. Several approaches have been taken to identify this domain within the approximately 430 amino acids of HifE. One is to

generate monoclonal antibodies to HifE, test the antibodies for their ability to block adherence, and identify the specific amino acids that form the epitopic domain of the blocking antibody. Sufficient amounts of HifE (from recombinant *E. coli*) have now been purified to immunize mice for monoclonal antibody production. Investigators also have developed ELISA assays with which to quantitate binding of the mouse antibodies to pili and HifE.

A second approach in attempting to identify the HifE adhesive domain is to create a series of NTHi isolates that carry single amino acid mutations in conserved regions of transformants displaying mutations in two amino acids in the –RA—HL—conserved region. Transformants possessing these mutations, Arg211—Ala and Leu217—Ala will then be tested to identify the role of these amino acids in binding. It is hoped that the identification of the epithelial cell-binding domain of HifE will provide new strategies to prevent NTHi colonization and infection, such as acute otitis media, sinusitis, bronchitis, and pneumonia.

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Influenza

Among viruses, influenza is notable in its ability to produce annual epidemics of disease in both developed and developing countries. Recorded as pneumonia and influenza (P&I) morbidity and mortality, the annual toll of P&I-related deaths in the U.S. typically ranges from 10,000 to 20,000, with estimates as high as 50,000 during severe outbreaks. Despite vaccination or prior infection, the population's susceptibility to infection is renewed annually because influenza viruses undergo two major forms of antigenic variation: antigenic shift and antigenic drift. The sudden appearance of a new antigenic subtype is considered a shift, whereas a more subtle antigenic variation within a subtype is considered a drift. Consequently, influenza viruses have the inherent capacity to change the antigenic makeup of their surface proteins. If the change is a major one with little or no cross-reactivity to previously circulating strains (i.e., an antigenic shift), serious epidemics can result because of low protective immunity in the population. Such changes are also responsible for variations in virulence, host range, and infectivity of the virus. These "chameleon-like" properties can result in serious epidemics. For example, during the catastrophic 1918 to 1919 epidemic, between 20 and 40 million people died worldwide, and 500,000 died in the United States (196,000 people during October 1918); it was the worst epidemic the United States has ever experienced.

Influenza disease has long been recognized as a major, uncontrolled health problem in the United States. In all age groups, vaccines continue to be the focus to control the risk of serious complications of influenza infection. Although annual influenza vaccination rates have risen in persons 65 years of age or older from 23 percent in 1985 to 58 percent in 1995, these older adults still account for 90 percent of deaths attributable to influenza. Most vaccine manufacturers and research institutions with interests in influenza vaccines are exploring new ways to improve vaccine performance.

NIAID is engaged in a multifaceted effort to improve the inactivated vaccine that is now licensed for use against influenza. NIAID intramural scientists conducted a series of phases I and II clinical studies to assist in the development of a live, attenuated influenza vaccine using the cold-adapted (*ca*) influenza virus that was initially discovered by Dr. Hunein Maassab of the University of Michigan. The unique characteristic of this attenuated strain is the ability to grow at 25° C and the inability to grow at less than 38° C (temperature sensitivity), allowing the virus to colonize the cooler upper respiratory tract but not the warmer lower respiratory tract. The *ca* vaccine presents an alternative vaccine approach that offers several advantages. This vaccine stimulates a wider range of antibodies inducing local, humoral, and cellular immunity, and it can be administered intranasally, at the site of infection. This *ca* vaccine has been studied clinically in a wide range of age groups as well as high-risk populations as a monovalent, bivalent, and trivalent preparation.

In 1995, NIAID signed a Cooperative Research and Development Agreement (CRADA) with Aviron. This ongoing CRADA provides a mechanism to complete development of the intranasal *ca* influenza vaccine, and, in the past 4 years, more than six clinical studies were designed and conducted to test the safety, immunogenicity, and efficacy of a trivalent formulation with a nasal spray-syringe delivery system (FluMist). On July 14, 1997, the initial results of a phase III efficacy study in more than 1,600 children who received 1 or 2 doses indicated that the *ca* influenza vaccine was approximately 93 percent efficacious in preventing culture-confirmed cases of influenza. The second year of the trial was initiated in fall 1997. Nine hundred-seventeen of the previous year's FluMist recipients and 441 placebo recipients received a single dose of FluMist or placebo, respectively. Subjects were followed for efficacy and immunogenicy through the 1997-1998 influenza season. During the second year of the trial, the 1997-1998 formulation of FluMist was 100 percent efficacious against culture-confirmed influenza for strains included in the vaccine and 86 percent efficacious against the mismatched/emergent strain A/Sydney/05/97 (H3N2). In the third year of the study (fall 1998), 650 previous FluMist recipients and 299 previous placebo recipients received an additional 1 or 2 doses of FluMist. The results of this series of studies have shown that the *ca* vaccine is safe, immunogenic, and efficacious in preventing culture-confirmed influenza virus illness. Although the vaccine was well tolerated in individuals who received repeated yearly doses and participants had reactogenicity profiles similar to those of first-time recipients, an additional study to further evaluate the safety and tolerability of the *ca* vaccine administered to recipients for a fourth consecutive season occurred in fall 1999.

In addition, Vaccine and Treatment Evaluation Unit (VTEU) multicenter studies have focused on understanding the potential effect of *ca* in high-risk groups, including HIV-infected adult and pediatric populations. Because a number of immunologic abnormalities have been described in HIV-infected individuals that may put them at risk from live attenuated vaccines, a clinical study was initiated during the summer of 1998 to evaluate the safety of immunizing asymptomatic or relatively asymptomatic HIV-infected adults with the *ca* vaccine. Fifty-seven HIV-infected adults (CDC class A1 or A2) and 54 non-HIV-infected adults ages 18 to 58 received either 1 dose of *ca* or placebo. There was a higher incidence of rhinorrhea on days 2 and 3 post-vaccination in both HIV- and non-HIV-infected subjects who received *ca* compared with placebo recipients; however, the rates of other local and systemic reactions were similar in HIV-infected and non-HIV-infected *ca* recipients as well as in *ca* recipients and placebo recipients. A similar multisite trial that was initiated during summer 1999 is underway to assess the safety, immunogenicity, duration of vaccine virus shedding, and the effect on HIV replication of the *ca* vaccine in relatively asymptomatic HIV-infected children between 1 and 7 years of age.

In fall 1998, a large multiyear clinical trial was initiated in Temple, Texas, to assess the potential of *ca* immunization to produce an indirect effect (herd immunity) and to define the proportion and characteristics of the persons in the community who should be vaccinated to control epidemic influenza. Nearly 4,300 children between 18 months and 18 years of age received the *ca* vaccine during the first year of the study. The investigators are comparing the rates of medically attended, acute respiratory illnesses, including upper respiratory illnesses such as otitis media and sinusitis, lower tract illnesses, and asthma, for vaccinated and unvaccinated children. The second year of this study began in fall 1999.

In an effort to evaluate the protective effect of a combination of the *ca* influenza vaccine and the currently licensed, inactivated, trivalent influenza vaccine, a randomized, double-blind, controlled trial in 662 elderly residents (mean age 84.2 years) of long-term health care units was conducted. The trial was designed to compare the protective efficacy of administering both vaccines to the efficacy of administering the inactivated influenza vaccine alone. Volunteers who received combined vaccination and who were subsequently exposed to influenza A virus had significantly lower rates of influenza A virus infection than those who received only inactivated vaccine. Although acute but mild influenza A virus infections were seen during all 3 years of the study, two cases of influenza A virus infection resulted in pneumonia and hospitalization. One of these two patients died, and both cases occurred in patients given only the inactivated vaccine. These findings suggest a potential adjunct role of *ca* vaccines in the high-risk elderly.

In May 1997, a 3-year-old boy in Hong Kong contracted an influenza-like illness and died 12 days later.

Molecular and serological techniques confirmed that the viral isolate was influenza A/Hong Kong/156/97 (H5N1)—a virus closely related to an avian virus responsible for the deaths of several thousand chickens earlier that year in Hong Kong. By January 11, 1998, 18 confirmed cases of H5N1 influenza in humans had been identified—of which 6 were fatal. When it was confirmed that infected chickens, ducks, and geese were the source of the infection, authorities contained the outbreak by slaughtering all the poultry in Hong Kong. This H5N1 epidemic in Hong Kong represented the first evidence of the direct transmission of an avian virus to humans. In December 1997, NIAID awarded a sole-source contract to Protein Sciences Corporation for the production of a single lot of recombinant H5 hemagglutinin (rH5HA) vaccine. A phase I study to evaluate the safety and immunogenicity of two doses (10μg or 20μg each) of this vaccine was conducted by NIAID in laboratory workers and other at-risk adults in 1998. A second phase I trial of rH5HA was initiated in fall 1998 to evaluate the dosage of vaccine, the immune response, the optimal interval between vaccinations, and the immunogenicity of low-dose boosting. A total of 146 subjects completed the second study. To date, all vaccinations with rH5HA have been well tolerated and none were associated with a significant increase in systemic symptoms. The final immunogenicity data from these trials are expected in early 2000.

NIAID continues to work with both independent investigators and pharmaceutical companies to evaluate alternative strategies to improve the use and effectiveness of the inactivated influenza vaccine. Previous collaborations have included clinical studies on the use of microencapsulation, oral dosing, and liposomes as vaccine delivery systems to improve the immunogenicity of the vaccine. Although preliminary results indicate that these vaccine delivery systems are safe, the consistency and stability of these products as well as the efficacy of these products remain uncertain. Strategies aimed at improving vaccine effectiveness by supplementing the current inactivated vaccine are also being investigated, and studies that will evaluate the potential role of recombinant neuraminidase used in combination with the currently licensed trivalent inactivated vaccine are planned for spring 2000.

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Measles

Measles is now rare in the United States but not in the developing world, where it still kills 1 million children each year. The reemergence of measles from 1989 to 1992 in the United States stimulated a rededication of efforts at control and eventual eradication. WHO has stated that after polio, the next disease targeted to be eradicated is measles, targeting 2010 as the measles eradication date. In some parts of the world, the most popular vaccine used for measles eradication efforts is the MMR (measles, mumps, and rubella) vaccine. Because mumps and rubella viruses appear to have no natural reservoir, they, like measles, potentially could be eradicated. The possibility of nationally, regionally, or globally eradicating all three diseases during the same time has recently been considered. However, as reviewed below, unlike measles, mumps and rubella have little scientific attention focused on them.

Between 1981 and 1988, about 3,000 cases of measles occurred in the United States consistently each year. This rate was a reduction of more than 99 percent from the 400,000 to 700,000 annual cases reported before the introduction of a vaccine in 1963. However, in the early 1900s, a resurgence of measles occurred in the United States. From 1989 to 1991, there were 55,165 cases with 123 deaths reported. The major cause of the

reemergence of measles in the United States was the failure to vaccinate children at the appropriate age rather than failure of vaccine efficacy. The United States undertook a major effort to increase vaccine coverage, and the number of cases in 1996 was reduced to 488. Many of these cases were imported cases, and, in the past few years, there have been periods when indigenous measles cases were not reported, and transmission of the virus appears to have been interrupted.

Worldwide, measles reporting is incomplete, but in 1996, the disease burden was estimated at 36.5 million measles cases and 1 million deaths. Measles remains a major health problem accounting for 10 percent of global mortality from all causes among children younger than 5 years of age. There is substantial underreporting of measles cases, but the number of cases officially reported to WHO has dropped from 1,356,992 in 1990 to 797,322 in 1996. The majority of these cases (445,949) are in the African region. Of the remaining cases, the European region accounted for 162,967, the Western Pacific region for 84,459, the Southeast Asia region for 81,477, the Eastern Mediterranean region for 20,361, and the American region for 2,109. This represented the lowest number of cases ever reported from the Americas. Forty countries reported no measles cases in 1996.

Measles vaccine coverage worldwide has gone from 5 percent in 1977, to 16 percent in 1983, to 81 percent in 1996. The success of the polio eradication campaign and the success in reducing measles in the Americas have led to a global call for increased efforts to control measles worldwide. The 1990 World Summit for Children adopted a goal of vaccinating 90 percent of children against measles by the year 2000, and in 1994, the Pan American Sanitary Conference resolved to eliminate measles from the Western Hemisphere by the year 2000. To accomplish these objectives, measles control has incorporated lessons learned from the polio eradication campaign. For measles, this approach has been termed the “keep-up, catch-up, follow-up” program, and it has been extremely successful in many countries, particularly in South America. However, some countries have used other control strategies, and the U.S. experience with a two-dose immunization schedule demonstrates that maintenance of high levels of routine immunization also can lead to successful interruption of virus transmission.

Unfortunately, two recent experiences with measles have illustrated that many challenges remain for measles elimination programs. In 1997, despite a well-coordinated measles control program, measles reemerged in Brazil. By the middle of the year, the state of Sao Paulo reported more than 400 cases, after having virtually eliminated measles for the previous 6 years. In 1997 in Canada, despite a successful change from a one-dose to two-dose schedule and extensive catch-up campaigns, measles reemerged. An epidemic started after importation of measles into a university setting and spread within the British Columbia and later to Alberta. By midyear, more than 500 cases had been reported. These epidemics are currently being studied to understand their cause and to fine-tune measles control strategies.

As a public health tool, the current vaccine has some deficiencies. It has a primary vaccine failure rate of about 5 percent, and thus, susceptible individuals accumulate in the population. This failure rate is higher if the current vaccine is given to children younger than 12 months of age, when maternal antibody interferes with vaccine efficacy. In developing countries, where measles continues to claim more than 1 million lives each year, infants are at greatest risk for serious disease and complications during the interval between loss of maternal antibody and receipt of vaccine, at 9 to 12 months of age. Because currently licensed vaccines have lower than desired efficacy in very young infants, research has been directed toward developing an effective vaccine that can be safely administered earlier in infancy. In addition, there is a potential need for an improved measles vaccine for future immunization schedules that will evolve to emphasize administration of vaccines at earlier ages in infancy and will make use of multiple combinations of vaccines.

Recent research to develop improved measles vaccines has concentrated on the selection of more potent measles vaccine strains, or the development of high-titer vaccine formulations, that might effectively immunize a higher percentage of vaccinees and might be given to infants at 6 months of age or younger. However, studies in some parts of the world showed that high-titer vaccines might be associated with an increase in childhood mortality during a period of up to 2 years following immunization at 6 months of age. Although the reasons for this are not known, it was suggested that the immunosuppression that results from natural measles might occur with high-titer vaccines as well. Consequently, in 1992, WHO recommended that high-titer measles vaccines not be used.

Unfortunately, measles is a difficult virus to study because there are no satisfactory animal models. Within the past 2 years, both basic and applied measles vaccine studies have been accelerated by complementary WHO and NIH funding for the development of a reliable measles monkey model. Considerable progress has also been made in applying basic molecular virology approaches to define the genetic, molecular, and antigenic characteristics of measles. After elucidation of the molecular structure of this virus, the major focus of research has been to express antigens (particularly antigenic sites on H, F, M, and N proteins) in a form suitable for use as a vaccine. A request for applications issued by NIAID in late 1992 stimulated measles research and resulted in the development of a number of potentially new measles vaccine candidates including ISCOMs (immunostimulatory complexes), nucleic acid vaccines, pox-vectored vaccines, viral subunit immunogens, and BCG (Bacille Calmette-

Guérin)-vectored vaccines. In addition, this research program helped advance the development of the new primate model systems. These systems have been used to directly compare the immunogenicity of potential new vaccine candidates in nonimmune monkeys and in monkeys passively given measles antibody to mimic maternal antibody. These primates have also been given standard vaccine, high-titer vaccine, and older killed vaccine (both frozen old stocks and recreated 1960s era products) in an attempt to use modern immunological tools to determine what caused the vaccine-related sequelae with inactivated vaccine in the 1960s and with live high-titer vaccine in the 1990s. Although data on nucleic acid vaccines in primates are incomplete, it currently appears that ISCOMs and the nucleic acid vaccine have the greatest potential for inducing a protective immune response in the presence of maternal antibodies.

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Mumps

Mumps vaccine was licensed in the United States in 1967; since that time, the number of cases has dropped more than 99 percent to 751 in 1996. This drastic drop in cases occurred because of an increasingly inclusive vaccination policy at the State and Federal levels. The recent introduction of a second immunization of measles using MMR vaccine has accelerated the reduction of mumps cases.

In contrast to the elimination of polio and measles, elimination of mumps has not been an important global health goal. However, a recent study indicates that elimination of mumps might not only eliminate the acute mumps illness but might also eradicate endocardial fibroelastosis. The study screened for the presence of genome material of various viruses in autopsy tissue from 29 pediatric patients with endocardial fibroelastosis. This study included tissue samples from 1955 to 1992, and more than 70 percent of the heart tissue contained genetic material from the mumps virus. Only 1 of 65 matched controls contained any viral material, and that was from an enterovirus. Endocardial fibroelastosis was once relatively common, occurring in 1 of 5,000 births, but the cases have declined sharply. Interestingly, almost all the tissue samples before 1980 contained mumps viral material, whereas none after 1980 did.

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Rubella

Worldwide, rubella remains a common benign febrile disease of childhood. The most serious effects of rubella—spontaneous abortions, miscarriages, stillbirths, and congenital rubella syndrome (CRS)—follow infection during early pregnancy. The currently licensed vaccine is highly effective, and its combined use with measles and mumps vaccines in childhood immunization programs has drastically reduced the number of cases of rubella in the United States. From 1969 to 1989, the number of cases of rubella reported annually dropped 99.6 percent. Although there was a slight reemergence of rubella cases between 1989 and 1992, from 1992 to 1996 an average of only 183 rubella cases occurred annually. Most recently, from 1994 to 1996, 18 States reported no rubella cases, and the majority of cases were clustered in 6 outbreaks scattered across the United States. It is estimated that the average cost of a single case of CRS is more than $500,000. Sixty-seven cases of CRS were reported in the United States in 1970, the year the vaccine was licensed, and except for the reemergence of CRS in the early 1990s (33 cases in 1991), CRS cases have steadily declined, with only 12 cases reported from 1994 to 1996. Encouraged by this success, indigenous rubella and CRS have been targeted for elimination in the United States by the year 2000.

It can be generally concluded that in the developing world, natural rubella infection occurs early in life and almost universally. In such a situation, unless the epidemiology of rubella changes, there is no pressing need to immunize against rubella. However, recently it has came to the attention of WHO officials that many countries have, on their own, purchased MMR for their measles campaigns, and thus have already started to alter the natural circulation of rubella. Once this interference has occurred and "natural immunization" with rubella is not universal, rubella immunization programs must be continued aggressively. Consequently, rubella control and eradication have again been catapulted into the public health spotlight.

The epidemiology of rubella in the United States has changed from the 1980s in that since 1994, 84 percent of the cases occur in patients older than 15. Apparently, most cases occur among unvaccinated adults. Ninety-three percent of cases were indigenous to the United States; many imported cases came from countries that do not routinely provide rubella immunization (e.g., Mexico). From 1991 to 1996, the percentage of cases among Hispanics increased from 19 percent to 68 percent. Therefore, future immunization programs will focus more efforts on adolescents and adults and on selected ethnic groups that have lower rates of immunization and close contact with people coming from countries without comprehensive rubella immunization programs. Attempts to eliminate rubella from the United States would clearly benefit from improved global immunization programs.

Although the total number of cases of rubella is low and the number of cases of CRS is limited, the recent reemergence of natural rubella led to a campaign to increase vaccination coverage in all U.S. age groups. Consequently, many adult women were immunized against rubella, and a longstanding concern was again raised about possible vaccine-associated arthritic complications in these women. Early reports of naturally occurring rubella epidemics noted an increased incidence of arthropathy, predominantly in adult women. Like natural rubella, there are reports that the rubella vaccine causes transient joint symptoms in a significant proportion of women vaccinees. Joint complaints have been reported in up to 25 percent of previously seronegative vaccinees; these symptoms may last from 1 day to 3 weeks after immunization. Investigators in Canada had reported preliminary data indicating that a small percentage of adult female vaccinees develop a more severe and persistent arthropathy. One suggestion was that these complications might increase with the age of the vaccinee or the presence of low or incomplete rubella immunity. The causal relationship of rubella vaccination to the acute type of arthritis was highlighted in a recent Institute of Medicine report on vaccine safety, but its relationship to chronic arthritis remains unclear. In the past few years, two large studies of immunized populations suggested that long-term arthritic complications are not commonly associated with rubella immunization. More basic research studies have shown that for rubella virus to replicate, it must bind to host cellular proteins. These cellular proteins are under investigation as to their potential role as autoantigens and their potential contribution to arthropathy.

Basic research on rubella is now proceeding at a reduced level of funding, and NIAID currently supports only one project dealing with rubella. This research is focused on identifying and characterizing virus gene products required for generating long-lasting immunity, as well as those associated with the expression of adverse effects.

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Meningococcal Diseases

Background

Neisseria meningitidis is the leading cause of bacterial meningitis and continues to be a major public health problem, not only in the United States but also worldwide. Although the disease has a more severe impact on children and young adults, all age groups are susceptible to infections. The disease is transmitted from person to person by close contact. In the United States there are an estimated 3,000 cases per year involving meningococcal serogroups B, C, and recently, Y. In other parts of the world, the number of cases is much higher. For example, in sub-Saharan Africa, during the 1996 epidemics caused by serogroup A, more than 200,000 cases were reported, with 20,000 deaths. Significant proportions of the children who survive infections caused by *N. meningitidis* have permanent sequelae such as deafness. The emergence of new strains of meningococci and penicillin-resistant meningococci in the United States has further complicated the picture and caused serious public health concerns.

A major gap in the understanding of the pathogenesis of meningococcal disease (MD) is the relationship between carriage of meningococci and invasive MD. Most meningococci possess a polysaccharide capsule, which forms the basis of classification into serogroups. The presence of the capsule helps the organism resist phagocytosis. Recent studies show that the capsule not only alters the adherence of the organisms to leukocytes but also alters the interaction with lysosomes within the cells. An additional virulence mechanism is the ability of meningococci to escape protective immunity by switching capsules. The organisms also carry pili that facilitate adherence to host cells and possess a large number of outer membrane proteins (OMP) including Opc and Opa, which appear to mediate invasion of epithelial cells. Further, some of these proteins (e.g., the pili and OMPs) show considerable antigenic variation. In addition, the organisms are capable of secreting proteins (e.g., FrpA and FrpC) that may act as toxins and IgA protease, which can cleave human IgA; however, the role of these molecules in pathogenesis is still not clear. Another important virulence factor is endotoxin. Unlike the endotoxin of Enterobacteria, this molecule contains short sugar chains and hence is termed lipooligosaccharide (LOS). Studies indicate that LOS is important for colonization in the nasopharynx. The release of meningococcal LOS also may contribute to the hypotension and shock associated with fulminant meningococcemia. LOS and other meningococcal components can induce a variety of cytokines and other mediators of the immune response that may have a significant impact on the course of the infections.

Although meningococci are carried asymptomatically in the nasopharynx of 5 to 10 percent of normal individuals during nonendemic periods, it is still not clear why some individuals become susceptible to invasive MD. It is known that individuals with complement deficiencies, those who are malnourished or immunosuppressed, and asplenic patients are at high risk for invasive MD. There is also evidence of a genetic predisposition to invasive MD based on a genetic inheritance pattern among families with respect to the amount of cytokines produced. These results also suggest that the type of cytokines produced may be associated with the risk of fatal disease. Other studies have demonstrated the presence of decreased plasma levels of coagulation factors and increased expression of cellular adhesion molecules in patients with invasive MD, and they have shown that interleukin-12, tumor necrosis factor, and interferon-gamma may contribute to natural immunity.

Current Status of Research and Development

Considerable efforts have been focused on vaccine development. The currently licensed vaccines, which contain purified capsular polysaccharides (PS) from four major serogroups (A, C, W135, and Y), are moderately immunogenic. However, the immune response is, in general, of short duration and cannot be boosted upon re-immunization, and the polysaccharide vaccines do not elicit an immune response in children less than 2 years of age. Interestingly, group A capsular polysaccharide vaccine is moderately immunogenic in this age group; the underlying mechanisms of this unique response are

not clear. Currently, an attractive strategy in vaccine development is to use polysaccharide-protein conjugate vaccines to enhance immunogenicity and to induce memory. In a recent study in the U.K., it was observed that meningococcal group C conjugate vaccine was immunogenic in infants and also induces a memory response.

Although major advances have been made in the development of vaccines for group A and C strains of meningococci, there are no licensed vaccines for group B meningococcal infections in the United States and the development of vaccines against group B strains remains problematic. Unlike the other meningococcal capsular PSs, the group B PS is poorly immunogenic in both infants and adults. Studies using x-ray crystallography suggest that the poor immunogenicity may be due, at least in part, to the fact that the conformational epitope of group B PS that is capable of inducing an immune response may not be stable under different physiological and pathological conditions. Because group B strains continue to be a major cause of meningococcal disease in the United States and several other countries, the development of an effective group B capsular PS vaccine would represent a major advance in the prevention of meningococcal disease. However, there are important concerns that such a vaccine might induce immunopathology such as the formation of cross-reactive autoantibodies to specific oligosaccharides found on mammalian cells. For example, anti-group B PS antibodies cross-react with the neural cellular adhesion molecule, a membrane glycoprotein involved in cell-cell adhesion. Therefore, it is possible that a group B PS-based vaccine may induce immunopathological side effects.

Such concerns have prompted the pursuit of alternative strategies for group B vaccine development including use of meningococcal OMPs, use of lactoferrin and transferrin-binding proteins, and modification of the sugar moieties on the capsular polysaccharide. Studies indicate that OMPs can induce protection. For example, it has been shown in an infant rat model that antibodies to PorA proteins are protective against meningococcal infections. Protein-based vaccines have been used in clinical trials in Cuba, Brazil, Chile, and Norway with efficacies ranging from 50 to 80 percent. Unfortunately, these vaccines induced no protection in children and the immune response was of short duration. Recent vaccine approaches include (1) a multivalent OMP vesicle vaccine in which vaccine strains were constructed by recombinant DNA techniques to express three different PorA proteins. This vaccine is currently undergoing clinical trials; (2) an A/B (chemically modified group B PS/C combination vaccine; and (3) an anti-idiotype group B vaccine.

Recent Accomplishments and Developments

Association of mannose-binding lectin and susceptibility to meningococcal disease

Although it is known that pathogenic strains of *N. meningitidis* are carried as harmless commensals in the nasopharynx of approximately 1 percent of the population, for some individuals meningococcal disease results in fatal illness. It has been postulated that this may be due to differences in the host response to infection. For example, studies have shown that individuals deficient in the terminal components of the complement system are susceptible to recurrent episodes of the disease. Previous studies also suggest that genetic variants of the mannose binding protein (MBP), a plasma protein involved in complement activation, may also be associated with susceptibility to the disease. Indeed recent studies show that individuals who have had meningococcal disease have a significantly increased frequency of MBP when compared to controls. It is conceivable that MBP is a plasma defense mechanism against meningococci.

Understanding the mechanisms by which Neisserial porins enhance immune responses

It is often desirable to enhance the immune responses of vaccine candidates by using adjuvants. Previous studies show that Neisserial OMPs (mainly porins) can augment the immune response to peptides, polysaccharides, and glycolipids. Recent studies indicate that meningococcal group C polysaccharide can generate a memory response when it is conjugated to neisserial porins. In an effort to examine the mechanisms by which porins augment the immune response, it was observed that blocking the co-stimulatory molecule, B-7, reduced the level of antibody produced. These studies indicate that the capacity of these porins to enhance the immune response is dependent on the expression of co-stimulatory molecules on antigens presenting cells.

Future Steps and Challenges

The development of a new and improved meningococcal vaccine, in the context of “optimal” adjuvant/delivery system, that is safe and immunogenic in children would have a tremendous impact in decreasing the incidence of the disease. Development of a vaccine against group B meningococci remains a major challenge. Another important task is to understand why certain individuals within a given population develop invasive MD. Studies using a number of adjuvants including monophosphoryl lipid A and Quil A, and neisserial porins to enhance the immune response to meningococcal vaccines represent a significant advance. In addition, basic research studies should be encouraged to analyze the biological, structural, and molecular aspects of potential virulence factors and to identify novel bacterial components that may serve as potential vaccine targets.

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Moraxella catarrhalis

Moraxella catarrhalis (M. catarrhalis) is an important cause of both upper and lower respiratory tract infections. This organism is recognized as the third-most common cause of otitis media and sinusitis in children, after *S. pneumoniae* and nontypeable *H. influenzae*. *M. catarrhalis* is also a major cause of lower respiratory tract infections in adults, especially those with chronic obstructive pulmonary disease (COPD), and may produce life-threatening pneumonia in elderly persons. The incidence of the diseases caused by *M. catarrhalis* appears to be increasing as well as the percentage of clinical isolates of *M. catarrhalis* that produce beta-lactamase. Currently, there is no vaccine for *M. catarrhalis.* As with other bacterial pathogens, serum or bactericidal antibodies appear to be involved in immunity against *M. catarrhalis* infections.

Lipooligosaccharide (LOS) is a major surface antigen of *M. catarrhalis* and may be a virulence factor. LOS has been shown to be a potential vaccine candidate because convalescent serum antibodies to LOS in patients with *M. catarrhalis* infections have bactericidal activity against this organism. The conjugation of LOS from *M. catarrhalis* either to a tetanus toxoid carrier or to high-molecular weight proteins from nontypeable *H. influenzae* produced significant amounts of anti-LOS IgG antibody against the homologous strain as well as heterologous strains of *M. catarrhalis*. Therefore, a detoxified LOS-protein conjugate may represent a reasonable candidate in both infants and adults for immunization against *M. catarrhalis* diseases.

Work in other laboratories has lead to the identification and characterization of two other potential vaccine antigens associated with *M. catarrhalis*. Outer membrane proteins CD and E are proteins that are abundantly present on the surface of *M. catarrhalis*. Several lines of investigation, including animal studies and *in vitro* assays indicate that antibodies to these proteins are protective. An important characteristic that distinguishes these proteins from other potential vaccine antigens of *M. catarrhalis* is the high degree of similarity in the amino acid sequences of protein CD and E among strains recovered from different patients in different geographic regions. This observation suggests that immunization with these outer membrane proteins would likely be highly effective at generating immune responses to many or all strains of *M. catarrhalis.*

Another important antigen of *M. catarrhalis*, the UspA1 protein, is an adhesin that allows this organism to attach to human eipithelial cells. UspA1 is probably involved in the process through which *M. catarrhalis* colonizes the human upper respiratory tract; this is the first important step in the production of disease by this pathogen. Therefore, antibodies to UspA1, produced in response to vaccination with purified UspA1 protein, might prevent colonization and infection of the respiratory tract by *M. catarrhalis*. Supporting this hypothesis is the recent finding that antibodies against the UspA1 protein can effectively block adherence of *M. catarrhalis* to epithelial cells. In addition, because the UspA1 protein is present on the surface of *M. catarrhalis,* antibodies directed against this protein can effectively bind to this pathogen and destroy it, as shown by several *in vitro* assays. Finally, nucleotide sequence analysis of the UspA1 gene indicates that the UspA1 protein is highly conserved among several different strains of *M. catarrhalis.* This finding suggests that it may be possible to use just one purified UspA1 protein to vaccinate against most or all strains of this organism.

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Mycoplasma pneumoniae

Background

In the United States, about 15 million respiratory infections, including atypical pneumonia and tracheobronchitis, are caused by *Mycoplasma pneumoniae* each year. *M. pneumoniae* is the leading cause of pneumonia in older children and young adults but also affects older adults and elderly individuals. This microorganism is responsible for 25 percent of all cases of pneumonia requiring hospitalization and 50 percent of all pneumonias in closed populations, and it is the second leading cause of tracheobronchitis in children. *M. pneumoniae* is also responsible for extrapulmonary complications such as arthritis and has been associated with chronic asthma. Related organisms, such as *M. hominis* and *Ureoplasma urealyticum,* may cause pulmonary diseases in neonates.

Mycoplasmas are wall-less prokaryotes that are biosynthetically deficient in several respects. Therefore, they must rely on the micro-environment provided by the host to obtain essential metabolites (nucleotides, fatty acids, sterols, and amino acids) needed for growth. Mycoplasmas possess a circular double-stranded DNA chromosome ranging from 600 to 1,300 kilobases, with complex genetic recombination systems and large genome families. The organism has a tremendous capacity to generate antigenic and phase variations that may be important in disease pathogenesis and tissue tropism, but this characteristic poses a special challenge for vaccine development. The complete genome of *M. pneumoniae* has been sequenced. It is anticipated that this information will significantly advance our understanding of the physiological and genetic characteristics of these organisms and may provide new leads for vaccine development.

Although mycoplasmas are responsible for a variety of important diseases in humans and various animal species, experimental vaccines have not affected the spread of infection, possibly because of the organism's ability to develop antigenic changes at high frequency. This difficulty in controlling mycoplasmal infection may also be due to our lack of understanding of the host response to this organism. Because previous studies indicate that patients with impaired humoral immunity suffer chronic sinopulmonary disease due to mycoplasma, it is generally believed that antibody plays a role in immunity. However, the role of cell mediated immunity has not been adequately investigated.

Current Status of Research and Development and Recent Accomplishments

Most of the current studies are focused on elucidating the molecular mechanisms of pathogenesis and the host responses, as well as efforts to improve the methods of diagnosis using PCR and modern immunological techniques. One area that has received considerable attention is understanding the mechanisms of mycoplasmal attachment to host epithelial cells, an event that has been described as cytadherence. The process of cytadherence is pivotal to the survival of *M. pneumoniae* and its ability to persist in the host. The mycoplasmal attachment organelle has been identified. Molecular characterization studies continue on the P1 adhesin protein, which is densely clustered at the attachment organelle, and on a series of cytadherence associated proteins, HMW1-3. Future studies will determine whether any of these proteins could represent vaccine targets.

Considerable efforts have also been made in the development of vaccines against infections caused by *M. pneumoniae*. Earlier challenge studies in the late 1960s conducted in human volunteers demonstrated that an inactivated *M. pneumoniae* vaccine was moderately protective, but these studies have not been pursued further. Studies in chimpanzees indicate that animals immunized with a formalin-inactivated vaccine or an acellular extract developed milder disease and lower colonization rates with mycoplasma compared with unimmunized controls. Because only partial protection was observed in such experiments, more studies are needed to increase the level of protection expressed. Other studies suggest that the manner in which immunogens are delivered may be an important factor in the generation of an optimal immune response.

An experimental *M. pulmonis* (the agent of murine mycoplasmosis) vaccine has been shown to induce protective antibodies, however, other approaches are still necessary. An oral *M. pneumoniae* vaccine is being developed to evaluate the role of sIgA antibody in the development of immunity. These preliminary studies may pave the way for rapid development of safe and effective vaccines against *M. pneumoniae* infections.

Future Steps and Challenges

Studies should continue to better define the epidemiology of diseases caused by *M. pneumoniae*, to better understand the molecular pathogenesis, and to develop improved diagnostic technology. In particular, it is critical that new targets for intervention are identified and appropriate animal models are developed. For example, it would be useful to understand the involvement of proteases in mycoplasmal growth and to evaluate the use of protease inhibitors on infections caused by mycoplasmas. There is a tremendous gap in our understanding of the role of the host immune system; for example, studies should be conducted to examine the role of Th1 versus Th2 type immune responses in resistance or susceptibility to infection. In addition, it is important that the role of cytokines and inflammatory mediators as well as cellular adhesion molecules and mechanisms of T-cell activation be clarified. Further, the significance of immunopathological reactions in the development of chronic diseases associated with *M. pneumoniae* needs to be investigated.

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Parainfluenza Virus

Four serotypes of human parainfluenza viruses (HPIV 1-4) are associated with respiratory illness. A variety of upper respiratory infections caused by HPIVs include otitis media, pharyngitis, conjunctivitis, and the common cold. These can occur alone or in combination with the following lower respiratory tract infections: croup, laryngitis, bronchiolitis, or bronchopneumonia. HPIV types 1-3 cause croup and laryngitis in infants and children. Although HPIV-1 and HPIV-2 generally cause disease in toddlers and preschoolers, HPIV-3 is unique among the parainfluenza viruses in its ability to infect young infants less than 6 months of age. The most common clinical syndromes caused by HPIV-3 are bronchopneumonia and bronchiolitis. HPIV-3 infections are second only to respiratory syncytial virus infections as a cause of serious respiratory tract disease in infants and children. HPIV-4 has been associated with mild upper respiratory tract disease in children and adults.

Antigenic subtypes are found within the HPIVs, however; temporal and progressive variability has been associated with both HPIV-1 and HPIV-3. There are two antigenic subgroups of HPIV-4, A and B, based on antigenic differences detected by hemadsorption-inhibition and monoclonal antibody reactivity. The antigenic variability may affect the efficacy of HPIV-1 and HPIV-3 vaccines being evaluated.

One approach to vaccine development has been purification of major outer membrane proteins (fusion [F] and hemagglutinin-neuraminidase [HN] glycoproteins) for use as immunogens in subunit vaccine candidates. One current effort involves using ion-exchange chromatography to purify HN and F detergent-solubilized proteins from HPIV 1, 2, and 3. When combined with the adjuvant, aluminum phosphate, this trivalent formulation evoked serum HPIV 1, 2, and 3 specific neutralizing antibody titers in mice.

Another approach involves the development of live-attenuated parainfluenza virus (PIV) candidate vaccines, including a bovine PIV-3 and cold-passaged (cp) HPIV-3 vaccines. Because cp-45 was more attenuated than other strains in nonhuman primate studies, the safety, infectivity, and immunogenicity of cp-45 were evaluated in children 6 months to 10 years of age. In this age group, the vaccine candidate cp-45 was well tolerated when given intranasally to PIV-3 seropositive and seronegative children. A dose-response study in seronegative children demonstrated infectivity and immunogenicity at doses of 10^2 to 10^5 pfu/

ml. Further evaluation of cp-45 will be supported by a collaborative research agreement between NIAID and Wyeth-Lederle Pediatric Vaccines.

A bovine PIV-3 vaccine was chosen as a candidate live-virus vaccine because it is antigenically related to HPIV-3, as shown by sequence analyses of bovine PIV and HPIV HN and F glycoproteins and cross-neutralization studies. The first phase I trial demonstrated that bovine PIV-3 was safe, infectious, immunogenic, and phenotypically stable when administered to 6- to 36-month-old PIV-3 seronegative infants and children. The second study evaluated the bovine PIV-3 vaccine in two age groups (2- to 6-month-old infants and 6- to 36-month-old infants and children). This vaccine was well tolerated in both age groups and infected 92 percent of infants younger than 6 months and 89 percent of infants and children older than 6 months. Serum hemagglutination-inhibition antibody responses to HPIV-3 and to bovine PIV-3, respectively, were detected in 42 and 67 percent of the younger infants, compared with 70 and 85 percent of the older group. Additional studies are needed to determine whether two or more doses will enhance the immunogenicity of the bovine PIV-3 vaccine in young infants.

No licensed PIV vaccines are available. Although several strategies have been examined, the approach that uses live-virus vaccine candidates is currently the most advanced. The effort to produce a live-attenuated HPIV-3 vaccine will be assisted by the recent generation of infectious HPIV-3 from a full-length clone of the HPIV-3 genome, an important research advance supported by NIAID. Analysis of mutations within the infectious clone will allow identification of mutations that attenuate the virus and could be used to develop new vaccine strains. In addition, it provides the tools to produce PIV-1 and PIV-2 vaccine candidates by substitution of the PIV-1 and PIV-2 HN and F glycoproteins for the PIV-3 glycoproteins in the PIV-3 cp45 backbone.

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Pseudomonas aeruginosa

Background

Pseudomonas aeruginosa is an opportunistic organism as well as a pathogen for patients with cystic fibrosis (CF), a disease that usually presents itself in early childhood. Although there has been considerable progress in the use of gene therapy to correct the basic genetic defect of CF at the molecular level, there is no evidence that gene therapy alters the course of *Pseudomonas* infection in this population. Therefore, preventive approaches, such as the development of safe and effective vaccines, are needed. Efforts to control these infections with antibiotics and better pulmonary therapy have done little to reduce the high mortality associated with *P. aeruginosa* pneumonia; however, immunotherapeutic interventions with active vaccination or passive therapy may have a significant impact on the development of sepsis and on survival. Indeed, the results of studies using passive immunotherapy with immune globulin enriched with anti-*P. aeruginosa* lipopolysaccharide antibodies indicate that this approach is effective.

While *P. aeruginosa* is a special problem for patients with CF, it also contributes to the high mortality rates (>50 percent) in patients with emphysema, cancer, AIDS, and serious burns. A recent study indicates that although *P. aeruginosa* is not the most common bacterial pathogen in AIDS patients, the presence of this organism is usually associated with increased mortality. In fact, it has recently been proposed that intervention strategies against *P. aeruginosa* be implemented in the management of AIDS patients. The reason for the extraordinary pathogenicity of *P. aeruginosa* in these patients is not clear. However, it is possible that a variety of virulence factors produced by *P. aeruginosa* may account for the high mortality rates. A major virulence factor produced by *P. aeruginosa* is an exopolysaccharide or alginate. Alginate not only encapsulates the infecting bacteria, protecting them from antibiotic treatment or from attack by host immune responses, but, in addition, enables the bacteria to adhere to epithelial cells of the lung, enhancing the opportunity for further colonization and invasion. Other virulence factors of *Pseudomonas* include cell-associated structures such as pili, as well as secreted products such as exotoxin A,

exoenzyme S, hemolytic phospholipase, and proteases. Expression of these virulence factors is highly regulated, which probably accounts for the ability of *P. aeruginosa* to cause a wide variety of infections in vastly different host environments.

Current Status of Research and Development and Recent Accomplishments

During the past 3 decades significant advances have been made in understanding the pathogenesis of pulmonary infections in patients with CF as well as in understanding the molecular and cellular basis of the CF defect. Recent studies have shown that cells expressing normal CF-transmembrane conductance regulator surface proteins internalize more *Salmonella typhi* organisms than cells expressing a CF mutation. Similar observations were made *in vivo* using mice expressing the CF mutation and the appropriate control mice. These studies suggest that the CF mutation may offer protection against disease manifestations such as diarrhea and typhoid fever that are caused by *S. typhi*. In addition, these studies have important implications in understanding the initial interaction between pathogen and host cells and the manner in which organisms gain access to body sites where they cause disease. It is also anticipated that understanding the processes involved would pave the way for the development of inhibitors that can prevent infection.

Other studies show that significant inflammatory changes are associated with *Pseudomonas* infections. It has been observed that CF patients have high levels of inflammatory cytokines (e.g., interleukin [IL]-8) in the lung environment relative to the levels in healthy individuals. By contrast, the levels of cytokines, such as IL-10, that decrease inflammation are low in CF patients as compared to those in healthy individuals. Recent molecular analyses of signal transduction mechanisms suggest that *P. aeruginosa* induces the epithelial cell production of IL-8 by activation of nuclear factor kappa B (NF-KB). Cells with CF mutations have significant endogenous levels of activated NF-KB. It is possible the mechanisms of signal transduction underlying the endogenous activation of NF-KB are different from the signals involved in the activation by *P. aeruginosa*. These inflammatory changes must be taken into account in the design of preventive strategies such as vaccines against *P. aeruginosa*. Recent studies now indicate that certain extracellular virulence factors are controlled by a system of quorum sensing molecules. Quorum sensing is a mechanism used by bacteria for cell-cell communication. It is also involved in the formation of bacterial biofilms, a major problem associated with *P. aeruginosa* infections. There are essentially two components to the system: a small diffusible signal molecule, typically N-acyl homoserine lactone in gram negative bacteria, and a second molecule, typically a transcriptional activator protein. Because of the increasing difficulty in treating this organism, particularly once the biofilms are fully formed, these findings, and the anticipation that these molecules may provide targets for therapeutic intervention, represent important advances.

Significant advances have been made in the development of vaccines against *P. aeruginosa*. Several surface proteins and polysaccharides have been demonstrated to be safe and immunogenic in small phase I/II studies, and have been shown to generate protective immunity in various animal model systems. For example, both high molecular weight polysaccharides and mucoid exopolysaccharide vaccine preparations have been tested in humans. Other vaccines based on outer membrane proteins or killed whole cell vaccine preparations have been found to be immunogenic in clinical trials. For example, studies have shown that recombinant outer membrane protein I (oprI) is highly protective in experimental animals; purified oprI was also found to be safe and immunogenic in clinical trials. In other studies, oral immunization with a killed *P. aeruginosa* vaccine preparation protected naive animals against challenge with live bacteria. Despite these encouraging results, most of the studies done to date that have demonstrated immunogenicity have failed to demonstrate protective efficacy.

