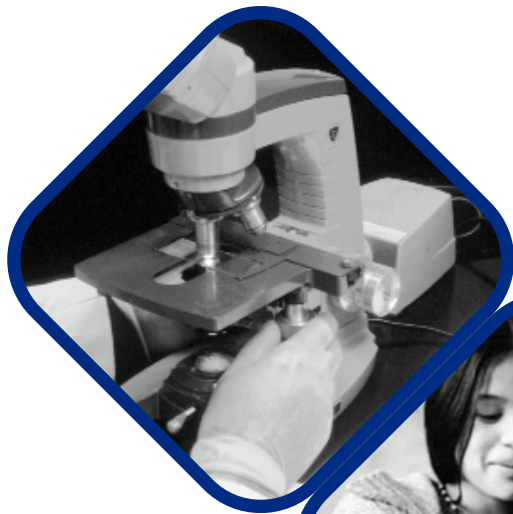




NIAID Biodefense Research Agenda for CDC Category A Agents

Overview of 2006 Progress Report



U. S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
National Institutes of Health
National Institute of Allergy and Infectious Diseases

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NIAID Biodefense Research on Category A Agents

Highlights of Progress

It has been three years since the National Institute of Allergy and Infectious Diseases (NIAID) released its first research progress report on Category A agents. Since that time, extraordinary progress has been made to increase scientific knowledge of these potentially deadly pathogens and to advance medical countermeasures along the research and development pathway.

To realize these accomplishments, NIAID greatly expanded its support of basic and applied research and advanced product development for biodefense agents including NIAID Category A-C Priority Pathogens, as well as microbes that cause naturally occurring emerging and re-emerging infectious diseases. This document outlines the components of the product devel-

opment pathway, provides highlights of scientific accomplishments, and explains the positive implications of NIAID's expanded biodefense research portfolio for improvements in global health. For detailed scientific advances beyond what is covered in this synopsis, please see NIAID Biodefense Research Agenda for CDC Category A Agents: 2006 Progress Report, which provides a comprehensive look at NIAID's accomplishments in biodefense and emerging infectious diseases research.

The Product Development Pathway

Developing products that can protect against potential biological threats is an integrated process that operates along a continuum that includes basic and applied research, and

THE PRODUCT DEVELOPMENT PATHWAY

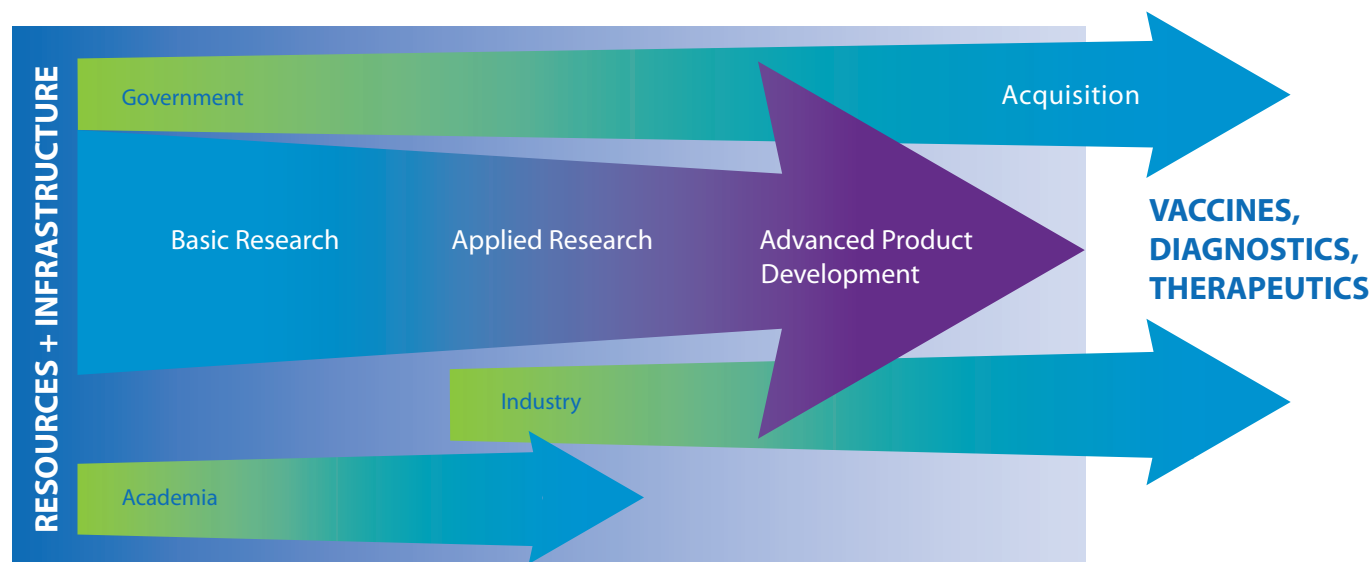


Figure 1. The product development pathway is an integrated process whereby concepts discovered through basic research are tested in practical settings and may generate candidate products: vaccines, diagnostics, and therapeutics. Academia participates in basic and applied research; industry participates in applied research and advanced product development. Government supports the entire process, including acquiring completed products. Resources available to scientists worldwide provide a foundation for the pathway. A strong infrastructure offers a network for creative approaches and sharing ideas. Knowledge gained along the way leads to new ideas to be explored through basic and applied research.

advanced product development (see Figure 1). Basic research lays the groundwork by developing new and innovative concepts. Scientists conducting basic research seek to better understand infectious agents and the response of host organisms by studying the cellular and molecular biology of pathogen and host, physiologic processes, and genome sequences and structures. Their findings elucidate pathogen entry mechanisms, survival strategies, and immune evasion techniques; evolutionary adaptations; activation of the host immune system; and cellular and whole organism responses to infection. Basic studies conducted at the macroscopic level by epidemiologists, population biologists, and ecologists shed light on transmission and disease patterns, habitat changes, and the relationship between organisms and their environments. Insights from basic research often yield concepts for new vaccines, drugs, and diagnostics.

Applied research builds on basic research by validating concepts in model systems, and developing and testing them in practical research settings. Advanced technologies such as genomics and proteomics help to identify and validate the most promising targets. At this stage, potential products are identified using relevant animal models and *in vitro* screening. Successful candidates move into advanced product development, where they are evaluated for safety and efficacy in relevant animal models. The most promising of these achieve IND status and move into further testing to determine whether they are safe and effective for preventing, diagnosing, or treating disease in humans. Successful products are then available for further development by industry or for eventual acquisition by the government.

Throughout the product development pathway, a comprehensive resource base provides expertise and services that allow scientists to take advantage of state-of-the-art technology and conduct the highest quality research. Likewise, a strong infrastructure provides a network of collaborating institutions that encourages sharing of ideas and creative approaches to scientific investigation.

