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EFFICACY OF A TECHNIQUE FOR EXPOSING THE MOUSE LUNG TO PARTICLES ASPIRATED FROM THE PHARYNX

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Recent studies have demonstrated that the mouse lung can be exposed to soluble antigens by aspiration of these antigens from the pharynx. This simple technique avoids the trauma associated with intratracheal instillation. In this study, the pharyngeal aspiration technique was validated for exposing the mouse lung to respirable particles. Using respirable fluorescent amine-modified polystyrene latex beads and beryllium oxide particles, we investigated the localization of aspirated particles within the lung and the relationship between the amount of material placed in the pharynx and the amount deposited in the lung. For exposure, mice were anesthetized with isoflurane in a bell jar, placed on a slant board, and the tongue was gently held in full extension while a 50-μl suspension of particles was pipetted onto the base of the tongue. Tongue restraint was maintained until at least two breaths were completed. Less than a minute after exposure, all mice awoke from anesthesia without visible sequela. There were no significant differences in particle distribution between the left and right side of the lung ($p = .16$). Particles were widely disseminated in a peribronchiolar pattern within the alveolar region. There was a linear and significant correlation ($r^2 = .99$) between the amount administered and the amount deposited in the lung. In beryllium-exposed mice, measurable lung beryllium was 77.5 to 88.2% of the administered beryllium. These findings demonstrate that following aspiration of pharyngeal deposited particles, exposures to the deep lung are repeatable, technically simple, and highly correlated to the administered dose.

Investigating the pulmonary toxicity of respirable agents requires that the toxic agent reach the target tissue in as physiological a manner as possible. The two most widely used techniques for experimental exposures of the lung to respirable particles are inhalation and intratracheal instillation. The comparative advantages and disadvantages of these methods have been extensively

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discussed (Brain et al., 1976; Dorries & Valberg, 1992; Hatch et al., 1981; Henderson et al., 1995; Leong et al., 1998; Pritchard et al., 1985; Ritz et al., 1993; Sherwood et al., 1988). Exposures by inhalation are expensive, require large amounts of the test agent, are technically difficult, and deposit poorly predictable amounts of the test agent. Under certain experimental conditions, the lung is exposed to particles using other methods, often intratracheal instillation, which deposits known amounts of test agent into the lung via the trachea. However, instillation by the intratracheal route requires exceptional technical skills (Driscoll et al., 2000) and, moreover, is associated with bolus effects, uneven pulmonary distribution, and invasiveness. In the mouse, trauma associated with intratracheal exposures often prevents repeated exposures to small concentrations of the test agent.

Aspiration is another method by which the respiratory tracts of animals and humans are commonly exposed to pathogens and particles (Brain and Valberg, 1979), but few studies described aspiration as a method for exposing animals to test agents under experimental conditions. Keane-Myers et al. (1998) briefly investigated the pharyngeal aspiration technique as a method for exposing the mouse lung to soluble antigen. Using pharyngeal aspiration of Evans blue dye to determine the tissue distribution of material aspirated by this technique, they observed none of the dye in the esophagus or stomach and only a small amount in the trachea. The remainder of the dye was in the lung. This suggested that restraining the tongue in extension under anesthesia prevents the swallowing of a pharyngeally deposited solution—the solution is aspirated into the lung.

Unlike intratracheal instillation, the pharyngeal aspiration technique does not require insertion of a needle or cannula through the laryngeal opening into the trachea. This eliminates the major source of trauma and technical difficulty associated with intratracheal instillation. No published studies have validated the pharyngeal aspiration technique for exposing the mouse lung to respirable particles. The purpose of this study was to determine if the aspiration procedure could consistently deliver a dose-dependent exposure to the deep lung and consistently expose both the left and right side of the lung. Our study examines the ability of this procedure to expose the mouse deep lung to two separate respirable particles: fluorescent beads and beryllium oxide.

