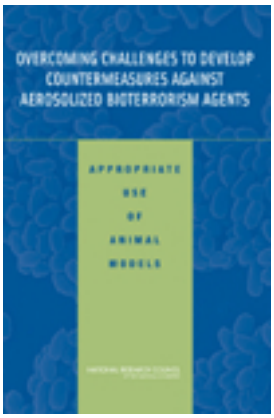

Overcoming Challenges to Develop Countermeasures Against Aerosolized Bioterrorism Agents: Appropriate Use of Animal Models

Committee on Animal Models for Testing Interventions Against Aerosolized Bioterrorism Agents, National Research Council

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OVERCOMING CHALLENGES TO DEVELOP COUNTERMEASURES AGAINST AEROSOLIZED BIOTERRORISM AGENTS

APPROPRIATE USE OF ANIMAL MODELS

Committee on Animal Models for Testing Interventions
Against Aerosolized Bioterrorism Agents

Board on Life Sciences

Institute for Laboratory Animal Research

Division on Earth and Life Studies

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Preface

The terrible events of September 11, 2001, and the dissemination of *Bacillus anthracis* by mail in October 2001, markedly increased awareness of the possibility of bioterrorism attacks and of the need for new vaccines and therapeutics to protect U.S. citizens from them.

Following this, Congress markedly increased the funding for research for new vaccines and therapeutics to protect the United States from a bioterrorist attack. Such research had largely been conducted by the U.S. Army Medical Research Institute of Infectious Diseases at Ft. Detrick, Maryland. Much of this research is now being directed by the National Institute of Allergy and Infectious Disease of the National Institutes of Health.

An integral part of the development of new vaccines and therapeutics is obtaining the necessary approvals from the U.S. Food and Drug Administration both for their initial use in people and their eventual licensure for general use. The present accelerated pace of development, however, has led to several additional needs: standardization of methods for the generation and characterization of aerosols of bioterrorism agents for use in animal studies (necessary for licensure of vaccines and therapeutics), characterization of the threat to the population, and expansion of the number of laboratories conducting the research. The Committee on Animal Models for Testing Interventions Against Aerosolized Bioterrorism Agents was convened by the National Research Council to address these issues. It was tasked by its sponsor, the National Institute of Allergy and Infectious Disease, to prepare a short consensus report that articulates the difficulties of testing countermeasures to aerosolized bioterrorism agents and considers whether there are opportunities for improving current approaches to animal testing of countermeasures against aerosols by applying knowledge from other fields of science.

Thus, the Committee organized a workshop, titled "Animal Models for Testing Interventions Against Aerosolized Bioterrorism Agents," which was held July 6th – 7th, 2005, in Washington, D.C. The Committee selected as participants scientists, from diverse disciplines, who made presentations that ultimately were integral to the development of this report.

As chairman, I thank the committee members for contributing their expertise and time to the committee, the workshop, and the report. And the entire committee thanks NRC staff members Kerry Brenner and Jennifer Obernier for their organizational skills and hard work in arranging the workshop and preparing the report. Thanks too to Seth Strongin for providing logistical support. The report would not have been possible without their assistance.

The report has been reviewed in draft form by individuals chosen for their diverse perspective and technical expertise, in accordance with procedures approved by the NRC's Report Review Committee. The purpose of this independent review is to provide candid and critical comments that will assist the institution in making its published report as sound as possible and to ensure that the report meets institutional standards for objectivity, evidence, and responsiveness to the study charge. The reviewers' comments and draft manuscript remain confidential to protect the integrity of the deliberative process. We wish to thank the following people for their review of the report:

Lynn Andersen, Johns Hopkins University, Baltimore, Maryland
Chris Gennings, Virginia Commonwealth University, Richmond, Virginia
Michael T. Kleinman, University of California, Irvine, California
Roger O. McClellan, Toxicology and Human Health Risk Analysis, Albuquerque, New Mexico
Matthew S. Meselson, Harvard University, Boston, Massachusetts
Stanley Perlman, University of Iowa, Iowa City, Iowa
David Y. H. Pui, University of Minnesota, Minneapolis, Minnesota
Chad Roy, U.S. Army Medical Research Institute for Infectious Diseases, Fort Detrick, Maryland

Although the reviewers listed above provided many constructive comments and suggestions, they were not asked to endorse the conclusions or recommendations, nor did they see the final draft of the report before its release. The review of the report was overseen by:

Peter Ward, University of Michigan, Ann Arbor, Michigan
Peter Palese, Mount Sinai School of Medicine, New York, New York

Appointed by the NRC, these individuals were responsible for ensuring that an independent examination of the report was carried out in accordance with institutional procedures and that all review comments were carefully considered.

Responsibility for the final content of the report, however, rests entirely with the authoring committee and the institution.

Charles H. Hobbs, *Chair*
Committee on Animal Models for Testing
Interventions Against Aerosolized
Bioterrorism Agents

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Glossary

aerodynamic diameter	An equivalent diameter for a particle defined as the physical diameter of a smooth solid sphere (of density 1 gram/cm^3) that has the same terminal settling velocity in still air (under standard laboratory conditions) as the particle in question.
aerosol	A relatively time-stable two-phase system consisting of finely-divided particles (that can be solids or liquids) suspended in a gas (which is usually air). Aerosol particles typically range in diameter from 0.001 to 100 μm.
bioterrorism agent	A microorganism or a toxin derived from a microorganism that causes human disease and is used to harm people, or to elicit widespread fear or intimidation, for political or ideological goals.
countermeasure	A drug, biological product, chemical, or other therapeutic technology that prevents or treats an illness caused by a bioterrorism agent.
dose	The amount (for bioaerosol particles this could be number, mass, viable units, or another metric related to biological effect) of an agent normalized to some property of the biological target (which could be mass, surface area or other

	descriptor of an individual, organ, or tissue). For example: mg of particles deposited in the subject; μg of particles deposited in the respiratory tract; μg of particles in the tracheobronchial region; or number of viable organisms in the alveolar spaces of the lung.
geometric standard deviation (GSD)	A measure of dispersion for a log-normal distribution that is analogous to the standard deviation for a normal distribution. The GSD is the ratio of the 84.13 percentile to the 50 percentile.
mass median aerodynamic diameter (MMAD)	For aerosols, the MMAD equals the particle diameter at which particles larger than the MMAD contribute half of the collected mass and particles smaller than the MMAD contribute the other half.

Summary

Incidents involving the dissemination of *Bacillus anthracis* and ricin through the U.S. postal service beginning in 2001 have led the federal government to focus attention on the importance of developing countermeasures[1] to agents of bioterrorism. The President's 2006 federal budget included $4.2 billion for the Department of Health and Human Services to address bioterrorism. $1.7 billion of that request was slated for the National Institute of Allergy and Infectious Diseases (NIAID) to accelerate the development of new and improved countermeasures against potential agents of bioterrorism (DHHS 2005).

The NIAID's Strategic Plan for Biodefense Research (2002) recognizes that bioterrorism agents[2] dispersed in aerosol form have the greatest potential to cause widespread disease. Therefore, NIAID's Strategic Plan gives highest priority to developing countermeasures to those bioterrorism agents that have a high infectivity in aerosol form (NIAID 2002). Since, during the course of studying bioterrorism agents, it is not ethically appropriate to deliberately expose human subjects to bioterrorism agents, development of countermeasures relies on the ability of the scientific community to adequately test the effectiveness of countermeasures in animal models.

There are many challenges associated with producing appropriate animal models for testing countermeasures. In many cases, there is little natural history

[1] "Countermeasure" is defined for the purposes of this report as a drug, biological product, chemical, or other therapeutic technology that prevents or treats an illness caused by a bioterrorism agent.

[2] "Bioterrorism agent" is defined for the purposes of this report as a microorganism, or a toxin derived from a microorganism, that causes human disease and is used to harm people, or to elicit widespread fear or intimidation, for political or ideological goals.

of aerosolized exposure in animals to guide development and characterization of new animal models. There are many methodological challenges in generating reproducible exposures, e.g. generation of viable aerosols, consistent exposure of animals, appropriate quantification of dose, and comparison of results among laboratories. Also, the use of animal models to demonstrate efficacy during the drug approval process is a relatively new approach for the regulatory community and there are issues that still need to be addressed.

CHARGE TO THE AUTHORING COMMITTEE

The NIAID approached the National Research Council with a request that it prepare a short consensus report that articulates the difficulties of testing countermeasures to aerosolized bioterrorism agents in animals and identifies opportunities that can be pursued to improve current testing efforts. As part of this project, NIAID requested that a workshop be organized to bring together select agent researchers, including researchers from NIAID and U.S. Army Medical Research Institute of Infectious Diseases, as well as leaders in complementary areas of science, particularly inhalation toxicologists, microbiologists, aerosol scientists, and statisticians. This report is based on the presentations and discussions held at this workshop on July 6 and 7, 2005, as well as further research and deliberations by the authoring committee following this workshop.

The committee approached its task by considering how to improve and standardize studies that generate efficacy data in animals. The committee generally focused on technical issues regarding the generation of consistent and reproducible exposures, which are key to producing scientifically-sound efficacy data, but also touched on issues to be considered in selecting an animal model. Studies to establish human dose response to a bioterrorism agent or conduct a risk assessment for a bioterrorism event are beyond the scope of the committee's charge.

The committee organized its efforts and report by focusing on four parts of the experimental design process: the selection of the animal model to be utilized, the generation and characterization of the aerosolized bioterrorism agent, characterization of dose, and selection and delivery of dose. For each part of the experimental design, the committee identified issues that potentially affect the quality and reproducibility of data, and then identified experimental approaches and technology that might overcome those challenges. The committee also took a holistic approach to their overarching task, and identified workforce, infrastructure, and regulatory issues that could present challenges to testing countermeasures to bioterrorism agents.

The following recommendations have been developed to address the major challenges identified by the Committee on Animal Models for Testing Interventions Against Aerosolized Bioterrorism Agents.

PRINCIPAL FINDINGS AND RECOMMENDATIONS

Development or Selection of an Animal Model when Testing Countermeasures

The development or selection of an animal model that approximates the human disease process is dependent on a robust knowledge of the natural history and pathogenesis of the disease. No single animal model will exactly replicate responses seen in humans; therefore endpoint-based findings in animals are extrapolated to humans. Accurate extrapolations are possible only when species-specific differences in pathogenesis at the cell, tissue, and organ level; pulmonary anatomy and physiology; and host-pathogen interactions are clearly understood. **The Committee recommends that additional data on experimental-animal airway anatomy and particle deposition and clearance be acquired to aid in developing new animal models and performing extrapolations to human populations. In particular, data are needed for various strains of mice and many species of nonhuman primates.**

Generation and Characterization of Aerosolized Agents

A key component of the effort to test countermeasures is generating consistent aerosol exposures. These aerosol exposures can then be reproduced, and data generated by different experiments or laboratories can be compared. However, studies involving aerosol inhalation exposures are technically difficult because the potency of the agent and the dose delivered are greatly affected by the aerosol generation equipment and the characteristics of the generated aerosol. **So that future studies to test countermeasures can be compared and reproduced, the Committee recommends that specific parameters be measured and reported as part of a standard operating procedure adopted by researchers studying aerosolized bioterrorism agents. Researchers should measure particle properties including aerodynamic size, size distribution, geometric size and shape, electrical charge, chemical composition, irritancy, and mass concentration (mass of particles per unit mass of air). Properties of the exposure environment should also be measured including temperature, relative humidity, osmolarity, airflow, and uniformity of the exposure in the breathing zone. In addition, information on aerosol generation and generation equipment, particle size and sizing instrument, impinger characteristics, exposure parameters and equipment, and animal characteristics and status should be recorded and reported in publications resulting from the work.**

Dosimetry

Dose is commonly reported as a median lethal dose (LD_{50}) or a median infectious dose (ID_{50}). However, these measures can be greatly affected by the method of delivery, the aerosol particle-size distribution, the site of deposition in the respiratory tract, and the species under study, making replication and interpretation of the study difficult. **Therefore, the Committee recommends that when a multiple of the LD_{50} or ID_{50} (e.g., 10 LD_{50}) is used to report dose, then sufficient additional data, including indices of viability of the agent and characteristics of the exposure, particle-size, and generation of the aerosol should be acquired and reported.**

Selection of Dose and Delivery

Studies to extrapolate a lethal or infectious dose often use a limited number of animals. This creates statistical concerns about variability and uncertainty that need to be addressed. In addition, the Committee found a wide variation in published LD_{50} values in the available literature making the selection of dose difficult. **Therefore, the Committee recommends obtaining statistical advice when designing an animal study to develop or test a countermeasure.**

The wide variation in published LD_{50} values in the available literature also makes it difficult to compare countermeasure efficacies, potency of different agent sources and strains, and response of different animal species and strains. Additional data on challenge doses may help alleviate this issue until reporting of dosimetry is standardized and sufficient additional data are generated. **Therefore, the Committee recommends that unclassified data from mortality and natural-history studies—including unclassified, unpublished data from U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID)—be published in the open literature for all agents. If publication in the open literature is not feasible, an inclusive database of unclassified government-sponsored studies should be established by NIAID[3].**

The committee considered several inhalation delivery methods including whole-body exposure, head-only exposures, nose-only exposures, or mouth-only exposures. The committee found that the use of apparatus specific to each type of delivery method required special considerations to lower variability during dosing. **For whole-body exposures, the committee recommends that individual cages without food be utilized to prevent animals from avoiding exposure by huddling or from increasing exposure through consumption of food available in the chamber. For other types of exposures, the committee recommends that neck seals, tubes, and masks be evaluated to ensure that**

[3] This recommendation is not intended to apply to research results or other data considered "sensitive but unclassified." Rather, the recommendation applies to data for which access would otherwise not be restricted that have not been published in a timely manner due to events such as personnel changes and changes in research priorities.

the equipment does not alter respiration, either directly or by stressing the animal. For all exposure systems the environmental temperature, humidity, and distribution of the agent should be regulated and samples be taken from the breathing zone nearest to the animal during exposure.

Resource and Regulatory Issues

Extensive data on the characteristics of many animal models and different strains of infectious agents are not available in the published literature. Access to such data could prevent unnecessary duplication, allow researchers to compare results between different experiments and laboratories, assure consistency by standardizing techniques, and allow data to be pooled for more rapid determination of results. **Therefore, the Committee recommends that an easily searchable central database registry (or registries) on animal model data be established. Further, the Committee recommends the establishment of a repository, which can supply investigators with a well-characterized sample of an agent. The American Type Culture Collection (ATCC) maintains such a repository, but additional information to facilitate comparisons of animal-model systems and ensure consistent results should be added.**

Finally, there are issues in the U.S. Food and Drug Administration (FDA) regulatory process for approval of countermeasures against bioterrorism agents that need to be addressed. This is especially true with respect to the new regulations, known as the "Animal Rule" (21 CFR 314 Subpart I and 21 CFR 601 Subpart H), which permits the agency to base its marketing-approval decision on animal efficacy data when the countermeasure cannot be tested for efficacy in humans. This Rule requires that the animal model used to generate efficacy data approximates and is predictive of the disease process in humans. The committee found that few animal models of bioterrorism agents have been shown to be predictive of the human disease process. In addition to demonstrating efficacy, considerable effort will have to be expended to establish the predictive value of an animal model, and there is widespread concern in the research community that animal models acceptable to the U.S. Food and Drug Administration cannot be developed for all of the select agents. **In order to address the need for rapid development of countermeasures, while striking an appropriate balance between efficacy and safety, the Committee recommends that the FDA improve the process by which new countermeasures are approved by working with researchers to draft and finalize practical guidelines to ensure that applicants can effectively meet approval requirements.**

1

Introduction

STATEMENT OF TASK

There are many challenges associated with producing appropriate animal models for testing countermeasures, including methodological issues in generating aerosols, dose delivery, and characterization of the model. Further, the use of animals in the drug approval process is a relatively new approach for the regulatory community, presenting issues that also need to be considered.

