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National Toxicology Program
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NTP Technical Report on the Toxicity Studies of *Aspergillus fumigatus* Administered by Inhalation to B6C3F1/N Mice

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Foreword

The National Toxicology Program (NTP), established in 1978, is an interagency program within the Public Health Service of the U.S. Department of Health and Human Services. Its activities are executed through a partnership of the National Institute for Occupational Safety and Health (part of the Centers for Disease Control and Prevention), the Food and Drug Administration (primarily at the National Center for Toxicological Research), and the National Institute of Environmental Health Sciences (part of the National Institutes of Health), where the program is administratively located. NTP offers a unique venue for the testing, research, and analysis of agents of concern to identify toxic and biological effects, provide information that strengthens the science base, and inform decisions by health regulatory and research agencies to safeguard public health. NTP also works to develop and apply new and improved methods and approaches that advance toxicology and better assess health effects from environmental exposures.

The Toxicity Report series began in 1991. The studies described in the NTP Toxicity Report series are designed and conducted to characterize and evaluate the toxicological potential of selected substances in laboratory animals (usually two species, rats and mice). Substances (e.g., chemicals, physical agents, and mixtures) selected for NTP toxicity studies are chosen primarily on the basis of human exposure, level of commercial production, and chemical structure. The interpretive conclusions presented in the Toxicity Reports are derived solely from the results of these NTP studies, and extrapolation of these results to other species, including characterization of hazards and risks to humans, requires analyses beyond the intent of these reports. Selection for study per se is not an indicator of a substance's toxic potential.

NTP conducts its studies in compliance with its laboratory health and safety guidelines and the Food and Drug Administration Good Laboratory Practice Regulations and meets or exceeds all applicable federal, state, and local health and safety regulations. Animal care and use are in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals. Studies are subjected to retrospective quality assurance audits before they are presented for public review. Draft reports undergo external peer review before they are finalized and published.

The NTP Toxicity Reports are available free of charge on the NTP website and cataloged in PubMed, a free resource developed and maintained by the National Library of Medicine (part of the National Institutes of Health). Data for these studies are included in NTP's Chemical Effects in Biological Systems database.

For questions about the reports and studies, please email NTP or call 984-287-3211.

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Peer Review

The National Toxicology Program (NTP) conducted a peer review of the draft *NTP Technical Report on the Toxicity Studies of* Aspergillus fumigatus *Administered by Inhalation to B6C3F1/N Mice* by letter in December 2020–January 2021 by the experts listed below. Reviewer selection and document review followed established NTP practices. The reviewers were charged to:

(1) Peer review the draft *NTP Technical Report on the Toxicity Studies of Aspergillus fumigatus*.

(2) Comment on NTP's interpretations of the data.

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Abstract

Aspergillus fumigatus is a thermotolerant, saprophytic fungal species that is ubiquitous in the environment. Mold was nominated to the National Toxicology Program (NTP) in response to public concern regarding suspected adverse health effects associated with personal exposure in indoor and occupational settings. *A. fumigatus* is of particular concern in the biowaste industry as the species can contaminate self-heating compost piles. Because of this potential for personal and occupational exposure and the lack of available toxicity data, toxicity studies were conducted in which male and female B6C3F1/N mice were exposed to *A. fumigatus* conidia (spores) two times a week for 3 months. All in-life procedures, including inhalation exposure, test article preparation, and hematology analysis, were completed by the National Institute for Occupational Safety and Health (NIOSH, Morgantown, WV). Battelle (Columbus, OH) conducted terminal necropsies, measured terminal body and organ weights, and evaluated gross lesions on-site at NIOSH. Tissue processing and histopathology were completed at Battelle. Grocott's methenamine silver (GMS) staining was performed at NIOSH. Genetic toxicology studies on mouse peripheral blood erythrocytes were conducted by Integrated Laboratory Systems, LLC (Research Triangle Park, NC).

Groups of 10 male and 10 female mice were exposed via nose-only inhalation to 1×10^5 *A. fumigatus* viable spores (viable *A. fumigatus*), 1×10^5 nonviable spores (heat-inactivated particle control), or to an air control. All male mice survived to the end of the study, whereas two female mice, one in the air control group and one in the heat-inactivated particle control group, died during the study. There was no effect of exposure on body weights. Gross lesions, observed at study termination, consisted of enlarged, gray bronchial lymph nodes in 5 out of 10 females exposed to viable *A. fumigatus*. Mean absolute and relative lung weights were significantly increased in male (43% and 47%, respectively) and female (68% and 75%, respectively) viable *A. fumigatus*-exposed mice compared to the air control groups.

Nonneoplastic lesions were observed in the larynx, lung, and bronchial lymph nodes. In the larynx, lesions presented primarily as epithelial squamous metaplasia at the base of the epiglottis in both males and females exposed to viable *A. fumigatus*; exposure to heat-inactivated control spores did not affect the larynx. The increased lung weights in the viable *A. fumigatus*-exposed groups correlated histologically with chronic active inflammation and hyperplasia of the bronchus-associated lymphoid tissue (BALT) and bronchiolar epithelium in a majority of males and females. Mice in the heat-inactivated particle control groups also showed BALT hyperplasia but at lower incidences as compared to viable *A. fumigatus*-exposed groups. The lungs of all mice exposed to viable *A. fumigatus* spores also showed medial hypertrophy in small- to medium-sized pulmonary arteries. GMS-stained lung sections of viable *A. fumigatus*-exposed mice revealed spores within the alveoli and alveolar macrophages. Hyperplasia, either of lymphocytes or plasma cells, was additionally observed in the bronchial lymph nodes of viable *A. fumigatus*-exposed mice and corresponded to the grossly enlarged bronchial lymph nodes in females.

A. fumigatus viable spores were tested in a bacterial mutation assay with and without exogenous metabolic activation (S9 mix); results were negative in each of the three strains tested. No increases in the frequencies of micronucleated erythrocytes were observed in peripheral blood samples from male and female mice obtained after 3 months of inhalation exposure to viable

A. fumigatus spores, indicating no chromosomal damage was induced in progenitor cells in the bone marrow of these mice.

Under the conditions of this 3-month study, target organs identified in B6C3F1/N mice following inhalation exposure to *A. fumigatus* spores were the larynx, lung, and bronchial lymph nodes. Significant differences were observed between viable *A. fumigatus* exposure and both air control and heat-inactivated particle control exposures. These results build on initial NIOSH pulmonary immunology studies using the same exposure parameters and demonstrate that the immunological responses and histopathology could be enhanced by the viability of the *A. fumigatus* spores.

Synonyms: *Aspergillus fumigatus* (*A. fumigatus*); NIH Strain B-5233; *Aspergillus fumigatus* Fresenius, anamorph (ATCC® 13073™)

Summary of Findings Considered Toxicologically Relevant in Mice Exposed to *Aspergillus fumigatus* Spores by Inhalation for Three Months

	Male Heat-inactivated Particle Control	Male Viable *A. fumigatus*	Female Heat-inactivated Particle Control	Female Viable *A. fumigatus*
Exposure Concentration (Estimated Lung Deposition)	1×10^5 heat-inactivated *A. fumigatus* spores	1×10^5 *A. fumigatus* spores	1×10^5 heat-inactivated *A. fumigatus* spores	1×10^5 *A. fumigatus* spores
Survival Rates	10/10	10/10	9/10	10/10
Body Weights	Similar to air control group	Similar to air control group	Similar to air control group	Similar to air control group
Clinical Findings	None[a]	None	None	None
Organ Weights	None	↑Absolute and relative lung weight compared to either air control or heat-inactivated particle control	None	↑Absolute and relative lung weight compared to either air control or heat-inactivated particle control
Hematology	None	↑Neutrophils compared to air control	None	None
Nonneoplastic Effects	Lung: inflammation, chronic (3/10); BALT, lymphocyte, hyperplasia (7/10)	Larynx: epiglottis, metaplasia, squamous (9/10) Lung: artery, medial, hypertrophy (10/10); bronchiole, epithelium, hyperplasia (9/10); inflammation, chronic active (10/10); BALT, lymphocyte, hyperplasia, (10/10); goblet cell, metaplasia (10/10) Bronchial lymph node: lymphocyte, hyperplasia (6/9); plasma cell, hyperplasia (5/9)	Lung: inflammation, chronic (7/10); BALT, lymphocyte, hyperplasia (8/10)	Larynx: epiglottis, metaplasia, squamous (9/10); epithelium, ulcer (1/10); squamous epithelium, hyperplasia (1/10) Lung: artery, medial, hypertrophy (10/10); bronchiole, epithelium, hyperplasia (8/10); inflammation, chronic active (10/10); BALT, lymphocyte, hyperplasia, (10/10); goblet cell, metaplasia (10/10) Bronchial lymph node: lymphocyte, hyperplasia (9/10); plasma cell, hyperplasia (8/10)
Genetic Toxicology				
Micronucleated Erythrocytes (In Vivo):	Negative in all exposure groups of male and female mice			
Bacterial Gene Mutations	Negative in *Salmonella typhimurium strains* TA100 and TA98 and *Escherichia coli* strain WP2 *uvr*A (pKM101), with and without S9			

BALT = bronchus-associated lymphoid tissue.
[a]None = no toxicologically relevant effects for this endpoint.

Introduction

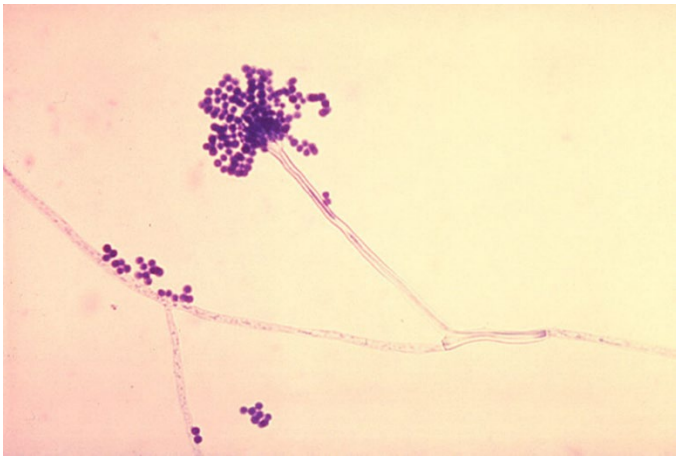

Figure 1. *Aspergillus fumigatus* Fresenius

Botanical Name: *Aspergillus fumigatus*. Synonyms: *Aspergillus fumigatus* (*A. fumigatus*); NIH Strain B-5233 (a clinical strain isolated in a case of invasive aspergillosis from a patient with leukemia); *Aspergillus fumigatus* Fresenius, anamorph (ATCC® 13073™). The light microscopic image depicts asexual *Aspergillus fumigatus* reproductive structures composed of a septate hyphae, conidiophore, and chains of conidiospores.
Photo courtesy of CDC; CDC Public Health Image Library ID #300.

Fungi, Indoor Environmental Quality, and Public Health

The fungal kingdom is a diverse lineage of eukaryotic microorganisms consisting of approximately 1.5 million species.[1] Fungi grow in a wide variety of lifeforms and include unicellular yeasts, filamentous hyphae, or a combination of both life cycles (dimorphism) that include species with the ability to switch between yeast and filamentous forms on the basis of temperature and nutrient profiles. Fungi reproduce via meiotic or mitotic processes and produce spores or conidia that vary morphologically and can be aerosolized into the breathing zone of a person following abiotic or biotic colony disturbance. In addition to spores, small submicronic fragments of hyphae can also be released from the colony, and, in some cases, these bioaerosols can be released in greater concentrations than spores.[2-5]

In the environment, fungi predominate as saprophytes; as such, they have positive and negative effects on the environment, industry, and human health. Fungi and fungal-derived products have broad industrial utility ranging from the production of food (yeast, fermentation), detergents (enzymes), and pharmaceuticals (penicillin).[6] The negative impacts that fungi have on society are more widely recognized by the public, however, especially after a cluster of infant pulmonary hemorrhage/hemosiderosis cases in the United States,[7; 8] as well as the effects on indoor environments resulting from natural disasters, including Hurricanes Katrina, Rita, Sandy, Harvey, Irma, and Maria. The growth of mold within the indoor built environment has led to public health and community concern over the potential pulmonary and neurological health effects associated with the inhalation of fungal spores, hyphae, and secondary metabolites such as mycotoxins and volatile organic compounds that may be released during fungal growth and colonization. Fungal secondary metabolites are frequently associated with sporulation and development and are produced late in the growth phase of the organism.[9]

Moisture infiltration in the indoor built environment can lead to mold growth; consequently, contamination of building materials has been the focus of numerous indoor air quality investigations over the last three decades. In 1994, epidemiological evidence of pulmonary hemorrhage/hemosiderosis in a cluster of Cleveland infants (n = 10)[7; 8] drew further attention to the negative impacts associated with fungal exposure in damp indoor environments. The investigation into the reported cases showed that infants with acute pulmonary hemorrhage/hemosiderosis were more likely to reside in water-damaged homes compared to non-damaged homes.[7] The investigation additionally identified the toxigenic fungal species *S. chartarum* (synonym *S. atra*) to be in higher concentrations in case homes compared to control homes.[7] A subsequent Centers for Disease Control and Prevention review of the Cleveland investigation[10] identified that the associations in the initial epidemiological investigations were not supported. The initial findings had a significant effect on the community, however, and fostered the citation of the study in a range of government, academic, and public forums that ultimately led to litigation and even the closure of buildings.[10]

Since the Cleveland investigation, consensus reports have been published by the Institute of Medicine (IOM), Committee on Damp Indoor Spaces and Health,[11] and the World Health Organization (WHO),[12] evaluating the role of indoor dampness and fungal exposures on human health. The reports presented sufficient epidemiological evidence of associations between personal exposure to damp indoor environments or the presence of mold indoors with several respiratory symptoms.[11; 12] Recent meta-analyses have further confirmed the IOM and WHO consensus reports and identified additional associations between visible mold, mold odor, and allergy/asthma in damp indoor environments,[13-15] although associations with individual fungal species and culturable fungi were limited.[16] A 2016 cost estimate analysis has placed the economic burden of fungal exposure-related illness to be as high as $3.7 billion for allergic rhinitis and $15.1 billion for asthma morbidity.[17] To date, the role of fungi and the physiological changes that follow exposure remain uncharacterized and in some cases completely misunderstood for indoor-contaminating fungal species. Numerous data gaps exist concerning the role of fungi in the areas of neurotoxicology, rheumatic diseases, reproduction, and cardiopulmonary disease. This lack of data has hindered understanding of the physiological consequences of fungal exposures and has resulted in no standardized methods or exposure limits for fungi.

To address some of these knowledge gaps, fungi were nominated to the National Toxicology Program (NTP) for investigation. In 2004, the NTP Board of Scientific Councilors and Executive Committee endorsed the study of fungi. The committee suggested studying fungal species commonly identified in indoor air, including *Aspergillus*, *Penicillium*, and *Stachybotrys* species. An expert review panel was convened in 2007 to discuss the study design for NTP subchronic toxicity studies. Several key areas that would have a direct bearing on study design were identified and considered, including colony growth conditions, life cycle, and biochemical makeup of the species. The panel recommended evaluating confluent fungal colonies derived from a single fungal species, as well as mixtures of fungal colonies co-cultured on building materials commonly used in the indoor environment. Variables related to the production, characterization, and reproducibility of the test material were also considered. The National Institute for Occupational Safety and Health (NIOSH) participated in the 2007 expert review and was identified as a partner to conduct the subchronic (13-week) fungal exposure studies due to their prior experience and expertise in microbial immunology and aerosol science.

As a part of an ongoing partnership with NTP, NIOSH initiated a series of pilot studies to develop and characterize an acoustical generation system (AGS) capable of simulating natural indoor and occupational fungal exposure. NIOSH has utilized the AGS and nose-only chamber in studies to characterize the pulmonary immune responses to dry viable and nonviable *A. fumigatus* conidia. In these studies, BALB/cJ mice were repeatedly exposed to 1×10^5 *A. fumigatus* conidia via inhalation twice a week for 4 or 13 weeks. In the 4-week study, histopathological analysis of lung sections showed moderate to severe inflammation, mucus production, bronchiolar pneumonia, and tissue remodeling/fibrosis. Increased incidence of neutrophils and eosinophils were observed surrounding the larger airways, and giant cell formation was apparent in mice 24 hours following the final (eighth) exposure. Flow cytometry analysis showed total CD8+ T cells were increased in bronchoalveolar lavage fluid (BALF) at 24 hours, and approximately 33% of the CD8+ T cell numbers were CD8+IL-17+ T cells (Tc17), which peaked at 24 hours.[18] Importantly, swollen conidia were observed at 4 hours, and by 24 hours, many conidia had begun to germinate. Emergence of hyphae from spores also correlated with an increase in Tc17 cell populations.[18; 19] This study was among the first to demonstrate in vivo *A. fumigatus* germination following spore exposures in an animal model.[18; 19]

NIOSH also completed a pilot 13-week subchronic study to examine pulmonary immune endpoints using B6C3F1/N mice.[20] In this study, mice were exposed twice a week for 13 weeks to dry viable *A. fumigatus* spores and compared to an air control group. Repeated exposures with viable spores revealed fungal dose-dependent pulmonary inflammation, cellular infiltration, goblet cell metaplasia, and peribronchial and perivascular collagen deposition. Flow cytometry of BALF showed CD4+ T helper (Th) cells expressing cytokines associated with an allergic phenotype [Th2; interleukin- (IL-) 5 and IL-13]. These cell populations were present in significantly increased numbers in the airways of animals dosed with viable *A. fumigatus* spores. The responses were accompanied by an increase in total serum immunoglobulin E (IgE) and development of immunoglobulin G (IgG) antibodies to *A. fumigatus*-specific proteins. The results of this study further supported the hypothesis that spore viability is a critical variable of fungal exposures that affected Th1/Th2 differentiation in mice. Compared with the 4-week repeated exposure study, these experiments demonstrated a shift toward a Th2-mediated response in animals that received 13 weeks of repeated exposures.

The overall goal in this series of studies was to characterize the hazards associated with exposure to *A. fumigatus*, a common fungal contaminant in indoor and occupational environments. The aim was to advance understanding of the toxicological and pulmonary immune responses associated with subchronic fungal exposures using a model that replicates human exposure conditions found in damp or water-damaged buildings. The data derived from these studies will provide further understanding of the species that can elicit adverse health effects. To address these experimental aims, NIOSH applied the same AGS and methodologies used in preliminary pulmonary immunological studies to characterize *A. fumigatus* toxicological endpoints following subchronic fungal spore exposures.

Chemical and Physical Properties

The genus *Aspergillus* includes a diverse group of approximately 250 species within the Ascomycota order Eurotiales.[21] *A. fumigatus* is environmentally ubiquitous and a common source of opportunistic fungal infections that were first described by Fresenius in 1863.[21] The

species is characterized by an anamorphic (asexual) life cycle (Figure 2) as well as a sexual phase (*Neosartorya*). The anamorphic phase is placed in the section *Fumigati* and is characterized by the production of chains of unicellular conidia (spores) that emerge from uniseriate phialides presented from conidiophores that consist of flask-shaped vesicles.[21; 22] *A. fumigatus* is abundant in indoor, outdoor, and occupational environments because it produces respirable-sized green echinulate globose-to-subglobose spores composed of a rodlet hydrophobic layer (2–3 µm spore diameter).[21-23] The spores are continuously generated via a rapid sporulation process and are easily aerosolized into the environment following abiotic or biotic disturbance.[21-23]

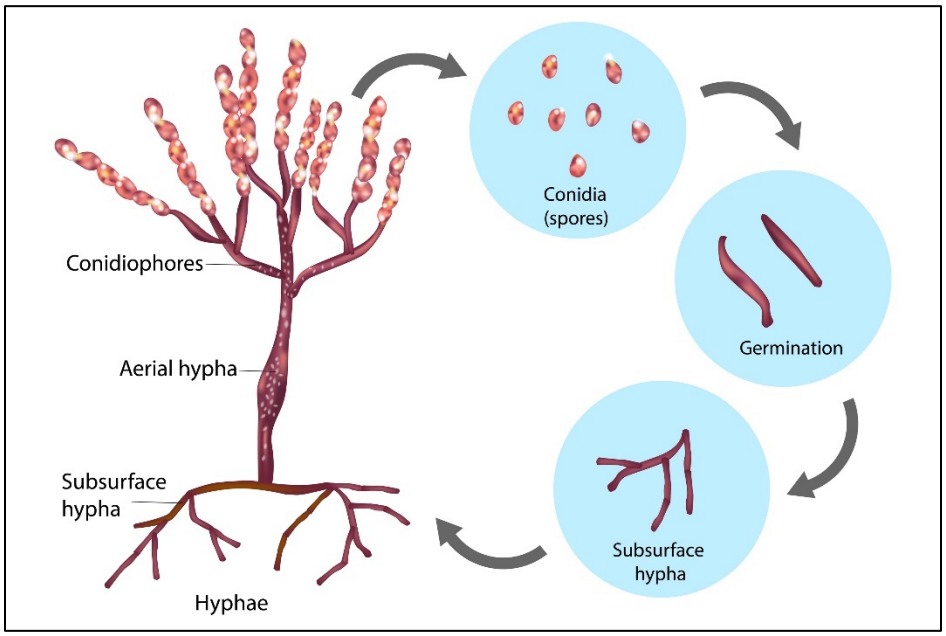

Figure 2. A Representative Life Cycle of Fungi in the Order Eurotiales

Production, Use, and Human Exposure

A. fumigatus is a ubiquitous saprophytic fungal species that has a worldwide distribution.[22; 23] It is found within the soil, where it is involved in the breakdown of plant-derived polysaccharides through the secretion of glycosyl hydrolases and proteases.[21-24] *A. fumigatus* is thermotolerant and can grow and proliferate in self-heating compost piles and waste management plants.[21; 23; 25] These growth conditions have become problematic in several occupational environments that produce compost. *A. fumigatus* has been identified as a harmful biological contaminant as well as an indicator organism within the composting industry.[26] In occupational environments, highest concentrations of fungi, including *A. fumigatus*, have been measured in dusty working areas during shredding and processing activities.[27] Airborne fungal concentrations are measured using multiple approaches, such as plating viable spores for determination of colony-forming units (CFU)/m^3 or microscopic spore counting of viable and nonviable conidia (conidia/m^3). Airborne *A. fumigatus* concentrations as high as 1×10^6 CFU/m^3 have been reported in occupational environments.[28; 29] Eleven secondary metabolites that were produced from cultured *A. fumigatus* strains collected in biowaste processing environments have been characterized.[30] Other occupational environments where *A. fumigatus* has been detected in elevated concentrations include residual waste collection,[31] the animal feed industry,[32] grass seed production,[33] baking,[34]

podiatry clinics,[35] swine and poultry processing,[36] ceramic production,[37] professional cleaners,[38] indoor workplaces,[39] hospitals,[40] and corn silage.[41] Spores of *A. fumigatus* have also been detected in other indoor environments. Although the relative abundance and concentrations may not be as high as those identified in some occupational settings, investigators have noted that *A. fumigatus* is ubiquitous in the environment and exposure occurs daily. Human exposure has been estimated at 1–100 conidia/m^3 in indoor environments.[22-24; 42] In damp indoor environments, exposure can be elevated due to the contamination of cellulose-based building materials.[43] A secondary source of *A. fumigatus* could also be from indoor plants;[44] however, anthropogenic and outdoor transport are the likely sources of most exposures.

