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Retention and Intracellular Distribution of Instilled Iron Oxide Particles in Human Alveolar Macrophages

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Bronchoalveolar lavage (BAL) was used to sample retention of particles within the alveolar macrophage (AM) compartment at various times from 1 to 91 d following intrapulmonary instillation of 2.6-μm-diameter iron oxide (Fe_2O_3) particles in human subjects. Particles were cleared from the lavagable AM compartment in a biphasic pattern, with a rapid-phase clearance half-time of 0.5 d and long-term clearance half-time of 110 d, comparable to retention kinetics determined by more traditional methods. The intracellular distribution of particles within lavaged AMs was similar in bronchial and alveolar BAL fractions. AMs with high intracellular particle burdens disappeared from the lavagable phagocytic AM population disproportionately more rapidly (shorter clearance half-time) than did AMs with lower particle burdens, consistent with the occurrence of a particle redistribution phenomenon as previously described in similar studies in rats. The rates of AM disappearance from the various particle burden categories was generally slightly slower in bronchial fractions than in alveolar fractions. The instillation of particles induced a transient acute inflammatory response at 24 h postinstillation (PI), characterized by increased numbers of neutrophils and alveolar macrophages in BAL fluids. This response was subclinical and was resolved within 4 d PI. **Lay, J. C., W. D. Bennett, C. S. Kim, R. B. Devlin, and P. A. Bromberg. 1998. Retention and intracellular distribution of instilled iron oxide particles in human alveolar macrophages. Am. J. Respir. Cell Mol. Biol. 18:687–695.**

Knowledge about the role of alveolar macrophages (AMs) in the long-term retention and clearance of particles deposited in the alveolar region of the lung has been derived predominantly from animal studies. These studies indicate that AMs rapidly phagocytize particles deposited on the alveolar surface (1–3). Although some particles may be transported to regional lymph nodes (4, 5) or become sequestered in the pulmonary interstitium or epithelium (especially at high particulate lung burdens), the vast majority are thought to eventually leave the lung as intracellular particles within AMs, predominantly by the tracheobronchial route (6, 7). Thus, particle clearance is intimately linked to egress of AMs from the lung. Such a scenario is presumed also to occur in clearance of particles from the human lung. Animal studies (2, 7, 8) indicate that a proportion of deposited particles remains sequestered in AMs for extended periods, possibly as a result of "redistribution" of particles among AMs. This might occur as the result of exocytosis of particles or release of particles from lysed senescent AMs and subsequent phagocytosis by new macrophages. Phagocytized particles may, thus, remain in the lung via passage from one macrophage to another instead of being cleared from the lung. In addition to physical transport out of the lung, some portion of a lung particle burden may be cleared by slow dissolution of particles within alveolar macrophages (9). The kinetics of particle clearance from the lungs of different species vary tremendously (10), with humans being the slowest and the rat being the most rapid in clearing a particle burden. The causes for this disparity between species are not known and may include differences in anatomy or fundamental clearance mechanisms.

Studies in human subjects have examined particle retention and clearance by monitoring radioactivity emitted from radiolabeled particles deposited in the lung (11–13). Many investigators have used inhaled particles with short-lived radiolabels to examine short-term clearance (tracheobronchial clearance and rapid-phase alveolar clearance)

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Abbreviations: alveolar macrophage, AM; analysis of covariance, ANCOVA; analysis of variance, ANOVA; bronchoalveolar lavage, BAL; bronchoalveolar lavage fluid, BALF; ferric ion, Fe^{3+}; iron oxide, ferric oxide, Fe_2O_3; *Limulus* amebocyte lysate assay, LAL; clearance rate constant, λ; postinstillation, PI; geometric standard deviation, σ_g; sterile physiologic saline solution, SPSS.

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during the first 24 to 48 h following deposition (14, 15). A few studies have used longer lived radiolabels to study long-term kinetics of particle retention and clearance (11, 13, 16). One study compared long-term pulmonary clearance of inhaled cobalt oxide in multiple species and found marked differences in particle retention between species over the 180 d following particle deposition (10). Pulmonary particle retention was definitively greater in humans and baboons than in the other species studied, clearly demonstrating the limitations of extrapolating animal-derived clearance data to humans. None of these studies have directly examined the role of AMs in the retention or removal of particles deposited in the alveolar region of the human lung.

The current study departs from more traditional methods and directly examines retention and distribution of particles within AMs lavaged from the lungs of human subjects following intrapulmonary instillation of particles. This approach, similar to that employed in previous animal studies (8), provides a means for evaluating possible particle redistribution within the human AM compartment. Our objectives were to quantify and compare the retention and intracellular distributions of insoluble particles within lavagable alveolar and airway macrophages as a function of time following instillation. By measuring relative rates of clearance of AMs having different intracellular particle burdens, we hoped to demonstrate whether there was a "redistribution" of particles between AMs in the human lung, similar to that seen in rats. In addition, after making certain assumptions, we hoped to estimate the overall particle retention and clearance kinetics for particles in the lavagable AM compartment.