To address these challenges, the National Institute for Allergy and Infectious Disease (NIAID) asked the National Research Council to convene a committee to consider these issues. The specific charge to the committee is as follows:

> The ability of the scientific community to develop new interventions to protect against biological terrorism agents hinges on the ability to test the effectiveness of interventions in animal models, because humans may not be deliberately exposed to the agents. For some pathogens, the disease produced when an agent is aerosolized is quite different than that produced via other exposures and thus it is necessary to test the effectiveness of interventions on animals that are exposed to the agents in aerosol form. Developing animal models of human exposure to inhaled infectious agents is a formidable endeavor for a number of reasons, including reproducibility issues that complicate interpretation of intervention studies. Under the direction of a committee, a 1-2 day workshop will be organized to bring together experts in using animal models to test interventions against aerosolized biological terrorism agents with specialists in other areas of biological

sciences, including aerosol toxicology, to encourage the application of the latest biological information, technology and experience to testing of aerosolized biological agents. The committee will use the workshop to inform their report, which will consider whether there are opportunities for improving current approaches to animal testing of interventions against aerosols by applying knowledge from other fields of science.

COMMITTEE PROCESS

On July 6th – 7th, 2005, the Committee on Animal Models for Testing Interventions Against Aerosolized Bioterrorism Agents convened a workshop at the National Academy of Sciences building in Washington D.C. (see Appendix A for agenda). Researchers experienced in using animal models to test countermeasures against aerosolized bioterrorism agents interacted at this workshop with specialists in other areas of the biological sciences. Some of the speakers helped to place the task in a larger context—analyzing, for example, the Animal Rule of the U.S. Food and Drug Administration (FDA)—while others provided more technical information such as methods for choosing appropriate animal models or characterizing the challenge aerosols. The workshop's presentations and discussions brought to the fore the latest pertinent scientific experience, information, and technology, which then informed the committee's report on whether opportunities exist for improving current approaches.

ANIMAL RULE

A serious set of limitations comes from the inherent difficulty of testing countermeasures against infections that are, by definition, naturally rare or nonexistent in the human population and that cannot ethically be tested for efficacy in humans because they might cause serious injury or even death. In response to these concerns, the FDA, which is responsible for licensure of medical products in the United States, developed the Animal Rule, which has been in effect since 2002. This rule states that the agency may grant marketing approval for a new drug product (21 CFR 314 subpart I) or biological product (21 CFR 610 subpart H) based on adequate and well-controlled animal efficacy studies when human efficacy studies cannot be conducted because it is unethical to expose human volunteers to the toxic substance—whether biological, chemical, or radiological—in question. Further details and implications of this important regulatory development will be discussed in Chapter 6.

Importance of Animals in Research on Aerosol-Mediated Disease

Compounding the research difficulties caused by prohibited studies with human volunteers is the fact that data acquired using computer simulations or

cell cultures and other in-vitro techniques cannot effectively address many of the efficacy and safety issues critical to the development of vaccines and other countermeasures.

Fortunately, several reliable laboratory animal models have been developed by members of the biomedical community who have studied a wide range of inhaled materials including infectious agents. Rodents are particularly useful in the initial phases of efficacy, toxicity, and lethality investigations, which are used to provide data for the design of more definitive studies. Larger mammals, including rabbits, pigs, sheep, ferrets, dogs, and nonhuman primates have proven invaluable as models in studies of chronic toxicity, infectivity, vaccine efficacy, and dermal and pulmonary physiology (Patterson and Carrion 2005). Nonhuman primates are phylogenetically closest to people (Sibal and Samson 2001) and thus are considered of great importance to these studies.

BIOLOGICAL WEAPONS CONVENTION

Work with bioterrorism agents imposes some unique difficulties. In addition to the numerous scientific concerns, such as developing appropriate models and ensuring suitable biosecurity and biosafety for work with dangerous pathogens, there are also legal and regulatory issues. Some constraints are imposed by legislation designed to limit access to such pathogens. At the national level in the United States, these laws include the Select Agent Rule (42 CFR Part 73) and the U.S. Patriot Act (2001), together designed to control the availability of dangerous pathogens and to regulate access to these agents. International controls are important as well. First signed in 1972, the Biological Weapons Convention (BWC), formally known as the 1972 Convention on the Prohibition of the Development, Production, and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on Their Destruction, was designed to prevent use of biological agents for offensive purposes. As of December 2004, 153 countries (including the United States) had signed and officially ratified the BWC, and 16 additional countries had signed but not yet officially ratified it (BWC 2004). The treaty is the key international agreement in this area, and is perceptually important even though it lacks specific enforcement measures (Taylor 1999).

Article I of the treaty states:

> Each State party to this Convention undertakes never in any circumstances to develop, produce, stockpile, or otherwise acquire or retain:
> (1) Microbial or other biological agents, or toxins whatever their origin or method of production, of types and in quantities that have no justification for prophylactic, protective, or other peaceful purposes;
> (2) Weapons, equipment, or means of delivery designed to use such agents or toxins for hostile purposes or in armed conflict.

In the global arena, it is essential that scientific research with dangerous pathogens comply with the BWC itself and, probably more difficult, avoid creating the perception throughout the international community that the countries involved in the research are attempting to evade the treaty. Careful attention to public information and openness is thus required.

KEY ISSUES EXAMINED

The key issues examined by the Committee and discussed in this report are:

1. Selection of appropriate animal models.
2. Generation and characterization of aerosols of bioterrorism agents.
3. Determination of the dose of select agents following inhalation.
4. Determination of the experimental designs for developing vaccines and therapeutics for inhaled select agents.
5. Resource issues related to inhalation studies of select agents.

The committee felt it was important to clearly define the animal studies that were considered as part of this report. The statement of task asked "whether there are opportunities for improving current approaches to *animal testing of interventions* against aerosols [emphasis added]." The committee focused on improving and standardizing animal studies that generate efficacy data. Due to scientific and regulatory demands, these studies require consistent and reproducible exposures that result in a disease state that approximates the human disease. Animal studies to establish human dose response or to simulate "real world" scenarios were outside the scope of the committee's task.

Seventy-two select agents, including biotoxins but excluding plant pathogens, are currently being regulated by the U.S. Centers for Disease Control and Prevention (CDC) or the U.S. Department of Agriculture (42 CFR 73, 7CFR 331, 9 CFR 121). In addition, the CDC has categorized these pathogens and biotoxins into three categories (A, B, and C), based on their potential for use as bioterrorism agents (Rotz and others 2002). Category A agents, being amenable to large-scale dissemination in a bioterrorism attack, have the greatest potential for mass casualties. As such, development of countermeasures to Category A agents have been given the highest priority; however, it is beyond the scope of the Committee's charge to discuss pathogens or biotoxins individually, except where data from specific studies are used as illustrations. Similarly, it is beyond the scope of the charge to make a specific recommendation for the appropriate challenge dose for any bioterrorism agent or to identify specific vaccines, therapeutics, or other countermeasures that could be developed.

2

Selection or Development of an Animal Model

Because bioterrorism includes malicious exposure of populations to a naturally occurring or human-modified infectious agent, such agents are by definition pathogens or toxins. Moreover, the "bio" prefix is appropriate as they are generally bacterial, viral, or fungal pathogens, or toxins of biological origin. The Animal Models for Testing Interventions against Aerosolized Bioterrorism Agents Workshop (held July 6th – 7th, 2005) focused on inhalation of a subset of these agents, the so-called select agents. Experiments designed to identify effective therapeutic countermeasures against an inhaled select agent requires knowledge of the bioaerosol and the experimental animal subjects.

Development or selection of an animal model that approximates the human disease process is dependent on a robust knowledge of the natural history and pathogenesis of the disease (Figure 2-1). However, a single well-characterized animal model may not suffice when testing countermeasures. For example, an animal model that proves predictive of the disease in healthy human adults may not approximate the disease course in immune-deficient adults, children, the elderly, infants, pregnant woman and the progeny they carry, or other susceptible subpopulations. Indeed, no single animal model will exactly replicate responses seen in humans, requiring extrapolation of endpoint-based findings in animals to humans. Accurate extrapolations are possible only when species-specific differences in pathogenesis at the cell, tissue, and organ level; pulmonary anatomy and physiology; and host-pathogen interactions are understood.

There are practical issues that need to be considered when selecting an animal model. Initial countermeasure strategies will likely focus on novel applications of existing, approved pharmaceutical agents. The pharmacodynamics and pharmacokinetics of these agents will have been

previously determined in preclinical studies using a relatively small number of animal models, most typically rodents, rabbits, dogs, and nonhuman primates. It is therefore anticipated that these commonly used species will be among the first to be used for the development of efficacious medical countermeasures for bioterrorism agents. It is also anticipated that nonhuman primates in particular may be extensively used in these studies, given that nonhuman primates are phylogenetically closest to humans (Sibal and Samson 2001). Nonhuman primates are not a homogeneous group, however. The primate order is composed of (1) prosimians (e.g., lemurs, loris, tarsiers) and (2) anthropoidia consisting of New World primates (e.g., marmosets, spider monkeys, cebus) and Old World primates further divided into monkeys (e.g., macaques, baboons) and greater and lesser apes (gorillas, chimpanzees, orangutans, and gibbons, respectively). Overall, there are more than 200 species of nonhuman primates, with approximately 30 species used in research (NRC 1998).

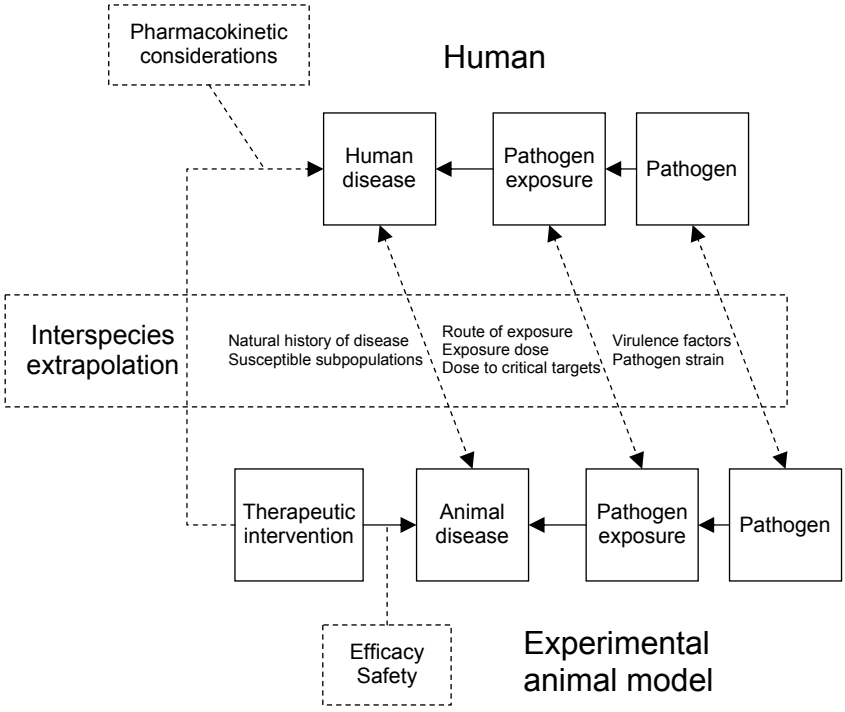


FIGURE 2-1 Factors that need to be considered when selecting an appropriate animal model.

PATHOGENESIS

The pathogenesis of certain human pathogens, including the hepatitis viruses, papillomavirus, polio virus, HIV-1, and measles virus, that can be studied in nonhuman primates cannot be easily studied in mice because mice are not normally susceptible to infection by these viruses. However, the identification and cloning of the human cellular receptors for some of the viruses have made it possible to create transgenic animals that express the corresponding receptors, thus potentially increasing their susceptibility to viral infection. For example, expression of CD46, the major cellular receptor for laboratory strains of measles virus in transgenic rodents, has been used to study immunosuppression caused by this virus (Manchester and Rall 2001).

Certain studies do not require the use of genetically altered rodents. For example, infant botulism—a neurological syndrome caused by the absorption of toxin produced by toxigenic organisms that colonize the intestinal tracts of infants under one year of age—can be replicated and studied in neonatal mice and rats (Moberg and Sugiyama 1980). In any case, reasonable application of well-characterized rodent models may accelerate development of efficacious and safe countermeasures for bioterrorism agents.

HOST-PATHOGEN INTERACTIONS

The selection of the appropriate animal model needs to also consider host-pathogen interactions. Host-pathogen interactions can result in a number of outcomes, including elimination through physical defenses or immune mechanisms, host-mediated or pathogen-mediated damage and disease, development of persistent infections and incapacitation, or death (Casadevall and Pirofski 1999). The ability to identify useful medical countermeasures for the treatment or prevention of disease associated with bioterrorism agents will depend on a robust knowledge of the pathogen and of the host response to that pathogen.

Selection of the appropriate strain of the agent is critical as strain characteristics may influence host responses. Various strains of microbes express different characteristics that can dramatically influence the virulence of the microorganism. In the case of *Bacillus anthracis*, the main virulence factors are the anthrax toxin proteins that, along with the capsule, are expressed largely under the control of the atxA gene (Abrami and others 2005; Brey 2005). In nonhuman primates, live attenuated pigmentation-deficient strains of *Yersinia pestis* (plague) are more virulent than is the wild-type strain when delivered as an aerosol (Welkos and others 2002). Disease pathogenesis varies following different routes of exposure. For example, the capsule is required for dissemination of inhaled *B. anthraci* spores within the host (Brey 2005); dissemination of these spores is enhanced by migration of phagocytic dendritic cells (Brittingham and others 2005).

But while selection of the appropriate strain of a given agent is clearly critical, this factor alone does not ensure clinical relevance. Experience with lentiviruses causing immunodeficiency provides one example where differences in host responses have been especially problematic. Simian immunodeficiency virus (SIV), feline immunodeficiency virus (FIV), and human immunodeficiency virus (HIV) share many molecular and structural features. Each of these viruses targets T cells and reduce CD4+ cell numbers in the host (Levy 1996), and xenoinfections can be produced experimentally. For example, cynomolgus monkeys infected with FIV reportedly develop depletion of CD4+ cells and weight loss (Johnston and others 2001). HIV-1 however, fails to replicate and cause disease except in humans or chimpanzees (Haigwood 2004). Thus the failure of alternative animal models, including other nonhuman primate models, to develop infection following HIV-1 infection has limited development of vaccines and other immunization approaches for this virus (Hu 2005).

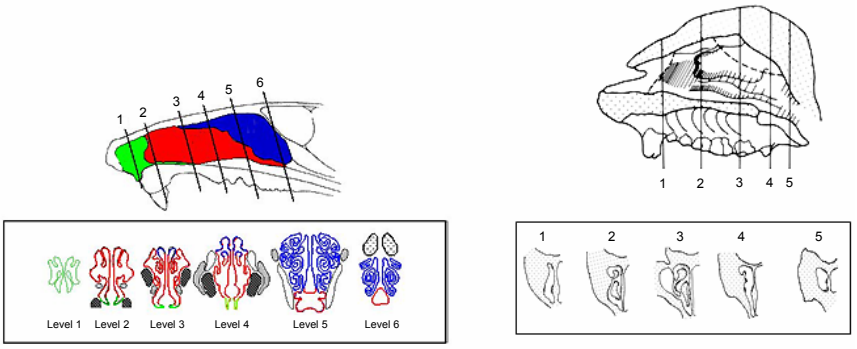
Significant differences in host response have also been noted for select bioterrorism agents. For example, several animal models have been investigated for use in testing vaccines protective against anthrax infection, including mice (Welkos and others 1986; Ezzell and others 1984), guinea pigs (Fellows and others 2001; Jones and others 1985), rabbits (Little and others 2004; Fellows and others 2001; Pitt and others 2001), and rhesus and cynomolgus monkeys (Vasconcelos and others 2003; Ivins and others 1998). Of these, macaques are the closest model to humans as they have analogous major-histocompatability complex (MHC) class I and II and cross-reactive IgG (Kennedy and others 1997). Differences in host response to an agent may also be influenced by the strain, age, or sex of the animal. For example, the A/J mouse strain is very susceptible to infection with unencapsulated anthrax strains (Welkos and others 1986)—the animal lacks a functional Hc gene, which encodes for complement component C5. The C5 protein attracts neutrophils and induces the release of histamine from mast cells and basophils. The absence of C5 results in a delay in the influx of macrophages to the site of anthrax infection, thereby allowing the bacteria to overwhelm the host before a suitable immune response can be mounted (Flick-Smith and others 2005).

