Anatomic localization of 24- and 96-h particle retention in canine airways

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Institutes for 1Inhalation Biology and 2Pathology, GSF-National Research Center for Environment and Health, D-85758 Neuherberg/ Munich; and 3Harvard School of Public Health, Department of Environmental Health, Boston, Massachusetts 02115

Kreyling, W. G., J. D. Blanchard, J. J. Godleski, S. Haeussermann, J. Heyder, P. Hutzler, H. Schulz, T. D. Sweeney, S. Takenaka, and A. Ziesen. Anatomic localization of 24- and 96-h particle retention in canine airways. J. Appl. Physiol. 87(1): 269–284, 1999.—Long-term retention of particles in airways is controversial. However, precise anatomic localization of the particles is not possible in people. In this study the anatomic location of retained particles after shallow bolus inhalation was determined in anesthetized, ventilated beagle dogs.

The aerosol bolus inhalation technique was introduced by Altschuler et al. (2) and has later been used successfully to test the first-in-last-out principle in respiratory physiology in humans (18, 45, 54) and dogs (50). An air volume inhaled at the beginning of a breath penetrates deeply into the lungs and is recovered late during exhalation, whereas an air volume inhaled at the end of a breath penetrates only slightly into the lungs and is exhaled first. It is therefore expected that particles deposited from an aerosol bolus penetrating into a volumetric lung depth (VLD) of the respiratory system that is less than the dead space volume are located in conducting airways. However, because of convective air mixing, the particles are not confined only to the bolus air while the bolus is transported through the respiratory system. The particles may be convectively transported into air adjacent to the bolus air, resulting in a more dispersed bolus on exhalation, with particles distributed in a much larger volume in the exhaled air than in the inhaled air (18). Therefore, convective air mixing transports particles deeper into the lungs than convective bulk transport so that the true sites of particle deposition can be different from those anticipated. It is therefore conceivable that long-term particle retention observed in the human lung after shallow aerosol bolus inhalation, and interpreted as prolonged retention of particles in ciliated airways, is actually retention of particles in nonciliated peripheral air spaces.
In this study, shallow aerosol bolus inhalation is used in dogs with radiolabeled or fluorescence-labeled monodisperse aerosols to both replicate the human studies noted above and to define, by subsequent microscopic analysis, the anatomic location of retained particles in the lungs. Because single-breath washout curves are the same in canine and human subjects, it was assumed that dogs would be well suited to this study (25, 28, 49, 51, 67). If a pattern of delayed clearance could be demonstrated with shallow bolus methodology in dogs, it would then be possible to analyze the location of the retained particles morphometrically and to identify the spatial distribution of retained particles in all anatomic sites, including different sizes of airways and alveoli.

MATERIALS AND METHODS

Experimental Methodology Considerations

Because each animal may have variably sized conducting airways, the dead space was precisely defined for each animal before the bolus inhalation, and then this measurement was used to determine the volume for shallow bolus insufflation into a breath. Also, a servo-controlled system was required for anesthetized and paralyzed animals to generate breathing maneuvers that were repeated in exactly the same manner for each breath.

To carry out these experiments, a sufficiently high number of radiolabeled, red fluorescence, and green fluorescence, 2-µm monodisperse particles were delivered in shallow boluses to permit their quantification anatomically at sites of retention. (The term shallow bolus inhalation will be used for a fractional volume containing aerosol particles inhaled at the end of the tidal volume.) Radiolabeled particles provided retention kinetics and gross distribution, whereas red and green fluorescent particles delivered 96 and 24 h before euthanasia yielded the anatomic site of retention. Two-micrometer-sized particles were used to minimize impaction and provide sufficient settling during the breath hold. A deep aerosol bolus inhalation using blue fluorescence-labeled 1-µm monodisperse particles served as a marker for the peripheral lung to further control the applied morphometric analysis. One-micrometer-sized particles were used for the deep bolus to ensure negligible settling and impaction in airways while ensuring adequate settling in alveoli. (The term deep bolus will be used to indicate a fractional volume containing aerosol particles inhaled at the beginning of the tidal volume.)

Definition of deposition and retention utilized external gamma radiation emission detection and imaging techniques (10, 21, 23, 33), macroscopic radiation distribution methodology at necropsy (59–62), and microscopic qualitative morphology for the ultimate analyses of particle localization.

Special emphasis was also placed on the technique of lung tissue fixation in these studies. Because liquid fixation by either intratracheal instillation or intravascular perfusion had the possibility of displacing particles from their location of retention, physical fixation methods were employed in which the lungs were microwave-dried during inflation at total lung capacity (59–62). In morphometric analysis, stratified random sampling was used with a microdissection technique for analysis of airways > 1 mm in diameter as well as stratified random sampling of sections of peripheral lung tissue to distinguish particle location between airways < 1 mm in diameter and alveoli.

Design of the Study

The study was divided into two parts. In the first part, the lung retention of technetium 99m-labeled polystyrene particles (99mTc-PSL) was followed for 2 days after shallow bolus inhalation to confirm a slowly cleared fraction in each dog and to determine the kinetics of fast and slow particle clearance from the lungs of each dog as schematically illustrated in Fig. 1A.

The second and main part took place several months later. A schematic flow chart (Fig. 1B) is provided to clarify this design. Four days (96 h) before euthanasia, each dog inhaled shallow boluses of red fluorescent PSL. Twenty-four hours before sacrifice, each dog inhaled shallow boluses of green fluorescent PSL and radiolabeled 99mTc-PSL. At the same time, blue fluorescent 1-µm PSL were delivered via deep aerosol bolus inhalation. Lung retention of 99Tc-PSL from the time of inhalation until sacrifice 24 h later was followed. For the first 2–3 h, retention was determined from the gross particle distribution as imaged by a gamma camera and later by external gamma counting by using a more sensitive gamma spectroscopy lung counter (10, 21, 23).

Fig. 1. A: flow diagram of 48-h, radiolabeled-particle, shallow bolus clearance study. B: flow diagram of comprehensive, 96-h, shallow and deep bolus deposition and clearance study. 99mTc-PSL, technetium 99m-labeled polystyrene particles; SPECT, single-photon-emission computed tomography.
Experimental Setup for Aerosol Bolus Delivery

The experimental setup was designed to deliver shallow or deep small-volume aerosol boluses to anesthetized dogs while the airborne particle number in the inhaled and exhaled air was measured. The dead space in each dog needed to be precisely defined. The experimental setup included a piston-type computer-controlled servo ventilator (28), the valve system for aerosol bolus delivery, a miniaturized in-line aerosol photometer for continuous monitoring of particle number concentrations (50), and a magnetic sector field mass spectrometer (M 3, Varian MAT, Bremen, Germany) modified as described by Scheid (43) for continuous monitoring of respiratory gases as well as the applied tracer gases, He and sulfur hexafluoride (SF₆).

