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Intracellular Particle Dissolution in Alveolar Macrophages

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Aerosol particles deposited in the lungs that are not readily soluble in the epithelial lining fluid will be phagocytized by alveolar macrophages (AM). Inside the phagolysosomal vacuole, the constituents of the plasma allow dissolution of a variety of compounds at a higher rate than dissolution in extracellular lung fluids. Chelator concentration and a pH value of about 5 were found to control intracellular particle dissolution (IPD). Hence, IPD is the initial step of translocation of dissolved material to blood, which is an important lung clearance mechanism for particles retained long term. IPD rates of uniform test particles determined in human, baboon, and canine AM cultures were similar to initial translocation rates determined in lung clearance studies of the same species after inhalation of the same test particles. IPD rate in cultured AM proved to be a sensitive functional parameter of AM, which was used to identify changes in the clearance mechanism of translocation during different exposure conditions.

Introduction

Translocation of deposited particles in the respiratory tract by particle dissolution and subsequent transfer of dissolved material to blood is well known and considered to be an important clearance mechanism for a large variety of ambient and occupational aerosol particles in the respiratory tract of man and various experimental animals (*1-3*). As a result, translocation of dissolved particle material from the respiratory tract to blood was incorporated in a first model of particle clearance from the respiratory tract by the International Commission for Radiological Protection (*4,5*), which was updated (*6*) and will be revised again incorporating the conclusions of recent lung clearance studies (*7-10*). In this new concept of lung clearance, translocation is considered to be an independent clearance mechanism competing with particle transport mechanisms. Although translocation is assumed to be similar in each compartment of the respiratory tract, the effectiveness of particle transport mechanisms varies considerably between extrathoracic airways, the tracheobronchial tree, and the alveolar region. Because particle transport from the alveolar region is slow, particularly in man (*11*), translocation of material from particles retained in the structures of the gas exchange region is recognized to be a very important clearance mechanism for the lung parenchyma. Moreover, recent studies have indicated that aerosol particles that were believed to be insoluble gradually dissolve in the lungs (*12-14*). This discovery, taken with the fact that retained particles are phagocytized by macrophages, indicated intracellular particle dissolution. In this paper the relevance and possible mechanisms of intracellular particle dissolution as the initial step of the lung clearance mechanism of translocation are reviewed.

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Modeling Particle Dissolution

Dissolution of material from particle surfaces has been described by a physical model (*15,16*), where the rate of dissolved mass (dm/dt) from particles of mass m is proportional to their total surface area, S:

$$\frac{dm}{dt} = -kS \tag{1}$$

where k is the dissolution rate constant of the chemical compound of the particle in the solvent. Hence, the fractional rate, f, of dissolved mass is proportional to the specific surface area, s, of the material (surface area per unit mass):

$$f = \frac{1}{m}\frac{dm}{dt} = -k\frac{S}{m} = -ks \tag{2}$$

Extracellular Particle Dissolution

The epithelium of the respiratory tract is covered with a lining fluid, and materials that dissolve in this fluid are readily transferred to blood. It was concluded that particles that were soluble in simulants of the lining fluid *in vitro* would dissolve in the epithelial lining fluid (ELF) and that this dissolved material would subsequently be transferred to blood (*17-20*); i.e., they would have been cleared by the mechanism of translocation. This was observed for soluble aerosol particles and was determined quantitatively for a number of soluble radioactive aerosols deposited in various experimental animals (*14-22*). This approach has also been applied to less soluble particles. Reasonable agreement was obtained when translocation *in vivo* was compared to dissolution of insoluble particles in lung fluid simulants (*17-28*). It has to be stated, however, that the particles used were not uniform and that their physical parameters, in particular their

specific surface areas, were not known exactly, resulting in considerable uncertainty. The *in vivo* translocation rate constants derived from lung clearance studies and the *in vitro* dissolution rate constants obtained from dissolution tests using ELF simulants varied within an order of magnitude at low levels of 1–10 × 10^{-8} g/cm²/day for a number of slowly dissolving materials.

