

NIAID Biodefense Research Agenda for CDC Category A Agents

Progress Report



August 2003



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**The National Institute of Allergy and Infectious Diseases (NIAID)
Biodefense Research Agenda for CDC Category A Agents***

Progress Report

In February 2002, the National Institute of Allergy and Infectious Diseases convened the first Blue Ribbon Panel on Bioterrorism and Its Implications for Biomedical Research. This panel of experts was brought together by NIAID to provide objective expertise on the Institute's future counter-bioterrorism research agenda for anthrax, smallpox, botulism, plague, tularemia, and viral hemorrhagic fevers, the pathogens commonly referred to as CDC Category A agents.

As a result of this meeting and the deliberations of the panel, a research agenda was developed and widely distributed to the scientific community. This agenda described the recommendations of the panel and NIAID's priorities for research on the Category A agents of bioterrorism (agenda available at <http://www.niaid.nih.gov/biodefense/research/biotresearchagenda.pdf>).

Tremendous progress has been made in the year since this report was released. A significant area of early emphasis for NIAID has been establishment of the research infrastructure necessary to support studies of biodefense pathogens, such as biosafety containment laboratories. In addition, NIAID has worked to attract the long-term interest and support of industry and academia in developing biodefense countermeasures. NIH has developed a number of grant and contract initiatives to help attract scientists to this area of research. In FY2002 and FY2003, NIAID developed more than 50 initiatives to stimulate biodefense research; approximately 75% of these are new initiatives and 25% are significant expansions of existing contracts. During this same time period, there was a 30% increase in the number of grant applications submitted and assigned to NIAID; the vast majority of these were in response to these biodefense initiatives.

In addition to increasing the breadth and depth of biodefense research, NIAID has made significant progress in meeting the specific goals and recommendations of the Blue Ribbon Panel. This progress report describes the progress that has been made toward addressing the immediate goals outlined in the research agenda. The first section of this report reviews progress on meeting the general recommendations made by the panel that apply to all areas of NIAID biodefense research. Research goals specific to each of the Category A pathogens are covered in individual chapters. Finally, the progress made thus far on immunology as it relates to biodefense is described in a separate chapter.

* The Centers for Disease Control and Prevention (CDC) Category A Agents of Bioterrorism include anthrax, smallpox, plague, botulism, tularemia, and viral hemorrhagic fevers. (<http://www.bt.cdc.gov/Agent/Agentlist.asp#categoryadiseases>)

Progress on General Recommendations of the Blue Ribbon Panel

Recommendation: Develop Regional Centers of Excellence for Bioterrorism and Emerging Infectious Diseases Research.

- In August 2002, NIAID announced an initiative to support large, multidisciplinary regional centers that are expected to provide the scientific information and translational research capacity needed to make the next generation of therapeutics, vaccines and diagnostics against Category A-C priority pathogens. NIAID expects to award 7-8 centers in late FY2003. A planning grant component was included in this initiative, and NIAID expects to award 2-3 planning grants in late FY2003.

Recommendation: Expand the capacity to conduct phase I, II, and III evaluations of candidate vaccines and treatments for agents of bioterrorism.

- NIAID has expanded the Vaccine Treatment and Evaluation Units (VTEUs) by approximately 60%. In the past year, eight clinical trials of various smallpox vaccines have been completed or are underway at VTEU sites (see Table 1 on the last page of this document). Additional clinical trials for recombinant protective antigen (rPA) vaccines are currently being planned for conduct by the VTEUs.
- Clinical trials are being conducted through NIAID's Vaccine Research Center (VRC). A clinical protocol has been approved that will allow DIR scientists to evaluate and treat persons with suspected exposure to, or infection with, anthrax, and to follow these individuals over time to assess potential complications of anthrax. DIR scientists have also developed attenuated viruses representing all four dengue subtypes for use as candidate vaccines. Phase I clinical testing of a live virus dengue 4 vaccine is nearing completion.
- NIAID has initiated two new programs, the Food and Waterborne Diseases Integrated Research Network and the Respiratory Pathogens Research Network. These programs expand the Institute's capacity to conduct clinical research studies of food and water-borne enteric pathogens and bacterial and respiratory pathogens, respectively.
- NIAID has expanded the Collaborative Antiviral Study Group (CASG) by approximately 20%. In the past year, a clinical protocol has been developed for the treatment of smallpox with cidofovir in the event of an outbreak or release. A phase I clinical study in normal volunteers is planned by the CASG in FY2004 to assess initial safety, tolerability and pharmacokinetics of a new oral derivative of cidofovir, a promising therapeutic for poxvirus infections.
- In order to support increased clinical research activities, NIAID has expanded contracts for regulatory support, assay development, immunology quality assurance and quality control, and clinical trial management.

Recommendation: Expand nonhuman primate capability to evaluate new therapeutic and vaccine products.

- *In vitro* and Animal Models for Emerging Infectious Diseases and Biodefense, a new initiative, will provide a range of resources for preclinical testing of new therapies and vaccines including nonhuman primate models. Awards are anticipated in late FY2003.
- NIAID has expanded the capacity to evaluate products for biodefense in nonhuman primates by expanding appropriate animal holding containment facilities as part of a cooperative research program with the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID).
- The NIAID intramural program has expanded a research support contract to provide additional nonhuman primates and animal BSL-3 facilities for biodefense research and studies of emerging and re-emerging diseases. Intramural BSL-3 capacity will expand in January of 2004 upon opening of the new Lab at Twinbrook in Rockville, Maryland.

Recommendation: Attract new scientific disciplines to counter-bioterrorism research, and expand the research training of a new cohort of investigators.

- The Rapid Response Grant Program on Bioterrorism-Related Research was developed to support exploratory, innovative research targeted at the design and development of specific diagnostics, therapies, and prevention strategies for Category A biological diseases. This program encouraged investigators from a diversity of disciplines to become involved in biodefense research. Sixty-nine grants were awarded in FY2002 in response to this initiative.
- NIAID recently made available new and expanded programs for supporting the training and career development of young scientists in biodefense research. Approximately 30% of the training grant applications received in FY2003 were specifically focused on training in biodefense.
- NIAID has expanded intramural biodefense investigations, which has provided new opportunities in NIAID laboratories for postdoctoral research in select agent biology and pathogenesis, and in development of vaccines, diagnostics, and therapeutics for biodefense.
- NIAID staff regularly present the institute's biodefense research agenda at scientific meetings and workshops to raise awareness of, and interest in, available funding opportunities. A biodefense website provides information on ongoing and new funding opportunities to the scientific community. In addition, NIAID co-sponsored a meeting with the American Society for Microbiology in March 2003, Future Directions for Biodefense Research: Development of Countermeasures, at which new biodefense research and training programs were discussed.

Recommendation: Expand extramural and intramural research and clinical infrastructure, including construction and renovation of BSL-3/4 laboratories.

- NIAID is supporting two initiatives for the development of extramural biocontainment laboratories. The National Biocontainment Laboratories (NBLs) and Regional Biocontainment Laboratories (RBLs) will provide funding for the design, building, and certification of comprehensive state-of-the-art BSL-3 and -4 laboratories. The NBLs will include BSL-4 capability, non-human primate capacity, and facilities and resources for

small scale phase I clinical trials. The RBLs will provide BSL-3 and BSL-2 facilities. These laboratories will serve as a national resource for preclinical and clinical research on biodefense agents and emerging infectious diseases. In the event of a bioterrorism emergency, these facilities will also serve as a resource to assist national, state, and local public health efforts. NIAID plans to fund 1-2 NBLs and 5-8 RBLs in FY2003.

- NIAID is providing support to current grantees for renovation/upgrade of their laboratories in order to ensure that appropriate containment facilities are available.
- NIAID has begun planning and preconstruction activities for three new integrated research facilities (IRF) that will expand NIAID's clinical, laboratory and animal biocontainment capacity. These include a BSL-2/3 IRF on the NIH campus, a BSL-2/3/4 IRF at Ft. Detrick in Frederick, Maryland, and a BSL-2/3/4 IRF at the Rocky Mountain Laboratories in Hamilton, Montana.

Recommendation: Expand the availability of animal models for preclinical research.

- The new initiative, *In Vitro* and Animal Models for Emerging Infectious Diseases and Biodefense, will provide a range of animal models for preclinical testing of new therapies and vaccines. Awards are anticipated in late FY2003.
- Under a coordinated network of contracts, NIAID supports the development of animal models and screening of compounds for activity against orthopoxviruses (murine models of vaccinia, cowpox, and ectromelia) and respiratory viruses (murine models of influenza A and B).
- Existing resources that support animal model development for emerging viral infections were expanded to include several viral hemorrhagic fevers and encephalitides. The new models are:
 - Bunyavirus: Punta Toro virus in mice
 - Arenavirus: Pichinde virus in hamsters
 - Flavivirus: Banzi virus in mice
 - Togavirus: Semliki Forest virus in mice

Recommendation: Develop rapid, inexpensive, and broad-based clinical diagnostics approaches using genomics and proteomics.

- NIAID has developed two initiatives that specifically support the use of genomic as well as non-genomic technologies for the development of the next generation of medical diagnostics to detect an infection and/or infectious agents: Biodefense Partnerships, and the Cooperative Research for the Development of Vaccines, Adjuvants, Therapeutics, Immunotherapeutics, and Diagnostics for Biodefense Program. Under these initiatives, 75 grant applications focused on the use of genomic/proteomic technologies for the development of medical diagnostics have been submitted. Awards are to be made before the end of FY2003.
- The FY2002 initiative, the Rapid Response Grant Program on Bioterrorism-Related Research, encouraged the development of medical diagnostics for biodefense agents.

Under this initiative, 10 applications related to the development of diagnostics were awarded.

- In FY2002, NIAID specifically encouraged the receipt of biodefense applications under the Small Business Innovative Research (SBIR) program using a special announcement (the Small Business Biodefense Program). Of the awards made, over 25% were related to the development of diagnostics. The SBIR program is ongoing, and applications related to diagnostics continue to be submitted.
- A new initiative, Identifying Targets for Therapeutic Interventions Using Proteomic Technologies, will support the development of innovative proteomic technologies and their application to understanding the pathogen and host cell proteome for the discovery and identification of novel targets for therapeutics, vaccines, and diagnostics. Awards are planned for FY2004.
- NIAID scientists have developed gene microarrays for several pathogens. These microarrays have led to the discovery of novel bacterial toxins, pathogen defense mechanisms, and potential targets for vaccines and therapeutics.

Recommendation: Encourage structural genomics and proteomics for the targeted development of drugs, vaccines, and diagnostics.

- NIAID has made a significant investment in the genome sequencing of microorganisms considered agents of bioterrorism. As a result of a coordinated federal effort with the Department of Energy (DoE), CDC, the United States Department of Agriculture (USDA), and the National Science Foundation (NSF), and international partners including the Sanger Center, genome sequencing projects are ongoing for at least one strain of every virus or protozoan on the list of Category A-C priority pathogens. To date, there is a completed or near completed genome sequence for every bacteria on the list, including *Bacillus anthracis*, *Clostridium botulinum*, *Yersinia pestis*, and *Francisella tularensis*. In addition, the coordinated federal effort has expanded the sequencing and annotation of variola major viruses. These sequences will be used for identifying potential microbial genetic signatures and targets for the development of drugs and vaccines against these agents.
- The NIAID-supported Pathogen Functional Genomics Resource Center (PFGRC) was expanded to provide the research community with the needed resources and reagents to conduct both basic and applied research on microorganisms responsible for emerging and re-emerging infectious diseases, including those considered agents of bioterrorism. Such resources will help researchers to identify targets and proteins for use in new diagnostics. (The Institute for Genomic Research [TIGR], Rockville, Maryland)
- The Poxvirus Bioinformatics Resource Center provides sequencing and functional comparisons of orthopox genes; designs and maintains of a relational database to store, display, annotate and query genome sequences, structural information, phenotypic data and bibliographic information; and serves as a repository of well-documented viral strains. This information is important for the development of drugs and vaccines. For example, sequence analyses of the variola DNA polymerase showed that this enzyme is highly conserved among all poxviruses. Thus, an antiviral drug that targets this enzyme and is effective in animal models infected with various poxviruses may also work against

variola. The center is a collaborative effort led by St. Louis University and the University of Alabama at Birmingham and supported by two federal agencies, the Defense Advanced Research Projects Agency (DARPA) and NIH; collaborators include the CDC, American Type Culture Collection, USAMRIID, the Medical College of Wisconsin, and the University of Victoria.