Methods

Study Population

Thirty healthy, nonsmoking volunteers (24 male, 6 female), 19.6 to 35.5 yr of age (mean age, 25.5 ± 4.3 yr) participated in the study. Potential subjects were excluded from participation if they had a history of smoking, asthma, allergy, cardiac disease, chronic respiratory disease, or recent acute respiratory illness. All potential subjects underwent screening procedures, including completion of the Minnesota Multiphasic Personality Inventory and medical history form, physical examination, chest radiographs, and routine hematological and serum chemistry tests. Accepted subjects were informed of the purposes, experimental protocol, and procedures of the experiments, as well as potential risk from participation, and each subject signed a statement of informed consent. This study was approved by the Committee on the Protection of the Rights of Human Subjects of the University of North Carolina School of Medicine (Chapel Hill, NC).

Study Design

Subjects were each assigned to one of five groups of six subjects each. Each subject underwent two bronchoscopy procedures. During the first bronchoscopy, inert, insoluble iron oxide (Fe_2O_3) microspheres suspended in nonpyrogenic, sterile physiologic saline solution (SPSS) (Baxter Healthcare Products, Deerfield, IL) were instilled into an identified subsegment of the lingula that could readily be wedged by advancing the bronchoscope. As a control, SPSS (without particles) was instilled into a segment (medial or lateral) of the right middle lobe. Subsequently, during the second bronchoscopy procedure, particles and cells were recovered by bronchoalveolar lavage (BAL) at a specified time (1, 2, 4, 28, or 91 d) postinstillation (PI). Total and differential cell counts, total recovered particles, and particles per cell were tabulated for the bronchial and alveolar wash fractions.

Iron oxide was chosen as the test particle for this study because of its safety, and because it is inert and relatively insoluble in aqueous suspension at neutral pH. It is reported to be nontoxic, nonfibrogenic, and noncarcinogenic (17), and has been used previously in humans for inhalation studies of mucociliary clearance (18–20).

Particle Generation

Spherical Fe_2O_3 particles (nonradioactive) were generated as previously described (21, 22) from suspensions of colloidal Fe_2O_3 (23) using a spinning disk aerosol generator (24), but without radioactive label. The count median diameter of the resultant particles was 2.6 μm, with a geometric standard deviation (σ_g) of 1.3. The particles were collected in SPSS in an impinger, concentrated by centrifugation, and then washed twice in SPSS. Initial particle batches were sterilized by autoclaving at 121°C for 30 min. Later batches were sterilized by baking the particles at 250°C for 3.5 h, which also ensured the destruction of any endotoxin activity that might be present in the particle suspension. Batches of all the particle suspensions were tested for endotoxin activity using a gelation-capillary method (Endotect; ICN Biomedical, Costa Mesa, CA) or using a semiquantitative method (performed by the University of North Carolina Tissue Culture Facility), both of which are based on the *Limulus* amebocyte lysate (LAL) assay. The capillary LAL method detects endotoxin concentrations as low as 0.06 to 0.10 ng/ml and provides only a positive or negative indication of the presence of endotoxin. The semiquantitative LAL method is equally as sensitive, and provides an indication of the actual concentration of endotoxin present. All particle suspensions tested by the capillary method were negative. Particle suspensions tested by the semiquantitative LAL method were $\leq$ 0.06 endotoxin units (1 EU = 0.1 ng/ml).

Just before instillation, concentrated particles were suspended in 2 to 3 ml of SPSS and placed in an ultrasonic bath for 30 min to disperse clumps of particles. Particles were examined and counted in a hemacytometer to assure the dispersion of clumps and to quantify particle numbers. Finally, 3.0×10^8 particles were suspended in 10 ml of SPSS and transferred to a sterile syringe for instillation.

Particle Instillation and Bronchoalveolar Lavage

Bronchoscopy and BAL were performed as previously described (25). Before bronchoscopy, all subjects were premedicated intravenously with 0.6 mg of atropine. The posterior pharynx was anesthetized by gargling with a saline solution containing 4% lidocaine, and the nasal passage was anesthetized with a lubricating jelly containing 2%

lidocaine. The larynx, trachea, and bronchi were anesthetized with topical 2% lidocaine instilled through a fiberoptic bronchoscope (BF type 1T20D; Olympus, Lake Success, NY) to control coughing. The total dose of instilled lidocaine was limited to no more than 300 mg.

To instill the particles into the distal airways and alveoli, the bronchoscope was passed to a wedgeable subsegmental bronchus in the lingula, but was not wedged. A Teflon catheter was passed through the biopsy channel and then extended 4 to 5 cm beyond the tip of the bronchoscope into the selected subsegmental bronchus. Subjects were instructed to take deep, slow, and regular breaths. Ten milliliters of SPSS containing 3.0×10^8 Fe_2O_3 microspheres was slowly instilled through the catheter coincident with inspirations to maximize placement of particles in the alveolar region. This was followed by an additional 10 ml of SPSS from a different syringe (for a total of 20 ml), with the intent of washing particles remaining in airways into the alveoli. As a control, 20 ml of SPSS (without particles) was similarly instilled into the medial segment of the right middle lung lobe. To assess the number of particles that were lost to the syringe and catheter, simulated instillations were performed *in vitro* by injecting particle suspensions through the catheter into a glass vial and counting the particles deposited in the vial. On the basis of these simulations, almost one-third of the particles ($31.4 \pm 2.2\%$) were lost to the syringe and catheter, so the actual number of particles instilled into the lung is estimated to be 2.06 ($\pm$ 0.07) $\times 10^8$ particles ($\sim$ 5 mg of Fe_2O_3).