The bolus valve system consisted of four electropneumatically driven valves supplying two pathways of ventilation (Fig. 2). One pathway was used to maintain normal ventilation, and the other for delivery of a 30-cm³ bolus. The aerosol bolus volume was inserted into the inhaled air at a preselected volume by computer-controlled switching of the valve system. To minimize aerosol particle deposition and subsequent reentrainment of the deposit during the next firing of the valve system, the aerosol valves were constructed with an actuated center-bore rod that provided an undisturbed aerosol flow without edges and bends.

Aerosol Generation

Monodisperse PSL of red, green, and blue fluorescence with diameters of 2.5, 2.2, and 1.0 µm, respectively, were obtained from Molecular Probes (Eugene, OR). In addition, monodisperse 2.4-µm PSL radioactively labeled with ⁹⁹mTc were produced as described earlier (42). The objective of this design was to facilitate the delivery of the highest possible monodisperse particle concentrations in the aerosol bolus. PSL were suspended in distilled water at a particle concentration of 7 × 10⁶ cm⁻³. The suspension was nebulized by using a concentric stainless steel nozzle (970-series, Schlick, Coburg, Germany) with the suspension fed through its inner tube by a syringe pump at 0.2 cm³/min and then sheared into an aerosol by the compressed air (pressure 2 × 10⁵ Pa, airflow 500 l/h) moving through the outer tube. The aerosol was quasi-neutralized by an ⁸⁵Kr source and dried by a silica-gel diffusion drier at the periphery of the aerosol generator column. At the end of this column, the aerosol was concentrated by a factor of 15–20 in a virtual impactor-like device (31) and then pulled into a 2-liter aluminum syringe. The aerosol concentration in the syringe was 2–3 × 10⁴ cm⁻³. After aerosol filling, the syringe was connected to the bolus valve system. Before each bolus delivery, an aerosol volume of 30 cm³ was very slowly injected into the bolus delivery tubing of the valve system by cranking the spindle-driven piston of the syringe. A schematic of the experimental system is shown in Fig. 2.

To define particle deposition and convective mixing in the dogs (51), an aerosol of di-(2)-ethylhexyl sebacate (DEHS) was generated from a condensation aerosol generator (Mage generator, Lavoro e Ambiente, Bologna, Italy) and delivered as a 30-cm³ bolus without a breath hold in the breathing maneuver. These particles had an aerodynamic diameter of 2.5 µm and were used with photometry measurements.

Animals and Anesthesia

Two female and six male healthy adult beagle dogs from the colony of the GSF were used in accordance with the animal protection standards of Germany and enforced by the Government of the District of Upper Bavaria as the local authority. Shallow bolus clearance was studied in all eight dogs, but only four of them were used for the complete study of shallow bolus deposition and retention including the
morphometric analysis. The other four dogs were used for development of the experimental methods. The mean body weight of all animals was 14.3 ± 2.6 kg; the mean age of seven dogs was 31 ± 6 mo, and the eighth was 88 mo old and was used only for clearance measurements and method development.

Each dog was anesthetized and paralyzed by intravenously delivered pentobarbitone (20 mg body wt) and alcuronium (0.2 mg/kg body wt) and maintained in a supine position. Intubation was via the oropharynx with a low-pressure cuffed endotracheal tube with an inner diameter of either 9 or 10 mm depending on the dog's tracheal size. The endotracheal tube was placed carefully such that it penetrated 6 cm into the trachea. The tube was connected to a servo ventilator as described earlier (50). The bolus valve system and the aerosol photometer were placed between the servo ventilator and the described earlier (50).

Aerosol deposition, convective mixing (bolus dispersion), and residual particles present in exhaled volumes at end expiration were determined from aerosol photometry in front of the endotracheal tube (51).

Shallow bolus inhalation. The red 2.5-μm PSL, the green 2.2-μm PSL, and the radioactively labeled 2.4-μm PSL particles were delivered to a shallow lung depth (shallow bolus), which was scaled to the individual airway volume of the dog, i.e., to the volume of phase I of the He-expirogram. The volumetric front of the shallow bolus was varied among the different dogs of the study, ranging from 45 to 85% of phase I. Although essentially no particles were detectable by photometry at the end of the expiratory volume of each breath, three subsequent breaths of clean air were given, and then the next bolus was delivered at the exact same volume in the breathing maneuver. Each dog usually inhaled 50 boluses within 25–30 min.

Deep bolus inhalation. The 1-μm blue PSL particles were delivered to the lung periphery. The front depth of the bolus was set to three times the series dead space of He (deep bolus), corresponding to a VLD of 350–500 cm3. Inspiration was again performed to 80% of TLC at a flow rate of 300 cm3/s. For this bolus there was a 10-s breath hold before exhalation. After three subsequent breaths of clean air, the next bolus was delivered. Fifty aerosol boluses during 20–25 min were delivered for this test aerosol, as was done for the others.

Determination of Lung and Airway Volumes

The He-rebreathing technique was used to measure the functional residual capacity (FRC) by use of a tidal volume of 450 cm3 at a rebreathing frequency of 40 breaths/min for 15 breaths. Total lung capacity (TLC) was defined as that lung volume at which tracheal pressure had reached +2.5 kPa at an inspiratory flow rate of 50 cm3/s.

Series dead space volumes for He and SF6 were determined by the single-breath washout technique (11). After equilibration of the lung with 1% He and 1% SF6 in air, a standardized single-breath maneuver was performed. After passive expiration, a test-gas-free air volume was inspired up to 80% of TLC at a flow rate of 300 cm3/s. Without a postinspiratory pause, expiration was performed to well below FRC. The volume of phase II of the expirogram was measured by least squares regression analysis and was used to determine the series dead space volume. The volume of phase I was also determined from the expirograms.

Aerosol Bolus Inhalation

For aerosol bolus inhalation, a standardized breathing maneuver was employed that was scaled to the lung size of each dog. After passive expiration, inspiration was performed up to 80% of TLC at a flow rate of 300 cm3/s that was followed by a breath-hold of 20 s to increase deposition probability. Expiration was performed at a controlled flow rate of 300 cm3/s until tracheal pressure reached +300 Pa. The dog was then allowed to expire passively to ensure that the end-expiratory lung inflation was at FRC. Test particles were introduced as a bolus during this breathing maneuver.