However, with two compounds of beryllium, the translocation rates in rats and baboons were not comparable with the *in vitro* dissolution rates in lung fluid simulants (*29*). Similarly, the model of particle dissolution in lung fluid simulants did not hold for moderately soluble, uniform Co_3O_4 particles (*14,30–32*). Although these particles were found to be almost insoluble *in vitro*, they were translocated from dogs' lungs to blood *in vivo* according to their specific surface area, s (Eq. 2), which varied over two orders of magnitude due to differences in the size and porosity of the particles (*14,30–32*). In fact, a translocation rate constant $k = 6.9 \times 10^{-8}$ g/cm²/day was determined for all canine lung clearance studies using Co_3O_4 particles (*32*). Interestingly, this constant was in the same range as the translocation rate constants found for other metal oxides in dogs (*21–28*). Similarly, a greater translocation of cobalt from uniform Co_3O_4 particles than observed *in vitro* was shown in man and six other laboratory animal species in a recent interspecies comparison of lung clearance (*33*).

Particle Phagocytosis and Retention

Under normal conditions, aerosol particles deposited on the alveolar epithelium of the lungs will be phagocytized within hours by alveolar macrophages (AM) (*34–37*). Each particle will be enclosed within a membrane-bound phagosomal vacuole onto which lysosomes will subsequently fuse to form secondary lysosomes or phagolysosomes. As a result, the phagolysosomes will contain proteolytic enzymes, oxygen radicals, chelators, precipitators, and, at a pH of about 5 (*20*), all may react with the enclosed particles.

Exhaustive total bronchalveolar lavages (BAL) in rodents up to half a year after particle administration (*38,39*) showed that more than 80% of the retained particles were still retained in AM on the epithelium. In large animals, partial BAL suggested particle retention in AM up to 500 days after inhalation (*14,31,40,41*).

Intracellular Particle Dissolution

Particle dissolution in cultured AM was first observed qualitatively by Lundborg and co-workers (*42*). AM obtained from total BAL of rabbits were incubated for up to 5 days with MnO_2 particles in full medium containing 15% rabbit serum. Phagocytosis of particles in AM was observed by light microscopy. The amount of dissolved manganese in the filtered media after 1–5 days of incubation was greater than the dissolved manganese determined from a control dissolution test with the same amount of MnO_2 particles in the same media but without AM. The fact that particles had been phagocytized and that the larger dissolved manganese fraction was found in the medium of the cell culture system than in medium only, clearly indicated that rabbit AM are able to dissolve MnO_2 particles. As a next step, the authors showed that human AM dissolved MnO_2 particles in a similar manner (*43*). A difficulty arose from the unkown fraction of phagocytized particles and the heterogeneity of the particulate material whose size varied from 0.1 to 0.5 μm with an unknown specific surface area.

At the same laboratory (*44*), the intracellular dissolution of two particulate compounds of arsenic was studied to test the effect of the acidic milieu in the phagolysosome of AM that was reported earlier (*45*). Whereas the solubility of lead arsenate was known to increase with decreasing pH, the solubility of arsenic trisulfide was known to decrease with decreasing pH. The dissolved arsenic fraction in the medium without AM at a pH of 7.4 was compared with the dissolved arsenic fraction in the AM culture suspension. In fact, more arsenic dissolved from the lead arsenate particles in the AM culture suspension than in the cell-free medium, while the opposite was the case for arsenic trisulfide particles. From these results the authors concluded that the pH value of the vacuolar sol inside the phagolysosome is an important factor of intracellular particle dissolution (IPD).

In another study, dissolution of polydisperse, radioactive $^{244}AmO_2$ particles was determined in cultured AM obtained from monkeys, dogs, and rats (*46,47*). Dissolution in the cell culture system increased linearly to 10% of the initial particle amount after 3 days of incubation, whereas dissolution of the AmO_2 particles in the culture medium only was constant at 1% of the initial particle amount. Because the fraction of phagocytized particles was not determined, the measured kinetics of dissolution resulted from both the increasing, time-dependent dissolution of the phagocytized particle fraction and the constant dissolution of the unphagocytized particle fraction. Interestingly, the cultured rat AM phagocytized about half of the particles that monkey and dog AM had phagocytized during the first 20 hr of incubation. However, in the cell culture system of each species, the same fraction (5% of the initial particle activity) dissolved after 20 hr of incubation. Hence, intracellular particle dissolution was faster in rat AM than in AM of the other species because rat AM had phagocytized a smaller fraction of particles than the AM of the other species at this time.