PULMONARY ANATOMY AND PHYSIOLOGY

The Upper Respiratory Tract

Upper airway anatomy, which differs significantly among species, can influence respiratory tract dosimetry of an inhaled toxin. Humans and certain nonhuman primates such as rhesus monkeys have relatively simple nasal anatomy in regions of the nose lined by the olfactory mucosa (Harkema 1990; Warwick and Williams 1973). In contrast, the rat, which is the animal species most frequently used in conventional toxicology studies, has a highly complex set of ethmoid turbinates (Méry and others 1994), as does the mouse, dog, and rabbit (Figure 2-2). Animal-model selection can be further complicated, as there

are known species differences in the distribution of luminal epithelial-cell populations along the nasal airway, in the intranasal airflow patterns (Morgan and Monticello 1990), and in the function of the nasal mucociliary apparatus (Harkema 1990). Species differences in nasal airway anatomy and airflow patterns influence the relationship between exposure concentration and pathogen dose delivered to either the lung or nasal cavity.



Rat
- obligate nose breather
- complicated turbinate structure
- 50% of nasal cavity lined by olfactory mucosa

Primate
- oronasal breather
- simple turbinate structure
- 3-5% of nasal cavity lined by olfactory mucosa

FIGURE 2-2 Nasal anatomy in rats and macaque monkeys and impact on airflow and particle distribution. Nasal epithelial subtypes are shown for the rat.

The size of the inhaled bioaerosol (or therapeutic agent) can also influence the sites at which deposition occurs. Because the nasopharynx compartment is a portal of entry into the lungs, particle removal by the nasal cavity will lower the fraction of the bioaerosol entering more distal airways. Removal of inhaled aerosols by the nasal cavity has been examined using inert particles. Nasal deposition of polystyrene particles with a mass median aerodynamic diameter (MMAD; see glossary) of 1 to 2.8 µm ranged from 12 to 20 percent in children and adults (Becquemin and others 1991), whereas rats have appreciably higher nasal deposition rates for comparably sized particles (Kelly and others 2001). Bacterial bioaerosols are likely to have an effective MMAD on the order of 1-10 µm (Louveau and others 2005; Nicas and others 2005; Jahrling and others 2004; Zaucha and others 2001) resulting in similar deposition patterns as that seen with similarly sized polystyrene particles. Viral bioaerosols; however, will be considerably smaller with aerodynamic diameters ranging from approximately

0.01 to 0.4 μm. Nano-sized particles are more effectively deposited in the nasal cavity (Martonen and others 2003). Differences in nasal anatomy and physiology also influence the delivery of a nasally administered pharmaceutical agent (Ugwoke and others 2001) that may be developed as a bioaerosol countermeasure.

The mode of breathing (e.g., obligate nasal versus oronasal), overall geometry of the nasal passages, relative nasal surface areas, proportions of nasal surfaces lined by various epithelia, mucociliary clearance patterns, and inspiratory airflow routes are also very different between rodents and humans. These anatomical differences may likewise influence the fate of an inhaled bioaerosol and thus influence the response seen in different animal species. This difference in response may become important for inhalation studies with spores or aggregates of bacteria that may deposit within the nasal cavity. Nasal deposition of bioaerosols is considered important diagnostically. For example, the use of nasal swabs in the diagnosis of human inhalational anthrax provides a dramatic example where deposition in the nasal cavity is clinically relevant in humans (Kiratisin and others 2002). In general, the complex structure of the rodent turbinates results in increased nasal deposition when compared with either nonhuman primates or humans.

The Lower Respiratory Tract

Differences in the structure of the lower respiratory tract are also an important consideration when choosing an animal model. There are marked differences in the tracheobronchial airways and lungs of various mammalian species, both at the macroscopic and microscopic levels, that entail not only obvious differences in size but also striking architectural differences. The latter include the branching system of the conducting airways (bronchi and bronchioles), the gross lobation of the lung, the amount of pleura and interlobular connective tissue, and the structural design of the pulmonary centriacinus, where the most distal conducting airways intersect with the alveolar gas-exchange regions in the lung parenchyma (Tyler 1983).

For example, the left human lung has two connected lobes, the superior and inferior, while the right lung is composed of three connected (fused) lobes—superior, middle, and inferior. In contrast, the right lungs of laboratory mammals, including nonhuman primates, are divided into four distinctly separated lung lobes (cranial, middle, caudal, and accessory). The left lung lobes of nonhuman primates are divided into cranial (with cranial and caudal portions) and caudal lobes, but rats and mice have a single undivided left-lung lobe. In humans, the visceral pleura on the outer surface of the lung lobes are thick and the interlobular connective tissue within the lobes is extensive. But in laboratory mammals, including nonhuman primates and rodents, the pleura of the lung are thin and the extent of interlobular connective tissue, if any, is modest.

In the human lower respiratory tract, there are seven generations of bronchi, and the tracheobronchial branching system is relatively symmetrical (i.e., the

parent airway divides into two smaller airways with relatively equal diameter). This is in contrast to most laboratory mammals, such as monkeys, dogs, rats, and mice, which have monopodial branching patterns consisting of daughter branches of unequal diameter (Tyler 1983). The lower respiratory tract in humans also contains several generations of nonrespiratory bronchioles—conducting airways without alveolarized outpockets (Weibel 1963). Considerably fewer generations of these small conducting airways are present in other mammalian species, including monkeys.

The most distal nonrespiratory bronchiole is defined as the terminal bronchiole, which connects to the aveolarized (respiratory) airways. This focal area where conducting and respiratory airways join is called the centriacinus, a common site of injury caused by inhaled toxic gases and particles. In humans and some laboratory mammals (e.g., monkey, dog), the terminal bronchioles end into several generations of respiratory bronchioles that are defined as bronchioles with a few, widely scattered, intramural alveoli (or alveolar outpocketings). This is in contrast to several small laboratory mammals (e.g., rat, mouse), whose terminal bronchioles end directly into one short segment of respiratory bronchiole or into airways with walls completely covered by alveoli (i.e., alveolar ducts) (Tyler 1983).

Though similar epithelial-cell types (e.g., ciliated, mucous, basal) line the tracheobronchial airways of mammalian species, the percentage of these cell types may differ markedly among these species and needs to be taken into consideration when selecting an animal model for inhalation studies. A striking example is the percentage of mucous goblet cells that are found in surface epithelium lining the tracheobronchial airways of human and nonhuman primates compared to the corresponding fraction in laboratory rodents (mice and rats). The primary secretory cell in the tracheobronchial airways of humans and nonhuman primates is the mucous goblet cell, while in similar airways of laboratory rodents the percentage of these secretory cells is very low compared to that of other secretory cell types (i.e., serous and Clara cells). In addition, while submucosal glands, which also contribute mucus to the airway luminal surface, are found throughout the tracheobronchial airways of humans and nonhuman primates, these glands are restricted to a very small portion of the proximal trachea in laboratory mice and rats. The density of mucus-secreting cells in either the surface epithelium or submucosal glands of the tracheobronchial airways will determine the amount and character of the airway lining fluid which serves as a critical protective interface protecting the airway epithelial cells from potential injury (or infection) from inhaled infectious agents.

Aerosol Deposition in the Respiratory Tract

Although it is clear that each species has its unique respiratory tract structure, an important consideration in selecting an appropriate animal model is how that structure affects particle (bioaerosol) deposition. Raabe and others

(1988; 1977) published aerosol-deposition data in common laboratory animals and in particular on the regional deposition of monodisperse particles (1-10 μm diameter) in Fischer 344 rats, Hartley guinea pigs, and New Zealand rabbits. The data describe deposition in several regions, including the naso-pharynx, larynx, trachea, bronchi, and pulmonary structures. A conclusion was that deep-lung deposition of particles with diameters greater than 3 μm is negligible, but that upper airway deposition is significant.

Schlesinger (1985) reviewed aerosol-deposition efficiencies in the human (for oral and nasal breathing), hamster, monkey, dog, guinea pig, rat, and mouse. The data for tracheobronchial deposition show relatively low, constant deposition in the experimental animals in the particle-size range of 0.1 to 5.0 μm. Alveolar deposition reached a peak at about 1 μm in experimental animals, but at about 2-4 μm in humans. Despite these differences, Schlesinger concluded that "the relationship between particle size and total respiratory tract deposition is quite similar in humans and most of the experimental animals presented." This conclusion must be qualified since total deposition may not reflect regional dose at the target site of interest.

Other dosimetrics can be considered. The ideal dose metric would need to be mechanistically associated with or closely correlated to the biological response. Internal dose may be accurately described by particle deposition alone if the toxin exerts its primary action on the epithelial surface tissues. Jarabek and others (2005; 1995) reviewed particle dosimetric adjustments for interspecies extrapolations, which underscore the importance of using quantitative morphometric measurements as input for mechanistic computer codes that calculate regional particle deposition. One important conclusion was that the magnitude of species differences in particle deposition depends strongly on whether the deposition is calculated for an entire anatomical region (e.g., tracheobronchial tree) or normalized to deposition per unit surface area. Use of dose metrics based on the number of retained particles and normalized to the number of alveoli, macrophages, or ventilatory units all gave rise to lower human equivalent concentrations than those based on the current default of particle mass normalized to pulmonary surface area.

Wolff (1996) reviewed differences in particle deposition and clearance measurements in several species, including humans, dogs, monkeys, and rats. A striking finding was that there were significant differences in pulmonary deposition of particles in the 0.1 to 10 μm diameter range; mouth breathing in humans led to large pulmonary deposition, and both dogs and monkeys had pulmonary depositions similar to that of nose-breathing humans. In addition to differences across species, strain differences may be important. Significant particle deposition efficiencies have been predicted for BALB/C mice versus B6C3F1 mice from respiratory tract morphometric measurements (Oldham and Phalen 2002).

Clearly, species differences in airway anatomy need to be considered when extrapolating results from animals to humans. Moreover, diverse responses may

arise when toxins are deposited in different airway regions (e.g., nose vs trachea or alveoli). Thus, local tissue dose needs to be considered, since similar total respiratory deposition among species does not necessarily lead to similar responses. Several tools are available to estimate the delivered dose to the airways of animals and humans (NCRP 1997; ICRP 1995). Computational models can also predict deposition and retention of particles in the lung (Brown and others 2005; Subramaniam and others 2003).

Clearance from the Respiratory Tract

Immunological or physical clearance of an agent from the lung may also be species-dependent. An inhaled pathogen or particle may become inactive or be cleared from the lung by either physical means (e.g., mucociliary clearance) or via immunological mechanisms (e.g., alveolar macrophage phagocytosis and subsequent removal). The physical processes demonstrate important species differences (Stober and McClellan 1997). The difference in rates of clearance of insoluble particles inhaled by several laboratory animals has been reviewed by Wolff (1996), who noted that rats and mice, in contrast to humans and dogs, exhibit an early and prolonged rapid clearance of material deposited in the alveolar region. This rapid clearance serves to protect the distal lung from particle accumulation, unless the particle load is great enough to overwhelm the physical-clearance mechanisms.

In addition to being influenced by mucociliary clearance, removal of a bacterial pathogen from the normal lung reflects the relative rates at which in-vivo bacterial multiplication and killing will occur. Marked differences in lung clearance have been reported with different strains of pathogens and may also be influenced by the strain of the animal model used (Jay and others 1976), as well as the immune status of the animal.

From the foregoing information, it is clear that pulmonary anatomy and physiology, at both the macroscopic and microscopic levels, differs significantly among species. This can influence the deposition of the inhaled agent within the lung, as well as the tissues and cells that will primarily be contacted by the agent. All of these differences can affect disease outcomes and whether the animal model will be predictive of the human response to a countermeasure.

It is clear that for the species and strains to be used in countermeasure research, additional information is needed on differences in respiratory-tract anatomy and particle deposition. **The Committee recommends that additional data on experimental-animal airway anatomy and particle deposition and clearance be acquired to aid in developing new animal models and performing extrapolations to human populations. In particular, data are needed for various strains of mice and many species of nonhuman primates.**

3

Generation and Characterization of Aerosolized Agents

Testing of aerosolized bioterrorism agents for research purposes does not require strict compliance with Good Laboratory Practice regulations (21 CFR 58), which require that investigators establish standard operating procedures for their testing procedures. However, if testing of aerosolized agents is performed in a standardized way—especially those procedures that may lead to application to the FDA for approval of therapeutics or vaccines—then laboratories can share their findings and compare outcomes. Judging by a number of aerosol generators and aerosol-characterization procedures that were described during the Workshop, it appears that they have not been standardized among laboratories. This lack of standardization is of concern, as it could lead to a high degree of variability between laboratories in terms of their results.

In this section of the report, several ways of generating aerosols are described and their advantages and disadvantages enumerated: so that scientists working in this field can be aware of the usefulness of these techniques. This section also offers a number of recommendations for addressing the various barriers to testing the effects of aerosolized bioterrorism agents, as well as methodological criteria that should be included in reports by laboratories working in this field.

PRINCIPLES FOR GENERATION OF AEROSOL

Bioterrorism agents can be formulated and generated as liquid aerosols or as dry powder aerosols. Monodisperse aerosols (aerosols having a narrow size distribution) are most useful for calibrating laboratory instruments or for answering basic questions related to aerosol deposition, but they may also be useful in bioterrorism research. Monodisperse aerosol generators include

spinning disk, vibrating orifice and condensation devices. On the other hand, polydisperse aerosols (aerosols that consist of particles of various sizes) usually more closely resemble what humans inhale and are often most relevant for countermeasure studies.

Liquid bioaerosols are usually generated by air-blast nebulizers (also known as compressed-air or jet nebulizers) or by ultrasonic nebulizers. The ultrasonic nebulizer produces aerosol particles through the vibration of a piezoelectric crystal, which forms a fountain of liquid that emits droplets from its tip. Although ultrasonic nebulizers produce a large number of droplets per liter of air, they are less applicable to the testing of bioterrorism agents than are air-blast nebulizers because the droplets tend to be larger—too large, in fact—and the heat produced during aerosolization can lead to the degradation of proteins that may be present in viral and bacterial agents. With the air-blast nebulizer, a liquid stream is drawn from a reservoir into the path of a jet of air that is under high pressure. As a result, the liquid shatters into large and small particles. These smaller particles exit the nebulizer and can be inhaled, while the larger particles impact on surfaces within the nebulizer and recirculate into its liquid reservoir. (Descriptions of the basic operation of many air-blast nebulizers can be found in Phalen [1984] and in Moss and Cheng [1995a; 1995b].)

The most commonly used aerosol generator for generating bioaerosols including bacteria, viruses and toxins at USAMRIID and Ft. Detrick has been the Collision nebulizer (Hartings and Roy 2004; Jahrling and others 2004; Roy and others 2003; Zaucha and others 2001; Pitt and others 2001; Johnson and others 1995; Larson and others 1980; May 1973; Henderson 1952). This generator produces droplet aerosols with mass median aerodynamic diameters of 1 to 3 μm. Other aerosol generators, such as the spinning disk aerosol generator, have also been used in some studies (Roy and others 2003). In the case of viruses, bovine serum at concentrations up to 10 percent have usually been added to the generator solutions as a stabilizer (Jahrling and others 2004; Zaucha and others 2001).

Nebulization is an extremely useful method for aerosolizing many substances and is therefore valuable for studying the consequences of inhaling aerosolized bioterrorism agents. Nevertheless, it presents a number of challenges to such studies. First, nebulization can lead to high shear stress levels, which may result in fragmentation and deactivation of bacteria and viruses. Air-blast nebulization can also result in the recirculation of the media and the formation of particle aggregates that are less inhalable than naturally occurring particles. In addition, during air-blast nebulization, the concentration of particles in the aqueous solution steadily increases. This means that, over time, the animal could be exposed to aerosols that contain increasingly concentrated amounts of the agent, resulting in an unrepresentative response. To circumvent these difficulties, researchers have used the following techniques: (1) placing the nebulizer reservoir on ice to reduce evaporative losses and help maintain the aqueous concentration at consistent levels; (2) nebulizing for short periods of time to keep the concentration more consistent and reduce the effects of shear

stress; (3) increasing the reservoir volume to reduce the change in concentration over time; and (4) using continuous fluid feed or recirculation with a large external fluid volume. An example of a continuous feed nebulizer is the TSI Model 3076 (TSI Inc., St. Paul, MN).