Regulatory Status

To date, no exposure limits for *A. fumigatus* have been proposed by NIOSH or the American Conference of Governmental Industrial Hygienists. An evaluation of human, animal, and epidemiological datasets by Eduard[45] of Norway's National Institute of Occupational Health has identified a lowest-observed-effect level of 1×10^5 spores/m^3. In 2010, the Environment Agency of the United Kingdom provided guidance on the acceptable levels of microbial contaminants emitted from composting operators.[29] Although not determined from dose-response relationships, airborne levels of *A. fumigatus* were set at 500 CFU/m^3 within 250 meters of a composting facility.[29] In Germany, the Federal Institute for Occupational Safety and Health set an occupational exposure limit of 50,000 CFU/m^3 for mesophilic fungal contaminants that also include *A. fumigatus*.[29]

Toxicity

Unlike low molecular weight chemicals that are traditionally studied by NTP, fungi are eukaryotic microorganisms. With fungi, the hyphae, fragments, spores, and reproductive propagules are all agents of personal inhalation exposure.[3] In addition to nonviable particles, an individual can be exposed to viable spores that can metabolically activate following in vivo deposition. Spore germination follows and can result in the release of immunologically active proteins and secondary metabolites.[3; 5; 6; 46] The cell wall of *A. fumigatus* contains numerous components, some of which stimulate the host's immune system, such as (1–3)-β-D glucan (or other high molecular weight proteins), and others that may suppress the host immune system, such as gliotoxin (or other secondary metabolites). These proteins and secondary metabolites, in combination with spore viability, result in a multifaceted pulmonary immune response involving a combination of innate and adaptive immune mechanisms.[18-20; 47]

Although numerous studies have identified and characterized fungal spore exposures in indoor, outdoor, and occupational environments, the toxicity of individual fungal proteins, secondary metabolites, and mycotoxins remains less clear. Personal exposure to *A. fumigatus* spores can result in a variety of adverse health effects, but the most commonly reported include invasive aspergillosis in immunocompromised subjects[22] and allergic bronchopulmonary aspergillosis.[48; 49] Other adverse respiratory conditions exacerbated by *A. fumigatus* include occupational rhinitis,[39] occupational asthma,[50] hypersensitivity pneumonitis,[34; 50] and organic dust toxic syndrome.[33; 51]

A. fumigatus produces approximately 100 proteins that can bind immunoglobulin,[22] as well as a broad diversity of secondary metabolites. Whole genome sequencing of *A. fumigatus* has

resolved 58 allergens and 9 additional predicted allergens that include secreted proteinases, glucanases, and cellulases.[52; 53] The International Union of Immunological Societies currently recognizes 23 *A. fumigatus* allergens. Ribotoxin (Asp f 1) is one of the most broadly characterized *A. fumigatus* allergens that is expressed during spore germination and emergence of *A. fumigatus* hyphae.[46] Exposure to Asp f 1 ranges from 7.6 to 29 ng/m^3 in outdoor air samples.[46] In addition to immunoglobulin binding proteins, *A. fumigatus* produces a variety of enzymes, including but not limited to, serine proteases, aspartic proteases, metalloproteinase, dipeptidyl peptidase, catalase, superoxide dismutase, and phospholipase.[42]

In addition to high molecular weight proteins, *A. fumigatus* produces toxic secondary metabolites capable of eliciting acute or chronic health effects.[54] Whole genome sequencing of *A. fumigatus* has identified 39 predicted secondary metabolism gene clusters,[55-57] although over 400 fungal mycotoxins or secondary metabolites have been characterized.[54] The pioneering chemical characterization studies by Guido Fischer[30; 58] and Frisvad and Sampson[59] provided preliminary insight into the species-specific secondary metabolite profiles derived from strains cultured from biowaste environments. In these studies, nine nonvolatile secondary metabolites were identified in culture and in conidial extracts and included gliotoxin, citrinin-like compound, fumagillin, fumigatin, fumiclavine A and C, fumitremorgin C, tryptoquivaline, trypacidin, and verruculogen.[30; 59]

Secondary metabolites produced by *A. fumigatus,* including ferricrocin, DHN melanin, fusarinine C, helvolic acid, and gliotoxin, have roles in mediating virulence and toxic effects.[55] Gliotoxin has been widely characterized for antimicrobial and immunosuppressive effects.[57; 60] Studies have shown gliotoxin to suppress cilia beat frequency, neutrophil function, T and B cell proliferation, protein synthesis, and phagocytosis, as well as inactivate alcohol dehydrogenase and cause epithelial damage.[54; 57; 60; 61] Toxicology studies have shown gliotoxin to be acutely toxic [as measured by median lethal dose (LD50)] to rabbits (LD50 = 45 mg/kg), mice (LD50 = 50 mg/kg), and rats (LD50 = 50–65 mg/kg).[62] In vitro studies have shown gliotoxin to have an IC_{50} of 0.3 µM in L-929 and A549 cell lines and 1.2 µM and 0.6 µM in Neuro-2a and HepG2 cell lines, respectively.[63] Waring and Beaver also reported that 0.1–3 µM gliotoxin resulted in DNA fragmentation in some immune cells and concentrations >10 µM induced apoptosis and cell death.[60] Other *A. fumigatus* secondary metabolites, such as fumagillin and helvolic acid, are also immunosuppressive but only at higher concentrations than gliotoxin.[54]

Trypacidin, a secondary metabolite localized on *A. fumigatus* conidia, has antiprotozoal activities and is a potential virulence factor.[64] In vitro studies have also shown trypacidin to reduce cell viability and to induce cell lysis at an IC_{50} of 7 µM, demonstrating toxicity to lung cells.[64] Helvolic acid (syn. fumigacin) is an antibacterial nortriterpenoid/metabolite produced by *A. fumigatus* and has also been shown to produce liver, peritoneal, and superficial lesions following repeated intraperitoneal injections.[65; 66]

Fumagillin is an additional *A. fumigatus* secondary metabolite that has been characterized to inhibit angiogenesis. Compared to gliotoxin, fumagillin is over 100-fold less cytotoxic to A549 cells but can inhibit endothelial cell proliferation and inhibit cilia beat frequency.[61; 63] Although oral administration of up to 2,000 mg/kg could be tolerated in rodent models,[65; 66] dermal exposure can be highly toxic and result in skin peeling from hands and feet as well as sensory disturbances.[65] Fumagillin has also been shown to have genotoxic effects by adducting DNA,[61; 63] resulting in a decreased mitotic index and increased micronuclei in erythrocytes.[67; 68]

Several secondary metabolites produced by *A. fumigatus* have specific central nervous system effects.[69] Tremorgenic mycotoxins are bioactive alkaloids that include fumitremorgin, tryptoquivaline, and verruculogen.[70; 71] Along with gliotoxin, verruculogen has the highest known toxicity of all the *A. fumigatus* secondary metabolites and can cause tremors.[58] However, the production of tremorgenic mycotoxins may be associated only with a growing mycelium and not with bioaerosols, suggesting that these secondary metabolites may not be an acute hazard in occupations such as the waste handling industry.[58] Animal studies have shown fumitremorgin to be a potent neurotoxin: Concentrations as low as 0.15 mg/kg elicited mild tremor following intravenous injection, and convulsion was induced with 0.3 mg/kg.[72] Fumitremorgin B and C administered to mice (1.0 mg/kg) and cockerels (25 mg/kg) were tremorgenic.[73] In vitro studies have additionally shown verruculogen to alter the electrochemical properties of human nasal epithelial cells,[74] and a dose of 2.4 mg/kg resulted in a lethality of 50%.[72] To date, little is known about toxicological responses to tryptoquivaline, and few studies have evaluated this secondary metabolite in occupational environments.[72]

The results of these combined immunological and toxicological studies demonstrate that *A. fumigatus* is a complex eukaryotic microorganism that produces a broad array of high molecular weight proteins, secondary metabolites, and mycotoxins with known toxicological effects. Each of these components varies in concentration, depending on the stage of the *A. fumigatus* life cycle.

Reproductive and Developmental Toxicity

Although several studies have evaluated reproductive and developmental toxicity for individual mycotoxins, NTP could not identify any studies following exposure to *Aspergillus* conidia.

Carcinogenicity

Although several studies have evaluated the carcinogenicity of individual mycotoxins, NTP could not identify any long-term studies that evaluated carcinogenic potential following exposure to *Aspergillus* conidia.

Genetic Toxicity

Little information has been published on the genotoxicity of *Aspergillus*-related chemicals, and none is available for *Aspergillus* conidia. Fumagillin, an antibiotic secreted by *Aspergillus*, has been tested in the form of fumagillin dicyclohexylamine salt for genotoxicity in vitro and in vivo. In vitro tests showed dose-related increases in structural chromosomal aberrations in human peripheral blood lymphocytes treated over a dose range of 0.34–9.20 µg/mL in a 1:1 water-sugar syrup vehicle.[68] Over this same dose range, these authors also reported significant, dose-related increases in micronucleated cells. The absolute increases in micronucleated cells were small (ranging from 0.62% in the control cultures to 0.89% at the highest concentration tested), however, and unlikely to be of biological significance, in contrast with the significant increases seen in the chromosome aberration assay. The dicyclohexylamine salt may itself possess genotoxic activity, having been reported to induce chromosomal aberrations in vitro,[75] thus confounding the interpretation of the genotoxicity test data reported for the fumagillin salt. In vivo, increases in micronuclei and chromosomal aberrations, along with cytostatic effects in bone marrow cells, were reported in BALB/c mice following administration of 25, 50, or

75 mg/kg fumagillin dicyclohexylamine salt in a water-sugar syrup once daily for 7 days via gavage.[76]

Study Rationale

Fungi were nominated to NTP for comprehensive toxicity studies due to continued public health concern about mold exposures in damp indoor environments or following natural disasters. NIOSH, in collaboration with NTP, utilized an AGS (Figure 3) that has enabled the production of a homogenous bioaerosol for delivery of fungal particles to mice housed in a multi-animal exposure chamber. The design and fabrication of the chamber allows inhalation exposures to test articles that simulate natural human exposures. *A. fumigatus* was selected as the initial test article for this series of inhalation toxicity studies given prevalence of the species in the environment, importance as an opportunistic pathogen, and occupational relevance as a contaminant in the biowaste industry.

Materials and Methods

Procurement and Characterization

Culture of *Aspergillus fumigatus*

Aspergillus fumigatus (NIH Strain B-5233) was acquired from the American Type Culture Collection (ATCC® 13073™). The strain was grown in bulk according to the culture methods presented in Appendix A. Fungal stock suspensions used throughout the study were prepared in sterile water and mixed with an equal volume of autoclaved 50% glycerol. The *A. fumigatus* suspensions were then transferred and stored at −80°C in 0.5 mL aliquots containing 25% glycerol to ensure the stability of the *A. fumigatus* strain.

Individual stock suspension aliquots were used weekly to inoculate two malt extract agar (MEA) plates for the cultivation of *A. fumigatus* according to the methods presented in Appendix A. Following 7–10 days of culture, *A. fumigatus* spores were liberated from individual MEA culture plates at weekly intervals using 2 mL of sterile water. The spore suspension was used to inoculate 10 g of autoclaved brown rice contained in a sterile petri dish with 7.5×10^6 *A. fumigatus* spores. A total of 15 petri dishes were inoculated each week. The rice culture plates were disturbed 1–2 times a day to prevent rice aggregation. Following 10–14 days of growth, the *A. fumigatus*-laden rice cultures were divided into two groups destined to become the heat-inactivated particle control and viable *A. fumigatus* test article. The *A. fumigatus* heat-inactivated spore exposure served as a biological particle control, as fungal spore germination has been identified in National Institute for Occupational Safety and Health (NIOSH) laboratory studies to be an important component associated with the pulmonary immune response to *A. fumigatus*.[18-20] *A. fumigatus* cultures for the heat-inactivated particle control groups were transferred to quartz plates and autoclaved for 15 minutes at 121°C according to the method in Appendix A. The plates for both exposure groups were then transferred to the NIOSH Inhalation Facility and stored in a desiccator for 3–4 days prior to study animal exposures.

Quality Assurance

Quality assurance and quality control of viable *A. fumigatus* consisted of testing cultures each week using internal transcribed spacer gene sequencing. This method allowed the amplification and detection of potentially contaminating mycobiota sources as described in Appendix A. The majority of identified fungal DNA belonged to *A. fumigatus* with no contaminating fungi detected in the viable test article and very little contaminating DNA detected in the heat-inactivated particle control. Cultures were also tested weekly to ensure reproducibility and production of nonviable *A. fumigatus* spores (Appendix A). Heat inactivation resulted in an average of 99.87% reduction in *A. fumigatus* spore viability throughout the course of the study (Table A-1).

Test Article Generation and Exposure System

As a part of an ongoing partnership with the National Toxicology Program (NTP), NIOSH initiated a series of pilot studies to develop and characterize an exposure system capable of simulating natural indoor and occupational fungal exposure. From these studies, the opportunistic fungal pathogen, *A. fumigatus*, was selected as a model organism due to its

widespread exposure potential in the biowaste industry, the production of respirable unicellular asexual spores, and the lack of available toxicity data. Previous animal models of fungal exposure have instilled or directly aspirated animals with protein extracts or spores washed in a buffered solution. Although these studies have provided initial insight into innate and adaptive pulmonary immune responses, they do not simulate fungal exposure in indoor, outdoor, or occupational environments. Unlike other microbial contaminants, fungi are viable organisms that grow as saprophytes in the environment and that produce a range of bioaerosols, including spores, hyphae, and submicron fragments.[3] Fungal bioaerosols are usually dry with the outer cell wall components intact. Immersion of spores and hyphal fragments in buffered solution may modify the outer cell wall, solubilizes outer cell wall antigens, and in some cases, may result in spore metabolic activation and germination prior to exposure of the animals. Each of these parameters could modulate toxicological responses to fungal particles following instillation or aspiration.

To control for these potentially confounding variables, NIOSH developed a computer-controlled acoustical generation system (AGS) that allows the aerosolization of dry fungal conidia (spores) into a chamber with the aim of replicating natural environmental exposures. Figure 3A shows the AGS based on a modified acoustically powered particle (Pitt-3) generator. The Pitt-3 generator, originally described by Weyel et al.,[77] was purchased from the University of Pittsburgh and evaluated under conditions recommended by Frazer et al.[78] The system was then fitted with a computer controller, and a number of design modifications were made[79; 80] for use by NIOSH to study the health effects caused by the inhalation of a wide variety of occupationally relevant dusts. In 2009, the AGS was used as the foundation for a new and improved system for dispersing nanomaterials,[77] which was subsequently modified for fungal spore aerosolization as described by Buskirk et al.[18] Fungi are grown on rice, a natural substrate, which is then placed on a rubber membrane in the AGS and aerosolized using acoustical energy. This method of fungal spore aerosolization mimics natural fungal exposures in the environment and enables the production of a homogenous spore suspension within the chamber (Figure 3B, Figure 3C). The AGS is linked to a multi-animal nose-only exposure chamber (Figure 3A). NIOSH has used this chamber in previous animal model studies that assessed diisocyanate exposures. The exposure chamber consists of a vertical cylindrical tower with animal tubes that radially project outward from the tower (Figure 3A). As spores are aerosolized in the exposure chamber, the mass concentration of spores can be monitored in real time and used to estimate the number of spores deposited within the animal's lung as described by Buskirk et al.[18]

To date, NIOSH has conducted several pilot studies that have characterized the aerosolization, deposition, and pulmonary immune responses to the heat-inactivated particle control and viable *A. fumigatus*. In these studies, reproducible growth of *A. fumigatus* on autoclaved rice was achieved, and test articles were generated by the AGS (Figure 3B, Figure 3C). Aerodynamic and microscopic analysis of the aerosolized *A. fumigatus* indicated the production of a respirable homogenous spore suspension (Figure 3B) with no ultrastructural changes (Figure 3C). Deposition studies then confirmed that *A. fumigatus* spores localized to the upper and lower respiratory airways of mice. The AGS and nose-only chamber were then used in proof-of-concept studies to determine an optimal dose for subsequent NIOSH and NTP studies evaluating dry *A. fumigatus* spores.[18] The pilot studies used a modified local lymph node assay to evaluate potential sensitization following exposures of approximately 1 hour per day, two times per week for 4 weeks. At a pulmonary deposition of 1×10^6 spores, viable *A. fumigatus* induced body

weight loss >10% and induced sensitization demonstrated by a 10-fold change in lymphocyte proliferation compared to the air control group in the pilot study. At 1×10^4 spores, the test article marginally induced sensitization at the requisite stimulation index of 10. Repeated exposures at 1×10^5 spores induced significant sensitization without body weight loss. An estimated pulmonary deposition of 1×10^5 spores was then selected as the optimal dose for all future NIOSH pulmonary immunology and NTP toxicology studies of *A. fumigatus*.

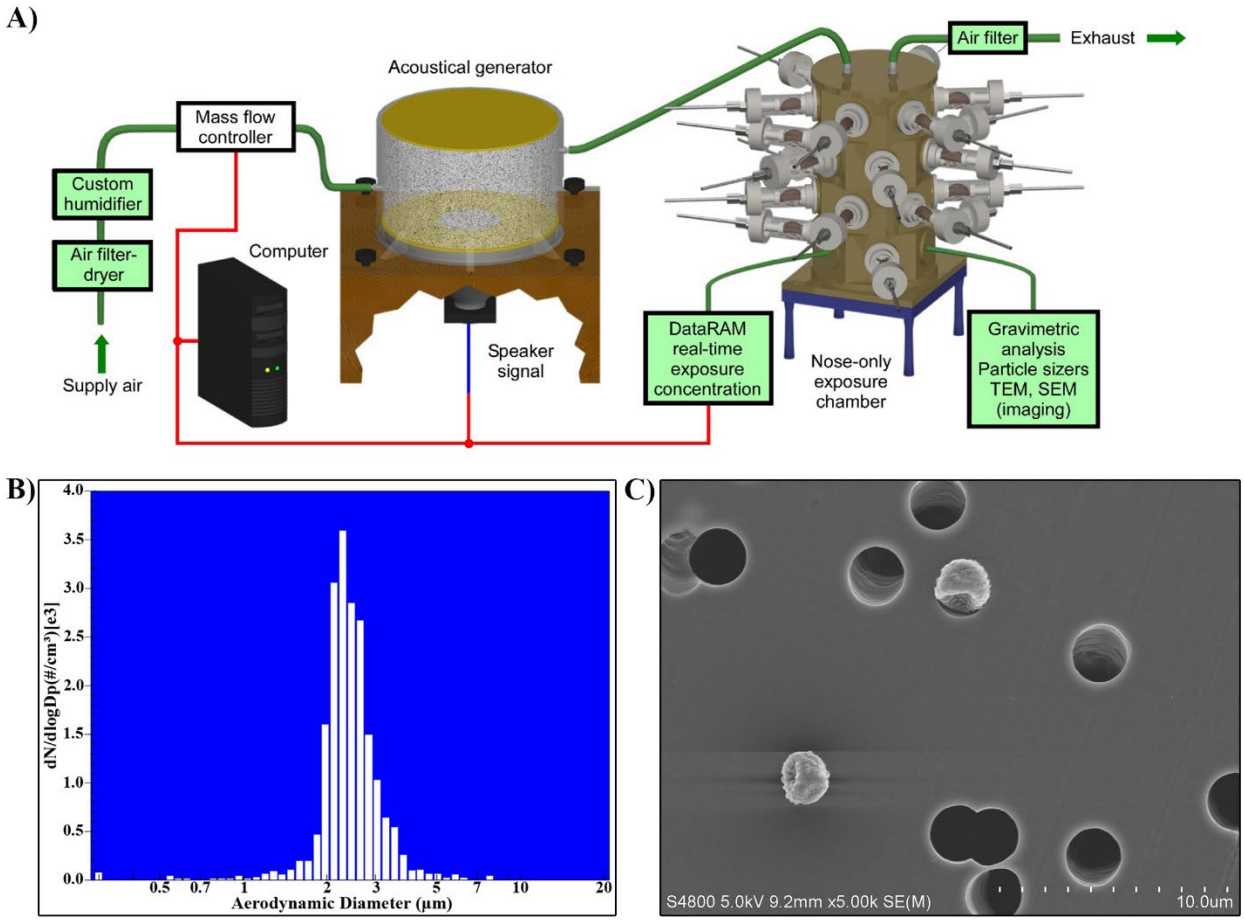

Figure 3. Illustration Showing the Acoustical Generator Nose-only Exposure System and *Aspergillus fumigatus* Particle Characterization

TEM = transmission electron microscope; SEM = scanning electron microscope. (A) HEPA-filtered supply air is directed into the acoustical generator, which vibrates at a designated frequency to form fungal spore aerosols from the fungus-coated rice grains. The fungal aerosol is directed into a nose-only chamber, and then the air is filtered before being sent into the exhaust system. A light-scattering instrument (DataRAM4; ThermoElectron Co., Franklin, MA) attached to the computer estimated the real-time aerosol mass concentration. This value was multiplied by a scale factor to determine the real-time number of spores deposited in the mouse lung based on the previously measured aerosol size distribution and a custom lung-deposition model. The software used that value to alter the amplitude of the speaker signal during exposures to obtain the desired chamber mass concentration. (B) The aerodynamic particle size distribution produced by the acoustical generator as measured with an aerodynamic particle sizer is shown (APS; TSI Inc., Shoreview, MN); 2–5 µm is the aerodynamic diameter of single or double aggregate *Aspergillus fumigatus* conidia. (C) Field emission SEM image is shown of *A. fumigatus* fungal conidia deposited on a polycarbonate filter sampled from the animal's breathing zone in the nose-only chamber.