MATERIALS AND METHODS

Animals

Specific-pathogen-free C3H/HeOuj $^{MMTV-}$ mice 6–16 wk old weighing 20–25 g were obtained from Jackson Laboratories, Bar Harbor, ME. The mice were maintained in a barrier facility in individually ventilated animal cages, housed on autoclaved hardwood beta-chip bedding, provided timed lighting assuring 12 h of light and 12 h of dark each day, and were provided ad libitum water and autoclaved feed. All procedures using animals were reviewed and approved

by the Institutional Animal Care and Use Committee and the animal program is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Aspiration of Particles from the Pharynx

Each mouse was anesthetized by placing it in a bell jar with a wire-mesh floor over gauze moistened with isoflurane (Abbott Laboratories, North Chicago, IL) and observed for signs of decreased mobility and unsteady gait for about 20 s. When fully anesthetized, the mouse was taken out of the jar and gently placed on a slant board similar to that previously described by others (Saffiotti et al., 1968). The animal was positioned so that its back was against the board while its neck and the lower cranium rested on a metal wire. Then the mouth was gently opened and the mouse was suspended from the upper thin rubber band by its incisor teeth. The tongue was gently pulled aside from the oral cavity and maintained in full extension by small forceps. A 50-μl suspension of particles was pipetted at the base of the tongue and the tongue restraint was continued until at least 2 deep breaths were completed but for not longer than 15 s. Following release of the tongue, the mouse was gently lifted off the board, placed on its left side on a flat surface, and observed for anesthetic recovery.

The distribution of aspirated particles within the lung was assessed using a pasteurized (50 °C for 1 day) aqueous suspension containing 2.5% 1-μm-diameter red (575/610 nm excitation/emission maxima) fluorescent amine-modified polystyrene latex beads. Intrapulmonary deposition of particles was determined in the 5 mice aspirating 50 μl of this bead suspension.

The dose-response relationship between particle administration and deposition in the alveolar region of the lung was assessed using pasteurized blue (360/420 nm excitation/emission maxima) 1-μm-diameter fluorescent amine-modified polystyrene latex beads that were serially diluted in phosphate-buffered saline (PBS), pH 7.4, to obtain 2.5%, 0.5%, 0.25%, 0.05%, and 0.025% aqueous suspensions representing 100%, 20%, 10%, 5%, and 1% of the original fluorescent bead number. Two mice received each bead concentration as a 50-μl pharyngeally deposited suspension, which was then aspirated into the lung.

The ability of the aspiration technique to deliver an occupationally relevant particle to the lung was assessed with suspensions of beryllium oxide particles (Aldrich Chemical Company, Milwaukee, WI). The particles were suspended in PBS, subjected to ultrasonic agitation for 48 h, and then diluted in PBS to obtain final beryllium oxide concentrations of 120, 60, 30, and 2.5 μg/50 μl (these represent elemental beryllium concentrations of 43.5, 21.75, 11, and 0.93 μg/50 μl). Seven mice were used to determine the relationship between administered and measured concentrations of beryllium in the mouse lung.

Preparation of Frozen Sections

The mice were euthanized with an overdose (>100 mg/kg) of intraperitoneal sodium pentobarbital (Veterinary Laboratories, Inc., Lenexa, KS). The lungs were removed from the thorax and inflated with 1 ml OCT compound (Tissue-Tek, Sakura Finetek USA, Inc., Torrance, CA)–Tris Buffered Saline (TBS) (2:1) mixture. Sections of the right and left lungs were placed in separate cryomolds (Tissue-Tek, Miles, Inc., Elkhart, IN), covered with OCT compound (Tissue-Tek, Sakura Finetek USA, Inc., Torrance, CA), and flash frozen in a container of prechilled 2-methylbutane (Fisher Chemicals, Pittsburgh, PA) in liquid nitrogen for about 20 s. Because the left lung of the mouse consists of a single lobe and the right lung of the mouse is divided into four lobes, a minimum of three lung lobes were present in the sections of right lung (4 lobes were present in the right lung sections of 12 mice and 3 lobes were present in the sections of 2 mice). The tissues were stored at −80 °C until further processing. For assessment of the distribution of fluorescent beads, the lungs were removed from the thorax 1 h after exposure. For assessment of the relationship between the administered and deposited amounts of fluorescent beads and beryllium, lungs were collected 4 h after exposure. The latter time point allowed additional time for mechanical clearance of particles from the conducting airways and provided a more relevant measure of the mass of beryllium deposited in the alveolar region of the lung.

Lung tissues were transferred to a −20 °C freezer overnight before sectioning. Sections were cut at 5 μm in a cryostat (Leica CM 3000).