Segmental BAL was performed at a specific time interval PI in the same lingular segment in which Fe_2O_3 was previously instilled and in the control segment in the right middle lobe. The lavage of each segment comprised six washes using a total of 270 ml of SPSS per segment. The first washing was done with only 20 ml of SPSS, and was considered to be enriched with materials from the peripheral airways (26). BAL fluid from the first wash was kept separate from five subsequent washings of 50 ml each. Similarly, the control subsegment was lavaged as described. BAL fluid and cells from each individual washing were collected in separate capped tubes and kept on ice during the bronchoscopy, and during processing.

Cell Preparation

BAL fluids from the particle-instilled segment (lingula) and control segment (right middle lobe) were processed separately, but in identical fashion, as described subsequently. BAL fluids were centrifuged at $250 \times g$ for 10 min. Cells from the bronchial fraction were kept separate, whereas cells from the five 50-ml washes (alveolar fraction) were pooled, washed twice with tissue culture medium (RPMI 1640; Sigma Chemical, St. Louis, MO), and used immediately for total and differential cell counts. Total cell counts were obtained by light microscopy, using a hemacytometer. Differential cell counts were obtained using slides prepared in a cytocentrifuge (Cytospin 3; Shandon, Pittsburgh, PA) at 500 rpm for 3 min. Slides were stained with a modified Wright's stain (Leukostat stain; Fisher Scientific, Fairlawn, NJ) and at least 300 cells were counted and evaluated.

Analysis of Particle Retention

We made the following assumptions regarding the recovery of particles by bronchoalveolar lavage in order to analyze and interpret the data we collected:

1. The vast majority of particles retained within the lung are located within alveolar macrophages located within lavagable airspaces.
2. Translocation of particles from the airspaces into the pulmonary interstitium is minimal during the 91-d course of the study.
3. Dissolution of particles is negligible during the 91-d course of the study.
4. The number of particles recovered by BAL is proportional to the number of particles present within the bronchial and alveolar airspaces accessible to lavage.
5. The efficiency of BAL in recovering particles is similar from one time point to another following particle instillation.

Counts of particles and intracellular particles per cell were performed by light microscopy, using a $\times 100$ oil immersion objective lens. One thousand AMs were counted and the number of Fe_2O_3 particles contained within the margins of each cell was tabulated. Particles located within the margins of the cell were assumed to be intracellular particles and all other particles were considered extracellular. Only intact cells with minimal degenerative cytological changes were included in the count. The number of extracellular particles encountered (per 1,000 macrophages counted) also was tabulated, as was the number of particles associated with degenerated cells and particles contained within neutrophils. The total number of particles recovered by BAL was then calculated using the total number of intracellular and extracellular particles counted and the total cell counts. Data from the six subjects at each time interval were averaged to provide a mean number of recovered particles for each time point (1, 2, 4, 28, and 91 d PI). Because we did not attempt lavage to recover particles immediately following instillation, a value for the number of particles that might have been recovered by BAL at $t = 0$ was estimated by extrapolation, using a single exponential decay function fit to data from Days 1 and 2 PI.

By a least-squares method, a double-exponential decay function (Equation 1) was fit to the data (total particles recovered versus time), using computer software (DeltaGraph 4.0; DeltaPoint, Monterey, CA):

$$\text{Number of particles} = Ae^{-\lambda_1 t} + Be^{-\lambda_2 t} \quad (1)$$

The half-time ($t_{1/2}$) for each of the two components of the curve was calculated (equation 2) as a function of the exponential decay constant (λ):

$$t_{1/2} = \ln 2/\lambda_i = 0.693/\lambda_i \quad (2)$$

Analysis of Intracellular Particle Distribution

The number of AMs containing various particle burdens (i.e., categorized as particles per cell: 1, 2–4, 5–7, 8–11, 12–19, $\geqslant$ 20 particles) was determined and expressed as a percentage of the number of AMs that had phagocytized

at least one particle (particle-laden AMs), rather than the total number of AMs recovered (total AMs). (This was necessary to allow comparisons of the particle distributions in the alveolar fraction with those in the bronchial fraction.) A single exponential decay function (Equation 3) was fit by the least-squares method to the mean percentage of AMs with a given particle burden to describe the loss of AMs from the various categories between Days 4 and 91 PI.

$$\text{Percentage AMs} = Ae^{-\lambda t} \quad (3)$$

The "one particle per cell" category was a special case that required a positive exponential function (Equation 4) to describe the change in percentage of AMs in this category between Days 4 and 91 PI.

$$\text{Percentage AMs} = Ae^{\lambda t} \quad (4)$$

The half-times or doubling times were calculated as described previously (Equation 2).