- In FY2003, NIAID initiated support for large, multi-disciplinary efforts focused on the proteomics of more than one microorganism. Under this program, NIAID is currently funding a Biodefense Proteomics Collaboratory, a multi-disciplinary team of scientists from both academia and the biotechnology industry who are using high-throughput proteomic technologies to identify protein targets from arenaviruses and Lassa virus. (University of Texas Medical Branch, Galveston)

Recommendation: Encourage industry participation to ensure the availability of rapid, sensitive, and licensed diagnostics to hospital clinical laboratories.

- NIAID has created several novel mechanisms to encourage research and development of diagnostics by the private sector. These initiatives include Biodefense Partnerships; the Cooperative Research for the Development of Vaccines, Adjuvants, Therapeutics, Immunotherapeutics, and Diagnostics for Biodefense Program; and the Small Business Biodefense Program.

Recommendation: Expand partnership opportunities with other agencies and governments.

- NIAID has established a cooperative program with USAMRIID to conduct mutually-agreed upon research projects related to biodefense. Ongoing projects under this agreement include the development of vaccines for viral hemorrhagic fevers using Ebola virus as a model and an evaluation of the efficacy of antibiotic treatment in a model of pneumonic plague in African green monkeys and an evaluation of MVA vaccine protection in a monkeypox challenge model. An evaluation of a recombinant plague vaccine candidate (F1-V) in a nonhuman primate parenteral challenge model is planned.
- NIAID and CDC, along with other DHHS agencies, participate regularly in several interagency working groups of technical experts to discuss and develop countermeasures for the Category A agents.
- NIAID is participating in the FBI-sponsored Scientific Working Group on Microbial Genetics and Forensics. Other participants include federal agency officials and scientists with expertise in genomics, bioinformatics, microbiology, and infectious diseases. The working group's mission is to define criteria and coordinate the development and validation of microbial forensic methods that will support criminal investigations.
- NIAID and the Department of Defense (DoD) are collaborating in development of overseas field sites for the testing of new therapeutics, vaccines, and diagnostics against Category A-C priority pathogens in endemic areas.
- The Regional Centers of Excellence (RCEs) for Biodefense and Emerging Infectious Diseases Research that will be established in late FY2003 will encourage cooperation with state and federal agencies as well as partnerships with the private sector. The

RCEs, together with the NBL/RBLs, could be used to augment CDC and state and local health department efforts for homeland defense.

- NIAID is collaborating with CDC for development of immunogenicity assays to be used for evaluation of a second-generation anthrax vaccine based on recombinant protective antigen (rPA).
- NIAID staff participate in an Interagency Working Group that coordinates science and technology efforts by federal agencies on biodefense diagnostics. The working group is a subcommittee of an Office of Science and Technology Policy/Homeland Security Council (HSC)-led interagency working group.
- NIAID continues to coordinate genomic and post-genomic initiatives, including those related to biodefense, with other federal agencies through both participation in the Microbe Project, a federal interagency working group that coordinates microbial genomic activities, and through contact with key scientists at specific agencies such as the Central Intelligence Agency (CIA), the Federal Bureau of Investigation (FBI), the Food and Drug Administration (FDA), CDC, DoD, DoE, NSF, and USDA.

Recommendation: Develop a centralized repository for reagents and clinical specimens for agents of bioterrorism.

- NIAID plans to support the acquisition, authentication, storage, and distribution to the scientific community of state-of-the-art research and reference reagents related to biodefense starting in late FY2003. Included will be the capability to validate, expand, and produce biological agents including cell lines, clones, proteins, monoclonal and polyclonal antibodies, and diagnostic tools.
- An existing NIAID-supported reference reagent repository has been expanded to specifically include reagents needed to develop and test improved vaccines, diagnostics, and therapeutics for anthrax. Under this expansion, the repository is now preparing and distributing the protective antigen, lethal factor, and edema factor proteins of anthrax.

Recommendation: Develop procedures and cGMP facilities capable of producing monoclonal antibodies, vaccines, and other immunotherapies for phase I and II clinical studies.

- NIAID plans to support an Antibody Production Facility starting in late FY2003. This facility will ensure that sufficient quantities of mouse, humanized, and/or fully human monoclonal antibodies against diseases caused by agents of bioterrorism can be produced for preclinical and clinical evaluations.
- In September 2002, NIAID awarded two contracts to develop, manufacture, and characterize a cGMP pilot lot of a *Bacillus anthracis* rPA vaccine for evaluation in a phase I clinical study (Avecia Ltd. of Manchester, UK, and VaxGen Inc. of Brisbane, California).
- In February 2003, NIAID awarded two contracts to develop, manufacture and characterize a cGMP pilot lot of a modified vaccinia Ankara (MVA) vaccine for evaluation in a phase I clinical study (Bavarian Nordic A/S of Copenhagen, Denmark, and Acambis Inc. of Cambridge, Massachusetts).

- Initiatives to support the intermediate-scale advanced development of rPA and MVA vaccines are planned for late FY2003 and early FY2004, respectively. These initiatives may include: production and release of cGMP-consistency lots; formulation, vialing, and labeling of vaccine; development of animal models in at least two species to support the FDA animal rule; process, assay and facility validation; and clinical evaluation in initial phase II trials.

Recommendation: Enhance adjuvant discovery and rational design of *Toll* system mediators.

- Under a new initiative, Innate Immune Receptors and Adjuvant Discovery, natural and synthetic ligands will be screened for their ability to stimulate innate immune receptors. This research is expected to establish a pipeline of new adjuvant leads. Awards are anticipated for late FY2003.

Recommendation: Identify and characterize innate and adaptive immune responses that occur after exposure to agents of bioterrorism and enhance basic research on mucosal immunology.

- Under the Biodefense and Emerging Infectious Diseases Research Opportunities initiative, NIAID is encouraging research on protective mechanisms against infection with the CDC Category A-C priority pathogens. One recently awarded grant is focused on the development of technology for probing innate immunology. It is hoped that a knowledge base of innate immune system activity will be developed to aid in identification of genetic changes and proteins that are triggered by encounters between innate immune cells and infectious pathogens. (Scripps Research Institute, La Jolla, California).
- NIAID recently initiated a new program, Cooperative Centers for Translational Research on Human Immunology and Biodefense, which will support an interactive network of investigators studying various aspects of immunity to biodefense pathogens and the development of new technologies to facilitate clinical immunology research.

Recommendation: Establish MHC-peptide and B-cell epitope databases that may be used to further define immune responses, including the identification of relevant immune polymorphisms, and maximize such responses.

- Under the Immune Epitope Database and Analysis Program, announced in FY2002, a publicly accessible immune epitope database will be designed, developed and maintained for the research community. An award is anticipated in FY2004.
- The Large Scale Antibody and T Cell Epitope Discovery Program, announced in FY2002, will promote the development of novel or high-throughput screening methods for antibody and/or T cell epitope identification. This should facilitate the design and development of improved immunotherapeutics and vaccines, and the prediction of host responses to antigens *in vivo*.

ANTHRAX

Bacillus anthracis, the agent that causes anthrax, has several characteristics that make it a formidable bioterrorist threat. These characteristics include its stability in spore form, its ease of culture and production, its ability to be aerosolized, the seriousness of the disease it causes, and the lack of sufficient vaccine for widespread use.

Human anthrax has three major clinical forms: cutaneous, inhalational, and gastrointestinal. If left untreated, all three forms can result in septicemia and death. Early antibiotic treatment of cutaneous and gastrointestinal anthrax is usually curative; however, even with antibiotic therapy, inhalational anthrax is a potentially fatal disease. Although case-fatality estimates for inhalational anthrax are based on incomplete information, the historical rate is considered to be high (about 75%) for naturally occurring or accidental infections, even with appropriate antibiotics and all other available supportive care. However, the survival rate after the recent intentional exposure to anthrax in the United States was 60% for the first 10 cases.

Scientific Progress

In the year since publication of the NIAID Biodefense Research Agenda for Category A Agents in February 2002, significant progress has been made in understanding the basic mechanisms by which *B. anthracis* causes disease, and in developing countermeasures against anthrax. Examples include:

Key features in the pathogenesis of anthrax identified; research may yield an antitoxin.

The anthrax bacterium causes illness and death by releasing toxins that kill cells and damage organs. Few people survive when the microbe spreads throughout the body, as is the case in the severe, inhalational form of the disease. This is because the toxin remains active in the bloodstream for several days, even if antibiotics kill the bacteria that are producing it. In one study, researchers elucidated how toxin binds to and enters healthy cells, and how it disrupts a cell's internal communications network. They identified key structures on the toxin molecule that could lead to the development of non-toxic analogs (decoys) to block the lethal effects of toxins, thereby resulting in a new approach for treating the infection. (Pannifer AD et al., Crystal structure of the anthrax lethal factor, *Nature* 2001;414:229-233)

Through genetic analysis, other scientists identified a protein on the surface of mammalian cells, the anthrax toxin receptor (ATR), and identified the specific region on ATR to which the toxin attaches. With this information they were able to produce a soluble version of this as well as another receptor with the toxin-binding domain. When these soluble receptors are mixed with mammalian cells in the presence of anthrax toxin, they act as decoys to absorb anthrax toxin before it can attach to target cells to produce its lethal effects. (Bradley KA et al., Identification of the cellular receptor for anthrax toxin, *Nature* 2001;414:225-229)

Both of these studies suggest that developing compounds to block the lethal effects of anthrax toxin, by one or both of the mechanisms described, will be of great value for treating anthrax.

Molecular mechanisms by which anthrax evades immune systems uncovered.

DIR and NIAID supported scientists have discovered one strategy that anthrax uses to avoid the host's immune reaction. In this research, the investigators determined that the critical virulence factor, anthrax lethal toxin, targets the MAP kinase intracellular signalling pathway in dendritic cells, an important class of antigen presenting cell in lymph tissues. This blocks the stimulation

of antigen-specific T cells and both T- and B-cell immunity are reduced. These data suggest a role for LT in suppressing host immunity during *B. anthracis* infections. Agrawal, A et.al., Impairment of dendritic cells and adaptive immunity by anthrax lethal toxin. *Nature* 2003; 424: 329–334; doi:10.1038/nature01794)

Researchers unravel anthrax genomes. *B. anthracis* is the microorganism that causes anthrax and is responsible for the illness and deaths associated with the deliberate exposure of civilian populations in the United States to this agent. Comparing the genome sequences of two strains of *B. anthracis* (the Ames strain and a clinical isolate from a patient in Florida exposed to anthrax), investigators identified four novel DNA sequence differences at the single nucleotide level not seen before using other molecular genotyping methods. These differences in sequence were used as genetic markers to screen a set of *B. anthracis* isolates. Analysis of the data revealed that the Florida isolate was likely to have been derived from the Ames strain. This study provides strong evidence to support the use of genome-based analysis in finding genetic variation at the single nucleotide level (SNP) for forensic strain identification. (Read TD et al., Comparative genome sequencing for discovery of novel polymorphisms in *Bacillus anthracis*, *Science* 2002;296:2028-2033)

Most recently, the complete genetic blueprint of *B. anthracis* has been solved. Investigators found a number of genes encoding proteins that *B. anthracis* may use to enter host cells and therefore may be important targets for vaccines and drugs. Using comparative genomics, investigators compared an Ames isolate with two closely related *Bacillus* bacteria and found remarkably little difference, highlighting the similarity of *B. anthracis* to related pathogens not associated with anthrax. Additional comparisons revealed similarities between genes of *B. anthracis* and pathogens that infect insects, suggesting that a recent ancestor of *B. anthracis* also may have infected insects. Information obtained from these efforts will help researchers to better understand the disease-causing capabilities of *B. anthracis* and to design new vaccines and treatments. (Read TD et al., The genome sequence of *Bacillus anthracis* Ames and comparison to closely related bacteria, *Nature* 2003;423:82-86)

Programmatic Progress in Addressing Immediate Goals

Goal: Establish capacity for the development and production of pilot lots of candidate anthrax vaccines.

