Chapter 29

AEROBIOLOGY: HISTORY, DEVELOPMENT, AND PROGRAMS

DOUGLAS S. REED, PhD*; AYSEGUL NALCA, MD, PhD†; and CHAD J. ROY, PhD‡

INTRODUCTION

CONCEPTUAL BASIS OF AEROBIOLOGY IN INFECTIOUS DISEASE

OFFENSIVE BIOLOGICAL DEVELOPMENT AND CLINICAL APPLICATIONS IN THE UNITED STATES
Camp Detrick, Black Maria, and the US Army Medical Unit (1954–1970)
Clinical Exposure Trials: Operation Whitecoat (1954–1973)

BIOLOGICAL AEROSOL EXPOSURE SYSTEMS
Exposure Systems
Sampling
Monitoring and Control
Dosimetry
Parameters Impacting Aerosol Dosimetry

SUMMARY

*Associate Professor, Aerobiological Manager, RBI, Department of Immunology, University of Pittsburgh, 3501 Fifth Avenue, Pittsburgh, Pennsylvania 15261; formerly, Microbiologist, Center for Aerobiological Sciences, US Army Medical Research Institute of Infectious Diseases, 1425 Porter Street, Fort Detrick, Maryland

†Chief, Department of Animal Studies, Center for Aerobiological Sciences, US Army Medical Research Institute of Infectious Diseases, 1425 Porter Street, Fort Detrick, Maryland 21702

‡Associate Professor, Department of Microbiology and Immunology, Tulane School of Medicine, 1430 Tulane Avenue, New Orleans, Louisiana 70112; and Director, Infectious Disease Aerobiology, Division of Microbiology, Tulane National Primate Research Center, 18703 Three Rivers Road, Covington, Louisiana 70433

INTRODUCTION

The concept of using inhaled infectious particles as biological weapons is not new. The significance of this route was first appreciated and truly understood in the early 20th century, although the concept of infection by inhalation has been intermittently influenced by the study of infectious disease epidemiology. The question of whether diseases are "air-caused" has had, in past centuries, ardent believers and equally passionate cynics. Historically, for example, the prevailing theory was that all infections originated from "miasma," or contaminated air. The cyclic nature of disease transmitted by aerosol among people living in groups is described in basic terms in ancient preserved artifacts. The Smith papyrus, dating from 1600 BCE and held in the Field Museum of Natural History in Chicago, describes prayers recited to gods of disease to purify the "winds" of the "pestilence of the time."[1] Epidemics were thought to be transmitted by aerosol even in the early days of medical science. Around 400 BCE, Hippocrates dictated that "airs, waters, and places" directly influenced the health of people, and he used the knowledge of seasonal change to guide diagnosis of differing ailments. In the Middle Ages, few pathogens impacted understanding of epidemic spread of disease as *Yersinia pestis*, the causative agent of the "black death." At-risk populations eventually learned that the only defense against infection and death was to avoid contact with victims dying or dead from the bacterial disease. Pneumonic plague, the corollary form of infection from an infected host, is now recognized to transmit from expectoration of respiratory droplets. People may have unwittingly avoided respiratory exposure to aerosolized *Y pestis* by avoiding contact with infected hosts and thereby not contracting the most feared (and deadly) form of the bacterial disease.

Advancements in the field of chemistry in the 19th century gave rise to the concept of miasmic theory of disease. Sir Edwin Chadwick (1850) in Britain advanced the public health practices associated with the avoidance of the malodorous vapors to preserve the overall health of at-risk populations. The concept of spontaneous generation of disease-causing agents in vapors, however, was countered and ultimately refuted by Louis Pasteur (1860) during the same era. Pasteur demonstrated the presence of living organisms that was the root cause of fermentation and decomposition. His work in this area was instrumental in the understanding that infection could only appear miasmic if airborne microorganisms were present. By the end of the 19th century, most communicable bacterial pathogens had been identified, and there were only a few effective airborne agents. At roughly the same time, seminal work in vector-borne disease, including demonstration of parasitic disease cycles for malaria and filariasis, further improved public health measures and reduced disease burden. The concept that the majority of disease agents affecting large numbers of people were food- or water-borne greatly minimized aerosol transmission as an important pathway of infection.

In the early 20th century, it was also shown that respiratory droplets from diseased individuals, never traveling more than an arm's length from the infected person, could readily transmit disease. The theory of large-droplet infection, coupled with the recognition that arthropods were vectors for disease, nearly negated the respiratory route of infection from consideration in natural endemic spread of disease.[2] It was not until the great influenza pandemic of 1917–1918 that airborne disease transmission was again considered a medically important infection route. The notion that near-instant dehydration takes place in the environment once numerous particles are expelled from an infected host, causing submicron infectious biological aerosols to "float" for hours, solidified the concept of ecological transport from an infected host to an otherwise naïve host and ultimately successful disease transmission. A more modern understanding of airborne contagion also dictated that the probability and rate of disease transmission through air differs from, for example, a contaminated well.[3] Although the number and tempo of infections from a contaminated well are horizontal, arithmetic, and limited to the number of interactions with a single source, airborne disease transmission is truly a vertical and geometric process and is not limited to interactions with one infected source, but rather the general vicinity of one or many infection sources.[4] The indoor environment that now comprises most of the modern world amplifies probability of vertical transmission from airborne pathogens.

There is a sharp distinction between naturally communicable airborne disease and those that are artificially induced through human-made biological aerosols. Modern military and ancillary industrial development activities, primarily associated with offensive biological weapon development in the 20th century, exploited the characteristics of aerosols that would promote maximum potential impact upon enemies.[5] This was primarily achieved by modern and sophisticated manipulation of the particulars, such as particle size distribution and environmental dehydration, to assure successful delivery to the respiratory system of the target host population. An early scientific

concept in the process of designing and producing biological aerosols as modern weapons was the research and understanding of naturally occurring airborne disease. A basic, empirically derived understanding of natural epidemics from human source generators (respiratory expectoration) and indirect sources (eg, fomites on bed sheets) was essential to better appreciate important environmental and physiochemical factors when designing biological aerosols. It was soon recognized that airborne infection, when left up to the natural transmission process, was an overwhelmingly variable process influenced by a number of intrinsic and extrinsic factors, many of which cannot be readily controlled. The process of natural spread of disease by the aerosol route was described in detail in studies predating World War II; comprehensive descriptions were first published in the eminent text, *Airborne Contagion and Air Hygiene,* by WF Wells.[1] Many of the early tenets of infection from droplet nuclei are presented in this work, with descriptions of experiments that demonstrate the most basic mechanisms dictating infection from an airborne microbial source.