Aerosolized viable *A. fumigatus* and the heat-inactivated particle control were delivered to male and female B6C3F1/N mice housed in a multi-animal nose-only exposure chamber via the AGS

(Figure 3A). Animals were exposed two times per week for 3 months via inhalation. In addition, an air control group received the same HEPA-filtered air that was being used to deliver viable *A. fumigatus,* and the heat-inactivated particle control group was exposed as previously described.[18]

Inhalation exposures were achieved by weighing and then placing the mice in a custom built nose-only multi-animal exposure carousel that was connected to the AGS (Figure 3A) as described in McKinney et al.[81] Mice were placed in random pods for each exposure to minimize the effects of small concentration differences observed within individual ports. Although the nose-only carousel houses up to 24 pods (Figure 3A), only 20 pods (10 male and 10 female mice) were used during each exposure. Additional ports served as sample ports for the collection and analysis of test articles. During exposures, air control and exposure animals were placed on different exposure carousels located in the same inhalation exposure room.

The B6C3F1/N mice were exposed to viable *A. fumigatus* or the heat-inactivated particle control until the DataRAM software estimated that a total of approximately 1×10^5 spores had been deposited within the lungs during the 1-hour exposure session. Figure 4 shows the estimated pulmonary deposition following each exposure for the duration of the study. The AGS successfully generated the aerosolized test article and was able to reproduce it over the course of this study (Figure 4). It is important to note that an inherent variability in the sporulation of the test article will always exist when working with biological organisms. In addition, heat inactivation via autoclaving can potentially result in some of the organism sticking to the rice, which will make the aerosolization of the heat-inactivated test articles more difficult compared to the viable test articles. The estimation software used a mouse-lung-deposition model developed by the NIOSH Inhalation Facility, which was based on deposition measurements from Raabe et al.[82] During viable *A. fumigatus* or heat-inactivated particle control exposures, lung-deposition calculations were achieved using real-time DataRAM mass concentration measurements and a previously described scaling factor that estimated the number of spores deposited in the lungs of mice in real time.[18; 19] The integrated value of mass deposition per unit time was calculated throughout the exposures. The AGS automatically turned off when the desired total spore deposition had been delivered to the mice; however, the mice continued to receive HEPA-filtered humidified (approximately 40% RH) air supplied at a flow rate of 6 L/minute until 60 minutes had been reached. The desired spore concentration for the viable *A. fumigatus* was usually reached more quickly than for the heat-inactivated particle control spores, but no exposure was longer than 60 minutes. Each exposure corresponded to a 60-minute average mass concentration of approximately 10 mg/m^3. Gravimetric filters were also collected during each exposure to continually monitor and adjust the DataRAM calibration. Air control mice received HEPA-filtered air supplied to the chamber at the same flow rate (6 L/minute) and time (60 minutes) as *A. fumigatus*-exposed mice. All environmental conditions were kept constant in each carousel between control and exposed (viable *A. fumigatus* and heat-inactivated particle control) animals.

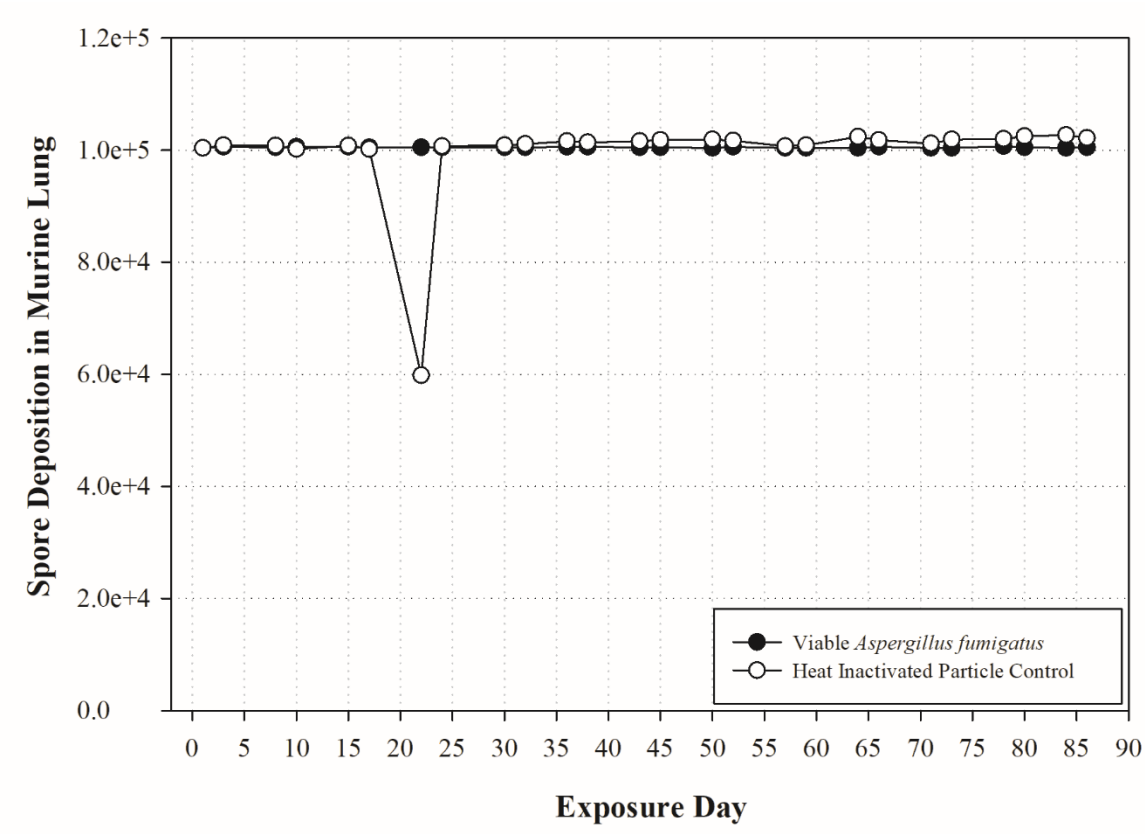

Figure 4. Estimated Pulmonary Deposition of *Aspergillus fumigatus* Spores for Each Exposure over the Course of the Three-month Study

Values represent the number of spores estimated to deposit in the lung for viable *A. fumigatus* (solid circles) and heat-inactivated particle control (open circles) exposure groups.

Animal Source

Male and female B6C3F1/N mice (n = 20/exposure group and 20 sentinel mice) were obtained via an NTP liaison from the NTP colony maintained at Taconic Biosciences, Inc. (Germantown, NY).

Animal Welfare

Animal care and use were in accordance with the Public Health Service Policy on Humane Care and Use of Animals. The NIOSH Inhalation Facility is an environmentally controlled barrier facility that is fully accredited by AAALAC. Studies were approved by the NIOSH Animal Care and Use Committee and conducted in accordance with all NIOSH animal procedures and policies.

Three-month Study

Mice were approximately 5–6 weeks old on receipt. The animals were weighed, distributed randomly into groups of approximately equal initial mean body weights, and assigned to viable *A. fumigatus*, heat-inactivated particle control, or air control groups. Mice were initially

identified via tail marking, using a permanent marker and a consecutive numbering system. Animals that weighed more or less than 10% of the mean of the group were placed in the sentinel group (n = 20). At 4 weeks and at the end of the study, serologic analyses for bacterial and viral pathogens were performed on 10 male and 10 female sentinel mice using the protocol of the NIOSH Sentinel Animal Program (Appendix D). Before the study, the mice were acclimated to the NIOSH animal facility for a minimum of 15 days. Then, to reduce potential stress from exposures, the mice were acclimated to the housing units used for the exposures (nose-only carousel) for 1 week (two 1-hour acclimations) before the initial fungal exposure. Study mice were placed individually in the carousel housing units (pods), and humidified HEPA-filtered air was supplied to the chamber for 1 hour on 2 separate days during the week before the initial exposures. After acclimation was complete and before the exposures began, the mice were tattooed using a consecutive numbering system.

Male and female B6C3F1/N mice were randomly assigned to viable *A. fumigatus*, heat-inactivated particle control, and air control groups. Using a modified NTP protocol, groups of 10 male and 10 female mice were placed in the exposure units on the carousel on the same 2 days of the week for 26 exposures (3 months). Male and female mice were exposed for 1 hour per day to 10 mg/m^3 (resulting lung dose = 1×10^5) viable *A. fumigatus* spores, 10 mg/m^3 (resulting lung dose = 1×10^5) heat-inactivated *A. fumigatus* conidia (particle control), or HEPA-filtered air (air control) that was delivered through the AGS into the nose-only exposure chamber at a flow rate of 6 L/minute. This estimated dose was also used in companion studies that evaluated pulmonary immunological responses to *A. fumigatus* spores.[20; 47]

Following acclimation and exposures, the animals were transferred from the individual carousel housing pods to a standard cage environment located in an adjoining room within the facility. Male and female mice were housed in HEPA-filtered ventilated polycarbonate cages on autoclaved hardwood chip bedding. Male B6C3F1/N mice were individually housed during acclimation and the life phases of the study due to social incompatibilities, whereas female mice were housed in groups of five. The ambient temperature of the animal facility was maintained between 67°F and 76°F, and the relative humidity ranged between 20% and 75% (Table 1). The light/dark cycle was maintained at 12-hour intervals. Mice were provided feed and autoclaved tap water ad libitum, except when housed in the pods during acclimation or exposures. Sentinel mice selected during the acclimation period were housed within the same room and were free of viral and bacterial pathogens (Appendix D).

Study animals were weighed upon initial receipt, and body weights were recorded weekly until the end of the study. Clinical observations were recorded daily for the duration of the study, once a day (morning) on nonexposure days, and twice a day (morning/afternoon) on exposure days. Information about the study design, facilities, and animal maintenance are provided in Table 1. The report on feed composition is provided in Appendix C.

At the completion of the 3-month study, the mice were injected intraperitoneally with a lethal dose of sodium pentobarbital (100–300 mg/kg). Peripheral blood for hematology and micronuclei determination was obtained via cardiac puncture. Whole blood (a minimum of 600 µL) was collected in tubes containing potassium ethylenediaminetetraacetic acid, and 400 µL was immediately processed for hematology using a ProCyte Dx hematology analyzer (IDEXX Laboratories, Inc., Westbrook, ME). Table 1 lists the parameters measured. A minimum

of 200 µL of blood was immediately refrigerated and shipped to Integrated Laboratory Systems, LLC (ILS; Research Triangle Park, NC) for micronuclei determination.

Necropsies were performed at the NIOSH site by Battelle staff (Columbus, OH) in random order on all animals surviving to terminal sacrifice. The organs weighed at terminal necropsy included the left and right epididymis, heart, left and right kidney, liver, lungs, left and right ovary, left and right testis, and thymus. Bilateral organs were weighed and recorded separately. At necropsy, the tissues in Table 1 were examined in situ for gross lesions, removed, and then fixed and preserved in 10% neutral buffered formalin (NBF). Exceptions included the eyes and testes (epididymis and vaginal tunics of testes), which were initially fixed in Davidson's solution and modified Davidson's solution, respectively, then transferred into 10% NBF. The collected and fixed tissues were then shipped overnight to the Battelle facility and further processed and trimmed, embedded in paraffin, sectioned at a thickness of 5 µm, and stained with hematoxylin and eosin (H&E). Histological sections of aorta, gallbladder, heart, liver, lung (all five lobes), bronchial lymph node, and spleen were stained with Grocott's methenamine silver (GMS) stain for fungal elements. Battelle laboratory pathologists conducted a complete histopathological examination on all air control, heat-inactivated particle control, and viable *A. fumigatus*-exposed mice. Table 1 lists the tissues and organs that were examined.

After a review of the laboratory reports and selected histopathology slides by a quality assessment (QA) pathologist, the findings and reviewed slides were submitted to an NTP Pathology Working Group (PWG) coordinator for a second independent review. Any inconsistencies in the diagnoses made by the study laboratory and QA pathologist were resolved by the NTP pathology peer-review process. Final diagnoses for reviewed lesions represent a consensus of the PWG or a consensus between the study laboratory pathologist, NTP pathologist, QA pathologist, and the PWG coordinator. Details of these review procedures have been described, in part, by Maronpot and Boorman[83] and Boorman et al.[84]

Table 1. Experimental Design and Materials and Methods in the Three-month Inhalation Study of *Aspergillus fumigatus* Spores

Three-month Study
Study Laboratories
National Institute for Occupational Safety and Health (Morgantown, WV) Battelle (Columbus, OH)
Strain and Species
B6C3F1/N mice
Animal Source
Taconic Biosciences, Inc. (Germantown, NY)
Time Held before Studies
20 days
Average Age When Studies Began
8 weeks
Date of First Exposure
September 15, 2014

Three-month Study
Duration of Exposure
3 months
Date of Last Exposure
December 9, 2014
Necropsy Date
December 10, 2014
Average Age at Necropsy
21 weeks
Size of Study Groups
10 males and 10 females
Method of Distribution
Animals were distributed randomly into groups of approximately equal initial mean body weights.
Animals per Cage
1 (male), 5 (females)
Method of Animal Identification
Tail tattoo
Diet
Irradiated NTP-2000 wafers (Zeigler Brothers, Inc., Gardners, PA), available ad libitum, changed at least weekly
Water
Autoclaved tap water (Morgantown, WV municipal supply) provided in water bottles, available ad libitum
Cages
HEPA-filtered, ventilated polycarbonate cages, in filtered and ventilated racks (Lab Products Inc, Seaford, DE). Cages that housed three or more female mice were changed weekly. Cages that housed two mice or fewer (males) were changed every other week.
Bedding
Autoclaved hardwood chip bedding (Teklad Certified Sani-Chips®; Envigo, Madison, WI); irradiated crinkle cut craft paper provided for nesting/enrichment (The Andersons, Inc.; Maumee, OH), changed with cage changes.
Animal Room Environment
Temperature: 71.31°F ± 1.44°F (min: 67.42°F; max. 75.17°F) Relative Humidity: 45.76% ± 11.49% (min: 22.09%; max: 74.55%) Room fluorescent light: 12 hours/day Room air changes: 10–15/hour
Test Article
Aspergillus fumigatus (*A. fumigatus*) spores: untreated (viable) or heat-inactivated particle controls NIH Strain B-5233; *Aspergillus fumigatus* Fresenius, anamorph (ATCC® 13073™)

Three-month Study
Exposure Concentrations
Estimated lung depositions: Air control: 0 (HEPA-filtered air only) Heat-inactivated particle control: 1×10^5 nonviable *A. fumigatus* spores (exposure chamber mass concentration: 10 mg/m^3) Test article: 1×10^5 viable *A. fumigatus* spores (exposure chamber mass concentration: 10 mg/m^3)
Type and Frequency of Observation
Observed once daily on nonexposure days (morning) and twice daily on exposure days (morning/afternoon). Clinical findings were recorded at each observation and at study termination. Animals were weighed initially and then weekly until the end of the study.
Method of Euthanasia
Intraperitoneal injection of a lethal dose of sodium pentobarbital (100–300 mg/kg; Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI) followed by exsanguination via cardiac puncture
Necropsy
Necropsies were performed on all study mice. Organs weighed were epididymis (left and right), heart, kidneys (left and right), liver, lungs, ovaries (left and right), testes (left and right), and thymus.
Hematology
Blood was collected from all surviving study mice via cardiac puncture at study termination. Hematological analyses included hematocrit, erythrocyte count, reticulocyte count, hemoglobin, mean cell volume, mean cell hemoglobin, mean cell hemoglobin concentration, platelet count, and leukocyte count and differential.
Histopathology
Complete histopathology was performed on all study mice. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal gland, aorta, bone marrow, brain, esophagus, eye, femur, gallbladder, Harderian gland, heart, intestine (large and small), kidney, larynx, liver, lung, lymph nodes (bronchial, mandibular, mediastinal, mesenteric), mammary gland, nose, pancreas, pancreatic islets, parathyroid gland, pituitary gland, salivary glands, skin, spleen, stomach, thymus, thyroid gland, trachea, and urinary bladder. Other male tissues examined included the epididymis, preputial gland, prostate gland, seminal vesicle, and testes. Other female tissues examined included the cervix, clitoral gland, ovary, uterus, and vagina.

Statistical Analysis

Calculation and Analysis of Nonneoplastic Lesion Incidences

The incidences of nonneoplastic lesions are presented as numbers of animals bearing such lesions at a specific anatomic site and the numbers of animals with that site examined microscopically. Fisher's one-sided exact test,[85] a procedure that uses the overall proportion of affected animals, was used to determine significance between all pairs of the three exposure groups.

Analysis of Continuous Variables

Two approaches were employed to assess the significance of pairwise comparisons between dosed and control groups in the analysis of continuous variables. Organ and body weight data, which historically have approximately normal distributions, were analyzed using a one-way analysis of variance with post-hoc Tukey HSD test for multiple comparisons between all pairs of the three exposure groups. Hematology data, which have typically skewed distributions, were

analyzed using the nonparametric multiple comparison method of Dunn.[86] Prior to statistical analysis, extreme values identified by the outlier test of Dixon and Massey[87] were examined by NTP personnel, and implausible values were eliminated from the analysis.

Quality Assurance Methods

The 3-month study was conducted in compliance with U.S. Food and Drug Administration Good Laboratory Practice Regulations whenever possible.[88] In addition, the 3-month study reports were audited retrospectively by an independent QA contractor against study records submitted to the NTP Archives. Separate audits covered completeness and accuracy of the pathology data, pathology specimens, final pathology tables, and a draft of this NTP Toxicity Study Report. Audit procedures and findings are presented in the reports and are on file at the National Institute of Environmental Health Sciences. The audit findings were reviewed and assessed by NTP staff, and all comments were resolved or otherwise addressed during the preparation of this Toxicity Report.

Genetic Toxicology

The genetic toxicity of *A. fumigatus* was assessed by testing the ability of an *A. fumigatus* extract to induce mutations in various strains of *Salmonella typhimurium* and *Escherichia coli* and to increase the frequency of micronucleated erythrocytes in mouse peripheral blood. The protocol for these studies and the results are given in Appendix B.

The genetic toxicity studies have evolved from an earlier effort by NTP to develop a comprehensive database that would facilitate critical anticipation of a chemical's carcinogenicity in experimental animals, using the results from several in vitro and in vivo short-term tests that measure functionally distinct genotoxicity endpoints. The short-term tests were originally developed to clarify proposed mechanisms of chemical-induced DNA damage based on the relationship between electrophilicity and mutagenicity[89] and the somatic mutation theory of cancer.[90; 91] Of note, however, is that not all cancers arise through genotoxic mechanisms.

Bacterial Mutagenicity

DNA reactivity combined with *Salmonella* mutagenicity is highly correlated with induction of carcinogenicity in multiple species/sexes of rodents and at multiple tissue sites.[92] A positive response in the *Salmonella* test was shown to be the most predictive in vitro indicator for rodent carcinogenicity (89% of the *Salmonella* mutagens are rodent carcinogens).[93; 94] Additionally, no battery of tests that included the *Salmonella* test improved the predictivity of the *Salmonella* test alone. However, these other tests can provide useful information on the types of DNA and chromosomal damage induced by the chemical under investigation. The protocol for these studies and the results are given in Appendix B.

Peripheral Blood Micronucleus Test

Micronuclei (literally "small nuclei" or Howell-Jolly bodies) are biomarkers of induced structural or numerical chromosomal alterations and are formed when acentric fragments or whole chromosomes fail to incorporate into either of two daughter nuclei during cell division.[95; 96] The predictivity for carcinogenicity of a positive response in acute in vivo bone marrow chromosome aberration or micronucleus tests appears to be less than that in the *Salmonella*

test.[97; 98] However, clearly positive results in long-term peripheral blood micronucleus tests have high predictivity for rodent carcinogenicity; a weak response in one sex only or negative results in both sexes in this assay do not correlate well with either negative or positive results in rodent carcinogenicity studies.[99] Because of the theoretical and observed associations between induced genetic damage and adverse effects in somatic and germ cells, the determination of in vivo genetic effects is important to the overall understanding of the risks associated with exposure to a particular chemical. The protocol for these studies and the results are given in Appendix B.

Results

Data Availability

The National Toxicology Program (NTP) and the National Institute for Occupational Safety and Health (NIOSH) evaluated all study data. Data relevant for evaluating toxicological findings are presented here. All study data are available in the NTP Chemical Effects in Biological Systems (CEBS) database: https://doi.org/10.22427/NTP-DATA-TOX-100.[100]

Three-month Study

All male mice survived to the end of the study. Two female mice, one in the air control group and one in the heat-inactivated particle control group, died on day 73 and day 84, respectively. Both mice were found dead when removed from nose-only pods following exposure. Gross examination showed discoloration of the lungs, but the cause of death could not be determined (Appendix E).