Quantifying Beryllium Concentration in Lung

For quantification of beryllium concentration in lungs of beryllium-exposed mice, lungs were excised, lyophilized for 48 h, and stored at −80 °C. For analysis, tissues were dissolved in nitric acid, heated to 150 °C, and diluted in ASTM type II water. Samples were analyzed using a Perkin Elmer Optima DV inductively coupled plasma spectrometer by a commercial laboratory (DataChem Laboratories, Inc., Salt Lake City, UT).

Digital Imaging and Morphometry

To assess the increase in 1-μm-diameter fluorescent beads within the deep lung, basic principals of morphometric analysis were applied (Baak et al., 1987). To measure the percent of microscopic lung fields occupied by the fluorescent particles, 18 to 20 microscopic fields were sampled in every mouse. Because particle density in the proximal and distal alveolar regions may differ during the first 24 h after inhalation exposure (Zeltner et al., 1991), the measurement of particle deposition was designed to include both random and proximal alveolar regions (the alveolar region anatomically closest to the terminal bronchiole). In the fluorescent bead distribution study, these were 5 random microscopic fields (0.13 mm^2) of alveoli and 5 microscopic fields (0.13 mm^2) extending from the proximal alveolar region to the distal terminal bronchiole for each side of the lung. In the fluorescent bead dose-response study, there

were 5 random microscopic fields (0.13 mm^2) of alveoli and 5 microscopic fields (0.13 mm^2) extending from the proximal alveolar region through the alveolar duct from each mouse where anatomically unambiguous fields were available from each side of the lung but not fewer than 18 fields per mouse. Samples were taken at random throughout each lung lobe. Microscopic fields were identified using an Olympus AX70 photomicroscope with fluorescence capabilities, and digital images were captured by a Quantix cooled color digital camera with 2000×2000 pixel resolution (Photometrics, Tucson, AZ), with QED acquisition software (QED Imaging, Inc., Pittsburgh, PA).

The area of each microscopic field occupied by fluorescent beads was quantified using commercial morphometry software (Metamorph Imaging System, Universal Imaging Corporation, Downingtown, PA).

Confocal Microscopy

Confocal images of lung tissue were recorded from cryosections using a Zeiss LSM 510 laser scanning confocal microscope (Carl Zeiss, Inc., Thornwood, NY). Areas of lung tissues were recorded as transmitted light images, whereas the fluorescent signal of the latex beads was displayed in a separate photodetector after ultraviolet (UV) excitation at a wavelength of 364 nm. Images of lung tissue and fluorescent latex beads were combined to reveal the position of the beads among lung structures.

Statistical Analysis

The mean percent areas occupied by beads in each microscopic lung region from each side of the lung were compared by a mixed model analysis of variance performed using SAS version 8.2 (SAS, Cary, NC) to assess regional distribution within the lung. SigmaStat (SPSS Science, Chicago) was used to conduct the regression analyses to determine the relationship between percent of the microscopic lung field occupied by particles and administered particle concentration. It was also used to determine the relationship between the mass of beryllium placed in the pharynx and the mass of beryllium recovered from the lung.

RESULTS

All the mice recovered within 1 min or less of the aspiration procedure. All mice exhibited normal behavior after completion of the procedure. No evidence of trauma was observed at necropsy.

In the distribution study, fluorescent beads were present in the left and right lung of all mice as free or aggregated particles (Figure 1). Within the lung, fluorescent beads were distributed to proximal and random alveoli. There were no significant differences in particle distribution between the right and left lung ($p = .16$), or between proximal alveolar regions and random alveolar regions ($p = .13$). Nor was there a significant interaction between side of the lung and region within the lung ($p = .48$). Low-magnification assessment of each