Investigators have also pursued the use of recombinant OMPs as vaccines against *P. aeruginosa*. The results of experiments using a hybrid vaccine, containing protective epitopes of outer membrane proteins F and I, indicate that the vaccine is highly immunogenic and protective against *P. aeruginosa* infections in mice, especially when expressed as a plant virus. In other studies, recombinant OMP I was also found to be safe and immunogenic in human volunteers. However, the use of OMPs as vaccines against *P. aeruginosa* infections requires further study.

Future Steps and Challenges

For CF patients a vaccine should induce an immune response that would prevent mucosal colonization of *P. aeruginosa* and/or elicit a response against virulence factors associated with adherence. Improved vaccine design is dependent on: 1) understanding the molecular regulation of *P. aeruginosa* virulence factors and, in particular, the interaction of *P. aeruginosa* with host cells, and 2) understanding overall host immune response to infections.

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Respiratory Syncytial Virus

Respiratory syncytial virus (RSV) is the single most important cause of severe lower respiratory tract infections in infants and young children. It is a common cause of winter outbreaks of acute respiratory disease and results in an estimated 90,000 hospitalizations and 4,500 deaths each year in the United States. The global annual infection and mortality figures for RSV are estimated to be 64 million and 160,000, respectively. RSV infects repeatedly and causes disease throughout life, including a wide array of respiratory symptoms from rhinitis and otitis media to pneumonia and bronchiolitis; the latter two diseases are associated with substantial morbidity and mortality. RSV infects nearly all children by 2 years of age. Reinfections during later childhood and adulthood are generally associated with milder disease. Outbreaks of RSV infections involving adults have been reported among institutionalized elderly patients and are often associated with pneumonia. Severe RSV infections are a problem in immunocompromised patients of any age, especially transplant recipients. There is also recent evidence of a possible link between RSV infection and the development of asthma.

The development of an RSV vaccine is difficult but a high priority. The major obstacle to developing a vaccine against RSV is the experience from clinical trials conducted in the 1960s in which a formalin-inactivated whole RSV vaccine was given to children. Recipients who were seronegative at the time of vaccination experienced lower respiratory tract disease of increased incidence and severity upon subsequent natural infection. Therefore, development of an effective vaccine will require an understanding of both the protective as well as the disease-enhancing immune responses to RSV. Research efforts have focused on the individual components of these responses, including cell-mediated events as well as production of serum and secretory antibodies. Although much has been learned about these components, a safe and effective vaccine that induces protective immunity and does not enhance natural infection is not yet available. An effective vaccine would reduce morbidity, the frequency of hospitalization, and the mortality from RSV infection. Vaccine candidates under development are evaluated in animal models first, followed by adults, immune children, older nonimmune children, younger nonimmune children, and, finally, susceptible infants.

Animal models that were developed to study RSV include the cotton rat, mouse, calf, lamb, and primates (baboon, bonnet monkey, African green monkey, chimpanzee). They have been useful in understanding and characterizing protective responses to RSV and vaccine candidates. In addition, data from these models are helping to elucidate the disease-enhancing reactions and are being used to formulate improved strategies for vaccine development. Recently a lot of formalin-inactivated RSV was made as a facsimile of the lot that caused enhanced disease in the 1960s clinical trials. This reagent has been available for investigations using animal models to further the understanding of the mechanism of disease enhancement.

There are two RSV strain subgroups, A and B. A successful vaccine would induce resistance to both subgroup A and B strains. Neutralizing antibodies are induced by F and G glycoproteins found on the surface of RSV. The F surface protein is highly conserved among the RSV subgroups and functions to promote fusion of the virus and host cell membranes. The major difference between RSV subgroups A and B is the G protein, which is responsible for attachment of RSV to a susceptible cell. Although there is 47 percent amino acid sequence diversity between RSV A and RSV B G proteins, the G protein contains a central conserved domain which is flanked by two hypervariable regions.

Purified F protein (PFP) has been developed as a potential vaccine candidate. PFP-1 is a subunit vaccine that contains 5 to 10 percent of non-F proteins consisting mainly of G protein. PFP-2 contains less than 2 percent of non-F proteins due to purification by ion-exchange chromatography. Both PFP-1 and PFP-2 have been

shown to be safe and immunogenic in studies with 12- to 48-month-old RSV-seropositive children. The efficacy of these vaccines could not be determined because of the small number of children in these studies. These subunit vaccines may be particularly useful in specific groups of high-risk children and adults. A study in children with cystic fibrosis demonstrated that PFP-2 vaccine induced a significant antibody response and a significant reduction in the number of lower respiratory tract illnesses. In addition, recent studies have demonstrated that PFP-2 vaccine is safe and immunogenic in ambulatory adults over 60 years of age and in seropositive children with bronchopulmonary dysplasia.

Maternal immunization using a purified F protein subunit vaccine is a strategy being evaluated to protect infants younger than 6 months of age from RSV disease. The rationale is based on reports of efficient transfer of specific maternal neutralizing antibodies to infants and demonstration of the prophylactic value of high-titer anti-RSV polyclonal antiserum administered to high-risk children. The maternal immunization strategy is based on the following: babies less than 6 months old are at high risk for RSV infection but respond poorly to vaccines; pregnant women respond well immunologically to vaccines; and placental transfer of maternal antibody occurs readily during the third trimester.

A preliminary phase I feasibility study on the use of the purified F glycoprotein in postpartum women was completed in 1993 at Baylor College of Medicine. This study demonstrated that the vaccine was only minimally reactogenic and was highly immunogenic. A second study comparing the safety and immunogenicity of the PFP-2 vaccine with a licensed trivalent inactivated influenza virus vaccine was undertaken at Baylor College in 1994–1995. As with the pilot study, the RSV PFP-2 vaccine was only minimally reactogenic and highly immunogenic in postpartum women and women of childbearing age. Data suggest that this vaccine could potentially provide protective serum antibody to newborn infants. A phase I immunization trial in third trimester pregnant women using PFP-2 will commence shortly.

A subunit approach has also been investigated using the G protein fragment of RSV-A Long strain. A novel recombinant vaccine candidate, BBG2Na, has been developed by fusing the conserved central domain of the G protein (G2Na) of RSV Long strain to BB (the albumin-binding region of streptococcal G protein). Animals were immunized intraperitoneally with BBG2Na and then challenged intranasally with RSV. A protective immune response was demonstrated in early life after murine maternal or neonatal vaccination, and immunized adult mice were protected against homologous and heterologous virus challenge. Another vaccine candidate under development is a synthetic peptide of the conserved region of the G protein. This vaccine is to be administered intranasally, either alone or in combination with cholera toxin (CT) as a mucosal adjuvant. Animals immunized with human derived G protein were protected against RSV challenge only when CT was included, but mice immunized with the bovine derived G protein were protected in the presence or absence of CT.

A live attenuated RSV vaccine that could be delivered to the respiratory mucosa has been the basis of another approach to vaccine development. Intranasal immunization with a live RSV vaccine has the potential to induce both systemic and local immunity and to protect against upper and lower respiratory tract disease. Early attempts at this approach included cold passage, cold adaptation, chemical mutagenesis, temperature-sensitive selection, and various combinations of these methods. Administration of live-attenuated virus preparations has not been associated with enhanced RSV disease upon subsequent natural reinfection. Problems that have impeded progress in this area are overattenuation, underattenuation, and concerns about genetic stability. As a result of recently developed technology, it is now possible to introduce individual mutations into a cDNA clone of RSV and recover infectious virus, thus providing a mechanism to determine the genetic basis for attenuation and construct defined attenuated vaccine viruses with improved genetic stability.

Scientists at the National Institutes of Health and in industry have been working with academicians to evaluate live-attenuated vaccine candidates. RSV strains that were shown to be attenuated and immunogenic in animal models have been tested in human clinical trials. RSV vaccine candidate cpts 248/404 (a cold passaged temperature, sensitive mutant of a human RSV A strain) has been shown to be safe and immunogenic when administered intranasally in a placebo-controlled, randomized, double-blind trial in RSV seropositive and seronegative infants and children. Because some residual nasal congestion was observed in 1- to 2-month-old infants who received this vaccine, work is in progress to genetically engineer this RSV strain to generate a vaccine for this age group that is slightly more attenuated.

The prospects for RSV vaccines are encouraging. Ongoing studies are focused on furthering the understanding of protection and immunopotentiation of RSV disease in order to provide the scientific basis required for the rational design of candidate RSV vaccines. Purified F protein subunit vaccines have been shown to be safe and immunogenic in seropositive children, postpartum women, women of childbearing age, and adults over 60. They have great potential for use in adults and specific groups of high-risk children and for protecting infants via maternal immunization. Different adjuvants are currently being studied to augment immunogenicity. Live-attenuated vaccine candidates have also been shown to be safe and immunogenic. New methods in biotechnology are now available to provide tools for designing vaccines with

defined mutations to achieve desired levels of attenuation that are genetically stable.

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Smallpox

Studies on smallpox immunization have high NIH programmatic relevance because of the growing concern of a bioterrorist threat and the lack of smallpox vaccine optimized for protection of the civilian population. Reports from former Soviet Union scientists indicate that the U.S.S.R. had an extensive smallpox program active into the early 1990s. There is concern that scientists from this program have migrated to other countries and that at least 10 countries now possess stocks of smallpox virus. Any release of smallpox virus would cause a global health emergency. Although a deliberate release is the major concern, an accidental release from a laboratory also poses a threat.

With eradication of the disease in the late 1970s, vaccination of the general population quickly ceased. Eventually all immunization programs in military populations were terminated as well. With no market for their vaccine, the private sector lost interest in the product and showed little interest in producing a next generation vaccine.

The classic smallpox vaccine licensed in the U.S. was prepared from calf lymph. This vaccine was made with 1950s methods and is not a sterile product. It produces significant side effects and is currently contraindicated in such populations as the immune-suppressed, pregnant females, and the very young. Currently available supplies of smallpox vaccine in the U.S. are limited to about 15 million doses, of which it is estimated only 7 million to 10 million immunizations could be performed. The anticipated need to control a U.S.

outbreak is 40 million doses, and the international need is undetermined but substantial.

Federally sponsored research to counter the threat of smallpox is progressing rapidly and will be accelerating in the year ahead. Major research efforts (cofunded by NIAID with CDC, (cofunded by NIAID with CDC and the Departments of Defense and Energy) include the following:

1) Developing and testing at least three antiviral drugs against smallpox and vaccinia viruses;

2) Extending the usefulness of the currently available, older vaccine (by doing human studies to determine whether we can "stretch" available stocks);

3) Developing a safe, sterile smallpox vaccine grown in cell cultures using modern technology;

4) Exploring development of a vaccine that can be used in all segments of the civilian population (i.e., the immune-suppressed, pregnant mothers); and

5) Increasing our knowledge of the genome of smallpox and related viruses.

Streptococcus pneumoniae

Streptococcus pneumoniae, a gram positive bacterium that colonizes the upper respiratory tract of humans, is the most common cause of community-acquired bacterial pneumonia throughout the world. Even before the emergence of antibiotic resistance, the pneumococcus ranked as the primary cause of morbidity and mortality due to respiratory infection. However, with the exception of the distinct polysaccharide capsule that distinguishes each of the 90 serotypes of *S. pneumoniae*, many of the virulence factors remain unknown. The current lack of understanding of the pathogenic mechanisms of the pneumococcus has hindered the development of a widely protective vaccine for the two most susceptible patient populations: children under 2 years of age and adults over 65. This report describes some of the current pneumococcal research projects under development and supported by NIAID.

Pneumococcus is the leading cause of meningitis; many survivors are left with long lasting deficits such as paralysis, seizures, motor deficits, and hearing loss. Investigators have determined that pneumococcal meningitis causes loss of brain cells by initiating an inflammatory response in the cerebrospinal fluid that results in a natural human cell death process called apoptosis. Knowing that this is the mechanism of brain cell death, it may be possible to inhibit this death process and thereby save brain function in survivors of meningitis. This has been demonstrated in an animal model using a broad-spectrum caspase inhibitor (z-VAD-fmk) and suggests that the application of this new agent(s) may be added to the therapy of this disease and, together with antibiotics, may improve clinical outcomes by specifically reducing brain damage.

Because *Streptococcus pneumoniae* is a re-emergent pathogen, there is a great deal of effort to develop pneumococcal vaccines effective among various high-risk groups including young children. Current vaccine development efforts are designed to elicit antibodies to the capsular polysaccharide following exposure to a protein-polysaccharide conjugate vaccine. A recent phase III trial conducted in infants with a 7-valent Wyeth-Lederle pneumococcal conjugate vaccine demonstrated overwhelming efficacy (100 percent) against bacteremia. A small but significant effect was also noted against the occurrence of otitis media. Based on these observations and considerable evidence indicating the safety of the vaccine, it is expected that the vaccine will be licensed by early 2000 for routine use in infants and children in the U.S. This vaccine, along with several other pneumococcal conjugate vaccines now under development, will be some of the most complex vaccines ever developed.

Additional studies in the elderly and immunocompromised groups are currently underway to determine whether these vaccines can replace the conventional 23-valent polysaccharide (PS) vaccine for controlling pneumococcal infection. Once these products are introduced into our vaccine armamentarium, many advantages are anticipated including: 1) the promise of better immunogenicity compared with PS vaccine, especially when two or more doses of conjugate vaccine are administered followed by a dose of PS vaccine; 2) the promise of better immunogenicity against serotypes that are poorly immunogenic in the PS vaccine; 3) the promise of better immunogenicity against virtually all of the serotypes that are associated with antibiotic resistance; 4) the possibility of inducing immunologic memory and protection that lasts longer than that associated with the PS vaccine; 5) the possibility that in at least some immunocompromised individuals, the conjugate will offer better protection than the PS vaccine; and 6) the possibility that by reducing nasopharyngeal carriage of pneumococci, exacerbations of chronic bronchitis and extension of nasopharyngeal colonization to others might be less frequent. Whether any of these potential advantages of conjugate vaccine over PS vaccine will prove to be real can only be determined by continued studies and additional clinical trials.

Pneumococcal surface protein A (PspA), located on the outer membrane of *S. pneumoniae*, has been shown in animal models to elicit protection against pneumococcal infection. To date, this has been the most successful of a group of noncapsular antigens that have been studied in an effort to develop a non-polysaccharide vaccine against pneumococcal infection. Such vaccines are considered important because the existing polysaccharide vaccine is not immunogenic in children less than 2 years of age in whom there are more than1 million pneumococcal

deaths per year worldwide. The polysaccharide vaccine also is not able to prevent 40 percent of the bacteremic pneumococcal pneumonia in adults. Conjugate vaccines with pneumococcal capsular polysaccharides and carrier proteins will undoubtedly improve protection in children and may improve protection in the elderly. Unfortunately, because these vaccines are expected to be very expensive, they may absorb a large fraction of State health department budgets in the U.S. and may be unaffordable in developing countries. The PspA vaccine, and others like it, need to become inexpensive to produce so that they can offer the prospect of providing worldwide protection.

The PspA comes in five major clades that comprise two PspA families. These two families include the PspAs of more than 97 percent of all pneumococci. Immunization with any member of either of these families provides protection in mice against all other pneumococci of the same PspA family. Thus, a vaccine to elicit protection against PspA should not need to contain more than two or three different PspA molecules. Because PspA evolves very slowly, it may not be necessary to reformulate the vaccine with different PspAs very often.

In collaboration with Pasteur-Merieux-Connaught, human safety trials have been conducted with a vaccine containing one family of PspAs. The results indicated that the vaccine was safe and less reactogenic than the licensed capsular polysaccharide vaccine. The results also demonstrated that the vaccine elicited extremely high titers of antibody to PspA. The human antibodies were able to protect mice from fatal pneumococcal infection. Moreover, the concentration of antibody elicited in human serum was more than 1,000 times the concentration required to protect mice from fatal infection. Pasteur-Merieux-Connaught is in the process of carrying out phase II and III studies with a vaccine containing more than one PspA family to determine the antibody dose-response of the vaccine necessary to prevent pneumococcal disease in humans. Followup studies are directed at developing a mucosal immunization technique with PspA and other pneumococcal proteins that will protect against bacteremia and sepsis as well as against nasopharyngeal carriage. If the vaccine can prevent carriage, it may be able to prevent the transmission of pneumococcal disease from healthy carriers to susceptible individuals.

Other studies of methods for boosting immune responsiveness to conjugate vaccines have also progressed during the past year. Data indicate that short DNA molecules that contain the dinucleotide sequence motif, CpG, can enhance certain aspects of the immune response to polysaccharide-CRM_{197} vaccines. Specifically, co-administration of these synthetic CpG-containing DNA molecules (CpG oligodeoxy-nucleotides), but not control oligodeoxynucleotides without CpG motifs, boosted the levels of total, IgG2a, and IgG3 polysaccharide-specific serum antibodies when BALB/c mice were immunized with 6B-CRM_{197} or 19F-CRM_{197}. For the 6B-CRM_{197} vaccine, CpG-containing (but not control) oligodeoxynucleotides also increased the levels of polysaccharide-specific serum antibodies of the IgG1 subclass. Further studies may help determine if these CpG-containing DNA molecules can overcome poor responses elicited by some of the conjugate vaccines.

The 23-valent pneumococcal polysaccharide vaccine was formulated to prevent invasive infection in the elderly and other high-risk populations from the most prevalent *Streptococcus pneumoniae* serotypes. However, the immunogenicity of all 23 vaccine polysaccharides has not been fully characterized in elderly adults. Previous reports indicated that whereas the majority of elderly subjects had vigorous immune responses to selected pneumococcal vaccine polysaccharides, a subset of elderly individuals responded to few of the vaccine serotypes after immunization. To determine whether these elderly low responders have a general inability to respond to pneumococcal vaccine, and to determine whether elderly low responders might be identified by their responses to a few polysaccharides, antibody responses to all 23 vaccine polysaccharides after pneumococcal immunization were measured in elderly adults. As a group, elderly subjects showed a significant rise after immunization in geometric mean antibody levels to all 23 vaccine serotypes. However, when individual rather than group immune responses were assessed, the 23-valent vaccine did not appear to be uniformly immunogenic in these elderly subjects. After vaccination, 11 elderly subjects (20 percent) had twofold increases in specific antibody to only 5 or fewer of the 23 vaccine polysaccharides, and they did not respond to the most prevalent serotypes causing invasive disease. Antibody responses to serotype 9N were found to reliably distinguish low vaccine responders from other elderly subjects. However, no particular group of vaccine polysaccharides could be used as markers for adequate immune responses if only post-vaccination sera were analyzed.

Development and/or improvement of the pneumococcal vaccines would be greatly facilitated with reliable surrogate assays for pneumococcal vaccine efficacy. In recent years, significant progress has been made on improving the surrogate assays for pneumococcal vaccine efficacy. Because of the inadequate specificity of the currently and commonly used ELISA for estimating the concentration of pneumococcal antibodies, efforts are underway to improve and standardize these ELISA assays by collaborating with investigators at the FDA. Several laboratories also are working on improved opsonophagocytic-killing assays that can simultaneously determine the opsonophagocytic titers for two different serotypes by using two strains of *S. pneumoniae* differing in their antibiotic sensitivity. The opsonophagocytic assay is a tedious and insensitive assay, but it is specific and, therefore, it is performed often due to the poor specificity

of the ELISA. The improved assay halves the work required for the conventional opsonophagocytosis assays and reduces the volume of sera needed from young children.

When the pneumococcus enters the lungs or the bloodstream, its ability to produce disease is combated by defenses in the human host. Foremost among these defenses is the third component of complement (C3), a protein that can be found not only in humans and other mammals, but even in primitive creatures like sea urchins. The fact that C3 is so widely conserved throughout nature tells us that its functions are very important for surveillance, targeting, and removal of invading microorganisms. It has been hypothesized that bacteria commonly residing in humans would have developed strategies to elude surveillance by proteins such as C3. Using techniques of column chromatography and molecular biology, researchers have now identified three such proteins that are made by *S. pneumoniae*: a C3-binding protein and two C3-degrading proteinases. The genes encoding these proteins have been completely sequenced and deposited in the NCBI database. The C3-binding protein entraps C3 so that it cannot target the pneumococcus for removal; the C3-degrading proteinases then digest C3 so that its functions are lost. The C3-binding protein is also able to elicit a molecule called interleukin-8 from the delicate cells lining the lungs; interleukin-8 is responsible for triggering the inflammatory response in the lung that results in the symptoms of pneumococcal pneumonia: fever, purulent sputum, and consolidation within the lung tissue. Humans recovering from pneumococcal infection produce antibodies to the C3-binding protein. Studies such as these are helping researchers understand the delicate balance between host and pathogen and are leading to new candidates for pneumococcal vaccines.

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Tetanus

Despite long-established and effective vaccines for tetanus, childhood and neonatal tetanus remain significant worldwide problems. A more simplified approach for immunizing children could greatly facilitate the delivery of these vaccines, decrease the barriers to immunization, and improve immunization rates.

One interesting area that has shown great promise is the use of the skin as a mechanism for the delivery of vaccines. Transcutaneous immunization (TCI) involves the introduction of antigens using a topical application to intact skin. This new technology offers many advantages over parenteral injections, such as eliminating the risk of needle-borne diseases and reducing the complications related to physical skin penetration.

Investigators are developing several novel approaches for the delivery of vaccines via the skin. One approach involves incubating expression vectors with the outer layer of skin in a noninvasive mode. Noninvasive vaccination onto the skin (NIVS) requires no needle injections and no specially trained personnel. These investigations have demonstrated that topical application of an adenovirus vector encoding either the tetanus toxin C-fragment (tet-C) or the influenza A virus hemagglutinin (HA) by using a patch could elicit specific humoral immune responses against either tet-C or HA in both rodents and nonhuman primates. Sub-fragments of antigen DNA can be found in other areas of the skin or in deep tissues after localized gene delivery by a patch. Results suggest that a transient but productive wave of antigen expression within the outer layer of skin may be able to broadcast with pre-exposure to adenovirus can still be vaccinated by adenovirus-mediated NIVS. It is conceivable that anti-adenovirus immunologic components may not be able to reach the surface of the skin in sufficient quantities for counteracting applied vectors. These studies suggest that vaccines may be inoculated by simply applying a “vaccine patch” containing concentrated adenovirus recombinants that encode specific antigens. The patch would be applied to the outer layer of skin, which is both a convenient target site and an immunocompetent area for the delivery of vaccines. The possibility of eliciting specific immune responses after the delivery of noninvasive vaccines provides the impetus for translating patch-based

noninvasive vaccination into routine vaccination programs in a wide variety of clinical settings.

Another approach in the development of this technology involves the use of cholera toxin (CT), that, when applied to the skin surface, acts as an adjuvant for the co-administered antigens diphtheria toxoid and tetanus toxoid. The observation that cholera toxin placed on the skin in a saline solution could induce a potent anti-CT response suggests that CT might act as an adjuvant for co-administered proteins on the skin. Studies are now in progress to determine the optimal dose and concentration of diphtheria and tetanus antigens required for transcutaneous immunization, the optimal ratio of antigen to adjuvant, and to determine whether co-administering various antigens with CT interferes with the immune response to each individual component.

Other novel antigen delivery systems have been developed in recent years including a new technique of antigen encapsulation that renders antigens, formerly ineffective when administered orally, into potent immunogens. This new encapsulation process avoids the use of organic solvents, protects the antigens during their passage through the stomach, and releases the antigens in a "burst" into the small intestine. With the aid of certain excipients, antigen presentation to Peyer's patches in sufficient quantity results in a vigorous immune response that is comparable to that produced by a parenterally administered antigen with an adjuvant such as alum. Numerous successful oral immunization studies have been carried out in mice with a number of antigens encapsulated by this new technique. In atopic humans, an encapsulated allergen (i.e., short ragweed extract) has been administered orally that induces significant immune responses.

Mice given three doses orally of encapsulated tetanus toxoid on days 0, 1, and 2 demonstrated an increase in the anti-tetanus toxoid antibody response after the primary immunization and a significant anamnestic response following a boost with the encapsulated vaccine on days 42, 43, and 44. Ragweed-sensitive patients, who received escalating or maintenance daily doses for up to 8 weeks, responded with a remarkable increase in allergen-specific IgG that was similar to the immune response observed with high-dose, long-term subcutaneously administered allergen. No toxicity was observed among the volunteers.

A phase I/II clinical trial with the encapsulated tetanus toxoid is now in progress in healthy adults with pre-vaccine anti-tetanus antibody titers $\leq$2 IU/ml. The objectives of this study are to show that the encapsulated antigens can elicit a booster response in humans and to compare the immune response following oral immunization to that obtained with standard intramuscular immunization with tetanus toxoid.

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Tuberculosis

Introduction

Mycobacterium tuberculosis, the bacterium that causes tuberculosis (TB), infects approximately 2 billion people worldwide (1 in every 3 persons), and last year killed an estimated 3 million individuals. When one includes deaths due to TB in HIV-positive individuals, TB is responsible for more deaths than any other single infectious agent. This devastating burden of disease continues despite the World Health Organization (WHO) Global TB Programme's substantial efforts to control TB in developing countries through Directly Observed Treatment Short-course (DOTS) and worldwide immunization of neonates by the WHO Expanded Programme of Immunization with BCG (Bacille Calmette-Guérin), a live-attenuated anti-TB vaccine.

M. tuberculosis-infected, immunocompetent individuals have an estimated 10-percent lifetime risk of contracting active TB disease. The majority of these persons will remain latently infected, not demonstrating any active symptoms of disease but exhibiting a positive delayed type hypersensitivity reaction on skin test challenge. HIV-infected and other immunocompromised individuals have a much higher risk of developing active TB disease—estimated at 8 to 10 percent per year. Amplifying the severity of this global problem is the fact that HIV and *M. tuberculosis* infections are synergistic. *M. tuberculosis* can increase the replication rate of HIV, and coinfection with *M. tuberculosis* speeds the progression and severity of AIDS in HIV-infected individuals. In 1996, TB became the leading cause of death in HIV-infected individuals worldwide, responsible for approximately one-third of all AIDS deaths. Conversely, HIV infection may increase susceptibility to primary infection with *M. tuberculosis* and dramatically increases

the risk of *M. tuberculosis*-infected individuals developing active tuberculosis. In parts of Africa and Asia, TB case rates are climbing rapidly as the HIV epidemic spreads. The chairman of the Kenya Association of Physicians reported that the number of TB cases in Kenya increased tenfold in the past decade.

The TB situation is serious not only in the developing world but in the United States and other developed countries as well. Improved control has been achieved overall in the United States since the resurgence of TB in the late 1980s and early 1990s—case rates for the country as a whole have decreased for the past 5 years. Nonetheless, the Centers for Disease Control and Prevention (CDC) reports 19,851 cases of TB in the United States in 1997; up to 15 million people in this country are believed to be currently infected with *M. tuberculosis*. Case rates remained level or increased in 17 States in 1997 compared with 1996. Approximately 38 percent of cases of TB in the United States are now found in foreign-born persons, and this percentage is increasing.

Multiple-drug-resistant TB (MDR-TB) is a major concern in the United States as well as in the rest of the world. CDC reported in September 1997 that whereas overall MDR-TB currently represents 2.2 percent of all the TB in the United States, a strain of MDR-TB that 6 years ago was present in only 13 States has now spread to 42 States. MDR-TB is both difficult and extremely costly to treat, and because treatment is often relatively ineffective, patients remain infectious much longer than do patients receiving proper treatment for drug-susceptible TB. Moreover, as with other forms of TB, MDR-TB can be spread by casual contact. WHO reports the prevalence of MDR-TB to be as high as 14 percent in some parts of eastern Europe. The imperative to control TB better is increased by the knowledge that drug-resistant tuberculosis is created almost entirely by delayed diagnosis and improper or inadequate therapy. Dr. James Musser and colleagues reported that greater than 95 percent of gene variation in *M. tuberculosis* is caused directly by human use of antibiotics.

For many reasons, the development of improved anti-TB vaccines has become a necessity for adequate control and elimination of tuberculosis. These reasons include the spread of MDR-TB, the global burden of the TB epidemic, the growing TB/HIV coepidemic in large areas of the world, the enormous practical barriers to controlling TB adequately through administration of what are complicated and costly treatment regimens, inadequate diagnostic methods, and the relative ineffectiveness of the current BCG vaccines.

BCG Vaccines

BCG was developed at the Institut Pasteur between 1906 and 1919 by serial passage of a mixed culture of *M. bovis* and was first used in humans in 1921. BCG is one of the six basic antigens of the Expanded Programme of Immunization, and Holland and the United States are the only two countries that do not recommend universal BCG vaccination of children. In 1997, more than 100 million infants were immunized with BCG; since 1921, more than 3 billion doses of BCG have been administered worldwide.

A review of the literature, including meta-analyses of BCG vaccination studies, demonstrates varying efficacy for BCG in providing protection against tuberculosis. Against miliary TB and TB meningitis in children, BCG's reported efficacy ranges from 46 to 100 percent. However, against pulmonary TB, which represents the majority of the burden of this disease, BCG's efficacy ranges from 0 to 80 percent.

Numerous factors have been cited as possible explanations for this variability, including methodological differences among the studies; variations among strains of BCG; differences among strains of *M. tuberculosis* in different parts of the world; nutritional factors; environmental factors; varying exposure to environmental, nonpathogenic mycobacteria; and genetic differences in host populations. Most likely, a combination of factors is responsible for the variable efficacies observed, with BCG strain differences and varying exposure to nonpathogenic mycobacteria perhaps playing the largest roles.

A recent report presents evidence that efficacy of a specific strain of BCG decreases as passage number increases, whereas induced delayed-type hypersensitivity (DTH) responses remain strong. Historically, manufacturers have selected for strong DTH responses while attempting to decrease reactogenicity/toxicity of BCGs, believing that DTH responses correlate with protective immune responses. However, more recent evidence suggests that protective immune responses do not necessarily correlate with DTH responses.

Efficacy of BCG vaccination appears to vary with geographic latitude—the farther from the equator, the more efficacious the vaccine. Environmental mycobacteria are more prevalent closer to the equator. Presumably, exposure to nonpathogenic mycobacteria induces a degree of protective immunity in the exposed human populations, masking any potential protection from BCG.

A comparative genomic analysis of strains of BCG, virulent strains of *M. bovis,* and virulent strains of *M. tuberculosis* has revealed 91 open reading frames of H37Rv (a virulent *M. tuberculosis* laboratory strain) that are absent from one or more virulent strains of *M. bovis* and an additional 38 open reading frames that are present in virulent strains of *M. bovis* but absent from some or all of the 13 strains of BCG examined. Further analyses are needed to assess the potential contributions of these genetic differences to the variable efficacy seen in human trials of BCG vaccines.

In addition to providing relative protection against extrapulmonary TB in children, BCGs have demonstrated efficacy against leprosy, another mycobacterial disease. As a result, it is difficult to imagine simply replacing BCG with a potentially better anti-TB vaccine, especially in the context of clinical trials. Rather, in most parts of the world, new, hopefully more efficacious, anti-TB vaccines will have to be tested and administered in addition or subsequent to BCG vaccination.

Status of TB Vaccine Development

Because BCG vaccination is unlikely to have significant impact on the TB epidemic, development of improved anti-TB vaccines is a high priority for NIAID and for the TB research community. In the past few years, numerous laboratories have undertaken the development of potential vaccine candidates, using a range of strategies. These approaches include development of modified BCGs, live-attenuated strains of *M. tuberculosis*, subunit vaccines, and naked DNA vaccines. A large number of candidate vaccines using all these strategies has been developed in academic and industrial laboratories in the past 3 to 5 years.

Evaluation of these candidate vaccines and their prioritization for use in human clinical trials will require investigations in animal models. The major animal models of tuberculosis currently available are based on the mouse, guinea pig, and rabbit. Efforts are also underway to establish useful models of human TB in cynomolgus monkeys and rhesus macaques. NIAID has established a contract-supported service for testing vaccine candidates in the mouse, guinea pig, and rabbit aerosol challenge models.

Modified BCGs

Recombinant DNA methodologies have been used to add expression of putative protective antigen(s) or cytokine(s) to BCG to boost its protective effect. This approach takes advantage of BCG's ability to persist intracellularly in the host, mimicking *M. tuberculosis* infection and its endogenous adjuvanticity. To date, several candidates of this type have shown protective efficacy in mouse or guinea pig models similar to, but not significantly better than, BCG.

BCG has also been modified through the creation of auxotrophic mutants. Currently, in some countries where BCG is otherwise administered universally, HIV-positive individuals do not receive BCG because of the fear of causing disseminated BCGosis in these immunocompromised hosts. It is hoped that vaccines based on auxotrophic strains of BCG or *M. tuberculosis* would be safe even for immunocompromised individuals. Doubly auxotrophic strains are currently being tested for their safety and efficacy in animal challenge models.

Attenuated Strains of M. tuberculosis

The recently completed sequencing of the *M. tuberculosis* genome, the newly developed techniques for *M. tuberculosis* mutagenesis and allelic exchange, and the recent availability of functional analyses in animal models should produce relatively rapid advances in the identification of *M. tuberculosis* virulence determinants. This information could then be used to rationally attenuate *M. tuberculosis* as the basis for vaccine candidates.

Subunit Vaccines

Candidate subunit vaccines consist of only a subset of *M. tuberculosis* antigens rather than the whole bacterium, which could potentially induce a strong protective immune response. These candidate vaccines would be attractive because of their perceived relative safety and potential ease of manufacture. Several investigators are trying to develop TB vaccine candidates of this type based on a variety of "culture filtrate proteins" (CFP)—that is, mycobacterial protein antigens visible to the T cell immune machinery.

Although some investigators are testing the ability of complex CFP preparations containing many proteins to induce a protective immune response in mice and guinea pigs, others are pursuing individual, purified proteins or defined mixtures of a relatively small number of purified proteins. To date, some of the most promising results have been seen in mouse and guinea pig aerosol challenge experiments following vaccination with a preparation of *M. tuberculosis* culture filtrate proteins combined with MPL adjuvant and cytokines interleukin (IL)-2 and IL-12. This combination provided protection at least equivalent to that of BCG in these animal models.

Rather than using whole protein preparations as vaccine candidates, some investigators are trying to identify peptide antigens that could evoke a protective immune response. Current data suggest that combinations of several such peptides will be more effective than any individual peptide. Prediction of major histocompatibility complex (MHC)-binding epitopes is now possible through computer algorithms, and efforts are underway to annotate the completed *M. tuberculosis* genomic sequence using this type of analysis.

A relatively small number of nonprotein antigens are currently under investigation as the basis of potential anti-TB vaccines. The most promising include mycobacterial cell wall mycolic acids and carbohydrate moieties, which are currently being investigated for antigenicity and the ability to induce protection. Elegant studies have recently demonstrated that human CD1 molecules present mycobacterial lipid antigens to cytotoxic T lymphocytes. More extensive work in this area is warranted.

DNA Vaccines

The potential efficacy of DNA vaccination against tuberculosis was first demonstrated by Silva and Lowrie, who showed that immunization of mice with the J774 macrophage cell line transfected with the *M. leprae* HSP60 gene provided protection against intravenous challenge with virulent *M. tuberculosis* and BCG. Direct DNA vaccination of mice, using DNA encoding the major secreted protein, Ag85A, HSP65, or the 36 kDa proline-rich mycobacterial antigen, has also been accomplished and demonstrated to provide some protection against challenge with *M. tuberculosis*. Recently, Lowrie, Silva, and their colleagues have demonstrated that HSP65 DNA vaccines can also have a significant therapeutic effect against established *M. tuberculosis* infections in mice, heightening the efficacy of the immune response and increasing bactericidal activity.

DNA vaccination offers several advantages, including enhanced safety as well as relative ease and low cost of production. Consequently, various approaches are being explored for increasing expression of the encoded antigens in an attempt to increase protective efficacy. These approaches include comparing expression vectors containing different signal sequences, altering codons to optimize codon usage, and mutagenizing potential glycosylation sites. It is believed that N-linked glycosylation of these bacterial antigens may interfere with their normal processing and presentation to the host immune system. Attempts are also being made to enhance their immunogenicity by coexpressing cytokines or altering the adjuvant(s) used.

"Whole genome" approaches to identifying mycobacterial protective antigens are also in progress. One technique pioneered by Stephen Johnston and known as "expression library immunization" attempts to screen all potential open reading frames for those that encode protective antigens using an animal challenge model. Such antigens, once identified, could form the basis of either a protein subunit or DNA-based vaccine.

Nonpathogenic Mycobacteria

Several nonpathogenic mycobacteria have been proposed as potential tuberculosis vaccines, including *M. habana*, *M. microti*, and *M. vaccae*. Most recently, *M. vaccae* has been evaluated as an immunotherapeutic agent in two relatively large human trials: a phase III trial in Durban, South Africa, and an NIAID-supported phase I/II trial in Kampala, Uganda. The data from these trials suggest that *M. vaccae* is not an effective immunotherapeutic for tuberculosis, at least when administered as a single dose in combination with standard antituberculous drug therapy in adults with newly diagnosed pulmonary, drug-sensitive tuberculosis.

Microbial Vectors

M. vaccae and *M. smegmatis* are also being used in vaccine candidates as vectors to overexpress mycobacterial antigens, including the 19 kDa, Ag85, and 45 kDa proteins. Other nonmycobacterial microbes, such as *Salmonella* and *Vaccinia,* are being investigated as potential vectors for overexpressing mycobacterial antigens.

Challenges to Improved TB Vaccine Development

A number of significant challenges face those interested in developing improved TB vaccines. NIAID is working with other U.S. Department of Health and Human Services (DHHS) agencies to develop and implement a national plan for TB vaccine development under the auspices of the office of the Assistant Secretary for Health, Dr. David Satcher. A key element of this plan is the long-term cooperation and collaboration with international partners including WHO, the International Union Against TB and Lung Disease, and national TB control programs in TB-endemic countries.

The past few years have been a time of incredible productivity with respect to laboratory development of potential TB vaccine candidates and in the testing of these candidates in short-term animal models. Additional animal models of persistence and reactivation are under active development. Discussions are underway within the TB research and control communities as to how best to design clinical trials of potential TB vaccines. The challenge for the next few years is to rationally prioritize the plethora of vaccine candidates for human clinical testing and to design the necessary trials and infrastructure.

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Sexually Transmitted Diseases

Overview

The human immunodeficiency virus (HIV) pandemic has focused attention on sexually transmitted diseases (STDs), both because HIV infection is a fatal STD and because other STDs such as chancroid, genital herpes, syphilis, trichomoniasis, gonorrhea, and chlamydial infection have been implicated repeatedly as risk factors for the sexual transmission of HIV. It is now clear that the risk of becoming infected, or infecting others, with HIV is increased substantially if one has an STD. More than 75 studies on the role of STDs in HIV transmission have been conducted; in 15 of these studies, both ulcerative and nonulcerative STDs increased risk of HIV transmission approximately threefold to fivefold, independent of the effect of sexual behavior. Although the risk of transmission of HIV in the genital ulcer diseases appears to be higher than in the discharge diseases, the high prevalence of discharge diseases results in a much higher population-attributable risk. A recent study in Mwanza, Tanzania, demonstrated that syndromic management of discharge diseases in men correlated with a 40 percent decrease in HIV infection. Implementation of programs to control STDs is, therefore, a logical next step in preventing the spread of HIV infection.

The control of STDs also is very important in HIV-infected people since more severe disease symptoms may enhance the infectivity of HIV. Recent studies in Malawi have demonstrated that treatment of gonorrhea coinfection in HIV-infected men significantly decreases HIV shedding in ejaculate. More than 80 reports on the impact of HIV infection on STDs suggest that, at a community level, HIV infection may increase the prevalence of some STDs (e.g., genital ulcers). If coinfection with HIV prolongs or augments the infectiousness of individuals with STDs, and if the same STDs facilitate transmission of HIV, these infections may greatly amplify one another. This "epidemiological synergy" may underpin the explosive growth of the HIV pandemic in some populations.

Apart from the HIV epidemic, STDs cause significant morbidity and mortality, as well as contribute greatly to increasing health care costs. Furthermore, STDs disproportionately affect the female, the fetus, and the newborn. Gonococcal and chlamydial infections cause pelvic inflammatory disease, infertility, and ectopic pregnancy. Several common STDs adversely affect pregnancy and result in spontaneous abortion, stillbirth, chorioamnionitis, premature rupture of membranes, preterm delivery, and postpartum endometritis. Neonatal infections include gonococcal conjunctivitis, which may lead to blindness; chlamydial pneumonia, which may lead to chronic respiratory disease; and herpes encephalitis. Moreover, genital infections attributable to human papillomavirus are causally associated with cervical cancer, the most common cause of cancer-related death in women throughout the world.

Despite recent global efforts in health education aimed at preventing the sexual transmission of HIV, STDs remain hyperendemic in many developing countries and in the inner-city populations of industrialized countries. Throughout the world, the majority of STDs are clustered in the resource-limited settings of urban and peri-urban areas where increasing numbers of adolescents and young adults, poverty, unemployment, lack of education, inferior status of women, and social disintegration fuel the epidemic spread of STDs.

A consensus has emerged that the prevention of sexually transmitted HIV infection and the prevention of the major sequelae of STDs in women and infants mandate a global initiative for the prevention and control of STDs. Among other things, this initiative will depend on the development of safe, effective vaccines that prevent infection, disease, and/or sequelae. Currently, except for hepatitis B virus (HBV) infection, no such vaccines exist.

Gonorrhea

Microbial Strategy: As an obligate pathogen of humans, the gonococcus has evolved to effectively avoid, subvert, or ignore the immunodominant host response using strategies that include:

- Phase variation: the ability to turn on or off the synthesis of a surface antigen, e.g., pili and opacity protein (Opa);
- Antigenic variation: the ability to synthesize a particular antigen from a large repertoire of antigenic types, e.g., pili, Opa, and lipopolysaccharide (LPS);
- Surface microheterogeneity: the ability to vary surface immunoaccessibility of antigens among organisms within a population; this appears to be characteristic of all gonococcal surface antigens;
- Elicitation of immunodominant responses that are not protective: the ability to present surface immunogens, the response to which is not protective, i.e., the generation of antibodies to the reduction modifiable protein (Rmp) that block the bactericidal activities of other antibodies; and
- Enzymatic alteration (sialylation) of gonococcal LPS by the host's enzymes; correlates with resistance to serum bactericidal activity.

Vaccine Strategy: Taking these observations into account, it is very likely that multiple immunogens will be included in an effective vaccine. Furthermore, protective immunogens undoubtedly will have one or more of the following characteristics:

- Be microbiologically essential throughout the life cycle, i.e., be expressed constitutively;
- Be phenotypically invariant within and between strains;
- Elicit bactericidal and/or opsonic antibodies;
- Induce functional immunity at the mucosal surface of the reproductive tract; and
- Not be contaminated with Rmp.

Several stable surface components have been identified. Genes for both types of the outer membrane porin protein (Por) have been cloned and sequenced, and common epitopes have been identified. Some epitopes elicit bactericidal antibodies. Chimeric genes encoding combinations of these epitopes have been expressed in *Escherichia coli*; such constructs are potential recombinant vaccines. Corresponding synthetic peptides also are being used as immunogens.

In wild-type strains, Rmp copurifies with Por; these heterogeneous antigenic preparations have elicited Rmp-specific blocking antibodies that interfere with the activity of Por-specific bactericidal antibodies. To circumvent this problem, the Por protein has been purified from genetically engineered mutants that lack the *rmp* gene. Purified Por has been incorporated into liposomes and screened for immunogenicity and protection in animal models. These preparations elicit bactericidal and opsonic antibodies. In 1998, phase I clinical trials using purified Por antigen began in the STD Clinical Trials Unit .

Three other components have been shown to be potential vaccine candidates on the basis of surface exposure, common epitopes, and the bactericidal action of antibodies directed at these targets. These are iron-binding proteins, proteins expressed anaerobically, and a lipoprotein unique to the pathogenic Neisseria, the H.8 antigen.

Recently, encouraging results have been achieved using a gamma-irradiated whole-cell vaccine as an immunogen. By combining parenteral priming with oral immunization, bactericidal activity, which is not complement-dependent, has been obtained. Vaccinated mice become very resistant to gonococcal infection when one of two animal models—the subcutaneous chamber model in mice or the estrogen-primed vaginal model in mice—is used. Recent studies have revealed that the basis for immunity is an antigen-induced peptide, similar to the antibiotic peptides, defensins, that have been described in other systems.