NIAID has expanded support for all stages of the product development pathway—a dynamic progression in which knowledge generated at each stage improves candidate products as they advance along the pathway, and generates new concepts for further exploration.

KEY RESEARCH ACCOMPLISHMENTS

This progress report provides highlights of NIAID research efforts and resulting scientific breakthroughs that have occurred over the last several years. It offers examples of scientific achievements that enhance understanding of basic microbial biology and host response, and that further the development of vaccines, diagnostics, and therapeutics. The report also highlights resources that NIAID has developed to provide a strong foundation for research. These include centers for genomics, proteomics, and bioinformatics; an *in vitro* and animal model screening program; and networks of institutions that offer an infrastructure for advanced biodefense and emerging infectious diseases research. The complete 2006 Progress Report presents more extensive accomplishments that meet the immediate goals identified in the 2002 research agenda. For a list of, and links to, NIAID strategic plans, research agendas, and previous progress reports, visit www.niaid.nih.gov/biodefense/research/strat_plan.htm.

Biology of the Microbe

NIAID-supported research is gradually shedding light on the varied and ingenious ways that microbes survive and multiply. By dissecting the infection process and discovering mechanisms that pathogens use to evade the immune system, scientists are developing new strategies for countermeasures.

- Scientists have discovered specialized genes and proteins that help poxviruses evade the immune system. For example, a smallpox gene allows the virus to co-opt protein production, ensuring that proteins essential to viral survival are generated; a gene from another poxvirus reduces host immune responses; and a common poxvirus enzyme increases viral transcription early in the infection process. Identifying the roles of particular genes and proteins can provide targets for products that prevent or treat infections.
- By mapping the three-dimensional structure of an anthrax toxin protein docked to a human cell receptor, researchers revealed how anthrax toxin enters human cells. This finding provides new approaches for developing anthrax anti-toxins. Because the toxin has potential medical applications for treating certain cancers, this finding may also aid in designing cancer therapeutics.

- Specialized proteins called cathepsins play an essential role in the ability of Ebola virus to enter and infect cells. Investigators at the NIAID Dale and Betty Bumpers Vaccine Research Center and their colleagues determined that inhibitors of cathepsins can block viral entry and offer promising new targets for anti-Ebola virus therapies.

Host Response

Qualitative and quantitative assessments of host response are crucial for creating vaccines and therapeutics. Recent research has helped scientists decipher how the immune system recognizes invading microbes, discriminates among them, and mounts a response. Enhanced understanding of complex host-microbe interactions opens new avenues for devising protective strategies.

- Recent studies helped to unravel one of the pathways that the innate immune system uses to discriminate among different types of bacteria. Toll-like receptors, molecules involved in early pathogen detection and immune activation, partner with a cellular protein to distinguish bacteria based on the types of lipids that the bacteria produce. Understanding this fine specificity for differentiating “bad bugs” may allow investigators to develop novel pathogen detection devices or methods to regulate immune responses.
- Plague bacteria, *Yersinia pestis*, inject particular proteins into host cells during infection. Studies using plague-infected mice found that the host cell types most often targeted by the bacteria are immune system cells. *Y. pestis* may make use of this mechanism to target and disable host immune responses during infection.
- Anthrax bacteria cause illness and death by releasing toxins that kill cells and damage organs. The toxins remain active in the bloodstream for several days, even if antibiotics kill the bacteria that are producing them. By determining how the toxins enter healthy cells and disrupt the internal communications network, scientists are paving the way for developing decoys to block the lethal effects of toxins, establishing a new approach for treating anthrax.
- Anthrax lethal toxin blocks a key immune pathway in the host, suppressing the immune response. Understanding the mechanism by which anthrax evades the immune system can advance research on protective strategies against the anthrax toxin.

Vaccines

Vaccine research and development has been a vital component of NIAID’s research agenda for more than 40 years. With the increased emphasis on biodefense research, NIAID is increasingly focused on working in partnership with industry to create new and improved vaccines against potential biothreats, especially Category A agents.

- Approximately 25 percent of the population cannot receive Dryvax®, the traditional smallpox vaccine, because they are at increased risk for post-vaccine complications. One alternative NIAID researchers are pursuing is a new, safer smallpox vaccine, known as modified vaccinia Ankara (MVA). MVA is unable to replicate in human cells and does not form a lesion at the site of vaccination, thus preventing many vaccine-related side-effects. Using an animal model, NIAID scientists and collaborators compared immunization with MVA against immunization with Dryvax. Researchers found that the immune response to MVA alone, or MVA and Dryvax, was equivalent to or higher than that induced by Dryvax alone. This shows that MVA may be an effective alternative to Dryvax.
- A weakened, recombinant vaccine against the deadly Marburg virus helped monkeys survive the virus when the vaccine was administered soon after infection. This result demonstrates that it may be possible to use these types of vaccines after infection to treat Marburg and similar viruses, such as Ebola, that cause severe hemorrhagic fevers.
- Developing effective countermeasures against tularemia is uniquely challenging because the vaccine currently being developed (known as LVS) generates an incomplete immune response, activating certain pathways but not others. In addition, the immune protection offered depends on several factors, including route of infection; LVS offers more protection from systemic than from aerosol infection. These findings may be crucial for developing effective vaccines for systemic and pulmonary manifestations of tularemia.

Diagnostics

Many of the initial symptoms caused by bacterial and viral infections are non-specific and do not lend themselves to a definitive diagnosis. This makes it difficult for both the clinician and patient to evaluate treatment options. In addition, the ability to rapidly determine whether an individual is infected by an organism posing a biological threat or a more innocuous pathogen is a critical component of public health preparedness, as are diagnostic tools to determine a pathogen's drug sensitivities.

- Multiplex diagnostics, products that can detect more than one pathogen in a single test, are essential to mount an effective response in the event of an urgent public health situation. By rapidly identifying infecting pathogens, multiplex diagnostics allow for timely implementation of containment measures. Many of these diagnostics platforms can also determine drug sensitivities such as antibiotic resistance, and distinguish pathogen subtypes and strains. These products are currently being tested in clinical settings and a small, low-cost, mobile unit is being designed to enable widespread use.
- Scientists have developed a rapid test to assist in diagnosing plague in patients presenting with pneumonia symptoms at a hospital. This will allow health care providers to identify and isolate pneumonic plague patients more quickly, reducing the spread of disease. The test can be used for both blood and sputum samples.

Therapeutics

The ability of pathogens to develop drug resistance makes establishing an arsenal of safe and effective anti-microbials especially challenging. Thus, NIAID is supporting a variety of approaches to identify potential targets for intervention and to engineer new therapies. Exciting progress is being made by screening existing products for activity against Category A agents and creating new therapies, such as those based on monoclonal antibodies.