CONCEPTUAL BASIS OF AEROBIOLOGY IN INFECTIOUS DISEASE

The basic mechanism for transmitting airborne disease is by droplet nuclei. Droplet nuclei have been described as small, air-suspended residues arising from the evaporation of droplets emanating from the mouth and nose. These nuclei-containing infectious microbes (bacteria or viruses) or toxic components collectively comprise biological aerosols that are medically important. Such aerosols are readily produced artificially by spraying or atomizing wet or dried preparations of microorganisms or toxins.

There are many experimental uses of aerosols, but those used for respiratory disease studies are especially important.[3] The study of disease pathogenesis in animal models can be more meaningful if subjects are infected by the same route that occurs naturally in humans. In contrast to intratracheal or intranasal instillation, infectious challenge with aerosol particulates greatly increases natural dispersion in the respiratory system and is consistent with "natural" aerosol infection. Dosage, aerosol particle size, age, environmental temperature, and humidity can all be measured, controlled, and analyzed to some extent.[6] Moreover, the interplay of these features can be studied in the context of microbial viability and resulting virulence.

There are disadvantages, however, that are inherent in aerobiological experimentation. The significance of aerosol age on airborne organism virulence is not fully known. Finally, respiratory doses are difficult to reliably calculate because the degree of lung retention of inhaled aerosol particles, while predictable, usually is not measured.[7] Experiments involving aerosol challenge of animals include determining the host species' susceptibilities, estimating or establishing dose-response curves, evaluating the effect of therapy or stress, and testing the efficacy of experimental vaccines.

These early studies made clear that measuring and controlling as many of the variables as possible associated with stability, viability, and corresponding infectivity of virulent biological aerosols was required for the first biological weapons produced using modern technological methods. Rapid industrialization of the microbiological and evaluation aspects of developing biological weapons was pursued by the militaries of world powers at the time, which ushered in an era of aerobiological research that was performed on a grand scale.

Military programs throughout the mid-20th century engaged in researching and developing biological weapons selected aerosol as the predominant modality and route of battlefield delivery to the enemy. A historic brain trust, comprised of the personnel and physical resources capable in this scientific area, was developed among the superpowers to support this effort. The extensive network that developed was uniquely qualified to harness and perfect the biological, physiochemical, and logistical characteristics preferential to aerosol stability and survival for industrial production and eventual delivery in munitions.

OFFENSIVE BIOLOGICAL DEVELOPMENT AND CLINICAL APPLICATIONS IN THE UNITED STATES

Camp Detrick, Black Maria, and the US Army Medical Unit (1954–1970)

In 1941, the Secretary of War asked the National Academy of Sciences (NAS) to review the risk of biological warfare if the United States were to become engaged in World War II. The War Bureau of Consultants from the NAS advised the Department of Defense to prepare for biological warfare and to provide the resources for both defensive and offensive capabilities. In the spring of 1942, the Army determined that the first US Army biological warfare laboratories would be located at Camp Detrick (Army Air National Guard [ANG] Airfield) in Frederick,

Maryland.[8] Before the offensive and defensive efforts were pursued at Camp Detrick, the Safety Division made great strides in developing capabilities for biocontainment, decontamination, and sterilization of hazardous disease agents. Biological weapon production and testing facilities were initially built at Camp Detrick for the purpose of producing anthrax and botulinum neurotoxin for weapons. The first research facility was located in the ANG hangar, which was modified to include laboratories. A seven-story pilot plant facility was built in 1943 to test fermenters to find the most optimal configuration for culturing large amounts of organisms such as *B anthracis*. A free-standing building was constructed to house this operation; it was covered with the most impervious material available at the time (tar paper), which gave the structure the appearance of an ominous black box and invoked the moniker "Black Maria." This facility was later dismantled to make way for larger, more modern buildings. The US expanded its offensive biological warfare efforts to include production and storage facilities at the Pine Bluff Arsenal in Arkansas, and in Terre Haute, Indiana, during the Cold War in the wake of World War II. The capability at Fort Detrick and ancillary production facilities throughout the United States provided the source material for initial efforts in preparing and packaging biological agents capable of being dispersed as aerosols via munitions. The microbiological expertise and industrial-sized production capabilities during these early efforts were essential for biological stability, which was required for continual production of microbial product that could survive the rigors of the aerosol environment. Maintaining strain virulence, toxin production, and corresponding lot comparability were critical to successful aerosol delivery.

In 1942, President Roosevelt dedicated an initial 126,720 acres of Utah desert land for use by the War Department. Another biological weapons laboratory was opened 6 days later at Dugway Proving Ground in Utah as a testing and evaluation facility. The remoteness and massive land area of this base was ideal for evaluating how aerosolized biological agents performed in the natural environment. A series of experiments were commenced to evaluate the utility of aerosol dispersal as a means of executing a biological weapon attack, including open-air experiments with active biological agents. Aerosolized organisms were detected as far as 30 or more miles away in large-scale aerosol tests. Clandestine dispersals of surrogate organisms, such as *Serratia marcescens* and *Bacillus globigii* (now *B atrophaeus*) were also conducted in a number of urban locations, including New York and San Francisco. Years later it was realized that these experiments actually resulted in a number of illnesses and possibly at least one death, despite the "harmlessness" of the bacteria used. These tests, while highly unethical, demonstrated the potential for an aerosol attack with a biological weapon.[9–11]

Clinical Exposure Trials: Operation Whitecoat (1954–1973)

Concurrent and subsequent to the massive operational efforts that were underway to produce and evaluate biological agents, limited human clinical studies began with a program called Camp Detrick-22 (CD-22) in 1954. Initially, this program of testing biological agents on human volunteers was to assess incapacitating agents' delivery and effects on soldiers, and was very similar to human evaluation of lachrymatory chemical agents (tear gas). The program was eventually expanded to test the efficacy of medical interventions and vaccines, and became known as Project or Operation Whitecoat. Program volunteers were primarily chosen from US enlisted soldiers who, based on their stated religious preference, were affiliated with the Seventh-day Adventist (SDA) church. These soldiers were promised to serve in the military only in noncombat positions if they were enrolled into Operation Whitecoat as volunteers for testing. In addition to SDA-affiliated soldiers, Ohio State Penitentiary prisoners also attended as volunteers of the program. Both soldiers and the prisoners signed the consent forms before they enrolled to the program. They were free to withdraw from the program any time and they were informed about the possible effects of each study. Overall, more than 2,300 volunteers were tested in 137 protocols to develop and test for safety, vaccines, and therapeutics against tularemia, Q fever, viral encephalitis, Rift Valley fever, sandfly fever, and plague between 1954 and 1973 during Operation Whitecoat.[9] Because this kind of testing is now recognized as unethical, Operation Whitecoat constituted one of the few times in history when aerosolized agent delivery was directly tested in the targeted host, the human being, rather than a surrogate animal species.[12] Although unethically obtained by modern standards, data from these early clinical studies remain highly relevant as true indicators of delivery of biological agents by the aerosol route in humans.