Studies on the protective function of LPS-induced anti-idiotype antibodies are under way. A conserved oligosaccharide epitope, expressed both *in vitro* and *in vivo,* has been identified. This epitope is not similar to the common gonococcal LPS/red blood cell antigen. When rabbits and mice are immunized with the anti-idiotype to this epitope, serum bactericidal antibodies are elicited.

Using the human challenge model to study the pathogenesis of urethritis, investigators have determined that mutants lacking the transferrin receptor are unable to establish infection, suggesting that this protein might be an effective vaccine candidate. In this model system it has recently been demonstrated that volunteers reexposed to the same strain of *N. gonorrhoeae* are susceptible to reinfection, indicating that short-term exposure, similar to disease in a symptomatic male treated shortly after infection, does not confer immunity.

Chlamydial Infection

Progress toward vaccine development has been aided by the realization that there are two types of immune responses to chlamydial antigens; one is protective (in an *in vitro* model) whereas the other, a deleterious component, is probably an integral part of the development of scarring, the hallmark of chronic chlamydial disease (demonstrated in a guinea pig model). Work is progressing on the molecular basis of these two responses; current efforts reflect the hypothesis that the chlamydial major outer membrane protein (MOMP) elicits neutralizing antibodies, whereas the heat shock protein elicits antibodies that correlate with—or perhaps mediate—scarring and chronic disease. Women with high titers of serum antibody to the chlamydial heat shock protein are at substantially higher risk for acute pelvic inflammatory disease if infected with *C. trachomatis.* Furthermore, HLA class II alleles DQA*0401/DQB*0402 are associated with high titers of antibodies to the heat shock protein, suggesting that host genetics may influence the development of chronic sequelae. Another heat shock protein, hsp10, has been associated with delayed hypersensitivity in the primate model.

Much attention is directed at developing strategies that will selectively stimulate a protective immune response. Antibodies to MOMP have been shown to block binding of infectious particles to host cells and to protect mice from death following injection of viable chlamydia. The MOMP genes from several serovars have been isolated and sequenced; a number of common regions and variable regions have been identified. Recently, vaccine experiments in monkeys, using a chimeric peptide containing both B- and T-cell epitopes of MOMP, have demonstrated production of neutralizing chlamydia-specific antisera. However, in a murine model of chlamydial infection by a human strain, an anti-idiotypic antibody provided protection against challenge with the organism; protective immunity to chlamydial infection was associated with antibodies targeted to exoglycolipid antibody but not to antibodies targeted to MOMP antigen.

Current efforts are focused on eliciting a protective mucosal immune response. Strategies include delivery

of antigen by alternative routes and alteration of vaccine formulations. Manipulation of lymphocyte trafficking is also being pursued as a creative approach to invoking mucosal responses with parenteral immunization.

Genital Herpes

Genital herpes is caused by herpes simplex viruses 2 and 1 (HSV-2 and HSV-1). It is estimated that between 40 and 60 million Americans are infected. Each year in the United States, there are 500,000 new infections and 10 million recurrences. As with other herpesvirus infections that involve the nervous system, the lesions are often extremely painful, and the psychological trauma and depression are often serious consequences for infected adults. The disease is insidious in that 80 percent of infected people have either no or mild clinical symptoms and are unaware of their infection; yet, they can reactivate and transmit the virus. Recent studies have demonstrated that people shed virus up to 20 percent of the days of each month. There are two fatal consequences of genital herpes: the transmission of infection to neonates at delivery and the acquisition of HIV infection, as discussed earlier. In neonates, unlike adults, infection is generally symptomatic and severe. Affected infants (approximately 1,500 in the United States each year) have almost a 50-percent risk of death or severe, permanent neurologic damage.

Herpes simplex virus vaccines have been refined dramatically since the 1920s, when patients were injected with untreated vesicular fluid. Recently, vaccines consisting of recombinant protein subunits, plasmid DNA, replication-defective viruses, and novel adjuvants have been used in human trials; several approaches are in development. No herpes vaccines are yet licensed for use in humans. The need is urgent; in the 17 years since HIV prevention efforts began, HSV-2 seroconversion rates, now estimated at 2 million annually in the United States, have increased 70 percent. Of the 2 million incident infections, 600,000 manifest as clinical disease. Transmission of HSV is probably driven by the large reservoir of asymptomatic carriers who frequently shed infectious virions. It is estimated that 40 to 60 million Americans are infected.

Until recently, patients were enrolled in two phase III trials of herpes simplex vaccines. Both trials were designed to test the efficacy of recombinant subunit vaccines, one developed by Chiron containing two glycoproteins, and the other by SmithKline Beecham containing one glycoprotein. Both vaccines consist of viral coat proteins produced in a recombinant bacterial system, together with an adjuvant. The vaccines utilize two different adjuvants—Chiron uses MF59, whereas SmithKline uses monophosphoryl lipid A immunostimulant (MPL), a bacterial product obtained in partnership with Ribi ImmunoChem Research. Chiron halted its trial because the results seen in phase I and II trials were not replicated in the preliminary results of the phase III trial. SmithKline continues to enroll patients, in the hope that the single glycoprotein and the cell wall adjuvant MPL will lead to an improvement in efficacy. The SmithKline vaccine actually employs several approaches to enhancing immunogenicity—the recombinant protein is first precipitated on alum, then suspended in an oil-in-water emulsion containing MPL. The results of the SmithKline trial were released in 1998.

The newer-generation vaccines, which often involve the introduction of engineered DNA into the human body, are beginning clinical trials. Some of these use traditional viral vectors, while others, termed DNA vaccines, employ naked plasmid DNA. Furthest along of the early investigational vaccines is the DISC (disabled infectious single cycle) herpesvirus vaccine, which is being developed by Cantab Pharmaceuticals. Subjects participating in the phase I trials are vaccinated with replication disabled HSV-2 virions that are indistinguishable from wild-type virus except for one protein. The difference is that the gene for glycoprotein H (gH), necessary for viral entry into the cell, has been deleted from the virus' genome. To compensate for this deficiency in the first replication cycle, the disabled virus is provided with the missing protein by growing it in monkey kidney cells transfected with the missing gene. The virus takes along only the protein product, not the gene for producing it, which remains in the kidney cells. The DISC herpes vaccine is being tested in the United States and the United Kingdom. This phase I study will form the basis for Cantab's therapeutic vaccine trial, and the results of this trial are expected this year. In phase II, the prophylactic efficacy of the vaccine will probably be tested in seronegative partners of discordant couples, at risk of contracting genital herpes.

A DNA vaccine made by Apollon began phase I trials in September 1996. A total of 40 healthy HSV-2-seronegative volunteers—including 20 who are seropositive for HSV-1 and 20 seronegative—will be enrolled in this ongoing double-blind controlled trial, designed to determine safety and begin to measure immunogenicity. Further clinical trials, this time in HSV-2-seropositive individuals, are soon to be conducted at the University of Washington. The vaccine consists of plasmid DNA, which encodes glycoprotein D2 (gD2). It contains no adjuvant, but a lipid is included as a "facilitator" to increase uptake of plasmid DNA into the vaccinee's muscle cells. Like gH, gD2 is necessary for viral entry into cells and is known to be one of the more immunogenic of the 75 gene products produced in the herpes-infected cell.

Virus Research Institute is currently developing a vaccine based on a replication-defective mutant of HSV-2, 5BlaZ, which contains an ICP8 gene mutation. Investigators at Harvard Medical School, originators of this vaccine strain, have recently shown that immunization with this mutant virus protects guinea pigs from primary as

well as recurrent disease following challenge with virulent HSV-2.

Pharmadigm, Inc., has developed a proprietary DNA vaccine construct containing a muscle-specific promoter and the gD2 gene. They have made a vaccine using this construct and 1,25-dihy- droxyvitamin D (1,25-D3) as an adjuvant. In rodent models, the plasmid induces production of gD2 in muscle cells. Pharmadigm has found, using a mouse model of primary HSV-2 infection, that when 1,25-D3 is injected along with the recombinant plasmid, the combination enhances protection from severe disease. The company also plans to experiment with dihydroepiandrosterone (DHEA) as an adjuvant; this is a precursor to many androgens and has been shown to restore antigenic responsiveness to stimulated senile immunocytes. Another innovation in the Pharmadigm DNA vaccine is the choice of plasmid. Researchers there achieved controlled expression of the gD2 gene with a muscle-specific promoter activated by myoD, a protein expressed preferentially in myotubes. It is hoped that this will alleviate the concern that nonspecific viral promoters might activate systemic tumor-inducing genes or other adverse genes. The company is currently modifying its plasmid to align with Food and Drug Administration regulations and is looking for corporate sponsors to help bring its technology to human trials.

Researchers at the Children's Hospital Medical Center in Cincinnati, Ohio, have evaluated a plasmid DNA vaccine produced by Vical. Their vaccine, which so far has only been tested in guinea pigs, uses an immediate-early cytomegalovirus gene promoter to express gD2. No component of this vaccine is separately classified as an adjuvant. However, it is believed that the plasmid DNA itself may induce a more rigorous response to the foreign gene product.

Scientists working at Cel-Sci and collaborators at Northeastern Ohio Universities College of Medicine have developed a group of peptides that theoretically work to preferentially stimulate cellular immunity, the immune response that is considered most likely to combat HSV effectively. The vaccine will make use of Cel-Sci's new heteroconjugate technology, in which small disease-associated peptides will be linked to T-cell binding ligands. The ligand theoretically presents the peptide to certain classes of T cells to induce specific immunity to fight herpes infections. The ultimate goal will be to develop a vaccine that protects the individual without the potential dangers of an attenuated virus or a DNA vaccine. The group is now using mice as a model to test its first batch of HSV-peptide heteroconjugate candidates.

Genital Warts and Cervical Cancer

In the United States, it is estimated that between 28 million and 40 million people are infected with human papillomavirus (HPV). HPV genital infections are associated with anogenital cancer, in particular with cervical cancer, one of the most common causes of cancer-associated death in women in the developing world and a cancer that kills 4,800 American women annually. Routine Pap smear screening is widely credited with reducing cervical carcinoma from the number one to the number eight cause of cancer death in American women, but the costs of providing Pap screening are considerable (estimated at $6 billion annually for American women). In addition, adequate screening is not available for all women, even in the United States. In developing countries, in the absence of screening programs, cervical cancer causes 250,000 cancer deaths in women each year.

The prevalence of HPV infection among sexually active women may range from 18 to 25 percent, especially in some populations of sexually active teenagers. Although most of these infections will not progress to cancer, many will cause cervical abnormalities. In each case, however, these women may transmit HPV to their partners or to their babies. In the neonate, HPV infection infrequently leads to warts in the oral cavity and upper respiratory tract. In HIV-infected immunocompromised adults, HPV infection appears to cause severe and rapidly progressing disease. This reinforces the evidence-based belief that the immune system plays a key role in ameliorating disease, if not preventing infection.

The disease rate for genital warts, also caused by HPV, is estimated to be 1 million Americans per year. Genital warts are sometimes difficult to treat; current treatment modalities (freezing, burning, and laser surgery) are associated with a 20- to 50-percent recurrence rate.

Vaccines against HPV—both the high-risk strains commonly associated with cervical cancer and the low-risk strains associated with genital warts—are a priority for a number of pharmaceutical and biotechnology firms. Two companies currently conducting clinical trials for these vaccines are Cantab and MedImmune. Merck has produced a vaccine that entered human trials in 1998. Apollon is also developing an HPV vaccine but has not begun clinical trials.

Cantab is developing three vaccines for the treatment or prevention of HPV-related disease. TA-HPV, for immunotherapy of cervical cancer, and TA-GW, for immunotherapy of genital warts, have demonstrated safety and immunogenicity in phase I and II clinical trials. TA-CIN, for the treatment of patients with cervical dysplasia, is in preclinical development.

TA-HPV is a live recombinant vaccinia virus engineered to express the E6 and E7 genes from HPV types 16 and 18, the principal viruses associated with cervical cancer. E6 and E7 proteins are believed to be involved in transformation of HPV-infected cells. The results of a phase I/II clinical trial of TA-HPV, conducted at the

University of Wales College of Medicine, Cardiff, U.K., and published in a June 1996 issue of *Lancet* were encouraging. The study, which successfully demonstrated that the vaccine causes no significant side effects, is paving the way for future studies of the vaccine's clinical efficacy. The clinical efficacy of TA-HPV could not be determined in the initial trial because the study group was too small and involved only patients with advanced disease. A followup trial using multiple doses on patients with less advanced disease has begun.

TA-GW is undergoing clinical trials in males with genital warts. TA-GW is a recombinant fusion protein made up of the L2 and E7 proteins of HPV type 6, produced in *Escherichia coli* and formulated onto alhydrogel adjuvant. There is marked homology between the proteins of HPV types 6 and 11, which may lead to immune cross-reactivity against type 11. (HPV types 6 and 11 are the principal viruses associated with genital warts and laryngeal papillomatosis.) The L2 protein makes up around 5 percent of HPV's viral coat. The vaccine has been shown to induce a serum IgG response in humans, as well as cellular immune responses, demonstrated by *in vitro* lymphocyte proliferation responses.

In December 1996, Cantab announced the conclusion of a phase IIa open-label trial using the TA-GW vaccine. The vaccine was used in 27 male patients, 16 with recurrent and 11 with new genital warts. The vaccine was given in three intramuscular injections at 0, 7, and 28 days. At week 8, six patients showed complete clearance. Of the 15 patients followed after week 8, 13 showed complete clearance of warts. So far, none of the patients who cleared the warts have relapsed. (In contrast, patients with genital warts have a 20- to 50-percent chance of recurrence after treatment with existing therapies.) Two other phase IIa trials of the vaccine—one using TA-GW in conjunction with cryotherapy and the other using the vaccine to treat laryngeal papillomatosis—are ongoing.

In July 1996, Cantab entered into a collaboration with SmithKline for the continued development and marketing of its therapeutic TA-GW vaccine for genital warts. The first SmithKline TA-GW-derived vaccine is under development, but the adjuvant has yet to be selected.

MedImmune's prophylactic vaccine for genital warts, MEDI-501, takes a different approach to vaccine development. The vaccine consists of recombinant HPV-11 L1 protein with an alum adjuvant. Recombinant L1 has the useful property of self-assembling into virus-like particles. This property is exhibited by the protein used in hepatitis B vaccine as well. Virus-like particles contain no viral DNA and are noninfectious, but when viewed through an electron microscope, they look very much like virus particles. More importantly, the particles stimulate production of antibodies that bind and neutralize infectious virus. In a *Proceedings of the National Academy of Sciences* paper published in December 1995, MedImmune investigators described a similar vaccine's performance in beagles. All seven beagles immunized with a vaccine made from canine oral papillomavirus (COPV) L1 protein were protected when exposed to a wart homogenate applied to excoriated buccal mucosa. Control animals injected with detergent-denatured L1 protein were not protected, indicating that the higher-order structure of L1 is important for protection.

MedImmune began phase I trials of MEDI-501 on February 3, 1997. By using healthy volunteers, MedImmune hopes to establish the vaccine's safety and immunogenicity in this year-long, placebo-controlled, dose-escalating trial.

Merck is also preparing a vaccine for clinical trials to prevent genital warts and cervical cancer. Like MedImmune's product, this vaccine uses recombinant proteins that self-assemble into virus-like particles. Merck is working with CSL (Australia) on a quadrivalent vaccine for HPV types 6, 11, 16, and 18—this is the only vaccine that uses recombinant protein from so many different strains of HPV. Preliminary experiments have found that recombinant L1 produced in the yeast *Saccharomyces cerevisiae* formed virus-like particles and, when formulated as a vaccine with an alum adjuvant, the virus-like particles efficiently protected rabbits from challenge with cottontail rabbit papillomavirus.

A group of investigators at the National Cancer Institute has entered into an agreement with the National Institute of Allergy and Infectious Diseases for phase I safety testing of an HPV virus-like particle L1 vaccine. Early results in preclinical animal models have been promising.

Investigators at Johns Hopkins University in Baltimore have developed an entirely distinct approach to a therapeutic vaccine for HPV-derived cervical cancer. They have engineered a vaccinia virus construct containing the transforming proteins of the oncogenic strains of HPV. The construct also contains a molecular signal that routes this protein antigen to the intracellular pathway that generates tumor immunity. These investigators have entered into an agreement with Pasteur Merieux Connaught for the development of vaccines based on this technology.

Merck is also developing an HPV vaccine using an entirely different technology—the naked plasmid DNA. In DNA vaccines, sequences from the viral genome are spliced into a plasmid that controls their expression. Efforts at creating a plasmid DNA vaccine for HPV are still in the preclinical stages. It is thought that delivery of a combination of capsid proteins as plasmid DNA would simplify the preparation of a multivalent HPV vaccine.

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Viral Hepatitis

Overview

Five distinct viruses are the known etiologic agents for hepatitis leading to fatigue, jaundice, liver damage, and in chronic cases, cirrhosis and even liver cancer. Hepatitis A is transmitted fecal-orally, and outbreaks of this acute infectious agent are common at day-care centers, nursing homes, and restaurants where inappropriate food handling might occur. Hepatitis B and C viruses are both blood-borne agents and both may cause chronic diseases. The infections produced by hepatitis B virus are more likely to be symptomatic and more likely to resolve spontaneously. The infections produced by hepatitis C virus have a high chronicity rate at all ages, and are far more likely to be asymptomatic despite ongoing liver disease. Until recently, the only licensed therapy for either hepatitis B or hepatitis C was interferon-alpha, which has low success rates for both diseases. Infection with hepatitis D virus is dependent on coinfection with hepatitis B virus and may lead to life-threatening superinfections. Hepatitis E virus, like hepatitis A virus, is transmitted via the fecal-oral route and produces an acute illness associated with a high mortality rate in pregnant women. Hepatitis E is reported primarily in developing countries; however, the CDC has determined by serologic screenings that more than 1 percent of the U.S. population has been exposed to hepatitis E.

In addition to these agents, there are some other viruses associated with hepatitis that have recently been identified. Hepatitis G virus (or GBV-C) is a flavivirus, related to hepatitis C virus, which is also a blood-borne agent that produces chronic carriage, but to date it has not been associated with a specific disease. Another similar virus, TTV, is found in about 7 percent of healthy blood as well as in patients with hepatitis as a coinfection. It is assumed to be transmitted via the fecal-oral route and has yet to be associated with a specific disease. An additional virus—S.E.N.-V—has recently been isolated from a patient with AIDS and does appear to produce hepatitis.

Several notable advances were recently made in the structural biology of hepatitis viruses. The structure of the core protein of hepatitis B, a molecule that does not

crystallize and was, therefore, incapable of being studied by x-ray crystallography, was determined by electron cryomicroscopy. Two other groups have published reports on the x-ray structure of the hepatitis C nonstructural NS3 protease, which is important for viral replication and is a target for antivirals. At least five companies are presently working on inhibitors for the hepatitis C virus protease. Finally, researchers identified the structure of the helicase of hepatitis C, an enzyme that is needed to uncoil the viral RNA and allow it to make a copy of itself for reproduction.

Recently, new therapies have been licensed for the treatment of chronic viral hepatitis—lamivudine (3TC, Glaxo Wellcome) for chronic hepatitis B and Rebetron™ (combination therapy of interferon alpha 2a and ribavirin, Schering Plough) for chronic hepatitis C. Recent studies in Japan and Italy demonstrated that half of the patients with hepatitis C-associated chronic liver disease also had a "silent" hepatitis B infection (lacking detectable surface antigen). These patients were much less likely to respond to interferon therapy and more likely to develop cirrhosis. Hepatitis C patients may do well receiving HCV-infected liver transplants of less-pathogenic genotypes. A novel tissue culture hepatitis C model, replicating only the nonstructural proteins in high numbers, may allow testing of targeted antivirals. The transgenic potato hepatitis B vaccine is in clinical trials. Due to possible associated adverse events, the safety of licensed hepatitis B vaccines for neonates is also under scrutiny. Meanwhile, the recent identification of a new hepatitis virus may explain remaining non A-E,G-chronic infections.

Hepatitis A

Hepatitis A virus (HAV) accounts for about 55 percent of acute hepatitis cases in the United States, with the highest incidence in the Southwest. There are approximately 132,000 cases per year, with elevated rates among American Indians, Hispanics, people of low socioeconomic levels, and those practicing risky lifestyle behaviors. Rates in males are 20 percent higher than in females, and prevalence of exposure (antibody to HAV) ranges from 11 percent in persons under 5 years of age to 74 percent in persons over 50 years of age. Most of the symptomatic disease is seen in 10- to 30-year-old patients. Person-to-person contact or sexual contact with a person infected with HAV accounts for most transmissions, but there is a viremic phase during acute infections when blood-borne transmission is possible. Asymptomatic infection is common below the age of 2 but becomes less common with increasing age. Fulminant disease may be fatal and accounts for 70 to 80 deaths per year among those between the ages of 30 and 49. Work-loss costs associated with acute HAV infection in the United States are $200 million (1991) each year.

Natural immunity levels in the United States have undergone a significant decline since 1980 and are currently in the 21- to 33-percent range. Two formalin-killed, licensed HAV vaccines are available for adults and children over 2 years of age—Havrix (SmithKline Beecham Pharmaceuticals) and Vaqta (Merck & Co.). Both Havrix and Vaqta contain inactivated viral particles (HM175 and CR326F strains, respectively) produced in infected human diploid fibroblasts. There are other inactivated hepatitis A vaccines that are not currently licensed in the United States. In addition, combination HAV and HBV vaccines are being studied in clinical trials.

As outbreaks of hepatitis A are transient and sporadic, recommendations for universal vaccination in the United States have not been instituted. The costs of hepatitis A vaccine are not covered by many health insurance policies, and the injections are expensive. Efforts are ongoing to create an attenuated live vaccine that would be cheaper and easier to administer, but so far no candidate is on the horizon nor is the demand necessarily that pressing. Immune serum globulin can be used within 2 weeks of exposure to prevent acute infection; however, timely recognition of exposure is critical. Not only is immune serum globulin much less expensive than the vaccine, but, in addition, immune serum globulin can be administered immediately before a trip lasting up to 3 months. Immune serum globulin can also be administered at the same time as the vaccine for additional protection. The hepatitis A vaccine is recommended for those already infected with other hepatitis viruses.

Hepatitis B

Hepatitis B virus (HBV) infections kill 4,000 to 5,000 Americans each year and 1 million people worldwide. Approximately 300 million people have chronic hepatitis B infections worldwide, with endemic areas primarily in Asia and Africa. HBV is highly contagious (100 times more contagious than HIV) and, like hepatitis A virus, is capable of producing fulminant disease. It is highly transmissible from HBV-positive mothers to their newborns. About 25 percent of infected adults become chronic carriers, and 20 percent of those patients develop cirrhosis or liver cancer. Perinatal infection of infants has a much higher chronicity rate of 70 percent resulting in a higher rate of subsequent cirrhosis and liver cancer. Each year, an estimated 20,000 infants are born to hepatitis B surface antigen-positive women in the United States. During the 1990s, from 200,000 to 300,000 new HBV infections were reported in the United States annually. Annually, hepatitis B accounts for 60,000 hospitalizations and 5,000 deaths for a total yearly cost of $800 million per year, excluding the cost of transplantation for end-stage liver disease. It is estimated that there are 1 million to 1.25 million chronic carriers of HBV in the United States.

HBV vaccines were first introduced in the early 1980s as either heat-inactivated or chemically inactivated small envelope viral (S) particles derived from chronic hepatitis B plasma. One vaccine, Heptavax, was licensed by

Merck, Sharp, & Dohme in 1981. Subsequently two recombinant vaccines were licensed and used: Recombivax HB (Merck & Co.) and Engerix-B (SmithKline Beecham). New recombinant vaccines containing both preS and S antigens (Hepagene, Medeva) are being developed with potentially increased immunogenicity in persons for whom the currently licensed recombinant vaccines are not effective. Other candidates still under development include a *Salmonella*-vectored vaccine and DNA vaccines that may be more efficient at inducing cytotoxic T cells as well as neutralizing antibodies. Transgenic potatoes genetically engineered to express the hepatitis B surface antigen have recently been demonstrated to produce mucosal immunity and systemic immunity when fed to laboratory mice. A clinical trial is ongoing at Roswell Park Cancer Institute to test this oral vaccine's ability to boost immunogenicity in previously vaccinated medical personnel in whom currently licensed recombinant vaccines were not effective.

Universal infant immunization is highly effective as demonstrated in Japan and most recently in Taiwan where the overall prevalence rate among children from 1 to 10 years of age has decreased from 9.8 percent in 1984 to 1.3 percent in 1994. Cancer incidence in 6- to 9-year-olds also dropped from 0.52 percent to 0.13 percent in the same report. A study of high-risk Taiwanese infants demonstrated that antibody levels remained high for at least 10 years suggesting that booster doses would not be needed. Although recently initiated, the beneficial effects of universal infant immunization in the United States are beginning to appear. An added benefit of universal immunization against hepatitis B is the prevention of coinfection by hepatitis D.

With increasing use of HBV vaccine there are increasing reports of adverse events temporally associated with vaccination; however, a causal relationship for most of these adverse events has yet to be established. Concerns about vaccine safety led to recent congressional hearings. In July, 1999, the Association of American Physicians and Surgeons (AAPS) called for an immediate moratorium on mandatory hepatitis B vaccines for schoolchildren pending further research on serious side effects. In the same month, the American Academy of Pediatrics issued precautionary recommendations to delay initiation of hepatitis B vaccination in healthy newborns due to possible ill effects of early exposure to the common vaccine preservative thimerosol.

Lamivudine now joins Interferon-alpha as a licensed antiviral for the treatment of hepatitis B infections in the United States. Other promising antiviral candidates currently in clinical trials include: BMS200475 (Bristol Myers Squibb), adevofir dipivoxil (Gilead), and L-FMAU (Triangle). Short-term therapy with lamivudine is associated with liver flare-ups and the return of hepatitis after cessation of treatment. Prolonged therapy with lamivudine may lead to the formation of escape mutants, but continued therapy has been shown to arrest liver fibrosis, prevent cancer, and, in at least one report, produce viral resolution in a relatively large proportion of patients after 3 years. Like HIV, drug cocktails targeting different viral mechanisms may be needed. L-FMAU, an agent developed by a NIAID grantee and presently being tested in a lifetime study in the NIAID-contracted woodchuck colony, has reduced viremia to levels detectable only by highly sensitive means, and unlike other nucleoside analogs tested, these low levels of viremia persisted for extended periods after treatment had been discontinued. Therapeutic vaccines such as Theradigm™, which induce a cytotoxic T lymphocyte response in patients with chronic infections, are still being developed. A recently published pilot study showed that administration of the current HBV vaccine to persons with chronically replicating HBV reduced viral replication and allowed for the induction of T-cell responses to the HBV envelope antigen.

Hepatitis C

Infection with hepatitis C virus (HCV) accounts for about 12 percent of the acute viral hepatitis in the United States. Approximately 35,000 cases occur annually (declining over the past decade from 180,000), with about 85 percent of those infected becoming chronic carriers at a total yearly cost of $600 million excluding transplants. Most carriers are asymptomatic. Many cases of hepatitis C can be attributed to the 1 million blood transfusions administered before 1990 from which an estimated 290,000 Americans became infected. A look-back study, expected to be completed by 2001, is currently alerting these patients to the potential risk. Most cases of HCV infection occur among young adults (especially injecting drug users), although among adults over 40 years of age, HCV is often the most common cause of acute hepatitis. Sexual transmission may account for as many as 20 percent of the cases. No risk factor can be identified for 10 to 30 percent of HCV carriers. Each year there are 8,000 to 10,000 deaths and 1,000 transplantations due to HCV infections. The current estimate, based on random serologic screenings of more than 21,000 serum samples, is that 3.9 million Americans are chronically infected with HCV—1.8 percent of the population, with higher rates in African Americans (8 to 10 percent) who are also more refractory to current therapies. Hemodialysis patients and hemophiliacs are at exceptionally high risk, and noninvasive person-to-person transmission has been documented. The World Health Organization estimates that 3 percent of the world's population has been infected and that there are 170 million chronic carriers at risk of developing liver cirrhosis and/or liver cancer.

Several investigators have reported a relatively high efficiency vertical transmission of HCV from mothers who were coinfected with HIV. On the other hand, some major studies in the United States and Europe have failed to

demonstrate transmission from HCV-positive mothers while others have provided compelling evidence that transmission occurs. Risk factors for transmission, which is assumed to occur *in utero,* include a high HCV RNA level in the mother and the presence of specific HCV variants. Results of a recent study of infants born to HCV-infected mothers demonstrated biochemical features of liver damage (ALT abnormalities) during the first 12 months of life, although HCV-associated liver disease is likely to be mild throughout infancy and childhood. Multivariate analyses of risk factors for cirrhosis and/or liver cancer with HCV infections demonstrated that increased age, male gender, and excessive alcohol consumption were all important factors. Additional risk factors for cancer were hepatitis B antibody positivity and HCV genotype. There was no relationship between the development of liver cancer and serum HCV levels.

The recently-licensed combination therapy of interferon alpha and ribavirin (Rebetron, Schering Plough) has demonstrated a considerable increase in disease resolution of HCV-infected patients compared to therapy with interferon alone. New therapies in early stages of testing include pegylated interferons (PEG-IFN, Hoffmann-La Roche)(PEG-Intron, Schering Plough), consensus interferon (Infergen, Amgen Inc.), and a polyclonal HCV antibody (Cicivir, Nabi). In addition, clinical studies are being performed of Interferon alpha at higher dosages, or more frequent dosages, and longer duration at therapy with some beneficial effects. Still lacking an infection tissue culture model, a group in Germany has produced a tissue culture model replicating non-integrated nonstructural viral proteins. If this model holds up, it should prove very valuable in screening targeted sites on non-structural proteins such as the protease and helicase. Consensus clones of the two most common and most virulent strains (1a and 1b) have been generated. However, to date only the chimp model is susceptible to infection. An infectious tissue culture model and a small animal model continue to be sought for studies of the natural history and pathogenesis of this pathogen.

Although HCV is the leading cause of chronic viral hepatitis in the United States, the development of an effective vaccine has been hindered by extensive genetic and possibly antigenic diversity among the different strains. New variants known as quasi-species arise quickly and frequently, thus allowing escape from neutralizing antibodies and cytotoxic T lymphocytes. Amino acid changes frequently observed in a region of about 27 amino acids, termed the hypervariable region 1 (HVR1), which is located at the amino terminus of the hepatitis C envelope protein E2, are postulated to lead to this viral escape from neutralizing antibodies. The identification of this most variable region of HCV, the HVR-1, as a critical neutralization domain poses a major challenge for the development of a broadly reactive vaccine against HCV. Early vaccine studies in chimpanzees using recombinant envelope glycoproteins showed limited protection upon challenge with the same virus. DNA vaccines are now being tested in chimpanzees using envelope as well as core protein constructs. Virus-like particles (VLPs) made up of structural HCV proteins have successfully been produced in insect cells and may serve as a potential vaccine model. Ribozymes, catalytic RNA molecules that bind specifically to target RNA by an antisense mechanism, are also being tested as a possible strategy for the treatment of HCV infection.

NIAID has expanded its efforts in both basic and applied HCV research and continues to interact with other institutes in joint funding efforts through the NIH HCV Working Group. One joint RFA brought in 87 applications of which NIAID will fund at least four of the top candidates. A funding plan for another joint RFA for small HCV animal models is being prepared. NIAID's multidisciplinary, multiproject cooperative research centers are being recompeted and expanded and will also include single projects and support from other NIH institutes. These centers will study viral replication, pathogenesis, and immune responses as well as the early stages of viral infection and host responses. A clinical component for multiprojects will still be required. The NIAID-sponsored Collaborative Antiviral Studies Group continues to review antiviral protocols for combination studies in both hepatitis B and C. This forum allows recruitment of patients from all over the country as well as some foreign sites for participation in clinical trials conducted in specific populations—pediatric patients, transplant patients, and patients coninfected with HIV.

Hepatitis D

The prevalence of hepatitis D virus (HDV) infection does not parallel that of HBV, although it is dependent on HBV for its transmission. It is highest in those with repeated percutaneous exposures, including IV drug users and hemophiliacs. Perinatal transmission is rarely reported (as yet undocumented in the United States). An estimated 70,000 people (4 percent of HBV cases) in the United States have chronic hepatitis D; there are 7,500 infections each year, and about 1,000 people die annually of HDV infections. There are three genetically different types: type I is found worldwide, type II in southeast Asia, and type III in northern South America. Vaccination against HBV prevents infection by HDV. As yet there are no proven therapies for coinfection with HBV and HDV.

Hepatitis E

In endemic areas, 7 to 17 percent of the population show evidence of previous Hepatitis E virus (HEV) infection. Unlike HAV, immune globulin has not prevented infection during outbreaks. HEV is transmitted fecal-orally and is usually seen in developing countries or in travelers. HEV can cause fulminant disease with high case fatality rates

(15 to 20 percent) commonly reported among pregnant women. Subsequent studies in pregnant rhesus monkeys failed to show a greater degree of severity than that seen in nonpregnant monkeys and it was not transmitted to their offspring. HEV can also infect and replicate in laboratory rats where antigens are seen in the liver as well as in the spleen, peripheral blood mononuclear cells, mesenteric lymph nodes, and small intestine. Efforts are ongoing to develop an infected tissue culture model for HEV.

HEV infections are rare in the United States but do pose a risk to persons who travel overseas to endemic areas. CDC developed a mosaic protein enzyme immunoassay, which, based on antibody titers, showed a 3-percent rate of recent exposure to HEV in a cohort of randomly screened patients in four geographic areas of the United States, none of whom had traveled abroad. A 1.2 percent rate of previous exposure among the U.S. population was also determined. There is a high seroprevalence among renal transplantation and hemophilia patient populations.

In NIAID studies conducted at the National Institutes of Health, cynomolgus monkeys were partially or completely protected against infection with HEV by both passive and active immunization. Convalescent serum was used for the passive immunization and a recombinant 55 kilodalton open-reading frame 2 protein known to induce antibody formation was used for the active immunization. These results pointed the way toward development of a vaccine but so far none has been licensed.

Hepatitis G or Hepatitis GB Virus-C

Much progress has been made in analyzing, sequencing, serotyping, and determining the prevalence of the blood-borne hepatitis G virus and hepatitis GB virus-C (HGV, HGBV-C). Discovered by Genelabs and Abbot Labs, respectively, these two viruses are now assumed to be different isolates of the same virus and are distantly related to another flavivirus, HCV (only 25 percent homology). Studies of stored serum specimens show that these viruses are not new. Their identification has awaited new methods of detection. They are endemic worldwide, though their potential for disease production remains unclear as most carriers are asymptomatic. Cases of fulminant hepatitis have been linked to these agents; however, they are not generally considered to be a cause of non-A-E hepatitis. Multiple strains of HGV/GBV-C have been found in dialysis patients; the virus is common in transplantation settings; and it is found in 10 percent of injecting drug users. HGV/GBV-C infection has often been associated with coinfection with certain strains of HCV (types 1a, 1b, and 3), but this additional infection does not seem to affect the patient.

Other Hepatitis Viruses

Four percent of acute cases of hepatitis are currently classified as non-ABCDE,G. A few years ago a novel fecal-orally spread form of hepatitis was named hepatitis F by a French team of researchers. No subsequent publications have appeared about HFV, but a second publication did refer to a novel hepatitis agent being detected from a screening of HEV-infected sera from an epidemic in the Andaman Islands.

TTV, a non-enveloped DNA virus discovered initially in a patient with hepatitis, appears to be following a similar path as HGV/GBV-C. It is still too early to determine whether it is a direct cause of hepatitis or merely a confounding coinfection.

Researchers in Italy have recently isolated a potential hepatitis agent from the blood of immunosuppressed HIV patients using an unusual “degeneration” technique for screening. The new virus that was isolated was called S.E.N.-V after the patient from whom it came. Extensive verification under code, as yet ongoing, found S.E.N.-V in a high percentage of previously unclassified hepatitis patients and in low numbers of healthy controls. The data are premature but promising.

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Human Immunodeficiency Virus Disease

Overview

As acquired immunodeficiency syndrome (AIDS) continues to take its toll globally, the development of a safe and effective vaccine against the human immunodeficiency virus (HIV) is critical to control the epidemic worldwide. Although educational and counseling efforts have had some success, it has become evident that these prevention activities alone are not sufficient to contain the spread of disease.

By the end of 1998, more than 33 million people worldwide were living with HIV/AIDS, according to estimates by the Joint United Nations Programme on HIV/AIDS (UNAIDS). An estimated 5.8 million new HIV infections occurred worldwide during 1998—approximately 16,000 new infections each day. More than 95 percent of these new infections occurred in developing countries. Alarmingly, in 27 developing countries, HIV prevalence more than doubled between 1995 and 1997. In 1998, HIV/AIDS was the fourth leading cause of mortality worldwide, resulting in an estimated 2.3 million deaths.

Although significant progress has been made in developing effective therapies for those infected with HIV, the cost and health infrastructure needed to ensure appropriate use of such treatments preclude their use for most of the HIV-infected world. The identification of effective, low-cost tools for preventing HIV infection is crucial to slowing the epidemic.

Researchers have shown that many approaches to HIV prevention can reduce the number of new infections, including education and behavior modification, the social marketing and provision of condoms, treatment of other sexually transmitted diseases, topical microbicides, and the use of antiretroviral drugs to prevent the transmission of virus from mother to infant. However, most researchers believe that a safe and effective vaccine for HIV infection is the best long-term solution to halting the spread of the disease.

Given the overwhelming need for a safe and effective HIV vaccine, President Clinton challenged scientists to develop a vaccine by the year 2007. Key to responding to that challenge will be the AIDS Vaccine Research Committee and the Dale and Betty Bumpers AIDS Vaccine Research Center, established by the National Institutes of Health (NIH). The AIDS Vaccine Research Committee, chaired by Dr. David Baltimore, is helping stimulate HIV vaccine research and assists NIH in developing a comprehensive research program aimed at expediting the discovery and development of a safe and effective AIDS vaccine. By bringing together intramural scientists from across NIH, the AIDS Vaccine Research Center will stimulate multidisciplinary research from basic and clinical immunology and virology through to vaccine design and production. The Center will complement the comprehensive extramural research activities of the Division of AIDS (DAIDS), National Institute of Allergy and Infectious Diseases (NIAID).

To hasten HIV vaccine discovery, many public and private agencies have dramatically increased the resources

devoted to HIV vaccine research. For example, at NIH, HIV vaccine funding increased by 93 percent between fiscal year (FY) 1995 and FY 1999. DAIDS receives a large proportion of these funds and supports basic, preclinical, and clinical HIV research toward the goal of a safe and effective vaccine for HIV/AIDS. In seeking to develop safe and effective vaccines for HIV, scientists are faced with unprecedented challenges because of lack of correlates of immunity, worldwide genetic HIV variability, lack of an ideal animal model, and multiple modes of transmission. To address these obstacles and facilitate HIV vaccine development, DAIDS supports a two-pronged strategy, which emphasizes both fundamental research and traditional empiric-based vaccine evaluation.

One approach to a vaccine candidate is through the discoveries that arise from fundamental research on HIV. The basic research fields of HIV pathogenesis, microbiology, immunology, virology, and animal model development all contribute essential knowledge to our understanding of how HIV establishes infection within an individual and causes disease. For example, the discovery of Beta chemokines and the role their receptors play in HIV infection has stimulated a wide range of new vaccine approaches. Much of this work is supported via traditional investigator-initiated research grants. A second path to development of vaccine candidates relies on a more empiric approach. Based on strong scientific rationale, investigators assemble and determine the immunogenicity of candidate vaccines.

NIAID has launched several initiatives, designed to help move vaccine concepts from basic research through clinical trials. One such initiative is the Innovation Grant Program for Approaches in HIV Vaccine Research, which encourages early exploration of novel and innovative vaccine concepts and attempts to attract scientists currently working in other areas. Since the program was first started in September 1997, more than 150 awards have been made. Another initiative is the HIV Vaccine Research and Design Program, which supports traditional midstage, targeted research, including animal model studies, to help develop promising vaccine concepts. A third initiative, the Integrated Preclinical/Clinical AIDS Vaccine Program, funds the iterative processes of product evaluation, optimization, and refinement, including animal and clinical studies. Other programs that will be implemented in the near future include the HIV Vaccine Design and Development Teams. This program will fund consortia of scientists with development experience from industry or academia that have (1) identified a promising vaccine concept and (2) envisioned a product worthy of targeted development and will assist in advancing vaccine concepts to the product stage. In addition, NIAID plans to support a program to encourage innovative research to improve technologies for evaluating immune responses.

NIAID is in the midst of restructuring its AIDS Vaccine Evaluation Group (AVEG) and HIV Prevention Trials Network (HIVNET). The reorganization will consolidate scientific responsibility and create two comprehensive networks for AIDS vaccine trials and nonvaccine HIV prevention research. A comprehensive web site (www.niaid.nih.gov/aidsvaccine) provides detailed information about these and other NIAID-supported activities.

In addition to the efforts of NIH are those of other Federal agencies including the Centers for Disease Control and Prevention (CDC), Department of Defense, and the Food and Drug Administration (FDA), as well as those of pharmaceutical companies and nongovernment agencies. Augmenting all these research activities are the HIV vaccine research activities sponsored by other countries.

Reasons for Optimism

There are sound scientific and strategic reasons that support these extensive efforts in HIV vaccine discovery and development.

- Immunization is a well-established method of reventing viral infections.
- The immune system may be capable of controlling HIV infection.
- Mucosal transmission is relatively inefficient.
- Passive transfer of anti-HIV or anti-SIV (simian immunodeficiency virus) antibodies to chimpanzees or monkeys has prevented infection under certain conditions.
- Some vaccines for HIV and related retroviruses have protected against challenge in certain animal models.
- To date, several HIV vaccine candidates in early human trials have been safe and immunogenic.
- Biostatistical modeling suggests that even a partially effective preventive intervention may have a powerful effect on the epidemic.

As a result of recent scientific advances, many of the obstacles to vaccine development appear to be much less formidable than had been previously thought. For example, it has long been feared that the variation of HIV within both populations and individuals would enable HIV to evade specific cytotoxic T lymphocyte (CTL) and antibody responses that may be crucial for protection against HIV/AIDS. Recent studies of HIV receptors have revealed a spectrum of coreceptors, including CCR5 and CXCR4, which may eventually form a basis for vaccines that can induce broadly neutralizing antibodies. A few monoclonal antibodies and sera from some infected individuals react against a variety of primary isolates of HIV. In addition, new methods for measuring CTLs have shown that CTLs from people infected with one subtype of HIV can recognize cells infected with HIV subtypes from other geographic regions. Furthermore, CTLs from vaccinated individuals can often recognize several genetic variants of HIV when

measured against a panel of isolates from around the world. This suggests that if CTLs (or other T-cell antiviral activities) are critical to controlling or eradicating HIV infection, one or a limited number of vaccines may be able to immunize diverse populations against many different strains of HIV. These new findings suggest that the remarkable genetic diversity of HIV may not be an insurmountable obstacle to developing a broadly reactive, preventive vaccine.

Basic research advances during the past year are also enhancing optimism that an AIDS vaccine is possible. For example, NIH-supported scientists have elucidated the three-dimensional crystal structures of two surface proteins of HIV, gp120 and gp41, which help the virus attach to cells. Studies of intermediate molecules formed during virus-cell fusion are helping explain the step-by-step process by which HIV enters the cell, which could lead to new vaccine approaches for inducing antibodies to block its entry. In another study, a NIAID-supported investigator modified the surface glycoprotein of HIV, which is covered with carbohydrate groups that may shield the protein from recognition by the immune system. Selectively deleting these carbohydrate groups from the viral protein of the macaque virus SIV, allowed immune responses to bring the mutant virus under control much faster than the wild-type virus. Therefore, using similarly modified HIV molecules as potential vaccines may improve the immune response.

In 1999, scientists developed a novel vaccine based on HIV fusion molecules exposed only by freezing the virus in the act of infecting a cell. This virus-cell fusion mixture, when injected into mice genetically engineered to ignore key human proteins on the cells, made antibodies against the virus-cell fusion mixture. In laboratory tests, these antibodies were able to neutralize 23 of 24 strains of HIV that represented a variety of different genetic subtypes and were all isolated from infected individuals.