- In a novel approach to anthrax therapeutics, scientists have designed an inhibitor that blocks anthrax toxin from attaching to receptors on the surface of host cells. The inhibitor, known as a "functionalized liposome," is a fatty bubble studded with small proteins that binds to sites on the two host receptors for anthrax toxin. The binding process blocks the toxin from entering the host cell and prevents it from

exerting its toxic effects. In animal studies, this new inhibitor was shown to be many times more potent than current anthrax therapies. It is especially promising as a countermeasure to antibiotic-resistant strains or as a potential adjunct to antibiotic therapy. The general concept could also apply to designing inhibitors for other pathogens.

- Scientists are using innovative monoclonal and polyclonal antibodies as the basis for developing therapeutics to treat botulinum neurotoxins, which are among the most potent and lethal toxins known. These human-compatible antibodies would be safer than existing therapies that rely on horse antibodies and can induce serious adverse reactions.
- Viruses are simple in their makeup, having only a small number of genes, and rely on being able to co-opt host cell machinery and cellular processes to replicate and spread. Two different studies have shown that the anticancer drugs CI-1033 and Gleevec each inhibit a late step in the life cycle of orthopoxviruses, the family of viruses that includes smallpox. By interfering with biochemical signaling pathways within the host cells, these drugs limit the spread of the virus and help to prevent poxvirus-induced disease. Because the drugs are aimed at host, not viral, targets, this strategy is less likely to cause drug resistance compared with conventional antimicrobial therapies. Over the past several decades, thousands of promising anticancer drugs have been developed that may hold promise as anti-poxvirus therapies. In addition, this approach may prove effective in treating other infectious diseases that rely on similar pathways.

Research Capacity and Infrastructure

To support the expansion of the Institute's research portfolio, NIAID is building a new infrastructure with the breadth and flexibility to meet changing research needs. This network includes research that could help the nation address the threat of emerging and re-emerging infectious diseases and prepare for public health emergencies. The expanded NIAID biodefense network now offers training programs and increased capacity to conduct clinical trials of candidate products. The network is being developed even further and will include high level biocontainment laboratories to support advanced research.

The network encompasses advanced research centers that draw upon expertise from some of the nation's finest institutions to participate in coordinated research efforts. For example, NIAID established ten Regional Centers of Excellence for Biodefense

and Emerging Infectious Diseases (RCEs) to support the development of medical countermeasures against the NIAID Category A-C Priority Pathogens as well as newly emerging and re-emerging infectious diseases. Each Center comprises a consortium of universities and complementary research institutions serving a specific geographic region. The primary objective of the RCEs is to support multifaceted research on scientific priorities identified in NIAID's biodefense research agenda. The Centers, located throughout the United States, are pursuing research to create the next generation of therapeutics, vaccines, and diagnostics, and are training scientists in the fields of biodefense and emerging infectious diseases. For a complete list of RCE sites, see Appendix A.

NIAID has also initiated a national network of high-level biocontainment facilities to complement the RCEs and support biodefense and emerging infectious diseases research. NIAID's 2 National Biocontainment Laboratories (NBLs) and 13 Regional Biocontainment Laboratories (RBLs) will serve as resources for conducting critical advanced testing of concepts and candidate vaccines, therapeutics, and diagnostics to protect the public from potential biological threats. These laboratories will also be prepared to assist national, state and local public health efforts in the event of a bioterrorism or infectious disease emergency. The facilities are being designed and built using the strictest Federal standards for safety and security to protect laboratory workers and the surrounding environment. For a list of NBL and RBL sites, visit Appendix A.

NIAID is constructing additional biocontainment facilities in Bethesda and Frederick, Maryland, and on the campus of its Rocky Mountain Laboratories in Hamilton, Montana. In May 2006, NIAID completed and inaugurated a new intramural biocontainment facility on the National Institutes of Health's (NIH's) main campus in Bethesda. The C.W. Bill Young Center for Biodefense and Emerging Infectious Diseases is a state-of-the-art research building that houses several NIAID programs and includes BSL-2 and BSL-3 laboratories and animal care areas. In Frederick, the Integrated Research Facility (IRF) at Fort Detrick will feature BSL-2/3/4 laboratories with unique aerobiology and BSL-4 imaging. An IRF at Rocky Mountain Laboratories will be part of the NIAID intramural research program. It will contain BSL-2/3/4 laboratories and house the Laboratory of Virology. Opening is anticipated for 2007.

To enhance clinical evaluation of candidate vaccines and drugs, NIAID has expanded its Vaccine and Treatment

Evaluation Units (VTEUs). This network comprises centers across the country with the capacity to conduct clinical trials on candidate products developed through NIAID's extramural research program. Lessons learned from experience in the field of biodefense product trials will be applied to future enhancements of the VTEU Program.

Research Resources

NIAID has developed key resources that enable scientists worldwide to use advanced technology and access the most current information that will further research on the spectrum of infectious diseases. For example, recognizing the significance of genomic sequencing to the biodefense effort, NIAID has provided genomic, bioinformatic, and proteomic resources to the scientific community. These include the Pathogen Functional Genomics Resource Center, Microbial Sequencing Centers, Bioinformatics Resource Centers, and Biodefense Proteomics Research Centers. The availability of microbial and human DNA sequences allows scientists to analyze the functions of genes and proteins in whole genomes and cells, as well as the host immune response and variation in genetic susceptibility to pathogens. By identifying microbial genes that play a role in disease, scientists can design targeted drugs to block those specific gene activities. Genetic variations can also be used to study the spread of a virulent or drug-resistant form of a pathogen. The complete genome of at least one strain of each Category A agent has now been sequenced through the combined efforts of publicly and privately funded investigators.

NIAID is currently embarking on a structural genomics initiative to use state-of-the-art technology to characterize proteins from NIAID Category A-C pathogens. The goal is to create a collection of three-dimensional protein structures that are available to scientists worldwide and can serve as a blueprint for structure-based drug development for infectious diseases.

The Biodefense and Emerging Infections Research Resources Repository (BEI Resources) is another important research resource established by NIAID. BEI Resources acquires, authenticates, and produces reagents that scientists need to carry out basic research and develop improved diagnostic tests, vaccines, and therapies. BEI Resources stores and ships biological reagents such as viruses, bacteria, antigens, antibodies, peptides, and nucleic acids directly to registered researchers to provide them with materials needed for basic and applied biodefense research.

NIAID also provides resources such as the Antiviral Testing Program, which offers screening systems and animal models to assess the safety and efficacy of potential therapeutic approaches for human viral infections. Researchers submit candidate compounds to the program for screening to identify promising compounds for further testing. This program facilitates identification of antiviral agents with potential for treating viral infections of public health importance, including newly emerging infections, and those diseases that may not be addressed by industry.