Final survival and body weight data are shown in Table 2. Terminal mean body weights of male and female mice exposed to viable *A. fumigatus* spores were not significantly different from the air control groups or the heat-inactivated particle control groups, nor were terminal mean body weights significantly different between the heat-inactivated particle control groups and air control groups. The body weight gain among groups of both male and female mice over the course of the study was similar, as illustrated in Figure 5. Clinical observations collected throughout the duration of the study revealed no remarkable findings (Appendix E).

Table 2. Summary of Survival and Mean Body Weights of Male and Female Mice in the Three-month Inhalation Study of *Aspergillus fumigatus* Spores[a]

Exposure Agent	Survival	Initial Body Weight (g)	Final Body Weight (g)	Change in Body Weight (g)	Final Weight Relative to Air Controls (%)
Male					
Air Control	10/10	25.7 ± 0.4	31.8 ± 0.7	6.0 ± 0.6	–
Heat-inactivated Particle Control	10/10	25.7 ± 0.5	32.1 ± 0.8	6.3 ± 0.7	101
Viable *A. fumigatus*	10/10	26.4 ± 0.7	30.8 ± 1.2	4.3 ± 0.6	97
Female					
Air Control	9/10[b]	20.4 ± 0.4	26.6 ± 0.9	6.2 ± 0.5	–
Heat-inactivated Particle Control	9/10[c]	20.9 ± 0.5	26.7 ± 0.8	6.0 ± 0.5	101
Viable *A. fumigatus*	10/10	20.8 ± 0.3	25.8 ± 1.0	5.0 ± 0.9	97

[a]Weights and weight changes are presented as mean ± standard error. One-way analysis of variance was conducted, and no significant differences were observed between groups.
[b]Day of death: 73.
[c]Day of death: 84.

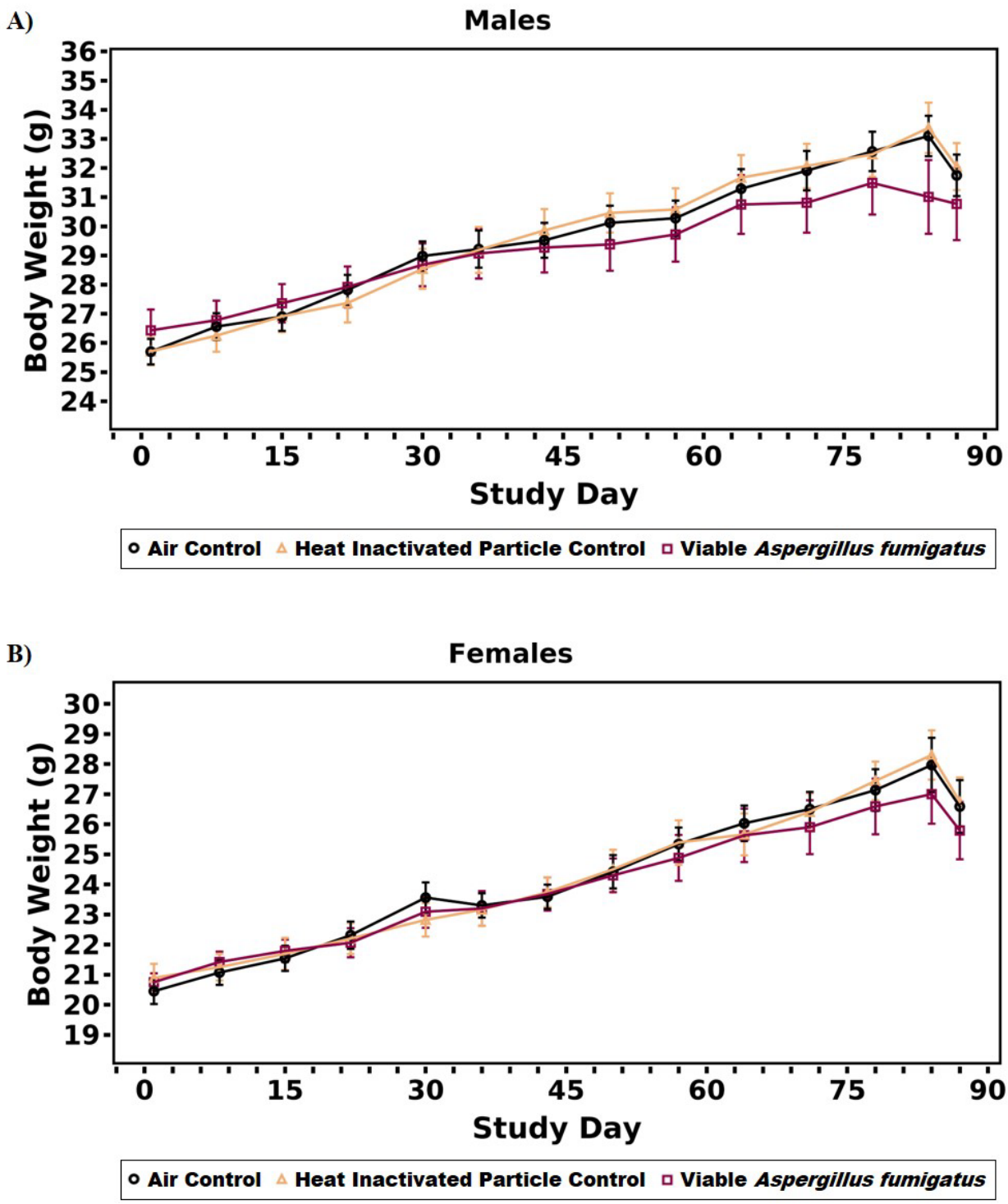

Figure 5. Growth Curves for Male and Female Mice in the Three-month Inhalation Study of *Aspergillus fumigatus* Spores

Data points represent mean body weight in grams, and error bars represent the standard deviation of the mean for male (A) and female (B) mice. Open circle: air control; open triangle: heat-inactivated particle control; open square: viable *A. fumigatus*.

Absolute lung weights were significantly increased in viable *A. fumigatus*-exposed mice compared to the air control groups (males: 43% and females: 68%) and compared to the heat-inactivated particle control groups (males: 36% and females: 52%) (Table 3). Similarly, mean relative lung weights of mice exposed to viable *A. fumigatus* were also significantly increased in male (47% and 45%) and female (75% and 59%) mice relative to the air control and heat-inactivated particle control groups, respectively (Table 3). The increases in absolute and relative lung weight between viable *A. fumigatus*-exposed and air control mice correlated with several histopathological lesions in the lungs of exposed mice.

Table 3. Summary of Select Organ Weights and Organ-Weight-to-Body-Weight Ratios for Male and Female Mice in the Three-month Inhalation Study of *Aspergillus fumigatus* Spores[a,b]

	Air Control	Heat-inactivated Particle Control	Viable *A. fumigatus*
Male			
n	10	10	10
Necropsy Body Wt. (g)	31.8 ± 0.7	32.1 ± 0.8	30.8 ± 1.2
Lung			
Absolute (g)	0.21 ± 0.01	0.22 ± 0.01	0.30 ± 0.01**[##]
Relative (mg/g)[c]	6.71 ± 0.26	6.81 ± 0.27	9.89 ± 0.60**[##]
Female			
n	9	9	10
Necropsy Body Wt. (g)	26.6 ± 0.9	26.7 ± 0.8	25.8 ± 1.0
Lung			
Absolute (g)	0.19 ± 0.01	0.21 ± 0.01	0.32 ± 0.01**[##]
Relative (mg/g)	7.17 ± 0.15	7.90 ± 0.35	12.53 ± 0.66**[##]

**Statistically significant pairwise difference from air control at $p \leq 0.01$.
[##]Statistically significant pairwise difference from heat-inactivated particle control at $p \leq 0.01$.
[a]Data are presented as mean ± standard error.
[b]Statistical analysis performed using one-way analysis of variance with post-hoc Tukey HSD test for all pairwise comparisons.
[c]Relative organ weights (organ-weight-to-body-weight ratios) are given as mg organ weight/g body weight.

A minimal (<4%) but statistically significant increase in the erythrocyte count in the female viable *A. fumigatus* group relative to the heat-inactivated particle control group was observed (Appendix E). There were no other changes in the female erythron and no changes observed in the male erythron (Appendix E). The minimal change in the female erythrocyte count was not considered related to exposure.

Relative to the air control groups, the neutrophil count was significantly increased in the viable *A. fumigatus*-exposed male mice (Table 4). In addition, the total leukocyte, neutrophil, lymphocyte, monocyte, and eosinophil counts were significantly increased in the viable *A. fumigatus* male mice compared to the heat-inactivated particle control group.

Table 4. Summary of Select Hematology Data for Male and Female Mice in the Three-month Inhalation Study of *Aspergillus fumigatus* Spores[a,b]

	Air Control	Heat-inactivated Particle Control	Viable *A. fumigatus*
Male			
n	10	10	10
Leukocytes (10^3/µL)	3.47 ± 0.16	2.87 ± 0.23	4.32 ± 0.37[##]
Neutrophils (10^3/µL)	0.56 ± 0.04	0.49 ± 0.04	0.97 ± 0.10*[##]
Lymphocytes (10^3/µL)	2.79 ± 0.14	2.28 ± 0.21	3.07 ± 0.26[#]
Monocytes (10^3/µL)	0.02 ± 0.01	0.02 ± 0.01	0.07 ± 0.03[##]
Eosinophils (10^3/µL)	0.10 ± 0.01	0.08 ± 0.00	0.20 ± 0.03[##]
Female			
n	8	9	9
Leukocytes (10^3/µL)	2.94 ± 0.33	2.85 ± 0.22	3.91 ± 0.42
Neutrophils (10^3/µL)	0.39 ± 0.05	0.43 ± 0.04	0.62 ± 0.07
Lymphocytes (10^3/µL)	2.47 ± 0.27	2.32 ± 0.20	3.07 ± 0.35
Monocytes (10^3/µL)	0.03 ± 0.01	0.03 ± 0.01	0.09 ± 0.04
Eosinophils (10^3/µL)	0.06 ± 0.01	0.07 ± 0.01	0.12 ± 0.02

*Statistically significant pairwise difference from air control at $p \leq 0.05$.
[#]Statistically significant pairwise difference from heat-inactivated particle control at $p \leq 0.05$.
[##]Statistically significant pairwise difference from heat-inactivated particle control at $p \leq 0.01$.
[a]Data are presented as mean ± standard error.
[b]Statistical analysis performed by the Dunn (pairwise) test.

Gross pathology findings can be found in Appendix E. Enlarged gray bronchial lymph nodes were identified in 5/10 females in the viable *A. fumigatus* group. This observation corresponded histologically to hyperplasia (either lymphocyte hyperplasia, plasma cell hyperplasia, or both). One female in the heat-inactivated particle control group and one female air control mouse, both of which were found dead after exposure, had red discoloration of the lungs. Overall, no gross lesions were identified in any of the other organs evaluated in male or female study groups.

The incidences of nonneoplastic lesions were increased in the larynx, lung, and bronchial lymph nodes of viable *A. fumigatus*-exposed mice compared to the heat-inactivated particle control or air control groups. The incidences of nonneoplastic lesions were increased in the lung of heat-inactivated particle control-exposed mice compared to the air control groups (Table 5, Table 6, Table 7; Appendix E).

In the larynx of males and females, the incidences of squamous metaplasia of the epiglottis were significantly increased in the viable *A. fumigatus* groups relative to the air control groups and the heat-inactivated particle control groups (Table 5). One female in the viable *A. fumigatus* group had an ulcer in the epithelium of the larynx, and another female in that group had hyperplasia of the squamous epithelium of the larynx (Table 5). Although not significantly increased, these lesions may have been related to exposure to viable *A. fumigatus*.

Microscopically, squamous metaplasia in the larynx was seen as replacement of the normal ciliated epithelium at the base of the epiglottis in level I sections by nonkeratinized squamous epithelium (Figure 6). The severity of squamous metaplasia was minimal in all groups where it occurred and affected <50% of the epithelium at the base of the epiglottis. The ulcer was characterized by a focal loss of the squamous epithelium overlying the arytenoid cartilage in a level II section of the larynx, and the squamous epithelium hyperplasia consisted of a thickening of the squamous epithelium overlying the arytenoid cartilage in level I.

Table 5. Incidences of Nonneoplastic Lesions of the Larynx in Male and Female Mice in the Three-month Inhalation Study of *Aspergillus fumigatus* Spores[a]

	Air Control	Heat-inactivated Particle Control	Viable *A. fumigatus*
Male[b]	10	10	10
Epiglottis, Metaplasia, Squamous[c]	3 (1.0)[d]	0	9**[##] (1.0)
Female	10	9	10
Epiglottis, Metaplasia, Squamous	0	1 (1.0)	9**[##] (1.0)
Epithelium, Ulcer	0	0	1 (1.0)
Squamous Epithelium, Hyperplasia	0	0	1 (1.0)

**Statistically significant pairwise difference from air control at $p \leq 0.01$.
[##]Statistically significant pairwise difference from heat-inactivated particle control at $p \leq 0.01$.
[a]Statistical analysis performed by the one-sided Fisher's exact test.
[b]Number of animals examined microscopically.
[c]Number of animals with lesion.
[d]Average severity grade of observed lesion in affected animals: 1 = minimal; 2 = mild; 3 = moderate; 4 = marked.

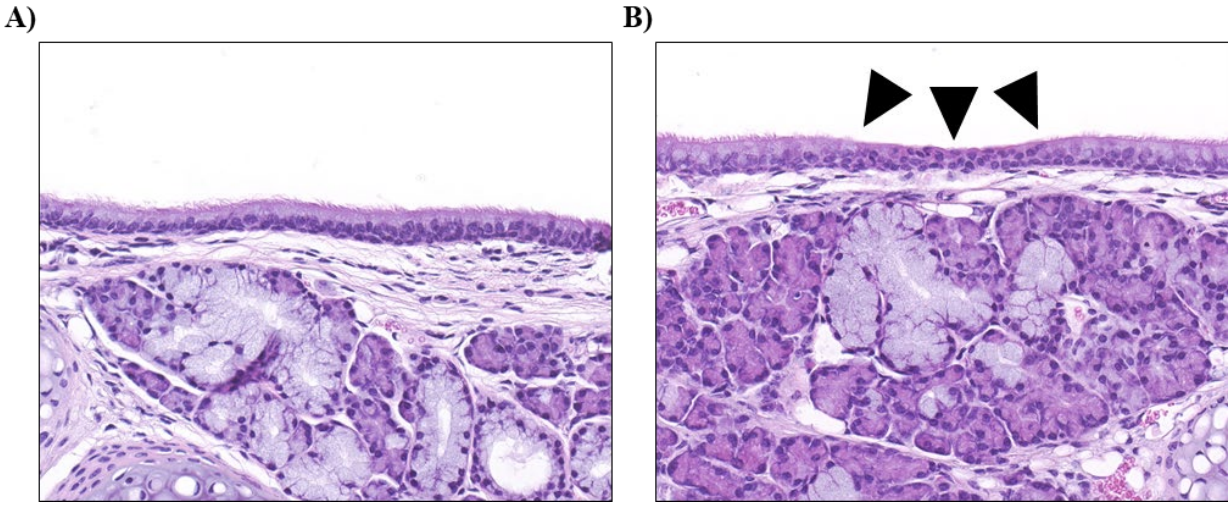

Figure 6. Histopathology Images of Larynxes Following Inhalation of HEPA-filtered Air or Viable *Aspergillus fumigatus* Spores for Three Months (H&E)

Larynx from an air control female mouse (A) and a viable *A. fumigatus*-exposed female mouse (B) at the base of the epiglottis (40x magnification). Squamous metaplasia at the base of the epiglottis of the larynx was seen as replacement of the normal ciliated epithelium (A, air control) by nonkeratinized squamous epithelium (B, arrowheads).

The lungs of the male and female viable *A. fumigatus*-exposed mice were characterized by numerous nonneoplastic lesions compared to those of the air control mice and the heat-inactivated particle control mice (Table 6). Nonneoplastic lesions were also observed in the heat-inactivated particle control mice at a higher incidence than in the air control mice.

In the lungs, the incidences of artery hypertrophy, medial, were significantly increased in the viable *A. fumigatus*-exposed males and females relative to either the air control groups or the heat-inactivated particle control groups (Table 6). Mild to moderate artery hypertrophy, medial, was present in all male and female viable *A. fumigatus*-exposed mice and was absent in all air control and heat-inactivated particle control mice (Table 6). Artery hypertrophy, medial, was characterized histologically by circumferential thickening of the tunica media of small and medium arterioles and arteries by concentric layers of hypertrophied and hyperplastic smooth muscle cells (Figure 7). Lesion severity was graded according to the proportion of the pulmonary arteries affected as follows: minimal severity (1) was <10% of the arteries affected, mild (2) was 10%–40% of the arteries affected, moderate (3) was 41%–75% of the arteries affected, and marked (4) was >75% of the arteries affected.

The incidences of bronchiolar epithelium hyperplasia were significantly increased in the viable *A. fumigatus*-exposed males and females relative to either the air control groups or the heat-inactivated particle control groups (Table 6). Minimal bronchiolar epithelium hyperplasia was seen in 9/10 males and 8/10 females exposed to viable *A. fumigatus*, and in 1/10 heat-inactivated particle control females (Table 6). Bronchiolar epithelium hyperplasia consisted of crowded, plump, often piled (2–5 cell layers thick), cuboidal, ciliated epithelial cells lining terminal bronchioles with occasional small papillary projections and extension into adjacent alveolar septa (Figure 7). Bronchiolar epithelium hyperplasia was considered to be of minimal severity (1) as it involved <10% of the bronchioles in all instances.

The incidences of chronic active inflammation in the lungs were significantly increased in the viable *A. fumigatus*-exposed males and females relative to either the air control groups or the heat-inactivated particle control groups (Table 6). Mild to moderate chronic active inflammation in the lungs was present in all male and female mice exposed to viable *A. fumigatus* spores and was absent in all air control mice and all but one of the females in the heat-inactivated particle control groups (Table 6). Microscopically, chronic active inflammation was seen as predominantly perivascular infiltrates of lymphocytes and abundant neutrophils and alveolar macrophages (Figure 7). The perivascular infiltrates frequently extended into surrounding alveolar septa and spaces and occasionally into vessel walls. The associated alveolar macrophages usually exhibited pale to brightly eosinophilic cytoplasm, which sometimes contained intracytoplasmic cell debris and/or variably shaped eosinophilic material. Multinucleated giant cells were often present in small numbers. The severity of chronic active inflammation in the lung was graded based upon the extent of lung involvement as follows: minimal severity (1) was <10% of the lung affected, mild (2) was 10%–40% of the lung affected, moderate (3) was 41%–75% of the lung affected, and marked (4) was >75% of the lung affected. This chronic active inflammation likely corresponded with the increased numbers of circulating neutrophils observed in the viable *A. fumigatus* groups compared to the air control groups (Table 4).

Chronic inflammation was seen in the lungs of 3/10 males and 7/10 females in the heat-inactivated particle control groups. In females, this chronic inflammation was significantly

increased in heat-inactivated particle control groups relative to the air control groups (Table 6). This inflammation differed in character and severity from the chronic active inflammation observed in all viable *A. fumigatus*-exposed males and females and the single heat-inactivated particle control female. Microscopically, chronic inflammation in the lung was minimal to mild in severity and consisted of infiltrates of lymphocytes that were often perivascular but frequently extended into surrounding alveoli, along with rare neutrophils and variable numbers of alveolar macrophages. The macrophages associated with the chronic inflammation in the heat-inactivated particle control mice were mononuclear and contained abundant, sometimes foamy, pale tan cytoplasm.

The incidences of bronchus-associated lymphoid tissue (BALT) lymphocyte hyperplasia in the lung were significantly increased in the viable *A. fumigatus*-exposed males and females relative to the air control groups, but not when compared with the heat-inactivated particle control groups (Table 6). Similarly, the incidences of BALT lymphocyte hyperplasia were significantly increased in the heat-inactivated particle control-exposed males and females relative to the air control groups (Table 6). Minimal to mild BALT lymphocyte hyperplasia was present in all males and females exposed to the viable *A. fumigatus* and in 7/10 males and 8/10 females in the heat-inactivated particle control groups—but in none of the air control mice (Table 6). BALT lymphocyte hyperplasia was characterized microscopically by an increase in the size and/or number of lymphoid aggregates of BALT compared to that observed in animals in the concurrent air control groups. Minimal severity was characterized by two or more small lymphoid aggregates in the lung, and mild was characterized by several larger, nodular lymphoid aggregates.

The incidences of goblet cell metaplasia in the lung were significantly increased in the viable *A. fumigatus*-exposed males and females relative to the air control groups and the heat-inactivated particle control groups (Table 6). Minimal to mild goblet cell metaplasia was present in all males and females exposed to viable *A. fumigatus*, as well as a single female in the heat-inactivated particle control group (Table 6). Goblet cell metaplasia affected most to all of the epithelium in an individual airway and was characterized by increased numbers of goblet cells in the epithelium lining the bronchi and/or bronchioles (Figure 7). Because this change often affected both bronchi and bronchioles in any given animal, a subsite was not used in the diagnosis. The severity of this lesion was graded based upon the proportion of airways affected as follows: minimal severity (1) was <10% of the airways affected, mild (2) was 10%–40% of the airways affected, moderate (3) was 41%–75% of the airways affected, and marked (4) was >75% of the airways affected.