The calculated coefficients (A), rate constants (λ), and clearance half-times ($t_{1/2}$) derived from Equations 3 or 4 were tabulated by particle burden category and the trend toward increasing or decreasing rate across the various categories was compared for alveolar and bronchial fractions.

Statistical Evaluations

All values are expressed as the mean ± standard error. Differences between particle-instilled versus control segments and for bronchial versus alveolar fractions for various parameters were analyzed using Student's *t* test for paired samples (27). Differences in various parameters (total particles recovered, particles per phagocytic cell, etc.) at different times PI were evaluated using analysis of variance (ANOVA) and post hoc hypothesis testing for differences between groups using the pairwise Tukey's honestly significant difference (HSD) test. Pearson's correlation coefficient was calculated to determine the significance of trends in clearance rate constants across particle burden categories, and analysis of covariance (ANCOVA) was used to compare the trends between bronchial and alveolar fractions. The rate constants were first transformed to their natural logarithms before doing this analysis. A *P* value of 0.05 was chosen as the level of significance for all statistical tests.

Results

Retention of Particles

Retention kinetics of particles recovered by BAL were assumed to be representative of the total burden of particles within the alveolar spaces (2). On average, no more than 10% of the particles instilled could be recovered by BAL (combined bronchial and alveolar) at any time PI. The mean number of particles recovered at 91 d PI was only 32% of that recovered at 1 d PI; however, this difference was not statistically significant (ANOVA, $P = 0.11$) owing to large variances in particle recovery from individuals within each group. A retention curve fit to the mean values (Figure 1), using an estimated value of 37.2×10^6 particles for t = 0, was best described ($r^2 = 0.91$) by a double-exponential decay function as follows:

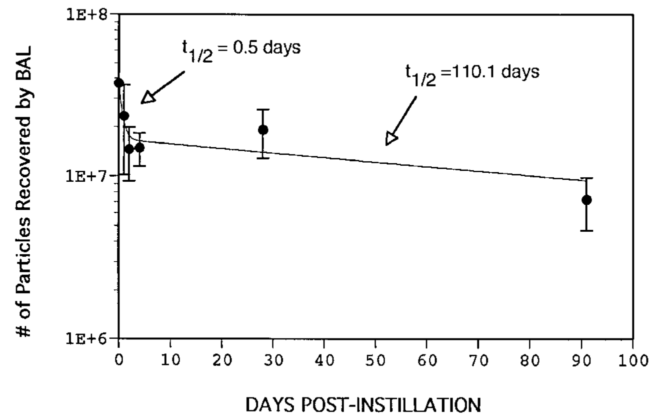

Figure 1. Mean number of particles recovered by BAL at various times PI: A double-exponential decay function fit to the data shows a very rapid clearance ($t_{1/2}$ = 0.5 d) during the first 2 d PI, followed by a prolonged slow clearance phase ($t_{1/2}$ = 110.1 d). The fitted equation is as follows: Number of particles = (1.671×10^7) $(e^{-0.006297t})$ + (2.068×10^7) $(e^{-1.4066t})$. Bars represent standard error.

$$\text{Number of particles} = (1.67 \times 10^7)(e^{-0.0063t}) + (2.07 \times 10^7)(e^{-1.41t}) \quad (5)$$

A rapid decrease in lavagable particles occurred during the first 4 d ($t_{1/2}$ = 0.5 d) and was followed by a much slower decay in lavagable particle numbers between 4 and 91 d ($t_{1/2}$ = 110.1 d).

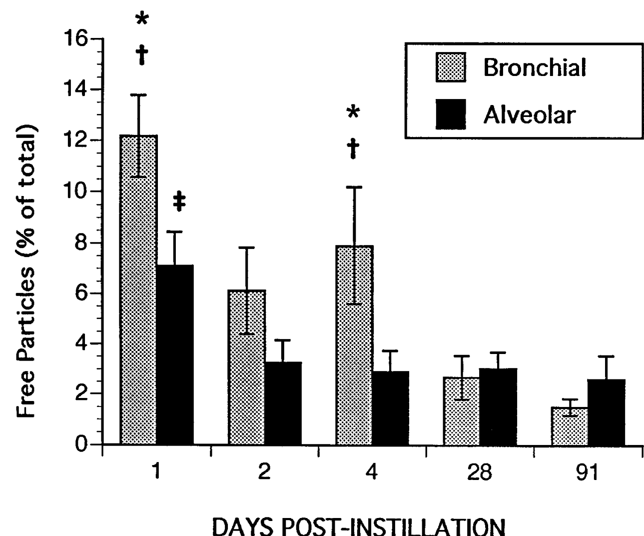

Figure 2. The proportion of free particles in the bronchial fraction (as the percentage of the total counted) tended to be greater in the early time points (1 to 4 d PI) as compared with the alveolar fraction and the bronchial fraction at 28 or 91 d PI. The percentage of free particles in the alveolar fraction was significantly elevated only at 1 d PI. Bars represent standard error. *Bronchial fraction statistically different from alveolar fraction (< 0.05, paired *t* test). †Bronchial fraction statistically different from bronchial fraction at 28 and 91 d PI. ‡Alveolar fraction statistically different from alveolar fraction at 4, 28, and 91 d PI.