Of the list of potential biological agents tested in this manner, only studies involving Q fever (*Coxiella burnetti*) and tularemia (*Francisella tularensis*) were considered safe enough for use in aerosol challenge in humans. Both agents produced infections that were not rapidly progressive, and antibiotic treatment (ie, chloramphenicol, streptomycin) was readily available

and proven to be effective. Consequently, aerosol studies in humans were performed with these agents, in which a 1-million-liter cloud chamber (Figure 29-1) was employed for the initial aerosol dispersion. This unique structure, with 1-¼-inch-thick steel walls, was truly remarkable in that it was one of the only configuration facilities where small munitions loaded with prepared biological agent could be detonated and aerosol dispersion could be studied over an appreciable amount of time. The black rubber bladders integrated in the otherwise gray exterior of the chamber, which absorbed the percussion from the detonation of the munitions used at the time, gave the enormous sphere its nickname, "Eight Ball." In addition to the study of agent survival estimates, aerosols from the interior could also be used for exposure studies with volunteers from Operation Whitecoat. These controlled clinical exposures were a critical aspect of the ongoing characterization of biological agents because they represented the only opportunity to study the interaction of aerosols originating from detonated munitions with the human respiratory system. These studies provided information on the physical size distribution, biological stability, and corresponding viability of the microbial payloads prepared for delivery on the battlefield. The Eight Ball was used for exposures first with *C burnetii* and then *F tularensis* aerosol trials, and decontaminated in 1970 during the decommissioning of the US offensive biological program. No longer in use, the testing chamber remains at Fort Detrick and was listed in 1977 on the US National Historic Register as a landmark site (National Park Service landmark 77000696). In addition to these aerosol trials, outdoor aerosolized *C burnetti* studies that emulated biological warfare scenarios at Fort Detrick were performed in Dugway Proving Ground, as well.

Figure 29-1. Design of the 1-million-liter sphere ball known as "Eight Ball," which was used to expose the Operation Whitecoat volunteers to *Francisella tularensis* and *C burnetii* at Fort Detrick, Maryland.
Photograph reproduced from US Army Medical Department, Medical Research and Materiel Command, Office of Public Affairs, Fort Detrick, MD. R3086, no. 1.

Studies with aerosolized *F tularensis* indicated that when the aerosol residence time increased, infectivity of airborne bacteria decreased. This information, critical to understanding the environmental susceptibility of an organism, opened the door to the development of an attenuated vaccine for *F tularensis*. Early killed and live attenuated tularemia vaccine testing studies with volunteers from the inmates of Ohio State Penitentiary used intracutaneous and respiratory challenge of *F tularensis*. Of the unvaccinated volunteers, 16 of 20 (80%) showed signs of disease following low-dose aerosol challenge ranging from 10 to 52 organisms.[10] Aerosol challenge of vaccinated volunteers resulted in signs of tularemia systemic infection in 8 of 14 (57%) killed vaccine vaccinated volunteers, while only 3 of 18 (16%) live attenuated vaccine vaccinated volunteers had any systemic signs of infection. [10,13]

The potency of an attenuated tularemia vaccine delivered as an aerosol against aerosolized *F tularensis* was tested during follow-up studies.[11] Minor systemic signs and symptoms, such as sore throat and cough, were seen in 30% of aerosolized live vaccine strain (LVS) vaccinated volunteers; pea-sized cervical lymphadenopathy was observed in all vaccinated subjects. Control group and aerosol-vaccinated volunteers were then exposed to approximately 2.5E+04 colony-forming units (CFUs) of aerosolized *F tularensis*; this challenge dose was estimated to be over 2,500-fold more than the minimum dose required to cause disease in humans. Almost all (94%) control group subjects had fever greater than 100°F after a 3- to 5-day incubation period. Following the clinical signs of sudden onset of fever (103°F/104°F), some patients had headache, chills,

and sore throat accompanied by malaise, noticeable myalgia and backache, nausea, and anorexia. Nearly all (89%) of the control group required treatment with antibiotics, while 70% of the vaccinated group had fever and only 23% required treatment. Other delivery routes (oral, cutaneous, and intradermal) for the tularemia vaccine were also evaluated against different challenge routes (intracutaneous, intradermal, aerosol).[13,14]

A similar study was performed to test prophylactic efficacy of tetracycline against aerosolized *F tularensis*.[15] Preceding a 2- to 6-day incubation period, all control group subjects (100%) experienced fever and the other hallmark clinical signs of the disease. The group receiving antibiotic 24 hours postexposure and continuing for 15 days showed no signs of disease. Interestingly, following the cessation of the treatment, 2 of the 10 (20%) volunteers developed acute tularemia. The group that received treatment 28 days initiating 24 hours after exposure did not experience any signs of the disease during or after antibiotic treatment. [10]

A portion of the ongoing clinical efficacy trials with Operation Whitecoat personnel involving Q fever (*C burnetti*) were performed with prisoners from the Maryland State House of Correction.[9] The efficacy of Phase I strain Henzerling and Phase II strain Nine Mile vaccine was tested against aerosolized *C burnetti* in these subjects. These studies indicated that a vaccine of adequate potency was effective in protecting humans against Q fever disease; the protection afforded by these vaccines lasted nearly 1 year after vaccination. Collectively, clinical studies using aerosol infection to develop offensive biological capabilities (at the beginning) and defensive biological capabilities (later) developed and improved medically important countermeasures (vaccines and therapeutic). These studies also contributed to a clear and scientifically realistic understanding of clinical disease progression, signs, symptoms, and diagnostic parameters of many of the priority biological pathogens of interest, namely *C burnetti*, *F tularensis*, sandfly fever, the alphaviral encephalitides, Rift Valley fever, and staphylococcal enterotoxins.[9] This line of investigation also provided clinical insight into the comparative pathophysiology of a disease experimentally induced through a non-natural route of exposure (aerosol), which was crucial for the viral disease agents that are naturally vector-borne (eg, alphaviruses).