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Clearance Kinetics Using Radioactive Test Particles

From the time immediately after 99mTc-PSL aerosol bolus delivery until euthanasia, lung retention was continuously measured. For the first 2–3 h, a SPECT gamma camera (Picker, Espekamp, Germany) collected planar images of the thorax from dorsal and ventral at 5-min intervals. Because mucociliary transport of 99mTc-PSL in the trachea was stopped by the cuffed balloon of the endotracheal tube, there was no loss of 99mTc-PSL to the gastrointestinal tract. Thus the radioactive measurements reflected the clearance and redistribution of a constant number of 99mTc-PSL particles within the lungs and trachea, including rapid particle transport into the trachea. Therefore, the gamma camera could be used to distinguish 99mTc-PSL retention in the lungs from 99mTc-PSL accumulation in the trachea at the point of the cuffed balloon. After ~2 h, a 40-min three-dimensional SPECT scan was performed to image the three-dimensional distribution of 99mTc-PSL in the lungs.

After the SPECT measurements, total retention measurements were continued with a gamma spectroscopic lung counter (10) because it had 30-fold higher sensitivity than the gamma camera. The collimated detectors of the lung counter were placed at 5-min intervals. Because mucociliary transport of 99mTc-PSL in the trachea was stopped by the cuffed balloon of the endotracheal tube, there was no loss of 99mTc-PSL to the gastrointestinal tract. Thus the radioactive measurements reflected the clearance and redistribution of a constant number of 99mTc-PSL particles within the lungs and trachea, including rapid particle transport into the trachea. Therefore, the gamma camera could be used to distinguish 99mTc-PSL retention in the lungs from 99mTc-PSL accumulation in the trachea at the point of the cuffed balloon. After ~2 h, a 40-min three-dimensional SPECT scan was performed to image the three-dimensional distribution of 99mTc-PSL in the lungs.

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After the SPECT measurements, total retention measurements were continued with a gamma spectroscopic lung counter (10) because it had 30-fold higher sensitivity than the gamma camera. The collimated detectors of the lung counter were positioned so that 99mTc-PSL lung retention was measured without significant interference from 99mTc-PSL either accumulated at the cuff of the endotracheal tube or swallowed into the gastrointestinal tract once the tube was removed. The 99mTc-PSL activity A(t) was corrected for decay, background, and sensitivity of the two gamma counters. Activity data were normalized to the initially deposited activity A(0). Lung retention data were fitted by the following function of two exponential terms to distinguish between fast and slow clearance

$$A(t) = A(0) = L_1 \exp(-\lambda_1 t) + L_2 \exp(-\lambda_2 t)$$

where $L_1 (i = 1, 2)$ are the fast and slow cleared fractions with $(L_1 + L_2) = 1$, and $\lambda_i (i = 1, 2)$ are the clearance rates. The initial long-term retention, $L_2$, is referred to as the A-value (54).
In the second method, the tissue of each compartment of each slice was then digested in bleach, the PSL particles in the digest were collected by filtering onto 0.2-µm pore-sized nuclepore-filters, and then the fluorescent particles on the filter were counted by using a fluorescence light microscope. To ascertain representative particle counts on the filter (50 mm diameter), 1) the suspension (volume > 50 ml) was rigidly stirred before pressurized filtration, 2) homogeneous deposition was grossly checked by low magnification, and 3) normally one quadrant of the filter surface was counted. (In the case of low particle numbers, the entire filter surface was counted; in the case of very high particle numbers, a series of fields of view along the diameter of the filter were counted making up one-tenth of the filter surface area.) Twenty-four-hour retention of the shallow bolus in each compartment was measured with the green fluorescent PSL; 96-h retention was measured with the red fluorescent PSL. Twenty-four-hour retention of the deep bolus was measured with the 1.0-µm blue fluorescent PSL. The filter area S_{obs} mentioned above was counted for particles kN_{obs} of a given fluorescence k and total numbers of particles per filter kN_{F} calculated from the filter area S_{F}. For each compartment (C), the number of each fluorescent PSL kN_{C} was integrated over the number of filters m

\[ \text{kN}_{C} = \sum_{m} \text{kN}_{F} = \sum_{m} \text{kN}_{obs} \text{S}_{F}/\text{S}_{obs} \quad (k = \text{Green, red, blue}) \]  

For a given fluorescent PSL, its fraction in each compartment was calculated as the number found in that compartment normalized by the total number found in all 1-mm slices analyzed, i.e., these data are expressed as fractions of the total number of these fluorescent particles recovered.

To compute retained PSL fractions of each compartment, these fractions were multiplied by the 24-h lung retention L(t = 24)/L(0) obtained from Eq. 1. To calculate PSL fractions relative to the particles inhaled, the retained fractions from Eq. 1 were multiplied by the deposition fraction as determined by aerosol photometry during aerosol bolus delivery. In toto, these methods provided PSL analysis of \(-1/33 = 0.03\) of the lung volume, because the total of all 1-mm slices was one-eleventh of the lung volume (paired 1- and 10-mm slices were cut), and every third 1-mm slice was analyzed.

Measurement of 24- and 96-h particle retention in the parenchyma and airways < 1 mm. Morphometric analysis is shown schematically in Fig. 3. The 10-mm lung slices were used to determine 24- and 96-h retention in the parenchyma and airways of known size. By using a dissection microscope, the slices were microdissected into four compartments: airways with a diameter > 5 mm, 2.5- to 5-mm airways, 1- to 2.5-mm airways, and the fourth compartment (mixed parenchyma) being the remaining tissue composed of airways < 1 mm and parenchyma. Because of the extensiveness of this dissection, only the tissue of every third slice was sampled, resulting in five slices to be dissected and analyzed. To reduce the risk of dislodging particles during dissection, several blades per slice were used, and forceps were frequently cleaned by using an ultrasonic bath. Two methods were used to measure retention in the four separate compartments of each slice. First, the radioactivity from retained 99mTc-PSL particles in each compartment was counted in a well counter. The retained fraction in each compartment was calculated as the 99mTc-activity of PSL found in that compartment normalized by the total 99mTc-activity found in all 1-mm slices.