Table 6. Incidences of Nonneoplastic Lesions of the Lung in Male and Female Mice in the Three-month Inhalation Study of *Aspergillus fumigatus* Spores[a]

	Air Control	Heat-inactivated Particle Control	Viable *A. fumigatus*
n	10	10	10
Male[b]			
Artery, Medial, Hypertrophy[c]	0	0	10**## (2.1)[d]
Bronchiole, Epithelium, Hyperplasia	0	0	9**## (1.0)
Inflammation, Chronic	0	3 (1.7)	0
Inflammation, Chronic Active	0	0	10**## (2.7)
BALT, Lymphocyte, Hyperplasia	0	7** (1.0)	10** (1.0)
Goblet Cell, Metaplasia	0	0	10**## (1.9)
Lung, Fungus	0	5*	1
Lung, BALT, Fungus	0	4*	0
Female			
Artery, Medial, Hypertrophy	0	0	10**## (2.6)
Bronchiole, Epithelium, Hyperplasia	0	1 (1.0)	8**## (1.0)
Inflammation, Chronic	0	7** (1.9)	0
Inflammation, Chronic Active	0	1 (3.0)	10**## (2.8)
BALT, Lymphocyte, Hyperplasia	0	8** (1.0)	10** (1.4)
Goblet Cell, Metaplasia	0	1 (1.0)	10**## (1.8)
Lung, Fungus	0	0	10**##
Lung, BALT, Fungus	0	1	0

*Statistically significant pairwise difference from air control at $p \leq 0.05$.
**Statistically significant pairwise difference from air control at $p \leq 0.01$.
##Statistically significant pairwise difference from heat-inactivated particle control at $p \leq 0.01$.
BALT = bronchus-associated lymphoid tissue.
[a]Statistical analysis performed by the one-sided Fisher's exact test.
[b]Number of animals examined microscopically.
[c]Number of animals with lesion.
[d]Average severity grade of observed lesion in affected animals: 1 = minimal; 2 = mild; 3 = moderate; 4 = marked.

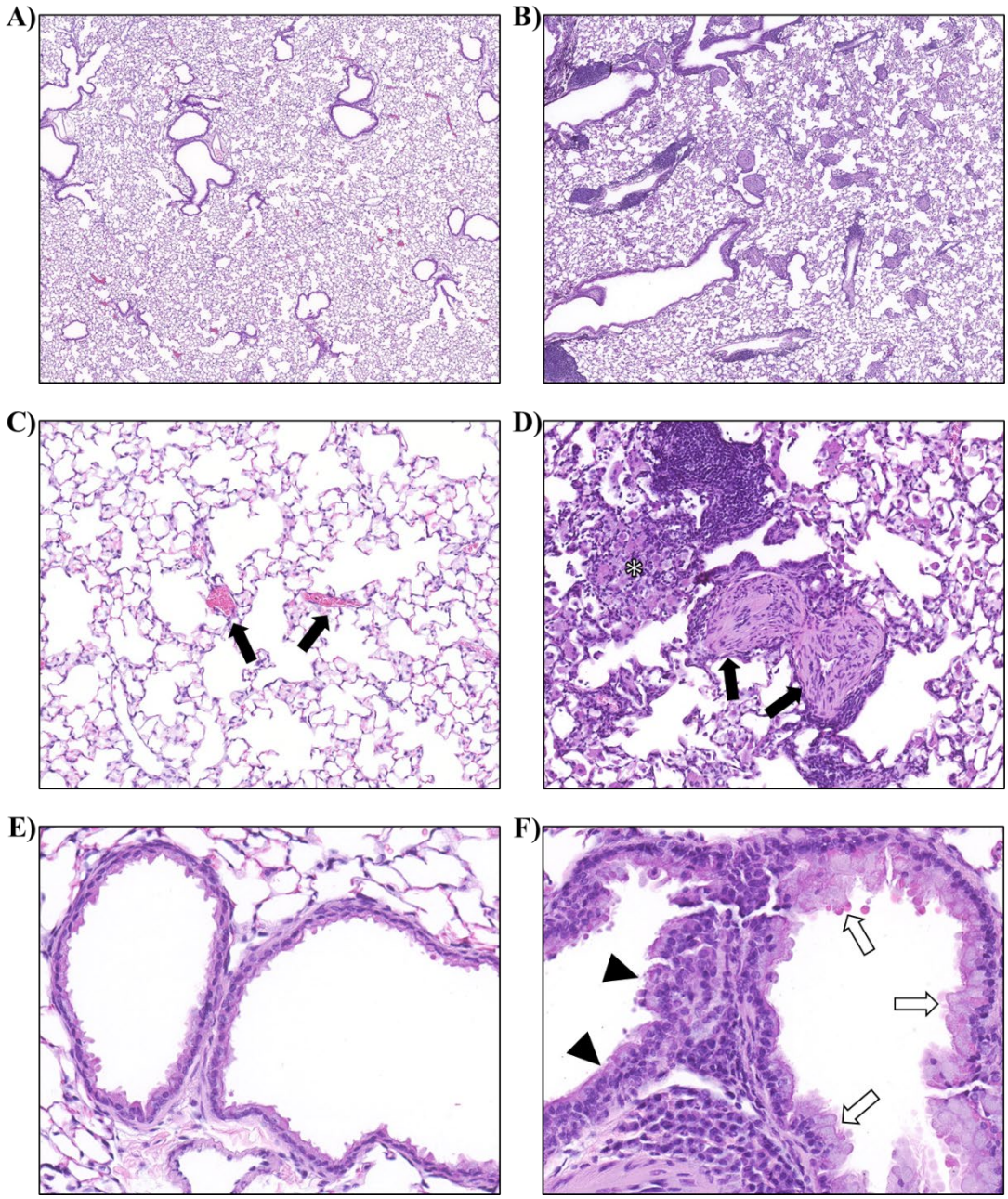

Figure 7. Histopathology Images of Lungs Following Inhalation of HEPA-filtered Air or Viable *Aspergillus fumigatus* Spores for Three Months (H&E)

Lung sections from air control female mice (A, C, E) and viable *A. fumigatus*-exposed female mice (B, D, F). At low magnification (4x), arterioles and inflammation are prominent in the viable *A. fumigatus*-exposed mouse lung (B) compared to the air control mouse lung (A). Compare the normal arterioles (arrows) with thin vessel walls in the lung from an air control female (C) to the arterioles with medial hypertrophy (arrows) in the viable *A. fumigatus*-exposed female (D). In the viable *A. fumigatus*-exposed female, there is circumferential thickening of the tunica media of arteries (arrows) by concentric layers of hypertrophied and hyperplastic smooth muscle cells, typically with narrowing of the vascular lumen (D) at 20x magnification. The viable *A. fumigatus*-exposed female mouse shows chronic active inflammation, seen as perivascular infiltrates of lymphocytes and neutrophils with neutrophilic infiltrates extending into vessel walls and histiocytic infiltrates in surrounding alveoli (white asterisk) (D). Compared to normal bronchioles in an air control female mouse (E), bronchiole epithelium hyperplasia (black arrowheads) and goblet cell metaplasia (white arrows) were seen in bronchioles of viable *A. fumigatus*-exposed mice (F) (40x magnification).

Histopathological analysis of lymph nodes showed no visible lesions in the mediastinal, mesenteric, or mandibular lymph nodes (Appendix E). In the bronchial lymph node, the incidences of lymphocyte hyperplasia and plasma cell hyperplasia were significantly increased in the viable *A. fumigatus*-exposed males and females relative to either the air control groups or the heat-inactivated particle control groups (Table 7). Bronchial lymph node lymphocyte hyperplasia occurred in 6/9 male and 9/10 female viable *A. fumigatus*-exposed mice and 1/10 heat-inactivated particle control female mice (Table 7). Bronchial lymph node lymphocyte hyperplasia was seen microscopically as increased numbers of lymphocytes within any of the compartments of the lymph node (Figure 8). Although the follicles were almost always involved, increased lymphocytes usually were present in the other compartments, such as the paracortex and medullary cords. Lymphocyte hyperplasia was considered minimal when the follicles, medullary cords, and/or paracortical regions were expanded by mature, medium-sized lymphocytes. Mild lymphocyte hyperplasia included the additional feature of small germinal centers rimmed by a prominent, but narrow, band of small mature lymphocytes. Moderate and marked hyperplasia were not seen in this study. The lesions of lymphocyte hyperplasia and plasma cell hyperplasia were often, but not always, present in the same bronchial lymph node. Lymphocyte hyperplasia and plasma cell hyperplasia accounted for the macroscopic enlargement of the bronchial lymph nodes observed in some viable *A. fumigatus*-exposed female mice.

Bronchial lymph node plasma cell hyperplasia occurred in 5/9 male and 8/10 female viable *A. fumigatus*-exposed mice (Table 7). Bronchial lymph node plasma cell hyperplasia was characterized by increased numbers of plasma cells within the medullary cords, sinuses, and/or paracortical regions that exceeded the number present in the bronchial lymph nodes of animals in the concurrent air control groups (Figure 8). Plasma cell hyperplasia was considered minimal when the sinuses, medullary cords, and/or paracortical regions were expanded by small clusters of plasma cells. Mild plasma cell hyperplasia included the features of minimal severity with the additional feature of continuous clusters of 15–25 cells. Increasing expansion by plasma cells would be a feature of moderate and marked infiltrates.

Additionally, minimal granulomatous inflammation of the bronchial lymph node was observed in a single viable *A. fumigatus*-exposed male mouse, which may have been related to exposure (Table 7).

Table 7. Incidences of Nonneoplastic Lesions of the Bronchial Lymph Node in Male and Female Mice in the Three-month Inhalation Study of *Aspergillus fumigatus* Spores[a]

	Air Control	Heat-inactivated Particle Control	Viable *A. fumigatus*
Male[b]	7	9	9
Lymphocyte, Hyperplasia[c]	0	0	6*[##] (1.8)[d]
Plasma Cell, Hyperplasia	0	0	5*[#] (1.6)
Inflammation, Granulomatous	0	0	1 (1.0)
Lymph Node, Bronchial, Fungus	0	2	0
Female	8	10	10
Lymphocyte, Hyperplasia	0	1 (1.0)	9**[##] (1.8)
Plasma Cell, Hyperplasia	0	0	8**[##] (1.5)
Lymph Node, Bronchial, Fungus	0	0	1

*Statistically significant pairwise difference from air control at $p \leq 0.05$.
**Statistically significant pairwise difference from air control at $p \leq 0.01$.
[#]Statistically significant pairwise difference from heat-inactivated particle control at $p \leq 0.05$.
[##]Statistically significant pairwise difference from heat-inactivated particle control at $p \leq 0.01$.
[a]Statistical analysis performed by the one-sided Fisher's exact test.
[b]Number of animals examined microscopically.
[c]Number of animals with lesion.
[d]Average severity grade of observed lesion in affected animals: 1 = minimal; 2 = mild; 3 = moderate; 4 = marked.

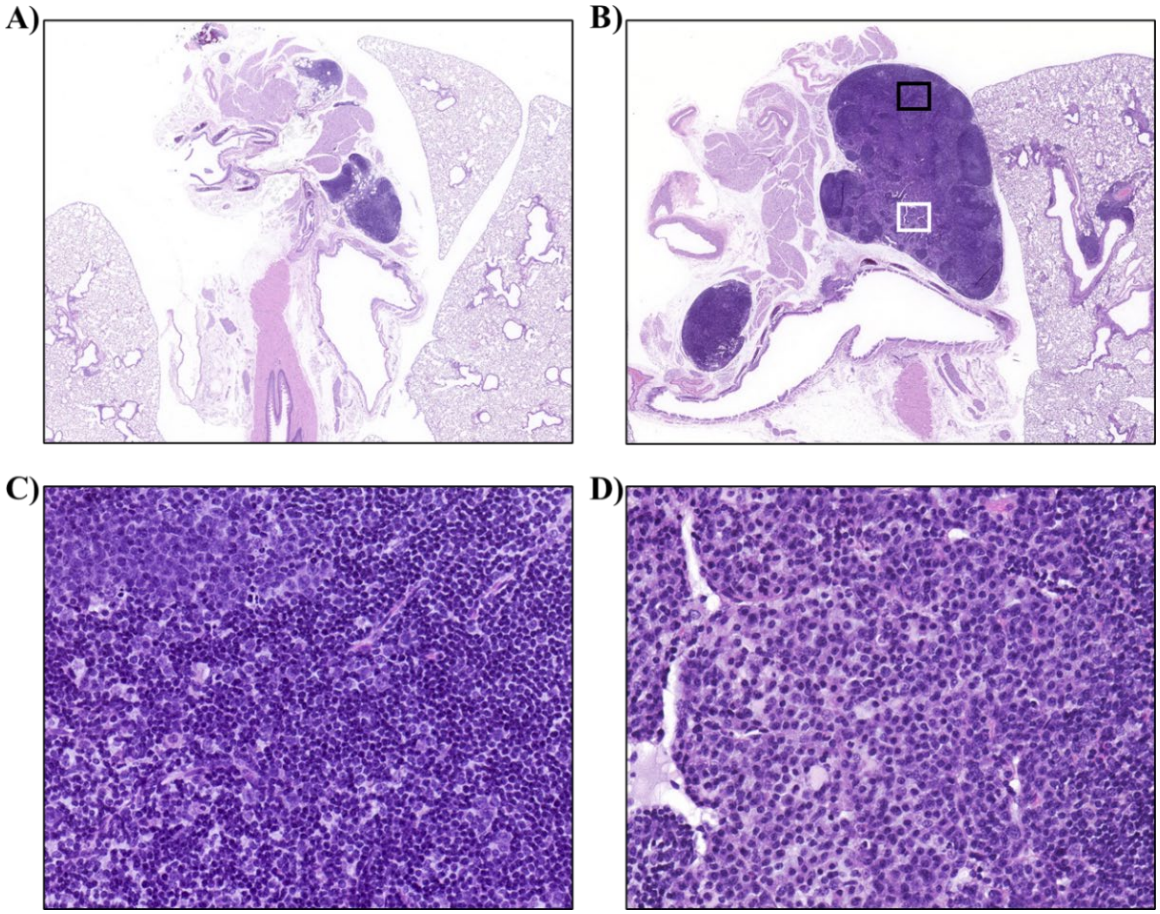

Figure 8. Histopathology Images of Lungs with Bronchial Lymph Nodes Following Inhalation of HEPA-filtered Air or Viable *Aspergillus fumigatus* Spores for Three Months (H&E)

Bronchial lymph node from an air control female mouse (A) and a viable *A. fumigatus*-exposed female mouse (B) showing enlargement of the bronchial lymph node (2x magnification). At high magnification (40x; C, black box inset in B), there is lymphocyte hyperplasia, which was generally seen as increased numbers of lymphocytes in multiple compartments, including the cortex, follicles, paracortex, and medullary cords. In the medullary cords at high magnification (40x; D, white box inset in B), there is plasma cell hyperplasia. The lesions of "hyperplasia, plasma cell" and "hyperplasia, lymphocyte" were often (as here), but not always, diagnosed in the same bronchial lymph node.

Fungal organisms were not observed in H&E-stained sections. The presence of fungal spores in histological sections of lung, BALT, bronchial lymph node, liver, spleen, gallbladder, heart, and aorta was evaluated using Grocott's methenamine silver (GMS)-stained sections. There were no gross or histological lesions observed in the standard three sections of the nasal cavity taken in NTP studies; therefore, GMS staining was not conducted on any nose tissue sections. GMS-positive structures consistent with fungal organisms (conidia) were observed in GMS-stained sections of lung, BALT, and bronchial lymph node in some mice from the heat-inactivated particle control groups and the viable *A. fumigatus* groups (Table 6, Table 7; Figure 9). No fungal organisms were observed with either stain in the liver, spleen, aorta, or heart. In viable *A. fumigatus*-exposed groups, fungal organisms were observed in the lung in 1/10 males and 10/10 females and in the bronchial lymph node in 1/10 females (Table 6, Table 7). In the heat-inactivated particle control groups, fungal organisms were observed in the lung in 5/10 males, in the BALT of the lung in 4/10 males and 1/10 females, and in the bronchial lymph node in 2/9

males (Table 6, Table 7). The fungal organisms were consistent with conidia, were 2–3 µm in diameter, and were present in extremely small numbers (i.e., <10 spores/section). They were most often intracellular within alveolar macrophages or multinucleated giant cells, but occasional extracellular spores in alveolar spaces were observed (Figure 9). Very rarely did the conidia appear to be germinating (Figure 9B).

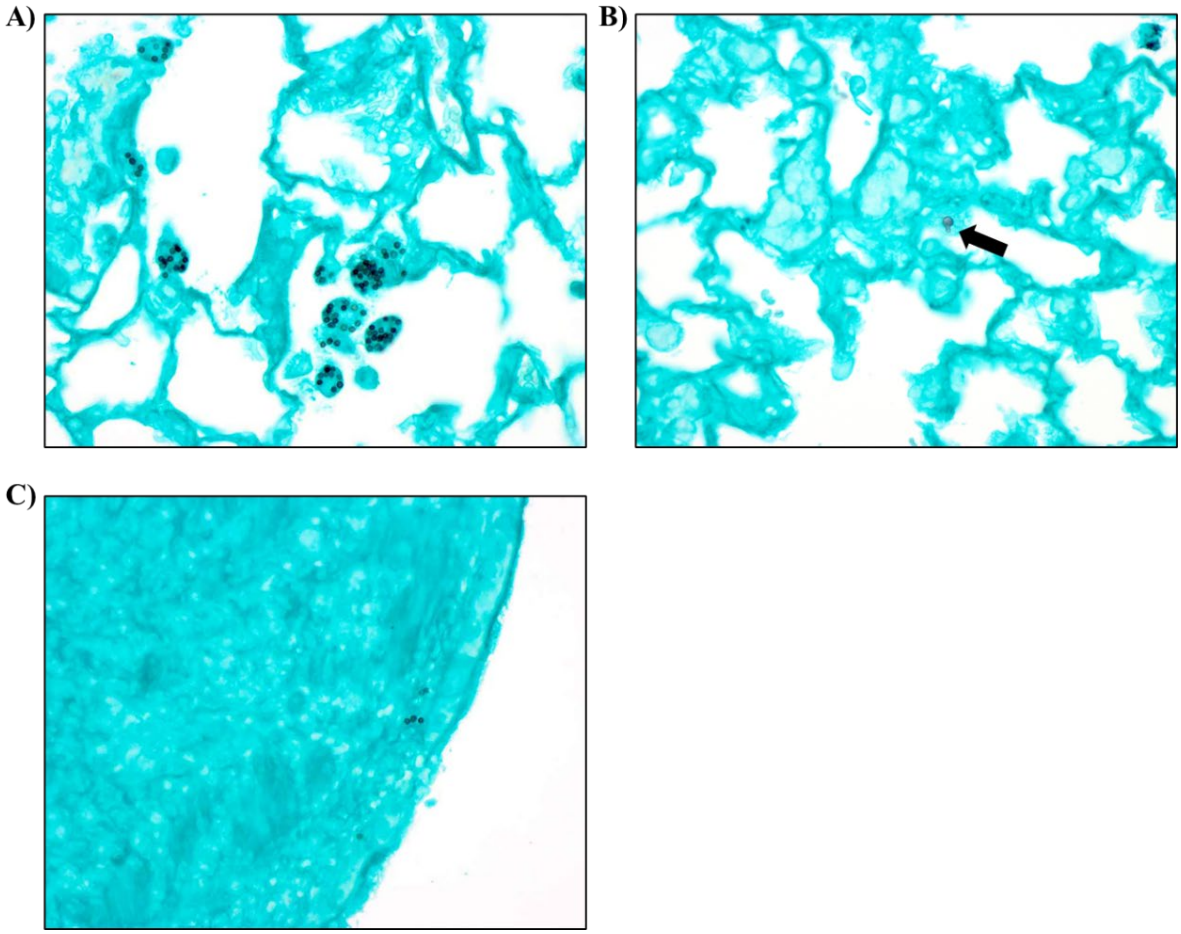

Figure 9. Images of Fungal Spores Observed within the Lungs of Mice Following Inhalation of *Aspergillus fumigatus* Spores for Three Months

Grocott's methenamine silver-positive structures consistent with *A. fumigatus* conidia were rarely observed, but, when present, were seen free within alveoli or within alveolar macrophages (A; heat-inactivated particle control male lung, 40x). Rarely, the fungal organisms appeared to be germinating (B; arrow, viable *A. fumigatus* female lung, 40x). Very low numbers of conidia were seen in bronchus-associated lymphoid tissue (C; heat-inactivated particle control male, 40x).

Genetic Toxicology

A. fumigatus viable spores were tested in a bacterial mutation assay with and without induced rat liver S9 mix; results were negative in each of the three bacterial strains tested (Table B-1). No increases in the frequencies of micronucleated polychromatic and normochromatic erythrocytes were observed in the peripheral blood of male or female B6C3F1/N mice exposed to either viable *A. fumigatus* or the heat-inactivated particle control spores relative to the air control mice (Table B-2). These negative results in the micronucleus test indicate an absence of exposure-induced chromosomal damage in progenitor erythrocytes in the bone marrow.