BIOLOGICAL AEROSOL EXPOSURE SYSTEMS

One of the cornerstones in the development of aerobiology capabilities during the former US offensive program and in the present-day defensive biological program is the operational capability to conduct animal studies that incorporate aerosol exposure as a modality for delivering biological agents. In contrast to the clinical studies that took place during the offensive biological program, animal studies presently serve as the only source for data on pathogenesis and performance of medical countermeasures to priority pathogens, such as Tier 1 select agents (those for which there is the most concern regarding their potential for use and the resulting consequences). Appreciation of the componentry in studies involving aerosol challenge is an essential part of the collective required for successful integration into animal experimentation, and remains a core competency of any infectious disease aerobiology program.

Significant efforts to place engineering controls to protect and contain biological aerosols were integrated early and remain the approach in modern facilitates engaged in this type of experimentation. In the modern era in the United States, experimenting with aerosol exposures with select agents requires approval of the Centers for Disease Control and Prevention's (CDC's) Division of Select Agent and Toxins (DSAT) and must follow the recommendations in the *Biosafety in Microbiological and Biomedical Laboratories* (BMBL) manual. Aerosol exposures of animals to infectious agents or toxins, particularly those that are potential biological threat agents, are performed in laboratory environments that are negatively pressurized and rigidly controlled, typically at biosafety level-3 (BSL-3) or higher. Most aerosol exposures are performed inside class III biological safety cabinets (BSCs), which are expensive, completely contained environments with HEPA-filtered supply and exhaust. However, this is not always the case. Some exposures are performed under standard class II BSC or in self-contained equipment, such as the Glas-Col (Terra Haute, IN) inhalation exposure chamber (which is typically used for tuberculosis studies and not select agents and toxins). In some institutions, aerosol exposures are performed in the same room where the exposed animals will be subsequently housed, while in others aerosol exposures take place in separate suites and animals are transported from the holding room to the exposure suite using negatively pressurized transport devices, such as a negatively pressurized and filtered mobile transfer cart. The use of a class III BSC in a separate suite provides the greatest flexibility for decontamination and reuse of the aerosol equipment between multiple pathogens or animal species. The different options also alter the need for personal protective equipment (PPE).

Using aerosols in a class III BSC in a dedicated suite separate from animal holding requires only minimal PPE, while other options, depending on the pathogen, typically require the use of N-95 or powered-air purifying respirators (PAPRs). Beyond the engineering controls and PPE described here, a number of other issues must be considered, including decontamination of the space and security and administrative controls (eg, standard operating procedures, training, and oversight). The laboratory space needed to prepare for aerosol exposures (both the pathogenic agent and the aerosol equipment) and the dose required should be considered and determined.

Exposure Systems

Henderson Apparatus

In 1952, David Henderson described an aerosol exposure system designed for ease of operation that could ensure reproducibility between experiments exposing animals to "clouds" containing infectious organisms.[16] This system also incorporated engineering controls to ensure the safety of those using it, to prevent exposure of laboratory personnel. It consisted of a spraying apparatus (an aerosol generator), an exposure tube (analogous to the exposure chambers used today), and an impinger (an aerosol sampling device), as well as a number of points for monitoring and controlling airflow, vacuum, and pressure. The system was dynamic, with air continuously pumped into and exhausted from the exposure apparatus throughout the exposure to eliminate effects resulting from aerosol decay of the organism, as would occur in a static system. As originally described, the system recirculated waste air that was filtered and reused as dilution air in the exposure.

Modern Exposure Systems

Most aerosol exposure systems used in present-day laboratories that perform bioaerosol studies with select agents are generally some derivation of the original Henderson apparatus.[17] Most are also dynamic and incorporate some measure of safety for laboratory personnel in addition to performing the aerosol in a BSC and with the primary engineering controls described above. These systems are designed with greater flexibility for incorporation of other aerosol generators, exposure chambers, or sampling devices, as well as improved monitoring and control of the aerosol. Exhaust air is filtered but, unlike the Henderson apparatus, waste air is not subsequently recirculated into the exposure loop.

Generators

Although a wide range of aerosol generators can be and are employed, the Collison nebulizer is by far the most commonly used aerosol generator for exposures using select agents (viruses or bacteria) and toxins. This generator has become a standard for three primary reasons: (1) Collison nebulizers are relatively inexpensive and easy to maintain; (2) the Collison generates a relatively uniform, nearly monodisperse particle distribution; and (3) aerosol particles in the size range generated by a Collison (approximately 1 to 2 μm in diameter) will reach the deep lung (alveolar regions) of most mammalian species with minimal deposition in the upper respiratory tract. However, other generators have been used, including in recent years the spinning top aerosol generator (STAG)[18,19] and flow-focusing aerosol generator (FFAG).[20–23] These generators allow for customization to larger particle size distributions, thereby allowing study of differential effects based on deposition in distinct compartments of the respiratory tract. Where it has been examined, in most instances particle deposition in the upper respiratory tract (as compared to deposition in the lower) increases the dose required to cause morbidity and mortality and alters the pathogenesis of the disease, and countermeasures are often more efficacious.[18,21–24] It has been postulated that deposition of encephalitic viruses in the upper respiratory tract might more readily lead to infection of the brain as a result of infection in the olfactory region, but the data accumulated to date is contradictory and needs further examination.[19,25–27]

All aerosol generators described in modern exposure systems utilize "wet," liquid aerosols rather than the dry powder aerosol systems that were used in the past during offensive development. The use of dry powder systems to aerosolize biologically active microbial aerosols raises concerns regarding the potential for "dual-use" research and harkens back to the type of technical expertise common during the now-decommissioned offensive biological development program. Dual-use research is defined by federal policy as, "life sciences research that, based on current understanding, can be reasonably anticipated to provide knowledge, information, products, or technologies that could be directly misapplied to pose a significant threat with broad potential consequences to public health and safety, agricultural crops and other plants, animals, the environment, materiel, or national security."[28] In particular, it is noted among the scope that experiments of concern include those that, "increase the stability, transmissibility, or the ability to disseminate the agent or toxin."[28]

Generation of dry-powder forms of virulent agents has been interpreted as falling into that category; however, that has not precluded the use of dry-powder research in biodefense, and in the pharmaceutical industry there is considerable interest and research into dry-powder delivery of vaccines and therapeutics.[29–37]