- In September 2002, NIAID awarded two contracts for the development and testing of next-generation anthrax vaccines based on recombinant protective antigen (rPA), including production of pilot lots (Avecia Ltd. of Manchester, UK, and VaxGen Inc. of Brisbane, California).

Goal: Conduct phase I and II trials with rPA anthrax vaccine candidates and alternative adjuvant formulations.

- An initiative to support the intermediate-scale advanced development of an rPA vaccine has been released. This initiative includes: production and release of cGMP consistency lots; formulation, vialing and labeling of vaccine; development of animal models in at least two species to support the Food and Drug Administration (FDA) animal rule; process, assay and facility validation; and clinical evaluation in initial phase II trials.

Goal: Expand clinical capability to accelerate phase I and II testing of candidate vaccines.

- Under the contracts for pilot lot production and evaluation, and intermediate-scale manufacturing and evaluation of rPA vaccine, multiple phase I and II clinical trials will be conducted.
- NIAID has expanded the Vaccine Treatment and Evaluation Units (VTEUs) by approximately 60%. A phase I trial of an rPA candidate vaccine is planned for late FY2003.
- In order to support increased clinical research activities, NIAID has expanded contracts for regulatory support, assay development, immunology quality assurance and quality control, and clinical trial management.

Goal: Screen existing FDA-approved antimicrobials and immunomodulators for efficacy against anthrax.

- Under the *In Vitro* and Animal Models for Emerging Infectious Diseases and Biodefense contracts, planned for award at the end of FY2003, NIAID will screen existing FDA-approved antimicrobials and immunomodulators for efficacy against inhalational anthrax. Five licensed antibiotics have been selected for study, with ciprofloxacin as a control. Studies will commence in FY2004.
- Under the *In Vitro* and Animal Models for Emerging Infectious Diseases and Biodefense contracts, and a cooperative agreement with the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID), NIAID will pursue studies to determine whether the course of antibiotic therapy can be decreased by vaccinating subjects with the rPA vaccine candidates currently under development. Studies are planned for early FY2004.
- Approximately 31 grants have been funded through both the Biodefense Partnerships and Small Business Innovative Research (SBIR) programs supporting work in this area.

Goal: Conduct comparative genomic sequencing of selected *Bacillus* strains to detect subtle differences in the pathogenesis and virulence associated with antigens or other factors.

- The Pathogen Functional Genomics Resource Center (PFGRC) completed the genome sequencing of 14 different species, strains, clinical isolates, and nearest neighbors of *B. anthracis*. In addition, comparative genomic analysis has been conducted and genetic variations and relatedness within and between species identified. Sequencing projects have been completed for the *Bacillus anthracis* strains, Kruger B and Western North America strain, and a related species, *B. cereus*. In addition, new genomic software tools have been developed to enhance comparative genomic analyses. (Also see the Scientific Progress section.) (The Institute for Genomic Research [TIGR])
- Rigorous protocols and standard operating procedures for high quality comparative genomic analysis for bacterial genomes have been developed by PFGRC. As a result, there will be standard tools for detecting genetic variation among different strains and clinical isolates, providing potential microbial genetic signatures for diagnosis.

Goal: Establish a centralized immunology laboratory to develop and validate tests required for the licensure of anthrax vaccines.

- NIAID is collaborating with the Centers for Disease Control and Prevention (CDC) for the development of immunogenicity assays to be used for evaluation of a second-generation anthrax vaccine based on rPA. CDC is establishing standard reagents and validating a number of critical serologic assays that will be used to evaluate samples from small animals, non-human primates, and humans.
- In April 2002, NIAID co-sponsored a workshop entitled Anthrax Vaccines that focused on anthrax vaccine history, histology of infection, animal models, human immune responses, assay validation, and approaches to establishing surrogate markers of immunity for licensing an anthrax vaccine. Co-sponsors included FDA, the Joint Vaccine Acquisition Program (JVAP), and the Department of Defense (DoD).

Goal: Develop and evaluate *in vivo* transmission and spore germination models.

- Under the new *In Vitro* and Animal Models for Emerging Infectious Diseases and Biodefense contracts, small animal models for anthrax will be developed and validated. Included in this activity will be safety, toxicology, and pharmaceutical testing in small and large animals, including the capability for conducting challenge studies.

Goal: Identify and characterize new virulence and pathogenicity factors

- NIAID is now funding more than 50 individual research projects on the identification of virulence factors to better understand the pathogenesis of *B. anthracis*. This includes research on the interactions between *B. anthracis* and macrophages; spore surface antigens and how spores survive and germinate within host cells; the characterization of anthrax toxins and how they produce their lethal effects; plasmids and their association with virulence and the production of toxins; as well as the genetic basis for all of these processes. Proteomics is being applied to identify suitable vaccine candidates and virulence factors that can be exploited for use in rapid and sensitive diagnostic procedures.

Goal: Identify targets within innate and adaptive pathways that can be used to modulate infection.

- Under a new initiative, Identifying Targets for Therapeutic Interventions Using Proteomic Technologies, NIAID will support the enhancement and/or development of innovative proteomic technologies and their application to better understand pathogen proteomes, including anthrax, and host cell proteomes, with the goal of developing targets for therapeutic development, including immunotherapies. Awards are planned for FY2004.

Goal: Identify and characterize innate and adaptive immune responses that occur after initial exposure to anthrax, including responses associated with spore germination.

- NIAID recently awarded grants to develop a knowledge base of innate immune system activity. As one example, researchers at Scripps Research Institute in La Jolla, California, will identify genetic changes and proteins that are triggered by encounters between innate immune cells and infectious pathogens and will study anthrax toxin interactions with innate immune receptors.

SMALLPOX

Smallpox, which is caused by the virus *variola major*, is considered one of the most dangerous potential biological weapons because it is easily transmitted from person to person, no effective therapy exists, and few people carry full immunity to the virus. Although a worldwide immunization program eradicated smallpox disease in 1977, small quantities of smallpox virus still exist in two secure facilities in the United States and Russia. However, it is likely that unrecognized stores of smallpox virus exist elsewhere in the world.

The symptoms of smallpox infection appear approximately 12 days (the range is from 7 to 17 days) after exposure. Initial symptoms include high fever, fatigue, headache, and backache. A characteristic rash, which is most prominent on the face, arms, and legs, follows in 2 to 3 days. The rash starts with flat red lesions (a maculopapular rash) that evolve into vesicles. Unlike chickenpox, the lesions associated with smallpox evolve at the same rate. Smallpox lesions become filled with pus and begin to crust early in the second week after exposure. Scabs develop, separate, and fall off after approximately 3 weeks. Individuals are generally infectious to others from the time immediately before the eruption of the maculopapular rash until the time scabs are shed. Smallpox spreads directly from person to person, primarily by aerosolized saliva droplets expelled from an infected person. Contaminated clothing or bed linens also can spread the virus. The mortality of smallpox infection is approximately 30%, and patients who recover frequently have disfiguring scars.

On December 13, 2002, President George W. Bush announced a plan to protect Americans against the threat of a smallpox bioterrorist attack. Under the plan, the Department of Health and Human Services (DHHS) is working with state and local governments to form volunteer "Smallpox Response Teams" who can provide critical services in the event of attack. To ensure that these teams can mobilize immediately in an emergency, health care workers and other critical personnel are being asked to volunteer to receive the smallpox vaccine. In addition, the Department of Defense has vaccinated certain military and civilian personnel who are or may be deployed in high-threat areas.

Scientific Progress

In the year since publication of the NIAID Biodefense Research Agenda for Category A Agents in February 2002, significant progress has been made in understanding the variola virus and how it causes disease, and in developing countermeasures against its intentional release. Key advances include:

Understanding of poxvirus pathogenesis has improved. Vaccinia virus, the immunizing agent used to help eradicate smallpox, encodes over 20 genes that regulate the immune response to the virus in the infected host. There has substantial progress in understanding these genes' pathogenic effects and mechanisms. Researchers determined that deletion of the vaccinia gene E3L, which is part of the interferon gamma evasion system in poxviruses, attenuates the virus to such an extent that it appears to be non-pathogenic in severe combined immunodeficiency (SCID) mice. This suggests that E3L-deleted viruses may be the basis for safer vaccines if modified vaccinia Ankara (MVA) proves to be not as efficacious as hoped. (Xiang Y et al., Deletion of vaccinia gene E3L makes virus apparently nonpathogenic in SCID mice, *J Virol* 2002;76:5251-5259)

In addition, researchers found that the domain of the E3L protein that binds to Z-DNA is important for pathogenesis; that is, Z-DNA binding per se is important for pathogenesis. This result has two important implications. Because variola virus contains a protein with a similar Z-DNA binding motif, Z-DNA binding could be a useful target for new anti-smallpox drugs. These results also may provide an opportunity to gain some insight into the role of Z-DNA in normal cells, which is currently not well understood (Kim Y-G et al., A role for Z-DNA binding in vaccinia virus pathogenesis, *Proc Nat Acad Sci USA*, in press).

Researchers have also made strides in understanding how these viruses' pathogenic potential can be altered either to decrease or increase virulence. Australian scientists showed that introducing the murine IL-4 gene into ectromelia (mousepox) virus increased the virus's pathogenicity to the extent that standard vaccines were no longer protective. Although publication of these results generated concern within the scientific community, they provide both an impetus and an opportunity to develop countermeasures against such engineered viruses. (Jackson RJ et al., Expression of mouse interleukin-4 by a recombinant ectromelia virus suppresses cytolytic lymphocyte responses and overcomes genetic resistance to mousepox, *J Virol* 2001;75:1205-1210; Buller M, personal communication)

Lastly, researchers better defined the mechanisms by which poxvirus virions are formed in and released from infected cells. Greater understanding of this process should lead to the identification of new targets for antiviral therapy and to a clearer picture of how the virus spreads from cell to cell in the infected host. Advances in this area include:

- Identification of a novel redox system encoded by virus that is responsible for forming disulfide bonds in virion proteins (Senkevich TG et al., Complete pathway for protein disulfide bond formation encoded by poxviruses, *Proc Nat Acad Sci USA* 2002;99:6667-6672)
- Identification of a virion-associated protease (Byrd CM et al., The vaccinia virus I7L gene product is the core protein proteinase, *J Virol* 2002;76:8973-8976)
- An understanding of the pathway by which virion membranes are formed (Szajner P et al., Vaccinia virus G7L protein Interacts with the A30L protein and is required for association of viral membranes with dense viroplasm to form immature virions, *J Virol* 2003;77:3418-3429)

Immune response to the vaccinia virus has been further characterized. Although the previously licensed smallpox vaccine was given to hundreds of millions of people in the past, routine vaccination was halted before the research tools needed to characterize the immune response to vaccinia became available. This lack of understanding of the details of the immune response to vaccination has hampered the development of new vaccines. Progress in this area is now beginning to emerge. Since most U.S. citizens over 30 years of age have been vaccinated against smallpox, the extent of their immunity is unclear. A small but significant study of such people showed that the duration of cell-mediated immunity in previously vaccinated individuals might be greater than previously thought. Vaccinia-specific CD8⁺ lymphocyte response in people vaccinated from 6 to 35 years previously averaged greater than one-half that of recently vaccinated individuals (4% of CD8⁺ lymphocytes responded to vaccinia vs. 6.5% in the recently vaccinated). This study suggests that previous smallpox vaccination, even if it occurred many years ago, may provide at least some protection. (Frey SE et al., Responses to smallpox vaccine, *NEJM* 2002;347:689-690)

In other findings, researchers in Germany have identified a vaccinia T-cell epitope that is conserved in variola. Vaccinated mice and humans both had specific T-cell responses to this

epitope, and in vaccinated mice, CD8⁺ responses specific to this epitope correlate with protection against lethal intranasal challenge with vaccinia. MVA-vaccinated mice have the same level of CD8⁺ responses to this epitope as DryVax-vaccinated mice. (Drexler I et al., Identification of vaccinia virus epitope-specific HLA-A*0201-restricted T cells and comparative analysis of smallpox vaccines, *Proc Nat Acad Sci USA* 2003;100:217-222)

Finally, NIAID-funded investigators identified two additional CD8⁺ T cell epitopes that are both highly conserved in vaccinia and variola. T cells recognizing these epitopes represented a relatively large percentage of all vaccinia-reactive cells, and were still detected as long as three years after immunization (Terajima M et al., Quantitation of CD8⁺ T cell responses to newly identified HLA-A*0201-restricted T cell epitopes conserved among vaccinia and variola (smallpox) viruses, *J Exp Med* 2003;197:927-932)

Existing supply of smallpox vaccine can be expanded to protect more Americans.