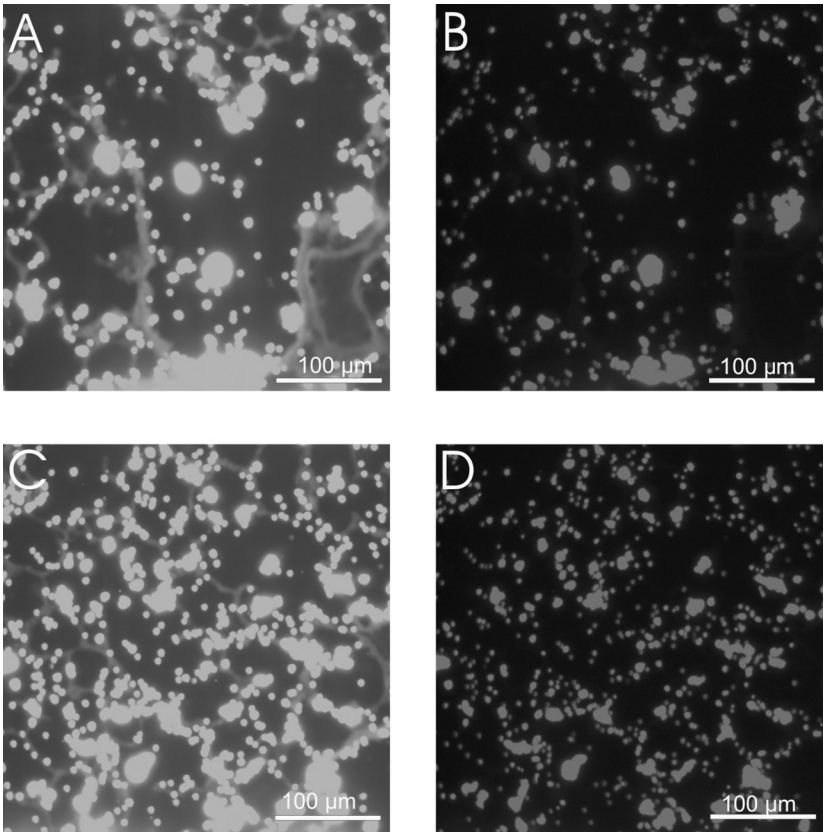

FIGURE 1. Distribution of the fluorescent beads in the alveoli near the terminal bronchioles and in the deeper areas of lungs of mice exposed to a 2.5% suspension of fluorescent beads. (A) Anatomical localization of alveolar structures using green autofluorescence. (B) Fluorescent beads in the same area visualized in A (bar = 100 µm). (C) Anatomical localization of alveolar structures using green autofluorescence. (D) Fluorescent beads in the same area visualized in B (bar = 100 µm).

lung confirmed that beads were distributed to areas immediately adjacent to airways, that beads extended into alveoli in the deeper lung, and that airways were not obstructed by the beads.

Low magnification assessment also identified areas of the lungs receiving fewer beads. These areas were generally at the greatest distance from the airways, indicating that the beads were widely disseminated into the deep lung with a bronchiolocentric pattern (Figure 2).

In the dose-response study, beads were present in the left and right lung of all mice. As the concentration of beads administered increased, the volume density of bead fluorescence in the alveolar region also increased (Figure 3). The volume density of fluorescent beads in the alveolar region was linearly and significantly associated with the bead concentration ($r^2 = .99$, Figure 4). Beads were distributed in alveoli and alveolar ducts as free or phagocytized particles.