Exposure Chamber

The choice of exposure chamber is greatly dictated by the animal species being exposed.[17] Rabbits and nonhuman primates are typically exposed one or two at a time using nose-only or head-only exposure chambers because of the animals' size and laboratory space limitations. Rodents (mice and rats) and ferrets are exposed using either nose-only or whole-body chambers. Nose-only exposure chambers deliver the aerosol to the respiratory tract without contaminating the surface of the animal with the pathogenic organism, alleviating concerns regarding infection via swallowing and/or fomites as opposed to true inhalation. However, the current designs of nose-only systems place far greater stress upon rodents as evidenced by increased corticosterone in the blood that could alter the outcome of infectious disease studies.[38] In addition, recent studies evaluating deposition and retention of select agents and toxins have demonstrated that the majority of what is initially inhaled is removed from the respiratory tract and ends up in the gastrointestinal system.[21–23] The choice of nose-only or whole-body exposure chambers should be carefully considered prior to initiating studies.

Sampling

Traditionally, aerosol sampling of infectious organisms and toxins has been done using liquid impingement as a means to collect a representative sample to quantify both concentration and viability of the pathogenic agent (or activity, if a toxin) in the aerosol at the time of exposure. Impingers come in a variety of shapes and sizes but invariably reply upon impaction of an aerosol into a liquid interface. Impingers allow assessment of viable bacteria or viruses in the aerosol but do not provide a means for assessing particle size or the number of bacteria or viruses per particle. Filters and cyclones are also routinely used for sampling bioaerosols to determine concentration of viable microorganisms.[39–41]

When selecting sampling devices, sampling efficiency should be evaluated, as well as the effects of sampling processes (eg, flow rate, collection media) on the viability of the organism being measured. Particle aerodynamic size can be measured during these types of exposures using either viable impactor-type devices, such as an Andersen cascade collector, or analytical devices that employ dual time-of-flight laser technology. Other optical technologies for particle sizing are also employed, such as laser-scattering type instruments (eg, Grimm Technologies, Incorporated [Douglasville, GA] or TSI Incorporated [Shoreview, MN] particle spectrometers). Size characterization of liquid bioaerosols, whose malleability depends on prevailing environmental conditions in the exposure chamber, and the density of the formulation used are usually expressed as the mass median aerodynamic diameter (MMAD). The MMAD provides a median size of the particle distribution based on the behavior of the particles through the air and the corresponding velocity, rather than an actual physical measurement of size. This type of characterization is appropriate for liquid-based aerosols whose size can be dynamic within the exposure systems. Great care should also be taken to sample in the "breathing zone" where the animal is likely to inhale particles, as particle size could be different outside of that zone. Larger particles can break into smaller particles or shrink through evaporation, which can greatly influence where particles will deposit in the respiratory tract. This can also influence the viability of the microorganism in the aerosol, as has been seen with *F tularensis*, in which increased sodium chloride concentration resulting from particle evaporation resulted in a loss of viability, thereby requiring higher concentrations to achieve a lethal dose.[42] Raising the relative humidity in the chamber improved bacterial viability in the aerosol, but it does not alter infectivity.[42,43] Particle density is also an important consideration because it will influence particle size based on the percentage of solids and volatile components that will be lost due to evaporation.[44]

Monitoring and Control

Older biological aerosol exposure systems allow for monitoring and control of environmental parameters, although typically in a crude fashion via a manually controlled instrumentation panel that must be continuously manipulated by a laboratory aerosol technician operator. Modern biological aerosol exposure systems, in contrast, are operated using fully integrated, process flow-control computer systems in addition to constantly monitoring and recording changes in environmental parameters (relative humidity, temperature, pressure) and flow rates (nebulizer, secondary air, exhaust, sampling).[45] This improves accuracy in exposure timing and control as well as improving dosimetry precision in nonhuman primates. With the increased monitoring

comes the improved ability to control and alter these parameters during the exposure to evaluate the impact on aerosol concentration.

Dosimetry

The most critical aspect of biological aerosol exposure systems is the ability to determine the dose delivered to the exposed animal based on the operational characteristics and performance of the system to the users' requirements. Aerosol "dose" is reported in one of three ways: (1) inhaled (also called presented dose, the total number of infectious organisms or mass of toxin inhaled), (2) deposited (the amount that deposits within the respiratory tract), and (3) retained (the amount that remains in the respiratory tract after a specific time).[17,46] The delineation between deposited and retained dose is time-dependent and is not fixed except within a given system. For example, in 1962 Harper and Morton defined retention of *Bacillus globigii* spores as the number of spores remaining in the lungs of guinea pigs 1 day after an aerosol exposure.[47] A considerable amount of what is inhaled is exhaled, removed, or destroyed by the host's innate mechanisms for clearing the respiratory tract (eg, the mucociliary escalator, mucin, defensins, and surfactants). Deposited and retained dose are difficult to measure for infectious organisms, which begin to replicate or escape from the respiratory tract into the circulation almost as soon as they deposit in the respiratory tract. Further, measuring deposited and retained dose requires sacrificing the animal and harvesting tissues in the respiratory tract. Understanding deposition and retention is useful for understanding the aerosol biology and pathology of infectious organisms, but the impact on the efficacy of medical countermeasures is less clear.[21,22,24,48,49] This must be evaluated on a pathogen- and host-specific basis, and the results from one system should not be generally applied to other systems (including other animal species infected with the same pathogen). Further research is desperately needed.

Parameters Impacting Aerosol Dosimetry

Aerosol Performance

System performance between aerosols is compared using the ratio between the aerosol concentration and the nebulizer concentration, also known as the spray factor. Spray factor can be used to determine the nebulizer concentration required to achieve a desired inhaled dose in future studies with that pathogen. Spray factors can only be compared within a given aerosol system for a particular agent, and different systems are not comparable using spray factor performance. Spray factor is essential to building a microbial database of relative aerosol viability within a particular aerosol system, and ultimately dictates the capability (and limitations) of dosing animal species within an aerosol system with that pathogen. Aerosols performed prior to animal exposures determine the spray factor as well as assess the impact of environmental parameters on aerosol performance. Relative humidity in particular has been shown to impact particle size distribution as well as viability of a number of bacteria and viruses.[43,50–57] Aerosol efficiency (the ratio of viable agent in the aerosol sample to the quantity of agent nebulized) and relative recovery (aerosol sampling of the challenge agent relative to a known standard included in the aerosol) have been used as alternatives to spray factor and serve an equivalent function.[50,58,59] These effects are pathogen- and system-specific and require careful evaluation in developing new systems or working with new pathogens.