Generation over a short time period will not eliminate recirculation of the media or all of its consequences. Investigators need to consider: (1) using a single-pass bubbling aerosol generator that does not recirculate the media and therefore may be a good alternative to the air-blast nebulizer (Mainelis and others 2005); or (2) continuously feeding the reservoir with fresh liquid to reduce the artifacts associated with recirculation. Continuously feeding the reservoir is particularly important for long exposures and when using generators that are not single-pass.

A final challenge is that freshly nebulized particles may be electrically charged, which affects deposition within the respiratory tract. In order to reduce this variability, investigators need to consider including a final discharge step for neutralizing the electrical charge (Ji and others 2004; Hinds and Kennedy 2000), unless charged particles are required to produce a specific disease state.

Liquid aerosols can also be generated by devices that utilize spinning disk or vibrating-orifice technology (Rubsamen 1997; Swift 1993) and a number of these devices are commercially available for the administration of therapeutic aerosols. In terms of testing for the effects of aerosolized bioterrorism agents, use of these devices could be an improvement over air-blast nebulization, as they allow for custom-design of the aerosol particle size. Thus they could be used to generate test aerosols of different particle sizes that could be compared in terms of their infectivities. Electrohydrodynamic spraying has also been used to disperse fine droplets (Chen and others 1995).

Solid-particle aerosols are typically generated by the pneumatic redispersion of a dry powder ("dry dispersion"). The output concentration of dry-dispersion generators ranges from milligrams per cubic meter to greater than 100 g/m3. The basic requirements for all dry-dispersion generators are: (1) a means of continuously metering a powder into the generator at a constant rate; and (2) a means of dispersing the powder to form an aerosol. Dispersability of the powder depends on the powder material, particle size and size range, particle shape, and moisture content. To fully disperse a powder, it is necessary to supply sufficient energy to overcome the attractive forces between the particles. Several dry-dispersion aerosol generators are commercially available. For more information about them, see Hinds (1999).

Additional technologies for aerosol generation, resulting from the need for improved therapeutic medications, have led to new formulations of dry powders and new devices for their aerosolization (Laube 2005; Rubsamen 1997). It is possible that these same approaches could be used to improve the animal testing of certain biological agents that can exist in dry-powder form. For descriptions of the basic operation of many dry-powder-inhaler designs, see Dunbar and others (1998).

In the case of microorganisms, the Committee recommends that investigators determine whether the generator fragments or inactivates the organism of interest. If so, this reduced viability would clearly impact the animal's response to the exposure; the researchers should then select another type of generator.

The Committee also recommends that investigators have some knowledge of the cellular targets of the agent under test within the human airways. They may then more appropriately choose an aerosol generator that will produce aerosol particles capable of reaching the appropriate anatomical regions in the animal model. For example, the cellular targets of a given bioterrorism agent are likely to be located in different regions of the respiratory tract (e.g., the tracheobronchial region versus the alveolar region). How much of the aerosol will be inhaled by a given animal and where in the respiratory tract it will deposit is a function of the aerosol's particle-size distribution. Techniques for characterizing the aerosol particle-size distribution from a particular aerosol generator will be discussed in detail in the next section of this chapter.

If one chooses the mouse as the animal model for early tiered testing of the effects of aerosolized bioterrorism agents, it is important to keep in mind that an aerosol consisting of 1-μm particles or smaller has the highest probability of depositing in the tracheobronchial and alveolar regions of the lungs of mice that are exposed during nose-only breathing (Raabe and others 1988). Similar results have been shown for Golden Syrian hamsters, Fischer 344 rats, Hartley guinea pigs, and New Zealand rabbits during nose-only breathing exposures (Raabe and others 1988). **Therefore, if one is testing an inhaled biological agent in animal models, the Committee recommends that the investigator choose a generator that can produce an aerosol that will reach the intended anatomical sites, which may be the nasopharyngeal, tracheobronchial or pulmonary regions.**

CHARACTERIZATION OF AEROSOL

Certain properties of inhaled particles, and of the exposure environment, can contribute to the agent's biological effects. The most important particle properties are its aerodynamic size, size distribution, geometric size and shape, electrical charge, chemical composition, irritancy, and mass concentration (mass of particles per unit mass of air). The most important properties of the exposure environment are its temperature, relative humidity, osmolarity, airflow, and uniformity of the exposure in the breathing zone. **Because one laboratory's well-characterized aerosols and exposure environments may not be readily comparable with those of another, the Committee recommends that these properties be quantified during each inhalation experiment and reported in all publications resulting from the work.**

It is convenient to express the size of an irregularly shaped particle by an equivalent spherical dimension—its "aerodynamic diameter" (D_{ae})—which is

defined as the diameter of a unit-specific-gravity sphere having the same settling velocity as the particle being studied. This dimension encompasses the particle's shape, density, and physical size.

A population of particles can be defined in terms of the mass carried in each particle-size range, and a measure called mass median aerodynamic diameter (MMAD) essentially divides that distribution of the mass in half. The distribution around the MMAD is expressed in terms of the geometric standard deviation (GSD). Aerosols with GSD >1.2 are considered to be polydispersed (i.e., the particles vary significantly in size).

In humans, particles in the range of 1-10 μm or <0.5 μm are most likely to deposit in the tracheobronchial and pulmonary regions of the lungs. However, particles ≤5 μm are thought to have the highest probability of entering the lower airways of the average adult during oral inhalation. Because the human nose is a much more efficient filter of particles compared to the mouth, only particles ≤3 μm have a high probability of entering the lower airways during nose breathing. As shown in Figure 3-1, maximal pulmonary deposition occurs when particles have diameters between 1 and 3 μm or <0.5 μm (NCRP 1997; ICRP 1995). However, there is great variation in the efficiencies and locations of particle deposition in humans, given individuals' diversity of airway sizes and breathing patterns.

A filter is the most common means of collecting an aerosol sample for assessment. That assessment might include gravimetric weighing on an analytical balance before and after sampling; or it might consist of visual characterization using an optical or electron microscope and a variety of analytical, chemical, or microbiological techniques. Membrane filters can retain particles effectively on their surfaces (good for microscopy and measurements of geometric size and shape), whereas fibrous filters provide in-depth particle collection and a high-load-carrying capacity (good for gravimetric assessment) (Vincent 1995). Specific filter requirements may be identified, depending on the characteristics chosen. For example, weight stability is important for gravimetric assessment and durability is needed for various extraction processes. Filters used for particle counting by optical microscopy need to be capable of being rendered transparent, and filters used for electron microscopy analyses need to allow good transmission of the electron beam.

Measurements of the MMAD and size distribution of a bio-aerosol can be achieved with a cascade impactor, wherein one determines the collected mass on each impactor stage as well as on the back-up filter (Lodge and Chan 1986). This approach, which can be done gravimetrically or by chemical analysis, requires time to obtain the sizing information. Other analyzers utilize optical techniques to provide particle-size information in real time. Such counters may be specifically designed for light scattered at low angles (low-angle devices), light scattered in the generally forward direction below 90º (forward-scattering devices), or light scattered at angles close to or beyond 90º (large-angle devices

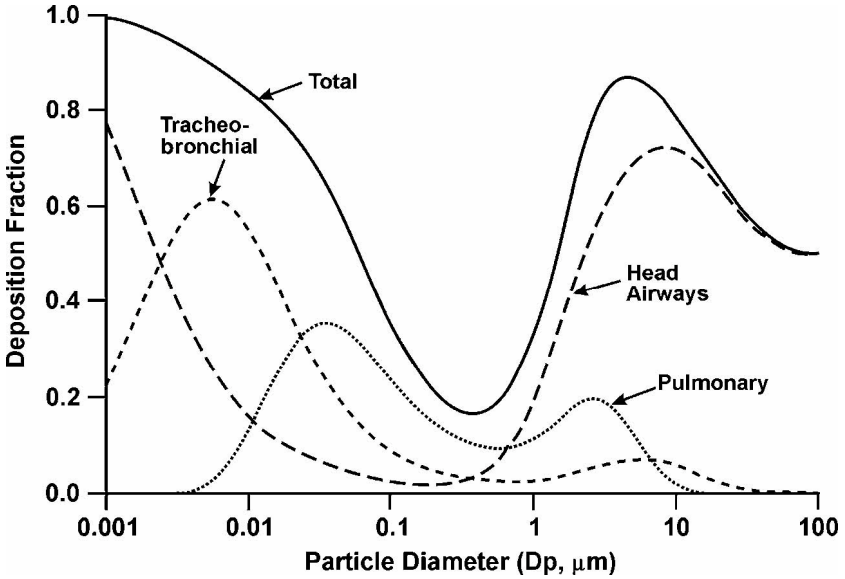


FIGURE 3-1 Fractional regional deposition of inhaled particles in the human (Snipes 1994). Reprinted with permission from Medical Physics Publishing, Madison, Wisconsin.

or devices that use aerodynamic properties of particles). Examples include Climet optical counters (Climet Instruments Company, Redlands, Calif.) and the Aerodynamic Particle Sizer (APS) (TSI Inc., St. Paul, Minn.).

Unfortunately, none of these optical particle counters can differentiate between those particles that contain microorganisms and those that do not, much less which particles contain viable versus nonviable organisms. **Thus, in the case of exposures that involve the inhalation of microorganisms, the Committee recommends that if investigators use an instrument that provides real-time particle-size information, they should also provide information regarding the size distribution of particles that contain microorganisms.**

In addition, it is important to obtain assessments of organism viability within the size distributions that are critical to deposition inside the lung. For these assessments, survival of the collected particles in their original airborne state is an important consideration in selecting the size analyzer. One factor that can adversely affect survival is desiccation, which usually occurs during particle sizing with a cascade impactor. An alternative to the impactor is the liquid impinger, though it has diminished collection efficiency for particles smaller than 1μm.

It is clear that several different approaches and instruments can be utilized when characterizing particle size and size distribution of inhaled bioterrorism agents. For more information concerning aerosol-sizing instruments, see Phalen (1984) and Lalor and Hickey (1997). For more information about aerosol sampling, see Vincent (1995).

To standardize aerosol characterization, the Committee recommends that measurement of certain parameters be incorporated into standard operating procedures to be adopted by researchers (Table 3-1). The Committee also recommends that specific information (Table 3-2) be included in reports of studies in order to standardize the reporting of aerosol characterization between laboratories.

TABLE 3-1 Recommended Measurements to be Included in Standard Operating Procedures for Generation of Aerosols

• Mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) of aerosol available for inhalation as close to the breathing zone of the animal as possible
• MMAD and GSD of aerosol available at the outlet of the aerosol generator in a test performed before administration
• Concentration of biologic agent as close to breathing zone of the animal as possible in an exposure chamber, or from an alternative to an inhalation delivery device
• Aerosol generation and exposure times
• Relative humidity and temperature of aerosol-exposure environment
• Estimation of the number of viable organisms (e.g., bacteria or viruses) in the aerosol exposure

TABLE 3-2 Recommended Information to be Included in Scientific Reports on Aerosols

Aerosol-generation information

1. Type of aerosol generator, including manufacturer name and location
2. Identification of aerosol vehicle (e.g., water, phosphate-buffered saline, glycerol, lactose)

Particle-size information, depending on type of instrument used

1. MMAD
2. GSD
3. Volume median diameter ($V_{0.50}$)
4. Type of particle-sizing instrument used, including manufacturer name and location
5. Sampling time and flow rate
6. Calibration method for the particle sizing instrument

Impinger information, if aerosolizing microorganisms

1. Type of impinger
2. Sampling time and flow rate
3. Indication of whether impinger was sterilized, and method used
4. Estimate of microorganism viability and how estimate was obtained

Exposure information

1. Did exposure occur during nose-only breathing, in a whole body chamber, or using an alternative to inhalation?
2. If animal was anesthetized, the anesthetic agent(s), dosage and route of administration
3. Animal's total aerosol-exposure time
4. Size and volume of exposure chamber
5. Were animals exposed separately or together?
6. Exposure period in relation to the animal's normal diurnal (light-dark) activity cycle
7. Temperature and relative humidity of exposure environment
8. Concentration of test agent in exposure environment
9. How the aerosol was charge-neutralized (or justification of why it was not neutralized)
10. Minute ventilation of animal, corrected to BTPS (body temperature and pressure, saturated relative humidity)
11. How minute ventilation was measured
12. Was animal loosely restrained or closely restrained during testing?
13. For bacterial and viral aerosols, the number of live organisms in the aerosol sample and how this information was obtained

Animal information

1. Species, age, and sex of the animals utilized
2. Health status of the animals utilized

4

Dosimetry Considerations

DOSE METRICS

The concept of a "dose metric" (measure of dose, also called an "indicator") has recently been sharpened in relation to monitoring air pollutants (EPA 1996). A measurable physical/chemical property that has several characteristics, the dose metric principally corresponds to an agent's ability to cause a biological effect of interest, such as the toxicity, infectivity, or efficacy of an agent. For chemical aerosols, the dose metric may be an aerosol mass fraction such as the inhalable mass fraction, the thoracic mass fraction, or the respirable mass (American Conference of Governmental Industrial Hygienists 2005). For infectious agents, the number of inhalable viable organisms, number of inhalable spores, or number of inhalable culturable units are potentially useful dose metrics. The size distribution of viable organisms also needs to be defined. In all cases, the dose metric needs to be directly measurable in a reproducible manner under a variety of laboratory and field conditions.

Defining the Dose/Deposited Dose

The concept of "dose" used in clinical medicine and routine toxicology is too simplistic to be of value in an aerosol-inhalation research setting, where dose refers to the amount of an agent (using the selected dose metric) that is presented to the specific tissue or tissues of interest (i.e., target tissues). Further, the dose is often normalized to some property of the target tissue, such as tissue mass or surface area, that relates to the potential for the presented dose to do harm. In inhalation toxicology, dose is often expressed in terms of exposure. Traditionally, an agent's airborne concentration (C) times the exposure time (T)

times the inhaled air volume per unit time ($\dot{V}$) has been used as a measure of the exposure dose (D_E). That is,

$$D_E = C \cdot T \cdot \dot{V} \quad (1)$$

However, this measure of dose is inadequate for several reasons. First, not all of an inhaled aerosol will actually deposit in the respiratory tract upon inhalation. Therefore the deposition fraction (F_T) in the target region of the respiratory tract needs to be included. Second, the inhalability (I), which is the sampling efficiency of the entrances to the respiratory tract, also needs to be considered. This depends on the particle size of the aerosol. Third, the concentration must be defined in terms of a proper dose metric (C_{DM}). Therefore, the deposition dose (D_D) becomes

$$D_D = F_T \cdot I \cdot C_{DM} \cdot T \cdot \dot{V} \quad (2)$$

The F_T and I are found by measurement under the experimental conditions, by reference to published values, or from dosimetry software (Brown and others 2005; ICRP 1995; Birchall and others 1991). The C_{DM}, T, and $\dot{V}$ can be directly measured during a study. However, $\dot{V}$ is often either calculated or based on values reported in the literature. In practical applications, the protocol for an exposure system can be calibrated for a specific species such that strict adherence to the protocol will provide a reproducible dose. Great care is necessary, however, to control unwanted factors such as electrical charges on the aerosol and on surfaces (e.g., chambers, piping, and animals), as these factors can affect aerosol size distribution, airflow rates, and losses in the exposure system.

Infectious Dose

Dose is commonly reported as a median lethal dose (LD_{50}) or median infectious dose (ID_{50}). However, LD_{50} and ID_{50}, which are indices of the potency of an agent for producing a response, are very procedure-specific. The potency of an agent can be affected by the method of delivery, the aerosol-particle-size distribution, the site of deposition in the respiratory tract, and the species under study. Therefore, defining an infectious dose for a given level of response presents a significant challenge, since the dose that causes the death (LD_{50}) or infection (ID_{50}) of 50 percent of the test group can be different in almost every experimental situation. This makes interpretation of a study or replication of an exposure difficult. **Therefore, the Committee recommends that when a multiple of the LD_{50} or ID_{50} (e.g., 10 LD_{50}) is used to report dose, then sufficient additional data, including indices of viability of the**

agent and characteristics of the exposure, particle-size, and generation of the aerosol should be acquired and reported.