Several investigators have been pursuing research on DNA vaccines. Most recently, a chimeric DNA vaccine made from harmless genes of HIV and SIV has shown promise in monkey studies. The vaccine, when combined with “booster shots” made from a specially engineered virus carrying the same genes, seemed to protect animals later given doses of a live hybrid virus made from HIV and SIV. The vaccine did not keep the animals from getting infected but kept the hybrid virus under control and undetectable in the blood.

In the past, researchers were concerned that it would be more difficult to prevent HIV infection from sexual transmission compared to parenteral transmission. Increasing evidence from animal models suggests that there is a short period during which the establishment of chronic infection may be prevented if infection occurs through vaginal, rectal, or oral exposure. Clinical and laboratory methodology for identifying binding antibodies and for cloning CTL from mucosal fluids or cellular specimens may help elucidate the local immune response during mucosal infection. In addition, genetically altered toxins and physical carriers are also being developed to enhance mucosal presentation, and they may be effective in inducing mucosal immunity in humans. Therefore, it may be more feasible than previously thought to prevent sexual or mucosally based transmission through either parenteral or mucosal immunization.

In addition to accomplishing these scientific achievements, individuals in the communities from which study volunteers will be recruited have been working to overcome fears and preconceived notions about vaccine trials. These volunteers will include both men and women at high risk of HIV infection because of their sexual behavior or drug use. Because vaccination can induce antibodies against components of HIV, some volunteers may test positive for HIV on standard HIV diagnostic tests. Although the vaccines being tested cannot result in HIV infection, volunteers may fear the social consequences and discrimination of falsely testing positive. NIAID continues to work proactively to minimize the potential for discrimination against study volunteers and over the past several years has developed strong linkages with various communities to address many of their questions and concerns about HIV vaccines. Public health and diagnostic laboratories, insurance companies, and manufacturers of diagnostic kits are also helping to address volunteers’ concerns.

Investigators continue to design and evaluate novel ways to present HIV proteins to the immune system as well as to examine new antigen/adjuvant and vaccine formulations. Traditional approaches to immunization (live-attenuated, whole-inactivated) when applied to HIV are technically complex and raise substantial safety concerns. Clinical trials involving candidates for prophylactic vaccines enroll volunteers who are not infected with HIV. Such trials have been conducted to test vaccines made through chemical synthesis or biotechnology: subunit vaccines, including envelope proteins or small particles; recombinant poxviruses bearing genes encoding one or more viral proteins; and DNA vaccines.

Since 1987, more than 3,000 non-HIV-infected volunteers have enrolled in 52 NIAID-supported preventive vaccine studies (50 phase I safety studies; 2 phase II safety and immunogenicity studies) involving 27 vaccines. These trials have been conducted primarily at six university-based AVEG sites nationwide. NIAID’s HIVNET, which includes international as well as U.S. sites, and NIAID investigators in Bethesda have also led or helped conduct some of these trials. As a result of these trials, three vaccines have proceeded to phase II trials: two different recombinant gp120 envelope proteins and one recombinant canarypox-HIV vaccine, which incorporates genetic material from the HIV envelope, *gag*, and protease genes.

In 12 years, HIV vaccine research has progressed from an early focus on HIV surface antigens (particularly envelope) and the role of neutralizing antibodies to increased attention to CTLs in HIV immunity. Many novel approaches to elicit anti-HIV neutralizing antibodies and CTLs are now under investigation. A brief description of the various types of HIV vaccines being tested and the status of research in each of these areas follows.

Combination Approaches/Adjuvants

The concept supported by NIAID that is furthest advanced in human testing combines different vaccine designs or different antigens; this results in additive or synergistic effects capable of greater, broader, or more prolonged immune responses. Different vaccines administered simultaneously (in the same or at different sites) or successively will (1) prime the immune system to different antigens, (2) prime for different immune functions, or (3) boost existing responses.

An HIV vaccine may most likely be successful if it combines (1) various antigens derived from single or multiple HIV strains, (2) different presentation methods that stimulate different immune effectors, and (3) adjuvants to enhance or modulate the resulting immune response. Recent knowledge from immunologic studies suggests that combining different vaccine antigens and adjuvants may result in both additive and synergistic effects. For example, induction of antibodies and T cell cytolytic responses are enhanced by the simultaneous inclusion of helper T cell antigens. Coadministration of immune-modulatory molecules (e.g., GMCSF, IL12) that increase antigen presentation through specific desirable pathways may also enhance the strength of immune responses. Combining HIV vaccines with "non-HIV" strong helper T cell antigens (e.g., tetanus toxoid) or with molecules that increase local induction of immune responses (e.g., cholera toxin) may also increase antigenicity.
Studies in nonhuman primates have confirmed the potential benefit of such approaches. The strategy that is most advanced in human testing is to incorporate *both* a live vector and recombinant protein as a way to stimulate both arms of the immune response. This strategy, called a "prime boost" approach, has usually been performed with the vector first and the protein second.

Several clinical trials have been conducted to ascertain the validity of combining vaccines. Clinical trials of an HIV/canarypox recombinant (PMC ALVAC vCP205) followed by, or simultaneously with, a gp120 subunit boost (Chiron SF2) have shown that this concept of immunizing volunteers with different antigens that target different presentation pathways results in responses that are at least additive. For example, the administration of pox-based vaccines ("prime") simultaneously with or followed by subunit antigens ("boost") has resulted in cytolytic activity, neutralizing antibody, proliferative responses (an indicator of T cell help), and antibody dependent cytotoxic activity (ADCA). Preliminary findings from a phase II study of this combination were recently reported at the International Society for Sexually Transmitted Diseases Research meeting in Denver. To date, these vaccines have induced anti-HIV immune responses in the majority of the volunteers. More than half the individuals receiving vCP205 alone, and more than 90 percent of those receiving the vaccine combination, have developed antibodies that can inhibit HIV in a laboratory assay. Thus far, about one-third of the volunteers receiving either vCP205 alone or the combination vaccine has developed anti-HIV CTL responses.

The same ALVAC vCP205 vaccine is being studied in a separate phase I trial in combination with GMCSF, a cytokine with potential adjuvant effects. Additional clinical trials are being planned to test a combination vaccine consisting of an HIV-DNA vaccine (Apollon) priming followed by boosting with either a gp120 subunit (VaxGen) or by a canarypox-based HIV recombinant (PMC).

A related vector comparison study is evaluating three potential products to determine which canarypox vector produces the most robust immune response. An additional trial will assess the best of these vectors combined with the best available protein boost. These studies, as well as additional data that emerge from basic research, will provide the information needed to determine which products will advance into efficacy trials.

Recombinant Live-Vector Vaccines

Recombinant live-vector vaccines are produced by engineering viral or bacterial genomes to express the desired HIV antigens. Viral vectors can be constructed that contain one or more HIV genes that cause infected cells to make the HIV-specific protein in native form. Recombinant viral vectors enter cells and allow the HIV or SIV proteins to be generated inside the cells; these proteins are then presented to the immune system in the same way that proteins from a virus-infected cell would be. As a result, vector-based vaccines induce both humoral and cellular immune responses. Importantly, immune responses can be generated to the vector as well as to the incorporated antigens. The immune responses to the vector could, however, limit the effectiveness of subsequent immunizations with the same vector. When given in combination with recombinant subunit products, live-vector experimental vaccines have been shown to prime the recipient for augmented immune responses.

Live infectious viral or bacterial vectors that are genetically engineered to express genes of HIV or SIV are being evaluated in animal models for their potential to prevent infection by HIV, SIV, or SHIV (a genetically engineered hybrid that has an HIV envelope and an SIV core). Poxvirus recombinants were the first to be evaluated in nonhuman primates. Vaccines based on vaccinia virus,

modified vaccinia Ankara or NYVAC (two attenuated vaccinia strains), have protected nonhuman primates from SIV, HIV-2, or HIV-1 infection when they were given alone or followed by immunization with purified envelope protein to boost the antibody response (prime-boost protocol). However, the results were variable; some animals were protected from infection, some only from disease, and some were not protected at all.

AVEG has conducted phase I clinical trials to evaluate recombinant vaccinia-HIV gp160 with and without boosting with one of six different candidate recombinant gp120 or rgp160 vaccines. By itself, the vaccinia-gp160 induced little antibody; however, one or two doses of it primed for both gp160-specific CTLs and anti-HIV neutralizing antibodies. The priming effect was strongest in those volunteers who were vaccinia naïve, that is, who had never been vaccinated against smallpox. A phase I trial of a recombinant vaccinia-HIV vaccine, incorporating *env, gag,* and *pol* antigens and boosted by rgp120 candidate vaccine, is underway in AVEG.

Vaccines based on the canarypox virus have also protected nonhuman primates from SIV, HIV-2, or HIV-1 infection when given alone or followed by a boost. These vaccines are considered safer than vaccinia vaccines because canarypox fails to replicate in mammalian cells. Recombinant canarypox-HIV vaccines have also been shown to induce both anti-HIV neutralizing antibodies and CTLs in humans, regardless of prior vaccination with vaccinia. Boosting or concomitant administration of rgp120 or rgp160 increases the production of HIV-neutralizing antibodies and may induce HIV-specific antibodies. Five different types of recombinant canarypox experimental vaccines (ALVAC) are undergoing testing in phase I trials in France and in the United States. In addition, a phase II trial of recombinant canarypox expressing *env*, *gag*, and protease, alone or in combination with rgp120, is being jointly conducted by AVEG and HIVNET in 420 uninfected (seronegative) volunteers, many recruited from populations at high risk of HIV infection. One form of canarypox has been developed that incorporates some genes from vaccinia; this significantly improves production of the HIV proteins in human cells without replication. One new vector contains *env* and *gag,* and another includes these two genes and portions of *pol* and *nef* genes. The new vaccines are being compared with the older canarypox vaccines in a phase I study.

An adenovirus-HIV envelope vaccine has also produced protection in nonhuman primates; it generated neutralizing antibodies and anti-HIV CTLs when administered in a prime-boost regimen with HIV envelope protein. The prime-boost regimen protected all chimpanzees from HIV infection when they were challenged shortly after the last immunization. These chimpanzees were also protected 1 year later despite not having received a boost and being challenged with a higher dose of the same challenge virus.

A non-disease-causing, nonreplicating Venezuelan equine encephalitis (VEE) virus particle (replicon) suitable for humans has been modified to carry selected HIV genes. When given to mice, this vaccine induced both antibodies to the virus and immune responses that killed HIV-infected cells. These experiments, and additional experiments in monkeys, demonstrate the potential for VEE-based replicon vaccines to stimulate both arms of the immune system and to control the level of virus. Another novel vector, a weakened form of vaccinia virus called MVA, has been developed and has been shown to induce good immune responses in rodents and monkeys. A variety of other vector-based approaches are also being developed for HIV vaccines and include recombinant poliovirus, mengovirus, herpesvirus, Semliki Forest virus, influenza virus, Salmonella, Bacille Calmette-Guérin (BCG), Shigella, lactococcus, listeria, yellow fever virus, measles virus, varicella-zoster virus, and cytomegalovirus. Poliovirus-SIV, BCG-SIV, and Semliki Forest virus-SIV recombinants have been tested in nonhuman primates. Much of this work is being performed through NIAID's Innovation Grant Program.

Additional research is needed in several areas regarding live recombinant vector AIDS vaccines. For example, NIAID is supporting studies to determine (1) whether increasing the level of expression of HIV proteins in the recombinant vector improves immune response; (2) whether vectors should contain genes from primary isolates or nonclade B isolates; (3) the optimum route, dose, and schedule of administration; and (4) the best way to combine recombinant vector vaccines with subunit, DNA, or other vector experimental vaccines to maximize or optimize the immune response.

Protein and Peptide Subunit Vaccines

Safety considerations surrounding other more conventional strategies have led some vaccine makers to pursue more modern approaches when designing an AIDS vaccine. Theoretically, virus-specific proteins, or portions of the proteins (peptides), could be used to induce virus-specific immunity without any possibility of inducing HIV disease. Protein subunit vaccines are made by genetically engineering HIV genes of interest into a laboratory culture system, such as bacteria, yeast, insect, or mammalian cell cultures, then purifying the resulting proteins from the culture media. Peptide vaccines are made by chemically synthesizing the region of interest. Generally, those regions include known T-cell or B-cell epitopes. Envelope subunit and peptide approaches were among the earliest attempts to make an HIV vaccine, based on the premise that the envelope protein would be the most important target because neutralizing antibodies in HIV-infected persons target the envelope.

Thus, virus-specific envelope proteins have been the primary target of these vaccine strategies with the goal of producing an immunogen that induces virus neutralizing antibodies. However, protein and peptide subunit strategies are also being pursued as a way of inducing T-cell immunity targeting both envelope and *gag*-gene specified virus structural proteins. Envelope protein vaccines, including insect, yeast, and mammalian cell-derived products, were some of the earliest preparations tested in human trials, with the full-length *env* gene product (gp160) and the surface glycoprotein (gp120) tested in phase I trials. The mammalian cell-derived gp120 product was later tested in phase II trials, and large-scale phase III trials are currently being carried out on two of these products.

The early optimism surrounding these vaccines was tempered by the observation that neutralizing antibodies elicited by these vaccines, although potent against laboratory-adapted strains of the virus, were unable to neutralize most primary isolates. The immunogens tested in phases I and II trials were modeled after laboratory-adapted strains of the virus, a choice that may have accounted for the laboratory-strain-specific neutralization properties of the antibodies elicited by these immunogens. Recent studies suggest that the virus must mutate its envelope gene to enable efficient replication in cell lines, and these mutations change the antigenic properties of the protein relative to wild-type virus. In addition, laboratory-adapted viruses use the cellular receptor CXCR4 for entry into host cells, whereas viruses most readily transmitted in humans preferentially utilize the CCR5 receptor. This receptor usage is a property of the virus envelope protein. Therefore, it is felt that a CCR5-using *env* protein may be more relevant for inducing functional antibodies. The vaccines currently being tested in phase III trials in the United States and Thailand include envelope proteins derived from CCR5-using primary isolates and are produced by VaxGen.

Many investigators believe that virus envelope immunogens need to be structurally folded to mimic their appearance in infectious virus particle to induce functional neutralizing antibodies. To pursue this theory, attempts are being made to generate envelope proteins that have been stabilized in their wild-type trimeric configuration. Recent molecular studies indicate that the envelope proteins undergo structural changes after binding to cellular receptors; this information has led some investigators to design immunogens that mimic these structural changes. In addition, some investigators are making envelope mutants, increasing or decreasing the number of glycosylation sites or removing portions of the coding sequence, in efforts to increase the immunogenicity of the proteins. Although none of these recent versions of envelope proteins have progressed to phase I testing, results of animal studies have been promising.

A number of approaches to increase the immunogenicity of peptide vaccines are being pursued. These include branch-chain peptides and conjugation of the peptide to lipid or other delivery molecules. Many of the earlier peptide approaches targeted the V3 envelope epitope of HIV. The resulting antibodies elicited by these immunogens were similar to those elicited by the monomeric envelope protein immunogens; the antibodies were reactive only against laboratory strains of virus and were generally strain specific. Recent studies suggest that the V3 epitope, although extremely immunogenic and exposed on laboratory strains of virus, is occluded on primary isolates and thus not a potent neutralizing target. Investigators are attempting to design peptide immunogens that exhibit key conformational properties of a functional envelope protein, but these peptide products have not yet reached the stage of human testing.

Live-Attenuated Vaccines

Historically, live-attenuated vaccines have been among the most efficacious viral vaccines. These vaccines use live virus that has been modified (attenuated) to make the virus less virulent. They induce both humoral and cell-mediated immunity and generally require only one or two doses because the immune responses induced by live-attenuated vaccines are durable. Because of safety considerations, live-attenuated HIV vaccines have not been tested in humans. Safety issues include the possibility that such a vaccine could cause AIDS, at least in some individuals; that attenuated virus in the vaccine could revert to the wild-type, disease-causing virus; or that long-term infection could cause autoimmune or malignant disease. These concerns are underscored by observations that small deletions in HIV accessory genes, those used to attenuate the virus, can be “repaired” after *in vivo* infection.

Live-attenuated AIDS vaccine studies have been conducted using SIV in macaques. These SIV studies have shown that both a naturally occurring attenuated virus with multiple mutations and one created by deletion of one or more genes can serve as effective vaccines. However, protective efficacy appears to vary inversely with the level of attenuation, increases over time after vaccination, and is strongest when the challenge virus is closely related to the vaccine. One of these vaccines, in high dose, caused death in neonatal macaques, and some individual juvenile or adult macaques also progressed to AIDS-like illness from these vaccines. In addition, a cohort of humans who received a naturally attenuated HIV through transfusions from an infected donor has been identified. Although many of them remained clinically healthy for up to 20 years after exposure, several, including the transfusion donor, have recently experienced notable declines in CD4 immune cells. These combined studies demonstrate that a live-attenuated design that is both effective and safe has not yet been identified.

Research is ongoing to determine the mechanisms responsible for protection with live-attenuated HIV vaccines and to find safer ways to attenuate the virus or mimic the immune responses they induce.

Particle or Whole-Inactivated Vaccines

Whole-inactivated viral vaccines are prepared by rendering the pathogenic virus unable to replicate, usually by a chemical treatment. These "killed vaccines" are potentially safer than live-attenuated vaccines; however, whole-killed vaccines often lose the potent, long-lasting immunogenicity of the live virus because the treatment used to inactivate the virus often destroys or alters important protective antigens. In addition, incomplete inactivation could result in infection of vaccinated individuals by residual pathogenic virus.

To overcome some of these obstacles, researchers have produced virus-like particle (VLP) or "pseudovirion" experimental AIDS vaccines by recombinant technology. These particles contain one or more structural proteins of HIV (or SIV) and are designed to mimic the native virus particle, in whole or part. However, pseudovirions do not contain the virus genome, so they cannot replicate and produce progeny virus. The manufacture of these VLPs is complex and given the genetic variability of HIV, these vaccines may need to be based on multiple strains of HIV.

To date, whole-killed HIV vaccines have not protected immunized chimpanzees against infection with the virus. Although macaques were protected against SIV challenge when immunized with inactivated SIV, the protection was the result of antibodies to human cell line xenoantigens present in the vaccine preparations and challenge stocks. Macaques that were immunized and challenged with SIV grown in monkey cells were not protected from infection.

Only one VLP experimental AIDS vaccine (p17/p24:TY) has been tested in prophylactic phase I trials. This particle contains only a portion of core, without envelope, so it does not mimic the entire HIV particle. Low levels of HIV-specific binding antibodies and T cell memory responses were generated in most of the volunteers after three or four doses. However, little HIV *gag*-specific CTL activity has been observed to date. In an ongoing study, volunteers receive intramuscular immunizations followed by boosts of the VLP orally or rectally to determine whether HIV-specific mucosal antibody responses are induced. Other recombinant particle vaccine candidates are in development or in the planning stages. One of these is a particle formed from the HIV p55 *gag* protein. Another recombinant pseudovirion candidate, derived from a primary HIV isolate, is likely to enter a phase I trial next year.

Researchers are examining new ways to develop a whole-inactivated HIV vaccine without destroying critical viral proteins. In one study, researchers were able to maintain conformation of important structures on HIV virions treated with a heat-inactivation procedure.

NIAID is pursuing additional studies of whole-killed HIV vaccine candidates and recently identified this as an area in need of further stimulation and support; special emphasis has been given to this area in grant funding by the Institute.

DNA Vaccines

In DNA immunization, the host is immunized by direct administration of viral genes; the genes are composed of DNA that encodes for the antigen that would normally be produced by the cell. Several experimental DNA vaccines for HIV/AIDS have been produced and tested in small animals and nonhuman primates. In general, the results of these studies have been promising. DNA vaccines delivered intramuscularly or by gene gun have been shown to induce both neutralizing antibodies and CTL responses against HIV and SIV antigens.

In one study, DNA immunization induced neutralizing antibodies and a vigorous CTL response. However, this immunization did not protect rhesus macaques from infection or disease on subsequent challenge with a pathogenic SIV after peak CTL and neutralizing antibody titers had waned. DNA immunization was successful in protecting chimpanzees against a nonpathogenic HIV infection and in protecting rhesus macaques against a nonpathogenic chimeric virus (SHIV). In the latter study, the animals were first immunized with a DNA vaccine expressing HIV-1 IIIB *env* and later received a booster immunization with a subunit protein vaccine composed of HIV-1 envelope protein before challenge.

Key issues for DNA vaccine development include optimizing antigen expression by DNA vaccine plasmids and optimizing immune responses to DNA vaccines in nonhuman primates and in human subjects. The development of new expression systems with more potent promoters, immunomodulatory cytokine genes, or costimulatory molecules, as well as the formulation of DNA vaccines with adjuvants, cytokines, or novel delivery systems, is also being evaluated as ways to enhance the immunogenicity of DNA vaccines. Research is also needed to determine the most effective routes of administration, optimize the timing of vaccine administration, and evaluate the utility of sequential immunization (prime-boost) strategies using DNA as a prime or boost with subunit protein or vector-based vaccines.

Phase I clinical trials of two DNA candidate vaccines, one containing an HIV-1 *env* and *rev* and the other a *gag-pol* construct, were conducted through NIAID's intramural program and AVEG, respectively. Both DNA vaccines were well tolerated. Further clinical tests of DNA vaccines

including the use of DNA as the primary or secondary immunogen in a "prime-boost" protocol with protein or viral vector-based vaccines are planned.

International

More than 10 distinct genetic types and various recombinants of these subtypes of HIV have been described worldwide. At this time, it is not known whether subtype-specific vaccines will be required or whether vaccines that induce broadly cross-reactive immune responses can be developed to protect against different subtypes. Vaccine research is proceeding along both paths with some encouraging developments in the past year. Two candidate vaccines based on local clinical isolates of subtype E are in clinical trails in Thailand, and findings from AVEG studies of a canarypox-vectored HIV vaccine suggest that CTLs are cross-reactive with viral core epitopes of several HIV subtypes. Basic research findings reported in the past year suggest that it may also be possible to design vaccines based on the HIV/host cell fusion complex that are capable of inducing broadly reactive neutralizing antibodies.

Clinical testing of HIV vaccines is underway at several international sites. Over the past few years, Thailand has completed a series of phases I and II trials on a variety of candidate vaccines. In 1999, Thailand began a phase III trial of VaxGen's clade B/E rgp120 in a population of intravenous drug users in Bangkok. Uganda began the first HIV vaccine trial in Africa in 1999, with a phase I study of PMC's canarypox-vectored clade B vaccine. A multisite phase II study of the canarypox with a gp120 boost (prime-boost) is planned to begin in Brazil, Trinidad, and Haiti in early 2000. These international sites and their international collaborators have demonstrated the capacity to address the many technical, logistical, and ethical issues that are involved in implementing vaccine trials, thereby establishing a model for future trials and the active participation of international sites in the development of HIV vaccines.

Assays To Measure Vaccine-Induced Immune Responses

Although scientists know more about HIV than any other virus, many challenges exist in designing a vaccine to prevent AIDS. Part of the problem is that, unlike the body's response to most acute viral infections, the natural immune response does not completely eliminate HIV. This failure makes it difficult for investigators to know what type of immune activity an effective vaccine should evoke.

To understand the biological mechanisms responsible for the success or failure of any candidate HIV vaccine in human trials, NIAID is attempting to develop a number of assays to evaluate the immune responses to these vaccines. Specifically, the assays in development must be accurate, precise, quality assured, and able to give consistent results regardless of where they are performed. Furthermore, it must be possible to use frozen cells or plasma to allow specimens to be stored and tested when appropriate. The assays being developed evaluate antibody and cell-mediated immune responses.

Assays for neutralizing antibodies must also be able to detect and define the spectrum of HIV variants that the vaccine-induced antibodies neutralize. The technologies to accomplish this have recently been explored by a working group of investigators, and refinements to the existing assays continue to be implemented. Additional tests such as antibody-dependent cellular cytotoxicity assays are also in development.

Methods to detect T-cell immunity have relied on the ^{51}Cr-release CTL-killing assay. Although considered the "gold standard," this method has significant limitations. One major problem is that a specific cell line must be established for every volunteer. The assay is also not consistently reproducible. NIAID is exploring a number of possible substitutes, such as gamma-interferon ELISPOT, intracellular cytokine assays, and class I HLA tetramers. These technologies, once standardized, will provide the essential methods to assess T-cell immunity in multicenter and international trials.

With the discovery of the essential role of the chemokine receptors in HIV infection, the need to determine vaccine-induced changes in chemokines has become an important measure of vaccination. Although these assays are not yet well developed, research is ongoing to improve and refine the methodology.

The other set of assays that are critical for understanding the mechanism of vaccine-induced protection is the detection and sequencing of both the virus circulating in the population where the trials are being performed and the virus that breaks through and infects a vaccinated individual. The combination of viral sequence information and a complete delineation of the immune responses to the vaccine will provide the necessary data to help elucidate the mechanisms of protection in controlled vaccine trials.

NIAID remains committed to the discovery and development of a safe and effective vaccine for the prevention of HIV infection or disease. Toward that end, NIAID will continue to pursue a comprehensive range of basic, preclinical, and clinical research and will continue to evaluate diverse vaccine strategies and products.

Selected Topics in Vaccine Research and Development

Transcutaneous Immunization

Dr. Gregory Glenn
Dr. David Lang
Dr. David Taylor

Introduction

Beyond the obvious need for new and improved vaccines against a number of infectious diseases lies the formidable task of making these products, once developed, accessible and easy to administer so that vaccine coverage can be expanded. Indeed, one of the goals of current vaccine development efforts is to develop delivery strategies that would be more acceptable to children and adults alike. This may be achieved by reducing or eliminating the number of vaccines that are delivered via injection. To that end, attention is being focused on the development of multivalent vaccines, effective adjuvants, oral or nasal delivery strategies, DNA vaccines, and edible vaccines. Another objective is to feasibly reduce the cost of effective vaccines so that they will be available to the millions of people in developing countries for whom the price of most current vaccines is prohibitively high. In this section, transcutaneous immunization (TCI) and some of the recent progress that has been made in developing this as an immunization strategy to consider in the future are described.

TCI is a new technique in which an antigen and adjuvant are administered to the skin to stimulate an immune response. This technique has made skin, or dermis, a new route of immunization and has important implications for vaccine development. Although TCI is a relatively new concept, the induction of immune responses after topical application to the skin has been known for many years through the immune pathologies created by contact sensitizers. The medical use of antigen-specific immune responses in the skin has traditionally been used for diagnostic purposes such as defining sensitizing agents or tuberculosis testing. The use of skin immune mechanisms for immunization began with scarification techniques (smallpox) and was followed by intradermal immunization, most notably efficient with rabies and hepatitis B vaccines. However, the recognition that topical application could capitalize on the potent immune system of the skin to stimulate systemic and mucosal responses that may be effective against infectious diseases is a recent observation that warrants inclusion in the array of new vaccine technologies.

Skin Immunization: Barriers and Targets

Topical administration of vaccine requires penetration of the vaccine components through the protective layers of the skin, an antigen presenting cell to initiate the the process that leads to an immune response, and a stimulus sufficient to induce a strong immune response. The stratum corneum, the outer protective layer of the epidermis, is composed principally of dead cells and lipids and is the principal barrier to penetration. Although the stratum corneum generally resists penetration, simple disruption can be accomplished by hydration of the skin, such as occurs after occlusion of the skin. Occlusion is widely used for enabling penetration of drugs with transdermal patches. However, in contrast to transdermal drug delivery, which generally requires delivery of the drug through the epidermis into the vasculature found in the deeper layers of the dermis, transcutaneous immunization appears to target only the most superficial layers of the skin, the epidermis. The epidermis contains immune cells known to be the most potent type of antigen-presenting cell, the Langerhans cells (LCs). LCs are known to increase their baseline rate of migration out of the skin in response to activating stimuli, such as contact sensitizers, LPS, or TNFα, and to travel to the draining lymph node where antigen presentation occurs to T cells. In immune pathologies, the T cells then migrate back to the skin and produce the inflammation characteristic of delayed-type hypersensitivity (DTH). The migration to the skin by T cells does not appear to occur in TCI, which differentiates this immune response from DTH. Extensive studies will be required to validate the current premise that LCs are the initial target for TCI, but it appears likely that LCs are involved because they are the only antigen-presenting cells found in uninflamed skin. The final requirement for TCI, a potent stimulus, is provided by inclusion of an adjuvant such as cholera toxin (CT). In fact, it appears that the role of the adjuvant is crucial to the induction of an immune response sufficiently robust to be effective. Thus, in contrast to the response contact sensitizers, the immune response induced by topical administration of adjuvants such as CT is characterized by more traditional vaccine-induced immune effector manifestations such as systemic and mucosal antibodies, T cells, and the absence of local skin eruptions, most notably DTH.

The Important Role of Adjuvants

The potent immune response induced via TCI is dependent on the presence of adjuvants, such as bacterial ADP-ribosylating exotoxins (bAREs), including CT, heat labile enterotoxin from *E. coli* (LT), their mutants, and other adjuvants. The bAREs have had extensive use as adjuvants via intranasal and oral routes, which has provided a wealth of applicable experience for their use on the skin. In fact, TCI appears to act in a manner similar

to intranasal or oral immunization in that the simple admixture of CT or LT with a coadministered antigen, such as tetanus toxoid or influenza hemagluttinin, results in far higher antibody levels than antigen alone and can simultaneously induce cell-mediated immunity. Point mutations and other techniques have resulted in mutant toxins that appear to have adjuvant activities similar to the native toxins but are less prone to induce potential side effects. The use of mutant toxins as TCI adjuvants may allay the possibly unwarranted concerns that are associated with the use of native toxins that may cause diarrhea on ingestion in fasting subjects in whom the gastric acid has been neutralized. However, the lack of local and systemic toxicity with the use of bAREs on the skin may allow the use of CT or LT with their matchless potency as adjuvants.

Immune Responses to Transcutaneous Immunization

Initial work has shown that CT acts as an adjuvant when applied to the skin, resulting in classic priming and secondary antibody responses to coadministered antigens. Mice immunized with CT or LT alone and then subsequently boosted demonstrate both strong priming and a typical secondary antibody response to the toxins. By contrast, when CT is coadministered with the vaccine antigens tetanus or diphtheria toxoids, minimal priming antibody responses are seen to the coadministered antigen; yet, after boosting immunizations, classic secondary antibody responses are observed. Most vaccines produce their protective levels of antibodies through boosting regimens; similarly, it appears that TCI elicits immune responses that can be readily boosted. Importantly, the humoral response to the adjuvant has been shown not to interfere with the response to coadministered antigens, and multiple immunizations with different antigens using the same adjuvant may be conducted. In addition, the antitoxin response to the adjuvant may also have a protective effect to prevent toxin-mediated diarrheas.

In general, antigen-specific T cells underlie the secondary antibody responses produced by immunization and can be expected to be induced using TCI. Consistent with this concept, mice immunized on the skin with diptheria toxoid, using CT as adjuvant, have been found to have proliferative responses in the spleen and draining lymph nodes. The proliferative responses to diptheria toxoid induced using TCI appear to be due to $CD4^+$ cells. Thus, it appears that TCI induces secondary antibody responses as well as T cell help that result in high levels of antibodies.

Intramuscular and mucosal immunization appear to induce immune responses in vastly different compartments. Mucosal immune responses are desirable for their ability to block pathogens at the point of entry at the mucosal lining or to neutralize toxins that induce pathology at the mucosal surface. Intramuscular and subcutaneous immunization generally result in systemic immune responses, with little or no mucosal component. However, intramuscular immunization can be effective against mucosal pathogens such as *Haemophilus influenza* type b, which has nearly been eradicated by the use of an intramuscularly injected vaccine (Jordan Report, 1988). Similarly, mucosal immunization may result in systemic antibodies and a response that extends well beyond locally produced secretory IgA. Transcutaneous immunization using bAREs may represent a similar type of response with both mucosal and systemic antibodies detectable after immunization. Thus, TCI may enhance efficacy through induction of both mucosal and systemic responses. The mucosal component of TCI will be an important topic of further research.

Delivery Options Using Transcutaneous Immunization

TCI appears to provide a new level of flexibility for delivery strategies. Although clinical studies using patches are underway, topical delivery of vaccines may not be restricted to patches but may involve gels, creams, or ointments as well. Other practical strategies include targeting multiple draining lymph nodes, delivery of multivalent vaccines, increasing the frequency of boosting, anatomical targeting, boosting subjects primed by other routes (intramuscular, intranasal), or by other immunization strategies (e.g., DNA immunization).

Optimization for Enhancement of the Immune Response

The earliest experiments conducted using TCI were performed using empiric antigen doses and no skin manipulation. The working hypothesis for TCI suggests that potent immune responses, comparable or superior to immune responses induced by standard routes, should be feasible because TCI uses gold standard mucosal adjuvants to target gold standard antigen-presenting cells, also known as “nature’s adjuvants” (Langerhans cells). Consistent with this expectation, optimization experiments have suggested that TCI can be enhanced by simple manipulations and can elicit responses comparable in magnitude to those seen in response to established routes of immunization. The model antigens, tetanus toxoid and diphtheria toxoid, have been used initially to define some of the optimization principles, but preliminary work has indicated that a broad variety of antigens may be used for topical immunization.

Enhanced immune responses after simple, practical skin manipulation were shown in experiments where alcohol swabbing of the skin before application of the immunizing solution enhanced the antibody response to CT as well as provided a more uniform immune response. In studies in which the skin was hydrated and alcohol swabbed before immunization with CT and DT, anti-DT

antibody responses comparable to anti-DT responses elicited by intramuscular injection with alum or intranasal immunization using LT were observed. Other studies have shown that lower doses of adjuvant and antigen than initially described can be used for TCI. Using 100 µg of antigen (diphtheria toxoid) and 100 µg of adjunct (CT) as the "standard" TCI dose, mice were administered various combinations of 10 µg of antigen or adjuvant on the skin. The mice that received 10 µg of adjuvant and 100 µg of antigen had anti-DT antibody responses that were equivalent to the mice receiving 100 µg of both antigen and adjuvant, and antibody responses to 10 µg of DT were readily detectable. This result and those of other studies suggest that lower doses of adjuvant and antigen may be used in the induction of immune responses using TCI and, in combination with simple skin manipulation, may induce responses equivalent to responses elicited using intramuscular or intranasal immunization routes.

The duration of time required for induction of an immune response via TCI has not been fully determined; however, if hydration of the skin and passive diffusion of the antigen into the epidermis are the underlying physical phenomena required for immunization, then a short period of immunization may be feasible. The time required for TCI was studied by applying an immunizing solution (CT+DT) for various times; after 15 minutes of application, the animals were well immunized, and by 30 to 60 minutes, maximal immune responses were obtained. These early studies suggest that simple optimization strategies may greatly enhance the immune response to TCI and that clinical strategies may anticipate low doses of adjuvant and antigen, as well as brief application periods that may be suitable during a clinic visit.

Conclusions

The use of topically applied vaccines may address the urgent need for needleless vaccine delivery, decrease the barriers to immunization, and allow flexible delivery for multiple boosting and multivalent vaccines. TCI appears to offer a new method for the delivery of vaccines with practical and immunological advantages. The exploitation of the immune system of the skin may improve the efficacy of established vaccines by eliciting both mucosal and systemic responses and may provide new possibilities for vaccines under development, not only for infectious disease, but for immunomodulation (such as required for cancer vaccines) as well. Clearly, there are substantial challenges ahead for the full development of TCI, but this technique has obviously entered into the current array of immunization and vaccine delivery strategies.

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Memories of Pathogens Past—Inborn Antigen Receptors and T Cell Affinity Maturation

Dr. Charles J. Hackett

Two hallmarks of the immune system are its ability to unequivocally distinguish foreign invaders and to establish and maintain immunological memory of previous encounters. Distinct immune components are involved: the innate immune system, which has evolved receptors to detect unique molecules of microbial pathogens; and the adaptive immune response, which selects B and T lymphocytes with receptors that precisely bind to specific foreign antigens. These two immune components interact closely—adaptive immune responses depend on innate immune recognition to identify dangerous invaders and provide the signals that specify the type of immune response needed. The development of robust memory T cells appears to depend on strong signals provided by sentinel cells that recognize pathogens. Recent advances in the understanding of innate pathogen recognition and T-cell memory are described here in the context of their importance for new vaccine approaches.

Toll Receptors Signal Innate Immune Recognition of Bacterial Cell Walls

Compounds unique to bacterial cell walls, especially lipopolysaccharide (LPS) of gram-negative organisms and peptidoglycan found in gram-positive organisms, are extremely potent inducers of cytokine release and cellular activation. In addition, bacterial cell walls signal the immune system that invaders are present, stimulating effective antibody and cellular immune responses. This latter function is exploited in certain immunological adjuvants that contain bacterial cells or cell wall molecules.

The discovery, more than 30 years ago, of the LPS-unresponsive C3H/HeJ mouse strain demonstrated that the biological activity and toxicity of LPS are due to genetically controlled host responses rather than to direct toxic properties of the bacterial cell walls. A long search for the mechanism by which cells detect and signal the presence of bacterial cell walls has led to a recently discovered group of molecules, the mammalian Toll-like receptors.

A Multiple-Receptor Pathway for LPS

Multiple receptors are involved in the cellular recognition of LPS. Ulevitch and coworkers described a pathway in which the serum factor LPS binding protein (LPB) conveys LPS to CD14, a molecule found both as a membrane molecule on macrophages and related cells and in a soluble form associated with the activation of endothelial and epithelial cells. Monocytes and macrophages activated by LPS secrete proinflammatory cytokines including interleukin (IL)-1, IL-6, and tumor necrosis factor. However, neither LPB nor CD14 is a signaling molecule, so those molecules clearly did not provide a means by which LPS binding could be communicated to cells.

Genetic and Phylogenetic Routes to Discovery of Mammalian Toll-Like Receptors for LPS

The discovery of the signaling molecules responsible for LPS recognition was preceded by the crucial observation that mammals possess homologs of the Toll proteins known to trigger the fruit fly Drosophila's antifungal defenses. Screening for molecules of the innate immune system that might be phylogenetically conserved, C.A. Janeway and coworkers identified a human molecule homologous to Drosophila Toll. When experimentally expressed on cells in a constitutively active form, the human Toll-like molecule, now known as human Toll-like receptor 4 (hTLR4), triggered the release of IL-1, suggesting that it could function as a membrane receptor of the innate immune system.

TLR4 was identified as the LPS signaling molecule by genetic analysis of mutations in the murine *Lps*^{d} allele associated with LPS nonresponsiveness in C3H/HeJ mice. By narrowing down their focus to a short region of mouse chromosome 4 that encompassed only two functional genes, B. Beutler and colleagues and D. Malo and coworkers were able to identify the mutated protein in C3H/HeJ, as well as in a second LPS-insensitive strain C57BL/10ScCr, as murine Toll-like receptor 4 (mTLR4). Genetic disruption of the mTLR4 gene by S. Akira and coworkers resulted in a strain of TLR4-/- mice that were defective in LPS responses, providing evidence that mutations confined to this molecule are responsible for the phenotype. Recently, J.C. Chow and colleagues reported that hTLR4 also mediated responses to LPS when the molecule was expressed in human cell lines, demonstrating that the human version of TLR4 is responsible for LPS recognition by human cells.

Therefore, TLR molecules serve as critical cell signaling molecules in antibacterial responses in both mice and humans. Because species differences exist in the recognition of variant LPS molecules, murine and human TLR molecules may exhibit distinct specificities for LPS; however, this remains to be demonstrated. It may also be possible that distinctive adapter molecules or coreceptors, much like CD14 and LPB, combine with LPS to form the precise complex that triggers TLRs, conferring species specificity. For example, although hTLR4 transfected into LPS unresponsive mouse cells

did not unilaterally confer LPS sensitivity, cotransfection of hTLR4 plus a second molecule, termed human MD-2, a newly-discovered protein that forms a complex with hTLR4 on the cell surface, resulted in LPS responsiveness. In addition, specific interactions with the mammalian plasma membrane may also have a role in LPS signaling because LPS uptake into cells and its localization near the Golgi are correlated with cellular responsiveness to LPS. Therefore, there are many additional complexities to LPS recognition that need to be understood, but TLRs provide a central focus for understanding the triggering of immune responses.

Versatile Role of Toll-Like Receptors in Innate Immune Responses

Recently, the gram-positive bacterial cell wall components peptidoglycan and lipoteichoic acid were shown to induce signaling in cells transfected with TLR2. Interestingly, TLR2 was shown to be specifically recruited to the phagocytic vesicles of macrophages that had engulfed gram-positive bacteria or yeast. Unlike LPS responses, LPB and soluble CD14 were not required for gram-positive component recognition, although the presence of CD14 enhanced the recognition of low concentrations of teichoic acid and soluble peptidoglycan. Conceivably, the TLRs may participate with other adapters and recognition molecules as part of complexes that respond to various microbial structures. At least one other peptidoglycan-binding protein, called peptidoglycan-recognition protein, has been demonstrated in mammals, but whether it is linked to TLR triggering remains to be established.

Bacterial lipoproteins, another unique class of microbial compounds known to trigger innate immunity, were recently shown to also signal through human TLR2. Again, CD14 enhanced the recognition of the bacterial lipoprotein, and, as with peptidoglycan recognition, the murine TLR4 did not appear to play a parallel role to the human TLR2.

These observations support the developing view that the innate immune system possesses a complex armament of proteins devoted to the recognition of unique microbial components. Many of these proteins may be adapter molecules that bridge or otherwise facilitate the interaction of microbial compounds with TLRs or other signaling receptors. At least six Toll-like receptor molecules are found in mammals, and they may serve various recognition functions in innate immune responses. Most likely, given the abundance of unique compounds expressed by pathogenic bacteria, fungi, and parasites, many more receptor systems may be expected to be uncovered.

Understanding the receptor pathways involved in triggering responses to microbial invaders should provide greater insight into how the innate immune system shapes T and B cell responses. For example, LPS and Toll-like receptor triggering generally leads to interleukin-12 production and T helper 1-type inflammatory responses, most likely mediated by the activation of distinct subsets of dendritic cells. Furthermore, hTLR2 triggering by bacterial lipoprotein was recently shown to also lead to programmed death of the responding monocytic cell, which may also contribute to the progression of an antibacterial immune response. The presence of alternative responding pathways triggered by different pathogens is well recognized, although the receptors involved have not yet been defined. Immunity to certain parasitic worms is strongly skewed toward antibody responses suggesting that receptor systems distinct from those involved in LPS recognition will be discovered.

Implications for Controlling Septic Shock and Vaccine Immune Responses

The discovery of Toll-like receptors as signaling molecules in bacterial recognition has many practical and theoretical implications for vaccines and therapeutics. Drugs that target Toll-like receptors or their associated molecules may offer an effective treatment for septic shock, the cause of thousands of deaths each year in the United States. Knowledge of the function and structure of these receptors should provide effective targets for drug discovery screening that could lead to a specific antagonist of the bacterial sepsis-signaling cascade. If, as indicated by data on gram-positive bacterial recognition, Toll-like receptors signal responses to a variety of bacterial cell-wall systems, Toll-blocking drugs may offer a generalized therapy for septic shock induced by a wide variety of bacterial pathogens.

Adjuvant design and development should also benefit from a better understanding of the innate immune recognition of bacteria. Although antigen-specific adaptive immunity is critical to successful vaccination and the effective control of most infections, T and B lymphocyte receptors by themselves cannot distinguish the presence of invading pathogens; they rely on signals from the innate immune system. LPS and other bacterial components are highly effective adjuvants that stimulate vigorous T and B cell responses. Toll-like receptor agonists might be designed that are able to trigger proinflammatory responses like LPS, but without the toxicity of LPS. Discovery of other receptors that function like Toll may provide the targets for controlling the induction of distinct types of immune responses, opening new avenues for the rational design of vaccine adjuvants.

Memory T Cells—Origin and Antigen Specificity

Successful vaccination is based on immunological memory—the ability of the immune system to respond with increased rapidity and potency to antigens previously experienced. T lymphocytes play a major role in

immunological memory. They persist for years after infection or vaccination and reactivate on antigenic challenge to provide accelerated T cell functions as potent effector cells as well as by promoting antibody and focusing cellular immune responses. All T cells are subject to complex selection processes for antigenic specificity, beginning in the thymus with the expansion of T cells that recognize class I or class II major histocompatibility complex (MHC) molecules, while eliminating those with autoreactive potential. The role of further selection in shaping T cells that have left the thymus is important for understanding and manipulating the specificity and vigor of memory T cell responses.