Anatomy and Physiology

Numerous studies have highlighted differences in the respiratory anatomy between mammalian species. In particular, the length and degree of branching in the bronchus and bronchioles vary greatly between species, getting smaller in length and with less branching as species get smaller.[60] Differences in the amount of branching have been noted between strains of inbred mice. There is also considerable difference in the thickness of bronchial epithelium and the production of mucus,[61] all factors that can impact particle deposition and retention in the respiratory tract.

Respiratory Function

To determine the inhaled dose requires measuring the respiratory minute volume (Vm) of the experimental animal.[62] For experiments with smaller animals like rodents and ferrets, where multiple animals are exposed at a time, minute volume is determined using a simplistic formula based on the animal's weight and corresponding surface area, and was developed by Arthur Guyton over six decades ago.[63] Other methods to determine respiratory function were developed by Bide and Alexander and are also used.[64,65] Respiratory function is typically measured using plethysmography if larger animals, such as rabbits and nonhuman primates, are employed in experimentation. Plethysmography is typically performed either immediately before or during the aerosol exposure. Plethysmography (and the aerosol exposure) of nonhuman primates is performed while animals are anesthetized, which can dramatically suppress respiratory function. Rabbits,

although not anesthetized, are typically restrained, which can increase respiration and minute volume between rabbits of similar size, age, and gender, and can vary dramatically. Because of space limitations in the class III BSC, plethysmography of rabbits and nonhuman primates can typically be done more easily before the aerosol exposure. If plethysmography is performed prior to exposure, minute volume is presumed not to change during exposure (or changes only minimally), although data have shown tremendous variation in minute volume in larger animals irrespective of weight and other factors such as age, gender, and level of anesthesia. If not accounted for, this can lead to tremendous variation in presented dose delivered to animals during exposure. However, most biological aerosol exposure systems do not account for individual variation in minute volume between animals, instead relying upon a fixed exposure time. This is an area that needs further development to ensure similar and consistent dosing between treated and control groups.

SUMMARY

The interface of aerobiology, infectious disease, and the transmissibility of disease are ever present. Harnessing, controlling, and delivering pathogenic agents by aerosol remains the primary and most predicted route of exposure for both military and state-sponsored terrorist acts using biological weapons. The threat of a biological agent being optimized for aerosol delivery holds the potential to reach a target population more efficiently and more completely than any other possible exposure modality available. Much of what is known in the scientific lexicon of aerobiology, as in many fields of study, is derived primarily from observation of nature and natural processes; namely the transmission of disease either through indirect sources, such as contaminated sewage aerosolized at a particle size distribution that approximates respirability, or direct sources, such as proximal contact with an infected host while coughing or sneezing.[4] The early challenge was to overcome the identified environmental and physiochemical factors that would most rapidly degrade or kill microbial preparations when in an aerosol form. Accordingly, modern development of aerobiological techniques was synthetically modeled after natural processes most efficient at disease transmission. Maintaining the physical characteristics and viability of a pathogenic organism for delivery into the environment by virtue of munitions or secondary direct aerosol generator was no small task, and by all accounts in the history of the offensive biological programs, overcoming these barriers required sophisticated approaches. Early biological weapons programs in the United States and Soviet Union focused initially on transferring laboratory bench-based microbiological propagation into industrial-class operational capability, first producing massive quantities of pathogenic agent. Preparation of live microbiological agents for airborne delivery relied heavily upon techniques for preservation and packaging that maintain viability and protect against environmental degradation once released. Concurrent to developing and perfecting industrial-class microbial propagation, preparation, preservation, and delivery techniques, significant efforts were made to determine pathophysiology and pathogenesis in animal models and even in limited human studies. Complex systems for testing and evaluating optimized microbial preparations using select animal species emerged at this time to better support this effort. Sophisticated testing systems that integrated aerosol delivery to a varied array of animal species developed during this time. The small modular aerosol exposure systems in use in many modern laboratories, which are mere shadows of the industrial versions of the past, continue to function under the same basic design and performance criteria. Conversely, the clinical studies that incorporated aerosol exposure with agents easily treated with available chemotherapeutic agents at the time were an advent that will forever remain in the annals of the offensive biological program. The massive dedication of scientific resources and infrastructure to respond to this effort was specifically focused on aerosol as the primary means of delivery to the enemy. This is an important consideration because a number of the biological agents selected for development were not naturally communicable through the airborne route; therefore no clinical experience with infection existed at the time. Predominant disease models and pathogens that catered to aerosol delivery emerged as cornerstones of the state-sponsored biological weapon programs. These very programs, at their zenith, optimized the industrial production, packaging, and prospective aerosol delivery of biological agents in a manner that history had never witnessed. The aerobiology resources and capabilities adjunctive to the biological weapon programs ultimately experienced a dramatic reduction, and a complete shutdown in many cases, that coincided with the signing of the biological weapons convention in 1969. Some countries, however, continued covert operations, including the aerosol research components, well into the 21st century.

Collectively, in the aftermath of the decommissioning of the offensive biological programs, much of the infrastructure needed to effectively perform research for medical countermeasures was effectively rebuilt, albeit on a much smaller scale with significant technical and engineering limitations in mind. Present-day research organizations that incorporate aerobiology resources and expertise, such as the program at the US Army Medical Research Institute of Infectious Diseases at Fort Detrick, Maryland, embody a small-scale, sophisticated support structure similar to many programs at other federally supported, contracting, and academic laboratories throughout the nation.

REFERENCES

1. Wells WF. *Airborne Contagion and Air Hygiene: An Ecological Study of Droplet Infections*. Cambridge, MA: Harvard University Press; 1955: 8-12.

2. Hatch T. Distribution and deposition of inhaled particles in respiratory tract. *Bacteriol Rev*. 1961:237–240.

3. Wolfe EJ. Quantitative characterization of aerosols. *Bacteriol Rev*. 1961:194–202.

4. Roy C, Milton D. Airborne transmission of communicable infection–the elusive pathway. *New Eng J Med*. 2004:1710–1712.

5. Goodlow R, Leonard F. Viability and infectivity of microrganisms in experimental airborne infection. *Bacteriol Rev*. 1961:182–187.

6. Yakovleva GS. *Certain Conditions Which Influence the Stability of a Viral Aerosol*. Wright-Patterson Air Force Base, Ohio: FT Division; 1973.