Morphometric Analysis

At euthanasia, the dog was anesthetized by using pentobarbital (30 mg/kg body wt) and exsanguinated via the cannulated carotid artery. The trachea was occluded, and the thorax was opened. The heart-lung block was excised, and the heart was separated from the lungs. The entire process took ~20 min. Then, the lungs were fixed and inflated to 3.5 kPa, which was considered to represent TLC. Immediately after fixation, the 99mTc-activity found in all 1-mm slices was separated from the lungs. The entire process took 20 min. Then, the 99mTc-activity found in all 1-mm slices was separated from the lungs. The entire process took 20 min.

For tissue sampling, the dried lung was sliced base to apex, after a random start, in alternating 10- and 1-mm-thick slices.

Measurement of 24- and 96-h particle retention in airways > 1 mm and mixed parenchyma. Morphometric analysis is shown schematically in Fig. 3. The 1-mm slices were used to measure 24- and 96-h retention in the parenchyma and airways of known size. By using a dissection microscope, the slices were microdissected into four compartments: airways with a diameter > 5 mm, 2.5- to 5-mm airways, 1- to 2.5-mm airways, and the fourth compartment (mixed parenchyma) being the remaining tissue composed of airways < 1 mm and parenchyma. Because of the extensiveness of this dissection, only the tissue of every third slice was sampled, resulting in five slices to be dissected and analyzed. To reduce the risk of dislodging particles during dissection, several blades per slice were used, and forceps were frequently cleaned by using an ultrasonic bath. Two methods were used to measure retention in the four separate compartments of each slice. First, the radioactivity from retained 99mTc-PSL particles in each compartment was counted in a well counter. The retained fraction in each compartment was calculated as the 99mTc-activity of PSL found in that compartment normalized by the total 99mTc-activity found in all 1-mm slices.

**Fig. 3.** Schematic diagram of morphometric studies to quantify particles in small airways and alveoli.
Thus a slice with an EI = 1 would have the average amount of activity for the whole lung (60).

In practice, the radioactivity in each 10-mm slice was measured by using a 1-liter well-type gamma counter. To fit into the counter well, the slices had to be first separated into left and right lungs and, when necessary, the caudal lobe. These sections were counted individually and then summed to compute the total radioactivity in each slice. To measure the volume of each slice, the lung pieces were reassembled into slices (by using as a guide a photocopied of the slices before they were separated) and mounted onto cardboard sheets. The volume of each lung slice was computed by multiplying its planar area and depth. The planar area was measured by morphometric point counting with a 0.615-mm² grid on the basis of the method of Cavalieri (30); depth was measured by using a caliper to measure slice thickness in three locations and taking the average.

To select the tissue samples for confocal fluorescence microscopy, the lung slices were arranged sequentially, and the tissue volume comprising airways <1 mm and parenchyma within each slice was measured by point counting with a 1-mm² grid. Usually, there were 17–18 slices. Then, the slices were systematically sampled (24, 34) after a random start, by taking about thirty 1-cm³ tissue blocks as described by Zeltner et al. (73). Finally, the resulting 30 tissue blocks were sliced into a 200-µm-thick section by using a vibratome (Vibratome 1000, Polysciences, St. Goar, Germany) and used for microscopic analysis. Care was taken to avoid cross-contamination of the PSL by frequently changing cutting blades and cleaning the forceps ultrasonically.

The 200-µm sections were used to count the fluorescent particles in the airways <1 mm in diameter and parenchyma with a confocal laser scanning microscope (LSM 410 invert, Zeiss Oberkochen, Germany). In each 0.2 × 10 × 10-mm section, all particles were counted in a volume defined by the entire cross section and the depth, which ranged from 15 to 160 µm, by taking scans at intervals of 5-µm depth. The first 15 µm of the section were not analyzed because of eventual artifacts caused by the first cuts; below 160 µm, the PSL signal appeared to be less clear in the microscope. Red PSL were excited with 543-nm laser light, and emission was observed at >590 nm; for green PSL, excitation was at 488 nm and emission was at 515–560 nm; and for blue PSL, excitation was at 360 nm and emission was at 400–430 nm.

Because airways could not be distinguished from blood vessels, all tubes were sized, and their volume was estimated. If the diameter of the tube was between 0.3 and 1 mm and there were no alveoli in the walls, then particles were assigned to the <1-mm airways compartment. To estimate the volume of each tube Vt, between 0.3 and 1 mm diameter, we assume that all airways are cylinders of circular cross section. If a cylinder is cut arbitrarily through its jacket, the shortest elliptical axis is the diameter da of the cylinder. Orthogonal to da is the elliptical axis a. If α is the angle at which the cylinder is tilted against the plain perpendicular to the cutting plane of the section, then a becomes

\[ a = d_a \cos \alpha \]

(5)

Similarly the length la of the jacket of the tilted cylinder is related to the analyzed depth h = 0.145 µm of the section

\[ l_a = h \cos \alpha \]

(6)

One then can show that the surface area Ma of the jacket of the tilted cylinder is

\[ M_a = U_a l_a \]

(7)

in which Ua is the perimeter of the circular cross section of the cylinder Ua = π da. Substituting Eqs. 5 and 6 into 7, the surface area of the cylinder jacket becomes

\[ M_a = \pi a h \]

(8)

The surface area of the small airways is overestimated because it includes the blood vessels. In beagle dogs (63), the volume density of blood vessels vs. that of bronchi including nonrespiratory bronchioli was determined to be 6.9 ± 1.9 and 5.3 ± 0.8%, respectively, of the lung volume. Presuming a similar distribution of blood vessels and bronchioli ratio over the whole size range and no PSL retention in blood vessels, the number of tubes analyzed between diameters of 0.3 and 1.0 mm represents 57% blood vessels and 43% nonrespiratory bronchioli.

The PSL surface density in small airways was obtained by the total number of PSL observed in all the tubes of all sections divided by the total surface area measured in all sections and corrected for the fraction 0.43 of nonrespiratory bronchioli.

Estimate of PSL surface density in parenchyma. The alveolar volume of each section Valsa was determined from the planar area of the field of view Sfov, the number of fields of view nfov per section, the analyzed depth h minus the volume of all tubes \( \Sigma V_t \) in this section

\[ V_{alsa} = S_{fov} n_{fov} h - \Sigma V_t \]

(9)

Then, the alveolar volumes of all sections were summed. Because we have not estimated the alveolar surface density in this study, the mean value of the alveolar surface density SDa = 600 cm²/cm³ was taken from Refs. 12 and 64. The alveolar surface area SA of each section is then the product of SDa and the analyzed volume Valsa. The PSL surface density in alveoli was obtained by the total number of PSL observed in the alveolar region of all sections divided by the total alveolar surface area measured SSA of all the sections.