Public-Private Partnerships

Implementing public-private partnerships continues to be a key factor in translating scientific advances into new medical countermeasures that will be available in the event of a public health emergency. To expand product development activities for biodefense, NIAID has increased partnerships between government and the academic and private sectors. The Institute has accomplished this through a variety of mechanisms by which NIAID provides support for research projects that industry would otherwise not pursue. This enables the Institute to play a greater role in industry-led projects that advance the biodefense research effort.

In a further effort to stimulate partnerships with the private sector, the federal government enacted the **Project BioShield Act of 2004**, which authorizes NIH to accelerate the award process for research on development of medical countermeasures; authorizes the U.S. Food and Drug Administration (FDA) to expedite approval for promising products; and authorizes acquisition of products from the private sector for addition to the Strategic National Stockpile (SNS) (see Figure 1). The SNS is a repository of medical supplies designed to supplement state and local public health agencies in the event of a public health emergency.

All three of the broad areas covered by Project BioShield remove some of the risk to industry and encourage industry investment in developing countermeasures to protect the United States against chemical, biological, and radiological threats. In FY 2004, NIAID began using authorities provided by Project BioShield to award grants and contracts to develop new and improved medical products against Category A agents of bioterrorism. Additional information on Project BioShield can be found at www.hhs.gov/ophep/bioshield.

CONCLUSION

The biodefense research pathway is an integrated process (see Figure 1). Basic and applied research conducted by scientists from academia and industry provide the building blocks for developing vaccines, diagnostics, and therapeutics. Knowledge gained as products move along the biodefense research pathway leads not only to improvements for those candidate products, but also to new ideas to be explored through subsequent basic and applied research.

In addition to expanding biodefense preparedness, NIAID's sizeable investment in the biodefense research pathway will have many positive implications for global public health. Studies of microbial biology and the pathogenesis of organisms with bioterror potential will lead to a better understanding of other more common and naturally occurring infectious diseases. For instance, advances in biodefense research are likely to have an enormous positive impact on our ability to diagnose, treat, and prevent major infectious killers such as malaria, tuberculosis, and HIV/AIDS. Furthermore, NIAID biodefense research promises to enhance the understanding of molecular and cellular mechanisms of the immune system, which may help in the search for new ways to treat and prevent a variety of immune-mediated diseases, such as type 1 diabetes and rheumatoid arthritis. New insights into the mechanisms of regulation of the human immune system will advance research on cancer, immune-mediated neurological diseases, and allergic and hypersensitivity diseases.

This report includes seven Stories of Discovery that describe how scientific advances are being translated into real-world applications that are transforming the fields of medicine and public health. From advanced genomic technologies used to characterize anthrax strains, to vaccines engineered to protect against some of the most feared pathogens, NIAID is enabling scientists to unlock the mysteries of infectious diseases.

While it is impossible to capture the true breadth of the NIAID biodefense research portfolio and accomplishments therein, the activities cited here and the comprehensive 2006 Progress Report most clearly demonstrate the determination and steadfastness of the Institute toward achieving the goal of developing new therapies, diagnostic tests, and vaccines.

Category A Progress Report

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NEXT GENERATION VACCINES FOR ANTHRAX AND SMALLPOX

The terrorist attacks of 2001 highlighted the need for effective countermeasures against potential agents of bioterrorism.

Although vaccines currently exist for anthrax and smallpox, both have negative side effects and can cause serious reactions. Therefore, novel strategies are being pursued to develop newer, safer vaccines that may be more suitable for civilian populations.

Background

Anthrax is caused by the exposure of a mammalian host to the spore-forming bacterium *Bacillus anthracis*. With an anthrax inhalation exposure, which is the most lethal form of exposure, a short incubation period is followed by symptoms that may include malaise, headache, fever, nausea, and vomiting. Shortly thereafter respiratory distress occurs, ending in shock and probable death with close to 100 percent mortality if not treated immediately with antibiotics.

Smallpox, caused by the variola virus, has devastated populations for centuries. With a mortality rate of over 30 percent, it has been estimated that throughout recorded history more people died of smallpox than from all other infectious diseases

combined. Fortunately, a vaccine was discovered that, when coupled with a global vaccination policy, led to the eradication of smallpox in 1979. Although the vaccine used to eradicate smallpox was highly efficacious, it was also associated with local and/or systemic reactions in the majority of recipients. Because of this, once smallpox was eradicated, most of the world discontinued routine smallpox vaccination in the 1970s.

Current Vaccines

The anthrax vaccine currently licensed in the United States, BioThrax®, is a crude mixture that consists of filtered *B. anthracis* culture supernatant treated with formaldehyde and mixed with an aluminum adjuvant. The vaccine includes a subunit of anthrax toxins called protective antigen (PA), which is known to generate an antibody response. A 2002 Institute of Medicine report, “The Anthrax Vaccine: Is It Safe? Does It Work?” recommended that research should be pursued and encouraged to develop other possible anthrax vaccine products that can be produced more consistently and that are less reactogenic than BioThrax®.

The smallpox vaccine presently in use, Dryvax®, is highly efficacious, but is associated with significant local and/or systemic reaction in over 90 percent of vaccinees. In addition, Dryvax should not be given to individuals who are immunocompromised, such as those with human immunodeficiency virus (HIV), as they are at increased risk for even more serious side effects. This leaves a significant portion of the U.S. population without access to a smallpox vaccine.

New Strategies

The National Institute of Allergy and Infectious Diseases (NIAID) Biodefense Research Program is funding the development of next generation vaccines to help ensure that safe and effective vaccines are available for the entire U.S. population, including for immunocompromised individuals.

To improve on the current anthrax vaccine, NIAID is supporting the development of vaccines composed of only PA produced by modern recombinant technology (rPA) and combined with

aluminum adjuvant. The rPA vaccines have been tested for safety and efficacy in rabbits and monkeys, and subsequently underwent clinical safety testing in people.

A post-exposure prophylaxis (PEP) scenario for emergency civilian usage has been integrated into animal model development and testing, as well as human testing of the vaccines. Following exposure or suspected exposure to anthrax spores a patient would immediately be treated with antibiotic therapy in conjunction with anthrax vaccine. This combination therapy is based on the premise that germinated anthrax spores would be eliminated by antibiotic therapy while spores undergoing latent germination, which is known to occur after cessation of antibiotic therapy, would be eliminated by a protective immune response generated by the vaccine.

To date, large-scale manufacturing capability of rPA vaccines has been demonstrated; the vaccines have been shown to be efficacious in a PEP animal model and are safe and immunogenic in people.

Meanwhile, NIAID is developing improved countermeasures against smallpox as well. One of the most promising is the modified vaccinia Ankara (MVA) vaccine. This vaccine does not replicate in humans, and therefore is likely to be safe for use in individuals who currently should not receive Dryvax. NIAID-supported researchers successfully performed small-scale manufacturing of the MVA vaccines and conducted small Phase I clinical trials in healthy volunteers. These early development studies showed that MVA could be manufactured in compliance with current laws and regulations, and that it was safe and immunogenic in healthy volunteers.