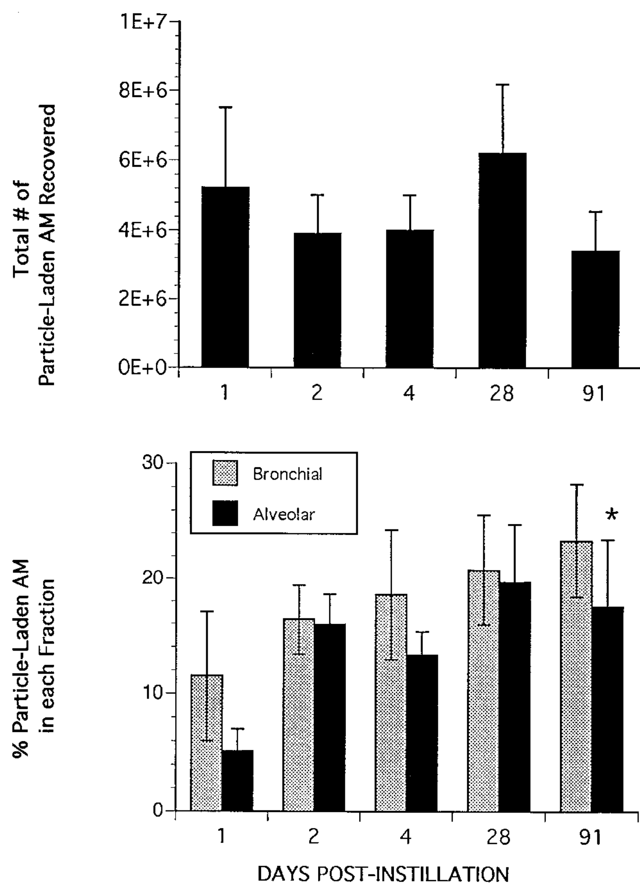

Figure 3. (*Top*) The total number of particle-laden AMs recovered by BAL (combined alveolar + bronchial fractions) decreased slightly between 1 and 91 d PI (no statistically significant change). Bars represent standard error. (*Bottom*) The percentage of particle-laden AMs appeared to increase slightly between 2 and 91 d PI in both alveolar and bronchial BAL fractions. The percentages of particle-laden AMs were lowest at 1 d PI, owing to the transient influx of AMs. *Alveolar fraction statistically different from bronchial fraction ($P = 0.035$, paired *t* test). Bars represent standard error.

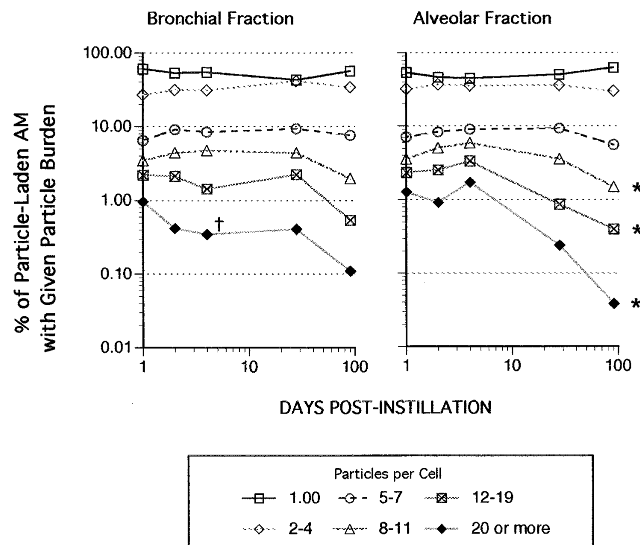

Figure 4. The distributions of particle-laden AMs in the various particle burden categories are similar in bronchial and alveolar fractions, especially for smaller particle burden categories. In the alveolar fraction, the percentage of cells in the larger categories clearly decreased more rapidly than in smaller burden categories. (Error bars were intentionally omitted to avoid excessive clutter.) *Significantly different from 4 d PI (ANOVA, $P < 0.05$). †Significantly different from corresponding value in alveolar fraction.

Extracellular Particles

The percentage of particles that were extracellular and not obviously associated with a cell disrupted during centrifugation was higher in the bronchial fraction and in the early time points, 1 to 4 d PI (Figure 2) relative to the alveolar fraction and also to the bronchial fraction at 28 and 91 d PI. The percentage of free particles in the alveolar BAL fraction also was elevated at 1 d PI ($P \leq 0.05$) relative to 4, 28, and 91 d PI.