Although smallpox was eradicated worldwide through a successful immunization program, and authorized samples of the virus are contained in only two laboratories in Russia and the United States, unauthorized sources of the virus are believed to exist, increasing the likelihood that smallpox could be intentionally released. The supply of smallpox vaccine available may be insufficient to adequately vaccinate all U.S. residents with the recommended dose. Thus, it is important to determine whether the current supply of the vaccine could be diluted to quickly increase the available number of doses and still provide effective protection.

An NIH-supported clinical study indicates that the existing U.S. smallpox vaccine supply—15.4 million doses—could successfully be diluted at least five times and retain its potency, effectively expanding the number of individuals it could protect. The study compared the effectiveness of full-strength smallpox vaccine to that of fivefold- and tenfold-diluted vaccine in 680 adults age 18 to 32 with no history of smallpox vaccination. More than 97% of all participants in the study responded with a vaccine "take," a blister-like sore at the injection site that serves as an indirect measure of the vaccine's conferred immunity. Most importantly, the investigators found no significant difference in the take rate of the three doses. (Frey SE et al., Clinical responses to undiluted and diluted smallpox vaccine, *NEJM* 2002;346:1265-1274)

Pill form of cidofovir developed for treatment of smallpox. The public faces a potential health threat from bioterrorist use of dangerous pathogens. The most feared agent in a biological terrorist attack is smallpox virus. Cidofovir has been identified as a potential treatment for smallpox and vaccine complications. This drug is of particular interest as a potential therapy for smallpox because it has already been approved for treatment of cytomegalovirus infection. In its current form cidofovir must be given by intravenously, a complicated approach to treating a large civilian population in an emergency situation. Development of a cidofovir pill would provide a more practical solution. Hostetler and colleagues developed a chemical process by which some lipids, or fats, are attached to the drug so it can be more easily taken up into cells following oral administration. Once in the cells, the lipid can be removed so that pure cidofovir is available. The researchers demonstrated that the modified drugs can significantly reduce the growth of virus in tissue culture. These drugs are currently being tested in animals. (Kern ER, Enhanced inhibition of orthopox virus replication in vitro by alkoxyalkyl esters of cidofovir and cyclic cidofovir, *Antimicrob Agents Chemother* 2002;46:991-995)

Programmatic Progress in Addressing Immediate Goals

Goal: Expand the existing supply of live and attenuated vaccines, with particular emphasis on vaccines with reduced reactogenicity.

- NIAID is conducting clinical trials to test safety and efficacy of Aventis Pasteur Smallpox Vaccine (APSV) and modified vaccinia Ankara (MVA) vaccine. These trials will help ensure a sufficient supply of usable and effective smallpox vaccine.
- New research initiatives encourage applications for the development of new smallpox vaccines: Biodefense Partnerships; the Cooperative Research for the Development of Vaccines, Adjuvants, Therapeutics, Immunotherapeutics, and Diagnostics for Biodefense Program; and the Small Business Biodefense Program.

Goal: Conduct phase I and II trials with new candidate smallpox vaccines, with particular emphasis on the cell culture vaccines currently under development.

- NIAID awarded contracts to two companies to develop, manufacture and conduct safety trials of MVA vaccine candidates (Bavarian Nordic A/S of Copenhagen, Denmark, and Acambis Inc. of Cambridge, Massachusetts).
- An initiative to support the intermediate-scale advanced development of MVA vaccine is planned for early FY2004. This initiative may include: production and release of consistency lots; formulation, vialing and labeling of vaccine; development of animal models in at least two species to support the FDA animal rule; process, assay, and facility validation; and clinical evaluation in initial phase II trials.

Goal: Initiate and expand clinical trials of existing smallpox vaccines.

- NIAID conducted eight clinical trials in healthy naïve and non-naïve adults evaluating DryVax and APSV vaccines.

Goal: Determine the correlates of immunity for smallpox vaccines through the detailed evaluation of immune responses to DryVax.

- NIAID is conducting and supporting studies to determine the correlates of immunity in samples collected from volunteers who have been administered DryVax and other vaccine candidates. This work has included the development of high-throughput virus neutralization assays, which have been optimized and are presently being validated. ELISA assays have also been developed and validated to determine general immune responses to vaccinia virus. Lastly, ELISPOT and internal cytokine staining assays to measure cellular responses to vaccines are under development.

Goal: Develop a centralized immunology laboratory to validate assays required for the licensure of smallpox vaccines.

- Through a contract expansion, NIAID is supporting the quality and validity of assays being done on clinical specimens collected through human clinical trials. Included as part of the laboratory support are the following activities: assessing a clinical site's ability to reliably perform immunological assays (proficiency testing); supporting comparative

evaluations of cytometric instruments, methods and reagents; facilitating the development, standardization, and assay characterization of immunological assays for implementation in multi-center investigations; acquiring, characterizing, storing, documenting, and disbursing quality-control materials and reagents; disseminating quality-assessment technical and scientific data; and maintaining a computerized system that supports the Quality Assessment Program.

Goal: Fully characterize activity of cidofovir against poxviruses and ensure that an adequate supply of the drug is available to treat complications from vaccination.

- In FY2002, NIAID developed a treatment IND to support the use of cidofovir as a backup to vaccinia immunoglobulin for the treatment of vaccination complications. If necessary, the protocol will be implemented by the Collaborative Antiviral Study Group (CASG) in people 13 and older. Additional protocols are being developed for children and people with renal impairment.
- A clinical protocol to assess activity of cidofovir as a treatment for complications related to smallpox vaccine has been developed and implemented in the Vaccine Treatment and Evaluation Units (VTEUs).
- A clinical protocol to assess activity of cidofovir against smallpox has been developed by the CASG and is ready for nationwide implementation.

Goal: Develop animal models for studying smallpox pathogenesis.

- Existing resources that support animal-model development for emerging viral infections including smallpox, were expanded.
- USAMRIID and CDC researchers have developed two models for studying smallpox in cynomolgus monkeys. After infection with monkeypox virus, these animals die of a disease that is very similar to human smallpox, but which progresses over a shorter period of time. Both models will likely be used in evaluation of new vaccines and therapies.

Goal: Expand *in vitro* and *in vivo* screening capability for oral antivirals, immunotherapies, and replacements for VIG.

- NIAID has expanded its *in vitro* and *in vivo* antiviral screening contracts. To date, over 650 compounds including most of the licensed antivirals have been evaluated for anti-poxvirus activities in cell culture and those with activity in animal models. So far cidofovir and its orally active derivatives appear to be the best candidates for treatment of both smallpox disease and vaccine complications.
- NIAID has fostered development of compounds with a mechanism of action different from cidofovir's via rational drug design and high-throughput screens. New targets for screens include viral DNA polymerase and processivity factor; topoisomerase; viral core protein protease; S-adenosylhomocysteine hydrogenase; inosine monophosphate dehydrogenase; E3L Z-DNA binding protein; mRNA capping enzyme; H1 phosphatase; and F10 kinase.

- NIAID Division of Intramural Research (DIR) scientists have initiated studies to develop a monoclonal antibody-based product for treatment of smallpox vaccine side effects and for prophylaxis or treatment of variola infection. Their approach involves the generation of monoclonal antibodies tailored to specific neutralizing epitopes of vaccinia; these could be used singly or in combination to formulate an immune globulin with increased potency, consistency, and specificity that is amenable to simple and scalable production methods.
- NIAID supported researchers have developed a new approach to antibody therapy for smallpox. This approach involves production of monoclonal antibodies directed against a virus-encoded pathogenesis factor rather than against one of the viral gene products that is required for replication. The specific target of this new therapeutic approach is variola virus-encoded growth factor, which is released from infected cells and stimulates cells that express the Epidermal Growth Factor Receptor. Studies in mice have shown that anti-growth factor antibody greatly increases the protective effect of a conventional neutralizing monoclonal antibody when the two types of antibodies are combined.
- A phase I clinical study to assess initial safety, tolerability, and pharmacokinetics of a new oral derivative of cidofovir in normal volunteers is planned by CASG in FY2004.

Goal: Validate current diagnostics for orthopoxvirus infections and other vesicular rashes (e.g., chickenpox).

- Bioinformatics tools are being used to develop forensic assays that may be capable of determining whether variola virus has been genetically modified. Multiple probes that align with different portions of the viral genome are being developed. Comparative sequencing has also paved the way for the development of rapid DNA-based diagnostic tests to differentiate between smallpox and other diseases with which it might initially be confused. Further, in collaboration with Affymetrix, microarray/chip approaches to sequencing viral genomes are being set up to rapidly examine viral genomes.
- NIAID is working with USAMRIID and CDC to develop real-time PCR assays to detect and distinguish between smallpox and other orthopox viruses.

Goal: Conduct comparative genomic sequencing of additional poxvirus strains to detect potential differences in pathogenesis and virulence.

- The Poxvirus Bioinformatics Resource Center provides sequencing and functional comparisons of orthopox genes; designs and maintains a relational database to store, display, annotate and query genome sequences, structural information, phenotypic data and bibliographic information; and serves as a repository of well-documented viral strains. The center is a collaborative effort led by two academic centers (St. Louis University and the University of Alabama at Birmingham) supported by two federal agencies (Defense Advanced Research Projects Agency and NIH). Collaborators include the CDC, American Type Culture Collection, USAMRIID, the Medical College of Wisconsin, and the University of Victoria.
- Eight distinct isolates of variola virus and three strains of vaccinia virus have been sequenced. In addition, NIAID has supported the sequencing of other important poxviruses. These include ectromelia (mousepox), rabbitpox, camelpox, and monkeypox.

viruses. The monkeypox virus sequence is of particular significance because monkeypox is known to infect humans and cause a disease that appears to be a mild form of smallpox. The availability of these sequences should provide a better understanding of poxvirus pathogenesis and differences between different isolates and strains.

- By mining the completely sequenced variola genome, NIAID-supported scientists have identified five genes whose protein products may be good targets for neutralizing antibodies that could stop smallpox infection. Approximately 10 promising antibodies to one smallpox protein are currently being tested.
- DIR scientists have cloned the entire vaccinia virus genome in a bacterial artificial chromosome, enabling the modification or deletion of vaccinia genes or the addition of foreign DNA via methods developed for bacterial systems. This advance will promote the development of genetically engineered and recombinant vaccinia viruses for use as vaccines and vectors.

Goal: Identify and characterize host factors and viral proteins that are involved in the production and maintenance of the two forms of infectious orthopoxviruses: intracellular mature virus (IMV) and extracellular enveloped virus (EEV).

- NIAID scientists, along with scientists from Spain and England, have begun to tease apart the mechanism by which intracellular orthopoxvirus virions are transported to the cell surface and are released into the extracellular environment. These investigators have shown that two viral proteins, A33R and A36R, first interact with each other and then with the cellular microtubule system. The interaction with the microtubule system results in the movement of virions to the cell surface. Since release of poxvirus virions from infected cells is an important step in the spread of virus throughout the body of an infected animal or human, this new data will inform the design of new vaccines and therapies.
- NIAID-supported scientists have shown that one of the first steps in the formation of new orthopox virions in infected cells is insertion of the viral protein A14 into cellular membranes. This membrane-bound protein then helps to recruit additional components into the developing virion structure. The function of A14 was shown to be regulated by certain modifications, including the addition of a phosphate group to the protein by another viral protein, F10 kinase. The kinase, as well as another viral protein identified by these researchers that removes phosphate additions from proteins, represent important new targets for development of anti-poxvirus drugs.
- NIAID scientists have discovered and characterized a novel poxvirus-encoded enzyme system that harnesses cellular energy carrier molecules to alter the structure of virion proteins while they are assembled into new virions. This newly discovered enzyme system also represents a potentially very important new target for antiviral drug development.