Following these successes, large-scale manufacturing of MVA was performed and Phase II clinical studies have been planned in both healthy individuals and those who are immunocompromised. The purpose of these studies will be to further assess the safety of these vaccines, and also begin to assess how effective the vaccines will be based on the immune response. Several of these clinical trials have started, including trials in volunteers with HIV.

Although MVA is likely to be much safer than Dryvax, particularly in immunocompromised individuals, it is still a live virus vaccine that may result in other, as yet unanticipated, adverse reactions. Accordingly, NIAID has also begun development of a smallpox vaccine that is based on protein subunits of the smallpox virus rather than a live virus. The idea behind such a vaccine is to use only the relatively few components of variola virus that are responsible for stimulating the protective immune response that occurs when the live virus vaccine is used, thereby limiting the adverse reactions that are usually caused by other components of the virus. Basic research carried out by NIAID scientist Dr. Bernard Moss has identified four poxvirus-encoded proteins that are critical for a protective immune response. In collaboration with investigators at the University of Pennsylvania, this has led to successful production of these four proteins in insect cells. Pilot experiments have shown that a vaccine composed of the four purified proteins, together with an appropriate adjuvant, induces immunity to poxvirus infections both in mice and in non-human primates. A commercial laboratory that has developed a proprietary method for producing proteins in insect cells was recently awarded a NIAID grant to manufacture large amounts of the four variola proteins for use in further developing this vaccine.

NIAID is also funding the development of several poxvirus animal models. These animal models will be used to demonstrate that the new smallpox vaccines can prevent pox diseases. As there is no current smallpox disease in the human population, data from these animal models, which will mimic the human disease, will be used to assess whether the vaccine is safe and effective. Several studies have already been conducted to develop the animal models, and additional data will be produced in the coming months.

MULTIPLEX DIAGNOSTICS: RAPID IDENTIFICATION OF EMERGING PATHOGENS

What Are Multiplex Diagnostics?

Many of the initial symptoms of infection caused by bacterial and viral agents are non-specific such as fever, headache, myalgia, and gastrointestinal or respiratory distress. Currently, most clinical diagnostic tests identify only one particular organism. Thus, clinicians must order a series of tests to identify disease-causing pathogens, resulting in higher costs and inconvenience to the patient. In addition, these single diagnostic methods are often slow and cumbersome, using multistep culture assays.

Multiplex diagnostic platforms-products capable of detecting more than one pathogen in a single test-can transform the process of identifying particular pathogens, allowing for treatment to begin promptly. Multiplex diagnostics have the potential to rapidly distinguish whether the infecting pathogen represents a biological threat agent or a common microbe that causes similar symptoms. Many of these diagnostics platforms can also identify multiple biomarkers for each pathogen, determine drug sensitivities such as antibiotic resistance, and distinguish subtypes and strains.

Biodefense Needs

These capabilities are especially important in the context of biodefense, when the cause of an outbreak must be identified rapidly in order to contain disease and implement public health measures. Until recently, diagnostic tests for NIAID Category A-C agents were limited to singleplex assays, detecting the presence or absence of a specific agent. The 2002 NIAID Strategic Plan for Biodefense Research called for new and improved clinical diagnostics for identifying agents of bioterrorism. Since then, advances in genomics and non genomics technologies have generated innovative multiplex diagnostic platforms that have the potential to be highly sensitive, specific, inexpensive, and easy to use.

Detecting Potential Biothreats

Multiplex diagnostic platforms should be automated systems that can perform multiple tasks including processing clinical samples to isolate the target analyte, performing the assay, and displaying the resulting interpretation. NIAID-supported scientists at the University of California Lawrence Livermore National Laboratory, in collaboration with the University of California Davis Medical Center, are developing just such a

system. Their multiplex diagnostic platforms for simultaneous detection of bioagents can distinguish pathogens that cause common respiratory infections from NIAID Category A-C priority pathogens.

For example, platforms developed at the Laboratory can detect either nucleic acids or proteins and can distinguish more than 20 different biothreat agents. The system uses both polymerase chain reaction (PCR) technology as well as multiplex immunoassays-a method of analysis based on binding of antibodies to antigens. It is currently being evaluated with point of care clinical partners including university medical centers, public health laboratories and the Centers for Disease Control and Prevention (CDC).

Identifying Bacterial Agents

NIAID researchers from the Regional Centers of Excellence for Biodefense and Emerging Infectious Diseases have developed multiplex diagnostic approaches for bacterial and viral agents. Investigators at The Johns Hopkins University have devised a system to initially determine if an infection is caused by bacteria, rather than other microbes such as viruses,

fungi, or parasites, and then to identify the precise bacterial organism responsible. The PCR-based system uses specific oligonucleotide primers, in this case coupled to fluorescent probes, to target the DNA of pathogens. The primer initiates multiple copies of a specific sequence of the pathogen's DNA to amplify the fluorescent signal and allow detection. All bacteria, but not higher organisms, contain a particular ribosomal RNA gene (16S) that has been conserved throughout evolution. Because this gene is found only in bacteria, an initial screening for its presence or absence, determines whether a bacterial agent is involved. Then a mixture of species-specific primers can be used to identify the exact bacterial organism causing the infection. This approach has been shown to be effective for differentiating between bacterial species that include *Bacillus anthracis* (anthrax); *Yersinia pestis*, (plague); *Francisella tularensis* (tularemia); and *Brucella ovis* (brucellosis).

Differentiating Viruses

The treatment of viral hemorrhagic fevers is limited to primarily supportive care, but early diagnosis is essential for appropriate response to a bioterrorism event or outbreak. A viral multiplex diagnostic system, developed by a group of investigators centered at Columbia University, uses PCR technology to differentiate between the possible causative agents

of viral hemorrhagic fevers. The approach employs a mixture of specific viral oligonucleotide primers to simultaneously discriminate between up to 32 DNA or RNA targets from clinical specimens (blood, oral swabs, etc.). Each probe is labeled with a distinct “mass tag” that consists of a molecule of defined molecular weight attached to the primer. Following amplification of the signal by PCR, mass spectrometry is used to distinguish among the mass tags and accurately identify the viral agent. The viruses that can be differentiated include Ebola virus, Marburg virus, Lassa virus, South American hemorrhagic fever viruses, Crimean-Congo hemorrhagic fever virus, Hantaviruses, yellow fever virus, dengue viruses, and others. A small, low cost, mobile unit is being designed to enable widespread use in clinical diagnosis.

Conclusion

Rapid, reliable identification of infectious agents is an important component of an effective response to a bioterrorism attack or an emerging infectious disease outbreak. New multiplex diagnostic methods will be an important addition to the tools available to physicians and public health officials in identifying and treating these potentially deadly diseases.