Responding T cell populations undergo a vast increase in numbers during acute infection; but once the infection is cleared, their numbers decline rapidly, leaving relatively few persistent memory T cells. New techniques for the accurate quantitation of antigen-specific T cells reveal that from several percent to nearly 90 percent of the total $CD8^+$ T cell population may be directed to a single pathogen soon after infection. However, only about 5 percent of those responding cells become memory T cells. Recent studies have focused on the differentiation of memory T cells from the pool of primary responding T cells and on their antigen specificity and affinity.

Memory T Cells Derive From T Cells Expanded in the Primary Immune Response

Molecules that are differentially expressed by memory versus naïve T cells have provided an important means of identifying memory T cells. However, tracking the development of memory cells requires a more sophisticated approach, a methodology to identify T cells early in an immune response that are destined to enter the memory T cell population. Recently, a genetically engineered reporter gene system was developed by Jacob and Baltimore that may now enable the identification of T cells that have the potential to become memory cells. The system employs transgenic mice bearing two distinctive genes whose expression marks only those T cells that are extensively activated in response to antigen-MHC. In an experimental viral infection of the transgenic reporter mice, the level of reporter-marked CD8+ T cells rose from undetectable levels before infection to 8 to 10 percent of the total CD8+ T cells. This was a significant increase, but it was only a fraction of the 50 to 70 percent of all CD8+ T cells that show antigen specificity at the peak of the response. Analyses of T cell function showed that both reporter-positive and -negative T cells could lyse virus-infected cells but that only the reporter-marked T cells were capable of cytolytic activity after long-term culture. Most important, cell transfers into naïve mice proved that only the reporter-positive population of CD8+ T cells could confer on recipients the ability to respond strongly to an initial infection, the hallmark of memory T cells. Thus, the reporter system identified a subset of responding T cells that could proceed to the memory state.

These data indicate that memory T cells must derive from T cells that are strongly activated in a primary immune response but that only a fraction of primary responders have this capability. P.G. Ashton-Rickardt and collaborators made similar observations, demonstrating that only the low percentage of cells that had gained effector activity during *in vitro* culture with antigen and antigen-presenting cells could give rise to memory T cells after transfer *in vivo*. Only after several cell divisions could memory T cell precursors be detected, suggesting that complete effector cell differentiation needed to be achieved before memory T cells could develop. The availability of these new experimental systems should facilitate the further analysis of the cellular and molecular basis of memory T cell development in infection and vaccination.

Antigen Receptor Affinity Selection in Memory T Cells

The strength of the interaction between antibodies and their specific antigens has long been known to significantly increase between primary and secondary immune responses. However, until recently it was unknown whether T cell receptors (TCR) likewise underwent such "affinity maturation." Estimates of the TCR affinity of bulk T cell populations were indirect, relying mainly on measurement of proliferative or cytotoxic T cell responses to graded doses of purified antigens. The recent application of peptide-MHC tetrameric reagents to affinity measurements allows research to focus on individual antigen-specific T cells and provides quantitative data on a large number of T cells taken directly from the responding organism, without complicated steps involving tissue culture. Two groups, led by E. Pamer, investigating class I MHC-restricted $CD8^+$ CTL, and M. Davis, studying class II MHC-restricted $CD4^+$ T cells, have recently reported tetramer affinity measurements. Both groups demonstrated that the dissociation rates of peptide-MHC tetramers binding to primary and secondary T cells could be used to evaluate TCR affinities for a large sample of T cells in responding populations.

In primary T cell responses, both $CD4^+$ and $CD8^+$ T cells exhibited a range of TCR affinities, with both high- and low-affinity T cells well represented. However, secondary T cells behaved more uniformly in their antigen-binding properties. Secondary T cells, both $CD4^+$ and $CD8^+$, exhibited an increased avidity for antigen-MHC as compared with their respective primary populations. Data suggest that a fast-decay component of the primary T cell response is absent in secondary T cell populations, resulting in a higher overall affinity of recalled memory T cell responses.

To extrapolate from our understanding of antibody affinity maturation in secondary responses, T cells that

are able to recognize lower concentrations of antigen may have a selective advantage and result in predominate responses. However, the precise mechanism by which this occurs is not yet known. Current data indicate that both affinity for antigen and other, as yet unknown, factors contribute to the selection of the subset of cells that can proceed to the memory pool. A great deal is being discovered about T cell recognition of antigen, especially the capacity of the TCR to distinguish subtle differences in peptide antigens and the role of adhesion and costimulatory molecules in T cell triggering. Although many complex cellular and molecular pathways need to be cleared, the availability of vastly improved methodology and the refined understanding of memory T cells will greatly accelerate the development of approaches to manipulate memory responses more effectively by vaccination.

Conclusions

These recent strides in the molecular understanding of pathogen recognition and T cell memory are two examples of basic immunological advances important to vaccine design and development. Knowledge of the initial triggering of the innate immune system by Toll-like molecules and similar receptors can be exploited to develop more effective immunogens and adjuvants. Identifying those responding T cells that have the potential to become memory cells should permit strategies to elicit longer lasting vaccine protection. Both the translation of basic findings into practical vaccine approaches and new basic studies focusing on vaccine-relevant topics will greatly benefit from increased interactions and communications between basic immunologists, microbiologists, and clinical researchers.

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Toward a Preventive Vaccine for Type 1 Diabetes

Dr. Charles J. Hackett

The Need for a Type 1 Diabetes Vaccine

Type 1 diabetes, previously known as insulin-dependent diabetes mellitus or juvenile onset diabetes, is an autoimmune disease that affects more than 13,000 children and young adults each year in the United States. Because the disease usually attacks people at a very young age, there is a lifelong burden on the affected individuals and their families imposed by the demands of disease management, care for deteriorating health, and the tragedy of a shortened lifespan. Certainly, we need improved treatments for those already suffering from type 1 diabetes. However, treatment of an existing disease is always less desirable than prevention.

The prospect for prevention of type 1 diabetes needs to be considered at this time. In its 1999 report, "Vaccines for the 21st Century: A Tool for Decision Making," the Institute of Medicine of the U.S. National Academy of Sciences concluded that the development of vaccines to treat or prevent prevalent autoimmune diseases, including type 1 diabetes, would bring exceptionally high economic and health benefits to our society [http://books.nap.edu/html/vacc21/]. The congressionally established Diabetes Research Working Group also issued a report in 1999 titled "Conquering Diabetes: A Strategic Plan for the 21st Century" that recommended intense research on the immunological basis and prevention of type 1 diabetes [http://mantis.cit.nih.gov/temp/diabetes/cd.pdf].

A diabetes vaccine—a preventive treatment conveniently administered to young children to provide long-term protection—requires major research and development efforts. There are currently no vaccines against any autoimmune diseases. Insufficient knowledge of immunization strategies and of key immune response parameters are major roadblocks to definitive clinical trials at this time. Nevertheless, recent success in preventing autoimmune diabetes in animal models, as well as the development of new methods to control undesired T cell responses in transplant graft rejection, suggests that a vaccine approach to the prevention of type 1 diabetes may eventually be possible. This report will summarize the rationale for considering a vaccine for type 1 diabetes at this time, including highlights of basic research needed to facilitate pivotal clinical trials.

Type 1 Diabetes—Cause and Insight Into Prevention

Disease etiology

Type 1 diabetes results from the destructive attack by the body's own immune system on the insulin-producing beta cells of the pancreatic islets of Langerhans. Once the beta cells are destroyed, survival depends totally on insulin supplementation. Leading the attack are thymus-derived T lymphocytes specific for self-major histocompatibility complex (MHC) molecules plus antigenic fragments derived from proteins of the pancreatic beta cells. Both CD4+ T cells that recognize antigen in context of MHC class II molecules and CD8+ T cells, which are class I MHC restricted, participate in diabetes autoimmunity. Distinct pancreatic antigens are known to be targeted by the autoreactive T cells, including insulin itself and glutamic acid decarboxylase enzymes (GAD). The currently known antigens may or may not include the true disease-initiating antigen(s) in humans and most likely do not constitute the universe of antigens that may be targets of autoimmune T cells in human disease.

There is a variable interval between the initial evidence of autoimmune responses to pancreatic antigens and the diagnosis of diabetes. During this time, autoantibodies to pancreatic antigens arise. Although the role of autoantibodies in the disease process is not yet clear, recent observations suggest that the presence of two or more antibodies to pancreatic antigens is a strong indicator that diabetes will eventually develop in at-risk individuals.

Why only certain individuals mount an autoimmune response to their insulin-producing cells is not yet fully understood; data suggest that both a genetic predisposition and outside triggering factors are required. Particular MHC types are more likely to develop diabetes, but there is less than 50 percent concordance for the development of type 1 diabetes between identical twins of diabetics, demonstrating that unknown precipitating factors also play a major role. The precipitating factors may be environmental agents or infections. Why a certain combination of genetic susceptibility and triggering events leads to an immune attack directed to specific pancreatic antigens is not well understood. Potentially, T cells that react with a pathogen may also cross-react with beta cell antigens in certain individuals, as has been proposed for Coxsackie B4 virus. Alternatively, a coincidental infection, particularly one that can infect the cells of the pancreas, might activate, by proinflammatory signals, autoreactive T cells in genetically susceptible individuals.

Which approach to prevention?

The three major factors in autoimmune disease development—genetic susceptibility, triggering events, and immune reactivity—represent potential targets for preventive strategies. Each component of disease development should theoretically be a therapeutic target; the central issue is feasibility. Control of genetic susceptibility seems to be out of reach of current techniques. Eventually, gene therapy methodology might

permit the insertion of protective versions of genes or replacement of faulty alleles in genetically at-risk people. However, considerable progress in understanding the functions of implicated genes as well as in gene delivery and expression is required for this to be a realistic approach. Similarly, prevention of disease-triggering events is not yet ready for consideration as a preventive strategy. There is too much uncertainty about the precipitating agents to focus on blocking them. At this time, control of immune responses to self-antigens is the most promising approach.

Immunological control of harmful T cell responses

Approaches designed to specifically control deleterious immune responses are now entering clinical studies. Unlike broad immunosuppression, only the undesired responses would be eliminated, leaving an otherwise intact immune system to defend against disease. The major safeguard against autoimmunity is the elimination of overtly autoimmune T cells in the thymus. However, many studies have convincingly shown that immune regulation of T cells outside the thymus occurs continuously throughout life. Insight into the regulation of immune responses throughout the body provides potential approaches that could be exploited to control immune-mediated diseases. “Immunological tolerance” is the general term for the group of approaches whose goal is to regulate the cells that participate in injurious immune responses.

Recent data from basic and preclinical studies demonstrate that it is possible to intervene in the molecular steps leading to antigen-specific T cell activation, disabling antigen-specific immune responses. In one of the best current examples, long-term tolerance of genetically unmatched kidney transplants has been accomplished in nonhuman primates by transiently blocking a needed signal, termed costimulation, that is required for naïve T cells to become activated in the presence of specific antigen. T cells that are exposed to the transplant where costimulatory processes are blocked are rendered inactive for prolonged intervals.

In addition to costimulation, the immune system possesses additional mechanisms for controlling T cell activity, offering a number of possible approaches to be exploited clinically. These include T cell receptor antagonism by altered antigenic peptides, opposition of inflammation by induction of regulatory cytokines, direct activation of negative signaling receptors on T cells, and deviation of responding T cell responses away from their harmful inflammatory activities.

Regulatory T cells can also be induced that exert a modulating effect on other T cells in the vicinity. A regulatory T cell of one antigenic specificity can control T cells of different antigenic specificities, as long as they are in close proximity; this is termed “bystander control.” The significance of this observation is that it is possible that not all the antigens involved in diabetes need to be discovered and targeted to successfully control immune responses.

How does bystander control of T cells work? There is a broad hypothesis that lack of activity is the default state in the immune system, unless pathogen invasion triggers the body’s inborn alarm system. This is logically and experimentally supported by observations that autoimmune diseases are relatively uncommon and that the body tolerates most foods, normal flora, and pregnancy, and because the immune system quickly returns to normal after conquering an acute infection. Data in animal model studies suggest that autoantigen presentation, in the absence of additional concomitant signals that indicate the presence of an infection, signals cells to become inactive rather than autoaggressive. In the case of diabetes prevention in naturally diabetic mouse strains, insulin or similar antigen presented in the absence of signals that would send the immune system on the attack results in the induction of T cells that, when they recognize antigen (which would in this case be in the pancreas locale), secrete substances that counteract inflammation. The cytokines interleukin-4 (IL-4) and TGF-$ are believed to be the major mediators of bystander control.

Bystander control of autoimmune diabetes in animal models

Successful prevention of diabetes has been demonstrated in animal models by administration of pancreatic antigens intravenously, subcutaneously, orally, or through the skin or nasal mucosa. Mechanistic analyses have shown that T cells induced by the successful protocols act to counter inflammatory processes in the pancreas. Individual pancreatic antigens such as insulin, GAD proteins, or their peptide fragments can block autoimmune diabetes in murine models. Although many different antigens participate in the autoimmune disease process and the true initiating autoantigen may not be known, biological control of T cell responses can be achieved by suitable administration of single antigens found in the pancreas and their ameliorating effects can spread to bystander T cells of different antigenic specificities in the pancreas.

Interestingly, pathogenic T cells may not be the direct targets of the regulatory T cells but rather may be affected indirectly via antigen-presenting cells (APC) that serve the pancreas and the associated pancreatic lymph nodes. Current data indicate that APC, chiefly tissue dendritic cells, hold the key to activation of T cells by their abilities both to present antigen-MHC and to provide the other signals needed for full T cell activation or, most important, for T cell inactivation. The IL-4 and TGF-$ secreted by T cells may signal APC that homeostasis should be maintained, and this may result in provision of appropriate molecular signals to T cells in the vicinity, leading to local control of immune reactions.

Take-home lessons from animal studies

How important are these findings in animals for the development of a human diabetes vaccine? There is a wide biological gulf between humans and the current animal models—mouse and rat strains—with no nonhuman primate models available to bridge the gap in preclinical studies. Furthermore, immunization approaches that work superbly in rodents are generally much less effective in humans. Understanding the basic differences between murine and human diabetes and immune responses is a high priority. Results from animal research have repeatedly shown, however, that the more refined the understanding of underlying mechanisms, the more generally applicable it is across species. For example, although few pathogens afflict both humans and mice, the mechanisms of immune responses are very similar between species, to the extent that, when refined to the cellular and subcellular levels, many components of murine and human immune responses are interchangeable. Another example is the way that class I MHC molecules are loaded with antigenic peptides to become targets for T cells. This process requires many complex enzymes, chaperone proteins, and molecular transport systems, and these components and the steps involved are similar for mice and humans. Therefore, if the underlying principle of an immunological mechanism can be refined to a molecular level in mice, it stands an excellent chance of being applied in humans, once the corresponding human components are identified and characterized. This suggests that a starting point for choosing a vaccination approach to move forward with should be one that is strongly supported on the basic mechanism level.

Current diabetes prevention trials in humans

Throughout the world, there are currently more than half a dozen sizeable clinical trials to test measures to delay or prevent the onset of type 1 diabetes (table 1). These include insulin administration by injection, oral routes, or inhalation; administration of nicotinamide; and cow's-milk-free diets. It is too early to tell whether any of these strategies has a positive effect, but these approaches are generally safe. Valuable information should accrue, perhaps providing an indication of effectiveness that could be further studied to provide a firmer basis for deciding future approaches. Furthermore, analyses of treated individuals' antibody and cellular responses should provide a basis for evaluating immunological parameters and possible immune mechanisms for application to future studies. These and other studies to predict individuals who will progress to diabetes, such as the Diabetes Autoimmune Study in the Young, in Colorado, provide important knowledge for defining the trial population and approach to pivotal clinical vaccine trials in the future. As will be discussed in the next section, mechanistic underpinnings provide a rational guide to many questions that cannot be approached empirically in diabetes prevention research.

Table 1. Treatments Currently Being Tested in Type 1 Diabetes Prevention Trials

Trial	Inclusion	Treatment	Location
DPT-1	Relatives, autoantibodies	Insulin subcutaneous or oral	USA
ENDIT	Relatives, autoantibodies	Nicotinamide	Europe/ Canada
TRIGR	Relatives, HLA	Cow's milk avoidance	Finland
DIPP	HLA, autoantibodies	Nasal insulin	Finland

Basic Immunology Behind Developing a Vaccine for Diabetes

What new research has to be done?

Many of the important principles needed for designing a preventive strategy for type 1 diabetes are known. Nevertheless, pivotal clinical trials are premature because potential approaches have not yet been optimized and adapted to humans, sufficient mechanistic understanding is not yet available so that problems arising in product development or trial protocols could be confidently dealt with, prediction of safety issues are incomplete, and the optimal approach to the gathering and interpretation of clinical data needs to be developed. The major step to addressing these important questions is the commitment to a long-range agenda aimed at pivotal diabetes vaccine trials. This does not mean delaying valuable smaller clinical trials that could produce important insights and uncover critical areas to be considered for larger trials; the major difference is that small clinical trials would be an integral part of the development process and would not decide the future programs. Similarly, animal research should continue vigorously on many fronts, from working out basic immune mechanisms to discovering a larger animal model of natural diabetes, but with the goal that the extrapolation to the practical human situation is paramount.

Ultimately, only a pivotal clinical trial will prove the value of a vaccine, and plans are needed for providing the knowledge needed to design an effective trial. For example, an initial phase could emphasize the human immunobiology of diabetes and its potential for immunoregulation, based on data from natural human disease, smaller clinical trials, and relevant animal model

data. From that information, revising and testing of improved approaches should lead to a strategy that could be adapted to human trials. In the second phase, clinical strategies including techniques and methods to measure relevant clinical parameters will be the focus. Information on the approach and methodology of evaluating clinical data should permit the formulation of an effective clinical trial design, including finalization of protocols, definition of the trial populations, and establishment of endpoints. The final phase will be the initiation of trial enrollment. More than one clinical trial may be envisioned; for example, increased knowledge of the human biology of diabetes obtained in the first phase might permit identification of a subset of individuals for efficient testing of specific approaches.

In 1999, the National Vaccine Advisory Committee published a report, "Lessons Learned From the Review of the Development of Successful Vaccines," which highlighted key areas shown by experience to be critical to the development of successful vaccines for infectious diseases. Importantly, all successful vaccines studied had a solid scientific base, which enabled the establishment of an optimal approach and well-defined endpoints. Forming such a knowledge base for an immunotherapeutic vaccine for an autoimmune disease will require continued and additional research efforts in immunology. The following topics in basic immunology research are especially relevant to the clinical development of approaches to the production of a type 1 diabetes vaccine.

Understanding the human immunology of type 1 diabetes and its potential for immunoregulation

Two major questions need to be answered: what immunological processes are occurring in human type 1 diabetes, and are they able to be controlled by vaccination? Current data indicate that T cells directed to pancreatic antigens are present in human diabetics, but additional evidence of altered immune homeostasis is beginning to emerge. For example, T cells belonging to a specific regulatory subset, termed NK T cells, are reduced in number in type 1 diabetic patients. This suggests that the abnormal NK T cells may play a role in the initiation or progression of autoimmunity. Whether NK T cells may be potential vaccine targets requires additional research. Another line of human immunology studies is characterizing nonprogressors—individuals who develop antibodies to pancreatic antigens but who are greatly delayed in overt disease development. Current data suggest that a subset of nonprogressors may inherit protective genes, but precisely how these may delay or prevent diabetes is not known. Further understanding of the immunological status of nonprogressors may provide additional insights into immune regulation relevant to vaccination. Basic studies of human immune responses in prediabetic, diabetic, and normal individuals, particularly monozygotic twins of diabetics, may yield many new insights into the control of autoimmune processes.

Approaches to preventing diabetes developed in animals need to be extrapolated to the human situation. Animal model research, especially using the NOD mouse, remains the most important guide for designing and developing a preventive diabetes vaccine. Mechanistic information from analyses using the tools of mouse immunology and molecular genetics can be used, not only to define new approaches but also to improve and optimize strategies. Even in NOD mice, prevention strategies are not 100 percent effective, and understanding how to improve responses in animals should be important for eventual human testing. Studies in animals need to be closely related to human information. Are human cells of predicted phenotypes and specificities demonstrable *in vitro* and *in vivo*? What is the longevity of vaccine responses in animal models, and how can that be extrapolated to humans? Do any results in mice permit definitive conclusions about which approaches may not work in humans? Development of nonhuman primate models of autoimmune diabetes would be invaluable in addressing the capacity to safely regulate autoimmune responses in humans.

Formulation of tools for clinical trials

Dosing**:** A rational guide to vaccine dosing is needed that goes beyond the conventional parameters of toxicity and immunogenicity. In immunoregulatory strategies, dose levels can swing responses from desired to undesired effects. The ultimate effect of dose level relates to the level of antigen presented by specific APC; methods to quantitate APC and presented antigen could help rationally establish a working dose range.

Monitoring response induction**:** Methods to track the development of desired responses to vaccination need to be established. Ideally, the actual cells that carry out the regulatory function induced by vaccination should be routinely identified and characterized in vaccine recipients. Newer methods, such as binding of tetrameric MHC-peptide reagents coupled with intracellular cytokine staining of cells from vaccines, may provide such information.

Evaluating the strength and longevity of vaccine-induced responses**:** Quantitative measurements of vaccine responses need to be developed that can be employed to assay vaccine-specific responses over time in individual vaccinees. Because prediction of disease onset is not precise, it is not possible to confidently define when "protection" is achieved. Because it will be impossible to "challenge" vaccine recipients as is ordinarily done in tests of vaccines for infectious diseases, it is important to know at what points vaccine-induced responses wane or flare up in order to evaluate the course of the response. Surrogate markers of vaccine responses over time may

also indicate whether a booster vaccination may be needed even while long-term observations of disease occurrence are being carried out.

Defining the clinical trial population: Knowledge is increasing about selecting potential vaccinees at highest risk of disease development. Data from current clinical trials should provide verification of the effectiveness of the inclusion criteria. Chief among these are the presence of autoantibodies. Presence of antibodies to insulin, GAD, and the islet of Langerhans antigens IA-2, especially if more than one antibody reactivity is present, and certain HLA DR and HLA DQ molecules predict 50 to 70 percent of relatives of type 1 diabetics who will progress to diabetes. Additional refinements to reduce false positives are needed as is integration of antibody observations with other criteria. T cell reactivities are not currently useful because T cells specific for pancreatic antigens can frequently be cultured from peripheral blood of normal individuals; these may represent T cells under control by normal immunoregulatory mechanisms.

Greater knowledge of the genetic factors that may influence diabetes susceptibility should improve the ability to define trial participants. Currently, more than a dozen human genes have been associated to greater or lesser degree with diabetes. At the top of the list are genes for certain class II MHC HLA DR and HLA DQ molecules, for insulin gene expression in various tissues, and for the lymphocyte regulatory molecule CTLA-4. However, none of these genetic associations or their occurrence in combination currently can predict type 1 diabetes with a high level of confidence. This may change, however, as more of the genes involved are discovered, the function of genes in diabetes becomes better understood, and the ability to measure gene activity in various tissues increases.

Evaluating vaccine effectiveness in recipients using reasonable milestones

The ultimate standard is whether a particular vaccination treatment prevents the development of diabetes. For type 1 diabetes, which can take long to develop, markers that indicate progression to disease need to be established. Current data indicate that the development of antibodies to multiple pancreatic autoantigens may be useful to predict disease before other markers of onset, but additional biomarkers of disease development would be particularly valuable for evaluating the success of a trial.

Significance for a New Health Policy

Unlike the vaccination strategies that led to the eradication of smallpox, and soon polio, vaccines for autoimmune diseases will need to be used permanently because the disease arises from within and is not transmitted to the individual. Nevertheless, the ability to prevent rather than treat autoimmune diseases would provide health and economic returns far beyond the research and development investment. Furthermore, a vaccine for type 1 diabetes would serve as a major proof of principle and prototype for developing preventive strategies for other major autoimmune diseases. For example, more than 1 percent of the U.S. population is affected by rheumatoid arthritis, an incurable, progressively debilitating, difficult-to-manage disease. Between 250,000 and 350,000 persons nationwide suffer from multiple sclerosis, an autoimmune disease of the central nervous system. Each disease has a distinct etiology and pathogenic mechanism that needs to be understood in depth to formulate a preventive strategy. Importantly, pathological T cells appear to be involved in both rheumatoid arthritis and multiple sclerosis, suggesting that many lessons learned from addressing type 1 diabetes prevention could also apply to developing vaccines for those diseases.

Acknowledgements

The author would like to thank Drs. Charles Janeway, Jr., Noel Maclaren, George Eisenbarth, Elaine Collier, and Helen Quill for insightful discussions.

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Cytomegalovirus Vaccine Development

Dr. Christopher E. Beisel

Introduction

The public health impact of human cytomegalovirus (HCMV) infection is arguably one of the most underappreciated of any infectious disease in the United States today. This is due, at least in part, to the normally inapparent nature of HCMV infections in healthy individuals. Unfortunately, this otherwise benign virus may exact a heavy toll when it infects unborn children or the immunocompromised.

Approximately 50 percent of the United States population is seropositive for HCMV. Seropositivity varies with socioeconomic status and geographic location: 40 to 60 percent in middle-income groups and up to 80 percent in lower socioeconomic groups (reviewed in Gershon et al., 1997). The outcome of HCMV infection is highly dependent on the immune status of the host. Primary infection in healthy individuals is likely to be asymptomatic, or it may cause a mild mononucleosis-like syndrome. However, in patients with deficient or immature immune systems, HCMV infection can be a serious, even life-threatening problem.

Congenital Infection

Congenital HCMV is the most common intrauterine infection in the U.S., occurring in 0.4 to 2.3 percent of all infants born alive (Stagno et al., 1986). It is estimated that 37,000 to 40,000 infants in the U.S. are born with congenital HCMV each year. Each year, about 3,000 to 4,000 infected newborn infants have symptomatic HCMV disease; of those who survive, most suffer from profound progressive deafness and/or mental retardation (Stagno et al., 1986). An additional 4,500 to 6,000 children who are asymptomatic at birth also develop serious handicaps (Fowler et al., 1992). The highest risk for congenital HCMV infection is among infants born to mothers who have had primary infection during pregnancy (Griffiths and Baboonian, 1984). In the U.S., congenital HCMV may be the cause of 20 to 40 percent of congenital deafness and is as frequent a cause of mental retardation as is the fragile X-chromosome. Putting this in an historical context, it has been estimated that congenital CMV infection causes more CNS damage than did either congenital rubella or *Haemophilus influenzae* type b meningitis prior to the routine use of vaccines against these infections. The cost of custodial care for severely affected children in the U.S. is estimated at $1.86 billion annually (Porath et al., 1990).

Organ Transplants

HCMV is the single most important infectious agent affecting organ transplant recipients, with at least two-thirds of these patients developing HCMV infection or reactivation 1 to 4 months after transplantation. In addition, about 15 percent of bone marrow transplant recipients develop HCMV pneumonia; without treatment, such infections are fatal about 80 percent of the time. Although less severe, active HCMV infection occurs in 20 to 60 percent of all liver transplant recipients (Demmler, 1991).

AIDS-Associated Infection

HCMV causes several distinct neurological syndromes in patients with AIDS. Earlier in the AIDS epidemic, HCMV retinitis occurred in 6 to 15 percent of AIDS patients, and HCMV enterocolitis occurred in at least 2.5 percent of AIDS patients. Recently, the number of new cases of HCMV retinitis has declined dramatically, most likely due to the introduction of highly active antiretroviral therapy (HAART) and consequent improved survival.

Coronary Artery Disease

HCMV was the first of several infectious agents associated with the development of coronary artery disease. Data from numerous studies of the native heart—incorporating serology, PCR, and assessments of active viral replication—are not entirely consistent, and the association of HCMV with primary heart disease remains controversial. A stronger association has been established between HCMV infection and coronary atherosclerosis after heart transplantation. Indeed, preliminary studies have shown that prophylaxis with the antiviral ganciclovir can reduce the incidence of post-transplant coronary artery disease (Valantine et al., 1999). Current investigations focus on possible cellular and molecular mechanisms by which HCMV might mediate arterial pathogenesis.

The Need for a Vaccine

The striking public health need for an HCMV vaccine is highlighted in a report by the Institute of Medicine (IOM) (Stratton et al., 1999). This report was commissioned by NIAID for the purpose of developing a model for prioritizing the Government's efforts in vaccine research and development. In their report, the IOM committee calculates the annual health care costs attributable to all HCMV infections to be approximately $4 billion. Based on the

potential of an HCMV vaccine to significantly reduce morbidity and mortality and to realize substantial savings in health care costs, the IOM committee designated an HCMV vaccine as one of seven "most favorable" for development.

Issues in HCMV Vaccine Development

Protective Immunity

Although the correlates of HCMV immunity are not precisely known, clinical observations suggest that cellular immunity plays the primary role in limiting viral replication and spread, with humoral responses possibly playing an auxiliary role. In AIDS patients with an intact HCMV-specific antibody response but impaired cell-mediated immunity, infection can lead to severe disease. In bone marrow transplant recipients, robust HCMV-specific $CD8^+$ T cell responses correlate with protection and recovery from disease. Further, infusion of *ex vivo* expanded HCMV-specific $CD8^+$ T lymphocytes reconstitutes immunity and provides protection against disease (Walter et al., 1995). Despite the dominance of cellular responses, humoral immunity may also contribute to protection. Maternal antibody in seropositive women appears to significantly reduce both the incidence and severity of congenital infection, and passive immunoglobulin therapy may benefit some transplant recipients (Fowler, 1992; Snydman et al., 1987). Similar observations regarding the roles of cellular and humoral responses have been made in the murine CMV (MCMV) model. Clearance of MCMV from all major organs, except the salivary gland, requires $CD8^+$ class I–restricted CTL (reviewed in Koszinowski et al., 1990). Antibody plays a role in limiting the dissemination of the virus but it is not required for resolution of primary infection.

The major HCMV protein target for neutralizing antibody is the surface glycoprotein gB. This protein induces the development of both antibody and T cell-mediated immunity, and the T helper-cell response to gB is HLA class II restricted (Rasmussen et al., 1991). Despite the presence of gB neutralizing antibodies, the virus can be reactivated and infections caused by other strains of HCMV can occur; in fact, multiple strains of HCMV have been identified. The primary target for cell-mediated responses appears to be the viral tegument protein pp65 (from the UL83 gene). This protein elicits strong CTL (McLaughlin-Taylor, 1994) and proliferative (van Zanten et al., 1995) responses, and is the target of the majority of CTL precursors following natural infections (Boppana and Britt, 1996; Wills et al., 1996). Other viral antigens, including the surface glycoprotein gH and additional early antigens, are also candidates for inclusion in a polyvalent HCMV vaccine.

Immune Evasion

HCMV employs several strategies that prevent the host immune system from recognizing infected cells and which could potentially hinder the ability of a live attenuated vaccine to stimulate a protective cellular immune response. HCMV interferes with MHC class I antigen presentation by protecting viral antigens from proteolysis; inhibiting transporter associated with antigen processing (TAP)-dependent peptide translocation; dislocating newly-synthesized MHC class I glycoproteins from the endoplasmic reticulum (ER) to the cytosol; and forcing retention of peptide-loaded class I molecules in the ER. More recent studies have also demonstrated interference with MHC class II expression and function. Recognition by $CD4^+$ T cells is inhibited by destruction of the HLA-DRα and DMα components of the MHC class II pathway, and possibly by blocking class II expression through disruption of the Jak/Stat signaling pathway. HCMV may also, in the face of reduced MHC class I expression, inhibit NK-cell-mediated killing through the expression of "decoy" molecules homologous to class I glycoproteins (reviewed in Farrell et al., 1999). Finally, HCMV may interfere with cytokine-mediated antiviral and immunoregulatory responses by blocking IFN-α signal transduction and by encoding functional chemokine receptors that sequester CC chemokines. The effect that any one of these immune evasion strategies may have on vaccine efficacy is unknown, and substantial further research is needed.

Animal Models

The fact that HCMV infects only humans complicates efforts to understand its pathogenesis and the nature of protective immune responses, and to evaluate potential vaccines prior to clinical testing in humans. Several model systems have been developed using related cytomegaloviruses derived from rodents and primates. These viruses differ from HCMV to varying degrees in their genetics, replication, and pathogenesis. The rodent and primate systems are useful for developing hypotheses related to vaccine development to be tested with the human virus. They cannot, however, be considered accurate predictors of how HCMV behaves in humans: the safety and efficacy of an HCMV vaccine can be assessed accurately only in humans.

Murine CMV (MCMV) is the best-characterized and most widely used cytomegalovirus animal model. MCMV has been particularly valuable for defining the immune responses to infection and for testing specific vaccine approaches to control infection, although not disease. Some aspects of MCMV pathogenesis are relevant to human disease (e.g., retinitis in the context of immunodeficiency), although it is not a model for congenital infection. The MCMV genome has been sequenced, revealing a substantial number of reading

frames that encode homologues of HCMV proteins. The availability of immunologic reagents and transgenic mice with specific immune defects has allowed the host immune response to MCMV infection to be dissected in great detail, and these findings generally parallel those seen in humans infected with HCMV. Viral clearance is mediated principally by $CD8^+$ T cells (reviewed by Koszinowski et al., 1990), and several viral genes specifically interfere with immune responses mediated by MHC class I, MHC class II, and NK cells. A variety of vaccine strategies have been shown to protect mice against lethal MCMV challenge, including live-attenuated virus, subunits (gB), plasmid DNA, inactivated virions, and peptides bearing CTL epitopes.

Guinea pig CMV (GPCMV), in contrast to MCMV, is a useful model for evaluating vaccination against congenital infection. Guinea pigs infected with GPCMV experience both transplacental transmission (reviewed in Griffith and Aquino-de Jesus, 1991) and hearing loss in congenitally infected pups (reviewed in Woolf, 1991). While GPCMV is less well characterized than MCMV, the homologues of the major HCMV immunogens gB and gH have been identified. Only limited vaccine studies have been done with GPCMV to date: tissue culture passaged attenuated virus protects against transplacental transmission (Bia et al., 1984), and purified viral proteins provide some protection against transmission (Harrison et al., 1995).

Rat CMV (RCMV) is often used for studies of CMV infection with transplantation and has been shown to exacerbate post-transplant coronary artery disease in rats (Hosenpud, 1999). No RCMV vaccine studies have been reported to date, but the model may be useful for studies of CMV-mediated heart disease.

Rhesus CMV (RhCMV) is not well developed as a model for vaccine studies, but may offer several advantages over the mouse and guinea pig systems. The pathogenesis of RhCMV is similar to HCMV regarding primary infection, congenital infection, and also as an opportunistic infection in the presence of simian immunodeficiency virus. Monkeys' humoral immune response to RhCMV infection parallels that of humans to HCMV (Baroncelli et al., 1997). While few studies have examined RhCMV genetics and replication, the limited sequence data available shows a genome organization generally collinear with that of HCMV, as well as better sequence conservation than MCMV. Additional sequence data and studies of the rhesus immune response are needed to determine whether the potential utility of the RhCMV model sufficiently outweighs the complications and expense associated with primate studies, and whether further development of this model is warranted.

SCID-hu mice have been used as a preclinical model for the analysis of several infectious agents in the context of human organ systems. For HCMV, striking differences have been seen between different strains in their ability to replicate in SCID mice implanted with human thymus and liver tissues (SCID-hu Thy/Liv; Brown et al., 1995). There is little correlation between a strain's ability to replicate in the SCID-hu mouse and its degree of pathogenesis/ attenuation in humans, but the model may, nevertheless, prove useful in the analysis of individual pathogenesis genes.

Status of HCMV Vaccine Development

Live-Attenuated Virus

A cell culture attenuated strain of HCMV, called Towne, stimulates both humoral and cellular immunity, although less so than natural infection (Plotkin et al., 1991), and it does not reactivate from latency (Plotkin and Huang, 1985). The efficacy of Towne has been evaluated in several clinical studies. In kidney transplant recipients, vaccination reduces the incidence and severity of reactivated HCMV infection (Sachs et al., 1984; Plotkin et al., 1994). In seronegative subjects, Towne provides some protection against experimental challenge with unattenuated HCMV, known as Toledo, but protection against congenital infection is less than that afforded by a natural infection (Adler et al., 1995). Improved live-attenuated vaccines are being sought through a rational design strategy. The concept has been tested in the mouse model: specific deletions engineered into MCMV can produce attenuated strains that protect against challenge (Morello et al., 1999).

Because the genes responsible for pathogenesis in HCMV are not as well defined as those of MCMV, investigators at Aviron (Mountainview, CA) are attempting to make the existing Towne vaccine more immunogenic by replacing selected parts of its genome with sequences from a non-attenuated strain of HCMV. They have identified numerous differences between the genome of the Towne strain and that of wild-type HCMV, including a large DNA segment present in the genomes of a virulent laboratory strain (Toledo) and of five clinical isolates, but not present in the Towne genome. The extensive variation in genome sequence observed between these strains may explain the differences that they exhibit in virulence and tissue tropism. The investigators have used this information to construct hybrid viruses that replace defined portions of the Towne genome with corresponding segments of Toledo (Kemble et al., 1997). The development of these and other live-attenuated candidate vaccines is complicated by several issues, including the inability to test these replicating viruses for safety in animals, and the possibility that establishment of latency could subject vaccinees to risks from reactivation disease or other long-term complications.

Adjuvanted Subunits

A subunit vaccine produced by Chiron Vaccines (Emeryville, CA), consisting of recombinant gB (produced in CHO cells) and the adjuvant MF59, has been evaluated in phase I and II trials. The vaccine is well tolerated and highly immunogenic in seronegative adults and toddlers, and it stimulates high levels of neutralizing antibody that cross-neutralize clinical isolates. Further studies are needed to establish the ability of this vaccine to prevent infection or disease.

Vectored Subunits

A canarypox vector expressing gB (ALVAC-CMV (gB)) has been constructed and tested by Pasteur Mérieux Connaught (Swiftwater, PA). In guinea pigs, the vaccine induces both neutralizing antibody and CTL. In phase I trials, the vaccine produced no adverse reactions, but induced little or no neutralizing or lymphoproliferative response in seropositive or seronegative subjects (Adler et al., 1999). However, a phase I trial has demonstrated that ALVAC-CMV(gB) can prime for induction of antibody responses by a subsequent immunization with the live-attenuated Towne vaccine. In subjects primed with two doses of ALVAC-CMV(gB), neutralizing titers and ELISA antibodies to gB developed sooner, were much higher, and persisted longer than for control subjects (Adler et al., 1999). The usefulness of this prime-boost strategy will need to be studied further with both live attenuated and subunit boosting vaccines.

Plasmid DNA

Immunization with DNA plasmids encoding the HCMV gB and the matrix (pp65) proteins has been evaluated in mice and induces both neutralizing antibody and CTL responses. Vical, Inc. (San Diego, CA) and Pasteur Mérieux Connaught have announced a collaborative agreement to further develop this approach, but no clinical studies have been reported to date.

Peptides

A pp65 nonapeptide has been found to induce cytolytic activity through *in vitro* immunization of human PMBC cells or *in vivo* in transgenic mice. No clinical trials have been reported.

Future Directions and Challenges

Substantial basic research remains to be done if candidate cytomegalovirus vaccines are to be designed and evaluated on a rational basis. Foremost, additional work is needed to define more precisely the key antigens and epitopes important in protecting against infection, primary disease, and reactivation. In addition, the role of immune evasion in the induction and response to host immunity needs to be clarified. To aid in the construction of live-attenuated vaccines, more information is needed on the genes that are critical for replication and pathogenesis. Finally, more information is needed on the role of HCMV in coronary artery disease and how it may affect vaccine safety.

There are several specific HCMV vaccines in the research and development pipeline that will continue to progress in their evaluation. Most developed at this point is the Chiron gB/MF59, which is presently in a phase II/III study to determine protective efficacy. A new preparation of the Towne vaccine is also undergoing phase II evaluation, with some evidence that variation in the manufacturing process may have yielded a preparation with better immunogenicity and efficacy than Towne has shown in previous trials.

Phase I testing is expected to begin soon for the Towne/Toledo chimeric live-attenuated vaccines developed by Aviron. Additional phase I and phase II testing is also likely for the prime-boost strategy, priming with Pasteur Mérieux Connaught's canarypox-expressed HCMV proteins and boosting with subunit (gB or pp65) or live attenuated HCMV. A DNA vaccine based on gB, pp65, or a combination of the two is also expected to enter phase I trials in the near future.

Finally, anticipating that a promising vaccine candidate will emerge from the R&D pipeline, researchers should plan to initiate phase III trials in support of licensure. Decisions must be made as to the best population to target for vaccination. Since the foremost public health objective is to prevent primary CMV infection during pregnancy, immunization of adolescents or women contemplating pregnancy has been suggested. Looking further ahead, researchers will need to consider approaches for immunizing seronegative solid organ transplant candidates.

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Development and Testing of *Streptococcus pneumoniae* Conjugate Vaccines

Dr. David Klein

Introduction

The pneumococcus presents one of the largest public health and economic impacts of any infectious disease. Globally, the pneumococcus remains a leading bacterial pathogen in adults and the foremost cause of morbidity and mortality in infants and children in developing countries. Patients recovering from viral infections such as measles or influenza and those already afflicted with chronic diseases such as HIV constitute especially susceptible hosts in whom mortality from the coinfecting pneumococcus is high. Overall, pneumococcal disease is a universal problem that has demanded an even greater amount of attention in recent years due to the advent of drug-resistant organisms.

Pneumococci are the largest cause of death by infectious disease in the elderly, and the cause of the majority of ear infections in young children (1,2). This organism is also an important cause of meningitis in young children and the elderly (3-5). Although ear infections in young children generally do not lead to meningitis or other serious pneumococcal diseases, they do result in costly clinic visits for the children and lost work time for their parents (6).

Although more than half of pneumococci remain susceptible to many antibiotics, many pneumococci have developed resistance to most of the antibiotics now in use—due to their indiscriminate use by physicians and health care providers. Unfortunately, this situation leaves physicians without a satisfactory means of treating patients infected with the highly resistant strains (7,8). Because laboratory confirmation of antibiotic susceptibility patterns may take several days to complete, clinicians are often forced to initiate treatment with broad-spectrum antibiotics such as cephalosporins and quinolones, thereby accelerating antimicrobial resistance (9,10). Preventing these infections with safe and effective vaccines will not only slow down the development of antibiotic resistance, but will be an extremely cost-effective way to control pneumococcal disease.

The present conventional vaccine contains a mixture of 23 different polysaccharides (11). This vaccine is not immunogenic in young children due to their inability to mount an adequate immune response to polysaccharide antigens. Additional data for groups that are considered high risk for life-threatening pneumococcal infections, such as the elderly, immunocompromised, splenectomized, sickle cell, and HIV-positive patients, also have shown only moderate to little efficacy when immunized with the conventional pneumococcal vaccine (12,13). Among the elderly population, vaccine efficacy to the licensed pneumococcal vaccine is estimated to be approximately 60 percent, but goes down appreciably with increasing age.

Much work has been done in developing pneumococcal conjugate vaccines as the next generation of promising vaccines against pneumococcal diseases. As with most T-independent antigens, the capsular pneumococcal polysaccharide vaccine induces an immune response that is short-lived and characterized by variable amounts of antibody that is disproportionately IgM (14). It also fails to produce high antibody affinity and a booster response upon repeated immunization. Because of these problems, attention has been given to the development of pneumococcal vaccines that more closely mimic protein vaccines currently used in infant and other high-risk populations. One approach that has been used to immunize infants against infections from encapsulated bacteria (e.g., *Haemophilus influenzae* type b vaccine) is to present the capsular polysaccharide antigens in a form that is more immunogenic, such as in a protein-polysaccharide conjugate vaccine (15). The coupling of polysaccharides to protein carriers such as diphtheria and tetanus toxoids, demonstrates that these polysaccharides can acquire new antigenic properties typical of "T dependent" protein antigens (16,17). They can stimulate a T cell response, as shown in experimental animals, by their ability to use T cell help to generate stronger "booster" responses on re-stimulation (18). The goal of a pneumococcal conjugate vaccine is, therefore, to convert the normally T-independent saccharide into a T-dependent antigen that will be immunogenic and efficacious in non-responsive populations (19).