Statistical Analysis

Least squares analyses for curve fitting were carried out by using commercially available software (SigmaPlot, Jandel Scientific Software, Jandels, Erkrath, Germany). Correlations were analyzed by using commercially available software (Statgraphics, Statistical Graphics, Manugistics, Rockville, MD). Comparison of 24- and 96-h retention were analyzed by Student’s t-test.
RESULTS

Aerosol Bolus Inhalation

In Table 1, lung volume parameters and aerosol bolus parameters are listed individually for all eight dogs. Although the total lung capacity and dead space volumes of the dogs varied considerably, the percentage of bolus penetration was maintained in a narrow range. Clearance kinetics of each dog were analyzed twice, first during part 1 of the study and again during part 2, which was at least 2 mo later. The VLD differed slightly in each dog for both of these measurements. Dispersion of the inhaled 30-cm³ bolus, as described by the SDs of the exhaled particle distributions, had a mean ± SD of 42.4 ± 3.5 cm³.

The fraction of particles present in expiratory volume after VLD was 2.4 × 10⁻⁵ ± 3.4 × 10⁻⁴, which is the mean fraction obtained from all eight dogs. This low fraction indicates the noise level of the aerosol photometer and the fact that no particles were contained in this part of the expired breath after the 20-s breath hold.

Tracheal Deposition at the End of the Endotracheal Tube

It was expected that the cross-sectional change from the endotracheal tube to the trachea might have enhanced PSL deposition in the trachea due to turbulence of the inhaled aerosol flow. Therefore, aerosol inhalation and ⁹⁹mTc-PSL deposition in the trachea were simulated by using a tube of the dimensions of the trachea and an endotracheal tube under standard inhalation flow conditions. PSL were collected on a filter at the end of the tube, and deposited fractions of the filter, the tube, and the endotracheal tube were analyzed by using the gamma camera. The deposited PSL fractions were 3 and 2% in the tube and the endotracheal tube, respectively. The gamma camera image of Fig. 6B shows the pattern for the 24-h study (part 2) for all four dogs analyzed morphometrically. The variations in the first lung retention points between the curves are related to variations in the VLD of the bolus and the time from the beginning of the inhalation to the first measurements. The variation in the latter was mainly caused by transportation to, and placement of the ventilated dog under, the SPECT gamma camera. Particles collected at the cuff of the endotracheal tube were considered in the mass balance of deposited particles but not considered to be retained in the lungs at that time point. Thus, when the bolus was most shallow, the particles often had already moved to the obstruction point created by the cuff of the endotracheal tube by the time the measurement was made (see planar image of Fig. 6B taken 2.5 h after the end of inhalation). The mass balance of particles either retained in the lungs or cleared to and retained at the obstruction point in the trachea was complete (as determined by the SPECT gamma camera) when the endotracheal tube was removed 4–5 h after particle inhalation.

Overall, the clearance patterns in both studies were quite similar. Dog 3, in the 24-h study, had the most shallowly delivered bolus, and therefore, the retained fraction was distinctively the lowest. Slow particle clearance in all eight dogs decreased with a median half-life of 63 h, with an interquartile range of 33 (25% quartile) and 158 h (75% quartile). Although the half-life of slow particle clearance increased slightly with the A-value, there was no significant correlation. There was also no significant correlation between the VLD and the half-life of slow particle clearance.

Analysis of all 16 clearance studies on the 8 dogs showed that the A-value increased with increasing

<table>
<thead>
<tr>
<th>Dog No.</th>
<th>Functional Residual Capacity, cm³</th>
<th>Total Lung Capacity, cm³</th>
<th>Series Dead Space Volume, cm³</th>
<th>Phase I, cm³</th>
<th>Tidal Volume, cm³</th>
<th>Bolus Penetration, %</th>
<th>Volumetric Lung Depth, cm³</th>
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<tbody>
<tr>
<td>1</td>
<td>794</td>
<td>1,754</td>
<td>174</td>
<td>129</td>
<td>575</td>
<td>77</td>
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<tr>
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<td>130</td>
<td>95</td>
<td>475</td>
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<td>124</td>
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<td>475</td>
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<td>120</td>
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</tbody>
</table>

Data are presented for the main part of the study (2nd measurement of each dog). Functional residual capacity, total lung capacity, series dead space volume of the airways, and phase I measurements were used to define the bolus parameters. The breath volume at which the aerosol bolus was inserted is given as a percentage of phase I. Volumetric lung depth is the volume into which the aerosol bolus penetrated.
bolus penetration. In Fig. 5, all the \( A \)-values are plotted against VLD expressed as a fraction of the series dead space volume of \( \text{He}(R) \). There is a significant linear correlation (\( r = 0.82; P < 0.05 \)) described by the following equation

\[
A = 1.76 \times \text{He}(R) - 0.56 \tag{10}
\]

In this relationship, an absolute difference in the front depths of \( -20 \text{ cm}^3 \) predicts the \( A \)-values to range from near 0 to 0.5. In Table 2, the \( A \)-values and the retentions of \( ^{99m}\text{Tc}-\text{PSL} \) 24 h after inhalation are given for the four dogs before morphometric analysis. The 24-h retention is presented as a fraction of both the deposited and the inhaled particles. Although the fraction of deposited particles varies between 24 and 50% of the inhaled PSL, the 24-h retention relative to the inhaled aerosol is 1–3% in three dogs, but dog 2 shows a value of 20%.

### Particle Distribution After 2- to 3-h Retention Using SPECT

In all dogs, SPECT scans 2–3 h after inhalation showed central but irregular \( ^{99m}\text{Tc}-\text{PSL} \) deposition patterns. The ratio of \( ^{99m}\text{Tc}-\text{PSL} \) deposition in the left vs. the right lung varied from 0.3 to 2 in different dogs but was reproducible within the two measurements of each dog as quantified by region-of-interest analysis of SPECT images. The \( ^{99m}\text{Tc}-\text{PSL} \) distribution is limited to the central slices starting at the first bifurcation expanding toward the basal lungs. However, within these central slices particle distribution may extend to the outer zones of the lungs. This is illustrated in Fig. 6 by a series of transverse, coronal, and sagittal slices of the lungs of dog 4. Retention of \( ^{99m}\text{Tc}-\text{PSL} \) is overlaid over a ventilation scan performed earlier. Note that the limited resolution of the SPECT gamma camera does not permit distinction between pulmonary airways and alveoli.