Intracellular Distribution of Particles

The mean percentage of the total AMs, which had phagocytized one or more particles (particle-laden AMs), appeared to increase slightly between 1 and 91 d PI in both bronchial and alveolar fractions (Figure 3). These apparent increases were not statistically significant. The mean percentage of particle-laden AMs appeared to be slightly higher in the bronchial fraction and was significantly higher than that of the alveolar fraction at 91 d PI (paired *t* test, $P = 0.04$). There was an apparent decrease of 34.6% in the total number of particle-laden AMs between 1 and 91 d PI (Figure 3); however, this change was not statistically significant owing to large variances within groups.

Alveolar fraction. Particles were observed almost exclusively in AMs and rarely in neutrophils. The distribution of particles within the lavagable AMs was heterogeneous, and changed slightly as a function of time PI (Figure 4). The vast majority of AMs at all time intervals contained no particles and, of the cells that had phagocytized particles, most contained only 1 or 2 particles, but as many as 72 particles were observed within a single AM. The proportions of AMs in all categories increased exponentially from zero as particles were phagocytized during the first 24 h PI, and continued to increase slightly between 1 and 4 d, with the exception that the "one particle per cell" category declined slightly between 1 and 4 d PI. This initial growth phase was followed by decline, which is evident between 4 and 91 d PI, most prominently in the higher burden categories. The proportion of AMs in the "one particle per cell" category, however, increased slowly between 4 and 91 d (doubling time, 155 days). In the lower burden categories (up to seven particles), the majority of the cell loss occurred between 28 and 91 d PI, whereas in larger particle burden categories (eight or more particles), loss of cells began earlier, between 4 and 28 d, and continued through 91 d PI. The percentage of cells in each of the three largest burden categories was significantly lower at 91 d than at 4 d PI (ANOVA and Tukey's HSD post hoc comparisons, $P < 0.05$). The coefficients, decay constants,

TABLE 1

*Fitted parameters for exponential decay function**

Category (*particle per cell*)	Bronchial Fraction A (*%*)	λ (*% day^{-1}*)	r^2	$t_{1/2}$ (*days*)	Alveolar Fraction A (*%*)	λ (*% day^{-1}*)	r^2	$t_{1/2}$ (*days*)
1	54.24	0.00036†	0.99	1,944‡	43.02	0.0047†	0.96	155‡
2 to 4	29.94	0.00098	0.98	707.9	36.48	0.0023	0.88	345.2
5 to 7	9.01	0.0017	0.88	414.0	9.84	0.0056	0.86	123.2
8 to 11	5.20	0.0095	0.98	73.0	6.20	0.017	0.99	40.3
12 to 19	2.64	0.013	0.92	53.4	4.15	0.054	0.98	12.8
20 or more	0.72	0.021	0.75	32.7	2.42	0.082	0.99	8.4

* These parameters represent the coefficients (A) and rate constants (λ) of long-term retention functions corresponding to Equations 3 or 4.
† Positive exponential.
‡ Value for 1 particle per cell category is doubling time, not half-time.

and half-times for long-term exponential functions fit to the various categories are listed in Table 1 for both alveolar and bronchial fractions. It is clear from these fitted parameters that there was a progressive increase in the rate of disappearance of particle-laden AMs as their particle burdens increased. On the basis of the calculated half-times, AMs in the 20+ particle category cleared approximately 40 times faster than AMs in the 2- to 4-particle category.

Bronchial fraction. As in the alveolar fraction, particles were observed almost exclusively in AMs and occasionally in neutrophils. Compared with the alveolar fraction, there were many degenerate cells in the bronchial lavagate, many of which were probably epithelial cells. Degenerated or lysed cells with associated particles were presumed to be AMs; however, it is possible that some were epithelial cells. We did not see particles associated with cells definitively identified as epithelial cells. The proportional distributions of AMs in the four lowest particle burden categories (1 through 8–11 particles) at the various times PI were similar to those of the alveolar fraction (Figure 4). In contrast to the alveolar fraction, the percentages of phagocytic AMs in the two highest particle burden categories (12 to 19, and 20 or more particles) in the bronchial fraction remained relatively constant between 1 and 28 d PI and diminished slightly between 28 and 91 d PI (not statistically significant, ANOVA). The percentage of phagocytic AMs in the 20-or-more particle category was significantly lower in the bronchial fraction compared with the alveolar fraction at 4 d PI (0.35 ± 0.26 versus 1.74 ± 0.48, paired *t* test, $P = 0.04$); however, there was no significant difference between bronchial and alveolar fractions at 91 d PI for either of these two highest particle burden categories.

Differences in disappearance of AMs from corresponding particle burden categories of bronchial and alveolar fractions are most apparent as differences in clearance rate constant (λ) and $t_{1/2}$ as listed in Table 1. Pearson's correlation coefficients for particle burden category versus λ were significantly greater than zero for both alveolar and bronchial fractions ($P = 0.003$ and $P = 0.019$, respectively), indicating trends toward increasing rates of clearance (AM disappearance) as a function of increasing particle burden. Regression lines demonstrating trends in rates for the two BAL fractions are plotted in Figure 5. The slopes of the two trend lines are slightly (but not statistically) different; however, the lines are different as defined by differences in their respective y intercepts (ANCOVA, $P = 0.006$).