The introduction of pneumococcal conjugate vaccines into the U.S. vaccine armamentarium will address many of the major concerns about changing patterns of pneumococcal infection. It is anticipated that these new second generation vaccines will help: 1) offset drug resistance and reduce antibiotic usage; 2) protect against the spread of uncontrolled invasive strains of pneumococci; 3) reduce the incidence of pneumococcal otitis media and tympanostomy; 4) reduce carriage and household/ community transmission of pneumococci; and 5) promote a significant degree of herd immunity. A more effective vaccine for pneumococcal infection, therefore, represents a major public health priority.

Conjugate Vaccine Development

Four major companies are involved in the development and manufacture of pneumococcal conjugate vaccines.

Each of the products vary slightly in their chemical composition and construct (e.g., different carrier proteins), and each of the products are in different stages of clinical development (Table 1). There is a select group of important serotypes that is now included in all conjugate pneumococcal vaccines due to their relative importance in the pathogenicity of the disease. The 7-valent vaccines contain serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F, while the 9-valent vaccines add serotypes 1 and 5, and the 11-valent vaccines add serotypes 3 and 7F. The protein carriers used to date are all functional entities and include tetanus toxoid, diphtheria toxoid, CRM 197 (a mutant protein of diphtheria toxoid), meningococcal outer membrane complex, and nontypeable *Haemophilus influenzae* outer membrane protein (Table 1).

Widespread vaccination against a select group of common and important serotypes of *Streptococcus pneumoniae* could reduce infant mortality and protect against antibiotic resistance by inducing titers of mucosal antibodies sufficient to eliminate nasopharyngeal carriage. Based on the use of a 7-valent vaccine, a highly efficacious vaccine would have the potential to prevent up to 85 percent of invasive pneumococcal disease and 65 percent of pneumococcal otitis media in U.S. children (20). Little clinical value will accrue for U.S. children from the addition of serotypes 1, 3, 5, and 7F to a 7-valent conjugate vaccine. The 9- and 11-valent vaccines were designed to benefit individuals in countries outside the United States as well as special high-risk groups (e.g., Eskimos and Native Americans).

When developing pneumococcal conjugates, the number of vaccine serotypes included in the construct is limited because of the need to conjugate each serotype as an individual entity. For now, the 11-valent vaccine appears to be the industry standard, although there has been some discussion to add an additional two serotypes to the mix. In order to avoid confusion within the medical community, the FDA would likely favor a uniform approach among the different vaccine manufacturers regarding the number of valences that are included in each of the multivalent pneumococcal conjugate vaccines.

Clinical Data that Contributed to the Characterization of Conjugate Vaccines

Some of the earliest and most significant clinical studies to date have been conducted in Finland. Table 2 shows the results of four separate clinical trials all conducted in a common setting using four different conjugate pneumococcal vaccines administered at 2, 4, and 6 months of age (21). Each trial used a similar protocol and incorporated common laboratory procedures that included a standardized ELISA assay. All four vaccines were considered safe, with no indications of any serious adverse events. After three immunizations at 2, 4, and 6 months, all four vaccines also induced significant levels of antibody to each of the serotypes tested. Some serotypes were more immunogenic than others (e.g., 14 and 19F), while serotypes 6B and 23F were considerably less immunogenic. This appears to be the trend for all the pneumococcal conjugate vaccines regardless of manufacturer. Unfortunately, it is not possible to make any true comparisons here since the studies were not done head-to-head and the vaccines were not optimal end-stage formulas, but early prototype vaccines.

Another study demonstrated the ability to boost a primary response among infants immunized with a 4-valent conjugate vaccine at 2, 4, and 6 months of age (Table 3)(22). One group of infants was primed with a conjugate vaccine and then boosted with a conjugate vaccine at 14 months, while a second group was primed with conjugate vaccine, but boosted with plain polysaccharide at 14 months of age. The data indicate that antibody levels declined significantly (two- to fourfold) by 14 months following the initial priming immunization. However, 1 month following a booster dose of vaccine, there was a six- to tenfold increase in antibody activity for each of the vaccine serotypes in both study groups. Furthermore, more than 90 percent of children in both groups had antibody concentrations >1.0 µg for all serotypes measured. The results indicate that pneumococcal conjugate vaccines confer properties of T-dependent antigens to polysaccharides and elicit immunological memory and that it is possible to boost with a polysaccharide vaccine after priming infants first with a conjugate vaccine.

The capacity of pneumococcal conjugate vaccines to prime for an anamnestic response was also observed in a study conducted in The Gambia (23). Children who were all previously primed at infancy with either two or three doses of a 5-valent pneumococcal conjugate vaccine or three doses of a control Hib conjugate vaccine were boosted at 2 years of age with a 23-valent polysaccharide vaccine. The data reveal that three priming doses were better than two in producing consistently higher antibody levels (51 µg/ml versus 28 µg/ml, respectively) (Table 4). Antibody concentrations were measured 2 years post priming, but immediately before the boost had dropped precipitously (Pre 3 levels). The rise in antibody concentrations 10 days following the polysaccharide boost was striking, regardless if two or three priming doses were used. It was surprising to see high antibody titers to serotype 6B in Gambian children given its status as a weak immunogen. Again, these results reinforce the idea that pneumococcal conjugate vaccines are capable of priming the immune system to respond to subsequent exposures to capsular polysaccharide antigens associated with the pneumococcal organism.

Several studies have been conducted in healthy elderly individuals to determine the impact of pneumococcal conjugate vaccines in this population (24, 25). In one

such study, adults received either one of four conjugate vaccines or a 23-valent polysaccharide vaccine (Fig. 1)(26). The results are expressed as a ratio of conjugate vaccine/ polysaccharide vaccine GMCs, with ratios >1.0 indicative of a better response for the conjugate vaccine and ratios <1.0 indicative of a better response for the polysaccharide vaccine. In general, IgG antibodies to serotypes 6B and 23F were greater following immunization with a conjugate vaccine (with one exception), and antibodies to serotype 14 were greater following immunization with a polysaccharide vaccine. This equivocal pattern of response has prompted additional studies in the elderly with newer versions of conjugate vaccines to determine whether other vaccine schedules may provide for a more favorable outcome.

In addition to measuring antibody concentrations following vaccination, it is important also to evaluate the qualitative characteristics of antibody production (i.e., avidity and opsonic antibody activity) that are important in assessing the effectiveness of pneumococcal conjugate vaccines (27). To address this issue, a study was conducted in which infants were primed with a tetravalent conjugate vaccine at 2, 4, and 6 months of age and boosted at 14 months with either the same conjugate vaccine or a 23-valent polysaccharide vaccine (28). An increase in the avidity of antibody to the vaccine serotypes was observed only among children boosted with the conjugate vaccine, but not among the recipients of plain polysaccharide (Fig. 2). Avidity was shown also to correlate with functional opsonic antibody activity. These results may have significant cost implications with regard to vaccine strategies. Although data indicate that boosting with a plain polysaccharide vaccine produces a significant increase in antibody activity to each of the serotypes contained in the priming vaccine, the functional activity of these antibodies compared to antibodies following a boost with a conjugate vaccine may be inferior and, therefore, provide for a lesser degree of protection. It is also possible that boosting with a conjugate vaccine may improve the duration of the protective antibody response (29).

There was concern whether children less than 2 years of age with recurrent respiratory infections who failed to respond to polysaccharide vaccine would be able to respond to a pneumococcal conjugate vaccine. It is estimated that between 5 to 10 percent of children with recurrent respiratory infections are unresponsive to the conventional polysaccharide vaccine (30). To examine the effectiveness of a conjugate vaccine in this population, children 2 to 13 years of age with no known immunodeficiencies were recruited into the study (Fig. 3). All the children received a pneumococcal polysaccharide vaccine followed 6 months later by either a heptavalent conjugate vaccine or plain pneumococcal polysaccharide (31). The results indicate that poor responders to the polysaccharide vaccine are able to respond more significantly, overall, to a single dose of conjugate vaccine, with increased antibody concentrations to all serotypes tested. Immunization with conjugate vaccines may, thus, represent a viable option for patients at high risk to recurrent respiratory infections who fail to respond initially to the currently available 23-valent polysaccharide vaccine.

Pneumococcal conjugate vaccines also have demonstrated tremendous value in other high-risk groups including Hodgkin disease patients (32) and HIV-infected children (33). In general, a significant increase was observed in the number of responders with antibody levels greater than 1.0 μg following one or more doses of conjugate vaccine. These results further suggest that priming with conjugate vaccines may represent a good strategy for high-risk populations.

An important consideration when administering pneumococcal conjugate vaccines to children is the potential impact they may have on colonization and transmission of the organism. A good example of the effect a multivalent pneumococcal conjugate vaccine has on nasopharyngeal carriage was observed in Israeli toddlers. Day-care children received either a single dose of a 9-valent conjugate vaccine or a conjugate meningococcal C vaccine (34). Figure 4 shows the nasopharyngeal carriage results 8 months following vaccination. Carriage rates were reduced for all vaccine serotypes as well as for penicillin resistant, multi-drug resistant, and non-resistant vaccine types, especially in children less than 24 months of age at the time of vaccination. These data indicate that conjugate vaccines are capable of reducing the colonization of vaccine-related serotypes significantly, as well as decreasing the burden of antibiotic resistant strains.

Similar studies were conducted in Gambian and South African infant populations (35, 36). Again, a reduction was observed in the pharyngeal carriage rates of serotypes associated with the 7-valent pneumococcal conjugate vaccine following its vaccination in infants (Fig. 5). Interestingly, Figure 5 also shows an increase in non-vaccine serotypes among infants in both locations who received the pneumococcal conjugate vaccine compared to a placebo (i.e., conjugate meningococcal C vaccine). These latter data suggest that replacement and/or unmasking of pneumococcal serotypes occurs in the host following immunization with a pneumococcal conjugate vaccine due to successful competition by non-vaccine serotypes. Whether this is truly a replacement phenomenon (i.e., new serotypes occupying available niches) or the unmasking of serotypes present previously, but not expressed due to the prevalence of more dominants types, or both, is unclear at this time. The real question and concern is whether replacement carriage will translate into replacement pneumococcal disease.

Recent Vaccine Trials That Define Efficacy

Currently, there are four efficacy trials in progress evaluating three different pneumococcal multivalent conjugate vaccines manufactured by two companies (i.e., Wyeth-Lederle Vaccines and Pediatrics and Merck Research Laboratories)(Table 5). The end points for these four trials vary considerably from acute otitis media to invasive disease to mortality. Recently, a large scale phase III clinical trial involving approximately 38,000 infants was unblinded (37). The trial involved the use of a Wyeth-Lederle pneumococcal conjugate vaccine and was conducted at a Northern California Kaiser Permanente HMO. The infants were randomized to receive either a pneumococcal conjugate vaccine or a meningococcal C conjugate vaccine, both produced by Wyeth-Lederle. The pneumococcal conjugate vaccine was formulated to protect against the seven most common strains of *S. pneumoniae* in the United States (polysaccharide serotypes 4, 6B, 9V, 14, 19F, 23F, and oligosaccharide serotype 18C) and included 20 μg of the carrier CRM197, a mutant form of diphtheria toxin. The infants were vaccinated at 2, 4, and 6 months of age and given a conjugate booster at 12 to 15 months of age and followed for disease. The trial was designed to have an initial look at the efficacy data once 17 fully vaccinated cases of invasive disease were documented. The results outlined in Table 6 are very encouraging and reveal 100 percent efficacy (95 percent CI = 75.7 – 100.0, $p<0.0001$) for the conjugate pneumococcal vaccine against invasive disease following either full vaccination or partial vaccination.

A randomized, double-blind safety and immunogenicity study was run concurrently with the efficacy study. The trial enrolled 212 healthy 2-month-old infants at four clinical sites. The infants received identical vaccines used in the efficacy trial (i.e., half received the pneumococcal conjugate vaccine and the other half the meningococcal C conjugate vaccine). Each child was given doses of vaccine at 2, 4, and 6 months, and those who remained in the study received a booster at 12 to 15 months of age. Results showed that the conjugate vaccine was highly immunogenic, produced a good antibody response against all seven strains of *S. pneumoniae*, was well tolerated with only minor reactions at the injection site, and caused only mild to moderate post-vaccination fever in some children (38).

During the next 12 to 18 months, several additional efficacy trials are expected to begin at four sites outside the United States (i.e., Israel, The Gambia, Philippines, and Chili) (Table 5). Many important issues will be addressed in addition to the question of vaccine safety and the affect these conjugate vaccines have on protective efficacy. These include: 1) determining laboratory correlates of protection; 2) examining interference with other childhood vaccines; and 3) evaluating the overall impact on ecology (i.e., herd immunity) and colonization. Many of the conjugate vaccines in these new trials will contain as many as 11 serotypes. In the near future, other phase II and phase III studies may be needed in special populations to adequately describe the ability of pneumococcal conjugate vaccines to serve as a good immunogens as well as protect against disease. These populations will include several high-risk groups such as sickle cell patients, the elderly, pregnant women in their third trimester, immunocompromised patients, and premature infants of low birth weight. The main objectives will be to determine whether conjugate vaccines offer any significant advantage over conventional 23-valent capsular polysaccharide vaccines with regard to safety and immunogenicity. Much of the existing data suggest that the pneumococcal conjugate vaccines will have their greatest impact in populations (e.g., infants and high-risk groups) that do not respond well to the 23-valent polysaccharide vaccine when used as a primary inoculum (39).

Alternative Pneumococcal Vaccine Strategies

The use of polysaccharide-protein conjugate vaccines, while overcoming many of the liabilities of polysaccharide vaccines, still involves a number of problems. First, new studies indicate that immunity induced by pneumococcal conjugate vaccines may be short-lived, especially in infants. Such a limitation would necessitate repeat vaccinations through the first several years of life—an expensive procedure even in relatively wealthy nations, but an even greater and prohibitive expense for the developing world where cost factors play a major role in deciding whether a vaccine gets used or not. Second, regional variations in the predominance of infecting pneumococcal serotypes necessitate the formulation of capsule-based vaccines that are appropriate to the local epidemiology. Such modifications are not only technologically difficult, but also exceedingly expensive. Third, because serotype coverage is severely restricted by the inclusion of only 11 serotypes, the capacity to promote wide protection against infections in developing countries is limited. At best, an 11-valent conjugate vaccine would cover only three-quarters of serotypes causing disease that may vary over time. Fourth, because the multivalent vaccines are made up of individual vaccines, a large total dose of carrier protein is required that may subsequently lead to carrier-induced suppression/ overload, or even anti-carrier proteins (40, 41). Last, the ability of pneumococci to change their capsular serotype as a result of uptake of heterologous DNA suggests that the protective effect of anti-capsular antibody may be all too temporary, as vaccine serotypes “deliberately” modify their surface polysaccharide in response to mucosal antibodies (42).

All of these considerations lead to the conclusion that new generations of pneumococcal vaccines will be needed to address many of these problems. Just as capsular polysaccharide vaccines have now given way to protein-

polysaccharide conjugates, the future predicts that pneumococcal surface proteins, which represent excellent virulence determinants that are immunogenic and conserved among global serotypes, will lead the way as third generation vaccines. These new surface protein vaccines most likely will be employed either as the carrier component in conjugate vaccines or as an individual vaccine combined with an adjuvant or cytokine (43-48). The use of such proteins as immunizing antigens might serve not only to prevent colonization in fully immunized hosts, but also to ameliorate the effects of breakthrough infections in incompletely protected populations such as infants.

Work in international laboratories has shown that several different pneumococcal surface proteins, such as pneumococcal surface protein A (PspA)(44, 48), pneumococcal surface adhesin A (PsaA)(45), neuraminidase (43), and autolysin (47), are able to elicit active protection against pneumococcal infection in mice when challenged with lethal doses of pneumococci. More recent data strongly indicate that antibody to PspA in human serum can protect mice from fatal pneumococcal infection (49). As a result of these studies, PspA is now being developed as a human vaccine by a major vaccine manufacturer and is currently in phase I trials. Due to the conserved nature of these surface proteins, it is possible they will provide a broader vaccine application and greater overall protection against serotypes causing pneumococcal disease than conjugate vaccines. In addition, surface protein vaccines may have the ability to stimulate a protective respiratory tract mucosal immune response when administered parenterally, something not observed generally with conjugate vaccines (50).

Even after the completion of the phase III trials and the possible licensure of pneumococcal conjugate vaccines, numerous outstanding issues and questions, both basic and clinical, remain to be addressed (as outlined in Tables 7 and 8).

Pneumococcal conjugate vaccines have evolved considerably over the past several years and appear to offer a number of opportunities for various high-risk groups compared to the licensed polysaccharide vaccines. What we know and can say about pneumococcal conjugate vaccines at this time is summarized below:

- All pneumococcal conjugate vaccines are not created equally. Furthermore, each conjugated antigen is a unique, separate vaccine with different immunological properties;
- Conjugate vaccines produce increased immunological responses compared to the plain polysaccharide vaccine;
- All pneumococcal conjugate vaccines have been found to be relatively safe and well-tolerated with no reported serious adverse events;
- Repeated injections following a priming immunization with conjugate vaccine elicits both IgG and functional antibody;
- Conjugate vaccines appear to work best in infants and high-risk groups;
- Conjugate vaccines induce immunological memory;
- Immunogenicity has been shown to vary significantly among serotypes in terms of magnitude and kinetics of response;
- Conjugate vaccines can decrease nasopharyngeal carriage rates and, thus, reduce the transmission of pneumococci in community and specialized settings;
- There are no antibody data that show what antibody concentrations are needed for protection;
- Pneumococcal conjugate vaccines may protect against the spread of uncontrolled invasive strains; and
- The routine use of pneumococcal conjugate vaccines may represent the most successful approach to decreasing the burden of antibiotic resistant strains of pneumococci.

Other new and unique pneumococcal constructs also are being developed that will induce much stronger anti-protein and anti-polysaccharide immunity following either subcutaneous or intranasal immunization and may even bypass the need for T cells and adjuvant. Mond et al. (35) have found that conjugation of lipidated molecules to polysaccharides induces high titer antibody that does not require T cell involvement. This would represent the first demonstration that immunological memory to a polysaccharide antigen can be induced in the absence of T cells. Mond and coworkers also have conjugated the hapten phosphocholine to the protein PspA using proprietary technology. This hapten has been shown to induce protective antibodies to *S. pneumoniae* infection. Immunization with this construct induces high titer antibody to both the phosphocholine and PspA components. Experiments are in progress to determine whether this antibody is more effective than antibody induced to either of the components alone. Additional approaches have been used to regulate the humoral antibody response to *S. pneumoniae* bacteria, both live and formalin fixed. The synthesis of cytokine fusion constructs (e.g., IL2 and GMCSF) with PspA produces a vaccine which when given intranasally or systemically induced a high-titer protective antibody response. Interestingly, no anti-cytokine antibodies were produced by these constructs (36).

Studies in laboratories looking at virulence factors other than polysaccharide capsule have identified pneumolysin as a major pneumococcal toxin (37).

Pneumolysin has a unique role in pneumococcal infection as it allows the bacteria to breach the tissue and mechanical barriers that otherwise confine infection to the respiratory tract. Consequently, pneumolysin is the principal means by which pneumococcus can disseminate from the lung into the blood and cause lethal infection. Because pneumolysin can induce good antibody responses, it is conceivable that future pneumococcal vaccines incorporating pneumolysin may be more effective at preventing invasive infection than the currently available licensed vaccines by its ability to neutralize pneumolysin toxin that causes lung injury (38). In this regard, pneumolysin may serve as an excellent carrier protein for future conjugate vaccines by providing additional protection to pneumococcal serotypes not included in the conjugate formulation (34). Pre-clinical studies with one such tetravalent conjugate vaccine containing 6B, 14, 19F, and 23F serotypes demonstrated both high-titered IgG ELISA antibody responses and functional (i.e. opsonophagocytic) antibody activity to both the pneumolysin and the various capsular polysaccharides (39). The antibody response to each of the pneumococcal serotypes was comparable to that observed in animals immunized with a tetravalent conjugate vaccine containing the same serotypes and a tetanus toxoid carrier.

Additional studies have revealed that interferon-gamma, a cytokine that regulates macrophage activity, is an important component of the early host defense against invasive pneumococcal infection (40). Serum concentrations of interferon-gamma rise in proportion to the virulence and degree of bacteremia produced by the pneumococcal strain in mice. Most importantly, animals that are rendered genetically incapable of producing interferon-gamma are less able to clear pneumococci from lungs and blood and are more susceptible to lethal pneumococcal infection. These studies suggest that the early mortality from invasive pneumococcal infection, which is largely unaffected by antibiotic therapy, might be reduced by augmenting host defenses through the administration of exogenous interferon-gamma at critical time points.

Capsular Transformation

For reasons that are incompletely understood, the overwhelming majority of penicillin-resistant multidrug-resistant *S. pneumoniae* isolates express a select few of the 90 different capsular types associated with the pneumococcus. These capsular types are predominantly types 6B, 14, 19F, and 23F (41). The restriction of these dangerous drug-resistant bacteria to such a few serotypes raised the hopes that appropriate conjugate vaccines, which include these few serotypes, could corner the most dangerous strains of *S. pneumoniae*. Recent work led to the discovery by which resistant bacteria could break out of this "corner" (42). The process appears to involve the acquisition of DNA molecules by multidrug-resistant strains (i.e., DNA molecules released by other pneumococci), and which carry genetic determinants of new capsular types. In a recent outbreak of multidrug-resistant pneumococcal disease among AIDS patients in New York, a most unusual phenomenon occurred—the appearance of a widely spread multidrug-resistant pneumococcal 23F strain that acquired the capsular type 3 (26). This bacterium was resistant to all antibiotics currently used against pneumococci except vancomycin. A simple test using a mouse model showed that these capsular type 3 "transformants" of the multidrug-resistant pneumococcus have increased their virulence capacity more than a million-fold over that of the same bacterium when it carries the usual 23F capsule. Evidently, the most frequent recipient of these spontaneous capsular switch events appears to be this 23F multiresistant epidemic clone of *S. pneumoniae*. Changing their surface polysaccharides can give a temporary *in situ* survival advantage to the bacterium *vis-à-vis* the immune system of the host. This can be accomplished in two ways: 1) provide a one-step mechanism to transfer resistance to several antibiotics to a new serotype, which may be a mechanism for the spreading of antibiotic resistance to other less often multidrug resistant serotypes; and 2) altering/transforming the surface properties of the bacterium and compromising immune recognition. Wide-scale deployment of pneumococcal vaccines containing the virulent serotypes may produce a selective pressure for this type of capsular switch among clinical isolates. The above finding emphasizes the importance of increased international surveillance for resistant pneumococci and should remind us all about the importance of regulated use of antibiotics not only in developed country settings, but in the international community as well (43). Understanding in molecular terms the capsular genes may also provide a rapid DNA-based typing technique as well as identify new antibacterial targets for drugs that could inhibit the production of capsular polysaccharides and, thus, make pneumococci avirulent (44).

Pneumococcal Carriage and Mucosal Immunity

Because *S. pneumoniae* is a respiratory disease, the role of mucosal immunity is critical in providing protection against pneumococcal infection. Unfortunately, little work has been done in this area. However, exciting new data have demonstrated that intranasal immunization with pneumococcal antigens can lead to protection against pneumococcal disease, and more importantly, against pneumococcal carriage in the nasal passages of mice (45). This discovery may be critical to the eventual control of pneumococcal disease. Pneumococci are spread by person-to-person contact. They are found/carried in the nasal passages of between 10 and 50 percent of humans, depending on their age and health status. High pneumococcal carriage rates are frequently found in young infants born to parents in developing countries. These rates are generally 2 to 3 times higher than among children

in industrialized countries and often involve more than one pneumococcal serotype. In most cases, carriage does not result in disease, but in some cases the pneumococci may overcome the normal defense mechanisms of the host and invade from the nasal tissue to cause ear and eye infections, pneumonia, or meningitis. Vaccines that could prevent carriage would, theoretically, be able to prevent the spread of pneumococci and ultimately its ability to cause disease. With this in mind, several studies have been conducted with conjugate vaccines demonstrating the ability of these vaccines to reduce the nasopharyngeal carriage of vaccine-type pneumococci in infants and children (46,47). This reduction has also been shown to involve drug-resistant pneumococcal species. There is some concern that this reduction in carriage may increase the prevalence of non-vaccine types of pneumococci due to the competitive replacement in the nasopharynx by different pneumococcal serotypes that colonize the host, but are not under vaccine control. This is an important phenomenon and must be examined seriously in the future since such replacement would be counter-productive, especially if it invoked new pathogenic types (47,48).

Population Shifts in the Colonizing Flora of Pneumococci Following Vaccination with Conjugate Vaccines

The findings reported earlier in the West Gambian and Israeli studies (46,47) each documented a very substantial decrease in the prevalence of vaccine type pneumococci in the nasopharynx following two or three doses of a 9-valent pneumococcal conjugate vaccine. The degree of change, in fact, approaches the effect of low concentrations of antibiotics. Therefore, the conjugate vaccine appears to exert a powerful selective pressure. It is not clear whether the reduction in the vaccine types of pneumococci involves elimination of the resident flora or protection against re-colonization.

In analyzing the population shift, one can envision at least three kinds of scenarios: 1) the pneumococci "replacing" the vaccine type bacteria may represent a quantitative replacement (i.e., occupation of the vacant ecological sites) by serotypes not included in the vaccine; 2) the serotype shift may represent the survival of pneumococci that were present as a minority population; and 3) the vaccine pressure may select for capsular transformants (i.e., original residents of the nasopharynx escaped the immune pressure by switching to a new capsular type through the acquisition of a new capsular determinant). Such a capsular switch may be advantageous for the bacteria since it is quite likely that the original pneumococcal residents of the nasopharynx are bacteria that carry an optimum constellation of genetic determinants required for effective adherence (and/or virulence). These determinants do not involve the polysaccharide capsule, but rather the cell wall and cell surface proteins.

Antibiotic-resistant strains of pneumococci are almost invariably restricted to the pediatric types 6B, 9V, 14, 19F, and 23F. Some of these penicillin-resistant lineages, particularly those associated with serotypes 9, 14, and 23, have achieved a virtual global spread and were shown to be capable of causing the entire spectrum of pneumococcal diseases (26). These serotypes were also shown to be the most frequent colonizers of the nasopharynx of children. As indicated in the previous section, spontaneous switch of capsular type has been reported with increasing frequency in the recent microbiological literature. These switches have involved capsular transformations in which type 23 pneumococci changed their capsule to either type 14, 9, 19, or 3. In this last serotype switch, the acquisition of type 3 capsule was accompanied by a 10^6-fold increase in mouse virulence for the strain. It is curious that in many of the cases observed so far, the recipient was a strain that expressed the 23F capsule and represented one of the most widespread penicillin resistant clones that have been recovered both in pediatric and adult invasive disease. This newly expressed clone is also a frequent colonizer of the nasopharynx of children attending day-care centers (42).

Relationship Between Pneumococci Causing Colonization and Those Causing Disease

While it is generally agreed that the nasopharyngeal flora of children represents the main ecological reservoir of *Streptococcus pneumoniae*, opinion is divided as to the relationship between bacteria colonizing the nasopharynx and bacteria that cause pneumococcal disease. On one level, expression of distinct genetic traits favoring colonization (i.e., adherence to epithelial cells) versus invasion has been described in pneumococcal strains in the form of a system of phase variation (49). On a different epidemiological level, recent studies have supported arguments in favor of the notion that disease-causing pneumococci are recruited from among bacteria that achieve prior colonization. Based on molecular fingerprinting techniques, data indicate that the same genetic lineages of penicillin-resistant pneumococci that are widespread among colonizing flora are also frequent among bacteria that can be recovered from sterile sties both in pediatric and adult disease (50). Nevertheless, the possibility exists that highly virulent pneumococci have only a very short residence time in the nasopharynx.

Role of Phosphorylcholine in Pneumococcal Pathogenesis

The most common use of antibiotics is in the treatment of respiratory tract infections. The specific etiologic agent(s) responsible for such infections is seldom identified and, therefore, any antibiotic would need to target the major pathogens of this site, *S. pneumoniae, Mycoplasma*

pneumoniae, and *Haemophilus influenzae.* Because these represent different classes of organisms, many of the antimicrobial agents used today are broad spectrum, which results in undesired effects on the normal flora and promotes the acquisition of resistance. A common mechanism among these bacteria that allows for colonization of the human respiratory tract would represent an ideal target for a highly specific antibiotic or vaccine. Recent work has described a genetic locus present in all strains of *H. influenzae*, which decorates its lipopolysaccharide with the host-like structure phosphorylcholine (ChoP)(51). This highly unusual bacterial structure is also found on the pneumococcus, meningococcus, and pseudomonas (52-54), and has recently been identified on the surface glycolipid of mycoplasma (55) as well as six additional pathogens that reside on the mucosal surface of the human respiratory tract (56).

The ChoP structure appears to be critical for the viability of *S. pneumoniae* in the host environment and the ability of both *H. influenzae* and *S. pneumoniae* to be carried within the nasopharynx (57). The three major pathogens of children—pneumococcus, meningococcus, and *H. influenzae*—all share a set of unusual characteristics. They colonize the nasopharynx and cause respiratory, vascular, and meningeal infections, they are naturally transformable, and they lyse and die in stationary phase. It is now clear that they also share choline on their surface that contributes to the ability of these pathogens to thrive in the human respiratory tract and interact with innate host defenses. Recent studies have suggested that this "formula" defines a highly regulated and successful program for pulmonary infection that is driven by the biology of lung fluid choline. The importance of ChoP in mycoplasma has not yet been addressed. Choline is not described in other species of medical significance with the exception of a few members of the genus Actinobacillus (58). The fact that this molecule may actually contribute to the success of several different microorganisms in colonizing the mucosal tract is extremely interesting and could provide important insights to understanding how microorganisms establish themselves in the host environment.

The key gene required for choline incorporation, *licA,* appears to be a choline kinase based on the presence of the putative reactive site for choline kinases in eucaryotes. This gene is also present in mycoplasma and a similar sequence has been identified in pneumococcus by PCR. Other than the active site, there is no significant sequence homology between the bacterial choline kinases and the eucaryotic choline kinases (including human). This bacterial choline kinase would, thus, represent a rational target for novel antimicrobial therapy and prophylaxis aimed specifically at treating or preventing lower respiratory tract infections due to the major pathogens *S. pneumoniae*, mycoplasma, and nontypeable *H. influenzae* (59,60). Inhibition of the choline kinase gene may specifically interfere with the pathogenesis of disease caused by these organisms without promoting microbial resistance.

Pathogenesis of Pneumococcal Meningitis and Identification of Genes Involved in Antibiotic Tolerance of *S. pneumoniae*

Bacteria travel from the nasopharynx to the lung, blood stream, and meninges by using adhesive proteins to bind to human cell receptors. Five adhesive proteins, the first ever described for pneumococcus, have been cloned, sequenced, and demonstrated to convey adherence for the bacteria (61). The regulatory mechanism affecting which adhesin is presented when in the growth cycle of the bacteria has also been identified. Surprisingly, the ability to stick to human cells is regulated as is the ability to take up DNA for transformation (which is key to developing antibiotic resistance) and the ability to lyse and die in stationary phase (a process downregulated in antibiotic tolerant bacteria)(62). Thus, a highly regulated and successful program has been charted as to how the pneumococcus enters and travels throughout the human host. A key aspect of the mechanism of this targeting is the presence on the bacterial surface of choline, an important constituent of human lung fluid, and of the inflammatory mediator, platelet activating factor (PAF)(63). Understanding the control mechanism and the proteins that participate in binding to the lung is a big step toward designing a pneumococcal vaccine and to finding non-antibiotic, novel ways to interrupt the development of pneumonia.

Translating this information to medical therapeutics has been undertaken by Tuomanen et al. (64). Antagonists of PAF receptor are available and when deployed in animals with pneumonia, the disease is cleared as effectively as with antibiotics. Furthermore, using an excess amount of the sugars that pneumococci bind to on human cells washes the bacteria out of the lung. These properties suggest ways to improve the outcome of disease that do not involve killing bacteria and ways to stop colonization from developing into disease that could be used to prevent the spread of virulent strains. Finally, the ultimate goal of understanding the participants in the trafficking of bacteria is the development of a pool of proteins from which a pneumococcal vaccine can be developed. This possibility has become much more tangible as the pool now stands at approximately 12 highly protective candidate antigens.

Tuomanen et al. (65) have also identified two bacterial gene clusters that are important in allowing penicillin to kill pneumococci. The proteins encoded by the genes act as triggers to dissolve the bacteria. Without them, penicillin does not work and if the proteins are added as drugs, they kill the bacteria and help penicillin work much better against strains not killed by penicillin alone. This is a new concept in antibiotic design and a new pathway

in bacterial physiology. These proteins, therefore, control the way cells are destroyed in response to antibiotics as well as play a significant role in determining the efficacy of antibiotics and the facility to generate resistance to antibiotics.

Genomic Research

One of the great technological advances of this generation has been the genome projects undertaken to decipher the DNA code of life. One of the landmarks has been the sequencing of free living organisms that began with the bacterium *H. influenzae* (66). The complete sequence of 12 additional bacterial genomes has now been completed (67), along with a eukaryote (the budding yeast, *Saccharomyces cerevisiae*)(68). The sequencing of more than 50 bacterial species is currently underway including the completion of two strains of *S. pneumoniae* (i.e., serotypes 4 and 23). In many respects, the sequencing and inspection of a bacterial genome may be the equivalent of a "walk on the moon," with molecular biologists emerging as modern "micronauts" (69).

The new challenges presented to the microbiologist are to use the genomic information contained on the computer to select for genetic elements to solve biological relationships. Several computer-assisted approaches have been taken to analyze bacterial genomes. Uptake sequences for genetic transformation in *H. influenzae* were identified in a computer-assisted search with known uptake sequences as templates (70). Loci associated with LPS biosynthesis in *H. influenzae* were identified by searching for protein homologues associated with this biosynthetic process found in other organisms (71). Using similar approaches, Masure et al. have identified a family of 12 surface proteins (choline binding proteins) that bind to the teichoic and lipoteichoic acid of the cell wall of pneumococcus using as a template the C-terminal repeat choline binding domain common to these proteins (72). These proteins were eventually shown to be linked to virulence determinants, potential adhesins, and several metabolic processing enzymes, all essential for designing new therapies to fight gram positive diseases, specifically those caused by pneumococcus. Because the choline binding proteins are surface exposed, they represent excellent candidates for the next generation of pneumococcal vaccines.

To identify elements related to genetic transfer, cis-acting regulatory elements were sought that matched a competence induce promoter (CIP). Six competence induced loci were identified that compose a regulon linked by the CIP consensus sequence TTTT(N9)TACGAATA. All of these genes appear to be activated during genetic transfer, they encode DNA processing proteins, and they are controlled by a two-component sensor regulatory system (73). Computer-assisted searches, therefore, represent an important tool in the effort to identify coordinately regulated genes in any organism using the product of genome sequencing.

Summary

The last decade has successfully exploited many of the advances in basic science research and applied them to the field of vaccines. During this time, scientists also have uncovered a number of potential obstacles, including the following:

- Data suggest that co-administration of different vaccines may be less effective than administration of the individual vaccine components;
- There are indications that priming with protein carriers has had variable effects upon subsequent immunization with protein-polysaccharide conjugate vaccine. Such priming has been shown both to enhance as well as suppress the response to the polysaccharide depending on the chemical and physical characteristics of the conjugate, timing of injection, and age of administration;
- The availability of new delivery systems and adjuvants has demonstrated a tremendous heterogeneity in vaccine efficacy, with some adjuvants enhancing the responses to some immunogens, while having no effect on the response to others;
- Accessibility of vaccines to countries of the developing world due to expanded technological expertise (e.g., conjugation of proteins to polysaccharides);
- Vaccines effective in neonates are not necessarily as effective in a geriatric population (e.g., conjugate vaccines) or in a T cell deficient HIV population; and
- Many effective vaccines are not suitable for mucosal immunization, which may prove to be a useful route of administration.

To overcome many of these problems, new novel approaches have been designed to address the above issues including the use of microencapsulated antigens, DNA vaccines, live vector vaccines, and the use of relevant cytokines to duplicate the physiologic immune system. These new approaches will hopefully allow for the development of biological systems that may lead to new generations of pediatric and geriatric vaccines for *S. pneumoniae.*

Table 1. Selected characteristics of glycoprotein conjugate pneumococcal vaccines

MANUFACTURER	PROTEIN CARRIER	LINKER	SACCHARIDE LENGTH	VACCINE SEROTYPES	CLINICAL STUDIES
Pasteur/Merieux/ Connaught	Tetanus toxoid/ diphtheria toxoid	Short linker	--	8 valent: 3,4,6B,9V,14,18C,19F,23F 11 valent: 1,3,4,5,6B,7F,9V,14,18C,19F,23F (D carrier-3,6B,14,18C) (T carrier-1,4,5,7F,9V,19F,23F)	Phase I/II Phase I/II
Wyeth/Lederle Vaccines & Pediatrics	CRM 197	Reductive amination (amine)	long	7 valent: 4,6B,9V,14,18C,19F,23F 9 valent: 1,4,5,6B,9V,14,18C,19F,23F 11 valent: 1,3,4,5,6B,7F,9V,14,18C,19F,23F	Phase III Phase III Preclinical
Merck Research Laboratories	OMP-meningococcus B	Bivalent linker (thioether)	long	4,6B,9V,14,18C,19F,23F	Phase II/III
SmithKline Beecham	Non-typeable H. influenzae OMP	--	long	4 valent: 6B,14,19F,23F 11 valent: 1,3,4,5,6B,7F,9V,14,18C,19F,23F	Phase I Phase I

CRM 197 = CRM - a nontoxic variant of diphtheria toxin

D - diphtheria toxoid

T - tetanus toxoid

OMP - outer membrane protein complex

Table 2. Immune response in infants to four different quadrivalent pneumococcal conjugate vaccines administered at 2, 4, and 6 months of age

Antibody concentration (µg/ml) at 7 months of age				
Vaccine	6B	14	19F	23F
Pn-CRM	0.40	2.50	0.79	1.10
Pn-OMP	1.30	8.27	9.85	1.90
Pn-D	0.88	2.30	5.29	0.88
Pn-T	0.77	3.06	3.20	0.67
Pn-CRM – Wyeth/Lederle Pn-OMP – Merck Research Laboratories Pn-D – Pasteur/Merrieux/Connaught Pn-T – Pasteur/Merrieux/Connaught				

Table 3. Infants primed with a 4-valent pneumococcal conjugate vaccine at 2, 4, and 6 months and boosted at 14 months

GMC µg/mL in infants at various months of age									
Vaccine				PS 6B			PS 23F		
Study Group	Primary	Booster	n	7	14	15	7	14	15
1	PncD	PncD	36	0.81	0.39	2.62	0.75	0.21	1.17
2	PncD	PncPS	35	1.45	0.49	3.03	0.71	0.29	2.79
PncD = conjugate of PSs of serotypes 6B, 14, 19F, and 23F to diphtheria toxoid PncPS = 23-valent pneumococcal PS vaccine									

Table 4. Antibody concentrations after a PncPS boost at 2 years of age in Gambian infants primed with either three doses of a Hib conjugate vaccine or with two or three doses of a 5-valent pneumococcal conjugate vaccine

Antibody Concentration (µg/ml)				
	None	Pre 3	Post 2	Post 3
6B	0.37	0.89	27.62	50.90
14	0.73	1.98	15.28	30.52
18C	1.62	0.30	5.82	7.26
19	0.54	0.99	5.70	10.33
23F	0.28	0.63	4.17	6.64

Obaro et al., *PIDJ* 1997; 16:1135.