In a more quantitative approach, the intracellular dissolution rates of monodisperse, porous Co_3O_4 particles with different specific surface areas were determined in cultured AM from man and dogs (*48–51*) and in AM of man, rats, and baboons (*52*) using slightly different methods. In the latter study, 260-mL culture flasks were used to culture all the AM together, and in the former study, the cells were incubated in 96-well cell culture plates, which allowed additional cell function tests in separate wells to be conducted during the entire incubation time. In both methods AM were purified and incubated in full medium containing fetal bovine serum and antibiotics with Co_3O_4 particles. Using inverted light microscopy, complete phagocytosis was observed within 1–2 days of incubation (*50*), which was obtained by a cell concentration in which cells covered 5–10% of the surface area of the well bottom and a 1:1 particle-to-cell ratio. The dissolved fraction of cobalt was separated from the particulate fraction by means of filtration. Initial leakage of a small amount of material from the particle surface was determined in parallel experiments in which particles were incubated in full medium without cells. IPD in cultured AM was determined by measuring the dissolved cobalt in the culture medium and the lysate of AM as a fraction of the initially added particle amount by means of the radioactive ^{57}Co label. An IPD rate was derived from measurements at

Table 1. Intracellular particle dissolution rates of cultures of alveolar macrophages of man, baboon/monkey, and dog.[a]

Particle size, μm	Man, %/d	Baboon, %/d	Monkey, %/d	Beagle dog, %/d	Mongrel dog, %/d
1.7	0.13 ± 0.02[b] (2)	0.18[b] (3)			0.16 ± 0.04 (6)
0.8	0.44 ± 0.03[b] (2)	0.29[b] (2)			0.33 ± 0.10 (4)
0.7	0.29 ± 0.01 (2)		0.46 ± 0.02 (2)	0.59 ± 0.14 (12)	0.40 ± 0.09 (6)
0.7		0.75 ± 0.02 (1)		0.76 ± 0.03 (50)	
0.5		1.2 ± 0.03 (1)		1.4 ± 0.05 (52)	
0.3	4.3 ± 0.5 (2)		4.3 ± 0.02 (2)	3.9 ± 1.1 (12)	4.8 ± 1.0 (6)

[a]Mean intracellular particle dissolution (IPD) rates (percent rate of administered particle dose, %/d) were determined in cultures of alveolar macrophages (AM) obtained from bronchoalveolar lavage of man, baboon/monkey, and dog using different monodisperse Co_3O_4 test particles. IPD rates and standard error of the mean were obtained from each AM culture of the AM harvested at the various bronchoalveolar lavages. Number of bronchoalveolar lavages are given in parentheses.
[b]Includes IPD measurements of André and co-workers (*51*).

various time points during 2 weeks of incubation (*50*). Because the integrity and function of the cultured AM is another essential condition for this assay during the 2 weeks of incubation, cell viability and phagocytic function were monitored at each sampling time to discard results from malfunctioning cell cultures. In Table 1, IPD rates are given for the various species studied and the different monodisperse, porous particles used. All the test particles were produced under the same conditions (*53,54*), and yet different batches show slight differences, as can be seen by the rates obtained from 0.8, 0.7, 0.5 particles of AM of beagle dogs. According to Equation 2, the fractional rates in Table 1 are proportional to the specific surface area, s, of the particles used. Therefore, the specific surface area of the various particles was estimated from the aerosol parameters measured during production as described previously (*32*). Figure 1 shows the good linear correlation ($r = 0.96$) between IPD rate and specific surface area. Because the number of determinations in man, baboon, and monkey are limited, no significant species differences have been determined so far.

Mechanisms of Intracellular Particle Dissolution

In the IPD studies (*50*), we have shown that no significant extracellular particle dissolution occurred by enzymes and/or other cell products that might have been released from the cultured macrophages: we observed no additional dissolution beyond the initial leaching when we suspended the $^{57}Co_3O_4$ test particles in conditioned media obtained from AM cultures that had phagocytized nonradioactive Co_3O_4 particles or latex particles. Based on protein precipitation studies, the dissolved cobalt did not appear to be bound significantly to either intracellular or extracellular protein. Therefore, we concluded that dissolved cobalt was in an ionic or chelated state in the culture media.