Discussion

Aspergillus fumigatus is a saprophytic fungal species with a worldwide distribution.[22; 23] As referenced in Figure 2, the asexual life cycle predominates and involves the production of long chains of unicellular spores (conidia).[21; 22] Abiotic or biotic disturbance to the asexual reproductive structures results in the aerosolization of respirable spores into the environment.[21; 22] *A. fumigatus* is thermotolerant and can grow and persist in a broad range of soil temperatures (12°C–65°C).[23] Because of these growth parameters, *A. fumigatus* has become an occupational hazard within the biowaste and general waste management industries.[25; 26] Viable *A. fumigatus* concentrations as high as 1×10^6 colony-forming units (CFU) per m^3 have been reported in working environments within the biowaste industry.[27-29] Although relative abundance and concentrations might not be as high as those identified in the biowaste industry, *A. fumigatus* spore exposures occur daily, and estimates of 100 conidia/m^3 indoor exposure have been reported.[22-24; 42] In damp indoor environments, exposure may be elevated due to fungal contamination of cellulose-based building materials with subsequent sporulation.[43]

To date, no reference limits for *A. fumigatus* have been proposed by the National Institute for Occupational Safety and Health (NIOSH) or the American Conference of Governmental Industrial Hygienists. Several European governmental agencies have suggested acceptable levels of *A. fumigatus*, including 500 CFU/m^3 (Environment Agency of the United Kingdom) and an occupational exposure limit (OEL) of 50,000 CFU/m^3 for mesophilic fungal contaminants (Germany's Federal Institute for Occupational Safety and Health).[29] Despite the number of exposure assessment studies and focus on *A. fumigatus* as an opportunistic fungal pathogen, limited datasets have characterized the toxicity of inhalation exposures to *A. fumigatus*. The present report describes a 3-month inhalation toxicology study of B6C3F1/N mice exposed to *A. fumigatus* viable conidia, with both heat-inactivated conidia as a particle control and an air-only control group. An estimated pulmonary deposition of 1×10^5 *A. fumigatus* spores twice per week was selected for a 3-month pulmonary immunology study,[19; 20] as well as the 3-month study presented in this report. The estimated lung burden in the mice in these studies would be equivalent to that of a worker breathing a constant workplace air concentration of 4.7×10^6 spores/m^3 (4.3 μg/m^3) for 1 week, working 8 hours per day for 5 days or equivalent to a worker breathing 3.2×10^4 spores/m^3 (0.029 μg/m^3) for 8 hours per day, 5 days per week over a 40-year working career. This daily exposure is comparable to exposures in various occupational environments,[45] including the biowaste industry,[27; 28] and is comparable to the German OEL of 50,000 CFU/m^3 proposed for mesophilic fungal contaminants.[29]

Inhalation exposure for 3 months to an estimated pulmonary dose of 1×10^5 viable *A. fumigatus* conidia did not elicit overt toxicity or result in >10% body weight loss compared to the air control group. There was evidence that *A. fumigatus* conidia produced histological changes in the larynx, lung, and bronchial lymph nodes. Although gross lesions were limited to the bronchial lymph nodes, a variety of nonneoplastic lesions were present in these organs in increased incidences in mice exposed to the viable *A. fumigatus* spores compared to mice in the heat-inactivated particle control groups and air control groups. Consistent lesions in the epithelium of the larynx at the base of the epiglottis were observed in both sexes of viable *A. fumigatus*-exposed mice. Low incidences of epithelial lesions were additionally reported overlying the arytenoid cartilage. Changes were characterized by squamous metaplasia of the larynx epithelium that included the loss of cilia and replacement of the cuboidal to columnar

cells by flattened stratified squamous epithelium. This type of metaplastic epithelial change is a biological response to chronic irritation or injury. Similar pathological changes in laryngotracheal histology have been described in mice that were exposed to *A. fumigatus* via an orotracheal tube.[101] Although human cases of tracheobronchial and laryngeal aspergillosis have also been reported in the literature,[102-108] fungal hyphae were not observed in the larynx histological sections in this study, indicating that there was no active laryngeal fungal growth or infection in the animals exposed to the viable *A. fumigatus*.

Statistically significant increases in absolute and relative lung weights were observed for both sexes exposed to viable *A. fumigatus* compared to air control mice. The increased lung weights corresponded histologically to chronic active inflammation that involved perivascular infiltrates that often extended into immediately surrounding pulmonary interstitial tissue as well as intra-alveolar infiltration of macrophages. These data corresponded to the increase in peripheral blood neutrophil counts in males in the viable *A. fumigatus*-exposed group compared to the air control group. Increased lung weights were also associated with bronchiolar epithelial hyperplasia, bronchus-associated lymphoid tissue hyperplasia, and medial hypertrophy of arteries and arterioles.

In addition to the increased neutrophil count observed in the male viable *A. fumigatus*-exposed group compared to the air control group, the total white blood cell and differential counts were increased in the *A. fumigatus*-exposed group when compared to the heat-inactivated particle control group. The relevance of the observed hematologic changes between the male heat-inactivated particle control group and *A. fumigatus*-exposed group was questionable, however, due to the exposure-related changes observed in the heat-inactivated control group. Exposure of the male mice to heat-inactivated particles caused histopathological changes, although at a lower incidence or severity compared to the *A. fumigatus*-exposed group. In addition, lung inflammation was observed in the male heat-inactivated particle control group, although it differed from the *A. fumigatus*-exposed group—chronic inflammation versus chronic active inflammation (i.e., more neutrophils), respectively. A closer look at the heat-inactivated particle control male hematologic data revealed that the white blood cell count was lower than the air control group, although this was not statistically significant. The reduction in white blood cell count was driven mostly by a decrease in the lymphocyte count; the eosinophil count was also lower. The combination of these changes is suggestive of a chronic stress leukogram[109] (i.e., exposure to the heat-inactivated particles could have caused chronic stress to the mice). In the *A. fumigatus*-exposed group, the inflammatory effects of exposure to the viable *A. fumigatus* outweighed any potential hematologic changes that one might observe from chronic stress of exposure. For these reasons, the observed hematologic pairwise changes between the male heat-inactivated particle control group and *A. fumigatus*-exposed group were considered to be due to biological variability and not an exposure-related effect.

Several rodent inhalation models of *A. fumigatus* pulmonary immunological responses have been published, reporting similar lung histopathological results, including increases in epithelial thickness, goblet cell metaplasia, and peribronchial collagen deposition compared to air control mice.[110; 111] The hybrid B6C3F1/N mouse strain used in this exposure study is resistant to disease and has low spontaneous disease rates compared with other strains and hybrids.[112] The B6C3F1/N mouse strain has been widely used to study systemic toxicological responses and was selected for consistency with other National Toxicology Program (NTP) toxicology studies. Although the female parent of the hybrid B6C3F1/N mouse strain, C57BL/6, is Th1-biased,

histopathological results of the airways are consistent with previously observed histopathology following fungal exposure in other strains of mice including BALB/cJ (Th2-biased)[18; 19] and ddY mice.[113] The histological results of the current study agree with an earlier pulmonary immunology study conducted in BALB/cJ mice that used the same pulmonary dosing and exposure schedule, including the heat-inactivated particle control exposure.[19] Histopathological analysis revealed fungal exposure-dependent pulmonary inflammation, cellular infiltration, goblet cell metaplasia, and peribronchial and perivascular collagen deposition. Flow cytometry analysis revealed significantly increased numbers of CD4+ T cells expressing cytokines that were associated with an allergic phenotype (Th2; IL-5 and IL-13). Following 13 weeks of *A. fumigatus* exposure, serum concentrations of immunoglobulin G (IgG1) and immunoglobulin E (IgE) were significantly increased in mice exposed to viable conidia compared to mice exposed to the heat-inactivated particle control or to air only.[20] Additionally, T cells (IFN-γ+ or IL-17A+) that co-expressed IL-13 were observed in the airways of viable *A. fumigatus*-exposed mice compared to air control mice.[19] Examination of the expressed genes confirmed expression of the *Il4*, *Il13*, and *Il33* genes in mice exposed to the viable *A. fumigatus*.[47] That preliminary study demonstrated a novel mechanism for immune responses to inhaled *A. fumigatus* and indicated the presence of germinating conidia in mice exposed to viable spores. The emergence of hyphae from germinating spores in the viable *A. fumigatus* group was hypothesized to lead to an allergic phenotype because similar immune responses were not observed in the heat-inactivated particle control group.[19] Croston and colleagues also showed germinating *A. fumigatus* spores to be associated with C-type lectin domain family 7 member A surface receptors (Dectin-1; increased expression of *Clec7a*), which were predicted to interact with the cytokines IL-13 and IL-33.[47] Spore germination may also be a precursor for the recruitment of neutrophils as shown by Mircescu et al.[114] and may explain the higher numbers of neutrophils in the lungs of viable *A. fumigatus*-exposed male mice compared to air control mice. Similarly, germinating *A. fumigatus* conidia expose cell wall (1–3)-β-D glucan that binds to the pattern recognition receptor, Dectin-1, mediating downstream NF-κB, proinflammatory cytokines, and reactive oxygen species by monocyte-derived macrophages.[115] In the current study, germinating *A. fumigatus* were directly observed (Figure 9B), albeit in very low numbers, and the neutrophilic perivascular infiltrates were only present in the mice exposed to viable *A. fumigatus*.

The data from the current study demonstrate that exposure to viable *A. fumigatus* was associated with the thickening of small- to medium-sized arteriole walls (medial hypertrophy) within the lungs of all exposed male and female mice. In male and female viable *A. fumigatus*-exposed mice, arterial medial hypertrophy was mild to moderately severe and affected approximately 10% to 75% of pulmonary arterioles. Remodeling of pulmonary arteries is characterized by the hypertrophy and hyperplasia of vascular smooth muscle and is defined as a lesion associated with pulmonary arterial hypertension[116] that can affect pulmonary arterial pressure.[117] Previous studies have shown that arteriole hypertrophy/hyperplasia is commonly associated with chronic inflammation such as hypersensitivity pneumonitis or chronic obstructive pulmonary disease, and immune-mediated processes have been implicated. Daley and colleagues showed that mice intranasally primed with *A. fumigatus* antigen developed thickening in the small- to medium-sized arteriole walls[116] similar to that observed in male and female viable *A. fumigatus*-exposed mice in the current study. Although the specific *A. fumigatus* antigen remains uncharacterized, a Th2 antigen-mediated response characterized by an influx of CD4+ T cells, IL-4 and IL-13, and additional effectors into the airways are criteria identified for the induction of arteriole hyperplasia.[116; 118] In addition, Shreiner and colleagues identified a potential role of IL-10,

independent of CD4+ T cells, as a driver of pulmonary arterial remodeling following repeated intranasal exposure to *A. fumigatus* conidia.[119] Subchronic inhalation exposure to viable *A. fumigatus* conidia that resulted in the same allergic phenotype as previously reported[19] is likely to be involved in the mechanism for the medial hypertrophy observed in all male and female mice exposed to viable *A. fumigatus* in the current study. A previously published study examining the pulmonary immune response following subchronic *A. fumigatus* exposure showed that inhalation of heat-inactivated particle controls did not result in medial hyperplasia (hypertrophy). As the responses following exposure to *A. fumigatus* are thought to be driven by the release of high molecular weight antigens during metabolic activation of spores, the lack of viability of the particle control or the destruction of antigens during the heat-inactivation process are variables that could contribute to the absence of medial hyperplasia/hypertrophy in the heat-inactivated particle control-exposed mice.[20] Subchronic studies that exposed mice to 1×10^4 *Stachybotrys chartarum* spores also showed similar arteriole lesions[113] that depended on Th2-associated inflammation.[120]

Increased incidences of lymphocyte hyperplasia and plasma cell hyperplasia were observed in the bronchial lymph nodes of viable *A. fumigatus*-exposed mice compared to air control mice. These histopathological lesions were associated with gross enlargement of the affected bronchial lymph nodes. In a previous subchronic *A. fumigatus* inhalation study conducted by NIOSH, gross enlargement and expansion of lymph node cellularity was also observed.[19] Flow cytometry analysis revealed lymph node preparations to consist primarily of B lymphocytes, CD4+ T cells, and CD8+ T cells that produced cytokine profiles similar to those obtained from bronchoalveolar lavage fluid.[19] Mice that were intratracheally exposed to *A. fumigatus* conidia and hyphae also showed the expansion of thoracic lymph node cellularity that was in part due to the migration of dendritic cells with phagocytized *A. fumigatus* to peripheral lymph nodes for antigen presentation.[121] The associated plasma cell infiltrates that expanded the medullary and subcapsular sinuses, as well as the medullary cords, supported bronchial lymph node enlargement through the proliferation of B cell lymphocytes in response to *A. fumigatus* antigen presentation.

A. fumigatus produces secondary metabolites with characterized toxicological effects.[55-57] One observation of the current study was metaplasia of the larynx epithelium, loss of cilia, and marked airway inflammation in viable *A. fumigatus*-exposed mice. Collectively, these results suggest the involvement of secreted *A. fumigatus* secondary metabolites, such as gliotoxin, helvolic acid, or fumagillin, that have been shown previously to mediate direct tissue injury and remodeling. Previous studies have demonstrated cytotoxicity of *A. fumigatus* preparations to lung cells that included inhibition of cilia beat frequency, epithelial damage, and induction of cell apoptosis.[54; 61; 64; 122] Similarly, tremorgenic bioactive alkaloids, including fumitremorgin, tryptoquivaline, and verruculogen, demonstrated in previous studies of *A. fumigatus*,[58; 70; 71] were not characterized in viable *A. fumigatus* preparations from the current study. Clinical observations associated with these secondary metabolites, however, also were not observed in mice exposed to the viable *A. fumigatus* conidia. Several of the secondary metabolites are additionally capable of adducting DNA, which could result in downstream genotoxic effects. In the current study, tests for micronucleus induction in bone marrow proerythrocytes showed no increases in the frequency of micronucleated polychromatic erythrocytes or micronucleated normochromatic erythrocytes in the peripheral blood of mice exposed to viable *A. fumigatus* or to the heat-inactivated particle control, indicating an absence of induced chromosomal damage.

Although individual secondary metabolites were not investigated in this subchronic toxicity study, the results of previous studies suggest that in vivo germination of *A. fumigatus* spores following respiratory deposition may be a potential source of secondary metabolites with possible pulmonary and genotoxic effects.[123]

Significant findings from the current 3-month inhalation study in B6C3F1/N mice included increased lung weights in viable *A. fumigatus*-exposed mice relative to the air control groups accompanied by increased incidence of nonneoplastic lesions. In males and females exposed to viable *A. fumigatus*, lesions were primarily restricted to the larynx, lung, and bronchial lymph nodes. Laryngeal lesions consisted of squamous metaplasia of the epithelium localized at the base of the epiglottis. Lung histopathology findings included chronic active inflammation, medial hypertrophy of arteries and arterioles, goblet cell metaplasia, and bronchiolar epithelial hyperplasia. Lymphocyte hyperplasia and plasma cell hyperplasia were observed in lymph nodes. In male mice, the increased lung weights and observed histopathology were consistent with the increased circulating neutrophil counts compared with the air control groups. The results of the current 3-month toxicity study corroborate the results of a recent subchronic *A. fumigatus* pulmonary immunology study conducted by NIOSH with the same exposure system that showed Th2 antigen-mediated allergic inflammation in response to subchronic *A. fumigatus* inhalation exposure.[19; 20] In the current study, NTP also provides evidence of medial hypertrophy of the pulmonary arterioles in *A. fumigatus*-exposed mice but not in heat-inactivated particle-exposed mice, which has been previously characterized to be mediated by an influx of CD4+ IL-13-producing T cells. The results of the micronucleus test in peripheral blood of exposed mice were negative and demonstrated an absence of chromosomal damage. In conclusion, the results of the subchronic inhalation exposure of mice to viable spores of *A. fumigatus* provide evidence that the targets of *A. fumigatus* included the larynx, lung, and bronchial lymph nodes. The magnitude of effects appeared to depend on the viability of *A. fumigatus* spores, with histopathology revealing enhanced toxicity with exposure to viable spores compared to heat-inactivated particle controls, possibly due to the germination of the viable spores.

Under the conditions of this 3-month study, target organs identified in B6C3F1/N mice following inhalation exposure to *A. fumigatus* spores were the larynx, lung, and bronchial lymph nodes. Significant differences were observed between viable *A. fumigatus* exposure and both air control and heat-inactivated particle control exposures. These results build on initial NIOSH pulmonary immunology studies using the same exposure parameters and demonstrate that the immunological responses and histopathology could be enhanced by the viability of the *A. fumigatus* spores.

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Appendix A. Culture, Characterization, and Delivery of *Aspergillus fumigatus*

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A.1. Reconstitution, Culture, and Glycerol Stock Preparation of *Aspergillus fumigatus*

The *Aspergillus fumigatus* strain (NIH Strain B-5233) used in this study was acquired from the American Type Culture Collection (ATCC® 13073™, lot 59030808, Manassas, VA) and stored at −80°C. The freeze-dried stock was reconstituted in 50 mL of sterile water according to the ATCC instructions. Once rehydrated, malt extract agar (MEA) culture plates were inoculated with 100 µL of the rehydrated fungus. MEA culture plates were incubated at 25°C for 10–14 days for optimal spore growth. *A. fumigatus* spores were harvested by liberating the spores in 2 mL of sterile water using an inoculating loop. The spore suspension was mixed with an equal volume of autoclaved 50% glycerol, and 0.5 mL aliquots of the suspension were prepared in cryogenic storage vials. Glycerol stock preparations were stored at −80°C.

A.2. Preparation of the Test Article

Aliquoted frozen glycerol spore suspension was thawed, and 100 µL was used to inoculate two MEA culture plates. Plates were incubated at 25°C for 7–10 days. *A. fumigatus* spores were harvested from each plate by liberating the spores in 2 mL of sterile water using an inoculating loop. The concentration of spores in the spore suspension was determined by diluting the suspension 1:100 and manually counting the spores using a hemocytometer. The spore suspension was diluted to 2.5×10^6 spores/mL using sterile water. Sterile petri dishes containing 10 g of autoclaved brown rice (15/week) were inoculated with 3 mL of the 2.5×10^6 spore/mL suspension. The petri dishes were wrapped in parafilm and incubated at 25°C for 10–14 days. The plates were shaken vigorously 1–2 times each day of culture until the rice grains were coated in fungi to prevent aggregation of the rice grains. Prior to aerosolization, viable cultures were placed in a desiccator for 3–4 days.

A.3. Heat Treatment of *Aspergillus fumigatus* Cultures for Creation of Nonviable Particle Control

After 10–14 days of culture, fungi-coated rice from two culture dishes were combined and then equally distributed to one sterile petri dish (viable *A. fumigatus*) and one sterile quartz plate (heat-inactivated particle control). This procedure was repeated for eight more plates for a total of four viable *A. fumigatus* and four heat-inactivated particle control plates. The quartz plates were autoclaved for 15 minutes at 121°C and allowed to cool. The fungi-coated rice grains were transferred from the quartz plates back to sterile petri dishes.

A.4. Determination of the Reduction in *Aspergillus fumigatus* Spore Viability in the Heat-inactivated Particle Control

Spores were harvested from heat-inactivated particle control cultures and viable *A. fumigatus* spore cultures in sterile phosphate buffered saline (PBS), pH 7.4. Spore suspensions were further diluted and plated on MEA culture plates to allow for counting of individual fungal colonies. For heat-inactivated particle control cultures, 1:1,000, 1:10,000, and 1:50,000 dilutions were used. For viable *A. fumigatus* cultures, 1:100,000, 1:500,000, and 1:1,000,000 dilutions were used. After 48–96 hours of growth at 25°C, fungal colony-forming units (CFU) were counted. Those

plates with 30–300 CFU were used to determine the number of viable *A. fumigatus* spores/mL PBS in the original stock solutions. These concentrations were used to determine the reduction in spore viability in the heat-inactivated particle control compared with the viable *A. fumigatus*. Reduction >98% was categorized as "nonviable."

A.5. Assessment of Fungal Contaminants in the Exposure Articles

Genomic DNA was extracted from the heat-inactivated particle control and viable *A. fumigatus* cultures each week for analysis of fungal contaminants. Spores were harvested in sterile, distilled, deionized water. Spores from 200 µL of the suspension were pelleted by centrifugation. Spores were then suspended in Roche Tissue Lysis buffer and transferred to tubes containing 0.3 µm glass beads. The tubes were processed in a bead mill homogenizer. The suspensions were centrifuged twice, and supernatants were transferred to new tubes in which 5 µL of CelLytic B Cell Lysis Reagent (Sigma Aldrich, St. Louis, MO) was added; tubes were incubated at 37°C for 30 minutes. Next, 40 µL proteinase K solution was added, and tubes were incubated at 70°C for 10 minutes. After the addition of 100 µL of isopropanol, extracted DNA was washed and eluted using the Roche High Pure PCR Template Preparation Kit (Roche, Indianapolis, IN). Internal transcribed spacer (ITS) regions of fungal ribosomal RNA were amplified using the Fun18Sf/ITS4R primer pair. Amplified DNA was purified, cloned into a pDRIVE vector, and transformed into chemically competent *Escherichia coli*. *E. coli* clones positive for the ITS amplicon insert were used to inoculate 96-well plates for generation of glycerol stocks. The glycerol stocks were sequenced with the T7 and SP6 primers using Sanger sequencing. Sequences were trimmed and assembled and then clustered into operational taxonomic units (OTUs)—groups of sequences with 97% or greater similarity. Representative sequences from each OTU were searched against the National Center for Biotechnology Information database. Sequences were then identified on the basis of identity with sequences banked in that database. Any sequence identified as a fungal species other than *A. fumigatus* or *Aspergillus* spp. were considered contaminants. The number of sequences analyzed depended on the number of viable spores in the sample. Very little DNA was obtained from heat-inactivated particle control samples due to the heat treatment. In Table A-1, the number of contaminant fungal sequences is shown relative to the number of sequences analyzed in the sample.

A.6. Aerosolization of the Exposure Articles Using the Acoustical Generator System

Prior to aerosolization of the exposure articles, the viable *A. fumigatus* and heat-inactivated particle control cultures were placed in a desiccator for 3–4 days to remove excess moisture and allow for optimal aerosolization. Fungal-laden rice was placed on a rubber membrane housed on top of a speaker within the acoustical generator system (AGS). The AGS consisted of a modified Pitt-3 generator[77] linked to a multi-animal nose-only exposure chamber with a vertical cylindrical tower of 24 ports that held animal pods radially projecting outward (Figure 3A). There were four levels of ports: ports 1–6 at the highest elevation, ports 7–12 at the next level, ports 13–18 at the next, and ports 19–24 at the lowest level. For this study, two AGS systems were used, one for the viable *A. fumigatus* exposures and one for the heat-inactivated particle control exposures. Air control mice were placed in an identical exposure chamber that was not attached to the AGS and received only HEPA-filtered air. Twenty animal pods were attached to

each exposure chamber, 10 for male mice and 10 for female mice. Animals were randomly placed in the pods for each exposure to minimize the effects of potential small variations in spore concentrations at each port. The additional ports were used to (1) measure the mass concentration with a light-scattering device (DataRAM 4, ThermoElectron Co., Franklin, MA), (2) acquire gravimetric measurements used to adjust the calibration of the DataRAM, (3) determine particle size using an aerodynamic particle sizer (APS; TSI Inc., Shoreview, MN), and (4) collect samples on filters for field emission electron microscopy analysis (FESEM; Hitachi S-4800, Tokyo, Japan).