### Distribution of Retained \( ^{99m}\text{Tc}-\text{PSL} \) on a Macroscopic Level in Lung Slices

The central 10-mm slices from the lungs of each dog around the first airway bifurcation had the highest \( ^{99m}\text{Tc}-\text{PSL} \) retention, and slices of the apical and caudal lobes contained the fewest \( ^{99m}\text{Tc}-\text{PSL} \). This is shown by the distribution of evenness indexes \( E_{\text{L}} \) of all slices.
Fig. 6. SPECT scan 3 h after inhalation. A: series of transversal (apex-to-base) and coronal (dorsal-to-ventral) slices of $^{99m}$Tc-PSL retention in lungs of dog 4 around level of carina. Slices were calculated at a distance of 2 mm apart. Retention of $^{99m}$Tc-PSL is overlaid in color scale over a ventilation scan in gray scale performed earlier. B: series of sagittal (right-to-left lateral) slices of $^{99m}$Tc-PSL retention in lungs of dog 4 and planar image from dorsal view of $^{99m}$Tc-PSL deposit in lungs and PSL accumulation in trachea at site of ciliary obstruction because of inflation of cuff of endotracheal tube. Planar image was taken at beginning of SPECT scan 2.5 h after end of inhalation.
in Fig. 7A. Count of slices (from base to apex) was normalized so that the slice containing the carinal ridge was slice 10. Mean EIs averaged for each slice from all four dogs are shown in Fig. 7A as a function of slice number.

Figure 7B shows the frequency histogram of EI, as function of values of EI observed. It showed marked nonuniformity with a large number of slices with low EI. Similarly, only a few of the slices had a high EI, indicating high 99mTc-PSL retention.

Retained Particle Fractions in Airways and the Alveolar Region

Figure 8 illustrates the particle fraction retained at 24 h (green and 99mTc-PSL) and 96 h (red PSL) in airways of various diameters and in alveoli after shallow aerosol bolus inhalation. Note the logarithmic scale of the ordinate. In Fig. 8, top, the fractions of the airway compartments >1 mm in diameter as determined by the microdissection analysis are illustrated. In the airway compartments >2.5 mm, ~2–6% of PSL particles retained for 24 h were found. Considerable variation among individual dogs was seen in these large airway compartments. Interestingly, although the fraction of PSL retained in the large airway compartments (>2.5 mm) for 96 h was lower, indicating that particle clearance continued after the first 24 h, this decrease between 24 and 96 h did not reach the level of significance.

The mixed parenchymal compartment (small airways <1 mm and alveolar structures) contained ~90% of the retained PSL at both 24 and 96 h. This mixed parenchymal fraction was further resolved by the analysis of 200-µm sections into the small-airway compartment with diameters between 0.3 and 1 mm and the alveolar compartment, which is shown in Fig. 8, bottom. About 55% of either retention, 24 or 96 h, was found in the alveolar compartment, whereas <35% was found in the small-airway compartment. There is no statistically significant difference (P > 0.05) between the particles retained for 24 h and those retained for 96 h in these two compartments, indicating that clearance in the intervening time between these measurements is quite slow. It also needs to be emphasized that
there was no statistically significant difference in the particle fractions observed in the various airway compartments and the alveolar compartment between dog 2, which had retained 20% of the inhaled particles at euthanasia after 24 h, and the mean fractions in the various compartments found in the other three dogs, which had retained only 1–3% of the inhaled particles at euthanasia.

Figure 9 shows the 24-h retention data obtained from deep aerosol bolus inhalations by using 1-µm PSL particles, which served as a positive control for deposition and 24-h retention in the deep lung. Data were analyzed in the same manner as described above for the shallow bolus inhalations. As expected, almost 96% of the retained PSL were found in the alveolar compartment. Interestingly, a very small particle fraction (0.08%) was found in the compartment of airways with diameters between 0.3- and 1-mm, whereas fractions of 3.1, 0.8, and 0.4% were found in the compartments of airways with diameters of 1–2.5 mm, 2.5–5 mm and >5 mm, respectively.

Particle Density in Small Airways and Parenchyma

Figure 10 shows the PSL density in small airways (diameter 0.3–1 mm) and alveoli, i.e., the number of PSL per calculated unit surface area. Because the surface area of the small airways is much smaller than the alveolar surface area, and the deposition probability was rather low in alveoli during shallow aerosol bolus inhalation, the PSL density in the small airways (~60 mm⁻²) is almost three orders of magnitude higher than in alveoli (0.01 mm⁻²). This distribution was found for both 24- and 96-h retention for shallow aerosol boluses.

In alveoli, the 1-µm PSL density after deep bolus inhalation was almost two orders of magnitude higher than after shallow bolus inhalation (green and red
PSL) because of the much higher deposition probability in the alveolar region during deep bolus inhalation. Very few blue particles were found in small airways (diameter 0.3–1 mm) after deep aerosol bolus inhalation. However, because the alveolar surface area is about two orders of magnitude larger than the surface area of small airways, the PSL density in small airways after deep bolus inhalation is on the same order of magnitude as the alveolar PSL density.

DISCUSSION

This study clearly shows that shallow aerosol bolus inhalation into a VLD less than the series dead space resulted in predominant airway deposition of particles. A fraction of 0.5–0.95 of the deposited particles was cleared within 24 h after shallow bolus inhalation by mucociliary action. The retention pattern 2.5 h after inhalation was irregularly shaped, usually located centrally at the level of the carina but occasionally reaching as far as the perimeter of the lungs. The remaining particle fraction of 0.05–0.5 was cleared slowly in all dogs after 24 h. This slowly cleared fraction increased linearly with increasing bolus front depth. Of the slowly cleared fraction, ~40% retained at 24 and 96 h were found in airways. Most of the particles retained in the airways were found in the small-airways compartment with diameters of 0.3–1 mm. Although there was a tendency of fewer particles in the large airways (>2.5 mm) after 96 h compared with 24-h data, there was no significant difference between 24- or 96-h retention. Therefore, we conclude that slow clearance from the airways represents clearance from small airways. More than 50% of the retained particles (2.5–25% of the particles deposited at t = 0) were in alveoli at 24 and 96 h.