PLAGUE

Plague is caused by the bacterium *Yersinia pestis*. Its potential for use as a biological weapon is based on methods that were developed to produce and aerosolize large amounts of bacteria and on its transmissibility from person to person in certain of its forms. An additional factor is the wide distribution of samples of the bacteria to research laboratories throughout the world. Infection by inhalation of even small numbers of virulent aerosolized *Y. pestis* bacilli can lead to pneumonic plague, a highly lethal form of plague that can be spread from person to person. Natural epidemics of plague have been primarily bubonic plague, which is transmitted by fleas from infected rodents.

Symptoms of pneumonic plague, including fever and cough, resemble those of other respiratory illnesses such as pneumonia. Symptoms appear within 1 to 6 days after exposure and lead rapidly to death. If untreated, pneumonic plague has a mortality rate that approaches 100%. Early, aggressive antibiotic treatment can be effective against plague, but there is no plague vaccine, licensed or investigational, available in the US.

Scientific Progress

In the year since publication of the NIAID Biodefense Research Agenda for Category A Agents in February 2002, significant progress has been made in understanding *Y. pestis* and how it causes disease, and in developing countermeasures. Key advances made in basic research on this pathogen include:

Genome sequence for the organism that causes bubonic and pneumonic plague has been completed. NIAID-supported investigators have completed the genome sequence of the KIM strain of *Yersinia pestis*, which was associated with the second pandemic of plague, including the Black Death. The publicly accessible genome sequence will provide a valuable research resource to the scientific community for identifying new targets for vaccines, drugs, and diagnostics for this deadly pathogen, considered an agent of bioterrorism. (Deng W et al., Genome sequence of *Yersinia pestis* KIM, *J Bacteriology* 2002;184:4601-4611)

Single gene change led to deadly plague organism. The plague organism, *Y. pestis* is highly infectious for humans and considered to be a bioterrorism threat. A relatively benign ancestor of *Y. pestis* picked up a gene called PLD from an unrelated organism only about 1,500-20,000 years ago. NIAID scientists discovered that this gene is required for the plague bacillus to survive in the midguts of fleas. Acquisition of the PLD gene thus explains how *Y. pestis* evolved from a form that caused only a mild intestinal illness, acquired through ingestion, to become a much more deadly pathogen transmitted by fleas. This work illustrates how microbes continually re-invent themselves to emerge as novel or more virulent agents of human disease. (Hinnebusch BJ et al., Role of *Yersinia murine* toxin in survival of *Yersinia pestis* in the midgut of the flea vector, *Science* 2002;296:733-735)

Genes in the yersiniabactin iron transport system have been identified. Nine genes and their respective products have been identified in the yersiniabactin (Ybt) iron transport system, a virulence determinant for *Y. pestis*. A more complete analysis of these genes and their products or interactions may advance the development of novel therapeutic approaches for the treatment of plague. (Perry RD et al., Iron and heme acquisition storage systems in *Yersinia pestis*, *Recent Res Dev Microbiol* 2001;5:13-27)

Programmatic Progress in Addressing Immediate Goals

Goal: Accelerate the search for candidate *Y. pestis* vaccines.

- New NIAID programs that encourage applications for the development of plague vaccines include: Biodefense Partnerships; the Cooperative Research for the Development of Vaccines, Adjuvants, Therapeutics, Immunotherapeutics, and Diagnostics for Biodefense Program; and the Small Business Biodefense Program.
- NIAID has established a cooperative program with U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID) to conduct mutually-agreed upon research projects related to biodefense. Evaluation of a recombinant plague vaccine candidate (F1-V) in a nonhuman primate parenteral challenge model is planned as part of the program. . NIAID scientists are nearing completion of an evaluation of this vaccine in their rodent model of plague.
- Contracts to support the advanced development stages of one or more candidate plague vaccines are planned for FY2004. These candidate vaccines are based on two antigens (F1 and V) of *Y. pestis* that appear to play a major role in mediating protective immunity.

Goal: Establish capacity for the development, refinement, production, and testing of pilot lots of candidate *Y. pestis* vaccines.

- During the past few years, USAMRIID investigators have conducted pre-clinical safety and efficacy studies on an F1-V fusion protein vaccine, while scientists in the United Kingdom have been conducting similar studies on an F1+V combined subunit vaccine. Plans for development of these products include: comparative studies to assess immunogenicity in various nonhuman primate animal models, as well as in phase I human trials; development and evaluation of surrogate markers of protective immunity generated after immunization; optimization of the immunization regimen to enhance the degree and duration of protective immunity for these and other promising vaccine candidates; validation of assays used for the assessment of protective immunity; and optimization and scale-up production of cGMP product.

Goal: Encourage exploration of new targets leading to the development of *Y. pestis*-specific chemotherapeutics and/or entities with novel modes of action (e.g., Ybt, TTSS, LcrV).

- Several new NIAID grant programs encourage the identification of new targets for the development of therapeutics for Category A agents including *Y. pestis*. These include the Cooperative Research for the Development of Vaccines, Adjuvants, Therapeutics, Immunotherapeutics, and Diagnostics for Biodefense Program; the Small Business Biodefense Program; and Biodefense and Emerging Infectious Diseases Research Opportunities.
- NIAID scientists completed pilot testing of the susceptibility of *Yersinia pestis* to a novel class of antimicrobial compounds being developed by researchers at Duke University. These compounds target bacterial enzymes required for the biosynthesis of lipopolysaccharide, the major component of the outer membrane of Gram-negative bacteria.

Goal: Screen existing FDA-approved antimicrobials and immunomodulators for efficacy against *Y. pestis*.

- In collaboration with USAMRIID and FDA, NIAID is supporting the testing of licensed antibiotics for efficacy against pneumonic plague. Five currently licensed drugs—gentamycin, doxycycline, ciprofloxacin, levofloxacin, and ceftriaxone—are being tested for efficacy in African green monkeys exposed to aerosolized *Y. pestis*. Studies are currently under way to determine the pharmacokinetics and toxicity of the drugs in this animal model.

Goal: Develop rapid, inexpensive, and broad-based clinical diagnostic approaches for plague.

- Several new NIAID grant programs encourage the development of diagnostics for the Category A agents including plague. These include: Biodefense Partnerships; the Small Business Biodefense Program; and the Cooperative Research for the Development of Vaccines, Adjuvants, Therapeutics, Immunotherapeutics, and Diagnostics for Biodefense Program.
- The Bioinformatics Resource Centers for Biodefense and Emerging Infectious Diseases will provide the research community with access to databases of genomic information. This information is important for the identification of putative targets for new diagnostics. Awards are planned for FY2004.

Goal: Develop standards for validation and comparison of potential plague diagnostics.

- In collaboration with USAMRIID, NIAID is supporting the development and standardization of pneumonic- and bubonic-plague animal models, and the development, standardization, and transfer of these research resources to a central repository.
- NIAID plans to support the acquisition, authentication, storage and distribution to the scientific community of state-of-the-art research and reference reagents related to biodefense starting in late FY2003. Included will be the capability to validate, expand, and produce biological reagents including cell lines, clones, proteins, monoclonal and polyclonal antibodies, and diagnostic tools. Plague reagents will be included in this repository.

BOTULISM

Botulinum toxin, which is produced by the spore-forming anaerobic bacterium *Clostridium botulinum*, is a highly toxic substance that presents a major threat from intentional exposure. The toxin is highly lethal, and easy to produce and release into the environment. Botulinum toxin is absorbed across mucosal surfaces and irreversibly binds to peripheral cholinergic nerve synapses. Seven antigenic types (A-G) of the toxin exist. All seven toxins cause similar clinical presentation and disease; botulinum toxins A, B, and E are responsible for the vast majority of foodborne botulism cases in the United States.

Exposure to the toxin induces symptoms that include muscle paralysis; difficulty in speaking, swallowing, or seeing; and, in severe cases, the need for mechanical respiration. People exposed to the toxin require immediate and intensive supportive care and treatment. The onset and severity of symptoms depend on the rate and amount of toxin absorbed into circulation. With foodborne exposure, incubation varies from 2 hours to 8 days but is generally limited to 72 hours. Symptoms subside when new motor axon twigs re-ennervate paralyzed muscles, a process that can take weeks or months in adults.

Scientific Progress

In the year since publication of the NIAID Biodefense Research Agenda for Category A Agents in February 2002, significant progress has been made in understanding *C. botulinum* neurotoxins and how they cause disease, and in developing medical countermeasures to protect exposed individuals. Key advances made in basic research on the organism and the neurotoxin include:

Sequencing of the *C. botulinum* Hall strain A bacterium genome has been completed. The genome was determined to be approximately 3.9 megabase pairs in size, with a G+C content of approximately 28.2%. There is also a plasmid of 16,344 base pairs. Genomic information is critical to the development of effective countermeasures against *C. botulinum* neurotoxins. (The Wellcome Trust Sanger Institute, available at www.sanger.ac.uk/Projects/C_botulinum)

Research provides a better understanding of botulinum toxin entry into cells.

Researchers have determined the role of gangliosides as receptors for neurotoxin entry. In addition, the mechanism used by the botulinum neurotoxin heavy chain to chaperone or carry the light chain across the endosomal membrane into the cytosol has also been further delineated. With this information researchers can develop drugs to block or disrupt toxin entry. (Koriazova LK and Montal M, Translocation of botulinum neurotoxin light chain protease through the heavy chain channel, *Nature Structural Biology* 2002;10:13-18)

Animals are protected after immunization with A and F toxins. Animals immunized intranasally were completely protected from intraperitoneal challenge with active *C. botulinum* neurotoxin A. (Park J-B and Simpson LL, Inhalational poisoning by botulinum toxin and inhalational vaccination with its heavy-chain component, *Infect Immun* 2003;71:1147-1154)

In other work, animals immunized with the heavy chain of botulinum neurotoxin expressed in *Salmonella enterica serovar Typhimurium* were partially protected from intraperitoneal challenge of active *C. botulinum* type F toxin. (Foyne S et al., Vaccination against type F botulinum toxin using attenuated *Salmonella enterica* var *Typhimurium* strains expressing the BoNT/F H_c fragment, *Vaccine* 2003;21:1052-1059)

Researchers have developed new methods for the expression and purification of recombinant catalytically active, non-toxic endopeptidase derivatives of *C. botulinum* neurotoxin type A. The recombinant protein was immunogenic in animals and induced neurotoxin neutralizing antibodies and thus could be used as an alternative to toxoid for producing antitoxin in animals. (Chaddock JA et al., Expression and purification of catalytically active, non-toxic endopeptidase derivatives of *Clostridium botulinum* toxin type A. *Protein Purification & Expression* 2002;25: 219-228)

These results provide important information regarding host response following immunization with vaccines against botulinum toxins.

Programmatic Progress in Addressing Immediate Goals

Goal: Process, produce, and conduct phase I and II trials with the heptavalent equine antitoxin.

- In order to expand the existing supply of antitoxin, CDC initiated the processing of frozen equine plasma to produce additional doses of heptavalent and pentavalent antitoxin. NIAID will determine the pharmacokinetics of botulinum toxins and antitoxins in several animal models including rats, guinea pigs, and non-human primates through its Food and Waterborne Diseases Integrated Research Network, which is planned for award in FY2003. This data will be helpful for the development of equine-based antitoxins and will serve as a basis for next-generation antitoxins.
- NIAID staff participated in an interagency working group to guide the formulation decisions for new equine antitoxin product.

Goal: Scale up production and phase I testing of three human monoclonal antibodies to toxin A.

- A combination of three monoclonal antibodies, which neutralize botulinum neurotoxin serotype A with a potency 90 times greater than human hyperimmune globulin, has been identified. If necessary, these antibodies could be produced by the new Antibody Production Facility, planned for award in late FY2003. (Nowakowski A et al, Potent neutralization of botulinum neurotoxin by recombinant oligoclonal antibody, *Proc Nat Acad Sci USA* 2002;99:11346-11350)
- NIAID will produce fully human monoclonal antibodies to toxin A in sufficient quantities to support safety and pharmacokinetic tests in phase I human trials. This work will be done through the new Antibody Production facility, funding for which will be awarded in FY2003.
- NIAID will support the research and development required for new therapeutic interventions within the framework of the Food and Waterborne Diseases Integrated Research Network, which is planned for award in FY2003.