Cell Counts in BAL Fluid

The instillation of Fe_2O_3 particles into the lingular subsegment resulted in a transient inflammatory response that was apparent in some of the subjects at 1 d PI. This cellular response was characterized by the influx of both AMs and neutrophils (Table 2). Although there were large differences in the mean number of cells recovered from control and particle-instilled segments, large variances in cell numbers in the lingular lavagate prevented the differences from being statistically significant. The number of AMs in BAL fluids from the particle-instilled segment returned to control levels by 2 d PI. The mean total number and percentage of neutrophils in the lingular alveolar BAL fluid

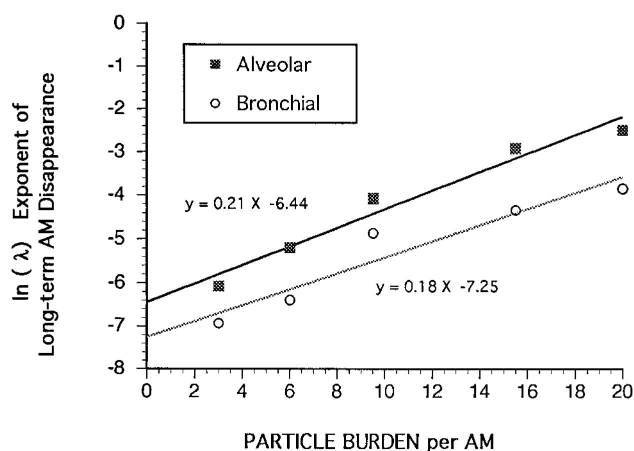

Figure 5. The rate constants governing the disappearance of AMs from the various particle burden categories tended to increase with increasing intracellular particle burden for both alveolar and bronchial fractions. Pearson's correlation coefficients (r) for alveolar (0.981, $P = 0.003$) and bronchial fractions (0.936, $P = 0.019$) are significant and positive. The slopes of the regression lines are similar, but y intercepts are significantly different ($P = 0.006$).

TABLE 2

*Total cells, alveolar macrophages, and neutrophils in BAL fluid from particle-instilled and control (saline-instilled) segments**

	Particle Instilled (10^7)	Saline Instilled (10^7)	Significance (P)[†]
Total cells[‡]	20.0 ± 7.72	2.95 ± 0.40	0.08
Alveolar macrophages[§]	9.68 ± 2.62	2.70 ± 0.39	0.05
Neutrophils[§]	9.35 ± 4.9	0.046 ± 0.015	0.11

* Twenty-four hours postinstillation. All values are mean ± standard error.
† Student's *t* test for paired measurements, P value ≤ 0.05 indicates statistical significance. Large variances in cell counts in the particle-instilled segment prevented the differences from being statistically significant.
‡ Combined bronchial and alveolar fractions.
§ Alveolar fraction.

was still marginally elevated at 2 d PI (although not statistically significant), and were no different from control values at 4 d PI and later.

Discussion

The relationship between lavagable particle burden and total particle burden has been clearly demonstrated by Lehnert and colleagues (8), who showed in rats that the lavagable burden was a proportional reflection of the total lung burden. Studies in sheep (7) and rats (2, 8, 28) showed that the bulk of the pulmonary particle burden is associated with AMs and that the AM population sampled by BAL is representative of the overall AM population with respect to the intracellular particle distribution. Realizing that we did not recover all the particles in the lung, or even all the particles in the lavagable spaces, we contend that in our study BAL resulted in recovery of a quantity of particles that is proportional to the total lung burden and that could be used to study changes in intracellular particle distribution and estimate retention and clearance kinetics for particles in the lavagable AM compartment.

Our comparisons of intracellular particle distributions at the various time points clearly demonstrate disproportionate rates of cell loss from the various categories in both alveolar and bronchial fractions. The rate of cell disappearance is dramatically greater in the high particle burden categories, similar to the findings in previous studies (8). The continued growth of the "one particle per cell" category in the face of decline in other categories might be explained by one of several mechanisms. The first is that cells in the higher categories may be preferentially cleared physically from the lung at a more rapid rate than cells in the smaller particle burden categories, thus the relative proportions of AMs in the smaller burden may actually continue to grow. This would require greater mobility and disproportionately more rapid movement onto the mucociliary apparatus or into the interstitium by AMs with high particle burdens. Evidence provided by other investigators, however, indicates that AMs heavily laden with particles actually tend toward decreased mobility (28, 29). A second explanation might be a change in the lavagability of AMs in the various particle burden categories with the passing of time. This seems unlikely because findings similar to ours were made by Lehnert and associates (8), who examined not only lavaged AMs, but also AMs remaining in the lung after lavage.

A third, and perhaps most probable, explanation might be that particles released by cells in the higher burden categories are rephagocytized by other cells, resulting in "redistribution" of particles from one cell to another. The possible mechanisms underlying cell-to-cell particle redistribution have previously been discussed in detail elsewhere (30). The cells that phagocytize the released particles are most likely to be cells that previously did not contain particles, because, proportionally, they are much more abundant than particle-laden AMs. This redistribution might occur following exocytosis of particles by viable cells, lysis of dead or degenerate cells, or phagocytosis of whole dead or degenerating cells. Our data seem consistent with the possible occurrence of a particle redistribution phenomenon in our human subjects. The true explanation for our observations may be a combination of mechanisms rather than just one single mechanism.