None = immunized 18 months previously with a Hib conjugate vaccine
Pre 3 = antibody levels 2 years after priming
Post 2 = received two doses of conjugate vaccine at 2 and 4 months
Post 3 = received three doses of conjugate vaccine at 2, 3, and 4 months

Table 5. Ongoing and planned efficacy trials with pneumococcal conjugate vaccines

Site	Vaccine	Starting year	Endpoint
USA (recently completed)	PncCRM - 7 Valent	1995	Invasive Disease
Finland	PncCRM - 7 valent PncOMPC - 7 valent	1995	Otitis media
USA (Navajo/Apache infants)	PncCRM - 7 valent	1998	Invasive Disease/Herd Immunity
South Africa	PncCRM - 9 valent	1998	Invasive Disease
Gambia (phase II ongoing)	PncCRM - 9 valent	1999	Mortality/Morbidity
Israel	PncD + T - 11 valent	1999	Otitis media
Phillipines	PncD + T - 11 valent	1999	Invasive Disease
Chili	PncD + T - 11 valent	2000	Invasive Disease

Table 6. Efficacy results for invasive pneumococcal disease

Number of Cases			
Group	Vaccine Gp.	Control Gp.	Serotypes
Fully Vaccinated	0	17	6B, 9V, 14, 18C 19F, 23F
Partially Vaccinated	0	5[1]	6B, 14, 19F, 23F
Non Vaccine Serotype	3[2]	5	3, 10F, 11A, 18B, 19A, 23A, 38

[1] three with one dose, two with two doses

[2] two fully vaccinated and one partially vaccinated

Table 7. Questions after current efficacy trials

- Persistence of antibodies and protection
- Impact on the epidemiology of pneumococcal infections
- Potential indications in other target groups:
  - Premature infants, pregnant women, elderly individuals, immunocompromised, individuals including HIV-positive infants, nephrotic patients, splenectomized subjects, and bone marrow transplant recipients
- Optimal use of the vaccine with regard to:
  - Schedule, combinations, immunization route
  - Possible interference with other childhood vaccines
  - Cost effectiveness
- Benefit of adding adjuvants to improve immune response
- Need for additional serotypes and improved formulations
- Standardization of laboratory methodology for total and functional antibody
- Role of CMI in protection
- Search for surrogates of protection
- Role of capsular transformation on conjugate vaccine effectiveness

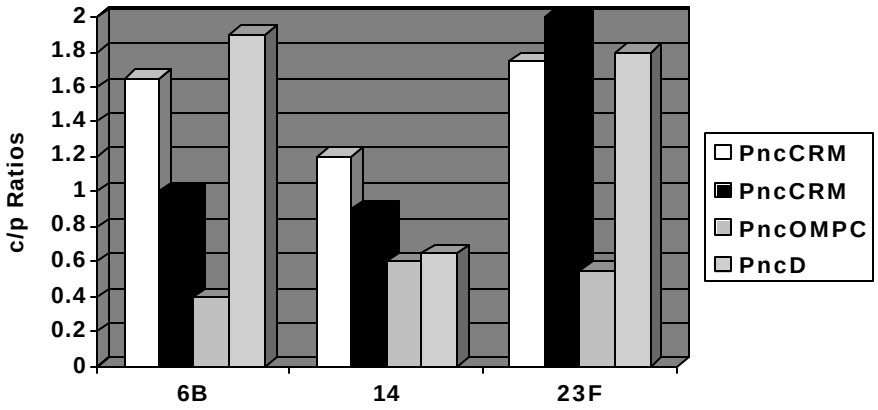
Table 8. Problems associated with pneumococcal conjugate vaccines

- Cost of producing vaccine and limited potential in developing countries
- Need for multivalent products made up of individual vaccines
- Need for different formulations, dosages, and combinations of serotypes to accommodate different populations, age groups, and geographical needs
- Problem with carrier-induced suppression or overload due to large total dose of carrier protein
- Current vaccine covers, at best, only three-quarters of types causing disease that may vary over time
- Majority of pneumococcal morbidity is associated with mucosal infections that conjugate vaccines do not address
- Each conjugated antigen is a unique, separate vaccine with different immunological properties - makes for a very complex product

Table 9. Summary

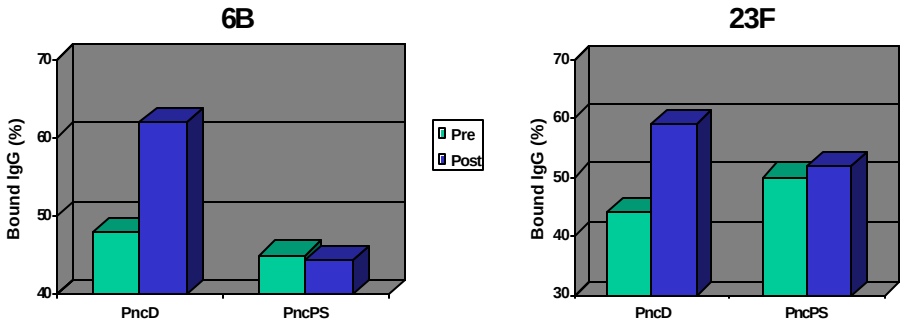
- Overall, conjugate vaccines produce increased immunological responses compared to the plain polysaccharide vaccine
- All pneumococcal conjugate vaccines have been found to be relatively safe & well tolerated with no reported serious adverse events
- Repeated injections following a priming immunization with conjugate vaccine elicits IgG and functional antibody
- Conjugate vaccines appear to work best in infants and high risk groups
- At least one pneumococcal conjugate vaccine has been shown to protect against invasive disease
- Conjugate vaccines induce immunological memory
- Immunogenicity has been shown to vary significantly among serotypes in terms of magnitude and kinetics of response
- All conjugate vaccine antigens are not created equally
- Conjugate vaccines can decrease nasopharyngeal carriage rates and thus reduce the transmission of pneumococci in community and specialized settings
- At this time, there are no antibody data that show what antibody concentrations are needed for protection
- May protect against the spread of uncontrolled invasive strains
- The routine use of pneumococcal conjugate vaccines may represent the most successful approach to decreasing the burden of antibiotic-resistant strains of pneumococci

Fig. 1 Ratio of GMCs (Conjugate/PS) After Pneumococcal Vaccination in Healthy Adults



Powers et al., *JID* 1996; 173:1014
Storek et al., *CID* 1996; 25:1255
Nieminen et al., *Vaccine* 1998; 16:313
Wuorimaa, unpublished

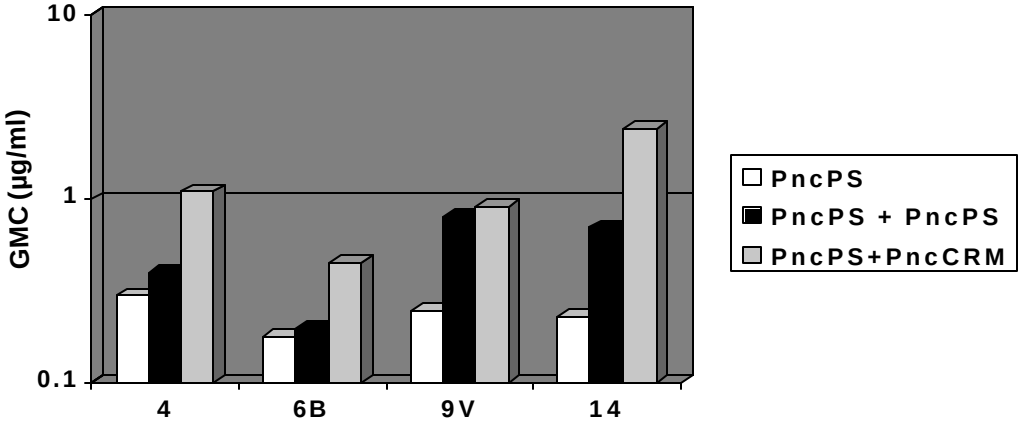
Fig. 2 Increase in Avidity of Antibodies After Booster Vaccination



PncD - PMC pneumococcal conjugate vaccine
PncPS - 23-valent pneumococcal polysaccharide vaccine

Anttila et al., *JID* 1998; 177:1614

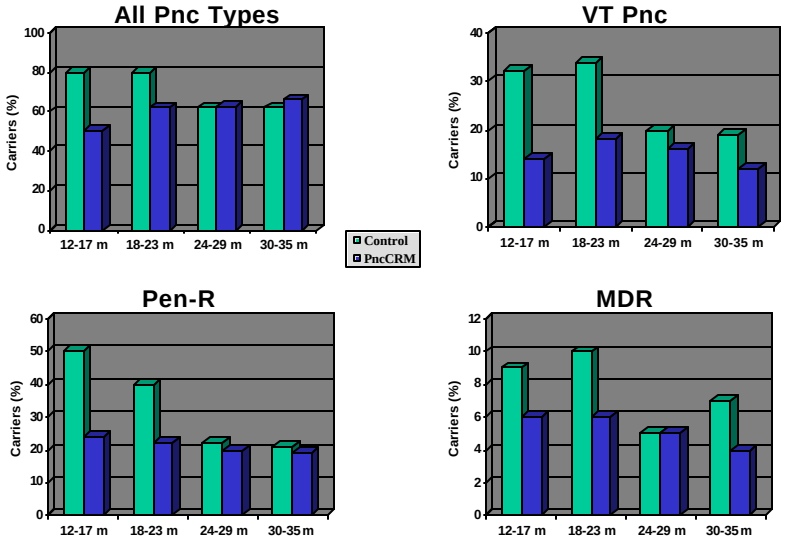
Fig.3 Response to PncPS or PncCRM in Children with Recurrent Infections



PncPS - 23-valent pneumococcal polysaccharide vaccine
PncCRM - Wyeth-Lederle pneumococcal conjugate vaccine

Sorensen et al., *PIDJ* 1998; 17:685

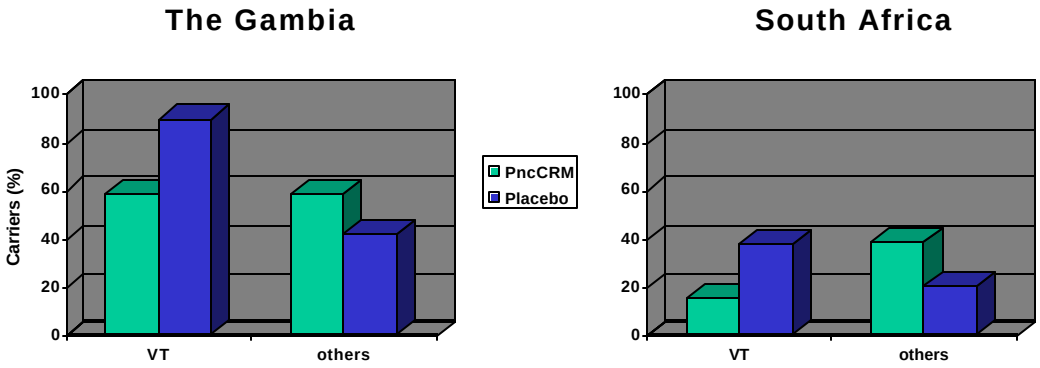
Fig. 4 Pneumococcal Carriage After PncCRM in Israeli Toddlers



PncCRM - Wyeth-Lederle pneumococcal conjugate vaccine
Pnc - pneumococci
VT Pnc - vaccine type pneumococci
Pen-R - penicillin resistant types
MDR - multi-drug resistant
Control - meningococcal C conjugate vaccine

Dagan et al., *ICAAC* 1998

Fig. 5 Reduction of Pharyngeal Carriage After PncCRM Vaccination in Infancy



Obaro et al., *Lancet* 1996; 348:271

Mbelle et al., *ICAAC* 1997

PncCRM - Wyeth -Lederle pneumococcal conjugate vaccine
Placebo - meningococcal C conjugate vaccine
VT - vaccine types
Others - non-vaccine serotypes

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Note: For additional references please contact the author

Global Alliance for Vaccines and Immunization—A Millennial Challenge

Dr. G.J.V. Nossal

Two programs are revered in the annals of world public health. They are the global eradication of smallpox and the Expanded Programme on Immunization (EPI) that took the coverage for the six common childhood diseases from 5 percent of children in developing countries to about 80 percent. Although the World Health Organization (WHO) had chief responsibility for the execution of these programs, there were and are many other important players in global vaccine deployment and in the search for new and improved vaccines. Recognizing this, five founding partners, WHO, the United Nations Children's Fund (UNICEF), The World Bank, the United Nations Development Programme (UNDP), and The Rockefeller Foundation, launched the Children's Vaccine Initiative (CVI) at the World Summit for Children in New York in 1990. CVI had as its main tasks applied research and development toward new and improved vaccines and strategic work in priority setting, coordination, and advocacy. Acting as an umbrella body for all the partners in the vaccine continuum, including developing country experts, foundations, nongovernmental organizations, national development aid agencies, pharmaceutical and biotechnology companies involved in vaccines, and the academic community, CVI performed many important studies, liaised with industry, and convened a major discussion forum every 2 years that allowed all relevant issues to be fully aired. Excellent work was done in all these areas, and great credit is due to CVI's sponsors and particularly to its coordinator, Dr. Roy Widdus.

Reinvigoration of Global Immunization Required

Nevertheless, by early 1998 it had become apparent that there were serious limitations in global vaccine programs. Immunization coverage had plateaued at about 80 percent in 1990 and, in some countries, was actually dropping. In many areas, the infrastructure for vaccine delivery was deteriorating. Although the World Health Assembly had mandated that hepatitis B and yellow fever vaccines should enter the EPI in relevant countries, this was happening far too slowly. UNICEF, the chief funder of universal childhood immunization, was seeking to deploy resources elsewhere. The World Bank, having identified vaccines as a most cost-effective public health tool, was doing very little to help immunization although 11 percent of its new lending was in health. Research into vaccines of interest predominantly to developing countries was lagging and in clinical trials was proving increasingly difficult to finance. Among the aid community, clear signs of donor fatigue were emerging. It was time to search for a new beginning.

In March 1998, the President of The World Bank, James D. Wolfensohn, convened a summit meeting in Washington to address these issues. The Director-General-Elect of WHO, Dr. Gro Harlem Brundtland, was present as was the Executive Director of UNICEF. Importantly, the five largest vaccine companies were represented, mainly at the chief executive level. There was general agreement that the imminent licensure of several new vaccines represented an opportunity to save lives that was just too good to miss. There was, however, a general skepticism that the necessary resources could be found quickly. A working party was formed to explore all possible issues in a systematic manner through a series of workshops and consultations involving all stakeholders, and the working party was asked to report back to a second summit meeting. This process took almost 1 year of hard work.

Bellagio Summit

In March 1999, the second summit took place at The Rockefeller Foundation's Study Center in Bellagio, Italy, under the author's chairmanship. It was in some respects a difficult meeting because some industry participants had been hoping that a large sum of money might have materialized to give renewed impetus to global immunization, and because some United Nations agencies feared that there might have been a hidden agenda for setting up some new and independent structure. At the meeting, a consensus emerged that what was required was a mechanism, not *independent* of but in fact intimately *dependent* on the chief players, an alliance subject to the interacting parties and to which the three chief UN agencies (WHO, UNICEF, and The World Bank) pledged themselves absolutely. This alliance was to be served by a small professional secretariat. A further outcome of the Bellagio Summit was the creation of three working groups on financing mechanisms, advocacy, and country coordination. The need for a fourth working group on research and development emerged through later discussions.

Embryology of GAVI

By June 1999, plans had evolved to the stage where it appeared prudent to wind up the CVI and to replace it with a Global Alliance for Vaccines and Immunization (GAVI). The distinguished Norwegian immunologist, Dr. Tore Godal, who had led WHO's Tropical Diseases Research Programme for more than a decade, was recruited to head the coordinating secretariat, and Dr. Brundtland was appointed to chair the GAVI Board for the

initial 2 years. In November 1999, another meeting in Geneva resolved to recommend to GAVI that a research and development component was required. The chief responsibilities of this component would be the commissioning of batches of candidate vaccine lots to be manufactured under Good Manufacturing Practice. In addition, this component would arrange for the conduct of clinical trials of such candidates, including the epidemiological studies, resolution of intellectual property issues, encouragement of collaboration between academic and industry sectors, and, where appropriate, technology transfer to developing countries.

At the time of writing (December 1999), the plan is to officially launch GAVI on January 31, 2000, at the World Economic Forum, in Davos, Switzerland. A full understanding of what is to be launched requires an explanation of the role of the Bill and Melinda Gates Foundation.

The Bill and Melinda Gates Children's Vaccine Program

In mid-1998, a separate but related agenda had begun to stir. Stimulated by WHO, Dr. Gordon Perkin, who was then the Health Adviser to the Bill and Melinda Gates Foundation, explored the possibility that immunization might become a priority activity for the Foundation, with the nongovernmental not-for-profit Program for Appropriate Technology in Health (PATH) acting as the implementing agency. A study chiefly conducted by Drs. James Maynard and Richard Mahoney devised a plan, and discussions with the President of the Foundation, William H. Gates, Sr., and eventually with his son, the Chairman of Microsoft, William H. Gates, Jr. (Bill), and his wife Melinda progressed rapidly. By December 1998, Bill and Melinda Gates were able to announce a $100 million program over 5 years to speed the introduction of new vaccines into developing countries. This initial seeding grant was not for the purchase of vaccines. Rather, it was for the assessment of disease burden in various countries through epidemiological and field studies, clinical trials of new vaccines, feasibility studies, the identification of possible roadblocks to vaccine introduction, and advocacy so that decisionmakers both in developing and industrialized countries could be better informed about the value of childhood immunization. A Strategic Advisory Council to the Bill and Melinda Gates Children's Vaccine Program (CVP) was formed, which the author has the great honour of chairing. Dr. Mark Kane, formerly of WHO, is the Director of CVP, and Dr. James Maynard is the Technical Director. The Council had its first meeting in Seattle in March 1999 and its second in September 1999. The third meeting will be in February 2000. The first meeting decided to place chief emphasis on four pathogens of global significance, namely, hepatitis B, *Haemophilus influenzae* type b, *Streptococcus pneumoniae*, and rotavirus, as well as two of regional significance, namely, Japanese B encephalitis and yellow fever.

By the time of the second meeting, the munificence of the Gates Foundation had expanded enormously. Another $50 million had been made available for research into a malaria vaccine, this to come under the CVP umbrella with the Advisory Council suitably expanded and Dr. Regina Rabinovich, formerly of the National Institute of Allergy and Infectious Diseases, Public Health Service, as the responsible officer. Independent of CVP, $25 million had been found for research into an HIV/AIDS vaccine, $25 million for a tuberculosis vaccine, and $50 million for eradication of poliomyelitis.

Global Vaccine Fund

Pivotally important was the decision to make available the huge sum of $750 million for the purchase of the newer (nontraditional EPI) vaccines, including one for hepatitis B. This was through a Global Vaccine Fund, which would sit within UNICEF but be subject to an independent board, which would take its technical instructions from the GAVI Board. Over the next few months, intense effort will go into enlarging the Global Vaccine Fund. Indeed, it is intended that the Gates benefaction be leveraged to the greatest possible extent. Development aid agencies, other foundations, high net-worth individuals, service organizations, and indeed all conceivable sources of philanthropy will be approached. However, it is not too early to make plans for the expenditure of some of the money that is available. The present intention is to solicit expressions of interest from countries that meet three criteria: a Gross Domestic Product (GDP)/head of population of less than $1,000; population size less than 150 million; and infant immunization coverage rate of at least 50 percent. This last criterion is to ensure that the country in question has at least a degree of commitment to immunization and a modicum of relevant infrastructure. It is probable that some countries will be selected for early pilot trials. In each country, national interagency coordinating committees will be encouraged to ensure the most effective implementation of the program, and country "ownership" of implementation will be strongly encouraged. About 41 of the poorest countries currently meet the criteria. Fully transparent processes of assessment are planned, and the GAVI Board will make the final choices.

At the moment, China, India, and Indonesia are ineligible to apply, and this draft policy requires some explanation. It is not intended to exclude poor children from these countries. Rather, it is felt that each of these countries has significant indigenous vaccine manufacturing capability, and it is preferable to strengthen this capability. Perhaps there is a special role here for The World Bank and the Asian Development Bank. GAVI will at all times keep in mind the concept that access to lifesaving vaccines is a human right. The moral imperative is to

minimize the gap in time between the introduction of an important new vaccine into industrialized countries and its availability in the poorer countries.

Eventually, it is hoped that the Global Vaccine Fund will contribute to research and development as well. Despite many expressions of goodwill from the vaccine industry, it is difficult for it to invest massively in research on vaccines for which there is no market in the richer countries. Current discussion centers on both "push" and "pull" mechanisms for the encouragement of such research. Push mechanisms relate to ways of directly supporting research and development by grants or loans, whereas pull involves a guaranteed market for the finished product if success is achieved.

Summary of GAVI's Tasks

GAVI's tasks divide into four priorities. The first is the global eradication of poliomyelitis. This is currently the world's largest public health program, and it has already achieved the remarkable goal of eradicating polio from the Americas, the Western Pacific-Southeast Asian region, and Europe. Transmission in Sub-Saharan Africa and the Indian subcontinent has also been materially reduced, but it is important to increase efforts in these two regions of the world, particularly in the countries impacted by conflict. It will not be possible to keep up the current expenditure of more than $300 million per annum forever. The next 2 years represent the best opportunity to finish the job. It should be remembered that when global eradication of poliomyelitis has been certified and when immunization ceases, it is the richer countries that will be the chief beneficiaries. Their annual savings will rapidly repay the investment. Nor should the public relations value of such a triumph be underestimated—a further and dramatic illustration of the power of vaccines. It is anticipated that at least part of this hoped-for "polio dividend" will be diverted to GAVI.

The second priority for GAVI will be to revitalize EPI. Coverage rates in many countries are simply unacceptable. There are real problems with infrastructure and in particular maintenance of the cold chain. Much of the equipment is deteriorating and in need of replacement. Countries of Latin America and the Caribbean have proven that vaccine coverage of 90 percent and higher is possible even in the face of poverty. It is important to learn from their example. There are also real problems of injection safety, with plentiful evidence that disposable syringes are in fact being reused and with further problems relating to safe disposal of syringes and needles. The ideal would be to employ solely single-use, auto-destruct syringes and needles, or, in the longer term, alternative routes of administration.

The third priority for GAVI will be to introduce newer vaccines into routine use so that the artificial distinction between "EPI" and "other" vaccines can disappear. The newer vaccines will of course be more expensive, at least until research and development costs have been recovered. The industry has pledged to make vaccines available to GAVI at reduced prices provided that their existing private-sector market in the more rapidly developing countries is not eroded and that leakage of contraband from poorest to more developed countries is prevented. Of course, the bigger and more secure the public-sector market, the lower the unit cost of production and thus the price. The Global Vaccine Fund could well break the "chicken and egg" situation of high prices because of small production runs and low sales volume, which presently pertains to some vaccines.

The fourth priority for GAVI relates to expanded research and development. Three diseases stand out above all others as desperately requiring an effective vaccine: HIV/AIDS, malaria, and tuberculosis. Bacillary dysentery (shigellosis) is not far behind, and as the licensed rotavirus vaccine has been suspended, its successors must also be mentioned. The newer cholera and typhoid vaccines work fairly well but could be improved. Group A streptococci remain a problem for many communities, and cancer-causing agents such as human papillomaviruses, hepatitis C, and *Helicobacter pylori* remain without an effective vaccine. Some authorities believe that pandemic "shift" variants of influenza would not be halted by the current influenza vaccine approach. Vaccines could augment drug control programs for parasites such as schistosomes or leishmaniasis. Every reader of *The Jordan Report* could add her or his favorite wish to the list. Perhaps the biggest single challenge will be to bridge the gap that separates preclinical vaccine research in academic institutions from the clinical research and extensive development work required before a licensed vaccine results.

Overall, expenditures on public health, preventive medicine, and positive health promotion represent about 5 percent of the total global health expenditure of about $2 trillion. With the success of these approaches in cost-benefit terms, including immunization, tobacco control, lifestyle changes for cardiovascular health, screening for cervical and breast cancer, and promotion of "safe sex," one senses that this area, traditionally a Cinderella of health science, is set to expand. Similarly, the total sales of vaccines represent less than 3 percent of all sales of prescription pharmaceuticals and biologicals. Because prevention is not only better than cure but also cheaper, logic suggests that a much greater global investment in vaccines would make a great deal of sense. One final reflection: there is no need to think that total eradication will be confined to smallpox and poliomyelitis forever. Eradication is feasible wherever there is a good vaccine and no animal or environmental reservoir. Measles could well be next on the agenda. Mumps, rubella, and varicella could follow. The unexpectedly great success of the Hib vaccine suggests that community control will be possible

for other encapsulated bacteria, including pneumococci and meningococci. GAVI will not be short of work for many decades. Readers of *The Jordan Report* are invited to dream on; only great dreams impel societal progress.

Appendix A

Status of Vaccine Research and Development, 2000

Target Agent	Vaccine	Basic R&D	Preclinical	Phase I	Phase II	Phase III
Ancyclostoma duodenale	Recombinant protein	+	+			
Bacillus anthracis	Recombinant subunit	+	+			
Bordetella pertussis	*B. pertussis* surface protein expressed by vector (e.g., *Salmonella* and					
	Vibrio cholerae)	+	+			
	Purified PT vaccine—acellular	+	+	+	+	+
	Recombinant PT vaccine—acellular	+	+	+	+	
	Purified PT and FHA—acellular	+	+	+	+	+
	Purified PT, FHA, pertactin, and agglutinogens 2 & 3—acellular	+	+	+	+	+
	Purified PT, FHA, pertactin—acellular	+	+	+	+	+
	Recombinant PT, FHA, pertactin—acellular	+	+	+	+	+
	PT peptides-CRM conjugates	+	+			
	Purified adenylate cyclase	+	+			
	DTP-Hib conjugate	+	+	+	+	+
	DTP-Hib conjugate-HBV	+	+	+	+	+
	DTP-IPV	+	+	+	+	
	DTP-Hib-conjugate-IPV-HBV	+	+	+	+	
	DTaP-Hib conjugate-HBV	+	+	+	+	
	DTaP-IPV—monovalent aP	+	+	+	+	
	DTaP-Hib conjugate-IPV-HBV—bivalent and trivalent aP	+	+	+	+	
	DTaP-Hib	+	+	+	+	+
	DTaP-Hib conjugatge-IPV	+	+	+	+	
Blastomyces dermatitidis	Purified yeast cell proteins (e.g., WI-1)	+	+			
	Recombinant proteins (e.g., WI-1)	+				
	WI-1 DWA	+	+			
	Live-attenuated strain	+	+			
Borrelia burgdorferi	Recombinant Osp A	+	+	+	+	+
	Osp A-based DNA vaccine	+	+			
	BCG-expressed Osp A	+	+			
	Purified Osp B, Osp C	+	+			
Brugia malayi	Purified parasite antigens (paramyosin, etc.)	+	+			
Calicivirus	Norwalk VLP's in transgenic potato	+	+	+		
	Norwalk VLP's orally delivered	+	+			
Campylobacter jejuni	Inactivated whole cell with mutant *E. coli* labile toxin (mLT) adjuvant, oral vaccine	+	+	+		
	Whole cell (intact)	+	+	+	+	
Chlamydia pneumoniae	Purified, major outer membrane protein, heat shock protein	+				
	Outer membrane protein-based DWA vaccine	+				
Chlamydia trachomatis	Major outer membrane protein (MOMP) viral vectors	+	+			
	Purified major outer membrane protein	+	+			
Clostridium botulinum	Toxoid	+	+	+	+	

Target Agent	Vaccine	Basic R&D	Preclinical	Phase I	Phase II	Phase III
Clostridium difficile	Formalin-inactivated toxins A and B	+	+	+		
Clostridium tetani	Recombinant toxin	+	+			
	Salmonella vector	+	+	+		
	Microencapsulation	+	+			
	Transcutaneous immunization	+	+			
Candida albicans	Cell surface oligomannosyl epitope	+	+			
Chikungunya virus	Live, attenuated	+	+	+	+	
Coccidioides immitis	Formalin-killed spherules	+	+	+	+	+
	Recombinant protein for Ag2, rAg2 (PRAg2)	+	+			
	Spherule homogenate (27kxg)	+	+			
	C-ASWS (Ag2)	+	+			
	Urease (recombinant and cDNA) (rURE)	+	+			
	Spherule outer wall glycoprotein (SOWgp)	+	+			
Corynebacterium diphtheriae	Recombinant toxin	+	+			
	Salmonella vector	+	+	+		
	Transcutaneous immunization	+	+			
Coxiella burnetti	Formalin inactivated	+	+	+	+	
Cryptococcus neoformans	Partially purified capsular polysaccharide	+	+			
	Glycoconjugate of capsular polysaccharide with tetanus toxoid	+	+	+		
Cytomegalovirus (CMV)	Live, attenuated strains (conventional)	+	+	+	+	
	Live, attenuated strains (engineered)	+	+			
	Glycoprotein subunit vaccine	+	+	+	+	
	Multiprotein subunit vaccine	+				
	Nucleic acid (DNA) vaccines	+	+			
	Canarypox vectored	+	+	+		
Dengue virus	Purified rDNA-expressed viral proteins	+	+			
	Infectious clone	+	+			
	Chimeric virus	+	+			
	Inactivated whole virus particle	+	+	+		
	Vaccinia vector (live)	+	+			
	Vaccinia subunit	+	+			
	Baculovirus subunit	+	+			
	Synthetic peptide	+	+			
	Micelle/ISCOM	+	+			
	Yeast subunit	+	+			
	Recombinant envelope (baculovirus and drosophila expression systems)	+	+			
	Live, attenuated dengue virus (monovalent)	+	+	+	+	
	Live, attenuated dengue virus (combined quadrivalent)	+	+	+		
	DNA vaccine	+	+			
Eastern equine encephalitis virus	Inactivated whole virus particles	+	+	+	+	
Endotoxin (Gram-negative sepsis)	Detoxified lipopolysaccharide from *E. coli* 0111:B4, Rc (J5)	+	+			

Target Agent	Vaccine	Basic R&D	Preclinical	Phase I	Phase II	Phase III
Entamoeba histolytica	Yeast subunit	+	+			
	Recombinant galactose-binding protein	+	+			
	Galactose-binding proteins expressed in *Salmonella*	+	+			
Enterohemorrhagic *Escherichia coli* (EHEC) [Shiga toxin-producing *E. coli* (STEC)]	Nontoxic mutant toxins	+	+			
	Intimin	+				
	LPS conjugates	+	+			
	Intimin expression in plants	+				
	Stx-1 beta-subunit in *Vibrio cholerae* vector	+	+			
Enterotoxigenic *Escherichia coli* (ETEC)	Killed cells and beta-subunit of cholera toxin	+	+	+	+	
	Nontoxigenic ETEC derivative, live, attenuated	+	+	+	+	
	Salmonella and *Shigella* vectored CFAs	+	+			
	Subunit synthetic toxoid (ST) and B subunit of heat-labile toxin (LT)	+	+			
	LTB expressed in potatoes	+	+	+		
	CFA II microencapsulated	+	+			
Epstein-Barr virus (EBV)	Glycoprotein subunit (gp350)	+	+	+		
	Vaccinia recombinant virus expressing gp350	+	+	+		
	Peptide induction of CTL	+	+	+		
Escherichia coli (urinary tract)	Anti-FimH adhesin	+	+			
Filoviridae (Ebola)	Recombinant subunit	+	+			
	Replicons	+	+			
Francisella tularensis	Live, attenuated	+	+	+	+	
Group A streptococcus	Glycoconjugate Group A polysaccharide with tetanus toxoid	+	+			
	M protein, multivalent type-specific epitopes	+	+	+		
	M protein epitope expressed in a commensal vector (*S. gordonii*)	+	+			
	Cysteine protease	+	+			
	C5a peptidase	+	+			
	Fibronectin-binding protein Sfb1	+	+			
	Streptococcal pyrogenic exotoxins	+	+			
Group B streptococcus	Glycoconjugate vaccines of type Ia, Ib, II, III, and V polysaccharides linked to carrier proteins		+	+	+	+
Haemophilus ducreyi	Major outer membrane protein	+	+			
	Hemolysin/cytotoxin	+	+			
	Hemoglobin receptor	+	+			
Haemophilus influenzae (nontypeable)	Recombinant protein subunit containing either P1, P2, or P6 proteins to serve as carriers in conjugate vaccine preparations	+	+			
	Recombinant protein subunit containing P4 and P6	+	+			
	Subunit Hi nontypeable 47 OMP (adjuvanted)	+	+			

Target Agent	Vaccine	Basic R&D	Preclinical	Phase I	Phase II	Phase III
Haemophilus influenzae (nontypeable) (continued)	Subunit lipoprotein D (nonacylated)	+	+	+		
	Subunit detoxified lipooligosaccharide conjugated to tetanus toxoid	+	+			
	Subunit detoxified lipooligosaccharide conjugated to HMW protein from *H. influenzae* (nontypeable)	+	+			
	OMP HiN47	+	+	+	+	
	Pili (HifE)	+	+			
Haemophilus influenzae type b (Hib)	Glycoconjugate of Hib PRP with CRM197	+	+	+	+	+
	Glycoconjugate of Hib PRP with diphtheria toxoid	+	+	+	+	+
	Glycoconjugate of Hib PRP with tetanus toxoid	+	+	+	+	+
	Hib-IPV-HBV	+	+	+	+	
	Glycoconjugate of Hib PRP with meningococcal type B outer membrane protein	+	+	+	+	+
	Glyconjugate Hib with meningococcal type A and/or C	+	+	+		
Hantaan virus	Vaccinia vector	+	+	+	+	
	Recombinant subunit	+				
	RNA replicons	+	+			
Helicobacter pylori	Recombinant *H. pylori* urease and cholera toxin—oral vaccine	+	+	+		
	H. pylori antigens and mutant CT or LT	+	+	+		
	Killed whole cells	+	+			
	Salmonella vectored *H. pylori* antigens	+	+			
Hepatitis A virus (HAV)	Inactivated HAV particles	+	+	+	+	+
	Live, attenuated HAV	+	+	+	+	+
	Virosome-formulated inactivated HAV	+	+	+	+	+
	Viral proteins expressed by vectors (baculovirus or vaccinia virus)	+	+			
Hepatitis B virus (HBV)	HBV core protein expressed by rDNA	+	+			
	HBV proteins expressed in yeast cells by rDNA	+	+	+	+	+
	Salmonella vector	+	+	+		
	Variants	+	+			
	Generation of cytotoxic T lymphocytes	+	+	+	+	
	DNA vaccines	+	+			
	rDNA, plants	+	+	+		
Combined HAV/HBV vaccine	Combined inactivated components	+	+	+	+	+
Hepatitis C virus (HCV)	rDNA-expressed surface proteins and epitopes	+	+			
	Generation of cytotoxic T lymphocytes	+	+			
	Nucleocapsid	+	+			
	DNA vaccines	+	+			
Hepatitis D virus (HDV)	Synthetic peptides	+	+			
	Baculovirus	+				
Hepatitis E virus (HEV)	Expressed proteins	+	+			
Herpes simplex virus types 1 and 2	gD2 recombinant protein	+	+	+	+	+
	gD2 and gB2 recombinant protein	+	+	+	+	+
	Disabled virus (gH deleted)	+	+	+		
	DNA encoding gD2	+	+	+		

Target Agent	Vaccine	Basic R&D	Preclinical	Phase I	Phase II	Phase III
Herpes simplex virus types 1 and 2	Heteroconjugate recombinant protein, T cell ligands with HSV-associated peptides	+	+			
(continued)	Vaccinia-vectored proteins glycoproteins	+	+			
Histoplasma capsulatum	Purified yeast cell proteins (e.g., His-62)	+	+			
	Recombinant proteins (e.g., His 62, H-antigen, hsp-70)	+	+			
Human immuno-deficiency virus, HIV-1	See Appendix C					
Human immuno-deficiency virus, HIV-2	Inactivated HIV-2	+	+			
	Live, attenuated HIV-2	+	+			
	rgp 125 or 130 (purified from virion)	+	+			
	rgp 160 (insect cells)	+	+			
	Highly attenuated, vaccinia HIV-2 gag-pol-env	+	+			
	Vaccinia HIV-2 env	+	+			
	Canarypox HIV-2 gag-pol-env	+	+			
	Salmonella HIV-2 env, gag	+	+			
Human papillomavirus (HPV)	Capsid protein	+	+			
	TA-HPV (live recombinant vaccinia) E6 and E7 (from HPV-16, and HPV-18)	+	+	+	+	
	TA-GN recombinant protein L2 and E7 (from HPV-6)	+	+	+	+	
	MEDI-501 recombinant VLP L1 from HPV-11	+	+	+		
	Quadrivalent recombinant VLP L1 (from HPV-6, HPV-11, HPV-16, and HPV-18)	+	+			
	DNA vaccine	+	+			
	LAMP-E7 (from HPV-16)	+	+			
Influenza virus	Cold-adapted live, attenuated	+	+	+	+	+
	Purified viral HA subunit	+	+	+		
	Liposome containing viral HA	+	+	+		
	Purified CTL specific peptides	+	+	+		
	Microencapsulated inactivated vaccine	+	+	+		
	Purified, inactivated viral neuraminidase	+	+	+		
	Baculovirus expressed recombinant HA subunit	+	+	+	+	
	Baculovirus expressed nucleoprotein	+	+	+		
	Transfection with nucleic acid (DNA) plasmid expressing HA subunit	+	+			
	Inactivated viral vaccines with novel adjuvants	+	+	+		
Japanese encephalitis virus	Whole, inactivated virus particles	+	+	+	+	+
	Infectious clone	+	+			
	Purified DNA expressed protein	+	+			
	Live attenuated virus	+	+	+	+	
	Vaccinia vector (live)	+	+	+		
	Chimeric virus	+	+	+		
Junin virus (Argentine hemorrhagic fever)	Live, attenuated	+	+	+	+	
Legionella pneumophila	Attenuated mutant	+	+			
	Purified bacterial surface protein	+	+			

Target Agent	Vaccine	Basic R&D	Preclinical	Phase I	Phase II	Phase III
Leishmania major	Attenuated or killed whole parasites	+	+	+	+	+
	Deletion mutagenized, attenuated parasite	+	+			
Multiple *Leishmania* spp.	Leishmanial surface antigens (gp63, 46 kD, and lipophosphoglycan)	+	+			
Measles virus	rDNA HA and fusion proteins	+	+			
	ISCOM	+	+			
	Live, attenuated	+	+	+	+	+
	High-titer live (multiple strains)	+	+	+	+	+
	Poxvirus vector (live)	+	+	+		
Moraxella catarrhalis	High molecular weight, outer membrane proteins CD, E, B1, and LBP for use in conjugate vaccines	+	+			
	Detoxified LOS conjugated to either tetanus toxoid or high MW proteins from nontypeable *H. influenzae*	+	+			
Mycobacterium leprae	BCG plus purified M. leprae antigens (35 kD)	+				
	Recombinant antigens in BCG	+	+			
	Live BCG expressing *M. leprae* antigens	+	+			
	BCG plus heat-killed *M. leprae*	+	+	+	+	+
	Heat-killed, purified *M. leprae*	+	+	+	+	+
	Mycobacterium w	+	+	+	+	+
	BCG	+	+	+	+	+
	ICRC	+	+	+	+	+
	Mycobacterium habana	+	+	+		
	Vaccinia virus vector expressing mycobacterial antigen	+	+			
Mycobacterium tuberculosis	BCG plus purified *M. tuberculosis* antigens	+	+			
	T cell reactive immunogens	+	+			
	Recombinant antigens in BCG	+	+			
	M. vaccae	+	+	+	+	
	Recombinant antigens in *M. vaccae*	+	+			
	M. tuberculosis culture filtrate proteins (CFP)	+	+			
	M. tuberculosis culture filtrate proteins and cytokines	+	+			
	Mycolic acids	+	+			
	BCG with CFP “boost”	+	+			
	Dendritic cells pulsed with for-met peptides	+	+			
	Transfected EL-4 cells	+	+			
	Recombinant *Salmonella* constructs	+	+			
	M. smegmatis expressing *M. tb* antigens	+				
	rBCG expressing cytokines	+	+			
	Auxotrophic mutant BCG	+	+			
	DNA vaccines	+	+			
	Auxotrophic mutant *Mycobacterium tuberculosis*	+	+			
	Live *Mycobacterium microti*	+	+			
Mycoplasma pneumoniae	Recombinant membrane-associated proteins	+	+			
	Purified outer membrane protein	+	+			
	Inactivated (heat-killed) oral vaccine	+	+	+		
Neisseria gonorrheae	Por (protein I)	+	+			
	Recombinant Por protein	+	+			
	Iron-binding protein (BPs)	+	+			
	PANS anaerobic proteins	+				
	H.8 lipoprotein	+				

Target Agent	Vaccine	Basic R&D	Preclinical	Phase I	Phase II	Phase III
Neisseria gonorrheae	LPS anti-idiotype	+	+			
(continued)	Whole cells	+	+			
Neisseria meningitidis	Glycoconjugate with tetanus toxoid	+	+			
(Group A)	Group A LOS	+				
Neisseria meningitidis (Group B)	Native outer membrane vesicle (NOMV)—intranasal route	+	+	+		
	OMP-dLPS liposome	+	+			
	Recombinant PorA outer membrane protein in liposomes	+	+			
	Outer membrane vesicles (OMV), high MW proteins, and C polysaccharide	+	+	+	+	+
	Hexvalent PorA outer membrane vesicle vaccine	+	+	+	+	
	Outer membrane vesicles (deoxycholate extracted)	+	+	+	+	+
	Recombinant transferrin binding protein (TBP1 and TBP2)	+	+			
	Recombinant low MW (NspA) outer membrane protein	+	+			
	Glycoconjugate modified polysaccharide with recombinant PorB protein	+	+			
	LOS micelle-based vaccine	+				
Neisseria meningitidis (Group C)	Glycoconjugate with tetanus toxoid	+	+	+	+	+
Neisseria meningitidis	Glycoconjugate A and C with CRM197	+	+	+	+	
A and C	Glycoconjugate A and C with DT	+	+	+		
Neisseria meningitidis	Combination glycoconjugate with					
A, B, and C	recombinant PorB	+	+			
Neisseria meningitidis A,B, C, and W-135	Glycoconjugate with DT	+	+	+		
Onchocerca volvulus	Recombinant proteins	+	+			
Paracoccidioides	Purified yeast cell proteins	+	+			
brasiliensis	Recombinant proteins	+	+			
	Synthetic peptide or multipeptide construction (P10, MAP-10)	+	+			
	DNA plasmid with gp43 gene	+	+			
Parainfluenza virus	Cold-adapted PIV3 attenuated virus	+	+	+		
	Purified HN and F protein subunit vaccine	+	+			
	Bovine attenuated PIV3 vaccine	+	+	+		
Plasmodium falciparum	Circumsporozoite antigen-based peptide or recombinant protein	+	+	+	+	
	Circumsporozoite antigen expressed in various vectors	+	+	+		
	Circumsporozoite antigen-based DNA vaccine	+	+	+		
	Noncircumsporozoite, pre-erythrocytic antigen-based constructs	+	+			
	Merozoite surface protein-1 (MSP-1) based recombinant protein	+	+	+		
	Non-MSP-1 asexual blood stage antigens	+	+			
	25 kD gametocyte antigen recombinant protein (TBV25H)	+	+	+		
	Other sexual stage antigens	+	+			

Target Agent	Vaccine	Basic R&D	Preclinical	Phase I	Phase II	Phase III
Plasmodium falciparum (continued)	Multivalent viral vector-based combination vaccines incorporating different stage -specific antigens (e.g., NYVAC Pf7)	+	+	+	+	
	Subunit (RTS,S)	+	+	+	+	
	DNA-based combination vaccines incorporating different stage-specific antigens	+	+			
	Combination vaccines incorporating different stage-specific antigens (e.g., SPf 66)	+	+	+	+	+
Plasmodium vivax	Circumsporozoite antigen-based peptide or recombinant protein	+	+	+		
	Asexual erythrocytic antigens	+	+			
Poliovirus	Reversion-stable attenuated OPV	+				
	Live (nonreverting)	+	+			
	Chimeric virus	+	+			
Pseudomonas aeruginosa	Purified bacterial proteins, including flagellar Ag, LPS-O, porins, several inactivated bacterial toxins, and high MW polysaccharide antigen and glycoconjugate	+	+	+		
	Inactivated whole bacteria—oral preparation	+	+	+		
	Synthetic peptides	+	+	+		
Pseudomonas (Burkholderia) cepacia	Purified bacterial proteins, LPS	+				
Pythium insidiosum	Sonicated hyphal antigens	+	+			
	Culture filtrate antigens	+	+			
	Purified proteins (e.g., 28, 30, 32 kD)	+	+			
Rabies virus	rDNA vaccinia virus recombinant for use in sylvatic rabies (veterinary vaccine)	+	+	+	+	+
	Inactivated mammalian brain	+	+	+	+	+
	Inactivated cell culture	+	+	+	+	+
Respiratory syncytial virus (RSV)	Live, attenuated *ts* and/or *ca* strains	+	+	+		
	Purified F protein subunit vaccine	+	+	+		
	G protein expressed vaccine	+	+			
Rickettsia rickettsii	Subunit vaccine containing major surface proteins (155 and 120 kD)	+	+			
Rift Valley Fever virus	Inactivated	+	+	+	+	
	Live, attenuated	+	+	+		
Rotavirus	Attenuated human rotavirus (cold-adapted)	+	+	+		
	Salmonella expressing VP4, VP7, or both	+	+			
	Attenuated bovine/human virus reassortants (WC3)	+	+	+	+	+
	Human nursery strains	+	+	+	+	
	Purified rotavirus proteins rDNA-derived virus-like particles (VLPs)	+	+			

Target Agent	Vaccine	Basic R&D	Preclinical	Phase I	Phase II	Phase III
Rotavirus (continued)	Vaccinia virus recombinant expressing VP4, VP7, or both	+	+			
	DNA vaccines	+	+			
Rubella virus	Live, attenuated	+	+	+	+	+
	Infectious clone	+				
	Synthetic peptide	+				
Salmonella typhi	Vi carbohydrate	+	+	+	+	+
	Vi carbohydrate-protein conjugate	+	+	+	+	
	Live, attenuated Ty21a vaccine	+	+	+	+	+
	Live, attenuated auxotrophic mutants	+	+	+	+	
Schistosoma mansoni, Schistosoma haematobium, Schistosoma japonicum	Purified larval antigens	+	+			
	Recombinant larval antigens	+	+			
Shigella dysenteriae	Live auxotrophic, attenuated mutants	+	+	+		
	Polysaccharide-protein conjugate	+	+	+	+	
Shigella flexneri	*E. coli* hybrids	+	+	+	+	
	Polysaccharide-protein conjugate	+	+	+	+	
	Live, attenuated oral vaccines	+	+	+	+	
	LPS proteosome (intranasal)	+	+			
Shigella sonnei	Live, attenuated (WRSS1) oral vaccine	+	+			
	LPS proteosome (intranasal)	+	+			
	Polysaccharide-protein conjugate	+	+	+	+	
	Nucleoprotein	+	+			
Staphylococcus aureus	Type 5/Type capsular polysaccharide (CPS) conjugate with *Pseudomonas aeruginosa* recombinant exoprotein A	+	+	+	+	
Staphylococcal enterotoxin B	Recombinant toxin	+	+			
Streptococcus pneumoniae	Glycoconjugate vaccine (1, 4, 5, 6B, 9N, 14, 18C, 19V, 23F) conjugated to meningococcal B OMP	+	+	+	+	+
	Glycoconjugate vaccine (1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, 23F) conjugated to CRM197	+	+			
	Glycoconjugate vaccine (3, 4, 6B, 9V, 14, 18C, 19F, 23F) conjugated to either tetanus toxoid or diphtheria toxoid	+	+	+	+	
	Glycoconjugate vaccine (6B, 14, 19, 23F) conjugated to tetanus toxoid	+	+	+	+	
	Glycoconjugate vaccine (4, 6B, 9V, 14, 18C, 19F, 23F) conjugated to CRM197	+	+	+	+	+
	Glycoconjugate vaccine (1, 4, 5, 6B, 9V, 14, 18C, 19F, 23F) conjugated to CRM197	+	+	+	+	+
	23-valent licensed vaccine with novel adjuvants (Quil A, QS21, MPL)	+	+	+		
	Glycoconjugate multivalent vaccine with novel adjuvants (e.g., MPL)	+	+	+		
	PspA	+	+	+		
	PsaA	+	+			
	Pneumolysin	+	+			

Target Agent	Vaccine	Basic R&D	Preclinical	Phase I	Phase II	Phase III
Streptococcus pneumoniae (continued)	Autolysin	+	+			
	Neuraminidase	+	+			
	Glycoconjugate vaccine (11-valent) linked to nontypeable *H. influenzae* OMP	+	+	+	+	
	Glycoconjugate vaccine (1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, 23F) linked to either tetanus or diphtheria toxoid carrier	+	+	+	+	
	Phospholcholine	+	+			
	Synthetic peptide epitopes and capsular polysaccharide combined	+	+			
	Genetic fusions (PspA-IL2 and PspA-GM-CSF)	+	+			
	CpG motifs cross-linked with 7-valent pneumococcal vaccine	+	+			
Tick-borne encephalitis virus	DNA vaccine	+	+			
	Inactivated, alum adjuvant	+	+	+	+	
Toxoplasma gondii	Recombinant parasite surface protein (p30)	+	+			
	Live, attenuated parasites	+	+			
	Parasite surface protein expressed in viral vector	+	+			
Treponema pallidum	Surface lipoproteins	+	+			
	Anti-idiotype/fibronectin	+	+			
Trypanosoma cruzi	Recombinant peptide	+	+			
Varicella zoster virus	Live, attenuated vaccine	+	+	+	+	+
	Subunit, glycoproteins	+				
	Vaccinia-vectored glycoprotein	+				
Venezuelan equine encephalitis	Inactivated, whole virus particles	+	+	+	+	
	Live, attenuated virus strain (TC-83)	+	+	+	+	
	Infectious clones	+	+			
Vibrio cholerae	Killed bacteria plus toxin B subunit	+	+	+	+	+
	Live, recombinant O1	+	+	+	+	+
	Live, recombinant O139	+	+	+	+	
	Conjugate lipopolysaccharide (LPS)	+	+			
Yellow fever virus	Live attenuated	+	+	+	+	+
	Infectious clone	+	+			
Western equine encephalitis virus	Inactivated, whole virus particles	+	+	+	+	
Yersinia pestis	Recombinant subunit	+	+			

Appendix B

Licensed Vaccines Currently Distributed in the United States and Reference Revocations, October 1999*

Product Name	Trade Name	License Date	Establishment
Acellular Pertussis Vaccine Concentrate (For Further Manufacturing Use)	No Trade Name	17-Dec-91	Takeda Chemical Industries, Ltd.
Acellular Pertussis Vaccine Concentrate (For Further Manufacturing Use)	No Trade Name	20-Aug-92	Research Fdn. for Microbial Diseases of Osaka University
Adenovirus Vaccine, Live, Oral, Type 4	No Trade Name	01-Jul-80	Wyeth Laboratories, Inc.
Adenovirus Vaccine, Live, Oral, Type 7	No Trade Name	01-Jul-80	Wyeth Laboratories, Inc.
Anthrax Vaccine Adsorbed	No Trade Name	12-Nov-98	BioPort Corporation
BCG Live	TheraCys®	21-May-90	Connaught Laboratories, Ltd.
BCG Live	Tice® BCG	10-Jan-95	Organon Teknika Corporation
BCG Vaccine (Reissued)	Mycobax®	09-Oct-98	Connaught Laboratories, Ltd.
BCG Vaccine	No Trade Name	10-Jan-95	Organon Teknika Corporation
Botulinum Toxin Type A	BOTOX®	09-Dec-91	Allergan, Inc.
Cholera Vaccine	No Trade Name	16-Jul-52	Wyeth Laboratories, Inc.
Diphtheria & Tetanus Toxoids & Acellular Pertussis Vaccine Adsorbed	Tripedia®	20-Aug-92	Connaught Laboratories, Inc.
Diphtheria & Tetanus Toxoids & Acellular Pertussis Vaccine Adsorbed	ACEL-IMUNE®	17-Dec-91	Lederle Laboratories, Div. of American Cyanamid Co.
Diphtheria & Tetanus Toxoids & Acellular Pertussis Vaccine Adsorbed	Infanrix®	29-Jan-97	SmithKline Beecham Biologicals
Diphtheria & Tetanus Toxoids & Acellular Pertussis Vaccine Adsorbed	Certiva®	29-Jul-98	North American Vaccine, Inc.
Diphtheria & Tetanus Toxoids & Pertussis Vaccine Adsorbed	No Trade Name	11-Sep-70	Wyeth Laboratories, Inc.
Diphtheria & Tetanus Toxoids & Pertussis Vaccine Adsorbed	No Trade Name	03-Jan-78	Connaught Laboratories, Inc.
Diphtheria & Tetanus Toxoids & Pertussis Vaccine Adsorbed	No Trade Name	12-Nov-98	BioPort Corporation
Diphtheria & Tetanus Toxoids & Pertussis Vaccine Adsorbed	TRI-IMMUNOL®	24-Jul-70 14-Aug-98†	Lederle Laboratories, Div. of American Cyanamid Co.
Diphtheria & Tetanus Toxoids & Biologic Pertussis Vaccine Adsorbed	No Trade Name	27-Jul-70 22-Dec-98†	Massachusetts Public Health Biologic Laboratories
Diphtheria & Tetanus Toxoids & Pertussis Vaccine Adsorbed and Haemophilus b Conjugate Vaccine (Diphtheria CRM197 Protein Conjugate)	TETRAMUNE®	30-Mar-93	Lederle Laboratories, Div. of American Cyanamid Co.
Diphtheria & Tetanus Toxoids Adsorbed	No Trade Name	18-Sep-84	Connaught Laboratories, Inc.
Diphtheria & Tetanus Toxoids Adsorbed	No Trade Name	11-Apr-97	Connaught Laboratories, Ltd.