Goal: Develop and test human monoclonal antibodies to toxins B, C, D, E, F, and G.

- NIAID is planning to support several grants to identify and validate therapeutic monoclonal antibodies that neutralize neurotoxin serotypes B-G in FY2003.

Goal: Produce and conduct phase I and II trials of recombinant vaccine for serotypes A and B.

- A recombinant vaccine to protect against serotypes A and B of botulinum neurotoxin is being developed by the Department of Defense (DoD).

Goal: Develop recombinant vaccines against neurotoxins A, B, C, D, E, F, and G.

- Several new NIAID initiatives encourage the development of vaccines for the Category A agents including botulinum neurotoxin. These include: Biodefense Partnerships; the Small Business Biodefense Program; and the Cooperative Research for the Development of Vaccines, Adjuvants, Therapeutics, Immunotherapeutics, and Diagnostics for Biodefense Program.
- NIAID plans to support several new grants for the development of recombinant vaccines against botulism in FY2003.
- In November 2002, NIAID convened a Botulinum Toxin Expert Panel to formulate specific goals regarding the development of second-generation therapies, vaccines, and diagnostics for biodefense. Discussions included the challenges posed by the rapid development of countermeasures, including vaccines, for *C. botulinum* toxins. (http://www.niaid.nih.gov/dmid/pdf/bot_toxins.pdf)

Goal: Develop rapid and inexpensive diagnostics for botulism toxins and their genes for use in multiple settings.

- An *in vitro* assay has been developed for botulinum toxin detection that utilizes embryonic spinal cord neurons. This assay has the same sensitivity as the standard mouse bioassay.
- Several new NIAID grant programs encourage the development of diagnostics for biodefense agents, including botulism. These include: Biodefense Partnerships; the Small Business Biodefense Program; and the Cooperative Research for the Development of Vaccines, Adjuvants, Therapeutics, Immunotherapeutics, and Diagnostics for Biodefense Program.
- In May 2003, NIAID convened an expert panel to focus on the development of next-generation diagnostics for botulinum neurotoxins. The panel discussed the most promising technical opportunities for the development of new diagnostics in the short, medium, and long term.

Goal: Develop effective cell culture systems to study toxin binding, internalization, and protease activity.

- A new NIAID grant program, Biodefense and Emerging Infectious Diseases Research Opportunities, encourages research on basic biology of *C. botulinum* neurotoxin.

TULAREMIA

Tularemia is a potential bioterrorist agent because of its high level of infectivity (as few as 10 organisms may cause disease) and its ability to be aerosolized. *Francisella tularensis*, which causes tularemia, is a non-spore-forming, facultative intracellular bacterium that can survive at low temperatures for weeks. Two strains of the organism have been characterized—type A, which is found in North America, is more virulent than type B, which is found in Europe and Asia. The disease is not transmitted from person to person; it spreads naturally from small mammals, arthropod vectors, or contaminated food, soil, or water to humans. Natural infection can also occur after inhalation of airborne particles. It is not known how this pathogen survives in nature to produce sporadic outbreaks in endemic areas.

Tularemia can take one of several forms, depending on the route of exposure. The disease resulting from the inhalation of airborne *F. tularensis* is of major concern with respect to biodefense since it results in an acute, nonspecific illness beginning 3 to 5 days after respiratory exposure; in some cases, pleuropneumonia develops after several days or weeks. If untreated, the disease could lead to respiratory failure. Treatment with antibiotics reduces mortality for naturally acquired cases by 2%-60%. A live-attenuated tularemia vaccine developed by the Department of Defense (DoD) has been administered under an investigational new drug (IND) application to thousands of volunteers. To date, use of this vaccine has been limited to laboratory and other high-risk personnel.

Scientific Progress

In the year since publication of the NIAID Biodefense Research Agenda for Category A Agents in February 2002, progress has been made in understanding *F. tularensis* and how it causes disease, and in developing countermeasures against its intentional release. Key scientific advances include:

Host defense mechanisms revealed in mouse model. Mice that were infected systemically with the live vaccine strain of *F. tularensis* have been used extensively in recent months to reveal host defense mechanisms against this pathogen. Such studies have demonstrated the critical need for neutrophils and interferon-gamma (INF-?) to combat early stages of systemic tularemia. However, these defenses do not appear to combat early pulmonary tularemia. This finding suggests that the effectiveness of particular antibacterial host defenses varies depending on the invasion site. (Telenev M et al., *Francisella tularensis* Toll-like receptor-mediated activation of intracellular signalling and secretion of TNF-alpha and Il-1 from murine macrophages, *Cell Microbiol* 2003;1:41-52)

Conjugating the O-polysaccharide of the lipopolysaccharide (LPS) of *Francisella tularensis* to bovine serum albumin (BSA) does not change the vaccine's effectiveness. Mice immunized with this conjugate vaccine, but not with BSA alone, were completely protected against intradermal challenge with a highly virulent type B strain of *F. tularensis*; they were only partially protected against aerosol challenge with the same strain. The same vaccination strategy failed to protect against aerosol challenge with a virulent type A strain. This suggests that the O-antigen of *F. tularensis* could be considered as a potential component of a subunit vaccine against type B, but not type A, strains of *F. tularensis*. (Conlon JW et al., Mice vaccinated with the O-antigen of *Francisella tularensis* LVS lipopolysaccharide conjugated to bovine serum albumin develop varying degrees of protective immunity against systemic or

aerosol challenge with virulent type A and type B strains of the pathogens, *Vaccine* 2002;20:3465-3471)

Programmatic Progress in Addressing Immediate Goals

Goal: Conduct comparative genomic sequencing of selected strains of *F. tularensis*, type A and B, LVS, and *F. novicida*, and develop genetic systems to correlate differences in pathogenesis and virulence.

- As a result of a coordinated Federal effort, genome sequencing projects funded by the NIAID, the Centers for Disease Control and Prevention (CDC), the Department of Energy (DoE), and the National Science Foundation (NSF) are ongoing for at least one strain of a bacteria or protozoan considered to be Category A organisms. To date there is a completed or near-completed genome sequence for every bacteria on the Category A list, including *F. tularensis*.
- The Bioinformatics Resource Centers for Biodefense and Emerging Infectious Diseases will provide the research community with access to databases of genomic information. Awards are planned for FY2004.

Goal: Develop a bacterial repository of *Francisella* species.

- NIAID plans to support the acquisition, authentication, storage, and distribution to the scientific community of state-of-the-art research and reference reagents starting in late FY2003. This repository will include *Francisella* species.

Goal: Characterize responses of *F. tularensis* to available chemotherapeutics *in vitro* and in animal models of infection and disease.

- A new initiative, *In vitro* and Animal Models for Emerging Infectious Diseases and Biodefense, will provide a range of animal models for preclinical testing of new therapies and vaccines against agents of bioterrorism, including *F. tularensis*. Awards are anticipated in late FY2003.

Goal: Develop new techniques to improve conditions for the culture of the microbe and for rapid determination of drug sensitivity profiles.

- Through many of the recently announced initiatives (e.g., Biodefense and Emerging Infectious Diseases Research Opportunities, and Collaborative Research for Vaccines, Therapeutics, Adjuvants and Diagnostics), NIAID is actively working to encouraging innovative research to develop new techniques for diagnosing tularemia.

Goal: Attract scientific researchers with expertise in a diversity of fields (e.g., immunology, microbiology, and lipid biochemistry) to the study of tularemia.

- The Rapid Response Grant Program on Bioterrorism-Related Research was developed to support innovative research targeted at the design and development of specific diagnostics, therapies, and prevention strategies for Category A biological diseases. This program encouraged investigators from a diversity of disciplines to become involved in biodefense research. Sixty-nine grants were awarded in FY2002 in response to this initiative; of these, two were for tularemia research.
- NIAID recently made available new and expanded programs for supporting the training and career development of young scientists in biodefense research. Approximately 30% of the training grant applications received in FY2003 were specifically focused on training in biodefense.
- NIAID continues a concerted effort to encourage investigators to become involved in research on biodefense pathogens through the use of targeted Requests for Applications and Program Announcements such as the Biodefense and Emerging Infectious Diseases Research Opportunities initiative. The response has been encouraging and a number of new grant applications focused on tularemia have been submitted. To date, four new awards in this area have been made.
- In 2002 NIAID, in collaboration with the Office of Rare Diseases, sponsored an international conference on the current status of vaccines against plague and tularemia. Experts from throughout the world participated in this conference, which also stimulated productive interactions between various groups of investigators.

Goal: Develop rapid, inexpensive, and broad-based clinical diagnostics approaches for tularemia.

- NIAID supports several new programs to foster the development of diagnostics for biodefense agents, including tularemia. These include: Biodefense Partnerships; the Small Business Biodefense Research Program; and the Cooperative Research for the Development of Vaccines, Adjuvants, Therapeutics, Immunotherapeutics, and Diagnostics for Biodefense Program. Two awards were made in the past year supporting work on tularemia diagnostics.
- The Bioinformatics Resource Centers for Biodefense and Emerging Infectious Diseases will provide the research community with access to databases of genomic information for the identification of putative targets for new diagnostics. Awards are planned for FY2004.

VIRAL HEMORRHAGIC FEVERS

Viral hemorrhagic fevers (VHFs) encompass a group of similar diseases caused by four types of viruses:

- Arenaviruses, associated with Argentine, Bolivian, and Venezuelan hemorrhagic fevers, Lassa fever, and Sabia-associated hemorrhagic fever;
- Bunyaviruses, including those causing Crimean-Congo hemorrhagic fever, Rift Valley fever, and hantaviral diseases;
- Filoviruses, associated with Ebola and Marburg hemorrhagic fevers; and
- Hemorrhagic flaviviruses, including those causing yellow fever, dengue hemorrhagic fever, Kyasanur Forest disease, and Omsk hemorrhagic fever.

These viruses pose a risk from intentional exposure because, with very few exceptions, no vaccines or proven treatments exist, and many of the diseases are highly fatal. Natural infections occur when people come in contact with animals or insects that are infected or act as vectors. After human infection occurs, some VHFs can be transmitted from person to person through close contact or contaminated objects, such as syringes and needles.

Initial symptoms of VHFs are nonspecific and include fever, muscle aches, and fatigue. Disease often progresses to bleeding under the skin and from body orifices and internal organs, followed by shock, coma, seizures, and nervous-system malfunction. Symptoms begin between a few days (in Ebola) and several weeks after exposure, depending on the particular virus. Mortality also varies widely among the diseases; often it is quite high. Some of these viruses also cause significant morbidity and mortality in economically important domestic animals.

Scientific Progress

In the year since publication of the NIAID Biodefense Research Agenda for Category A Agents in February 2002, significant progress has been made in understanding how the organisms responsible for VHFs cause disease, and in developing countermeasures against their intentional release. Key scientific advances include:

Accelerated vaccine for Ebola protects monkeys. VRC scientists, in collaboration with USAMRIID, reported that a single shot of a fast-acting, experimental Ebola vaccine successfully protected monkeys after only one month. In this study, the VRC scientists immunized eight monkeys with a single boost injection, consisting of attenuated carrier viruses containing genes for important Ebola antigens. The monkeys were then delivered to USAMRIID where they were injected with an Ebola virus strain obtained from a fatally infected person from the former Zaire in 1995. The single vaccine injection completely protected all eight animals against Ebola infection, even those who received high doses of the virus. This finding suggests that it might be possible to quickly contain Ebola outbreaks with ring vaccination. (N Sullivan *et al.* Accelerated vaccine for Ebola virus hemorrhagic fever in non-human primates. *Nature* 424(6949):681-84 (2003).