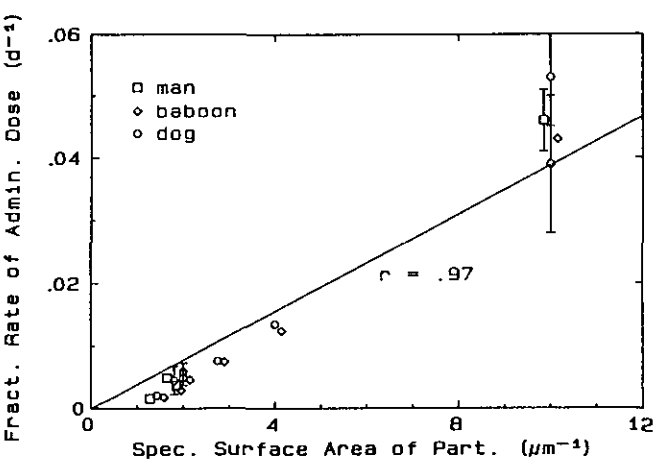

FIGURE 1. Intracellular particle dissolution rates in alveolar macrophages of man, baboon/monkey, and dogs versus the specific surface area, s, of uniform Co_3O_4 test particles. Mean fractional rates and SEM are given according to Table 1. Linear correlation was forced through the origin according to Equation 2.

We also investigated the effect of temperature on IPD in AM (*50*). When the incubation temperature was reduced from 37°C to 20°C after 4 days of incubation, viability and phagocytic function of AM in culture did not change from day 4 to 14 compared to the control cell cultures maintained at 37°C. No further dissolution occurred, however, and the dissolved fractions remained constant and did not increase when the cultures were incubated at 20°C. At the same time, particle dissolution in media without cells did not differ significantly for the two incubation temperatures.

The pH in phagolysosomes was shown to effect IPD (*43*). In addition, we showed that chelators in the phagolysosomes were important cofactors in controlling IPD (*51*). In Figure 2, the kinetics of dissolution of 0.3 and 0.7 μm $^{57}Co_3O_4$ particles are shown in various cell-free buffered saline solvents (RPMI 1640) using the same incubation method as for AM culture systems. For comparison, IPD data for canine AM are also plotted. At a pH value of 7.2, the particles showed a constant leaching fraction. Changing the pH value to 5.0 by a phosphate buffer had the effect of increasing the dissolved percentages slightly with time. Using a citrate buffer to obtain a pH value of 5, the dissolved percentages increased more rapidly with time, resulting in similar slopes compared to IPD in AM. The chelating citrate concentration in the medium was in the millimolar range. Interestingly, similar or somewhat steeper slopes were obtained with 1 mM EDTA, another chelating agent, in phosphate-buffered medium at a pH of 5. However, in medium with 50 mM EDTA at pH 5, particles dissolved even faster. At a pH of 3.5, particles dissolved very quickly, irrespective of whether chelating agents were present or not. Particles with a diameter of 0.3 μm were completely dissolved after 3–8 days, and dissolution curves for 0.7-μm particles were obtained that were steeper than those for 0.3-μm particles (Fig. 2).

These studies showed that the kinetics of IPD could be simulated by the presence of a chelator and the appropriate pH value in the solvent. Because there are chelating agents present in the phagolysosomes of AM (*20*) and the pH value is in the