DENGUE VACCINE DEVELOPMENT: MEETING THE CHALLENGE

Dengue is the most prevalent mosquito-borne viral illness in the world, with more than 2.5 billion people at risk of infection in tropical regions, particularly in Southeastern Asia and Central and South America. Dengue is transmitted to humans primarily by the urban-dwelling *Aedes aegypti* mosquito, which also transmits the yellow fever virus. World War II era programs to eliminate yellow fever had the added benefit of controlling dengue in targeted areas for nearly a generation. In the past three decades, cessation of these programs and rapid urbanization in tropical areas have allowed dengue's major mosquito vector to expand geographically and dengue to re-emerge.

Today, dengue is present in 100 countries and is a serious public health problem in the Americas, where its incidence and severity are increasing dramatically. Dengue, along with other viral hemorrhagic fevers also represents a potential bioterror threat.

The dengue virus has four serotypes (virus subtypes distinguishable by serum antibody tests), each capable of infecting the same person. Dengue infections can be asymptomatic or cause a spectrum of illness ranging from a debilitating fever (breakbone fever) to a potentially fatal hemorrhagic fever and shock syndrome (DHF/DSS). Severe disease is more common among children. There is no licensed vaccine or antiviral treatment for dengue, and DHF mortality can be as high as 15 percent, averaging 5 percent where supportive care for DSS is available. Worldwide, the World Health Organization estimates 50 million to 100 million cases of dengue fever, 500,000 cases of severe dengue disease, and more than 20,000 deaths each year. The lethality of DHF/DSS and dengue's pandemic re-emergence account for its status as an National Institute of Allergy and Infectious Diseases (NIAID) high-priority pathogen.

Most experts believe a vaccine is the only practical solution to the increasing threat from dengue; however, dengue vaccine development is complicated by several factors. Chief among them is that while infection with one dengue serotype results in lifelong immunity against that serotype, at best it provides only temporary cross- protection against the others. Worse yet, subsequent infection with a different dengue serotype can be much more severe, a phenomenon called antibody-dependent disease enhancement (ADDE). Therefore, a dengue vaccine

must be tetravalent, that is, simultaneously protective against all four dengue serotypes. A vaccine protective against only one serotype could trigger ADDE in a vaccinee infected with a different dengue serotype. A second major impediment to vaccine development is the lack of an animal model of dengue that mimics human disease. The effectiveness of a vaccine candidate is inferred by the levels of antibodies it induces and by determining the level of dengue wild-type virus detected in the blood of vaccinated animals (vs. unvaccinated controls) following challenge with wild-type dengue virus.

Researchers in the United States and abroad have worked to develop a tetravalent dengue vaccine for years. Most have used the live, attenuated (weakened) vaccine approach. This approach is used in the successful yellow fever vaccine and is also the most economical method of vaccine development, an important consideration for encouraging the manufacture and distribution of the vaccine in developing countries where dengue is epidemic. However, this approach is especially slow and difficult for dengue because of the need to develop four different vaccine viruses that must be assessed separately and in combination both in vitro and in animals before advancing to clinical trials. Classic in vitro tests such as plaque reduction assays do not reliably predict the behavior of dengue viruses in animals, which, in turn, are imperfect models of dengue in humans. And often a single serotype vaccine shows promise in clinical trials but fails when included in a tetravalent vaccine as it competes with the other three serotypes to stimulate immunity.

As with other live, attenuated vaccines, success lies in making the viruses sufficiently weak to be safe to administer yet still able to induce a protective immune response. Two other qualities are important in a dengue vaccine as well: the vaccine viruses should not be transmissible to mosquitos that bite a vaccinee, and the viruses should be cultivable to high titer in a cell line that permits cost-effective manufacturing.

Dengue researchers have used several methods to weaken the vaccine viruses, including serial infection in cell cultures or animals and reverse genetics to introduce attenuating nucleotide deletions and point mutations. Chimerization, a method developed by NIAID researchers in the early 1990s, also has been found to attenuate flaviviruses such as dengue. Chimerization involves replacing the genes of an attenuated virus (the backbone or recipient virus) with those of another (the donor virus) to develop a third virus-a chimera-that is

attenuated but induces immunity to the donor virus. This method has been used to develop vaccines against other flaviviruses, such as West Nile virus, Japanese encephalitis virus, and tick-borne encephalitis virus.

NIAID researchers have used both recombinant DNA techniques and chimerization to develop a tetravalent dengue vaccine slated for clinical trial in the near future. In addition, they continue to create dengue viruses with novel attenuating mutations for use in the event that ongoing clinical trials suggest additional changes in the tetravalent formulation are needed. Each of the four components of NIAID's dengue tetravalent vaccine has a large, attenuating, and genetically stable 30-nucleotide deletion in its genome. Because chimerization is also attenuating, the two chimeric viruses in this tetravalent vaccine are even more stable and less likely to revert back to non-attenuated forms, as well as less transmissible to mosquitoes.

NIAID's chimerization methodology has been licensed to Acambis, whose tetravalent vaccine appears promising in early clinical trials. NIAID is working with scientists in India and Brazil to further dengue vaccine efforts in those countries as well. Using different attenuation techniques, investigators from Mahidol University in Thailand, Walter Reed scientists, and others continue their long-term work toward development of a successful dengue vaccine. The obstacles are daunting, but there are good reasons for optimism.

BOTULISM: DISCOVERING AND DEVELOPING NEW TREATMENTS

Botulism is a life-threatening disease caused by exposure to the most potent toxins known to mankind: the botulinum neurotoxins. These toxins may be ingested in accidentally contaminated food and cause a descending paralysis that may eventually lead to an inability of the patient to breathe unassisted. Patients may require mechanical ventilation in a respiratory intensive care unit for weeks to months. Due to improved food processing techniques, however, less than two dozen cases of naturally occurring food-borne botulism are reported each year in the United States. In contrast, the deliberate exposure of civilian populations to botulinum neurotoxins through an act of bioterrorism has the potential to cause significant illness and death in large numbers of people.

Unfortunately, the only treatment available for botulism is an anti-toxin derived from the blood of horses immunized with the toxins. The anti-toxin is difficult to produce and carries a significant risk of both mild and serious side effects. Production of anti-toxin in horses requires years. The horses need to be immunized many times in order to develop high enough concentrations of the specific antibodies that will effectively neutralize the botulinum neurotoxin. The final therapeutic product contains a mixture of the botulinum-specific antibodies but also a large amount of nonspecific horse antibodies, which have no therapeutic effect. The human body sees these horse proteins as foreign and may have a severe response to them.