The fungus-laden rice was allowed to settle within the AGS prior to the start of exposures. Acoustical energy aerosolized fungal spores, which were then delivered into the exposure chamber at a flow rate of 6 liters per minute. Mass concentration was monitored in real time using the DataRAM software. That software shut down the AGS once the mice had been exposed to an estimated pulmonary deposition of 1×10^5 spores, equating to approximately 10 mg/m^3 fungal spores inhaled over 1 hour. Pulmonary deposition was calculated using a mouse lung-deposition model adapted from Raabe et al.[82] The mice remained in the pods for a full 60 minutes and received HEPA-filtered air if the desired lung deposition was reached before the 60-minute exposure interval. Table A-2 and Table A-3 describe the measured concentrations of each exposure. Aerodynamic particle size data are shown in Table A-4, and Figure A-1 demonstrates spores within the respirable range of 2 to 3 µm. FESEM analysis showed that the aerosol primarily consisted of single spores or doublets (Figure A-2).

Table A-1. Weekly Evaluation of *Aspergillus fumigatus* Heat-inactivated Particle Controls and Viable *A. fumigatus*

	Heat-inactivated Particle Control	Viable *A. fumigatus*
Viability		
Viable Spore Concentration[a]	1.18×10^4 spores/mL (± 2.12×10^4)	1.49×10^7 spores/mL (± 1.07×10^7)
Reduction in Viability	99.87% (± 0.33%)	–
Contamination[b]		
Week 1	1/22	0/48
Week 2	0/24	0/48
Week 3	4/11	0/72
Week 4	0/11	0/70
Week 5	0/12	0/70
Week 6	2/12	0/71
Week 7	0/6	0/69
Week 8	4/6	0/70
Week 9	0/10	0/71
Week 10	0/7	0/72
Week 11	0/12	0/70
Week 12	0/12	0/71
Week 13	0/12	0/72

[a]Spores were harvested from nonviable and viable cultures in phosphate buffered saline (PBS). These suspensions were plated at various dilutions. Colony-forming units were then counted to determine the spores per mL of PBS in each of the original, undiluted suspensions. Data presented as mean ± standard deviation.
[b]Although no fungal contamination was visible within the cultures, DNA derived from other fungal species was detected in the heat-inactivated particle control but not in the viable *Aspergillus fumigatus* cultures on weeks 1, 3, 6, and 8. Sequence analysis revealed these species to include *Leptosphaerulina chartarum, Davidiella* spp., *Sordariomycetes* spp., *Piptoporus beultinus,* and *Alternaria alternata*.

Table A-2. Weekly Viable *Aspergillus fumigatus* Exposure Concentrations

Exposure Day	Chamber Concentration (DataRAM [mg/m^3])	Chamber Concentration (Filter [mg/m^3])	Filter Weight (mg)	Number of Spores Deposited in Lung
1	9.89	6.99	0.4335	1.004×10^5
3	9.93	10.33	0.6405	1.006×10^5
8	10.24	9.84	0.5906	1.005×10^5
10	9.38	8.73	0.5778	1.006×10^5
15	10.26	10.28	0.6169	1.006×10^5
17	9.30	9.21	0.6092	1.005×10^5
22	10.27	5.94	0.3562	1.005×10^5
24	9.93	8.84	0.5482	1.005×10^5
30	9.61	9.43	0.6041	1.005×10^5
32	10.19	10.02	0.6014	1.005×10^5
36	10.11	9.91	0.5943	1.006×10^5
38	9.01	8.76	0.5983	1.006×10^5
43	9.57	9.57	0.5700	1.005×10^5
45	10.25	7.89	0.4733	1.005×10^5
50	10.24	9.23	0.5538	1.004×10^5
52	9.03	9.72	0.6640	1.006×10^5
57	10.22	9.24	0.5543	1.004×10^5
59	9.90	11.45	0.7100	1.004×10^5
64	9.66	10.49	0.6722	1.004×10^5
66	9.36	10.08	0.6669	1.006×10^5
71	8.69	7.95	0.5599	1.004×10^5
73	10.26	13.07	0.7843	1.004×10^5
78	10.29	10.40	0.6237	1.007×10^5
80	9.29	8.83	0.5843	1.005×10^5
84	9.92	8.24	0.5109	1.004×10^5
86	10.30	9.62	0.5770	1.005×10^5
Mean[a]	9.81 ± 0.48	9.39 ± 1.39	0.588 ± 0.085	$1.005 \times 10^5 \pm 87.09$

[a]Data are presented as mean ± standard deviation of the measurements over the course of the study.

Table A-3. Weekly *Aspergillus fumigatus* Heat-inactivated Particle Control Exposure Concentrations

Exposure Day	Chamber Concentration (DataRAM [mg/m^3])	Chamber Concentration (Filter [mg/m^3])	Filter Weight (mg)	Number of Spores Deposited in Lung
1	10.26	8.79	0.5275	1.004×10^5
3	10.24	8.44	0.5062	1.009×10^5
8	10.30	9.81	0.5887	1.008×10^5

Exposure Day	Chamber Concentration (DataRAM [mg/m^3])	Chamber Concentration (Filter [mg/m^3])	Filter Weight (mg)	Number of Spores Deposited in Lung
10	10.24	9.50	0.5699	1.002×10^5
15	9.69	8.85	0.5668	1.008×10^5
17	8.76	9.20	0.6480	1.002×10^5
22	5.62	4.86	0.2918	5.985×10^4
24	10.00	6.45	0.3998	1.007×10^5
30	10.31	12.80	0.7682	1.009×10^5
32	9.94	10.72	0.6648	1.011×10^5
36	10.39	12.88	0.7727	1.016×10^5
38	10.07	12.37	0.7673	1.014×10^5
43	9.78	10.31	0.6600	1.016×10^5
45	10.03	9.83	0.6099	1.018×10^5
50	10.35	9.75	0.5849	1.019×10^5
52	9.42	8.79	0.5820	1.017×10^5
57	10.28	10.75	0.6449	1.007×10^5
59	10.31	8.20	0.4917	1.009×10^5
64	10.45	10.95	0.6572	1.024×10^5
66	10.40	11.20	0.6719	1.018×10^5
71	9.38	8.02	0.5305	1.012×10^5
73	10.39	8.55	0.5812	1.019×10^5
78	10.42	13.80	0.8281	1.020×10^5
80	10.48	16.24	0.9741	1.025×10^5
84	10.49	15.71	0.9424	1.027×10^5
86	10.45	14.34	0.8604	1.022×10^5
Mean[a]	9.94 ± 0.97	10.43 ± 2.69	0.642 ± 0.156	$9.978 \times 10^5 \pm 8{,}173.36$

[a]Data are presented as mean ± standard deviation of the measurements over the course of the study.

Table A-4. Aerodynamic Particle Size Statistics for Aerosolized *Aspergillus fumigatus* Spores from Aerodynamic Particle Sizer Measurements[a]

	Number (Particle Size)	Surface (Particle Size)	Mass (Particle Size)
Viable *A. fumigatus*			
Median (µm)	2.25	2.32	2.35
Mean (µm)	2.26	2.37	2.43
Geometric Mean (µm)	2.23	2.34	2.40
Mode (µm)	2.29	2.29	2.29
Geometric Standard Deviation	1.19	1.16	1.17
Heat-inactivated Particle Control			
Median (µm)	2.31	2.38	2.44
Mean (µm)	2.36	2.52	2.75
Geometric Mean (µm)	2.34	2.46	2.60
Mode (µm)	2.29	2.29	2.29
Geometric Standard Deviation	1.15	1.21	1.33

[a]The columns represent the three different methods of producing size distribution curves (as seen in Figure A-1). The aerodynamic particle sizer totals the particles in each particle size by number, by the surface area of the particles in each size bin, and by the mass of the particles in each size bin.

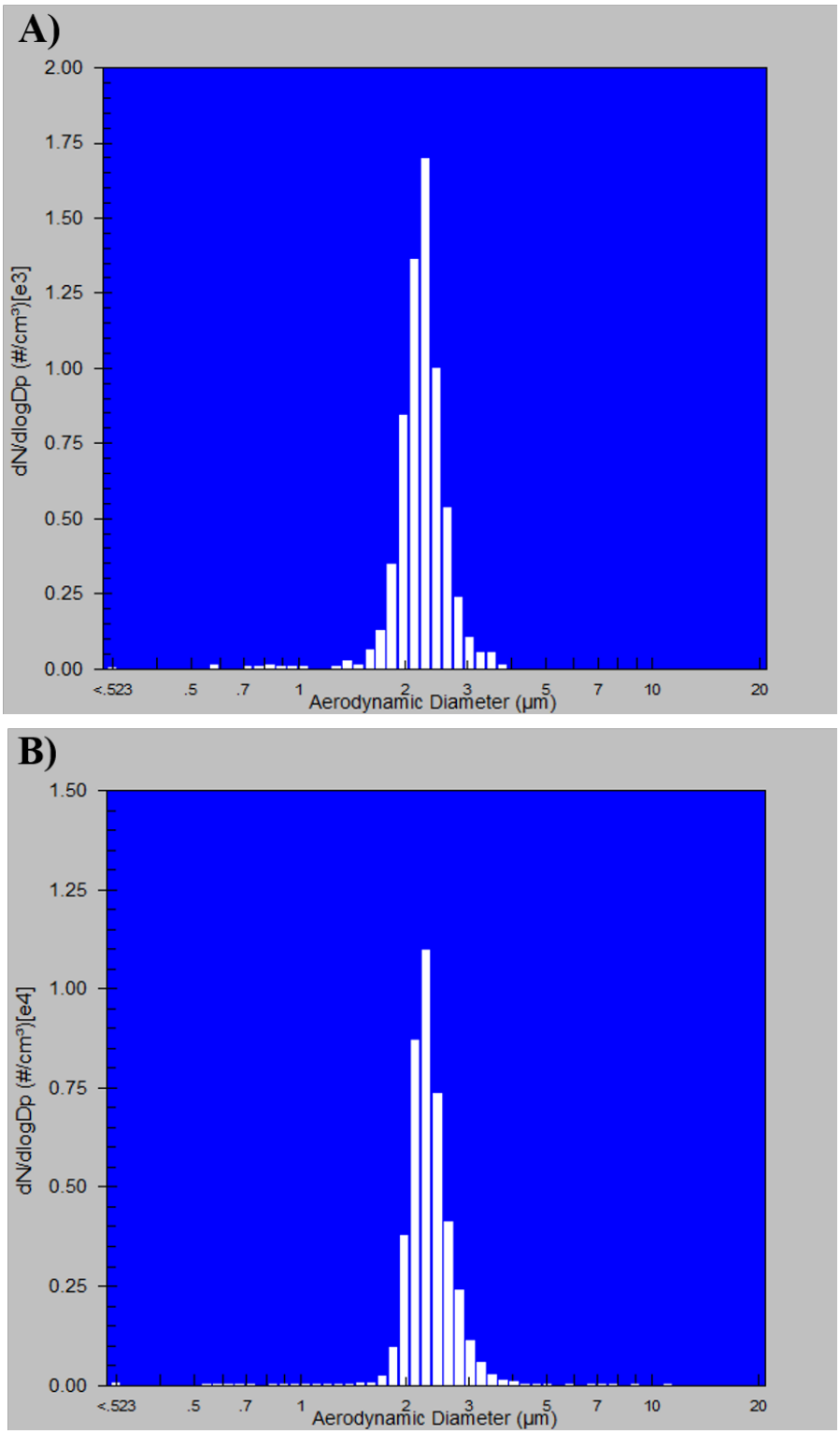

Figure A-1. Aerodynamic Particle Size Measurements Following Aerosolization of *Aspergillus fumigatus* Spores

The images depict the size distrution of particles measured with the aerodynamic particle sizer following aerosolization of (A) viable *A. fumigatus* spores and (B) the heat-inactivated particle control (nonviable *A. fumigatus* spores).

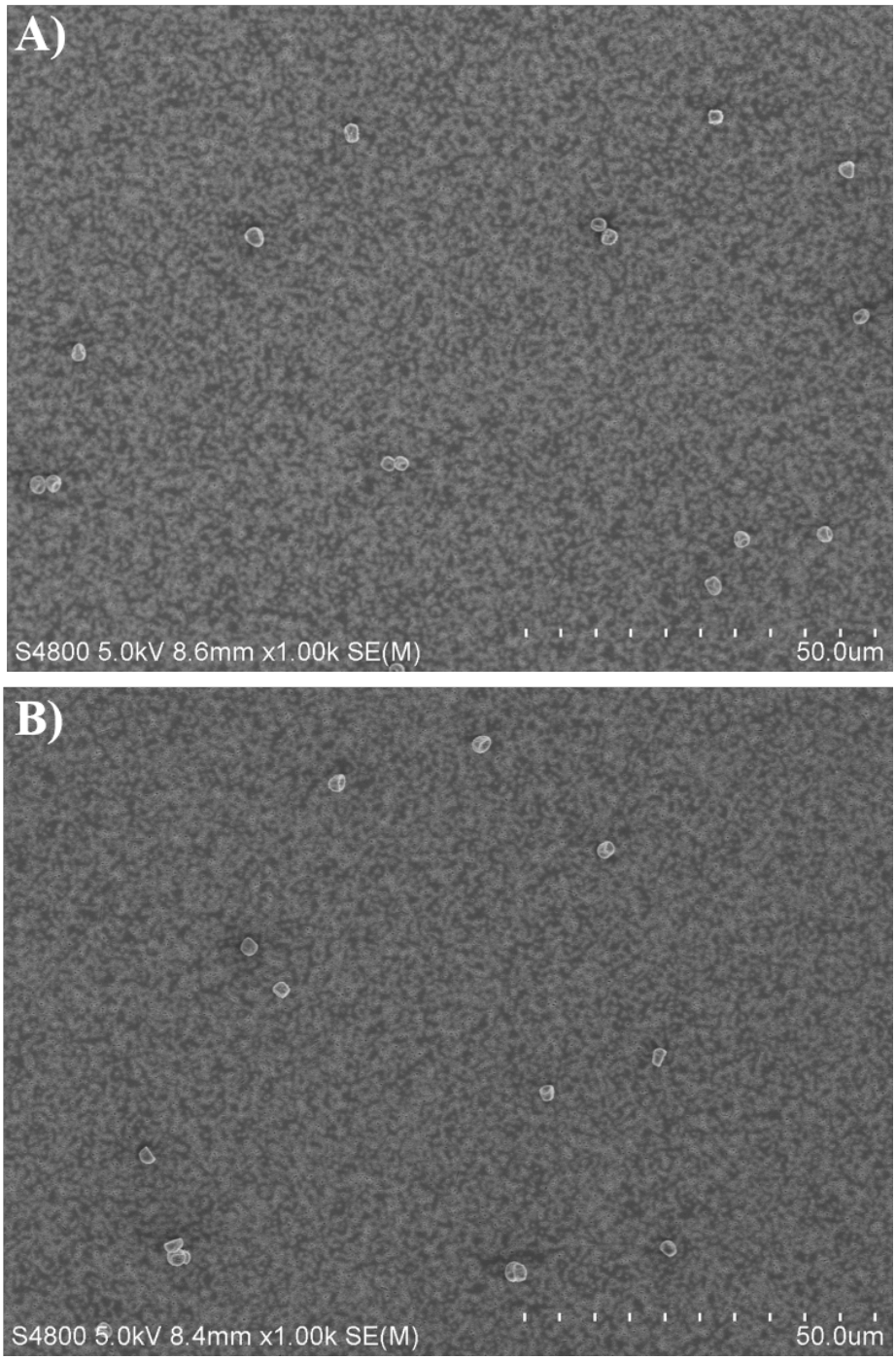

Figure A-2. Electron Microscropy Images of *Aspergillus fumigatus* Spores Aerosolized Using the Acoustical Generation System

Field emission electron microscopy images of spores collected on filters following acoutstical generation system aersolization of (A) viable *A. fumigatus* spores and (B) the heat-inactivated particle control (nonviable *A. fumigatus* spores). Spores in both exposure groups were aerosolized as single spores and doublets.

Appendix B. Genetic Toxicology

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B.1. Bacterial Mutagenicity Assay

B.1.1. Bacterial Mutagenicity Test Protocol

Testing procedures were modified from those originally reported by Zeiger et al.[124] Coded samples of viable *Aspergillus fumigatus* (the same test article used in the 3-month bioassays) dissolved in dimethylsulfoxide were incubated with the *Salmonella typhimurium* (TA98, TA100) or *Escherichia coli* (WP2 *uvr*A [pKM101]) tester strains, either in buffer or S9 mix (metabolic activation enzymes and cofactors from phenobarbital/benzoflavone-induced male Sprague Dawley rat liver), for 20 minutes at 37°C. Top agar, supplemented with *L*-histidine (or tryptophan for the *E. coli* strain) and *d*-biotin, was added, and the contents of the tubes were mixed and poured onto the surfaces of minimal glucose agar plates. Histidine- or tryptophan-independent mutant colonies arising on these plates were counted following incubation for 2 days at 37°C.

Each trial consisted of triplicate plates of concurrent positive and negative controls and of at least five doses of viable *A. fumigatus*. The highest concentration tested was limited by toxicity in strain TA100; the other two strains were tested up to the assay limit dose of 6,000 µg/plate. All trials were repeated.

In this assay, a positive response is defined as a reproducible, dose-related increase in histidine-independent (revertant) colonies in any one strain/activation combination. An equivocal response is defined as an increase in revertants that is not dose-related, reproducible, or of sufficient magnitude to support a determination of mutagenicity. A negative response is obtained when no increase in revertant colonies is observed following treatment with spores. No minimum percentage or fold increase is required for a test article to be judged positive or weakly positive, although positive calls are typically reserved for increases in mutant colonies that are at least twofold over background.

B.1.2. Results

A. fumigatus viable spores were tested in a bacterial mutation assay with and without induced rat liver S9 mix; results were negative in each of the three bacterial strains tested (Table B-1).

B.2. Micronucleus Assay

B.2.1. Peripheral Blood Micronucleus Test Protocol

Peripheral blood samples were analyzed by Integrated Laboratory Systems, LLC (ILS; Research Triangle Park, NC) for determination of erythrocyte micronucleus frequencies. Briefly, EDTA-stabilized peripheral blood samples were shipped on ice packs immediately following terminal necropsy from the National Institute for Occupational Safety and Health to ILS. Before ILS received the samples, microcentrifuge tubes containing anticoagulant (heparin) were prepared and stored at 4°C, and 15 mL conical tubes containing fixative (methanol) were prepared and stored at −80°C ± 5°C.

Upon arrival, blood samples were fixed in ultracold methanol using a MicroFlowPLUS Kit (Litron Laboratories, Rochester, NY) according to the manufacturer's instructions. Fixed samples were stored in a −80°C freezer until analysis. Thawed blood samples were analyzed for

frequency of micronucleated immature erythrocytes (polychromatic erythrocytes or PCEs, reticulocytes) and mature erythrocytes (normochromatic erythrocytes or NCEs) using a flow cytometer[125]; both the mature and immature erythrocyte populations were analyzed separately by employing special cell surface markers to differentiate the two cell types. In mice, both the mature and immature erythrocyte populations can be evaluated for micronucleus frequency because the mouse spleen does not sequester and eliminate damaged erythrocytes. Damaged erythrocytes achieve steady state in the peripheral blood of mice following 4 weeks of continuous exposure. Approximately 20,000 immature erythroytes and 1×10^6 mature erythrocytes were analyzed per animal for frequency of micronucleated cells, and the percentage of immature erythrocytes (% PCE) was calculated as a measure of bone marrow toxicity resulting from test article exposure.

For most NTP studies, the statistical methods for micronucleus assays employ a trend test and a Levene's test for equal variances to determine which type of pairwise test (Williams or Dunn) is more appropriate. Due to the design of this study (with one exposed group and two control groups), no trend test is needed, and the use of the Williams test would be inappropriate due to its step-down nature. Therefore, the Dunn test was used for the three-way pairwise comparison of the treatment and exposure groups. The Dunn test has a built-in correction for multiple comparisons so that the overall false positive rate of the experiment can be maintained. Pairwise comparisons with the control groups are considered statistically significant at $p \leq 0.025$.

Historical control data are used to evaluate the biological significance of any observed response. Both statistical significance and biological significance are considered when arriving at a call. Ultimately, the scientific staff determines the final call after considering the results of statistical analyses, the reproducibility of any effects observed (in acute studies), and the magnitudes of those effects.

B.2.2. Results

No increases in the frequencies of micronucleated polychromatic and normochromatic erythrocytes were observed in the peripheral blood of male or female B6C3F1/N mice exposed to either viable *A. fumigatus* or the heat-inactivated particle control spores compared to the air control mice (Table B-2). In addition, no significant alteration in the % PCE was observed in these mice, suggesting no effects on hematopoiesis. These negative results in the micronucleus test indicate an absence of exposure-induced chromosomal damage in progenitor erythrocytes in the bone marrow of mice, along with an absence of generalized bone marrow toxicity.