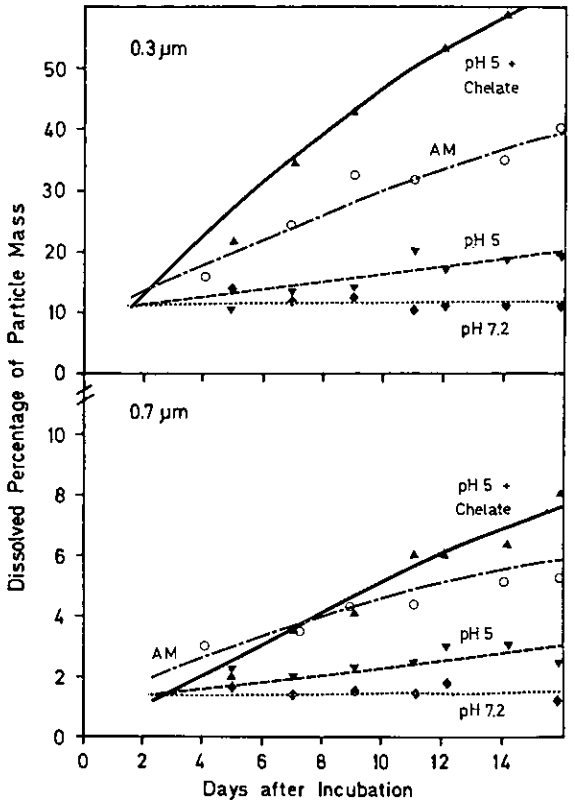

FIGURE 2. Simulation of the kinetics of intracellular particle dissolution. Dissolved percentage of the administered particle mass in full medium containing RPMI 1640, serum, and antibiotics without cells during 2 weeks of incubation for uniform 0.3- and 0.7-µm Co_3O_4 test particles. Solvents of pH 5 were buffered either by phosphate or citrate, which is a chelator. For comparison, the kinetics of intracellular particle dissolution in canine AM is given by the dashed and dotted line (AM).

range of 5 (*45–60*), it is plausible to conclude that the intracellular mechanism of particle dissolution is controlled by these two parameters. However, at this moment we cannot exclude other mechanisms of IPD resulting from proteolytic enzymes, oxygen radicals, chelators, precipitators, etc., that are contained in the phagolysosomal plasma (*20*).

Intracellular Particle Dissolution and *in Vivo* Translocation

The *in vitro* IPD rate in AM (Table 1, Fig. 1) was similar to the initial *in vivo* translocation rate when the same particles were inhaled and subsequently cleared from the lungs. In Table 2, initial translocation rates obtained from man, baboon, and dog are given for the various Co_3O_4 test particles. In Figure 3, IPD rates and initial translocation rates obtained from dogs show the same correlation with the specific surface area of the particles used. The species differences of the initial *in vivo* translocation rates found in the interspecies comparison are slightly indicated by the *in vitro* IPD rates in Table 1 but less prominent.

I emphasize, however, that translocation also involves the transfer of the dissociated material from the cell to blood through intra- and extracellular fluids and across the various membranes

Table 2. Initial translocation rates found in man, baboon, and dog.[a]

Particle size, µm	Man, %/d	Baboon, %/d	Beagle dog, %/d
1.7[b]	0.19 ± 0.06 (2)	0.16 ± 0.05 (2)	0.33 ± 0.01 (2)
1.7			0.21 ± 0.01 (4)
0.8[b]	0.33 ± 0.07 (2)	0.37 ± 0.08 (2)	0.49 ± 0.05 (2)
0.7			1.5 ± 0.40 (4)
0.3			6.3 ± 1.9 (2)

[a]Mean initial translocation rates (percent rate of the initial alveolar dose, *%/d*) were taken from previous lung clearance studies on dogs (*32*) and determined in the interspecies comparison of lung clearance (*31,33,40,61*) for man, baboon, and dog using different monodisperse Co_3O_4 test particles. Mean rates and standard errors of the mean were calculated from the translocation rates found in each subject observed during the first 1–2 weeks after inhalation. Number of bronchoalveolar lavages are given in parentheses.

[b]Determined in the interspecies comparison of lung clearance (*31,33,40,61*).