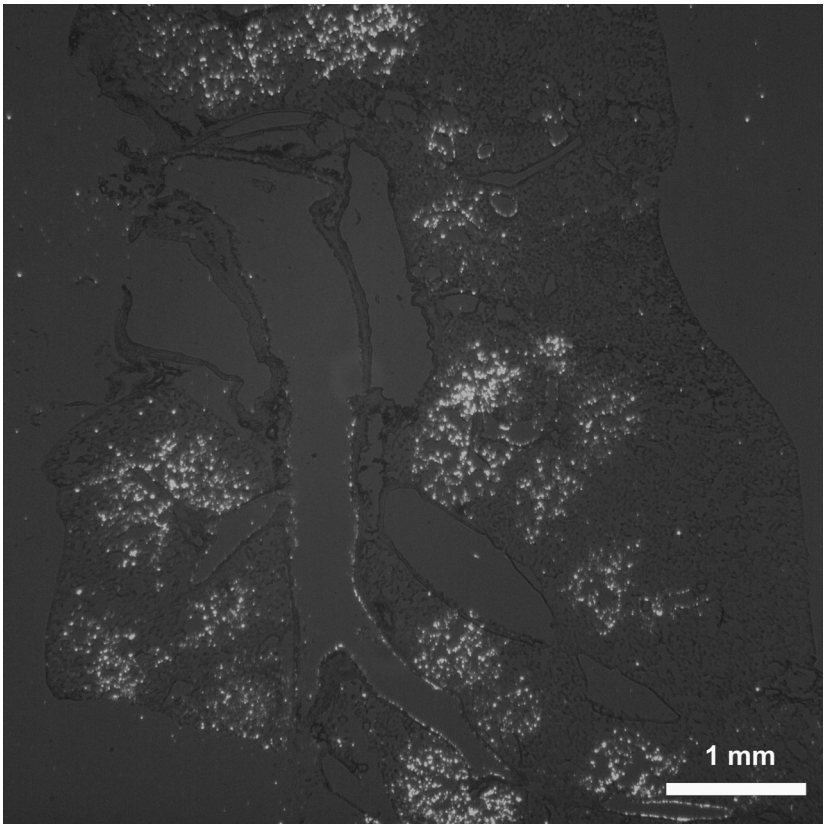

FIGURE 2. Low-magnification image demonstrating widespread dissemination of fluorescent beads in a bronchocentric pattern in the left lung of a mouse exposed to a 2.5% suspension of fluorescent beads during the distribution study. Fluorescent beads are shown against the dark unstained outline of the lung (bar = 1 mm).

Similarly, the occupationally relevant particle, beryllium oxide, reached the lung after aspiration of a pharyngeally deposited suspension. The amount of beryllium deposited in the lung was linearly and significantly correlated with the amount of beryllium administered in the pharynx (Figure 5). The percentage of administered beryllium measured in the lungs ranged from 77.5 to 88.2% (Table 1).

DISCUSSION

Using 1-μm-diameter fluorescent beads to visualize aspirated particles in the lung, particles consistently entered the lung of each exposed mouse. Further, the material distributed in a bronchocentric pattern, extended into the alveolar region, and did not obstruct airways. For both of our two test particles, fluorescent