The biodefense program of the National Institute of Allergy and Infectious Diseases (NIAID) is supporting development of new treatments that can be more quickly and reproducibly manufactured and have the potential to be much safer than the current horse-derived anti-toxin. The Department of Defense (DoD) has long been concerned with the threat that botulinum neurotoxins used as weapons pose to the war fighter. Indeed, the DoD supported the earliest programs to discover alternative treatments for botulism. In 2002, NIAID provided a significant increase in resources to the field of botulism research, and alternative treatments in particular, to accelerate the discovery and subsequent development of safer and more effective treatments.

The most advanced program is the discovery and development of human compatible monoclonal antibodies that bind and remove the neurotoxins from the patient's blood, preventing the neurotoxin from entering nerve cells where it causes paralysis. These antibodies are genetically engineered to bind very tightly to the neurotoxin. Because the antibodies are also engineered to be "human-like," it is unlikely that they will be seen as foreign and should be much safer than the horse-derived antibodies. In addition, only botulinum-specific antibodies are included in the treatment product so that no unnecessary protein is given to the patient.

An investigator at the University of California San Francisco engineered three highly potent monoclonal antibodies that, when combined, completely protect animals challenged with very high doses of botulinum neurotoxin. Through a competitive process these three monoclonal antibodies were selected for further development. In 2005, NIAID awarded a contract for manufacture of the antibodies in sufficient amounts for further evaluation in animals and eventually in humans. All three of the monoclonal antibodies were successfully manufactured and the contract has been extended to include formulation of the three-antibody mixture.

Currently there are no licensed drugs that include a mixture of three monoclonal antibodies. The development of this product, therefore, presents novel challenges for which there is no prior history. In addition, because the final efficacy of this treatment cannot be evaluated in humans (there are not enough cases of naturally caused botulism each year), specific animal models that mimic the disease and treatment in humans need to be developed and utilized. NIAID is also supporting research to develop animal models that can be used for the final evaluation of the monoclonal antibodies' effectiveness as a treatment.

DECODING ANTHRAX

Anthrax is one of the most genetically homogenous species known. Little genetic variation exists among species and strains, and traditional molecular methods have been limited in their ability to distinguish among them. With *Bacillus anthracis* a potential biological weapon, the ability to identify, differentiate, and forensically track strains is crucial and new research tools are critical. Genomic sequencing and comparative genomic analysis are among the most promising techniques being used. Rapid genome analysis offers not only the ability to quickly differentiate anthrax strains and determine strain relationships, but also has promise as a new type of public health diagnostic.

Substantial effort is being put forth to develop techniques to help differentiate anthrax strains. For example, scientists at Northern Arizona University and the National Institute of Allergy and Infectious Diseases Pathogen Functional Genomics Resource Center (PFGRC) at The Institute for Genomic Research (TIGR) used a combination of whole genome sequencing, comparative genomic analysis, and comparison of single nucleotide polymorphisms (SNPs) to define detailed phylogenetic lineages of *B. anthracis*. Five diverse strains of *B. anthracis* were chosen for analysis, based on relationships obtained from previous research using multiple variable-number tandem repeat (VNTR) analysis. With newly developed bioinformatic software tools, the team compared the sequence data from each genome to determine a set of 990 SNP markers, or single nucleotide variations in the genome. The study determined three major lineages (A, B, C) of *B. anthracis*, with the ancestral root located between A+B and C branches, and provided a model that could be used for examining other agents of bioterrorism. Even more importantly, however, the new DNA biosignatures have the potential to be used in the development of more sensitive diagnostic assays for *B. anthracis*.

The complexities of diagnosing *B. anthracis* were highlighted recently when a collaborative research team from TIGR and the Centers for Disease Control and Prevention (CDC) discovered that the genes for the anthrax toxins of *B. anthracis* are also found in *Bacillus cereus*, an opportunistic pathogen known to cause food poisoning. The strain of *B. cereus* examined was isolated from a patient who had a life-threatening pneumonia similar to inhalation anthrax and was phylogenetically characterized by the CDC as *B. cereus*. PFGRC at TIGR sequenced the complete genome of this *B. cereus* (G9241) strain and performed comparative genomic analysis with previously sequenced strains of both *B. anthracis* and *B. cereus*. The rapidly produced genome sequence and analysis found that the virulent *B. cereus* strain included a small circular piece of DNA that was identical to a *B. anthracis* plasmid, pX01, which contains the anthrax toxin genes. These studies provided evidence that the two species shared genes, including those that create the deadly anthrax toxin, and showed that a species related to *B. anthracis* could cause an inhalation anthrax-like disease, thus complicating anthrax diagnosis.

Although strain differentiation and diagnosis of anthrax has proven to be a public health challenge, genomic sequencing combined with comparative genomic analysis has provided a new approach to tackling the organism's complexity. Rapidly identifying strains and determining strain relationships may be crucial forensic tools in the event of a bioterrorism attack. In addition, rapid genomic sequencing and analysis have demonstrated novel methods in public health diagnostics, providing insight into the correlation between an observed clinical phenotype and its genetic basis.

INNOVATIVE THERAPEUTICS AGAINST SMALLPOX

Smallpox has terrorized mankind for at least three thousand years because it is highly infectious, has a mortality rate of about 30 percent, and usually leaves survivors covered with disfiguring scars. Consequently, despite the unprecedented achievement of global eradication of this disease, the fear remains that smallpox may return: either accidentally from one of the two authorized storage facilities where the virus still resides, or, more horribly, intentionally as the result of an act of bioterrorism. While most of the historical devastation caused by smallpox, such as the decimation of the Aztecs, was the result of accidental introductions, smallpox has been used as a weapon, most notably by the British against the Indians during the French and Indian wars.

Vaccination has been essential in the eradication of smallpox. However, the containment of future potential outbreaks may rely on effective therapeutics. Ring vaccination, the final successful strategy in smallpox eradication, involved identifying and vaccinating all family members and contacts of infected people to break the cycle of transmission. In the event of a terrorist-caused outbreak of smallpox, there may be too many people infected initially for this to be effective, especially if they are geographically scattered as would be likely with an airport aerosol exposure. Furthermore, the number of people who are vulnerable to the vaccine's serious side effects has increased tremendously in the last 50 years, eliminating a significant portion of the population from immunization eligibility. Finally, a vaccine will not help those infected in the initial event. Therefore, safe and effective smallpox therapeutics will be crucial in preparing for possible bioterror outbreaks.

A survey of the small number of known antiviral drugs approved by the U.S. Food and Drug Administration (FDA) quickly identified cidofovir as having great potential in use against smallpox as well as against an array of viruses that are

closely related to the smallpox virus. However, cidofovir was not an ideal solution. The drug had to be given intravenously, making it inconvenient for an emergency situation. In addition, it had the potential to cause serious kidney damage unless the patient's hydration level was carefully monitored, another factor often not feasible during times of emergency.