This study directly examined the question of airway retention of particles. It was designed to assess particle retention in airways of all sizes and used the shallow aerosol bolus approach as a means to preferentially deposit particles in a very central location. As such, it is also a test of the capability for shallow boluses to achieve this pattern of deposition, as well as a test of our ability to predict the anatomic and/or functional distributions of a labeled aerosol inhaled into a volume less than the anatomic dead space. Thus the design of this experiment was crucial. The choice of the experimental animal rested on a number of considerations. Small animals, such as rodents, were not feasible because of the need for reliable external imaging of the deposition pattern, limitations on usable volumes with the bolus delivery system, and lung geometry. Larger animals, such as canines, had advantages on the basis of the size of their respiratory tract. Anatomic and geometric design considerations of the respiratory tract in terms of similarities to and differences from humans were also considered. Single-breath washout curves in human and canine subjects are very similar and indeed are functionally the same (25, 28, 67). Similarly, in bolus studies, it appears that bolus penetration is into the same anatomic regions (48–51). However, one known difference is that measurements of shallow bolus dispersion in the anesthetized and ventilated dog show a smaller degree of convective mixing than do similar studies in humans (49, 51). Thus in the canine lungs the site of bolus deposition should be more related to bulk transport and less influenced by convective mixing, which would carry particles deeper into the lungs. Finally, experiments carried out in canine lung casts had precisely defined bolus deposition and effect of flow rate on bolus penetration (9, 44).

The degree of ventilation nonuniformity is an important issue in these studies both in terms of differences between humans and animals as well as in the capability of bolus methodology to confine inhaled particles to the conducting airways. A major confounding effect is that ventilation nonuniformities can transport particles to lung depths deeper than the VLD, where they will readily deposit due to smaller dimensions in the deep lung. One transport mechanism operates when the lung fills unevenly due to nonuniformities in compliance (35). Another possible transport mechanism is the nonslug inspiratory flow profiles that arise from the repeated division of the gas flow at bifurcations (47, 65).

In theory, the more uniform the lung ventilation and the more symmetric the lung, the better the ability of the bolus method in confining the inhaled particles to the desired VLD within the conducting airways. A symmetric lung has dichotomous branching equal-caliper airways, a geometry that has an equal path length (and volume) from the larynx to each airway of the same generation. Because these ideal geometric conditions are not present in the human and in the monopodial canine airways (17, 19, 69, 72), the lungs of both species do exhibit nonuniform ventilation (25, 28, 67). Thus some particles may reach alveolar air spaces, although the VLD is smaller than the anatomic dead space volume of the airways. Because we directly quantified the location of particles, the important fact is the observed slowly cleared fraction in airways and not so much this alveolar penetration of particles, which may be specific to the canine species, and/or due to specific differences in the experimental protocol between the human and the canine bolus inhalation.

The findings of this study suggest that, in healthy large airways, mucociliary clearance quickly and effectively removes almost all particles. However, in healthy small airways, mucociliary clearance is less effective and there are even sites of longer-term retention. Mucus transport velocity is highest in the trachea and decreases with increasing generations of airways (70, 71). Measured mucus transport velocity in the human trachea is ~5 mm/min (37). In contrast, model estimates on the basis of water and ion balances suggest that the mucus transport velocity in terminal bronchioi is only ~10−3 mm/min (7). Thus, for a typical terminal bronchioi that is 2 mm long, the calculated particle retention times will be >35 h. Another potential factor leading to longer-term retention is that the ciliated airway epithelium may not be continuous, particularly in small airways. Although there is agreement that the human trachea is covered by a continuous ciliated epithelium, controversy remains how these
cells diminish with increasing generations of airways (27). The lack of cilia and the nature of the mucous layer in these areas need further definition. Nevertheless, if particles deposit onto nonciliated areas, it is less likely that they will be cleared by mucociliary transport. In this case, they are likely to be phagocytized by either alveolar macrophages (14, 15) or epithelial cells themselves (68), resulting in prolonged retention of the particles in the airways.

Another mechanism of airway retention of particles has been proposed by Gehr and co-workers (13, 14), who showed in vitro that particles deposited on top of the mucous gel covered with a bimolecular layer of surfactant are likely to be pulled into the gel phase because of gradients of surface tension forces at the interfacial layer. In addition, findings of microscopic particles among the cilia on the surface of ciliated epithelial cells in hamster airways led to the hypothesis that particles may be moved even further into the sol phase by those gradients of surface tension forces (15, 16). Particles located at the base of cilia appear to be immobilized and are likely to be retained for prolonged times. Also at this location, particles may be subject to phagocytosis by either airway macrophages or epithelial cells. The present study offers some support for these hypotheses in small airways but little support in the largest airways.

Another possible explanation for the observed longer-term retention is that particles observed in small airways were initially deposited in alveoli and then transported to small airways by alveolar macrophages. However, such a mechanism is not consistent with alveolar macrophage particle transport rates from the alveolar region and the small airways to the larynx at 24- and 96-h retention. Particle transport from the alveolar region of the dog’s lungs has been estimated to be 0.002 ± 0.0002 d⁻¹ (daily particle fraction normalized to the particles contemporarily retained in the lungs) (22, 53). Multiplying the observed alveolar-retained fraction of 0.55 by this transport rate shows the fraction of particles that could have left the alveolar region was only 0.001 at 24 h (for both shallow and deep boluses) and 0.004 at 96 h (shallow bolus). Even if no particle transport would occur in the proximal airways, these fractions are still negligible compared with the observed 24-h fractions of 0.33 in airways <1 mm in diameter and 0.15 in airways >1 mm in diameter (see Fig. 8). Also, if the PSL retention in <1-mm-diameter airways and in the alveolar region are taken together, the fractions of transitional particles in large airways would increase to 0.002, which is still negligible compared with the observed fraction of 0.15 of retained PSL.

Another approach to the question of airway penetration of particles was used in recent studies (3, 8, 58). These investigations sought to enhance particle deposition in airways proximal to the alveolar region during extremely slow inhalation of radiolabeled aerosols (50 cm³/s airflow) by healthy subjects. An intermediate, slowly cleared particle fraction (half-life 30 d⁻¹) was seen which was not observed after inhalation at normal air flow. This fraction was found between rapid clearance within a few hours and slow clearance (half-life > 100 d⁻¹). The observed particle fraction cleared at an intermediate, slow half-life is considered to represent particle retention in small airways and supports the findings of the human aerosol bolus studies. Our study, which anatomically defined the location of these particles in small airways, is supportive of these conclusions.