The proportions of AMs in the various particle burden categories were similar in alveolar and bronchial fractions, although there were apparent differences in the rates of AM disappearance from corresponding categories in the two BAL fractions. We interpret this to indicate that many of the AMs in the bronchial fraction are most likely egressing AMs that have moved from the alveolar region into the airways, resulting in an apparent lag in clearance from the bronchial region. This also suggests that mobility of AMs toward the bronchial airways may not be influenced by their particle burdens.

The fast clearance phase ($t_{1/2}$ = 0.5 d) found in our study is much faster than that ($t_{1/2}$ = 12–19 d) found by others in rats (8, 31) and is representative of the early clearance (0 to 4 d), which probably includes mucociliary clearance of particles from small peripheral airways in addition to rapid-phase alveolar clearance. Our data do not allow for the resolution of a distinct intermediate phase of relatively rapid alveolar clearance comparable to that described in the rats (8). It is incorporated into the long-term clearance phase and undoubtedly influences our calculated value for the long-term clearance half-time. Additional subjects and groups examined between 4 and 28 d PI and beyond 91 d PI would probably allow for more confidence in resolving fast and slow components. Given the marked disparity in long-term clearance rates between humans and rats (10), we would have expected to see a much slower long-term clearance half-time in our human subjects as compared with rats (8).

The long-term clearance half-time of 110 d found in our BAL study is within the range of half-time values (62 to 300 d) found by others using radioactive tracer or magnetic methods to measure clearance of iron oxide particles (32–34), polystyrene (12, 13), or Teflon particles (20). This is much faster, however, than the mean 272-d half-time found by Bailey and coworkers (11) for clearance of radiolabeled 3.9-μm fused aluminosilicate particles in humans, in which it was found that the calculated half-time increased with increasing length of the study. Thus, the calculated half-time in our study may have been affected by the relatively short length of the study period.

Our technique only samples the retention of the lavagable cells and particles; whereas the external monitoring of inhaled radiolabeled particles assesses retention of all particles, including those sequestered within epithelial cells, the interstitium, and thoracic lymph nodes. Thus, our method tends to underestimate retention and overestimate clearance of particles when there is significant movement of particles into the interstitium. It is likely that the large number of particles and the transient inflammation in our study may have resulted in greater than usual access of particles to the interstitium, where they would be inaccessible to lavage. Studies in mice (35) and rats (36), however, indicate that instilled inert particles of at least 1-μm diameter are unlikely to penetrate to the interstitium. Even so, because the majority of inhaled particles become associated with AMs (2, 7) and because the lavagable particle burden is proportional to the total burden (8), it is likely that sampling by BAL reflects the kinetics of retention and clearance of particles in the AM compartment and within the alveolar spaces.

A transient cellular influx, similar to that in the current study, has been observed following instillation of polystyrene (37) or carbon particles (38) and following inhalation of ferric oxide dust (39). This inflammation may result from release of neutrophil chemoattractants by AMs (40), complement activation (41), or the presence of small amounts of soluble ferric ion (Fe^{3+}) (42) that may have been associated with the particles used in our study. The sudden introduction of large numbers of particles and subsequent phagocytic activity by AMs also may result in accidental release of lysosomal contents (hydrolases, proteases, reactive oxygen species, and other products) during phagocytosis (premature fusion of lysosome with phagosome), leading to inflammation.

Studies have examined and quantified particles within lavaged AMs in attempts to index pulmonary particle burdens in occupationally exposed individuals (43). Because occupational dust exposures generally involve nonradioactive materials, estimation of pulmonary particle burden and clearance kinetics is difficult. The current study demonstrates that sampling of the lavagable particle burden by BAL over a period of time can yield an estimate of particle retention kinetics comparable to that obtained by other, more traditional methods, such as external monitoring of radiolabeled particles. BAL, thus, may be useful not only for assessing pulmonary particle burdens, but also for predicting particle retention kinetics following occupational inhalation exposure to insoluble dusts.

This is the first study to employ intrapulmonary instillation of particles and BAL to study kinetics of pulmonary particle retention and clearance in human subjects. It demonstrates the usefulness of BAL for studying intrapulmonary particle burdens by sampling lavagable AMs, which are crucial for clearance of particles from the alveolar region and that also serve as a compartment for storage or sequestration of a large proportion of the total lung burden of insoluble particles. With this technique we demonstrated disproportionate "clearance" (disappearance) of AMs with high particle burdens, suggestive of a particle redistribution phenomenon similar to that described in rats. In addition, we were able to estimate a long-term clearance half-time comparable to that determined by more traditional methods. Finally, we demonstrated a transient acute inflammatory response to the particles at 1 d PI, a finding that may be important in the light of studies showing acute adverse health effects of ambient particulate air pollution.

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