7. Wright G. Structure and function of respiratory tract in relation to infection. *Bacteriol Rev*. 1961:219–227.

8. Covert NM. *Cutting Edge: A History of Fort Detrick, Maryland*. 4th ed. Frederick, Maryland: US Army Medical Research and Materiel Command; 2000.

9. Clendenin RM. *Science and Technology at Fort Detrick 1943–1968*. Ft Detrick, Maryland: Technical Information Division; 1968.

10. Saslaw S, Eigelsbach HT, Prior JA, Wilson HE, Carhart S. Tularemia vaccine study. II. Respiratory challenge. *Arch Intern Med*. 1961;107:702–714.

11. Hornick RB, Eigelsbach HT. Aerogenic immunization of man with live Tularemia vaccine. *Bacteriol Rev*. 1966;30(3):532–538.

12. Martin JW, Christopher GW, Eitzen EM. History of biological weapons: from poisoned darts to intentional epidemics. In: Dembek ZF. *Medical Aspects of Biological Warfare*. Washington, DC: Borden Institute; 2007.

13. Saslaw S, Eigelsbach HT, Wilson HE, Prior JA, Carhart S. Tularemia vaccine study. I. Intracutaneous challenge. *Arch Intern Med*. 1961;107:689–701.

14. Hornick RB, Dawkins AT, Eigelsbach HT, Tulis JJ. Oral tularemia vaccine in man. *Antimicrob Agents Chemother (Bethesda)*. 1966;6:11–14.

15. Sawyer WD, Dangerfield HG, Hogge AL, Crozier D. Antibiotic prophylaxis and therapy of airborne tularemia. *Bacteriol Rev*. 1966;30(3):542–550.

16. US Department of Health and Human Services, Centers for Disease Control and Prevention, and National Institutes of Health. Chosewood LC, Wilson DE, eds. *Biosafety in Microbiological and Biomedical Laboratories*. 5th ed. Washington, DC: US Government Printing Office; 2009. http://www.cdc.gov/biosafety/publications/bmbl5/BMBL.pdf. Accessed December 28, 2013.

17. Henderson DW. An apparatus for the study of airborne infection. *J Hyg (Lond)*. 1952;50(1):53–68.

18. Roy CJ, Pitt LM. Infectious disease aerobiology: aerosol challenge methods. In: Swearengen JR, ed. *Biodefense Research Methodology and Animal Models*. 2nd ed. Boca Raton, FL: CRC Press; 2012: 65–80.

19. Roy CJ, Hale M, Hartings JM, Pitt L, Duniho S. Impact of inhalation exposure modality and particle size on the respiratory deposition of ricin in BALB/c mice. *Inhal Toxicol*. 2003;15(6):619–638.

20. Roy CJ, Reed DS, Wilhelmsen CL, Hartings J, Norris S, Steele KE. Pathogenesis of aerosolized Eastern Equine Encephalitis virus infection in guinea pigs. *Virol J*. 2009;6:170.

21. Thomas RJ, Webber D, Hopkins R, et al. The cell membrane as a major site of damage during aerosolization of Escherichia coli. *Appl Environ Microbiol*. 2011;77(3):920–925.

22. Thomas R, Davies C, Nunez A, et al. Influence of particle size on the pathology and efficacy of vaccination in a murine model of inhalational anthrax. *J Med Microbiol*. 2010;59(Pt 12):1415–1427.

23. Thomas RJ, Webber D, Collinge A, et al. Different pathologies but equal levels of responsiveness to the recombinant F1 and V antigen vaccine and ciprofloxacin in a murine model of plague caused by small- and large-particle aerosols. *Infect Immun*. 2009;77(4):1315–1323.

24. Thomas RJ, Webber D, Sellors W, et al. Characterization and deposition of respirable large- and small-particle bioaerosols. *Appl Environ Microbiol*. 2008;74(20):6437–6443.

25. Day WC, Berendt RF. Experimental tularemia in Macaca mulatta: relationship of aerosol particle size to the infectivity of airborne Pasteurella tularensis. *Infect Immun*. 1972;5(1):77–82.

26. Danes L, Rychterova V, Kufner J, Hruskova J. The role of the olfactory route on infection of the respiratory tract with Venezuelan equine encephalomyelitis virus in normal and operated Macaca rhesus monkeys. II. Results of histological examination. *Acta Virol*. 1973;17(1):57–60.

27. Danes L, Kufner J, Hruskova J, Rychterova V. The role of the olfactory route on infection of the respiratory tract with Venezuelan equine encephalomyelitis virus in normal and operated Macaca rhesus monkeys. I. Results of virological examination. *Acta Virol*. 1973;17(1):50–56.

28. Steele KE, Davis KJ, Stephan K, Kell W, Vogel P, Hart MK. Comparative neurovirulence and tissue tropism of wild-type and attenuated strains of Venezuelan equine encephalitis virus administered by aerosol in C3H/HeN and BALB/c mice. *Vet Pathol*. 1998;35(5):386–397.

29. US Department of Health and Human Services, National Institutes of Health Office of Science Policy. *United States Government Policy for Oversight of Life Sciences Dual Use Research of Concern*. Bethesda, MD: NIH. http://osp.od.nih.gov/office-biotechnology-activities/dual-use-reasearch-concern-policy-information-national-science-advisory-board-biosecurity-nsabb/united-states-government-policy-oversight-life-sciences-dual-use-research-concern. Accessed June 9, 2014.

30. Baron PA, Estill CF, Deye GJ, et al. Development of an aerosol system for uniformly depositing bacillus anthracis spore particles on surfaces. *Aerosol Sci Tech*. 2008;42(3):159–172.

31. Kunnil J, Sarasanandarajah S, Chacko E, Swartz B, Reinisch L. Identification of Bacillus spores using clustering of principal components of fluorescence data. *Aerosol Sci Tech*. 2005;39(9):842–848.

32. Smith DJ, Bot S, Dellamary L, Bot A. Evaluation of novel aerosol formulations designed for mucosal vaccination against influenza virus. *Vaccine*. 2003;21(21-22):2805–2812.

33. Wong YL, Sampson S, Germishuizen WA, et al. Drying a tuberculosis vaccine without freezing. *Proc Natl Acad Sci U S A*. 2007;104(8):2591–2595.

34. Ohtake S, Martin RA, Saxena A, et al. Formulation and stabilization of *Francisella tularensis* live vaccine strain. *J Pharm Sci*. 2011;100(8):3076–3087.