The results of this study are in conflict with the concept that, in humans, a bolus delivered to a shallow lung depth deposits predominantly in the airways and not in the peripheral lungs (45, 54). However, there has been no anatomic localization of particles in the human studies. These studies are all based on the ability of subjects to breathe in a defined pattern to ensure the replication of the protocol, the delivery of boluses to a portion of the breath corresponding to volumes predicted to represent conducting airways, and external measurements of radiolabeled particles (5, 45, 54). The study reported herein uses the same type of particles used in some of the human studies (45), so the particles are unlikely to account for any differences between human and canine studies. The differences in morphology between the human and the canine lungs have been discussed, and these are likely to account for similarities rather than differences.

Another difference between the study reported herein and human studies is the delivery of the aerosol bolus inhalation. The human subjects were breathing actively and visually guided to achieve a constant flow. The animals were anesthetized, paralyzed, and ventilated with positive pressure in a supine position. This system allowed greater control of breathing maneuvers than could be expected even in highly trained human subjects. There are no proven differences between inspiration by positive pressure and negative pressure in both species (39, 40). In dogs, ventilation by a positive servo ventilator results in a more uniform distribution of the inspired air compared with active breathing in humans (39, 41). For example, the active inspiration in humans is usually maintained by the action of the diaphragm, resulting in a predominant ventilation of the lung base. This is enhanced by the gravity-dependent filling of the lungs in the upright position (6). This implies that a shallow aerosol bolus is more likely to be delivered to the airways and parenchyma of the lung base in an actively breathing human, but it is more uniformly distributed in the airways of a ventilated dog. It is notable that these differences would suggest that particles were less likely to be transported beyond the dead space of conducting airways in the ventilated dog compared with the actively breathing human subject.

Another factor is that, in human studies, shallow bolus inhalations at flow rates of ~250 cm³/s delivered to a lung depth of 50–75 cm³ require that the flow be instantaneously stopped 0.2–0.3 s after bolus delivery. It is likely that this level of control is not achievable by an actively breathing human subject. Although closing the valve of the inhalation apparatus instantaneously...
stops inhalation at the preselected tidal volume, some degree of negative pressure is still created within the lung before the inspiratory muscles relax. Because in dogs a small variation in volume fraction creates a large difference in the A-value, i.e., a change of 20 cm$^3$ in the VLD increases the slowly cleared fraction (A-value) from 0.05 to 0.5, it is possible in humans that small increases in inhaled volume, due to the short period of negative pressure created at the end of inspiration, caused redistribution of PSL among the anatomic compartments. These phenomena can certainly be excluded in the dog model, where the lungs of the paralyzed animal exactly follow the prescribed volumes of the computer-controlled ventilator system.

The effect of anesthesia on the experiment is potentially problematic. Alveolar deposition and retention fractions suggest that macrophage function was intact, and there is ample evidence in this study to suggest that alveolar function was maintained, as shown by the rapid particle transport to the obstruction point of the trachea caused by the cuffed endothelial tube (Fig. 6). In this study, oxygen saturation was maintained at a physiological level, as were the expired concentrations of oxygen and carbon dioxide. However, there is neither direct nor indirect evidence to determine whether the ability of epithelial cells to take up particles was maintained in these dogs, which could potentially explain, in part, the observed low airway retention in large airways.

Nevertheless, it must be remembered that all dogs in each of the two clearance studies show a slowly cleared particle fraction. In both humans and dogs, particle deposition patterns after shallow bolus inhalation are central and irregular as observed by gamma camera imaging (5). Although in humans the deposited particle fractions are consistently larger in the left lungs at 75% TLC bolus inhalation maneuvers, the ratio of left-to-right deposited fractions varies widely in the eight dogs studied. Furthermore, in humans the slowly cleared particle fraction of $A = 0.4$ for 2-µm particles is basically independent of the VLD in a range of 50–110 cm$^3$, corresponding to 0.4–0.8 of the anatomic dead space. This is clearly different from the canine lungs, which show a linear correlation between VLD in the same range of the anatomic dead space, and the slowly cleared fraction of $A$ ranging from 0.05 to 0.5.

Because slow clearance of particles from small airways has been shown morphometrically in this study, the relevance of this clearance phenomenon for normal breathing needs to be estimated. For deposition measurements of large aerosol volumes in beagle dogs, using the same aerosol photometry system, Schulz and co-workers (51) found a total deposited fraction of 0.36 ± 0.04 of the inhaled monodisperse 2.0-µm DEHS particles after inhalation of a tidal volume corresponding to 75% of TLC. After bolus delivery of the same-size particles to a VLD of 120 and 160 cm$^3$, they observed deposited fractions of 0.05 and 0.11, respectively. On the basis of the results of this study and by using an average cleared fraction of 85% within 24 h found in the present study, a conservative estimate of the fraction of particles deposited and slowly cleared from small airways would be 0.003–0.007 after tidal volume inhalation. Hence, ~0.8–2% of the totally deposited 2-µm particles may be subject to slow clearance from small airways. Because of the similarity of total deposition of 2-µm particles in human and canine lungs, a similar fraction of slowly cleared particles from small airways would be expected from the human lungs.

Conclusion

Shallow aerosol bolus inhalation applied to a canine model under controlled conditions provides a suitable tool for preferentially depositing particles in the dead space of conducting airways. The similarity between the functional particle clearance patterns in human and canine lungs during the first 2 days after inhalation supports the use of canine lungs as a model for studying slow-particle-clearance phenomena associated with the tracheobronchial tree. The morphometric analysis proved that particles are cleared slowly from small airways (diameters between 0.3 and 1 mm) of canine lungs. This finding suggests reiteration of the results of earlier regional particle clearance studies on canine lungs. This finding suggests reiteration of the basis of functional time-dependent analyses and has potentially important disease-risk implications for the lower bronchial tree, a well-identified target site of pulmonary disease processes associated with inhaled particles.

The authors express gratitude for intellectual support and fruitful discussions and suggestions by Drs. Joseph D. Brain (Boston, MA), Willi Stahlhöfen (Frankfurt, Germany), Mariann Geiser (Bern, Switzerland), and Anne Schultz (Munich, Germany). This study required considerable technical support, and efforts were provided with particular care by Gunter Eder, Franz Erbe, and Gaby Schumann (GSF lab, Munich, Germany) and Gopala Gazula Krishna Murthy, (Boston, MA).

This work was supported by the US-German cooperative program in pulmonary research of the US Public Health Service, National Institutes of Health (NIH), Division of Lung Diseases, National Heart Lung and Blood Institute, and the Bundesministerium für Forschung und Technologie, as well as NIH research Grants ES-00002, HL-31012, HL-19170, and ES-08219.


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Received 21 July 1998; accepted in final form 2 March 1999.

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