* Prepared by Alice D. Knoben, R.Ph., Viral Vaccines Branch, DVRPA, OVRR, CBER. For revisions to this chart, please contact Ms. Knoben at 301-827-3070.

† License revocation date.

Product Name	Trade Name	License Date	Establishment
Diphtheria & Tetanus Toxoids Adsorbed	No Trade Name	27-Jul-70	Massachusetts Public Health Biologic Laboratories
Diphtheria & Tetanus Toxoids Adsorbed	No Trade Name	12-Nov-98	BioPort Corporation
Diphtheria & Tetanus Toxoids Adsorbed	No Trade Name	29-Jul-70	Lederle Laboratories, Div. of American Cyanamid Co.
Diphtheria & Tetanus Toxoids Adsorbed	No Trade Name	11-Sep-70	Wyeth Laboratories, Inc.
Diphtheria Toxoid Adsorbed	No Trade Name	12-Nov-98	BioPort Corporation
Haemophilus b Conjugate Vaccine (Diphtheria CRM197 Protein Conjugate)	HibTITER®	06-Dec-94	Lederle Laboratories, Div. of American Cyanamid Co.
Haemophilus b Conjugate Vaccine (Diphtheria Toxoid Conjugate)	ProHIBIT®	22-Dec-87	Connaught Laboratories, Inc.
Haemophilus b Conjugate Vaccine (Meningococcal Protein Conjugate)	PedvaxHIB®	20-Dec-89	Merck & Co., Inc.
Haemophilus b Conjugate Vaccine (Tetanus Toxoid Conjugate)	ActHIB® OmniHIB®	30-Mar-93	Pasteur Mérieux Sérums et Vaccins, S.A.
Haemophilus b Conjugate Vaccine (Meningococcal Protein Conjugate) & Hepatitis B (Recombinant) Vaccine	Comvax®	02-Oct-96	Merck & Co., Inc.
Haemophilus b Polysaccharide Vaccine	HibVAX®	20-Dec-85	Connaught Laboratories, Inc.
Hepatitis A Vaccine, Inactivated	HAVRIX®	22-Feb-95	SmithKline Beecham Biologicals
Hepatitis A Vaccine, Inactivated	VAOTA®	29-Mar-96	Merck and Co., Inc.
Hepatitis B Vaccine (Recombinant)	RECOMBIVAX HB®	23-Jul-86	Merck & Co., Inc.
Hepatitis B Vaccine (Recombinant)	Engerix-B®	28-Aug-89	SmithKline Beecham Biologicals
Influenza Virus Vaccine	Fluzone®	03-Jan-78	Connaught Laboratories, Inc.
Influenza Virus Vaccine	FluShield®	13-Dec-61	Wyeth Laboratories, Inc.
Influenza Virus Vaccine	Fluogen®	20-Apr-98	Parkedale Pharmaceuticals, Inc.
Influenza Virus Vaccine	Fluvirin®	3-Nov-98	Medeva Pharma, Ltd.
Influenza Virus Vaccine	FLU-IMUNE®	07-Dec-45	Lederle Laboratories, Div. of American Cyanamid Co.
Japanese Encephalitis Virus Vaccine Inactivated	JE-VAX®	10-Dec-92	Research Fdn. for Microbial Diseases of Osaka University
Lyme Disease Vaccine (Recombinant OspA)	LYMErix®	21-Dec-98	SmithKline Beecham Biologicals
Measles and Mumps Virus Vaccine Live	M-M-Vax®	18-Jul-73	Merck & Co., Inc.
Measles and Rubella Virus Vaccine Live	M-R-Vax® II	22-Apr-71	Merck & Co., Inc.
Measles, Mumps, and Rubella Virus Vaccine Live	M-M-R® II	22-Apr-71	Merck & Co., Inc.
Measles Virus Vaccine Live	ATTENUVAX®	21-Mar-63	Merck & Co., Inc.
Meningococcal Polysaccharide Vaccine, Group A	Menomune®—A	03-Jan-78	Connaught Laboratories, Inc.
Meningococcal Polysaccharide Vaccine, Group C	Menomune®—C	03-Jan-78	Connaught Laboratories, Inc.

Product Name	Trade Name	License Date	Establishment
Meningococcal Polysaccharide Vaccine, Groups A, C, Y and W-135 Combined	Menomune®—A/C/ Y/W-135	23-Nov-81	Connaught Laboratories, Inc.
Mumps Virus Vaccine Live	MUMPSVAX®	28-Dec-67	Merck & Co., Inc.
Pertussis Vaccine	No Trade Name	03-Jan-78	Connaught Laboratories, Inc.
Pertussis Vaccine Adsorbed	No Trade Name	12-Nov-98	BioPort Corporation
Plague Vaccine	No Trade Name	05-Oct-94	Greer Laboratories, Inc.
Pneumococcal Vaccine Polyvalent	PNEUMOVAX® 23	21-Nov-77	Merck & Co., Inc.
Pneumococcal Vaccine Polyvalent	PNU-IMUNE® 23	15-Aug-79	Lederle Laboratories, Div. of American Cyanamid Co.
Poliovirus Vaccine Inactivated (Human Diploid Cell)	Poliovax®	20-Nov-87	Connaught Laboratories, Ltd.
Poliovirus Vaccine Inactivated (Monkey Kidney Cell)	IPOL®	21-Dec-90	Pasteur Mérieux Sérums et Vaccins, S.A.
Poliovirus Vaccine Live Oral Trivalent (Sabin Strains Types 1, 2 and 3)	ORIMUNE®	25-Jan-63	Lederle Laboratories, Div. of American Cyanamid Co.
Poliovirus Vaccine Live Oral Type I	No Trade Name	27-Mar-62	Lederle Laboratories, Div. of American Cyanamid Co.
Poliovirus Vaccine Live Oral Type II	No Trade Name	27-Mar-62	Lederle Laboratories, Div. of American Cyanamid Co.
Poliovirus Vaccine Live Oral Type III	No Trade Name	27-Mar-62	Lederle Laboratories, Div. of American Cyanamid Co.
Polyvalent Bacterial Antigens with "No U.S. Standard of Potency"	Staphage Lysate®	31-Aug-59	Delmont Laboratories, Inc.
Polyvalent Bacterial Vaccines with "No U.S. Standard of Potency"	MRV® Mixed Respiratory Vaccine	27-Apr-76 02-Jun-99†	Bayer Corporation
Polyvalent Bacterial Vaccines with "No U.S. Standard of Potency"	No Trade Name	02-Jun-99	Hollister-Stier Laboratories, LLC
Rabies Vaccine	RABIE-VAX®	27-Dec-91	Connaught Laboratories, Ltd.
Rabies Vaccine	Imovax® Rabies	09-Jun-80	Pasteur Mérieux Sérums et Vaccins, S.A.
Rabies Vaccine	RabAvert	20-Oct-97	Chiron Behring GmbH & Co.
Rabies Vaccine Adsorbed	No Trade Name	12-Nov-98	BioPort Corporation
Rotavirus Vaccine Live, Oral, Tetravalent*	RotaShield®	31-Aug-98	Wyeth Laboratories, Inc.
Rubella and Mumps Virus Vaccine Live	BIAVAX®	30-Aug-70	Merck & Co., Inc.
Rubella Virus Vaccine Live	MERUVAX®	09-Jun-69	Merck & Co., Inc.
Smallpox Vaccine	No Trade Name	03-Jan-78	Connaught Laboratories, Inc.
Smallpox Vaccine	Dryvax®	19-May-44	Wyeth Laboratories, Inc.
Tetanus & Diphtheria Toxoids Adsorbed for Adult Use	No Trade Name	27-Jul-70	Massachusetts Public Health Biologic Laboratories
Tetanus & Diphtheria Toxoids Adsorbed for Adult Use	No Trade Name	03-Jan-78	Connaught Laboratories, Inc.
Tetanus & Diphtheria Toxoids Adsorbed for Adult Use	No Trade Name	11-Sep-70	Wyeth Laboratories, Inc.

* On October 15, 1999, Wyeth Lederle Vaccines announced that it had withdrawn RotaShield® from the market.

Product Name	Trade Name	License Date	Establishment
Tetanus & Diphtheria Toxoids Adsorbed for Adult Use	No Trade Name	29-Jul-70	Lederle Laboratories, Div. of American Cyanamid Co.
Tetanus Toxoid	No Trade Name	03-Jan-78	Connaught Laboratories, Inc.
Tetanus Toxoid	No Trade Name	14-Jan-43	Connaught Laboratories, Ltd.
Tetanus Toxoid	No Trade Name	19-May-44	Wyeth Laboratories, Inc.
Tetanus Toxoid Adsorbed	No Trade Name	11-Sep-70	Wyeth Laboratories, Inc.
Tetanus Toxoid Adsorbed	No Trade Name	29-Jul-70	Lederle Laboratories, Div. of American Cyanamid Co.
Tetanus Toxoid Adsorbed	No Trade Name	12-Nov-98	BioPort Corporation
Tetanus Toxoid Adsorbed	No Trade Name	11-Dec-70	Swiss Serum and Vaccine Institute Berne
Tetanus Toxoid Adsorbed	No Trade Name	03-Jan-78	Connaught Laboratories, Inc.
Tetanus Toxoid Adsorbed	No Trade Name	29-Jul-70	Massachusetts Public Health Biologic Laboratories
Typhoid Vaccine	No Trade Name	16-Jul-52	Wyeth Laboratories, Inc.
Typhoid Vaccine Live Oral Ty21a	Vivotif Berna®	15-Dec-89	Swiss Serum and Vaccine Institute Berne
Typhoid Vi Polysaccharide Vaccine	Typhim Vi®	28-Nov-94	Pasteur Mérieux Sérums et Vaccins, S.A.
Varicella Virus Vaccine Live	Varivax®	17-Mar-95	Merck & Co., Inc.
Yellow Fever Vaccine	YF-VAX®	03-Jan-78	Connaught Laboratories, Inc.

APPENDIX C

AIDS Vaccine Candidates in Development

Vaccine Candidate	Expression System/ Production Method	HIV Strain(s)	Adjuvant or Delivery System	Stage of Development	References
Subunits:					
rgp160	Vaccinia/Mammalian cell	MN/BRU, MN/LAI, MN/LAI-2	alum, IFA, polyphosphazene	PhI-P*	1-7
rgp160 (VaxSyn TM)	Baculovirus/Insect cell	LAI	± alum	PhI-P*, PhI&II-T*	8-23
rgp160	Vaccinia/Monkey kidney cell	IIIB (LAI)	alum + DOC	PhI-P*, PhI&II-T*	24-29
rgp160	Vaccinia/Monkey kidney cell	MN	alum + DOC	PhI-P*, PhI&II-T	24, 30-31
rgp160	Vaccinia/Mammalian cell	LAV (IIIB)	IFA, ISA724	PhI-P, PhI-T	32-36
rgp160	Vaccinia/Mammalian cell	LAI	Oil/water, 3-deacyl monophosphoryl lipid A	PCT-C	37
rgp160	Baculovirus/Insect cell	MN, Thai Clade E	alum	PCT-SA	8, 38
rgp160, Oligomeric	Vaccinia/Mammalian cell	IIIB (LAI)	alum, MPL, IFA, MPL-AF, RIBI, polyphosphazene, proteosomes, liposomes, emulsomes, cholera toxin B	PCT-SA,M	39-43
rgp120 Bivalent Clade B (AIDSVAX ™)	Chinese hamster ovary cell	MN, GNE8	alum	PhIII-P	44-46
rgp120 Bivalent Clade B/E (AIDSVAX ™)	Chinese hamster ovary cell	MN, A244	alum and/or QS-21	PhI-P*, PhIII-P	44-46
rgp120	Chinese hamster ovary cell	MN GM-CSF	none, alum and/or QS-21,	PhI&II-P*,PhI/II-T*	47-54
rgp120 Bivalent Clade B/E	Chinese hamster ovary cell	SF-2, CM235	MF59	PhII-P	55-56
rgp120	Chinese hamster ovary cell	CM235 (Thai Clade E)	MF59	PhI-P	57-58
rgp120	Chinese hamster ovary cell	SF2	alum, MPL, liposome-encapsulated MPL with alum, MF59 ± MTP-PE, SAF-2 ± MDP	PhI&II-P*, PhI-T*	59-64
rgp120	Chinese hamster ovary cell	W61D	QS21 + MPL, alum; ± “emulsion”	PhI-P	65-68
rgp120	Chinese hamster ovary cell	US4		BR&D	69
rgp120 (Env 2-3)	Yeast	SF2	MF59 ± MTP-PE	PhI-P*, PhI-T	70-73
rgp120	Chinese hamster ovary cell	IIIB (LAI)	alum	PhI-P*, PhI-T	74-79
rgp120	Baculovirus/Insect cell	LAI	Oil/water, 3-deacyl monophosphoryl Lipid A	PCT-C	80
rp24	Yeast	SF2	MF59	PhI-P*	58, 81
rp24	Baculovirus/Insect cell	LAI	alum	PhI-P, PhI-T*	12-13, 82
rRT	Recombination		cholera toxin	PCT-SA	83
RT-VCG	*E. coli* plasmid/*Vibrio cholera*	LAI	*V. cholera* ghosts (VCG)	PCT-SA	84-85
Tat, chemically inactivated (“toxoid”)	Carboxymethlyated tat		ISA 51	PhI-P, PhI/II-T	86-88
Peptides:					
V3-T helper epitope peptides (PCLUS 3-18 MN, PCLUS 6-18 MN)	Synthetic chimeric	MN	IFA	PhI-T	89-95
V3 sequences in single peptide (TAB9)	Recombinant *E. coli*	BRVA, JY1, LR150, RF, MN, LAI	Montanide-ISA720	PCT-SA, PhI-P	96-98
C4-V3 peptides (T1SP10 MN(A))	Synthetic chimeric	MN, RF, CAN0, EV91	IFA	PhI-T*, PhI-P*	99-104

Vaccine Candidate	Expression System/ Production Method	HIV Strain(s)	Adjuvant or Delivery System	Stage of Development	References
p17-KLH (HGP-30W™)	Synthetic p17 peptide coupled to Keyhole Limpet Hemocyanin (KLH) carrier protein	SF2	alum	PCT-SA, M, C; PhI & II-P	105-113
p24-V3 peptide, linear monomeric (CLTB-36)	Synthetic chimeric	LAI (p24), MN (V3)	alum or QS21	PhI-P	114-117
V3-MAPS, octameric, monovalent (Synvac™)	Synthetic	MN	alum	PhI-P*, PhI&II-P	118-121
V3-MAPS, octameric, multivalent	Synthetic	15 strains/5 clades	alum	PhI-P*	122-123
V3-MAPS, microparticulate, monovalent	Synthetic	MN	microparticulate	PhI-P*	124-126
V3 linear peptide	Synthetic	MN	alum or IFA	PhI-P	127
V3 peptide (RP400c)	Synthetic	MN	alum	PhI-T	128
V3-PPD, monovalent	V3 peptide coupled to PPD	MN		PhI-P	129-131
V3-PPD, multivalent	V3 peptide coupled to PPD	5 strains		PhI-P	131
V3-Toxin A	V3 peptide coupled to Pseudomonas aeruginosa toxin A	MN	none	PhI-P	131-132
V3 peptide coupled to Mycobacterium protein	Synthetic	MN	10K Mycobacterium protein	PhI-P	131, 133
V3 PND, p18 CTL epitopes, Th epitopes	Synthetic peptides	LAV (LAI)	ISA724	PhI-P	133
gag-lipopeptide (P34541b)	Synthetic	LAI	lipopeptide	PhI-P*	134-146
lipopeptides + nef (2), gag (2) and V3 peptides	Synthetic	LAI	QS21	PhI	137-138
gag, pol and nef (2) peptides + tetanus toxoid peptide (LIPO-6T)	Synthetic	LAI	none	PhI	139
pol-tetanus toxoid polypeptide (CY2301)	Synthetic	Multiple	lipopeptide	PhI-T	140
gp120, gp41, gag p7 peptides	Synthetic		embedded in proteosomal granules; ISA724	PhI-P, PhI-T	141-143
V3-HA	Recombinant baculovirus	MN	Influenza virus haemagglutinin (HA)	PCT-SA	144
V3 loop-T helper epitope peptides, conformationally constrained	Synthetic		alum, IFA	PCT-SA	145
V3-CD4 binding site-gag peptides, Multicomponent (VC1)	Synthetic	IIIB, Thai A, Thai B, LAI	CFA	PCT-SA	146-148
CD4 Binding domain peptomer	Synthetic, conformationally constrained	MN	alum	PCT-SA	149
gp120-derived multiple chain peptide	Synthetic chimeric	Clade B consensus	CFA/IFA	PCT-SA	150
gp41 Katinger epitope-Hepatitis B virus surface antigen	Recombinant Pichia pastoris	LAI	Hepatitis B virus surface antigen	PCT-SA	151
p24-hsp70 fusion protein	Recombination	LAI	Mycobacterium tuberculosis heat shock protein (hsp) 70	PCT-SA	152
p24 23-mer peptides	Synthetic	Conserved sequence across clades A-G	CFA	PCT-SA	153

Vaccine Candidate	Expression System/ Production Method	HIV Strain(s)	Adjuvant or Delivery System	Stage of Development	References
V3/gag peptide	Synthetic			PCT-SA	3
gp160, gp120, gp41, p24, p17 peptides (NFU.Ac. HIV[JM])	HIV-1/Mammalian cell treated with detergent, formaldehyde, ultracentrifugation, acetone	GB8		PCT-SA	154
gag, pol, vpu, nef, rev, *tat* multiple CTL epitope peptides (HIV-Peplotion™)	Synthetic chimeric		Lotion applied to skin or mucous membranes	BR&D	155
V3 peptide, Constrained	Synthetic	MN/B consensus		BR&D	156
V3 peptides, Multiple cyclic	Synthetic	MN		BR&D	157
Particles:					
HIV-1, whole inactivated, gp120-depleted (Remune™)	Inactivated with betapropiolactone and irradiation	HZ321	IFA	PhI, II, III-T*	158-166
HIV-1, whole inactivated, RNA-depleted (HIVIONS)	Stabilized with formaldehyde		none, P40	PhI-T	141, 143, 167
p17/p24:Ty-VLP (p24 VLP)	p17/p24+ yeast transposon product	LAI	± alum	PhI-P*, PhI&II-T	168-174
env, gag, pol pseudovirions	plasmid/Mammalian cell	BX08	alum, QS-21	PCT-SA, M	175-177
p55 gag particle	Baculovirus/Insect cell	LAI	alum	PCT-SA	178
V3:Ty-VLP transposon product	V3-peptide+yeast	LAI	± alum	PCT-SA	179-182
V3-HBcAg particles	E. coli	MN (HBcAg)	Hepatitis B core antigen	PCT-SA	183
gag-V3 virus-like particles	Baculovirus/Insect cell	LAI		PCT-SA	184-187
HIV-1, whole inactivated	Inactivated with betapropiolactone, BEI, formaldehyde	RF, IIIB	none, Digitonin	PCT-SA	188-189
env, gag, protease, pseudovirions	Moloney murine leukemia virus/Mammalian cell Clade B	LAI/MN, Primary	CFA, IFA	PCT-SA, M	188-189
env, gag, pol pseudovirions	Vaccinia/Mammalian cell	IIIB	alum	PCT-SA, M	180-192
Recombinant live vector:					
Adenovirus-HIV-1 env	Recombinant adenovirus (Ad4, Ad5, Ad7 vaccine strains)	MN		PCT-C	193-195
ALVAC-HIV gp 160 (vCP125)	Attenuated recombinant canarypox	MN		PhI-P*	4, 7, 196-199
ALVAC-HIV env, gag, protease (vCP205	Attenuated recombinant canarypox	MN/LAI	none, GM-CSF	PhI&II-P*	115-117, 196-207
ALVAC-HIV env, gag, protease and pol and nef epitopes (vCP 300)	Attenuated recombinant canarypox	MN/LAI		PhI-P*	196-199, 208-210
ALVAC-HIV env, gag, protease and pol and nef epitopes (vCP 1433)	Attenuated recombinant canarypox	MN/LAI		PhI-P*	137
ALVAC-HIV env, gag, protease and pol and nef epitopes (vCP 1452)	Attenuated recombinant canarypox	MN/LAI		PhI-P*, PhI-T	137

Vaccine Candidate	Expression System/ Production Method	HIV Strain(s)	Adjuvant or Delivery System	Stage of Development	References
ALVAC-HIV env, gag, protease and pol and nef epitopes (vCP 1551)	Attenuated recombinant canarypox	Clade E/B		PCT	211
ALVAC-HIV env, gag, protease and pol and nef epitopes	Attenuated recombinant canarypox	Clade A		BR&D	137
BCG-HIV-1 env peptides	Recombinant Bacillus Calmette-Guerin	LAI		PCT-SA	212-214
BCG-HIV-1	Recombinant Bacillus Calmette-Guerin	LAI		BR&D	215-216
BCG-V3	Recombinant Bacillus Calmette-Guerin	LAI		PCT-SA	217
BCG-V3 (rBCG-THA13)	Recombinant Bacillus Calmette-Guerin	Japanese consensus, Clade E		PCT-SA	218-220
Brucella abortus-V3	Recombinant B. abortus	MN		PCT-SA	221-222
Fowlpox-HIV gag, prot, pol	Recombinant Fowlpox			PCT-M	223-225
Influenza HIV-1 V3	Recombinant influenza virus	LAI		PCT-SA	226
Influenza gp41 Katinger epitope	Recombinant influenza	LAI		PCT-SA	227-229
Lactococcus-HIV-1-V3 peptide	Fusion of V3 peptides to TT fragment C in Lactococcus lactis	MN		BR&D	230
Lactococcus HIV-1 peptides	Recombinant L casei or L lactis			PCT-SA	231
Listeria monocytogenes HIV-1 *gag*	Recombinant Listeria monocytogenes	LAI		PCT-SA	232-234
Mengovirus-HIV-1-nef	Recombinant mengovirus (attenuated M16 murine strain)	multiple		PCT-SA	235
Mengovirus-HIV-1 V3, C4 peptides	Recombinant attenuated mengovirus	MN		PCT-SA	236
Modified vaccinia Ankara (MVA)-HIV gag + multiple epitopes	Recombinant Modified vaccinia Ankara	Clade A		PCT-M	235-238
Moloney murine leukemia virus-HIV-1 *env, rev*	Recombinant Moloney murine leukemia virus	LAI		PhI-T	239-241
Poliovirus-HIV-1	Recombinant poliovirus	LAI		BR&D	242-243
Poliovirus-HIV-1 envelope Peptides	Recombinant dicistronic poliovirus	LAI		BR&D	244-245
Poliovirus-HIV-1 env, gag or pol minireplicons, encapsidated	Recombinant poliovirus	LAI		PCT-SA	246-247
Rhinovirus-HIV-1 V3, V4 peptides	Recombinant human rhinovirus (HRV14)	multiple		PCT-SA, C	248-249
Salmonella-HIV-1 gp120 (VVG203)	Recombinant attenuated Salmonella typhi (CVD 908 vaccine strain)	LAI		PhI-P*	250-254
Salmonella-HIV-1 gp120, p24, *nef*	Recombinant attenuated Salmonella typhi (CVD 908 vaccine strain)	LAI, MN		PCT-SA	250-254

Vaccine Candidate	Expression System/ Production Method	HIV Strain(s)	Adjuvant or Delivery System	Stage of Development	References
Salmonella-HIV-1 V3 peptide	Recombinant attenuated Salmonella typhimurium, aroA strain	LAI		PCT-SA	255
Semliki forest virus-HIV-1 envelope (gp120 and gp160)	Recombinant Semliki forest virus	LAI		BR&D	256
Vaccinia-HIV-1 gp 140	Recombinant vaccinia	23 isolates		PCT-SA, C; PhI-P	257-259
Vaccinia-HIV-1 env, gag, pol (TBC-3B)	Recombinant vaccinia	LAI		PhI-P*	260
Vaccinia-gp 160 (HIVAC-1e™)	Recombinant vaccinia	LAV-1 (LAI)		PhI-P*	261-268
Vaccinia-gp 160	Recombinant vaccinia	LAV (IIIB)		PhI-P, PhI-T	33, 269
Vaccinia-HIV-1 env, gag, pol** (NYVAC)	Attenuated recombinant vaccinia	LAI, MN		PCT-SA	270-272
Venezuelan equine encephalitis virus-HIV-1 matrix/capsid coding domain of *gag*	Recombinant Venezuelan equine encephalitis virus replicons	HXB2		PCT-SA	273-274
Venezuelan equine encephalitis virus-HIV-1 matrix/capsid coding domain of *gag*	Recombinant attenuated Venezuelan equine encephalitis virus replicons	Clade C		PCT-SA	275
Venezuelan equine encephalitis virus-HIV-1 *env, gag, pol* replicons	Recombinant Venezuelan equine encephalitis virus replicons	Clade C		PCT-SA	276
Vesicular stomatitis virus-HIV-1 gp 160	Recombinant vesicular stomatitis virus	89.6		BR&D	277
DNA:					
env (gp160), rev DNA (APL 400-003; GeneVax™)	Plasmid	MN	bupivacaine; ± jet injection	PhI-P*, PhI-T	278-290
gag + pol DNA (APL 400-047, GeneVax™)	Plasmid	HXB2	bupivacaine; ± jet injection	PhI-P*, PhI-T	291-298
gp160, *nef* ± *rev* ± *tat* DNA	Plasmid	LAI		PCT-SA, PhI-T	299-305
gag DNA	Plasmid	Primary HIV optimized sequence		PCT-M	306-307
gp120 DNA	Plasmid	SF2, CM235 (Thai E), US4 (B)	± gene gun	PCT-SA	308-310
gp160, gp140, gp120 DNA	Plasmid	ADA	± gene gun	PCT-SA, M	311-317
gp160 DNA	Plasmid	IIIB	alpha 25 dihydroxy-cholecalciferol	PCT-SA	318
gp120 DNA	Plasmid	LAI	± gene gun	PCT-SA, M	319-321
env (gp160)-*rev* DNA	Plasmid	IIIB	MPL-A, QS-21, mannan-coated diC14-amidine, ubenimex	PCT-SA	322-324
gp 120 DNA	Plasmid	JR-FL, Bal, HXB2, SP5, SP6	± gene gun	PCT-SA	325
gp 120 DNA	Plasmid		bupivicaine, cardiotoxin	PCT-SA	326
gp120 or rev-gp160 DNA	Plasmid	MN, LAI	± gene gun	PCT-SA, M	327-332

Vaccine Candidate	Expression System/ Production Method	HIV Strain(s)	Adjuvant or Delivery System	Stage of Development	References
Cellular:					
Autologous, allogeneic dendritic cells pulsed with HIV antigens	Cells pulsed with rgp160 MN, *gag* and *env* peptides	MN		PhI-T	333
Moloney murine leukemia virus-HIV-1 env, rev vector transduced autologous fibroblasts	Recombinant Moloney murine leukemia virus/ transduction	LAI		PhI-T	334
Disrupted autologous HIV+ PBMC & plasma (DROVAC)	Disruption of PBMC; plasma containing virions	Clade B	Sendai virus envelope-derived adjuvant (SDE)	PhI-T	335-337
Autologous CD8+ cells, killed after in vitro culture with HIV-1	Cells fixed and gamma irradiated	LAV (IIIB)		PhI-T	141
Autologous cells, killed after in vitro infection with recombinant vaccinia-HIV-1 gp160	Recombinant vaccinia-HIV-1 gp160 (vv25). Cells fixed and irradiated	LAV (IIIB)		PhI-T	142
Autologous B lymphocytes, killed after in vitro infection with recombinant vaccinia-HIV-1 gp160	Recombinant vaccinia-HIV-1 gp 160 (vv25). Cells fixed and irradiated	LAV (IIIB)		PhI-T	338-339
CD4 as immunogen:					
rCD4	Recombinant protein		IFA	PhI-T	340
Anti-idiotype approach:					
Anti-gp 120 (C39)	Murine monoclonal antibody		SAF-M	PhI-T	341-343
Anti-CD4 idiotype (IOT4a)	Murine monoclonal antibody		alum	PhI & II-T	344-345
Plant produced:					
Alfalfa mosaic virus-HIV-1 V3	Recombinant alfalfa mosaic virus/tobacco plants	MN	CFA/IFA	PCT-SA	346
Cowpea mosaic virus-HIV-1 gp41 (Kennedy epitope)	Recombinant cowpea mosaic virus/plants		LAI	PCT-SA	347
Cowpea mosaic virus-HIV-1 env peptides	Recombinant cowpea mosaic virus/plants			BR&D	348
Live attenuated:					
Live, attenuated HIV	Mutations and deletions	Multiple		PCT-SA, M	349-357
Live, inactivatable attenuated HIV-1	Insertion of ganciclovir susceptibility gene into HIV-1, deletion of nef gene	Multiple		BR&D	358-359
Live, attenuated Simian/ Human Immunodeficiency Virus (SHIV)	Recombination, mutation, deletion	Multiple (SHIV)		PCT-M	360-361

Stage of Development

BR&D, basic research and development
PCT-SA, preclinical testing in small animals
PCT-M, preclinical testing in monkeys
PCT-C, preclinical testing in chimpanzees
Ph-I, Phase I clinical trials
Ph-II, Phase II clinical trial
Ph-III, Phase III clinical trial
P, prevention trial
T, treatment trial
* clinical trials conducted by NIAID

HIV Strains

LAI, group of closely related HIV isolates that includes LAV, IIB, BH10, and BRU

Vaccine Candidates

MAPS, multiple antigen presentation systems
VLP, virus-like particle
MoMLV, Moloney murine leukemia virus
BCG, Bacillus Calmette-Guérin

Adjuvants

alum, aluminum hydroxide, aluminum phosphate, aluminum hyroxide gel
CFA, Complete Freunds Adjuvant
DOC, deoxycholate
GM-CSF, granulocyte-macrophage colony-stimulating factor
IFA, Incomplete Freund's Adjuvant, mineral oil + mannose monooleate
ISA 720, a mixture of a natural metabolizable oil and a highly refined emulsifier
ISA 724, a mixture of mineral oil and metabolizable oil
MDP, threonyl muramyl dipeptide (Termutide; N-acetylmuramyl-L-threonyl-D-Isoglutamine)
MF59, microfluidized oil-in-water emulsion
Montanide ISA720, see ISA720
MPL, a detoxified form of lipid A, a component of bacterial lipopolysaccharide endotoxin
MPL-A, a detoxified form of bacterial lipid A in a 0.25% squalene emulsion
MPL-AF, see MPL
MTP-PE, muramyl tripeptide (MTP) linked covalently with dipalmitoyl phosphatidylethanolamine (PE)
P40, Protein 40, Corynebacterium extract
PCPP, polyphosphazene (PCPP) is a synthetic microsphere hydrogel
PPD, purified protein derivative of Mycobacterium tuberculosis
QS-21, a nontoxic saponin derivative from the bark of the soapbark tree Quillaja saponaria
RIBI, see MPL
SAF-2, see Syntex Adjuvant Formulation
SAF-M, Syntex Adjuvant Formulation, an oil in saline emulsion

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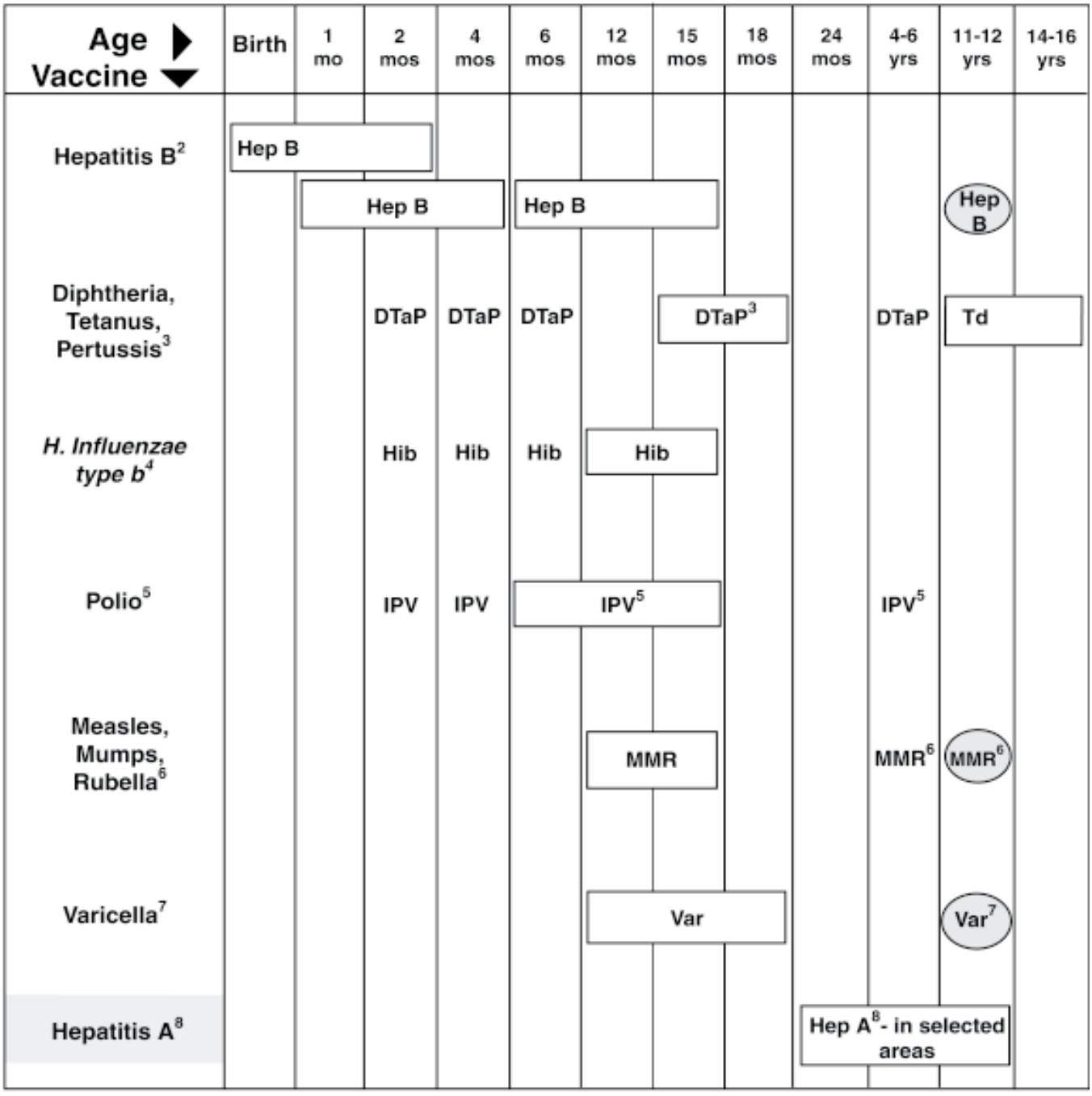
APPENDIX D

Recommended Childhood Immunization Schedule United States, January - December, 2000

Vaccines[1] are listed under routinely recommended ages. Bars indicate range of recommended ages for immunization. Any dose not given at the recommended age should be given as a "catch-up" immunization at any subsequent visit when indicated and feasible. Ovals indicate vaccines to be given if previously recommended vaccines were missed or given earlier than the recommended minimum age.



Approved by the Advisory Committee on Immunization Practices (ACIP), the American Academy of Pediatrics (AAP), and the American Academy of Family Physicians (AAFP).

In October 1999, the Advisory Committee on Immunization Practices (ACIP) recommended that Rotashield (RR V-TV), the only U.S. licensed rotavirus vaccine, no longer be used in the United States (MMWR Morb Mortal Wkly Rep. Nov 5, 1999;48(43):1007). Parents should be reassured that their children who received rotavirus vaccine before July are not at increased risk for intussusception now.

1. This schedule indicates the recommended ages for routine administration of currently licensed childhood vaccines as of 11/1/99. Additional vaccines may be licensed and recommended during the year. Licensed combination vaccines may be used whenever any components of the combination are indicated and its other components are not contraindicated. Providers should consult the manufacturers' package inserts for detailed recommendations.

2. Infants born to HBsAg-negative mothers should receive the first dose of hepatitis B (Hep B) vaccine by age 2 months. The second dose should be at least 1 month after the first dose. The third dose should be administered at least 4 months after the first dose and at least 2 months after the second dose, but not before 6 months of age for infants.

   Infants born to HBsAg-positive mothers should receive hepatitis B vaccine and 0.5 mL hepatitis B immune globulin (HBIG) within 12 hours of birth at separate sites. The second dose is recommended at 1 month of age and the third dose at 6 months of age.

   Infants born to mothers whose HBsAg status is unknown should receive hepatitis B vaccine within 12 hours of birth. Maternal blood should be drawn at the time of delivery to determine the mother's HBsAg status; if the HBsAg test is positive, the infant should receive HBIG as soon as possible (no later than 1 week of age).

   All children and adolescents (through 18 years of age) who have not been immunized against hepatitis B may begin the series during any visit. Special efforts should be made to immunize children who were born in or whose parents were born in areas of the world with moderate or high endemicity of hepatitis B virus infection.

3. The fourth dose of DTaP (diphtheria and tetanus toxoids and acellular pertussis vaccine) may be administered as early as 12 months of age, provided 6 months have elapsed since the third dose and the child is unlikely to return at age 15 to 18 months. Td (tetanus and diphtheria toxoids) is recommended at 11 to 12 years of age if at least 5 years have elapsed since the last dose of DTP, DTaP, or DT. Subsequent routine Td boosters are recommended every 10 years.

4. Three *Haemophilus influenzae* type b (Hib) conjugate vaccines are licensed for infant use. If PRP-OMP (PedvaxHIB or ComVax [Merck]) is administered at 2 and 4 months of age, a dose at 6 months is not required. Because clinical studies in infants have demonstrated that using some combination products may induce a lower immune response to the Hib vaccine component, DTaP/Hib combination products should not be used for primary immunization in infants at 2, 4, or 6 months of age unless FDA-approved for these ages.

5. To eliminate the risk of vaccine-associated paralytic polio (VAPP), an all-IPV schedule is now recommended for routine childhood polio vaccination in the United States. All children should receive four doses of IPV at 2 months, 4 months, 6 to 18 months, and 4 to 6 years. OPV (if available) may be used only for the following special circumstances:
   1. Mass vaccination campaigns to control outbreaks of paralytic polio.
   2. Unvaccinated children who will be traveling in less than 4 weeks to areas where polio is endemic or epidemic.
   3. Children of parents who do not accept the recommended number of vaccine injections. These children may receive OPV only for the third or fourth dose or both; in this situation, health care professionals should administer OPV only after discussing the risk for VAPP with parents or caregivers.
   4. During the transition to an all-IPV schedule, recommendations for the use of remaining OPV supplies in physicians' offices and clinics have been issued by the American Academy of Pediatrics (see *Pediatrics,* December 1999).

6. The second dose of measles, mumps, and rubella (MMR) vaccine is recommended routinely at 4 to 6 years of age but may be administered during any visit, provided at least 4 weeks have elapsed since receipt of the first dose and that both doses are administered beginning at or after 12 months of age. Those who have not previously received the second dose should complete the schedule by the 11- to 12-year-old visit.

7. Varicella (Var) vaccine is recommended at any visit on or after the first birthday for susceptible children, i.e., those who lack a reliable history of chickenpox (as judged by a health care professional) and who have not been immunized. Susceptible persons 13 years of age or older should receive 2 doses, given at least 4 weeks apart.

8. Hepatitis A (Hep A) is shaded to indicate its recommended use in selected States and/or regions; consult your local public health authority. (Also see *MMWR Morb Mortal Wkly Rep.* Oct 1, 1999;48(RR-12); 1-37).