Acquiring data necessary to interpret or replicate the experiment requires careful attention to the biological material used and experimental conditions. Information about the viability of the agent should be acquired, for example obtaining a measurement of the number of viable organisms, the number of colony-forming unites, or the number of plaque-forming units. Specifics about the experimental design should also be noted or measured, including, details of the generation of the aerosol, particle-size, and exposure characteristics (see Tables 3-1 and 3-2).

5

Experimental Design

DELIVERY OF DOSE

Inhalation

In this chapter, we discuss the advantages and disadvantages of existing methods for delivering a dose of an aerosolized bioterrorism agent in animal models.

The first method is inhalation delivery, which often entails the whole-body approach. Other approaches involve head-only, nose-only, or mouth-only exposures, where the aerosol is generated in a smaller volume and the animals are individually constrained such that their heads, noses, or mouths project into the test environment of interest.

In the case of whole-body exposure, test animals are placed singly or together in cages into which the desired aerosol environment is introduced. Attention needs to be paid to maintaining a diurnal light cycle in multi-day studies. Whole-body exposure chambers are usually of the type in which a continuous flow of throughput air is maintained; and they tend to be constructed of stainless steel, mainly because stainless steel does not build up localized electrical surface charge (which can affect dosing) and it is sterilizable. This type of exposure has several advantages. It can accommodate a large variety and number of animals for long periods of time, and it does not require restraint or anesthesia during exposure. However, this type of exposure can lead to highly variable dosing because animals can come into contact with the aerosolized material in a variety of ways. In addition to inhalation, exposure can occur through the skin, mouth, and eyes as well as from the animals' licking their fur or cage material and eating their food. Animals can also receive an additional

aerosol dose from their own fur or that of other animals. **To reduce variability, the Committee recommends that food should be removed during aerosol exposure.**

In chambers without individual cages, animals can avoid high dose exposures by huddling together and covering their noses with their neighbors' fur. **To avoid this possibility, the Committee recommends the use of chambers with individual cages.** A good example of this approach is a Hinners-type of exposure chamber, which allows for the exposure of a small number of animals in subdivided individual sections (Steinbach and others 2004; Moss and Cheng 1995b). Another disadvantage of the whole-body approach is that the desired level of exposure may take some time to stabilize in the chamber (Phalen and others 1984). **When using a whole-body chamber, the Committee also recommends that the environmental temperature and humidity be regulated and the spatial and temporal distribution of the aerosolized material be uniform.** Uniformity can be achieved by fitting the chamber with a cone or pyramid-shaped entry and exit and by either mixing the throughput air or by rotating the cages during exposure. The Rochester-type of chamber has a tangential inlet at the top that rotates the chamber air and provides good uniformity of exposure (Leach and others 1959). **The Committee further recommends that samples for characterization of the exposure environment should be taken from the breathing zone nearest to the animal during an actual exposure.** For more information regarding the use of whole-body exposure chambers, see Phalen and others (1984) and Moss and Cheng (1995b).

An alternative to whole-body exposure is head-only exposure, which requires that the head or neck region of the animal be firmly restrained. In contrast to the whole-body chamber, this type of exposure reduces the number of ways the aerosolized material can enter the animal and reduces variability in dosing. **For this type of exposure, the Committee recommends that a good neck seal, which does not interfere with blood flow or ventilation, be utilized. In addition, environmental air temperature, humidity, and levels of carbon dioxide and oxygen need to be properly regulated.** Other alternatives to whole-body exposures are nose-only or mouth-only exposure systems, which limit the entry of the aerosolized material to either the nose or oral cavity. An advantage to head-only, nose-only, or mouth-only exposure is that the amount of aerosolized material per animal is reduced compared to whole-body exposure and the concentration of the exposure material can be rapidly changed. Inhalation exposures by nose or mouth can be achieved using masks or cylindrical tubes (with a conical end to accommodate the head and one end open to the exposure environment) (Phalen 1984). **The Committee points out that it is important to design and validate the tubes and masks properly so that exposure using these systems does not lead to stress on the part of the animal and altered respiration, which can affect responses to test agents.**

Aerosol can also be delivered directly to the lungs via the mouth by introducing the aerosol through an endotracheal tube (Phalen and others 1984). This approach has been shown to eliminate losses in the upper airways and

significantly increase aerosol delivery to the lungs of rhesus macaques compared to nebulization-only delivery to their mouths (Beck and others 2002). However, losses still occur in the tubing, and normal protective mechanisms such as deposition of particles in airways of the head are bypassed. Other disadvantages include the need for general anesthesia, mechanical trauma to the larynx and trachea, interference with normal airflow characteristics, and loss of normal humidification and thermal regulation of the inspired air (Phalen and others 1984). Loss of humidification can be overcome by warming and humidifying the aerosol (without increasing particle size) to near-physiological values.

Alternatives to Inhalation Delivery of Aerosol

It is clear that inhalation exposure is the gold-standard approach for studying inhaled agents. However, there may be instances when alternative techniques for delivering a dose of an aerosolized agent may be useful, such as when there is a need for improved quantification of delivered dose. If use of alternative techniques for delivering an aerosolized agent is necessary, clearly documenting and justifying the alternative exposure technique can aid in the interpretation and replication of the study.

Several issues can influence which alternative exposure method is chosen. The Committee has developed this section to provide researchers with the most up-to-date information on alternative exposure methods to facilitate the decision-making process and to maximize the effectiveness of available delivery strategies for current testing.

As mentioned above, inhalation delivery results in significant losses of the exposure material on the head or body of the animal, as well as multiple entryways for exposure, including the eyes, oral cavity, nasal cavity, and gastrointestinal tract. Moreover, because deposition measurements within the lung compartment after exposure is difficult, quantification of the delivered dose is commonly estimated from calculations that include the inhaled particle concentration, or by measurement of biomarkers. These estimates may or may not provide the precise dosing information necessary for determining the relationships that will lead to the development of therapeutic agents that prevent or treat the biological response to inhaled bioterrorism agents. Compared to inhalation delivery, each of the alternative delivery methods described below provide for a higher degree of quantification of the dose delivered to the lower respiratory tract. However, their distributions of material in the respiratory tract may be dissimilar to the distribution during inhalation delivery (Beck and others 2002).

Alternatives to inhalation delivery include intratracheal or transtracheal instillation, during which a solution or suspension of the agent can be placed directly into the lumen of an airway. The dose delivered can be precisely controlled by the use of an injection syringe. Of the two methods, intratracheal instillation is preferred because it avoids the need for surgical penetration of the trachea, which requires humidification of the inspired air and postsurgical care

to prevent infection (Phalen and others 1984). Both approaches suffer from the effect of gravity on the instilled fluid, which distributes unevenly, running down into the dependent areas of the lungs (Brain and others 1976). This can lead to high local concentrations of the bioterrorism agent, local tissue damage, and biological responses that may be unique to this method of exposure.

Another delivery alternative is microspraying, which involves the passing of a small-diameter tube through the oral or nasal cavity and delivery of particles in the form of a spray to the tracheal carina, or into specific lung regions. The spray is produced by means of an injection syringe from a solution or suspension. Microsprayers are commercially available for mice and larger mammals, including nonhuman primates, and can be introduced into the lungs inside of a bronchoscope, which also aids visualization and targeting. Like intratracheal instillation, the delivered dose can be precisely controlled. Unlike instillation, the distribution of the dose is considerably more predictable and uniform (Beck and others 2002). Nevertheless, particles generated during microspraying can be significantly larger than traditionally nebulized particles, with aerodynamic diameters averaging >4 μm for some products and >20 μm for others. These large particles may target different cells and receptors than those that are targeted by smaller-particle generators, leading to differences in the biological responses.

Yet another alternative to inhalation delivery is aspiration. With this approach, a known amount and concentration of a solution or suspension can be pipetted either into one of the nares or into the distal part of the oropharynx of a lightly anesthetized animal, as described previously in mice and rabbits (Steinbach and others 2004; Miller and others 2002; Foster and others 2001; Larsen and others 2001; Gelfand and others 1997). Within minutes, the solution or suspension is aspirated by the animal and deposits in the nasal cavity and/or lungs. There are several differences between the aspiration approach and inhalation delivery in terms of the dose deposited and the distribution of the dose within the lung. Sequential gamma camera images of the lungs of mice, following oropharyngeal aspiration of a radiolabeled liquid, suggest that aspirated particles deposit in the alveolar region of the lungs (Foster and others 2001). In addition, initial images of the lungs indicate that the inhalation method delivers significantly less radiotracer to the lungs compared to aspiration; and images of the stomach and esophageal regions indicate more radiotracer in these regions with the inhalation method compared to aspiration.

EXPERIMENTAL DESIGN AND SELECTION OF DOSE

One of the major concerns in developing new countermeasures against bioterrorism agents involves the ability to demonstrate their effectiveness: to show that the countermeasure provides a statistically significant level of improvement in some physiologically relevant outcome—often, survival—versus the response in the countermeasure's absence. The outcome of interest can be dependent on a variety of factors, including the type of agent and its route

of administration, as well as the species in which the tests will be conducted. In the case of potential bioterrorism agents such as anthrax or other infectious diseases, the route of administration—should the agents be used in a conflict setting or terrorist attack— would be by inhalation following aerosolization.

The evaluation of countermeasures will include testing in model animal species to evaluate efficacy, and the value of those experiments will be dependent to a large extent on the experimental design. The first consideration is the dose of agent to use for evaluating the level of efficacy provided. This is often done by first determining the median lethal dose (LD_{50}) of an agent in untreated animals, and then determining a protective ratio—i.e., the ratio of the LD_{50} in untreated animals versus the LD_{50} in a population of animals treated with the countermeasure of interest.

Multiples of the LD_{50} (i.e., 100 LD_{50}) have often been used to set the challenge dose of select agents. In some cases, sufficient data have been presented so that the challenge dose in colony-forming units (bacteria), plaque-forming units (viruses), or mg per liter of air is also known, while in other cases only the multiple of the LD_{50} given. It is not always clear why a certain multiple of the LD_{50} is used (i.e., 10 LD_{50} versus 100 LD_{50}) for some agents in some studies but not for others. This situation underscores the need for well-designed experiments that would allow for reliable inter-laboratory comparisons.

A statistical calculation made from the experimental data, the LD_{50}, is the dose expected to kill 50 percent of the animals from the infection or toxicant within a defined time (often, 30 days). This can be determined by a classic probit-type design (Burn and others 1950), an up-down design (Dixon and Mood, 1948), or generated in sequential stages using the methods described by Feder and others (1991a; 1991b; 1991c). In some instances, the challenge dose is reported to be the LD_{99} (the dose that would be predicted to kill 99 percent of the animals). An alternate approach is to estimate a dose of the agent that produces the toxic effect in all animals tested, and then test increasing doses of the countermeasure until complete or a statistically significant level of protection is achieved.

It is important to recognize that the LD_{50} and LD_{99} (and any other LD value) is estimated from experimental data and has variability associated with it. Confidence limits (e.g., 95 percent confidence limits) express the uncertainty in the estimate. As the studies used to determine the LD values usually use a relatively limited number of animals (especially in the case of nonhuman primates), these upper and lower bounds estimated from a probit analysis are very large. Thus it is important to regard the LD values as estimates that are useful but not absolute. Moreover, even if the LD_{99} is determined in a way that minimizes error; one potential problem with the approach is that the dose of agent may be so great that it cannot be reversed by the countermeasure under investigation.

It is also very important to recognize that many factors, such as the strain or substrain of the animal model, the strain or substrain of the select agent, and even environmental factors, can markedly affect the LD values for a given

study. Other metrics of dose, including an infectious dose (e.g., ID_{50}) or effective dose (ED_{50}) can also be used to set a challenge dose. Thus it is important to actually determine such a dose, perhaps using the sequential-stages method of Feder and others (1991a; 1991b; 1991c) or fixed-design method and not extrapolate it from a probit analysis designed to evaluate the LD_{50}. Though the sequential-stages and fixed design methods provide a more accurate estimate of dose, there is a level of uncertainty (variability) associated with the estimate, which can be expressed as a confidence interval. Determining whether a sequential-stages or fixed-design method is best depends on the steepness of the dose-response curve and the length of the steps in the sequential method. **These concerns emphasize the importance of statistics in the experimental design of aerosol exposures, and the Committee recommends obtaining statistical advice when designing an animal study to develop or test a countermeasure.**

Ultimately, two approaches are generally used: (1) fix the concentration of agent at some finite level that produces an adverse response, and then vary (increase) the dose of the countermeasure until protection is achieved; or (2) use a fixed dose of the countermeasure and vary the concentration of the agent across a concentration range (that one expects to encounter) until protection is overwhelmed. The relative advantages of either approach are usually determined by the type of outcome being measured—e.g., for aerosol exposures the former approach may be more practical because of the necessary observation time of the subject animals to make sure that an outcome is valid. In either event, the up-down or sequential-stages approaches will reduce the number of animals needed for a statistically valid response, which is an important consideration.

Given animal-welfare concerns, it is hoped that more sophisticated metrics of dose will replace use of the classic LD_{50} determination in most studies using animal models; and the practice of humanely euthanizing moribund animals prior to their death is recommended to reduce or eliminate pain and suffering (Toth 2000). As previously noted, the outcome chosen need not be lethality; and often it may be preferable to evaluate efficacy by some other criteria (Toth 2000). In the case of infections diseases, for example, delay in time to onset or a reduction in clinical severity may be very acceptable outcomes that would support a claim of efficacy to the regulatory body, particularly if approval is being sought under the newly instituted Animal Rule.

The following are some examples of inhalation LD_{50} values and their use. A 1966 study (Glassman 1966) reports an inhalation LD_{50} for anthrax spores of 4,130, with 95 percent confidence limits of 1,980 to 8,630 spores. This was based on 1,236 cynomolgus monkeys (*M. fascicularis*). The strain of *Bacillus anthracis* was not given, but it may have been Vollum 1B (Albrink and Goodlow 1959). At the Animal Models for Testing Interventions Against Aerosolized Bioterrorism Agents Workshop, some details were presented on the LD_{50} that the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID) has used in publications on spores since 1991. The LD_{50} for the Ames strain was 54,687 cfu, with 95 percent confidence limits of 44,809 to 8,300,000, in rhesus monkeys (*M. mulata*). The LD_{99} was 130,000, with 95

percent confidence intervals of 78,229 to 9,100,000,000. Thus there was a factor of about 2.4 between the LD_{50} and the LD_{99} (Pitt 2005). A contemporary report from another laboratory gave an LD_{50} for the Ames strain of 61,800 (95 percent confidence intervals 34,800 to 110,000) cfu in *M. fascicularis* (Vasconcelos and others 2003). However, at the workshop an LD_{50} for a new batch of Ames-strain spores was reported to be 7,221 for rhesus monkeys and 8,294 for African Green monkeys (Pitt 2005). Thus even for anthrax, which has been comparatively well studied, fairly large variations in the LD_{50} via inhalation in non human primates have been reported. In addition an inhalation dose of 161 to 760 LD_{50} of Ames-strain spores of *B. anthracis* has been used as the challenge dose of spores for studies on vaccines (Phipps and others 2004). However, prophylactic use of antibiotics to prevent anthrax in rhesus monkeys was reported following an exposure of about 8 LD_{50} of Vollum 1B spores (Friendlander and others 1993). This variability makes it very difficult to evaluate apparent differences in protection afforded from vaccines and the efficacy of therapeutics and sort out whether they result from variations in spore lots, spore strains, or monkey strains and substrains. The logic used to select challenge doses for studies on vaccine efficacy and therapeutic agents is also difficult to discern from available information.