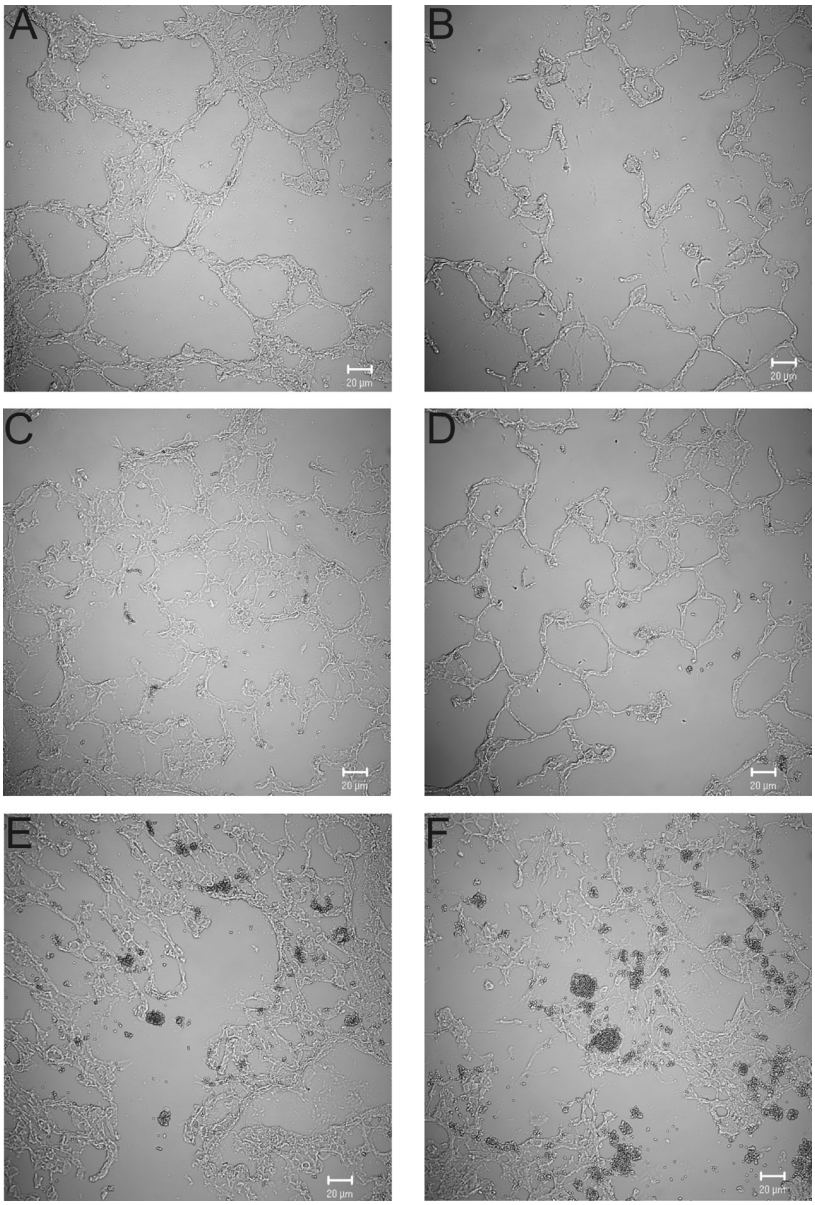

FIGURE 3. Confocal microscopic images of the left and right sides of the lung exposed to varying concentrations of fluorescent beads (bar = 20 µm). (A) Left lung of a mouse receiving a 0.025% suspension of beads. (B) Right lung of a mouse receiving a 0.025% suspension of beads. (C) Left lung of a mouse receiving a 0.25% suspension of beads. (D) Right lung of a mouse receiving a 0.25% suspension of beads. (E) Left lung of a mouse receiving a 2.5% suspension of beads. (F) Right lung of a mouse receiving a 2.5% suspension of beads.

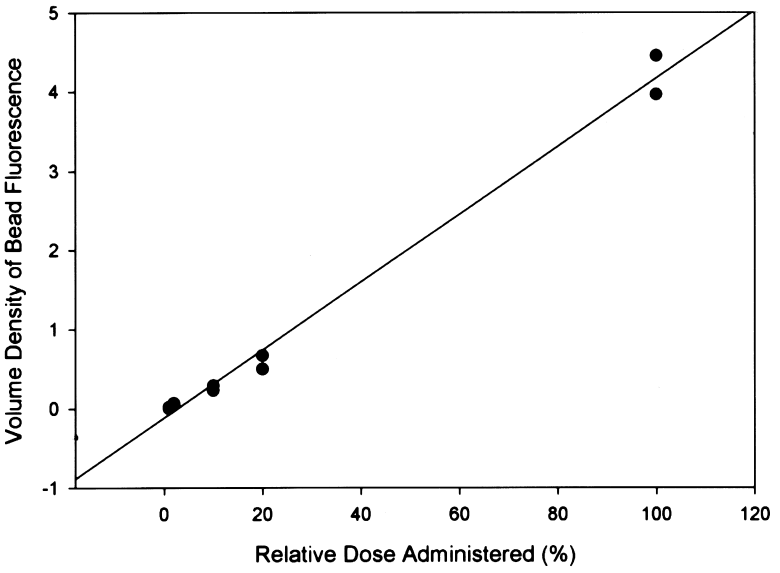

FIGURE 4. Dose-response relationship between the relative dose administered (% of original suspension) and the mean volume density of bead fluorescence in the alveolar region of the lung.

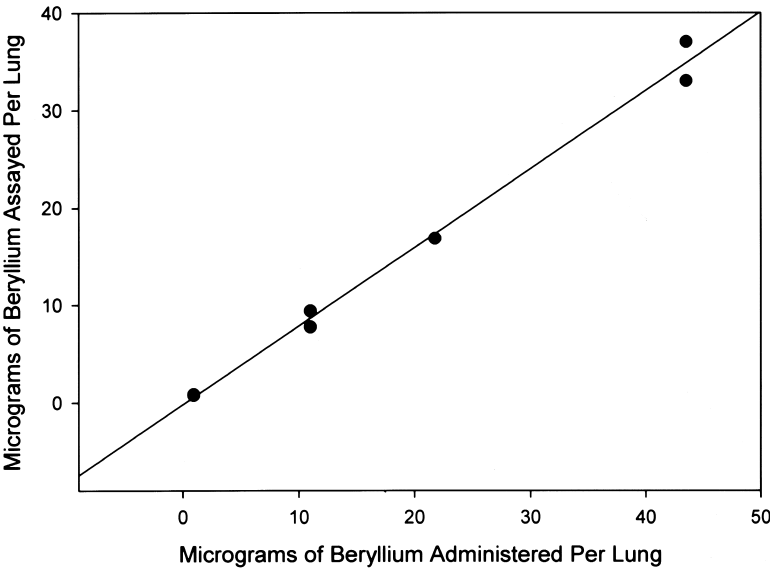

FIGURE 5. Dose-response relationship between the micrograms of beryllium aspirated and the micrograms of beryllium measured in the lung.