35. Ohtake S, Martin R, Saxena A, et al. Room temperature stabilization of oral, live attenuated *Salmonella enterica* serovar Typhi-vectored vaccines. *Vaccine*. 2011;29(15):2761–2771.

36. Matinkhoo S, Lynch KH, Dennis JJ, Finlay WH, Vehring R. Spray-dried respirable powders containing bacteriophages for the treatment of pulmonary infections. *J Pharm Sci*. 2011;100(12):5197–5205.

37. Jin TH, Tsao E, Goudsmit J, Dheenadhayalan V, Sadoff J. Stabilizing formulations for inhalable powders of an adenovirus 35-vectored tuberculosis (TB) vaccine (AERAS-402). *Vaccine*. 2010;28(27):4369–4375.

38. Corbanie EA, Remon JP, Van Reeth K, Landman WJM, van Eck JHH, Vervaet C. Spray drying of an attenuated live Newcastle disease vaccine virus intended for respiratory mass vaccination of poultry. *Vaccine*. 2007;25(49):8306–8317.

39. Gater ST, Peters KN, Kocsis AG, Dhariwala MO, Anderson DM, Anderson PE. Host stress and immune responses during aerosol challenge of Brown Norway rats with *Yersinia pestis*. *Front Cell Infect Microbiol*. 2012;2:147.

40. Dybwad M, Skogan G, Blatny JM. Comparative testing and evaluation of nine different air samplers: end-to-end sampling efficiencies as specific performance measurements for bioaerosol applications. *Aerosol Sci Tech*. 2014;48(3):282–295.

41. Fabian P, McDevitt JJ, Houseman EA, Milton DK. Airborne influenza virus detection with four aerosol samplers using molecular and infectivity assays: considerations for a new infectious virus aerosol sampler. *Indoor Air*. 2009;19(5):433–441.

42. Dabisch P, Yeager J, Kline J, Klinedinst K, Welsch A, Pitt ML. Comparison of the efficiency of sampling devices for aerosolized *Burkholderia pseudomallei*. *Inhal Toxicol*. 2012;24(5):247–254.

43. Hood AM. Infectivity of *Pasteurella tularensis* clouds. *J Hyg (Lond)*. 1961;59:497–504.

44. Faith SA, Smith LP, Swatland AS, Reed DS. Growth conditions and environmental factors impact aerosolization but not virulence of Francisella tularensis infection in mice. *Front Cell Infect Microbiol*. 2012;2:126.

45. Hinds WC. *Production of Test Aerosols. Aerosol Technology—Properties, Behavior, and Measurement of Airborne Particles*. New York, NY: Wiley & Sons; 1999.

46. Hartings JM, Roy CJ. The automated bioaerosol exposure system: preclinical platform development and a respiratory dosimetry application with nonhuman primates. *J Pharmacol Toxicol Methods*. 2004;49(1):39–55.

47. Lippmann M, Yeates DB, Albert RE. Deposition, retention, and clearance of inhaled particles. *Br J Ind Med*. 1980;37(4):337–362.

48. Harper GJ, Morton JD. A method for measuring the retained dose in experiments on airborne infection. *J Hyg (Lond)*. 1962;60:249–57.

49. White JD, Rooney JR, Prickett PA, Derrenbacher EB, Beard CW, Griffith WR. Pathogenesis of experimental respiratory tularemia in monkeys. *J Infect Dis*. 1964;114:277–283.

50. Thomas RJ, Davies C, Nunez A, et al. Particle-size dependent effects in the Balb/c murine model of inhalational melioidosis. *Front Cell Infect Microbiol*. 2012;2:101.

51. Kim SW, Ramakrishnan MA, Raynor PC, Goyal SM. Effects of humidity and other factors on the generation and sampling of a coronavirus aerosol. *Aerobiologia*. 2007;23(4):239–248.

52. Walter MV, Marthi B, Fieland VP, Ganio LM. Effect of aerosolization on subsequent bacterial survival. *Appl Environ Microbiol*. 1990;56(11):3468–3472.

53. Marthi B, Fieland VP, Walter M, Seidler RJ. Survival of bacteria during aerosolization. *Appl Environ Microbiol*. 1990;56(11):3463–3467.

54. Stephenson EH, Larson EW, Dominik JW. Effect of environmental factors on aerosol-induced Lassa virus infection. *J Med Virol*. 1984;14(4):295–303.

55. Cox CS. Inactivation kinetics of some microorganisms subjected to a variety of stresses. *Appl Environ Microbiol*. 1976;31(6):836–846.

56. Schaffer FL, Soergel ME, Straube DC. Survival of airborne influenza virus: effects of propagating host, relative humidity, and composition of spray fluids. *Arch Virol*. 1976;51(4):263–273.

57. Cox CS, Goldberg LJ. Aerosol survival of Pasteurella tularensis and the influence of relative humidity. *Appl Microbiol*. 1972;23(1):1–3.

58. Cox CS. Aerosol survival of Pasteurella tularensis disseminated from the wet and dry states. *Appl Microbiol*. 1971;21(3):482–486.

59. Appert J, Raynor PC, Abin M, et al. Influence of suspending liquid, impactor type, and substrate on size-selective sampling of MS2 and Adenovirus aerosols. *Aerosol Sci Tech*. 2012;46(3):249–257.

60. Stewart SL, Grinshpun SA, Willeke K, Terzieva S, Ulevicius V, Donnelly J. Effect of impact stress on microbial recovery on an agar surface. *Appl Environ Microb*. 1995;61(4):1232–1239.

61. Hyde DM, Miller LA, Schelegle ES, et al. Asthma: a comparison of animal models using stereological methods. *Eur Respir Rev*. 2006;15(101):122–135.

62. Patton JS, Byron PR. Inhaling medicines: delivering drugs to the body through the lungs. *Nat Rev Drug Discov*. 2007;6(1):67–74.

63. Guyton A. Measurement of the respiratory volumes of laboratory animals. *Am J Physiol*. 1947;150:70–77.

64. Bide RW, Armour SJ, Yee E. Allometric respiration/body mass data for animals to be used for estimates of inhalation toxicity to young adult humans. *J Appl Toxicol*. 2000;20(4):273–290.

65. Alexander DJ, Collins CJ, Coombs DW, et al. Association of Inhalation Toxicologists (AIT) working party recommendation for standard delivered dose calculation and expression in non-clinical aerosol inhalation toxicology studies with pharmaceuticals. *Inhal Toxicol*. 2008;20(13):1179–1189.