Given the extent of variation in published LD_{50} values for anthrax, **the Committee recommends that unclassified data from mortality and natural-history-of-disease studies—including unclassified, unpublished data from USAMRIID—be published in the open literature for all agents[1].** In each case, the materials and methods section should include the source of the agent or toxin, the characterization of the agent or toxin (including the number of passages), and the media used to prepare the agent or toxin. It should also include the species, stock, and strain of the laboratory animal used. In the case of nonhuman primates, it is necessary to include the source and country of origin of the animals (Flick-Smith and others 2005). **If publication of past, current, and future studies in the open literature in detail is not feasible, an inclusive database should be established by the National Institute of Allergy and Infectious Diseases. All data from unclassified government-sponsored studies should be placed in this database[1].**

In the design of efficacy studies for new or potential countermeasures against bioterrorism agents, care needs to be taken to ensure that the outcomes are in some way related to defining, or supportive of defining, the mechanism of action of the countermeasure. Given that the route to approval by the FDA could be the Animal Rule (21 CFR 314 Subpart I and 21 CFR 601 Subpart H), the major requirements are: that efficacy be shown in more than one species; that the mechanism of action of the proposed countermeasure be understood or

[1] This recommendation is not intended to apply to research results or other data considered "sensitive but unclassified." Rather, the recommendation applies to data for which access would otherwise not be restricted that have not been published in a timely manner due to events such as personnel changes and changes in research priorities.

defined; that the indication chosen be related to the desired outcome in humans (i.e., survival); and that there be sufficient pharmacokinetic and pharmacodynamic data in animals to extrapolate to a dose for use in humans.

These requirements often constrain the ability of researchers to use "surrogate" markers as end points in the design of their efficacy studies—for example, reduced fever as an endpoint for countermeasure effectiveness would not be a good surrogate marker if the fever was not known to be the direct result of exposure to the agent. In contrast, if the agent was a microorganism and the countermeasure reduced the level of the organism in a biological system known to cause its transmission in vivo—e.g., reduction of the malarial parasite *P. berghei* in red cells following countermeasure administration—that could be a useful demonstration of efficacy based on an understanding of the mechanism of the disease. Such an approach also allows for dose-dependent efficacy studies, which are always useful in seeking licensure of a new countermeasure, as they can be used to estimate a dose of countermeasure that would cause a similar response in humans. In addition, the route of exposure is of less importance than the appearance of the agent in a biologically important site, so the ability to test a countermeasure against multiple routes of exposure is enhanced. Similar examples can be found for the development of countermeasures to viral infections.

Most of the preceding discussion has assumed that standard models of exposure are available for aerosolized agents—i.e., a fixed method for generation of an aerosolized agent and a set route of administration. Validation of such models across species is needed so that one is not comparing intranasal administration in one animal model with aspiration in another. This challenge, previously discussed, is important to address. Otherwise it will be difficult to convince the regulatory agency of comparable levels of agent and the ability of a countermeasure to provide an efficacious outcome that is understood at the pharmacological level in at least two species.

Although a variety of techniques exist for determining outcomes that either predict efficacy or measure it directly through the survival of animals following antidote administration, emerging technologies may offer more precise and less invasive ways to determine positive outcomes. The ability to noninvasively measure antidote concentrations via new imaging technologies for biodistribution studies, or the application of new human clinical technologies to measure efficacy of a drug at the cellular level (e.g., toxicogenomic or proteomic studies of lung tissue or lavage fluid as a predictor of a new antidote to prevent injury), offers great promise in the design of new antidote-evaluation studies.

6

Resource Issues

Beyond the technical considerations addressed in the preceding chapters of this report, a number of resource and regulatory issues also limit the development of appropriate animal models for countermeasures against aerosolized bioterrorism agents. These issues include personnel needs and training, infrastructure limitations on integrating advances in technology, and coordination with federal agencies. This chapter will consider these broad structural needs and some possibilities for addressing them, based in large part on discussions at the Animal Models for Testing Interventions Against Aerosolized Bioterrorism Agents Workshop.

PERSONNEL NEEDS AND TRAINING

Development and testing of animal models for countermeasures against aerosolized bioterrorism agents requires collaboration between the several different communities of scientists and clinicians with interests in aerosol models. Many of these professionals, however, have historically worked along parallel tracks. The infectious-disease and microbiology communities, for example, have long focused on the relevant diseases, but most experts in these areas lack the expertise, facilities, or interest to develop aerosol-inhalation techniques, say, to the necessary degree of rigor. Thus the characterization and standardization of the biological (both animal and microbial), aerosol, and dose-measurement properties of the countermeasures-development system requires experts in the diverse disciplines to understand each others' needs and terminologies and work together to advance the state of the art.

In general, the Committee envisions a team approach, in which each team member has sufficient general understanding of the others' disciplines to be able

to contribute usefully to the overall project. The Committee was impressed by the efforts made in this regard by some of the organizations (e.g., USAMRIID) represented at the July 2005 Workshop, and it commends these efforts. They illustrate what can be done when scientists with the appropriate combination of expertise collaboratively interact. The Committee also feels that building a community in this field in the near future is important, given the urgent needs and expectations in biodefense and the funding available from multiple agencies..

The various experts at the Workshop represented a cross-section of the skills that need to be brought together, their exchanges were highly informative, and these communications were welcomed by virtually all who participated. While that event was a salutary beginning, it was also apparent that further and continuing opportunities are needed to exchange information and to sustain the effort of building a broader community.

Accordingly, the Committee recommends:

- **sufficient cross-training of physical and biological scientists with expertise in the aerosol, infectious-disease, microbiology, and aerosol-medicine fields to facilitate their ability to collaborate productively;**
- **targeted ongoing opportunities for information exchange among these disciplines in order to encourage the formation of a community of researchers; and**
- **the development of interdisciplinary teams to collaborate closely in the long-term; these teams should include strong biostatistical support.**

Information exchange and community building could be facilitated through a consortium of scientific societies (such as the American Association for Aerosol Research, the American Society for Microbiology, the International Society for Aerosols in Medicine, and the Society of Toxicology, among others) to develop targeted meetings or joint sessions at appropriate professional meetings (such as the Emerging Infectious Diseases or Biodefense Research meetings organized by the American Society for Microbiology). Federal partners can also play a key role in building the community, as several agencies, including the Department of Health and Human Services, the Department of Homeland Security, the Department of Defense, the Environmental Protection Agency, the Department of Energy, and the Department of Agriculture, have interests in this area (the specific role of FDA will be discussed later in this chapter). Meanwhile, the National Institute of Allergy and Infectious Disease (NIAID) has an extensive program in biodefense, including academically based Regional Centers of Excellence (RCEs) in Biodefense and Emerging Infectious Diseases as well as Regional Biocontainment Laboratories (RBLs) that can help by serving as unifying platforms for training and research. Indeed, several of

these centers are already developing training in selected areas, including laboratory-safety and emergency-response requirements (Connel, J., personal communication, 2005).

In addition to the need to build a well-trained community, there are other personnel issues that can impede the development of this field, including the need for personnel protection and biosecurity. For example, vaccine availability for biomedical researchers is an issue. Although second-generation vaccines based on newer technologies are in the works or on the drawing board, none are generally available at this time. Moreover, the traditional anthrax vaccine is in short supply, while a previously licensed plague vaccine is no longer available in the United States. To add to the difficulty, it has generally been doubted that the previously licensed plague vaccine would be effective against aerosol exposure (Adamovicz and Andrews 2005). While these points only serve to emphasize the need for the kind of work on countermeasure development and validation that is the subject of this report, they also indicate some of the barriers to performing such work.

INFRASTRUCTURAL ISSUES

Biosecurity

Closely related to personnel problems is the matter of infrastructure support. Many of the aerosolized agents require high levels of personal protection, such as gloves, masks, and biocontainment in secured facilities. There are currently two components to laboratory biosecurity: (1) biosafety, which includes physical containment and safe handling of the agent; and (2) physical security. Biosafety precautions are typically ranked in terms of Biosafety Levels 1–4 (with 4 being the highest). They "consist of combinations of laboratory practices and techniques, safety equipment, and laboratory facilities"; and the recommended biosafety level for an agent "represent those conditions under which the agent ordinarily can be safely handled" (CDC and others 1999).

Work with select agents (see Table 6-1) imposes additional requirements for enhanced physical security of the laboratories and storage areas, for shipping of agents, and for controlled access. Such facilities therefore are specially designed and very expensive to construct and operate. Aerosol generation and exposure equipment, itself expensive and specialized, when used with the agents needs to be carefully adapted for containment and decontamination, further adding to the costs.

Animal Resources

The cost of the laboratory animals themselves is another important factor to be taken into account. As discussed elsewhere in this report, many investigators feel that the FDA Animal Rule will place more emphasis on using primates than

TABLE 6-1 Select Agents and Toxins Identified by the U.S. Department of Health and Human Services and U.S. Department of Agriculture

HHS Select Agents and Toxins

Abrin
Cercopithecine herpesvirus 1 (Herpes B virus)
Coccidioides posadasii
Conotoxins
Crimean-Congo haemorrhagic fever virus
Diacetoxyscirpenol
Ebola virus
Lassa fever virus
Marburg virus
Monkeypox virus
Reconstructed replication competent forms of the 1918 pandemic influenza virus containing any portion of the coding regions of all eight gene segments (Reconstructed 1918 Influenza virus)
Ricin
Rickettsia prowazekii
Rickettsia rickettsii
Saxitoxin
Shiga-like ribosome inactivating proteins
South American Haemorrhagic Fever viruses (Flexal, Guanarito, Junin, Machupo, Sabia)
Tetrodotoxin
Tick-borne encephalitis complex (flavi) viruses (Central European Tick-borne encephalitis, Far Eastern Tick-borne encephalitis, Kyasanur Forest disease, Omsk Hemorrhagic Fever, Russian Spring and Summer encephalitis)
Variola major virus (Smallpox virus)
Variola minor virus (Alastrim)
Yersinia pestis

USDA Plant Protection and Quarantine (PPQ) Select Agents and Toxins

Candidatus Liberobacter africanus
Candidatus Liberobacter asiaticus
Peronosclerospora philippinensis
Ralstonia solanacearum race 3, biovar 2
Schlerophthora rayssiae var *zeae*
Synchytrium endobioticum
Xanthomonas oryzae pv. *oryzicola*
Xylella fastidiosa (citrus variegated chlorosis strain)

USDA Select Agents and Toxins

African horse sickness virus
African swine fever virus
Akabane virus
Avian influenza virus (highly pathogenic)
Bluetongue virus (Exotic)
Bovine spongiform encephalopathy agent
Camel pox virus
Classical swine fever virus
Cowdria ruminantium (Heartwater)
Foot-and-mouth disease virus
Goat pox virus
Japanese encephalitis virus
Lumpy skin disease virus
Malignant catarrhal fever virus (Alcelaphine herpesvirus type 1)
Menangle virus
Mycoplasma capricolum/ M.F38/*M. mycoides Capri* (contagious caprine pleuropneumonia)
Mycoplasma mycoides mycoides (contagious bovine pleuropneumonia)
Newcastle disease virus (velogenic)
Peste des petits ruminants virus
Rinderpest virus
Sheep pox virus
Swine vesicular disease virus
Vesicular stomatitis virus (Exotic)

Overlap Select Agents and Toxins

Bacillus anthracis
Botulinum neurotoxins
Botulinum neurotoxin producing species of *Clostridium*
Brucella abortus
Brucella melitensis
Brucella suis
Burkholderia mallei (formerly *Pseudomonas mallei*)
Burkholderia pseudomallei (formerly *Pseudomonas pseudomallei*)
Clostridium perfringens epsilon toxin
Coccidioides immitis
Coxiella burnetii
Eastern Equine Encephalitis virus
Francisella tularensis
Hendra virus
Nipah virus
Rift Valley fever virus
Shigatoxin
Staphylococcal enterotoxins
T-2 toxin
Venezuelan Equine Encephalitis virus

small animal species; and in many other cases only nonhuman primate models are sufficiently characterized to use for aerosol studies. Unfortunately, while use of nonhuman primate models may sometimes be necessary, nonhuman primates are expensive and in short supply (Robinson and Beattie 2003). The National Institutes of Health has recognized that acute shortages in availability of nonhuman primates may hamper biomedical studies, and it is anticipating the establishment of more sources of these animals (Robinson and Beattie 2003). **The National Institutes of Health and other federal agencies involved in research utilizing nonhuman primates should coordinate their efforts to ensure adequate supplies of rhesus and cynomolgus macaques and other nonhuman primates.**

Though nonhuman primates are widely favored as test subjects in studies of aerosolized bioterrorism agents, they are often difficult to handle in high-containment laboratories; this description applies especially to macaques (Patterson and Carrion 2005). Requirements for social interactions, environmental enrichment, and other unique needs of nonhuman primates are also critical factors to be considered and adequately addressed. The number of research facilities that are capable of performing inhalation studies with these animals and meet these ancillary needs is quite limited. Programs to develop or expand inhalation research facilities may be needed to support bioterrorism studies. Because programs that focus entirely on the use of macaque models could prove extremely expensive, one interim solution may be the development of well-accepted and well-characterized alternative nonhuman primate models for these studies. At the Workshop, Dr. Leah Scott and her colleagues at the Defence Science and Technology Laboratory (Porton Down, United Kingdom) discussed the marmoset as an alternative nonhuman primate species with which they have worked successfully for the past two decades; and Dr. Louise Pitt and her colleagues at USAMRIID discussed their work in developing the vervet (African green monkey, Cercopithecus aethiops) model for pneumonic plague. An important common denominator was the amount of effort these investigators devoted to ensuring that the new animal model would be well characterized—a status that includes natural history (pathogenesis) studies, dose-response data, comparisons with past results (under comparable conditions) in other nonhuman primate species, and availability of needed reagents (such as those for immunological markers). Guidelines for validation, which need to include baseline pathogenesis and pathology studies, are therefore of great importance in animal-model development, and they are probably essential for any anticipated applications under the FDA Animal Rule. Such information is also of direct scientific value, lending additional insights into the host-pathogen interaction.

It is also ethically, scientifically, and economically prudent to obtain the greatest amount of feasible data, with the least possible stress to the animal, from each experiment. Several participants in the Workshop—including Dr. Scott, Dr. Pitt, and their colleagues—described how their animals were monitored (by whole-body plethysmography) during the experiments, as well as their subsequent use of remote telemetry to provide electrocardiogram,

electroencephalogram, electromyography, blood pressure, heart rate, body temperature, respiration, and activity data. Remote telemetry, coupled with a scoring system, is particularly promising for determining endpoints, thereby reducing distress among the animals and minimizing the handling of infected animals under containment conditions. As more advanced technologies become validated for assessing an animals' dose and physiological state, these technologies can be readily incorporated. For example, there was considerable discussion at the Workshop about methods to determine actual inhaled dose of agent. The traditional approaches involve calculations from input and chamber samples, or sacrificing some of the animals to determine actual numbers of the agent in the lung.

While these approaches are still necessary, recently developed technologies using bacteria with inserted light-emitting genes or other markers are making it possible to determine the number of inhaled bacteria by direct imaging in the living animal (Advance Research Technologies and GE Healthcare 2005; Contag and Bachmann 2002). At the moment, these imaging methods are more easily used with small animals than with larger species, but practical applications for larger species may well become available in the near future. This is but one example of a new advanced technology that has the potential to greatly improve the quality of animal-model data. It is likely that other new and equally useful technologies will become available in the foreseeable future. It is critical to develop suitable systems and resources for testing, validating, and rapidly incorporating useful new technologies as they become available.

Availability of Data and Materials

Given the limitations in expert personnel and resources, their collaborative use seems essential. Resource- and information-sharing also appear to the Committee as highly desirable ways to achieve greater leverage, reduce unnecessary duplication, and accelerate the process of developing new products. The Committee believes that effective data-sharing is one of the most critical (and potentially the most easily implemented) areas for immediate development. The Internet, after all, was originally created for just this purpose (Hafner and Lyon 1996). Information technology, already changing the way the biomedical community works, needs to be fully utilized in this regard.

For example, extensive data on the characteristics of many animal models used in testing countermeasures are not available in the published literature. Access to such data could prevent unnecessary duplication, allow researchers to compare results with different animal models, help determine the relative advantages and disadvantages of those models, assure consistency by standardizing techniques, and allow data to be pooled for more rapid determination of results. **Therefore, the Committee recommends that an easily searchable central database registry (or registries) on animal model data be established.** Determination of the exact data types and format is probably best left to a working group, but data could include standard operating

procedures, efficacy established on meta-analysis, comparative analysis of different models, and countermeasure and animal-model failures for each agent, among others.