TABLE 1. Beryllium Assayed in Lung Tissue After Aspiration of Pharyngeal-Deposited Beryllium

µg Beryllium instilled (as beryllium oxide)	*n*	Mean µg beryllium measured in the lung	Percent
0.93	2	0.82	88.2
11	2	8.58	78.05
21.75	1	16.86	77.5
43.5	2	35.04	80.6

beads and beryllium oxide, microscopic deposition within the lung was linearly related to the dose originally deposited within the pharynx. Importantly, these results reflect the methods used in our study, particularly maintaining the tongue in extension to prevent swallowing until at least two breaths were completed. Because the mouse respiratory efforts actually bring the material into the lung, a reproducible exposure cannot be assured for mice not completing two breaths. With completion of the minimum of two breaths, exposures were remarkably consistent.

The comparison between the areas occupied by the fluorescent material in the proximal alveolar region and the random alveolar regions did not show any statistically significant difference. This is consistent with dissemination of particles from the airways into the lung. Slight statistical trends for differences in distribution were detected and were expected due to anatomical differences between the multiple lobes of the right lung and the single lobe of the left lung, between the alveoli closer to the source of exposure and the more abundant alveoli further from the terminal bronchioles. Indeed, low-magnification images of the exposed lungs clearly demonstrate that the particles are widely disseminated in the alveoli but that not all alveoli receive the same or similar exposures. Such nonuniform particle deposition, particularly between alveolar duct regions and alveoli in the first day after exposure, is also observed in animals exposed by inhalation (Sweeney & Brain, 1991).

An important consideration with pharyngeal aspiration of experimental material from the mouse pharynx is that the material is initially deposited well above the tracheal bifurcation. With intratracheal instillation, insertion of a cannula too deep into the trachea can cause the cannula to pass into the mainstem bronchus of the left or right side of the lung, resulting in unilateral lung exposure. As expected with the pharyngeal administration site well above the tracheal bifurcation, unilateral deposition was not observed when particles were administered by aspiration. Thus, aspiration of test material from the mouse pharynx was not associated with many of the complications of intratracheal instillation: possible unilateral administration of the test agent, possible instillation of the esophagus instead of the trachea, and possible trauma to the larynx or trachea. However, the material is initially administered above the larynx and a percentage of the material cannot be recovered from the respiratory tract. While the exposures were consistent and reproducible, the 11.8–22.5%

of the administered beryllium that did not enter the respiratory tract after pharyngeal aspiration is a safety consideration. With toxic materials, personal protection of laboratory personnel during exposures is indicated. Thus, exposure of the mouse respiratory tract by pharyngeal aspiration avoids many of the technical difficulties of intratracheal instillation but requires increased safety precautions, especially when working with highly toxic materials. In contrast, the generation of aerosols needed for inhalation exposures has a much greater potential for exposure of nonrespiratory tissues in the mouse and requires the generation and use of much greater quantities of test agents with much greater potential for contamination of equipment.

The technical simplicity and lack of invasiveness of the pharyngeal aspiration techniques are its major advantages. The technique provides an additional tool for researchers investigating particle toxicity in the mouse lung. Evidence indicates that the technique (1) vastly simplifies the exposure of mice to respirable particles, (2) permits controlled exposures to scarce or hazardous particles such as beryllium, and (3) enables exposure to the deep lung over a wide range of particle concentrations. Thus, the aspiration procedure represents a noninvasive and technically simple method for reproducibly exposing the mouse deep lung to respirable particles. It represents a noninvasive alternative to the intratracheal instillation procedure. Because the mice are not traumatized and the technique is reproducible, it is suitable for repeated exposures.

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