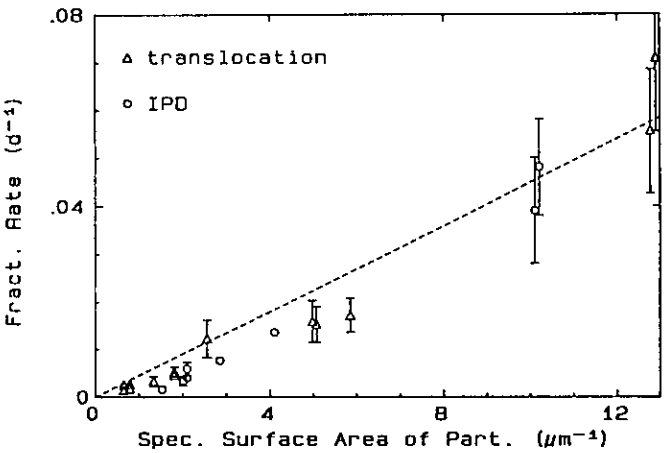

FIGURE 3. Comparison of *in vitro* intracellular particle dissolution (IPD) rates and *in vivo* translocation rates of dogs. *In vitro* IPD rates in canine alveolar macrophages and *in vivo* initial translocation rates obtained from lung clearance studies on dogs versus the specific surface area, *s*, of uniform Co_3O_4 test particles. Mean fractional rates and SEM are given according to Tables 1 and 2. Linear correlation was forced through the origin according to Equation 2.

of cells and lung tissues. In case of the Co_3O_4 test particles, we have shown for dogs and hamsters (*30,32,62*) and for various animal species studied (European Late Effects Project Group, personal communication) that transfer was fast compared to IPD. Therefore, IPD was the rate-determining mechanism of translocation for these test particles. In case of other particles with different chemical compositions, the dissociated material might be precipitated and/or transformed such that it will be not readily transferred to blood but retained in the lungs.

Intracellular Particle Dissolution: Functional Test of Lung Clearance

IPD in AM and, hence, translocation, might be altered under certain exposure conditions. Recently (*63*), we used IPD measurements as an indicator for the lung clearance mechanism of translocation. We compared IPD in AM obtained from beagle dogs that were housed in ambient air with IPD in AM from dogs living in clean air. IPD was applied to a longitudinal study in which AM were obtained from sequential bronchoalveolar

Table 3. Intracellular particle dissolution rates of canine alveolar macrophages under different exposure conditions.[a]

Study	Exposure	Run no.	Lavage no.	Relative IPD rate ± SD
Longitudinal	Control	30	7	1.00 ± 0.22
	Ambient	36	10	0.73 ± 0.19*
Cross-sectional	Control	17	7	1.00 ± 0.23
	Ambient	12	4	0.64 ± 0.19*

[a]Mean relative intracellular particle dissolution (IPD) rates obtained from alveolar macrophages of dogs exposed to either clean air (control) or ambient air. IPD rates were normalized to the mean of the control values. Three dogs were incorporated in the longitudinal study and eight dogs in the cross-sectional investigation. Multiple determinations were carried out at each bronchoalveolar lavage.

*$p < 0.001$ significance level at the 95% confidence interval that data are different from control data.

lavages carried out on three dogs during 1 year as well as a cross-sectional investigation where AM were obtained from eight beagle dogs. During the longitudinal study, the dogs were housed in exposure chambers ventilated with clean air during the first 4 months and later with ambient air in the normal kennels of our animal facilities. For the cross-sectional study, AM were harvested from dogs housed either under clean air conditions in exposure chambers or at ambient air by one or more bronchoalveolar lavage. Results are summarized in Table 3. In both the longitudinal and the cross-sectional study, the mean IPD rates in AM obtained from dogs living in ambient air were about 30% lower than under clean air exposure.

However, when eight dogs were exposed chronically to a sodium bisulfite aerosol for 9 months, the preliminary results obtained from seven sequential lavages of each dog during the control period and five lavages of each dog during exposure period indicated that IPD rates increased significantly by 20% compared to control values under clean air conditions (*64*). It is unclear why IPD rates were lowest under ambient air conditions and highest under pollutant exposure, with intermediate IPD rates under clean air conditions. Based on current knowledge, we hypothesize that constituents of the phagolysosomal plasma in AM such as chelator and proton concentration were changed under the different exposure conditions.

Conclusions

Intracellular particle dissolution of a variety of compounds was faster than extracellular dissolution in solvents with composition similar to lung fluids. IPD in AM is the initial step of translocation, which is an important lung clearance mechanism for long-term retained particles.

IPD proved to be a sensitive functional parameter of AM. Because AM play an essential role in the clearance of inhaled particles, IPD of test particles can be used to indicate changes of the lung clearance mechanism of translocation.

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