Methods developed to study individual proteins from these viruses in regular, low containment laboratories. Investigators have been able to transfer important genes from these viruses to benign vector or carrier viruses where they can be safely expressed in cells to produce proteins. As a result, the function of these proteins can be studied separately and

safely, in the absence of the infectious virus particle. These new methods provide a powerful tool for dissecting the virus life cycle, examining virus assembly, and understanding the role of viral proteins in pathogenicity and the interplay of viral proteins with components of the host cell immune response. These new methods will also help open new avenues to develop live attenuated virus vaccines and vaccine vectors. (Jasenosky LD et al., Ebola virus VP40-induced particle formation and association with the lipid bilayer, *J Virol* 2001;75:5205-5214)

Development of a novel assay for the detection of human antibodies to Ebola using reverse genetic systems. In this assay, which uses reverse genetics and de novo synthesis of negative sense viruses from cloned cDNA, researchers were able to safely determine antibodies that are reactive with all subtypes of Ebola. The assay uses particles prestained with a dye so that detection of binding can be directly determined by visual inspection. The assay is both simple and economical. This accomplishment has particularly helped foster the development of a broad-based, novel assay for the detection of Ebola. A reverse-genetics system for Crimean-Congo hemorrhagic fever virus is also under development. (Neumann G et al., Reverse genetics demonstrates that proteolytic processing of the Ebola virus glycoprotein is not essential for replication in cell culture, *J Virol* 2002;76:406-410)

Novel mechanism of antibody-dependent enhancement discovered for Ebola. NIAID supported researchers determined that infection with the Ebola Zaire virus induces antibodies that enhance viral infectivity. When plasma or serum from convalescing patients was mixed with primate kidney cells, the infection of these cells was enhanced. This enhancement was mediated by antibodies to the viral glycoprotein and by complement C1q. This finding raises additional considerations for vaccine development. (Takada, A. et.al., Antibody-dependent enhancement of Ebola virus infection. *J Virol* 2003; 77, 7539-7544)

Programmatic Progress in Addressing Immediate Goals

Goal: Develop animal models that mimic human disease for studying VHF pathogenesis in humans.

- NIAID animal model resources were expanded to include evaluation of several viral hemorrhagic fevers and encephalitides. The new models are:
 - Bunyavirus: Punta Toro virus in mice
 - Arenavirus: Pichinde virus in hamsters
 - Flavivirus: Banzai Nile virus in mice
 - Togavirus: Semliki Forest virus in mice

Goal: Expand the effort to determine the correlates of immunity for VHF vaccines by using appropriate models of natural infection.

- In FY2002, NIAID awarded two contracts under the U.S. Based Collaboration in Emerging Viral and Prion Diseases program that include projects on viral hemorrhagic fevers. Studies with Rift Valley fever virus (a bunyavirus) focus on genetic structure determination and development of rapid detection assays and animal models of disease; studies with dengue fever virus (a flavivirus) include novel approaches for the detection and control of vectors and disease. (Health Research Inc., New York, and the University of Texas Medical Branch, Galveston)

Goal: Advance the development of VHF vaccine candidates (e.g., Rift Valley fever and Junin fever).

- Investigators at the NIAID Vaccine Research Center (VRC), with scientific collaborators at the U.S. Army Medical Research Institute for Infectious Diseases (USAMRIID) and the Centers for Disease Control and Prevention (CDC), have developed a potentially effective vaccine strategy for Ebola virus infection in non-human primates. Previous VRC studies have shown that a combination of DNA vaccination and boosting with adenoviral (ADV) vectors that encode viral proteins was protective against Ebola viral challenge and generated cellular and humoral immunity in cynomolgus macaques. In addition to testing preventive vaccine candidates, the VRC is currently testing a vaccine that may be useful in an acute outbreak setting. A second-generation product will also be evaluated that would provide coverage for Marburg and possibly Lassa virus.
- Division of Intramural Research (DIR) investigators have developed attenuated viruses representing all four dengue subtypes for use as candidate vaccines. Phase I clinical testing of their first live virus dengue 4 vaccine is nearing completion. Animal testing of a new type 4 vaccine as well as of vaccines for types 1 and 2 is well under way, and an attenuated dengue 3 virus has just been recovered. DIR investigators are also using an attenuated dengue 4 virus as a backbone for substituting homologous genes from other flaviviruses (or multiple dengue subtypes) to produce live-attenuated chimeric vaccines. Hybrid dengue 1/4, 2/4, and 3/4 vaccines have been made and testing in rodents or non-human primates is almost complete. Two tetravalent dengue vaccines will be studied in non-human primates in FY2003.
- NIAID scientists used Langkat virus (LGT) strain TP21 to construct a chimeric candidate tick-borne encephalitis virus (TBEV) vaccine comprising LGT genes for pre-membrane and envelope glycoprotein and all other sequences derived from dengue type 4 virus (Langkat virus is a naturally avirulent tick-borne flavivirus that shares protective epitopes with highly virulent, closely related TBEV). The LGT-dengue hybrid protected mice against the most virulent TBEV and subsequent studies in rhesus monkeys suggest that the hybrid is attenuated, immunogenic, and able to induce a protective immune response.
- In FY2002, a grant was awarded for the development of a Marburg virus (filovirus) vaccine under the Biodefense Partnerships Program. (Alphavax Human Vaccines Inc., Research Triangle Park, North Carolina)

Goal: Establish capacity for the development, refinement, and production of pilot lots of candidate VHF vaccines.

- The VRC has begun development of a candidate vaccine for West Nile virus, a growing public health concern. The VRC proposes to use codon-modified gene-based DNA plasmids to make DNA constructs that express West Nile virus proteins. Vectors have been constructed and preclinical immunogenicity and viral challenge studies will be performed in the near future. The VRC plans to produce clinical grade plasmid DNA for future phase I trials. This work will have relevance for many other VHFs.

Goal: Develop a centralized immunology laboratory for the validation of tests required for licensure of priority VHF vaccines.

- As candidate vaccines for VHFs are developed, NIAID will expand existing infrastructure in order to meet requirements of licensure.

- The NIAID Vaccine Research Center has developed overlapping peptides to glycoprotein and nucleoprotein for strains of Ebola virus. These peptides will be produced and validated under GMP and will be used in ELISA assays by the centralized immunology laboratory.

Goal: Screen antibodies to evaluate their possible use as immune therapy for VHF.

- VRC scientists have developed a neutralizing human monoclonal antibody, which protects guinea pigs from lethal Ebola Zaire virus challenge. This antibody represents a proof of principle that such antibodies, generated in cell culture, might be promising candidates for immunoprophylaxis of Ebola virus infection.

Goal: Obtain clinical samples from patients with VHF to help validate potential diagnostics and aid the development of new vaccine therapies.

- A new contract, the World Reference Center for Emerging Viruses and Arboviruses, includes a provision for collection of reference clinical material from anonymous donors where appropriate. (University of Texas Medical Branch, Galveston)

Goal: Expand the *in vitro* and *in vivo* screening capability for antivirals against VHF.

- NIAID has expanded its *in vitro* and *in vivo* antiviral screening contracts. To date, hundreds of compounds have been evaluated for *in vitro* activity against hemorrhagic fever viruses: over 400 for Yellow Fever virus, over 100 for Pichinde (model for Lassa and other arenaviruses), and over 350 for Punta Toro (model for Rift Valley fever and hantaviruses). Compounds with *in vitro* activity (evidenced by reduced cytopathogenic effect or growth in cell culture) are then evaluated in animal models of disease.

Goal: Encourage the exploration of new targets for antiviral therapies against VHF.

- Several new NIAID grant programs encourage the development of antivirals for biodefense agents, including those that cause VHF. These include: Biodefense Partnerships; the Small Business Biodefense Program; and the Cooperative Research for the Development of Vaccines, Adjuvants, Therapeutics, Immunotherapeutics, and Diagnostics for Biodefense Program.
- In FY2003, NIAID is initiating support for large, multi-disciplinary efforts focused on the proteomics of more than one microorganism. Under this program, NIAID is currently funding a Biodefense Proteomics Collaboratory, a multi-disciplinary team of scientists from both academia and the biotechnology industry who are using high-throughput proteomic technologies to identify protein targets from arenaviruses and Lassa virus. (University of Texas Medical Branch at Galveston)

Goal: Complete the genomic sequencing of representative members and strains of the VHF, and compare them to detect differences in pathogenesis and virulence.

- NIAID has made a significant investment in the genomic sequencing of microorganisms considered agents of bioterrorism. As a result of a coordinated federal effort with the Department of Energy, the CDC, United States Department of Agriculture, and the National Science Foundation, and international partners including the Sanger Center,

genome sequencing projects are ongoing for at least one strain of every virus on the list of Category A-C priority pathogens.

Immunity and Biodefense

The key strategies of vaccination, immunotherapy, and passive antibody treatment rely upon effective manipulation of the immune system in the face of highly virulent pathogens. New understanding of how the immune system recognizes and takes action against pathogens opens new avenues for devising protective strategies. Most important is the elucidation of the innate immune system's response to microbial invasion and its interaction with the adaptive immune system, leading to the effective development of antibodies and cellular responses that clear the infection and provide long-term protection.

Innate immune defenses are based both upon cells permanently located within tissues and upon the migration of additional cells to the site of infection as needed. The innate immune system network includes Langerhans cells of the skin, tissue dendritic cells and macrophages, and tissue-associated lymphocytes such as natural killer cells and gamma-delta receptor T cells. In addition, many epithelial cells can sense and respond to microbes. Responses include the secretion of antimicrobial peptides that directly act on microbes, as well as the expression of cytokines and chemokines that call additional cells into action, including antigen-specific T and B cells. The key to rapid innate responses is the presence of highly specialized receptors, including the family of receptors known as the Toll-like molecules, which trigger cellular activation in response to various microbial structures. These "pattern-recognition receptors" detect and signal the presence of a broad range of microbes and viruses. For example, ligands of the Toll-like receptors include bacterial lipopolysaccharides, bacterial cell wall teichoic acid, flagellin, cell wall lipoproteins, highly mannosylated polymers, and bacterial nucleic acids that contain unmethylated CpG sequences.

In addition to innate immunity, the host relies on the induction of acquired immune responses to provide long-lived, pathogen-specific defense against infectious diseases, including those caused by the agents of bioterrorism. Acquired immunity is distinguished from innate immunity in several ways: specificity, reaction time, and duration of response. Acquired immune responses are directed against specific pathogen components, broadly termed antigens. Development of a protective response requires exposure to the antigen, either by natural infection or through vaccination strategies, and can take up to two weeks to reach maximum protection. However, once generated, most acquired immune responses persist for the life of the host, a phenomenon known as immune memory. Hallmarks of immune memory include the presence of antibodies against the original antigen and a decrease in the time required for the protective T-cell response after secondary or subsequent exposure to an antigen. Immune memory is the main mechanism by which vaccines provide protection to the host.

Scientific Progress

Protein switch for both bacterial and viral infections identified. NIAID supported researchers have determined that a single protein acts as a key switch point in frontline immune system reactions to both bacterial and viral infections in mice. This finding explains why certain symptoms, such as fever, occur regardless of the cause of infection. The protein, called Trif, is a critical first-line signal for the mouse innate immune system and alerts toll like receptors to the presence of infectious agents. Once activated by invading pathogens, TLRs relay the alarm to other actors in the immune system. The innate immune system then responds with a surge of chemicals that together cause inflammation, fever and other responses to infection or injury. This finding provides a new target for the development of broad-spectrum antibiotics. (K Hoebe *et al.* *Identification of Lps2 as a key transducer of MyD88-independent TIR signaling.* *Nature*. Published online July 20, 2003. DOI: 10.1038/nature01889.)