The committee also recognizes that the infectious agent or toxin being studied should be well characterized—for example, it is very important that the strain being studied, together with its natural history, be well documented. **The Committee recommends the establishment of a repository, which can supply investigators studying a particular agent with a well-characterized sample of that agent. The American Type Culture Collection (ATCC) maintains such a repository, but additional information to facilitate comparisons of animal-model systems and ensure consistent results should be added.** There is also considerable precedent for reagent repositories, such as the AIDS Reagent Repository developed by NIAID.

AGENCY CONSIDERATIONS

Several federal agencies have major roles in biodefense—as scientific partners, regulators, or potential customers. Good interagency coordination is therefore highly desirable, as is good communication and collaboration between these agencies and biodefense researchers in government, academe, and industry.

Probably no federal agency has a more critical role in the development of new countermeasures against bioterrorism agents than the FDA, the principal U.S. regulatory agency for medical countermeasures. As discussed earlier in this report, researchers are hopeful about the use of the FDA Animal Rule (21 CFR 314 Subpart I and 21 CFR 601 Subpart H), which permits the agency to base its marketing-approval decision—of a candidate vaccine, therapeutic, or diagnostic for a bioterrorism agent—on submitted animal efficacy data when the countermeasure cannot otherwise be tested for efficacy in humans.

There are four general scientific requirements for submission of efficacy data under the Animal Rule:

> "1. There is a reasonably well-understood pathophysiological mechanism for the toxicity of the chemical, biological, radiological, or nuclear substance and its amelioration or prevention by the product;
> 2. The effect is demonstrated in more than one animal species expected to react with a response predictive for humans, unless the effect is demonstrated in a single animal species that represents a sufficiently well-characterized animal model (meaning the model has been adequately evaluated for its responsiveness) for predicting the response in humans;
> 3. The animal study endpoint is clearly related to the desired benefit in humans, which is generally the enhancement of survival or prevention or major morbidity; and

4. The data or information on the pharmacokinetics and pharmacodynamics of the product or other relevant data or information in animals and humans is sufficiently well understood to allow selection of an effective dose in humans, and it is therefore reasonable to expect the effectiveness of the product in animals to be a reliable indicator of its effectiveness in humans" (21 CFR 601.91a and 21 CFR 314.610a).

The committee believes that the Animal Rule and the apparent intent of the Rule—to provide flexibility for marketing approval of a new countermeasure against a bioterrorism agent—are laudable accomplishments and a major step forward in ensuring the nation's preparedness against bioterrorism attacks. Once the Animal Rule was passed in June 2002, it took less than a year for pyridostigmine bromide to receive FDA marketing approval as a prophylactic treatment against soman. Prior to the passage of the Animal Rule, the Army had been seeking FDA approval for pyridostigmine for more than 15 years (McNeil 2003).

Many obstacles in the FDA regulatory process still need to be addressed, however. The power of the Animal Rule is that the efficacy of a countermeasure can be demonstrated in a nonhuman species *whose responses mimic and are predictive of the disease process in humans*. However, the current lack of animal models is an impediment to gaining FDA approval; few animal models have been established that predict the human disease process associated with Category A select agents. This means that in addition to the efforts necessary to demonstrate the efficacy of a countermeasure, considerable time and resources will first need to be expended to establish the predictive value of the animal model.

In addition, the FDA approval process requires that an animal model mimic or predict the human response to an agent at the disease stage for which the countermeasure is expected to be used. For regulatory purposes, countermeasures are categorized in one of three ways—(1) as prophylactics (administered before an exposure; e.g., most vaccines); (2) as post-exposure prophylactics (administered after an exposure but prior to the onset of the disease process; e.g., antibiotics, antivirals, and some vaccines); or (3) as symptomatic treatments (e.g., antibiotics and antivirals) (FDA 2002). Unfortunately, an acceptable animal model of exposure is not necessarily an acceptable model of the disease process. In the case of inhalational anthrax, there is a proposed rhesus macaque model for testing post-exposure prophylactics (FDA 2002); however, the disease progression and many of the clinical manifestations of inhalation anthrax in humans differ from those of the rhesus macaque (Vasconcelos and others 2003; Shafazand and others 1999; Ivins and others 1998; Zaucha and others 1998; Fritz and others 1995; Friedlander and others 1993). Therefore the FDA may or may not accept efficacy data for symptomatic treatments tested in the macaque model. Though efforts are being turned toward development and characterization of other

animal models, there is considerable concern that animal models acceptable to FDA cannot be developed for all of the select agents.

The lack of acceptable animal models, if that turns out to be the case, could be a significant impediment to the creation of new countermeasures against bioterrorism agents. Without an acceptable animal model, there would be no pathway for achieving FDA approval of these countermeasures through the Animal Rule, thereby discouraging pharmaceutical industry efforts in this area. Already, the market for countermeasures against bioterrorism agents is considered modest, there are product-liability issues, and the cost of research, development, and testing of antibiotics—which have a 90-percent failure rate during that process—is high (Cassell 2002).

However, countermeasures that cannot be approved through the Animal Rule because of a lack of acceptable model can be approved through the Emergency Use of an Investigational New Drug (IND) Rule (21 CFR 312). Under this rule, in an emergency situation the FDA may authorize use of an unapproved drug for specified use without submission of an IND. This authorization hinges on the declaration of a domestic emergency by the Secretary of Homeland Security, a military emergency by the Secretary of Defense, or a public health emergency by the Secretary of Health and Human Services. This rule is particularly advantageous when a drug that has already received FDA marketing approval for use against another disease or condition needs emergency-use authorization. In that case, safety has already been established for the drug and the focus of emergency approval is on indications of efficacy.

Though countermeasures that have not previously received FDA marketing approval can be approved through the emergency-use authorization rule, it does not remove the fiscal barriers to the pharmaceutical industry's development of novel countermeasures. Companies will not likely invest resources in researching and developing a novel countermeasure that may win FDA approval only if an emergency situation has been declared. Similarly, regarding countermeasures that have already received marketing approval, there is little incentive for companies to perform extensive efficacy testing for uses against bioterrorism agents.

The practical implications of implementing the Emergency Use Rule also need to be considered. In the event of an emergency, will the recommended safety, efficacy, manufacturing, and alternative products data, discussions of risks and benefits, fact sheets for health care providers and recipients, and proposed labeling all be available for submission to the FDA in a matter of hours or days? If symptomatic patients are the first indication that a bioterrorism event has occurred, will there be sufficient time for the FDA to perform a review of the submitted data and information? Finally, it seems unlikely that a pharmaceutical company would manufacture and store an investigational drug in quantities sufficiently large enough to address a national bioterrorism incident. There may be a role for the U.S. Centers for Disease Control and Prevention and U.S. Department of Health and Human Services—which maintain and manage

the National Strategic Stockpile—in addressing this particular issue, in terms of working with the FDA to focus new product development in areas of critical national need.

Further, as both the Animal Rule and Emergency Use Rule are relatively new, there is very limited experience with approvals. For example, as of September 2005, only pyridostigmine bromide had been approved for a new label indication by use of the Animal Rule. **The Committee therefore recommends that the FDA work with investigators to draft and finalize practical guidelines to help applicants ensure that they can meet the approval requirements.**

Though the Committee focused most of the report on technical and methodological issues, the resource and regulatory issues outlined in this chapter can hamper or facilitate progress. Access to information on animal models and previous research, as well as access to well characterized samples of agents, will directly affect decisions regarding the experimental design of countermeasure testing. In addition, collaboration between the research community and the FDA regarding the scientific requirements of the Animal Rule could increase the rate at which new countermeasures are tested and approved. The Committee recognizes that the FDA is in a critical position to help advise researchers involved in countermeasure testing, and the agency appears willing to serve this vital role.

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About the Authors

Charles H. Hobbs (Chair) is Director of Toxicology at Lovelace Respiratory Research Institute in Albuquerque, N.M. He is also Vice-President of the Lovelace Biomedical and Environmental Research Institute. Dr. Hobbs' primary research interests are the long-term biological effects of inhaled materials and the mechanisms by which they occur. His experience covers inhaled nuclear and chemical toxicants as well as infectious diseases, with research in such areas as physical and chemical characterization of airborne toxicants, in vitro mechanistic and toxicologic studies, and long-term studies in laboratory animals (particularly regarding the relationships between dose to critical tissues and resulting biological effects and the active mechanisms in determining these relationships). Dr. Hobbs has also been heavily involved in research management, focusing on the direction and use of multidisciplinary teams to address complex problems. He received his D.V.M. with high distinction at Colorado State University.

David C. Dorman is Director of the Biological Sciences Division at CIIT Center for Health Research in Research Triangle Park, N.C. (CIIT was previously known as the Chemical Industry Institute of Toxicology). Dr. Dorman is nationally recognized for his research on the nasal toxicity, neurotoxicology, and pharmacokinetics of inhaled chemicals. He conducted studies to evaluate the pharmacokinetics of inhaled methanol in normal and folate-deficient monkeys, for example, and to determine the neuroteratogenic effects of methanol in rodents. He is an adjunct professor of toxicology at North Carolina State University, Duke University, and the University of North Carolina. Dr. Dorman holds a Ph.D. in Veterinary Biosciences/Toxicology from

the University of Illinois (Urbana-Champaign) and a D.V.M. from Colorado State University.

Diane E. Griffin is Professor and Department Chair of Molecular Microbiology and Immunology at the Johns Hopkins Bloomberg School of Public Health. Griffin is a world leader in the study of the pathogenesis of viral infections, the viral determinants of virulence, and the host responses to infectionviral pathogenesis. She has elucidated mechanisms that control Sindbis virus neurovirulence, and her pioneering work on measles virus has revealed the bases of the profound immunosuppression caused by measles infection and of the development of severe atypical measles. She was elected both to the Institute of Medicine and the National Academy of Sciences in 2004. She currently serves as a member of the Editorial Board for *Proceedings of the National Academy of Sciences* and on the Committee on Defense Intelligence Agency—Technology Forecasts and Reviews. Dr. Griffin holds a Ph.D. and M.D. from Stanford University.

Jack R. Harkema is Director of the Laboratory for Experimental and Toxicologic Pathology in the Department of Pathobiology and Diagnostic Investigation, College of Veterinary Medicine, at Michigan State University. He is also Director of AirCARE 1 (Mobile Air Research Lab). Dr. Harkema's primary research interests are the cellular and molecular mechanisms responsible for the pathogenesis of airway epithelial injury, adapation, and repair after exposure to inhaled toxicants. His team is investigating the roles of inflammatory cells and their mediators in the pathogenesis of airway epithelial alterations in the upper and lower respiratory tract after exposure to inhaled xenobiotic agents. He holds a D.V.M. from Michigan State University and a Ph.D. in comparative pathology from the University of California, Davis. He currently has an NIH grant to study mechanisms of species-dependent environmental lung injury and has done research on rodents, dogs, and nonhuman primates.

Beth L. Laube is Associate Professor of Pediatrics at the Johns Hopkins University School of Medicine. Her research focuses on the relationship between particulate distribution within the human lung and its response to inhaled allergens, nonspecific stimuli, and aerosolized medications. Her approach involves in vivo quantification of the deposition and removal of particulates in healthy and diseased lungs using radiolabeled aerosols and gamma scintigraphy. Computer analyses of scintigraphic images of the lungs following the inhalation of radioaerosols provide assessments of deposition pattern and mucociliary clearance. These radioimaging assessments can be combined with functional measurements of changes in airway responsiveness to provide a new method for assessing the efficacy of a variety of inhaled medications that are administered to the lung as the target organ or through the lung with the systemic circulation as the target. The principles that are basic to

this approach are being applied to preventing and treating asthma, cystic fibrosis, dysphagia, and diabetes. Dr. Laube holds a Ph.D. from the Johns Hopkins Bloomberg School of Public Health. She is currently President of the International Society of Aerosols in Medicine and advises the WHO Product Development Group on the aerosolized measles vaccine project.

David E. Lenz is Research Chemist and Team Leader at the U.S. Army Medical Research Institute of Chemical Defense (USAMRICD). He is responsible for a research program to develop human enzymes capable of acting as novel prophylactic drugs for protection against chemical warfare nerve agents. He also coordinates a molecular biology project for biological scavengers against nerve agents. Previously, Dr. Lenz served as Team Leader in the Biochemical Pharmacology Branch, researching the pharmacokinetics of soman in animals; his was to develop a model to allow interspecies extrapolation of pharmacological data. He serves as Chairman of the NATO Technical Group on Prophylaxis and Therapy Against Chemical Agents and Chairman of an Integrated Product Team for pyridostigmine bromide; and he sat on the Scientific Advisory Panel to the EPA on Cholinesterases. He holds a Ph.D. in chemistry from Boston University.

Stephen S. Morse is Founding Director of the Center for Public Health Preparedness at the Mailman School of Public Health of Columbia University and is Associate Professor in the Epidemiology Department. He also holds an adjunct faculty appointment at the Rockefeller University. Dr. Morse returned to Columbia in 2000 after four years in government service as Program Manager at the Defense Advanced Research Projects Agency of the Department of Defense. In that position, he co-directed the Pathogen Countermeasures program and subsequently directed the Advanced Diagnostics program. Dr. Morse was Chair and principal organizer of the 1989 NIAID/NIH Conference on Emerging Viruses and has served as an adviser to the World Health Organization, the Pan-American Health Organization, CDC, FDA, and other agencies. He was the founding Chair of ProMED (the nonprofit international Program to Monitor Emerging Diseases) and was one of the originators of ProMED-mail, a network inaugurated by ProMED in 1994 for outbreak reporting and disease monitoring using the Internet. He currently serves on the Steering Committee of the IOM's Forum on Emerging Infections and was previously a member of other IOM committees. Dr. Morse received his Ph.D. from the University of Wisconsin-Madison.

Robert Franklynn Phalen directs the Air Pollution Health Effects Laboratory at the University of California, Irvine (UCI). He also holds two academic appointments in the School of Medicine at UCI: Professor in the Department of Community and Environmental Medicine; and Professor in the Center for Occupational and Environmental Health. His research is in several areas, including: lung modeling for predicting doses from inhaled particles; lung

morphometry for growing mammals; health effects of inhaled air pollutants; and applied aerosol physics. In 1971, he obtained a Ph.D. in biophysics, with specialization in inhalation toxicology, from the University of Rochester. His postdoctoral research in aerosol physics and inhaled-particle deposition modeling was conducted at the Inhalation Toxicology Research Institute (now the Lovelace Respiratory Research Institute) in Albuquerque, N.M. In 1972, Dr. Phalen joined the College of Medicine at UCI to establish the Air Pollution Health Effects Laboratory, which conducts studies relating to the toxicology of air pollutants. He has served on the editorial boards of three scientific journals and as an editor of one. He has published over 100 scientific papers, authored a book titled *Inhalation Studies: Foundations and Techniques* (CRC Press, 1984), edited another book on a similar topic (CRC Press, 1996), and authored *The Particulate Air Pollution Controversy* (Kluwer Academic Publishers, 2002). Dr. Phalen has organized four major international scientific conferences on the health effects of particulate air pollution. He recently received the Public Education Award from the California Biomedical Research Association and the Career Achievement Award from the Inhalation Specialty Section of the Society of Toxicology. He is a Fellow of the Academy of Toxicological Sciences.

Appendix A

PUBLIC WORKSHOP AGENDA

July 6-7, 2005
National Academy of Sciences Building
2101 C St., NW Washington D.C.