Table B-1. Mutagenicity of Viable *Aspergillus fumigatus* Spores in Bacterial Tester Strains[a]

Strain	Concentration (µg/plate)	Without S9	Without S9	Without S9	With 10% Rat S9	With 10% Rat S9	With 10% Rat S9	With 10% Rat S9
TA100								
	0	108 ± 3.3	102 ± 3.2	100 ± 14.3	105 ± 4.2	97 ± 8.3	59 ± 5.5	81 ± 4.4
	10	114 ± 12.4	101 ± 4.7	–	–	–	–	–
	20	112 ± 7.3	99 ± 1.9	–	89 ± 4.3	82 ± 4.7	–	–
	50	108 ± 6.2	93 ± 7.4	–	91 ± 4.6	84 ± 4.1	–	–
	125	101 ± 9.2	130 ± 2.0	–	108 ± 5.1	104 ± 12.3	–	–
	250	120 ± 4.8	96 ± 8.2[c]	–	96 ± 7.6	95 ± 4.7	–	–
	500	88 ± 11.3[c]	92 ± 3.3[c]	71 ± 3.8[c]	70 ± 9.9	59 ± 4.6[c]	–	36 ± 4.4
	750	–	–	49 ± 1.3[d]	–	–	–	30 ± 2.0
	1,000	–	–	–	–	–	58 ± 4.8	–
	1,500	–	–	38 ± 4.8[d]	68 ± 4.9[c]	69 ± 0.9[c]	47 ± 13.2	33 ± 2.0
	2,000	–	–	–	–	–	62 ± 3.8	–
	2,500	–	–	–	–	–	68 ± 8.0	–
	3,000	–	–	32 ± 3.3[d]	–	–	67 ± 9.1	74 ± 4.5
	6,000	–	–	17 ± 9.8[d]	–	–	–	64 ± 10.5[c]
Trial Summary		Negative	Negative	Negative	Negative	Negative	Negative	Negative
Positive Control[b]		857 ± 14.7	862 ± 40.8	528 ± 27.8	1,847 ± 138.6	2,181 ± 126.2	572 ± 35.1	709 ± 35.0
TA98								
	0	34 ± 4.2	19 ± 4.8	16 ± 3.8	40 ± 5.4	36 ± 1.5	24 ± 0.9	26 ± 2.7
	10	18 ± 1.3	19 ± 1.2	–	–	–	–	–
	20	31 ± 3.6	20 ± 4.8	–	44 ± 0.7	29 ± 4.4	–	–
	50	16 ± 3.8[c]	18 ± 3.2[c]	–	42 ± 4.4	34 ± 5.9	–	–
	125	18 ± 3.2[c]	21 ± 0.6[c]	–	43 ± 2.0	33 ± 4.0	–	–
	250	13 ± 0.9[c]	15 ± 2.6[c]	–	31 ± 0.9	39 ± 8.1	–	–
	500	19 ± 0.6[c]	20 ± 2.7[c]	14 ± 1.2[c]	33 ± 4.1	29 ± 2.3[c]	–	22 ± 4.5
	750	–	–	13 ± 1.5[c]	–	–	–	30 ± 3.3
	1,000	–	–	–	–	–	26 ± 4.6	–

Strain	Concentration (µg/plate)	Without S9	Without S9	Without S9	With 10% Rat S9	With 10% Rat S9	With 10% Rat S9	With 10% Rat S9
	1,500	–	–	7 ± 1.5[d]	25 ± 2.3[c]	23 ± 2.8[c]	22 ± 2.2	33 ± 2.7
	2,000	–	–	–	–	–	20 ± 0.9	–
	2,500	–	–	–	–	–	16 ± 2.4	–
	3,000	–	–	6 ± 0.7[d]	–	–	25 ± 3.3	25 ± 3.3[c]
	6,000	–	–	7 ± 1.2[d]	–	–	–	21 ± 0.3[c]
Trial Summary		Negative	Negative	Negative	Negative	Negative	Negative	Negative
Positive Control		473 ± 25.4	719 ± 13.2	444 ± 2.7	1,721 ± 57.6	1,082 ± 58.1	2,290 ± 293.6	1,794 ± 95.2
***Escherichia coli* WP2 *uvrA* (pKM101)**								
	0	181 ± 9.9	183 ± 13.5	144 ± 31.5	206 ± 8.2	199 ± 8.7	216 ± 22.0	220 ± 8.5
	10	165 ± 2.0	172 ± 6.7	–	–	–	–	–
	20	159 ± 9.2	165 ± 2.6	–	198 ± 3.4	222 ± 19.0	–	–
	50	170 ± 4.6[c]	129 ± 11.7[c]	–	205 ± 0.6	213 ± 10.1	–	–
	125	184 ± 2.9[c]	134 ± 6.8[c]	–	195 ± 9.7	207 ± 3.8	–	–
	250	153 ± 1.0[c]	156 ± 5.8[c]	–	193 ± 5.0	182 ± 3.2	–	–
	500	135 ± 16.2[c]	147 ± 3.5[c]	146 ± 9.5[c]	187 ± 3.4	194 ± 12.7	–	223 ± 7.2
	750	–	–	140 ± 10.5[c]	–	–	–	217 ± 8.5
	1,000	–	–	–	–	–	163 ± 13.6	–
	1,500	–	–	145 ± 1.8[c]	220 ± 10.7	225 ± 13.0	163 ± 3.6	204 ± 13.2
	2,000	–	–	–	–	–	204 ± 31.9	–
	2,500	–	–	–	–	–	206 ± 29.6	–
	3,000	–	–	149 ± 5.2[c]	–	–	149 ± 2.8	213 ± 14.3
	6,000	–	–	132 ± 3.7[c]	–	–	–	162 ± 5.2[c]
Trial Summary		Negative	Negative	Negative	Negative	Negative	Negative	Negative
Positive Control		909 ± 20.1	1,033 ± 47.8	1,716 ± 100.3	1,431 ± 150.2	1,287 ± 50.6	1,560 ± 33.5	1,881 ± 42.9

[a]Studies performed at Integrated Laboratory Systems, LLC. Data are presented as revertants/plate (mean ± standard error) from three plates; 0 µg/plate designates the negative control.
[b]The positive controls in the absence of metabolic activation were sodium azide (TA100), 2-nitrofluorene (TA98), and methyl methanesulfonate (*E. coli*). The positive control for metabolic activation with all strains was 2-aminoanthracene.
[c]Precipitate on plate.
[d]Slight toxicity and precipitate on plate.

Table B-2. Frequency of Micronuclei in Peripheral Blood Erythrocytes of Mice in the Three-month Inhalation Study of *Aspergillus fumigatus* Spores[a]

	Micronucleated PCEs/1,000 PCEs[b]	P Value[c]	Micronucleated NCEs/1,000 NCEs[b]	P Value[c]	PCEs (%)[b]	P Value[c]
n	5	5	5	5	5	5
Male						
Air Control	2.699 ± 0.219	–	1.463 ± 0.052	–	1.550 ± 0.063	–
Heat-inactivated Particle Control	2.390 ± 0.080	0.283	1.395 ± 0.028	0.483	1.623 ± 0.057	0.433
Viable *A. fumigatus*[d]	2.550 ± 0.154	0.818/0.716	1.379 ± 0.025	0.269/1.000	1.587 ± 0.049	0.594/1.000
Female						
Air Control	1.730 ± 0.080	–	0.931 ± 0.014	–	1.614 ± 0.100	–
Heat-inactivated Particle Control	1.960 ± 0.206	0.323	0.959 ± 0.032	1.000	1.883 ± 0.273	0.135
Viable *A. fumigatus*	1.684 ± 0.108	1.000/0.343	0.903 ± 0.021	0.387/0.344	1.864 ± 0.122	0.206/1.000

PCE = polychromatic erythrocyte; NCE = normochromatic erythrocyte.
[a]Study was performed at Integrated Laboratory Systems, LLC.
[b]Data are presented as mean ± standard error.
[c]P values for all endpoints were calculated using the two-sided Dunn's test, comparing all three groups simultaneously. For the PCEs (%) endpoints, the log-transformed PCE/NCE ratios were used.
[d]P values for the viable *A. fumigatus* groups are presented as: p value (viable *A. fumigatus* compared to air control)/p value (viable *A. fumigatus* compared to heat-inactivated particle control).

Appendix C. Ingredients, Nutrient Composition, and Contaminant Levels in NTP-2000 Mouse Ration

Tables

Table C-1. Ingredients of NTP-2000 Mouse Ration.....C-2
Table C-2. Nutrient Composition of NTP-2000 Mouse Ration.....C-2
Table C-3. Contaminant Levels in NTP-2000 Mouse Ration.....C-4

Table C-1. Ingredients of NTP-2000 Mouse Ration[a]

Ingredients	Percentage by Weight
Ground Whole Wheat	23.00
Ground No. 2 Yellow Corn	22.44
Wheat Middlings	15.00
Oat Hulls	8.50
Alfalfa Meal	7.50
Solka Floc-40	5.50
Soy Protein Concentrate	4.00
Fish Meal (60%)	4.00
Soybean Oil	3.00
Corn Oil	3.00
Dried Brewer's Yeast	1.00
Calcium Carbonate	0.90
Dicalcium Phosphate	0.40
Salt	0.30
DL Methionine	0.20
Premixes	1.26

[a]Ingredient list is based on the formulation listed on the packaging.

Table C-2. Nutrient Composition of NTP-2000 Mouse Ration[a]

Nutrient	Amount
Crude Protein[b] (% by Weight)	14.60%
Crude Fat[b] (% by Weight)	8.40%
Crude Fiber[b] (% by Weight)	9.86%
Ash[b] (% by Weight)	5.09%
Amino Acids (% of Total Diet)	
Arginine	0.75%
Lysine	0.65%
Methionine	0.44%
Cystine	0.21%
Tryptophan	0.17%
Histidine	0.32%
Leucine	1.04%
Isoleucine	0.58%
Phenylalanine	0.59%
Tyrosine	0.45%
Threonine	0.52%

Nutrient	Amount
Valine	0.66%
Minerals	
Calcium	0.99%
Phosphorus	0.56%
Potassium	0.69%
Sodium	0.19%
Magnesium	0.22%
Iron	132.00 ppm
Zinc	47.00 ppm
Manganese	44.00 ppm
Copper	10.00 ppm
Cobalt	0.12 ppm
Iodine	0.33 ppm
Vitamins	
Vitamin A	9.70 IU/g
Vitamin D_3	1.00 IU/g
α-Tocopherol	145.00 IU/g
Thiamine	9.30 ppm
Riboflavin	5.70 ppm
Niacin	54.90 ppm
Pantothenic Acid	19.00 ppm
Choline	2,326.00 ppm
Pyrodoxine	11.00 ppm
Folic Acid	1.80 ppm
Biotin	0.32 ppm
Vitamin B_{12}	53.50 Mcg/kg
Vitamin K	1.60 ppm

[a]Nutrient content is based on the formulation listed on the packaging unless otherwise noted.
[b]Nutrient content for protein, fat, fiber, and ash were calculated from the analysis of lot number 07-01-114 used in the study.

Table C-3. Contaminant Levels in NTP-2000 Mouse Ration[a]

Contaminant	Amount
Contaminants	
Aflatoxins	
B1	<0.500 ppb
B2	<0.500 ppb
G1	<0.500 ppb
G2	<0.500 ppb
Arsenic	0.211 ppm
BHA	<1.0 ppm
BHT	<1.0 ppm
Cadmium	0.054 ppm
Daidzein	<10.0 ppm
Daidzin	<10.0 ppm
Genistein	<10.0 ppm
Genistin	10.7 ppm
Glycitein	<10.0 ppm
Glycitin	11.0 ppm
Lead	0.158 ppm
Mercury	<0.01 ppm
Nitrate Anion	73.4 ppm
Nitrite Anion	<0.5 ppm
N-nitrosodibutylamine	<1.0 ppb
N-nitrosodiethylamine	<1.0 ppb
N-nitrosodimethylamine	2.2 ppb
N-nitrosomorpholine	<1.0 ppb
N-nitrosopiperidine	<1.0 ppb
N-nitrosopyrrolidine	6.9 ppb
Selenium	0.196 ppm
Aerobic Plate Count	<10 CFU/g
Coliform	<3 MPN/g
Escherichia coli	<3 MPN/g
Salmonella	Negative
Pesticides	
α-BHC	<0.0125 ppm
β-BHC	<0.0125 ppm
γ-BHC	<0.0125 ppm

Contaminant	Amount
δ-BHC	<0.0125 ppm
Acephate	<0.0400 ppm
Aldrin	<0.0125 ppm
Arochlor 1254	<0.2000 ppm
Azinphos-methyl	<0.0400 ppm
Captafol	<0.0315 ppm
Captan	<0.0500 ppm
Chlorfenvinphos	<0.0400 ppm
Chlorothalonil	<0.0125 ppm
Cis-permethrin	<0.0375 ppm
Coumaphos	<0.0500 ppm
Cypermethrin	<0.0940 ppm
DCNA	<0.0185 ppm
DCPA	<0.0185 ppm
Demeton-S	<0.0250 ppm
Diazinon	<0.0200 ppm
Dichlofenthion	<0.0300 ppm
Dicofol	<0.0315 ppm
Dieldrin	<0.0125 ppm
Dimethoate	<0.0200 ppm
Disulfoton	<0.0250 ppm
Endosulfan I	<0.0125 ppm
Endosulfan II	<0.0185 ppm
Endosulfan Sulfate	<0.0185 ppm
Endrin	<0.0185 ppm
EPN	<0.0400 ppm
Et-chlorpyrifos	<0.0250 ppm
Ethion	<0.0200 ppm
Et-parathion	<0.0200 ppm
Fenitrothion	<0.0250 ppm
Folpet	<0.0315 ppm
Fonofos	<0.0250 ppm
HCB	<0.0065 ppm
Heptachlor	<0.0125 ppm
Heptachlor Epoxide	<0.0125 ppm
Malathion	0.5850 ppm

Contaminant	Amount
Me-chlorpyrifos	0.2640 ppm
Me-parathion	<0.0200 ppm
Me-pirimiphos	<0.0250 ppm
Methamidophos	<0.0150 ppm
Methidathion	<0.0300 ppm
Methoxychlor	<0.0315 ppm
Mevinphos	<0.0250 ppm
Mirex	<0.0125 ppm
Omethoate	<0.0350 ppm
Oxadiazon	<0.0375 ppm
p,p'-DDD	<0.0185 ppm
p,p'-DDE	<0.0125 ppm
p,p'-DDT	<0.0200 ppm
PCNB	<0.0100 ppm
Phosalone	<0.0400 ppm
Phosmet	<0.0350 ppm
Propetamphos	<0.0300 ppm
Propyzamide	<0.0250 ppm
Prothiophos	<0.0300 ppm
Ronnel	<0.0200 ppm
Tech Chlordane	<0.0500 ppm
Tecnazene	<0.0125 ppm
Telodrin	<0.0200 ppm
Tetradifon	<0.0185 ppm
Thimet	<0.0200 ppm
Toxaphene	<0.1000 ppm
Trans-permethrin	<0.0250 ppm
Trithion	<0.0300 ppm
Vapona	<0.0150 ppm
Vinclozolin	<0.0250 ppm

BHA = butylated hydroxyanisole; BHT = butylated hydroxytoluene; CFU = colony-forming units; MPN = most probable number; BHC = hexachlorocyclohexane or benzene hexachloride; DCNA = 2,6-dichloro-4-nitroaniline; DCPA = dimethyl tetrachloroterephthalate; EPN = ethyl p-nitrophenyl thionobenzenephosphate; HCB = hexachlorobenzene; DDD = dichlorodiphenyldichloroethane; DDE = dichlorodiphenyldichloroethylene; DDT = dichlorodiphenyltrichloroethane; PCNB = pentachloronitrobenzene.
[a]NTP-2000 Rodent Diet, Wafer lot number 07-01-114 was irradiated.

Appendix D. National Institute for Occupational Safety and Health Sentinel Animal Program

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Table D-1. Methods and Results for Sentinel Animal Testing in Mice D-2

D.1. Methods

The sentinel animal program at the National Institute for Occupational Safety and Health monitors for common rodent pathogens in each study room to ensure study mice are healthy and pathogen-free. Pathogens are monitored through the collection of blood and feces from sentinel mice housed in the study rooms.

For this 3-month subchronic inhalation toxicology study, mice weighing more or less than 10% of the mean weight of all male or female study mice were selected for the sentinel group (n = 20; 10/sex). Females were group-housed with five per cage. Male mice were single-housed. For each week the mice were on study, sentinel animals were exposed to dirty bedding from study animals. The 4-week sentinels were evaluated for viral antibodies and *Helicobacter* 24 hours following the second exposure in the fourth exposure week. The 3-month sentinels were evaluated only at study termination. All sentinels were euthanized at the end of the study and evaluated for abnormalities by gross necropsy.

Whole blood was collected via tail vein bleed for 4-week sentinels or via cardiac puncture postmortem for 3-month sentinels. Three to four drops of whole blood were placed onto an Opti-Spot card (IDEXX Laboratories, Inc., Westbrook, ME) for serology tests. These cards were submitted to IDEXX BioResearch for analysis using the Opti-HM serology profile. The profile included: *Mycoplasma pulmonis*, ectromelia virus (ECTV), epizootic diarrhea of infant mice virus (EDIM), lymphocytic choriomeningitis virus (LCMV), mouse hepatitis virus (MHV), minute virus of mice (MVM), mouse parvovirus (MPV), mouse norovirus (MNV), pneumonia virus of mice (PVM), reovirus type 3 (REO3), Sendai virus (SV), and Theiler's murine encephalomyelitis virus (TMEV).

One fecal pellet from each sentinel mouse was collected with sterile forceps, and all pellets for each sample group were placed in a single sterile sample tube for analysis (maximum of 10 pellets/tube). Samples were submitted to IDEXX BioResearch for polymerase chain reaction (PCR) analysis using the *Helicobacter* profile. The profile included: *Helicobacter bili*, *H. ganmani*, *H. hepaticus*, *H. mastomyrinus*, *H. rodentium*, *H. typhlonius*, and *Helicobacter* spp.

Table D-1. Methods and Results for Sentinel Animal Testing in Mice

Three-month Study				
Collection Time Points	**Four-week Males**	**Four-week Females**	**Three-month Males**	**Three-month Females**
n	5	5	5	5
Method/Test				
Serology Profile[a]				
Ectromelia virus	–	–	–	–
Epizootic diarrhea of infant mice (mouse rotavirus)	–	–	–	–
Lymphocytic choriomeningitis virus	–	–	–	–
Mycoplasma pulmonis	–	–	–	–
Mouse hepatitis virus	–	–	–	–

Three-month Study				
Collection Time Points	**Four-week Males**	**Four-week Females**	**Three-month Males**	**Three-month Females**
Mouse norovirus	–	–	–	–
Mouse parvovirus	–	–	–	–
Minute virus of mice	–	–	–	–
Pneumonia virus of mice	–	–	–	–
Reovirus type 3	–	–	–	–
Theiler's murine encephalomyelitis virus	–	–	–	–
Sendai virus	–	–	–	–
PCR Evaluation[b]				
Helicobacter	–	–	–	–

– = negative; PCR = polymerase chain reaction.
[a]Serology profiles were conducted on whole blood samples of individual sentinel mice (n = 5/group).
[b]PCR evaluations of *Helicobacter* species were conducted on pooled fecal samples for each group.

D.2. Results

All test results were negative.

Appendix E. Supplemental Data

Tables with supplemental data can be found here: https://doi.org/10.22427/NTP-DATA-TOX-100.

E.1. Three-month Study – Mice

I01 – Animal Removal Summary
C08022_I01_Animal_Removal_Summary.pdf

I02 – Animal Removals
C08022_I02_Animal_Removals.pdf

I03 – Growth Curve
C08022_I03_Growth_Curve.pdf

I03C – Growth Curve
C08022_I03C_Growth_Curve.pdf

I04 – Mean Body Weight Summary
C08022_I04_Mean_Body_Weight_Summary.pdf

I04G – Mean Body Weight Gain
C08022_I04G_Mean_Body_Weight_Gain.pdf

I05 – Clinical Observations Summary
C08022_I05_Clinical_Observations_Summary.pdf

PA02 – Neoplastic Lesion Summary with Percent Incidence
C08022_PA02_Neoplastic_Lesion_Summary_with_Percent_Incidence.pdf

PA03 – Non-Neoplastic Lesion Summary with Percent Incidence
C08022_PA03_Non-Neoplastic_Lesion_Summary_with_Percent_Incidence.pdf

PA05 – Incidence Rates of Neoplastic Lesions with Systemic Lesions Abridged
C08022_PA05_Incidence_Rates_of_Neoplastic_Lesions_with_Systemic_Lesions_Abridged.pdf

PA06 – Organ Weights Summary
C08022_PA06_Organ_Weights_Summary.pdf

PA10 – Statistical Analysis of Non-Neoplastic Lesions
C08022_PA10_Statistical_Analysis_of_Non-Neoplastic_Lesions.pdf

PA14 – Individual Animal Pathology Data
C08022_PA14_Individual_Animal_Pathology_Data.pdf

PA18 – Incidence Rates of Non-Neoplastic Lesions by Anatomic Site with Average Severity Grade
C08022_PA18_Incidence_Rates_of Non-Neoplastic Lesions by Anatomic Site with Average Severity Grade.pdf

PA43 – Hematology Summary
C08022_PA43_Hematology_Summary.pdf

PA46 – Summary of Gross Pathology
C08022_PA46_Summary_of_Gross_ Pathology.pdf

E.2. Individual Animal Data

Individual Animal Body Weight Data
C08022_Individual_Animal_Body_Weight_Data.xlsx

Individual Animal Clinical Observations Data
C08022_Individual_Animal_Clinical_Observations_Data.xlsx

Individual Animal Gross Pathology Data
C08022_Individual_Animal_Gross_Pathology_Data.xlsx

Individual Animal Hematology Data
C08022_Individual_Animal_Hematology_Data.xlsx

Individual Animal Histopathology Data
C08022_Individual_Animal_Histopathology_Data.xlsx

Individual Animal Organ Weight Data
C08022_Individual_Animal_Organ_Weight_Data.xlsx

Individual Animal Removal Reasons Data
C08022_Individual_Animal_Removal_Reasons_Data.xlsx

E.3. Genetic Toxicology

G06 – Ames Summary Data
G10334_G06_Ames_Summary_Data.pdf

In Vivo Micronucleus Summary Data
G08022_In_Vivo_Micronucleus_Summary_Data.pdf

Individual Animal In Vivo Micronucleus Data
G08022_Individual_Animal_In_Vivo_Micronucleus_Data.xlsx

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