Immune-evasion strategies determined for anthrax, smallpox, and plague. An understanding of the strategies that pathogens have evolved for evading innate immunity is critical for the rationale design of immunotherapeutic approaches. Scientists have now shown that anthrax lethal factor selectively inhibits a key receptor-signaling pathway needed for macrophage survival, resulting in the death of these key cells of the innate immune system. Variola (smallpox) virus possesses an extraordinarily effective inhibitor of complement, one of the key tools of innate immunity involved in viral clearance. Poxviruses also possess several evasion strategies aimed at defeating interferon, including at least four gene products that counteract interferon. *Yersinia pestis* secretes an outer membrane protein that attaches to the innate immune receptors CD14 and Toll-like receptor 2, triggering an abnormal immune system suppression. The prevalence of these evasion strategies points to the importance of the innate immune system in protection against infection, and underscores the need to search for approaches to bolster innate immunity as a strategy for biodefense. (Sing A et al., *Yersinia V*-antigen exploits toll-like receptor 2 and CD14 for interleukin 10-mediated immunosuppression, *J Exp Med* 2002;196:1017-1024)

New clues are discovered on how innate immune system is regulated. A full understanding of how innate immune responses are regulated by the body so that they do not result in overwhelming septic shock is a major challenge. The regulatory mechanisms of innate immunity are being defined. For example, the molecule IRAK-M was recently found to negatively regulate Toll-like receptor signaling. (Kobayashi K et al., IRAK-M is a negative regulator of Toll-like receptor signaling, *Cell* 2002;110:191-202)

Prophylactic and post-exposure strategies involving innate immune stimulation can prevent or ameliorate bacterial and viral infections in animal models. Researchers recently demonstrated that administration of CpG-containing nucleic acids primed animals to resist lethal infection with intracellular bacteria. Fast-acting, broad-spectrum immunotherapeutics are being developed using knowledge about mechanisms for stimulating Toll-like molecules and other innate immune receptors. The next step will be preclinical development and testing to evaluate whether innate immune stimulation could be safely and effectively manipulated. (Katze MG et al., Viruses and interferon: a fight for supremacy [Review], *Nat Rev Immunol* 2002;2:675-687)

Stimulation of Toll-like receptors allows immune system to overcome natural suppression. For many years adjuvants have been used to enhance vaccination responses by a process now understood as the innate immune triggering of adaptive immunity. Immature dendritic cells activated via their Toll-like receptors undergo a maturation process that includes migration to the local lymph node, expression of co-stimulatory molecules needed for lymphocyte activation, and an enhanced ability to present engulfed protein antigens in the context of major histocompatibility complex (MHC) class I and class II molecules. Recently, investigators have shown that adjuvant activation of innate immune molecules promotes increased survival of activated T cells. Stimulation via Toll-like receptors also temporarily relieves natural immune suppressive mechanisms in the body, thus enabling more vigorous responses. This knowledge will help scientists in developing new, more specific adjuvants that are safer and more powerful. (Sing A et al., *Yersinia V*-antigen exploits Toll-like receptor 2 and CD14 for interleukin 10-mediated immunosuppression, *J Exp Med* 2002;196:1017-1024)

Cellular protein that plays a critical role in humoral immunity is identified. Humoral immunity is provided by B cells, which differentiate into antibody-producing cells (i.e., plasma cells) upon exposure to antigen. Long-lived plasma cells and memory B cells are vital components of the protection induced by most vaccines. Researchers recently identified a cellular protein, SAP, that is critical for the development of long-term humoral immunity.

Understanding SAP's role in humoral immunity may lead to the manipulation of SAP-mediated signaling pathways for therapeutic benefit. (Crotty S et al., SAP is required for generating long-term humoral immunity, *Nature* 2003;421:282-287)

Advances in antibody engineering technology provide knowledge to craft more effective and safe passive antibody treatments. Studies of a panel of engineered anthrax toxin-neutralizing antibodies demonstrated that passive transfer of high-affinity antibodies provides post-exposure protection in animal models. These high-affinity antibodies may be of therapeutic value in alleviating symptoms of anthrax toxin in infected individuals, and for prophylaxis to infection. Passive antibody treatment requires the production of large quantities of safe, effective product. In the past, and currently for some applications, antibodies for passive treatment were isolated from immune donors or immune animals, such as the horse. Antibodies produced in animals have the potential of inducing an adverse reaction in the recipient. Transgenic animals are being generated that produce "human" polyclonal and monoclonal antibodies in response to immunization to alleviate this problem. (Maynard J et al, Protection against anthrax toxin by recombinant antibody fragments correlates with antigen affinity, *Nat Biotechnol* 2002;20:597-601)

Passive administration of antibodies can be used to control infections. Recent advances by domestic and international researchers reinforce the idea that passive antibody treatments can be used to control human infections, with both prophylactic and therapeutic applications. For example, administration of a monoclonal antibody that neutralizes vaccinia virus is effective as both a prophylactic and therapeutic treatment of vaccinia-infected mice. (Ramirez JC et al., Administration to mice of a monoclonal antibody that neutralizes the intracellular mature virus form of vaccinia virus limits virus replication efficiently under prophylactic and therapeutic conditions, *J Gen Virol* 2002;83:1059-1067)

Similarly, monoclonal antibody epitopes have been identified that recognize specific sites on the Ebola virus glycoprotein. Passive administration of a cocktail of these antibodies completely protects mice from lethal Ebola infection (Casadevall A, Passive antibody administration [immediate immunity] as a specific defense against biological weapons [Review], *Emerg Infect Dis* 2002;8:833-841).

A novel protein that plays a critical role in antigen processing identified. Antigen-presenting cells (APCs) rely on internal enzymes to digest, or process, the antigens. Recently, investigators identified a novel protein, gamma interferon-inducible lysosomal thiol reductase (GILT), which plays a critical role in antigen processing. Most proteins are globular in form and are held together by chemical bonds. Normally, GILT breaks down one type of chemical bond that is commonly used by certain viruses, bacteria, and parasites to hold their proteins together. These studies show that immune responses are significantly diminished in GILT-free mice compared to normal mice that have the GILT protein. This work defines a critical antigen-processing component whose function contributes to the development of protective immunity. A clearer understanding of the antigen processing pathway may lead to the design of novel vaccines and therapeutics to combat infectious diseases. (Maric M et al., Defective antigen processing in GILT-free mice, *Science* 2001;294:1361-1365)

T cell binding is driven by survival of the fittest. Antigen processing and presentation are critical steps in the development of a protective immune response because T cell recognition of the peptide-MHC complexes results in T-cell activation and pathogen clearance. Recent studies have shown that T cells that bind very tightly to peptide-MHC prevent weaker-binding T cells from binding and being activated. This competition results in the preferential activation and

growth of strong-binding T cells, which may be more efficient at clearing infections from the host compared to weak-binding T cells. These results help explain the observation that responding T cell populations become less diverse upon second infection with the same microbe, and may be exploited to improve vaccine design and delivery strategies. (Tangri S et al., Structural features of peptide analogs of human histocompatibility leukocyte antigen class I epitopes that are more potent and immunogenic than wild-type peptide, *J Exp Med* 2001;194:833-846)

Molecular modifications result in more effective cytotoxic T lymphocyte (CTL)

recognition. Another promising approach to enhancing immune responses is to design vaccines that contain specific viral or bacterial peptides that can be recognized by CTLs. Frequently, however, the natural peptides derived from these pathogens do not stimulate T cell responses efficiently. Investigators have now modified these peptides, resulting in more effective CTL recognition than the natural, unmodified peptide. In addition, in some cases, far less modified peptide was needed to obtain the same immune response as compared with the natural peptide. When tested in mice, the modified peptides efficiently elicited CTLs. These observations suggest that systematic molecular alterations of peptides may prove a viable approach for designing vaccines that elicit potent CTL responses. This finding can also be exploited to design subunit vaccines that will induce long-term protection without the risk of side effects that live or attenuated vaccines can induce. (Drexler I et al., Identification of vaccinia virus epitope-specific HLA-A*0201-restricted T cells and comparative analysis of smallpox vaccines, *Proc Natl Acad Sci USA* 2003;100:217-222)

Programmatic Accomplishments

- NIAID recently awarded a multi-component grant to create an “encyclopedia” of innate immunity: a comprehensive and detailed picture of this ancient, essential first line of defense against bacterial and fungal diseases. Under this award, researchers from Scripps Research Institute in La Jolla, California, Rockefeller University in New York; and the Institute for Systems Biology in Seattle will focus on the discovery of new ways to study the immune system in living tissue in real time, and to provide materials and information to the scientific community. Knowledge generated could help scientists develop treatments for septic shock, certain autoimmune disorders, and diseases caused by potential agents of bioterrorism.
- Administrative supplements were awarded to existing NIAID-supported research programs in FY2002 to support studies on:
 - o Genes important for macrophage phagocytosis of anthrax
 - o Anti-influenza responses in the elderly
 - o Cloning of viral genes that subvert the effects of interferon
 - o Protective immune responses in children receiving influenza vaccine
 - o Protective effects of a DNA vaccine for anthrax
 - o CD8⁺ T cell responses to pox and dengue viruses
 - o Development of monoclonal antibodies against botulism toxin
 - o Characterization of potent anti-influenza T-memory cells in the lung

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
National Institutes of Health



National Institute of Allergy and Infectious Diseases

NIH Publication No. 03-5432
August 2003
<http://biodefense.niaid.nih.gov>

DMID/NIAID Smallpox Vaccine Clinical Trials

Title	Objectives	VTEU Sites	Status -June 2003
A randomized, double-blind Phase 2 trial to evaluate the safety and lesion formation (take) of three doses of Dryvax vaccine against Smallpox in previously unvaccinated adults	<ul style="list-style-type: none"> • Determine take rates for undiluted, 1:10, and 1:100 dilutions of Dryvax • Assess safety profile • Evaluate immunogenicity • Assess viral shedding at site of vaccination 	St. Louis University	Frey SE, Newman FK, Cruz J, Shelton WB, Tennant JM, Polach T, Rothman AL, Kennedy JS, Wolff M, Belshe RB, Ennis FA. Dose-related effects of smallpox vaccine. N Engl J Med. 2002 Apr 25;346(17):1275-80
A multicenter, double blind, randomized dose-response study of Dryvax vaccine against smallpox in previously unvaccinated adults.	<ul style="list-style-type: none"> • Determine take rates for undiluted, 1:5, and 1:10 dilutions of Dryvax • Assess safety profile • Evaluate immunogenicity 	St. Louis University Baylor University University of Rochester University of Maryland	Frey SE, Couch RB, Tacket CO, Treanor JJ, Wolff M, Newman FK, Atmar RL, Edelman R, Nolan CM, Belshe RB. Clinical responses to undiluted and diluted smallpox vaccine. N Engl J Med. 2002 Apr 25;346(17):1265-74.
A pilot, double-blind, randomized dose-response study of Dryvax vaccine against smallpox in previously vaccinated adults.	<ul style="list-style-type: none"> • Define the proportion of previously vaccinated individuals who respond to vaccination with a "major" reaction or "take." 	St. Louis University	This trial is fully enrolled. Analysis is ongoing.
A multicenter, double blind, randomized dose-response study of Dryvax vaccine against smallpox in previously vaccinated adults.	<ul style="list-style-type: none"> • Determine the precision of take undiluted, 1:5, and 1:10 dilutions of Dryvax • Determine the safety profile • Measure humoral and cellular immune response 	University of Rochester Kaiser, Northern California Stanford University Kaiser, Southern California St. Louis University University of Maryland Duke University	This trial is fully enrolled. Analysis is ongoing.
A Phase I/II study to evaluate the safety and preliminary efficacy of various concentrations of Aventis Pasteur's Smallpox Vaccine, USP (APSV) in vaccinia-naïve adults	<ul style="list-style-type: none"> • Determine take rates of Dryvax at undiluted, 1:3.2, 1:32 and 1:100; Aventis 4136 at undiluted, 1:3.2, 1:10 and 1:32; Aventis APSV undiluted, 1:32. and 1:10. • Define the safety profile. 	Baylor University University of Iowa Kaiser, Northern California Vanderbilt University	This trial is fully enrolled. Analysis is ongoing.
A Phase I study of the immunogenicity of Aventis Pasteur's smallpox vaccine and Dryvax in previously immunized adults	<ul style="list-style-type: none"> • Characterize the immunogenicity of the APSV and Dryvax vaccines in previously immunized adults. 	Baylor University	This trial is fully enrolled. Analysis is ongoing.
A multicenter, double-blind, randomized, study of the safety and efficacy of Aventis Pasteur's Smallpox Vaccine, USP (APSV) in vaccinia-naïve adults	<ul style="list-style-type: none"> • Determine clinical take rates with undiluted and two dilutions of APSV vaccine. • Define the safety profile. • Evaluate immunogenicity. 	Vanderbilt University University of Iowa University of Cincinnati	This trial is fully enrolled. Analysis is ongoing.
Evaluation of human immune responses to smallpox vaccine (<i>Vaccinia Virus</i>).	Detailed characterization of the cellular and humoral immune response to vaccinia virus infection and comparison of the responses to primary and secondary infections with assessment of the durability of immune memory.	University of Rochester	This trial is fully enrolled. Analysis is ongoing.