Wednesday, July 6, 2005

8:30 – 9:00 am	**Chuck Hobbs**, Lovelace Respiratory Research Institute Charge to the Committee Goals of the Workshop
9:00 – 9:40 am	**Michael Moodie**, Chemical and Biological Arms Control Institute Issues Related to the Biological Weapons Convention
9:40 – 10:20 am	**Lew Schrager**, FDA Issues Related to Animal Rule
10:40 – 11:20 am	**Charles Plopper**, UC Davis Comparative Anatomy for Various Animal Models
11:20 – 12:00 pm	**Joe Mauderly**, Lovelace Respiratory Research Institute Comparative Lung Physiology for Various Animal Models

1:00 – 1:40 pm	**Leah Scott**, DSTL, Porton Down, UK Making Biodefense Products Happen: Emerging models
1:40 – 2:20 pm	**Gareth Griffiths**, DSTL, Porton Down, UK Making Biodefense Products Happen: Correlates of protection
2:20 – 3:00 pm	**Victor DeGruttola**, Harvard University Statistical Issues in Validating Surrogate Endpoints in Clinical Trials
3:10 – 3:50 pm	**Eric Harvill**, Pennsylvania State University Pathogenesis of Bordetella
3:50 – 4:30 pm	**Maryna Eichelberger**, Virion Systems, Inc. Pathogenesis of Respiratory Viruses
4:30 – 5:30 pm	**Panel Discussion**

Thursday, July 7, 2005

8:30 – 8:35 am	**Jack Harkema** (committee member), Michigan State University Review of Day 1 and Overview of Day 2
8:35 – 9:15 am	**Mark Hernandez**, University of Colorado Characterizing Microorganisms in Aerosols
9:15 – 9:55 am	**Brian Wong**, CIIT Considerations in the Generation and Characterization of Bioaerosols
9:55 – 10:35 am	**Beth Hutchins**, Schering Plough Biopharma Standardizing Agent Characteristics between Labs: The adenovirus reference material model
10:45 – 11:25 am	**Chad Roy**, USAMRIID Inhalation Exposure Systems
11:25 – 12:05 pm	**Mike Foster**, Duke University Routes of Exposure for Bioaerosols
12:40 – 1:20 pm	**Louise Pitt**, USAMRIID Biology of Appropriate Dose in Animals vs. Humans

1:20 – 2:00 pm	**Chris Gennings**, Virginia Commonwealth University Experimental Design and Statistical Approaches to Dose-response
2:00 – 2:40 pm	**Richard Corley**, Pacific Northwest National Laboratory Modeling/imaging of Airways
2:40 – 3:20 pm	**Robert Phalen**, University of California, Irvine Particle-deposition Patterns
3:20 – 4:00 pm	**Panel Discussion**

Adjourn

BIOGRAPHICAL INFORMATION ABOUT THE SPEAKERS

Richard A. Corley, a Staff Scientist for Biological Monitoring and Modeling in the Environmental Technology Directorate of the Pacific Northwest National Laboratory, received his Ph.D. in environmental toxicology from the University of Illinois in 1985. Prior to joining PNNL in 1996, he spent 11 years at the Dow Chemical Company's Toxicology Research Laboratory. While at Dow, he functioned as the Technical Group Leader of the chronic toxicology laboratory and inhalation toxicology laboratory and served as a toxicology advisor for several industrial research organizations. Dr. Corley's general research interests are the development of physiologically based pharmacokinetic and pharmacodynamic models of environmental/industrial chemicals and these models' applications in human health risk assessment. Current research activities include: the development of three-dimensional computational fluid-dynamics models of the respiratory system for gas, vapor, and particulate dose-response realtionships; kinetics and mechanisms of action of industrial chemicals; dermal and respiratory bioavailability of volatile organics; and development of models for embryo/fetal dosimetry. Dr. Corley has served on numerous workshops or advisory panels for the National Academy of Sciences, National Institutes of Health, Environmental Protection Agency, American Chemistry Council, Agency for Toxic Substances and Disease Registry, and International Life Sciences Institute, and he has written over 160 peer-reviewed publications, book chapters, technical reports, and published abstracts in pharmacokinetic modeling and toxicology.

Victor DeGruttola is Professor of Biostatistics at the Harvard School of Public Health. His research activities focus on the development of statistical methods required for appropriate public health response to the AIDS epidemic, and he has worked in particular on transmission of the human immunodeficiency virus (HIV), natural history of infection with HIV, and clinical research on AIDS therapies. These efforts involve not only statistical methodology but also public health surveillance systems, medical issues surrounding HIV infection, and concerns of communities most affected by the epidemic; and their goals include forecasting future AIDS incidence, developing strategies for clinical research on HIV infection, and evaluating the public health impact of antiviral treatment. The statistical issues in which Dr. DeGruttola has been engaged include evaluating the degree to which the treatment response of markers of HIV infection constitute adequate evidence for clinical efficacy; and he has also worked on projections of AIDS incidence using data from the New York City Department of Health. A special focus of this activity was estimation, using data combined from a variety of sources, of the risk that children of HIV-infected mothers would develop AIDS in the first 10 years of life.

Maryna Eichelberger of Virion Systems, Inc. (Rockville, Md.) uses the cotton rat as animal model to study respiratory viral pathogenesis and immunity. She is also an adjunct assistant professor in the Department of Microbiology and Immunology at the Uniformed Services University in Bethesda, Md. Dr. Eichelberger received her B.Sc. and M.Sc. degrees from the University of Natal, Republic of South Africa, and her Ph.D. from the University of Alabama at Birmingham. There she studied the structure and function of influenza virus neuraminidase in the laboratory of Gillian Air, and she continued her interest in viral immunity as a postdoctoral fellow with Peter Doherty at St. Jude Children's Research Hospital in Memphis, Tenn. Dr. Eichelberger was an assistant professor in the Department of International Health at the Johns Hopkins Bloomberg School of Public Health before taking a position in the biotech industry. Her primary interests are in the development of vaccines to prevent—and therapeutic agents to treat—diseases caused by respiratory viral pathogens.

W. Michael Foster, a Research Professor of Medicine in the Division of Pulmonary and Critical Care Medicine at Duke University Medical Center, received his Ph.D. in physiology from New York University. Dr. Foster's laboratory performs research on humans and animal models and investigates the biological effects of inhalational hazards (particulate and gases) on airway and parenchymal lung tissues. These efforts focus on barrier function of the respiratory epithelial membrane—the primary location at which inhaled gases and particulate initially impinge upon lung tissue. Imaging and exposure techniques developed for human study have also been integrated with initiatives that utilize lab models. Dr. Foster's specific areas of interest and expertise include: (1) in vivo tissue response of the lung (human and animal model) using inhalation of inert, sterile, radiolabeled test particles and noninvasive radioimaging techniques—an approach essential to characterizing the epithelial barrier system of the airway surface; and (2) effects of oxidant-type air pollution (e.g., ozone at ambient urban concentrations) on lung epithelial membrane physiology.

Chris Gennings is a Professor in the Department of Biostatistics at Virginia Commonwealth University (Richmond, Va.) and a founding Principal in Solveritas, LLC, a company focusing on analysis/assessment of chemical mixtures. She has been working on statistical issues associated with chemical mixtures for almost 20 years, with funding sources that include the U.S. Army Medical Research Institute of Chemical Defense, Health Effects Institute, Environmental Protection Agency, National Institutes of Health, and World Health Organization. Dr. Gennings is the Principal Investigator on a training grant from the National Institute of Environmental Health Sciences, titled "The Integration of Chemical Mixtures Toxicology and Statistics," that fully supports five doctoral students in her department who are developing statistical methods

associated with mixtures issues. Her research goals include bridging the gap between statistical methods development and their applications and use in real world problems.

Eric Harvill is an Assistant Professor of Microbiology and Infectious Disease at Pennsylvania State University. His primary research interest is in the interactions between bacterial pathogens and the host immune system, and his group investigates the molecular bases for bacterial virulence factors and host immune functions using the tools both of bacterial genetics and mouse molecular immunology. They focus on bordetella bacteria, which are highly infectious, cause a range of respiratory diseases, and persist for the life of the animal despite an active immune response. These characteristics are indicative of a highly evolved bacterium-host interaction. Dr. Harvill has studied basic immunology since earning his Ph.D. at the University of California, Los Angeles.

Mark Hernandez is Associate Professor of Environmental Engineering at the University of Colorado, Boulder. His research interfaces classical industrial hygiene and sanitary engineering with recent advances in molecular biology to study airborne primary biological materials and the microbial ecology of aerosols under in situ conditions. Dr. Hernandez teaches courses on introductory environmental engineering, wastewater engineering, and applied environmental microbiology.

Beth Hutchins is Director of Process Sciences at Schering Plough Biopharma (formerly Canji, Inc., and DNAX Research Institute). SP Biopharma is Schering Plough's center of excellence for discovery research and early development of biologics, including gene therapy, monoclonal antibodies, and recombinant proteins. Dr. Hutchins received her Ph.D. in molecular biology from Washington University in St. Louis in 1982 and was a postdoctoral fellow at Stanford University, where she studied xenotransplantation of islets for diabetes. Dr. Hutchins then worked for Syva Company (the diagnostic arm of Syntex Corp.) for five years prior to moving to Genentech in 1988. There she was responsible for immunochemistry-based analytical methods for recombinant protein development; her primary contributions were the methods supporting development of Herceptin®. Dr. Hutchins joined Canji, Inc., in San Diego in 1993 as Director of Quality and Analytical Sciences, responsible for setting up the Quality Assurance Unit and developing analytical-methods support for product candidates. Today, as part of SP Biopharma, her department is responsible for production and characterization of preclinical materials, preclinical sample analyses, and development of process and analytical methods to support new-product candidates and characterize research reagents.

Joe L. Mauderly is Vice President of the Lovelace Respiratory Research Institute (LRRI) in Albuquerque, N.M.; President of one of its subsidiaries, the

Lovelace Biomedical and Environmental Research Institute; and Director of one of its research programs, the National Environmental Respiratory Center. He was Director of the Inhalation Toxicology Research Institute before its merger with LRRI. After receiving his Doctor of Veterinary Medicine degree from Kansas State University and brief periods in clinical practice and the U.S. Air Force, Dr. Mauderly specialized in research on comparative respiratory physiology, comparative pulmonary responses to inhaled toxicants, and the human health hazards of materials inhaled in the workplace and environment. During the past decade his research has focused on disentangling the physical-chemical interactions of complex mixtures of air contaminants, including engine emissions, that cause health effects. He has authored or co-authored over 250 articles, chapters, and books, and published technical reports. Dr. Mauderly is on the editorial boards of *Inhalation Toxicology, Experimental Lung Researc*h, and *Environmental Health Perspectives*. He is a member of the Particulate Matter Panel of the Clean Air Scientific Advisory Committee and the National Research Council's (NRC) Committee on Changes in New Source Review Programs for Stationary Sources of Air Pollutants. He also serves on the advisory committees of several research centers and programs. Dr. Mauderly is an Adjunct Professor of Medicine at the University of New Mexico and a member of the joint LRRI/UNM NIEHS Environmental Health Center's Environmental Lung Disease Research Core and Internal Advisory Committee. Among his past appointments, he was Chairman of the Clean Air Scientific Advisory Committee of the EPA Science Advisory Board, Chairman of the NRC Committee to review the NARSTO review of particulate matter science, member of the NRC Committee on Research Priorities for Airborne Particulate Matter, Chairman of the Environmental and Occupational Health Assembly of the American Thoracic Society, President of the Inhalation Specialty Section of the Society of Toxicology, member of the Research Committee of the Health Effects Institute, Chairman of the Air Pollution Health Advisory Committee of the Electric Power Research Institute, and Associate Editor of *Fundamental and Applied Toxicology*.

Michael Moodie is co-founder and President of the Chemical and Biological Arms Control Institute. In this capacity, he is responsible for all aspects of the Institute's activities, including oversight of programs, design and implementation of projects, outreach, administration, and publications. In government, Mr. Moodie served from 1990 to 1993 as Assistant Director for Multilateral Affairs at the U.S. Arms Control and Disarmament Agency (ACDA), where he was responsible for, among other issues, chemical and biological arms control. He has also served as Special Assistant to the Ambassador and Assistant for Special Projects at the U.S. Mission to NATO. In the policy research community, Mr. Moodie has held senior research positions at the Foreign Policy Research Institute, the Institute for Foreign Policy Analysis, and the Center for Strategic and International Studies, where he was also Senior Advisor to the President. He has been a Visiting Lecturer at Georgetown

University's School of Foreign Service and a consultant to the President's Foreign Advisory Board, the U.S. Navy, and ACDA.

Robert Franklynn Phalen directs the Air Pollution Health Effects Laboratory at the University of California, Irvine (UCI). He also holds two academic appointments in the School of Medicine at UCI: Professor in the Department of Community and Environmental Medicine; and Professor in the Center for Occupational and Environmental Health. His research is in several areas, including: lung modeling for predicting doses from inhaled particles; lung morphometry for growing mammals; health effects of inhaled air pollutants; and applied aerosol physics. In 1971, he obtained a Ph.D. in biophysics, with specialization in inhalation toxicology, from the University of Rochester. His postdoctoral research in aerosol physics and inhaled-particle deposition modeling was conducted at the Inhalation Toxicology Research Institute (now the Lovelace Respiratory Research Institute) in Albuquerque, N.M. In 1972, Dr. Phalen joined the College of Medicine at UCI to establish the Air Pollution Health Effects Laboratory, which conducts studies relating to the toxicology of air pollutants. He has served on the editorial boards of three scientific journals and as an editor of one. He has published over 100 scientific papers, authored a book titled *Inhalation Studies: Foundations and Techniques* (CRC Press, 1984), edited another book on a similar topic (CRC Press, 1996), and authored *The Particulate Air Pollution Controversy* (Kluwer Academic Publishers, 2002). Dr. Phalen has organized four major international scientific conferences on the health effects of particulate air pollution. He recently received the Public Education Award from the California Biomedical Research Association and the Career Achievement Award from the Inhalation Specialty Section of the Society of Toxicology. He is a Fellow of the Academy of Toxicological Sciences.

Louise Pitt is Director of the Center for Aerobiological Sciences at the U.S. Army Medical Research Institute of Infectious Diseases. She earned her Ph.D. from the University of Witwatersrand, South Africa.

Charles Plopper is Professor of Anatomy, Physiology, and Cell Biology at the University of California, Davis. The goal of his research is to identify and characterize the cellular mechanisms that define the response of the respiratory system to environmental toxicants. Research efforts have defined the subpopulations of pulmonary cell phenotypes that are susceptible to specific model compounds, and the laboratory work has been carried out on a microenvironment-specific basis that clearly defines the heterogeneity in the response of different subpopulations of each cellular phenotype to short-term exposure. Two related research projects investigate postnatal development of the respiratory system (specifically the Clara cells) and the development of an investigative strategy that will allow characterization of cellular and metabolic processes involved in cell-specific lung toxicity in situ in local environments.

Chad Roy is in the Center for Aerobiological Sciences at the U.S. Army Medical Research Institute of Infectious Diseases. He earned his Ph.D. in inhalation toxicology from the University of Iowa in 1999.

Lewis K. Schrager is a Lead Medical Officer in the Division of Counterterrorism, within the Office of Counterterrorism and Pediatric Drug Development of the FDA's Center for Drug Evaluation and Research. He oversees a research portfolio that includes support for: studies of antibiotic efficacy against pneumonic plague in an African green monkey model, in collaboration with the U.S. Army Research Institute of Infectious Diseases and the National Institute of Allergy and Infectious Diseases (NIAID); and studies of gentamicin efficacy against naturally occurring human plague, in collaboration with the Centers for Disease Control and Prevention. Dr. Schrager is a board-certified internist and infectious-disease specialist. Prior to joining the FDA he served for nearly 10 years with NIAID's Division of AIDS. He received his B.A. from Johns Hopkins University and his M.D. degree from Vanderbilt University Medical School. Dr. Schrager served as a resident in internal medicine at the University Hospital/Bellevue Medical Center in New York City; and he was an infectious-disease fellow at the Harvard University Joint Fellowship in Infectious Diseases and at the Albert Einstein College of Medicine/Montefiore Medical Center.

Leah Scott is in the Biomedical Sciences Department at the Defence Science and Technology Laboratory at Porton Down, United Kingdom. She was awarded the GlaxoSmithKline laboratory animal welfare prize for developing and championing remote monitoring of laboratory animals, particularly primates.

Brian Wong is Senior Research Investigator in the Division of Biological Sciences and a Supervisor of Inhalation Services at the CIIT Centers for Health Research (CHR). His research in inhalation toxicology has included: development of specialized equipment for conducting inhalation studies; and programming for the automation of exposure control and data collection. He has a special interest in the application of aerosol-science technology to study the deposition and retention of aerosols in the respiratory tract, especially to validate mathematical models under development at the CIIT CHR. He earned his Ph.D. in environmental engineering science from the California Institute of Technology.