Recognizing the need for improved smallpox therapeutics, National Institute of Allergy and Infectious Diseases (NIAID)-supported scientists began ground-breaking research on two innovative new drugs. At the Veterans Hospital at the University of California San Diego (UCSD), researchers took cidofovir and engineered it to overcome its shortcomings. By adding a lipid (fatty component) to the drug structure, researchers converted the drug from one that had to be given intravenously to one that could be given by mouth. Animal model testing found that the new drug, HDP-cidofovir or CMX-001, was just as active as its parent against poxviruses and does not accumulate in the kidney like the older version, making it unlikely to cause problems with kidney toxicity.

Meanwhile, at Siga Technologies, Inc., NIAID-supported researchers developed a second potential poxvirus therapeutic, ST-246. Like CMX-001, ST-246 was effective in all the animal models in which it was tested as a treatment for poxvirus infections. Furthermore its preclinical toxicity profile was comparatively clean so it may be safe to give to the majority of the population. Even better, its viral target is different from that of CMX-001; thus, each drug should retain activity against a virus that has developed resistance to the other.

While both CMX-001 and ST-246 appear to work well alone, an added bonus is that they could potentially offer even more potent therapy when used together. Currently, both drugs are in Phase I clinical studies and are excellent candidates for eventual licensure by the FDA to treat smallpox infection in humans.

DEVELOPING A FAST-ACTING EBOLA VACCINE

Ebola virus is a deadly microbe producing viral hemorrhagic fever (VHF), with lethal results in the vast majority of infected individuals. No vaccines or proven treatments exist for Ebola, a virus that periodically attacks African villages, and is known to have been “weaponized”, i.e., adapted for use as a bioterrorism agent. Although other viral diseases claim more lives each year, the potential threat posed by Ebola virus has warranted a high priority for development of a vaccine against this killer.

Scientists at the National Institute of Allergy and Infectious Diseases’ (NIAID) Vaccine Research Center (VRC) have recently developed a single shot, fast-acting experimental Ebola vaccine that successfully protects monkeys from this deadly virus after only one month. If this vaccine proves similarly effective in humans, it may one day allow scientists to quickly contain Ebola outbreaks with ring vaccination—the same strategy successfully used in the eradication of smallpox. With ring vaccination, everyone who has been in contact with a patient, as well as all members of the patient’s household, are vaccinated. The ring strategy, which requires a fast-acting vaccine, not only protects people who may have been exposed to the virus but also creates an added barrier of immunity around them, thereby protecting the entire community.

Ebola Virus

Ebola virus, and the closely related Marburg virus, are the only known members of the filovirus family, one of four distinct virus families that cause viral hemorrhagic fevers. The onset of Ebola hemorrhagic fever is abrupt and is characterized by the sudden onset of fever, weakness, muscle pain, headache, and sore throat, followed by vomiting, diarrhea, rash, both internal and external bleeding, and often death. Three of the four known strains of the Ebola virus—Zaire, Sudan, and Ivory Coast—are highly lethal to humans. The fourth strain, Ebola-Reston, is known to infect, but not cause clinical disease in humans, though it is deadly to monkeys.

Ebola virus, along with other viruses that cause hemorrhagic fever, are classified by the Centers for Disease Control and Prevention (CDC) as Category A agents. Such Category A agents are considered to pose the greatest risk to public health due to their properties of pathogenesis (disease producing ability) and the likelihood of weaponization. Category A

agents can be easily disseminated or transmitted from person to person, result in high death rates, and have the potential for major public health impact.

Ebola outbreaks have occurred sporadically since its discovery in 1976 in the Democratic Republic of the Congo, with most subsequent outbreaks occurring in Central Africa. The exact origin, locations, and natural reservoir for Ebola virus remain a mystery. Available evidence and the nature of similar viruses have led scientists to speculate that it is maintained in an animal host native to the African continent. Since the natural reservoir is not known, environmental control and avoidance strategies are not currently possible.

While the human exposure route for Ebola virus is unknown, scientists suspect that the virus may be introduced to potential victims through contact with an infected animal. Once a person becomes infected, the virus spreads rapidly to other persons in close contact with the infected individual. Because no effective treatment for Ebola VHF exists, development of an effective vaccine against Ebola offers the best hope for preventing this dreaded disease.

Developing Ebola Vaccines

NIAID VRC scientists continue to pursue the development of safe and efficacious Ebola vaccines, in collaboration with other federal scientists. In 2000, VRC scientists, in collaboration with CDC researchers, developed a candidate vaccine that protected monkeys against Ebola virus. Using a vaccine strategy called “prime-boost,” they combined two different vaccines. The first vaccine, the prime, consisted of strands of DNA containing the gene that encodes Ebola glycoprotein, a protein found on the outside of the virus, from each of the three most lethal strains of Ebola virus—Zaire, Sudan, and Ivory Coast. The second vaccine, the boost, consisted of a weakened form of a common cold virus called adenovirus, which had been modified to produce Ebola virus glycoprotein. The researchers found that the immune response initiated in response to the DNA vaccine was increased (boosted) by the second vaccine. When this two-step prime-boost vaccine was tested in monkeys, all vaccinated animals mounted a strong and long lasting immune response, and survived exposure to a lethal dose of Ebola virus.

Though the prime-boost strategy generated potent and lasting immunity, the course of vaccination consisted of multiple injections administered over the course of six months. While

this vaccine may prove useful in the prevention of the disease in individuals at risk of exposure to the virus (e.g., healthcare workers in regions where Ebola is endemic), this slower acting prime-boost vaccine would not be an effective tool for containing an outbreak. For such an outbreak, a fast-acting vaccine would be needed.

Building on their previous results, scientists at the NIAID's VRC, in collaboration with researchers at the U.S. Army Medical Research Institute for Infectious Disease (USAMRIID), have developed an accelerated Ebola vaccine. While developing the prime-boost vaccine, the scientists noticed that monkeys administered a single injection of the modified adenovirus (the boost) produced an immune response against Ebola. Though the magnitude of the response was not as great as that of the prime-boost vaccine, immunity developed rapidly. Working with their colleagues at USAMRIID, VRC scientists tested whether the immune response mounted against the boost component alone would be sufficient to protect monkeys against Ebola infection. The VRC scientists immunized eight monkeys with a single boost injection. The monkeys were then transferred to USAMRIID where they were injected with Ebola virus 28 days after vaccination. All eight animals vaccinated with the boost survived, even those who received high doses of the virus.

If the results of this animal study hold true for humans, the new fast-acting vaccine may be just the tool public health officials need to contain epidemic outbreaks of Ebola, arising from either natural infection or as a result of a bioterror event. Even though the fast-acting boost alone appears to be effective, VRC scientists are continuing to refine their prime-boost vaccine strategy because it is likely to elicit a stronger immune response in humans, and may lead to the development of a vaccine that will protect hospital workers at high risk of exposure to the virus.