Experimental Lung Research

ISSN: 0190-2148 (Print) 1521-0499 (Online) Journal homepage: http://www.tandfonline.com/loi/ielu20

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To cite this article: R. A. Harper, C. Stirling, K. M. S. Townsend, W. G. Kreyling & G. Patrick (1994) Intracellular Particle Dissolution in Macrophages Isolated from the Lung of the Fischer (F-344) Rat, Experimental Lung Research, 20:2, 143-156, DOI: 10.3109/01902149409064379

To link to this article: http://dx.doi.org/10.3109/01902149409064379

Intracellular Particle Dissolution in Macrophages Isolated from the Lung of the Fischer (F-344) Rat

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Alveolar macrophages were removed from male F-344 rats by bronchoalveolar lavage and maintained in vitro for 14 days. Over this period the macrophages remained viable as judged by propidium iodide exclusion, lysosomal uptake of acridine orange, and phagocytosis of 1.75-μm latex beads. After 7 and 14 days of culture the cells contained lipid droplets. The macrophages were shown to ingest and dissolve monodisperse $^{57}Co_3O_4$ particles, which were relatively insoluble in extracellular medium. The fraction of $^{57}Co_3O_4$ dissolved intracellularly was determined at intervals during the culture period. The mean dissolution rate (± SEM) was 0.36 ± 0.02% per day for the F-344 rat. This was lower than 1.4 ± 0.05% per day estimated by Kreyling in macrophages from the beagle dog. The significance of this difference is discussed.

Keywords *alveolar, cobalt, culture, dissolution, F-344 rats, macrophage*

To assess the risk to humans from aerosols containing noxious particulate matter, the effect of exposure on various regions of the lung is important. A clearance model proposed by Cuddihy provides a means by which regional clearance of toxic materials from the human lung can be estimated [1, 2]. However, although the model allows for species variability

Received 30 March 1993; accepted 22 October 1993.

This work was coordinated by the European Late Effects Project Group (EULEP) under contract BI6-099 from the Commission of the European Communities.

From the MRC Radiobiology Unit, Chilton, Didcot, Oxon, United Kingdom; and the GSF-Forschungszentrum für Umwelt und Gesundheit GmbH, Projekt Inhalation, Neuherberg, Oberschleissheim, Germany.

Address correspondence to R. A. Harper, PhD, MRC Radiobiology Unit, Chilton, Didcot, Oxon OX11 ORD, UK.

Experimental Lung Research 20:143–156 (1994)

0190-2148/94 $10.00 + .00

in mechanical transport from the lung, absorption (translocation) to blood is assumed to be similar in all species [1].

Dissolution of inhaled test particles of cobalt oxide, with the dissolved material being translocated to the blood, was shown to be an important clearance mechanism in vivo in a variety of species [3]. However, the observed interspecies differences mean that direct comparison to humans can be difficult. Particles deposited on the epithelial surface of the lung will be phagocytosed by macrophages [4]. It would be of interest to know whether the difference in translocation seen between species was due to physiological variation in the rate of intracellular particle dissolution (IPD) within the alveolar macrophages themselves.

In view of (1) the known differences between species in translocation rates in vivo, and (2) the ability of cultured alveolar macrophages from the dog to dissolve monodisperse test particles such as cobalt oxide Co_3O_4 [5–7], it was of interest to know if similar in vitro studies could be performed with macrophages from another species. The Fischer rat was selected for study here mainly because it had also been used in the in vivo interspecies comparison. The time-dependent rate of absorption to blood obtained in the Fischer rat was quite different from that of other species, including the beagle dog, and was also similar in its time-course to that seen in humans [3]. However, the present report does not constitute a rigorous interspecies comparison of IPD in vitro.

METHODS

Preparation of $^{57}Co_3O_4$ Particles

Monodisperse ^{57}Co-labeled Co_3O_4 particles were prepared with a count median diameter of 0.5 μm and a geometric standard deviation (σ_g) of 1.1, using a spinning top aerosol generator and heat degradation at 800°C [8, 9]. The resulting spherical particles were porous, with a surface area 10 times larger than their calculated spherical area [9]. The activity at the time of the study was 0.61–0.93 mBq per particle. These particles were from the same batch prepared by Kreyling and coworkers [7]. They were stored dry before use. Experience in both laboratories has shown that this assures reproducible measurements of IPD over extended periods of time. For example, in a recent study with dog macrophages, the same $^{57}Co_3O_4$ particles gave uniform IPD rates over 400 days, during which the dogs were maintained under constant clean air conditions [10].

Bronchoalveolar Lavage

Under halothane anesthesia, 9 adult male Fischer F-344 rats were exsanguinated via the dorsal aorta prior to induction of a pneumothorax. The

trachea was then exposed and the lungs were cannulated with a 14-gauge cannula. Alveolar macrophages were obtained by instillation and drainage of 8 × 8 mL of 1× modified Dulbecco's phosphate-buffered saline without calcium and magnesium (PBS) (ICN Biomedicals Ltd, High Wycombe, UK) at 4°C.

The lavaged fluid was filtered through a 125-μm gauze to remove debris and mucus. A differential count of approximately 400 lavaged cells was performed on Wright-Giemsa stained cytocentrifuge preparations and the remaining sample was centrifuged at 900*g* for 10 min to concentrate the cells. Following resuspension in PBS, the absolute cell number was determined using a hemocytometer.

Culture Conditions

For culture the macrophages were suspended in RPMI 1640 medium (obtained without L-glutamine) supplemented with 1.25 μg/mL amphotericin β, 200 IU/mL penicillin–streptomycin, 0.292 μg/mL L-glutamine (ICN Biomedicals Ltd, High Wycombe, UK), and 5% autologous F-344 serum. The serum was prepared from donor animals by removal of blood by cardiac puncture into cold glass containers. Following a period of 30 min at 4°C, the blood sample was centrifuged at 1000*g* for 10 min and the serum was removed. The serum was then recentrifuged at 1000*g* for 10 min and heat-treated for 25 min at 56°C. The alveolar macrophages were placed in 96-well cell culture dishes (Nunc, Gibco BRL, Paisley, UK) and kept in a humidified incubator at 37°C with 95% air and 5% CO_2. The minimal number of macrophages required for total phagocytosis was 20,000 cells/well, as demonstrated by Kreyling et al. [6]. On day 0 sufficient cells were added to ensure that this number remained on day 14. This gave an adherent cover of between 5 and 10% of the well bottom.

The change of medium and addition of $^{57}Co_3O_4$ particles followed the method of Kreyling et al. [6]. After an initial incubation of 2 h, the unattached cells were removed and 200 μL fresh RPMI medium containing antibiotics, serum, and the radioactive $^{57}Co_3O_4$ particles was added to each well. The number of particles added was such that an initial concentration of approximately 1 particle per cell was obtained. A comparable plate containing particles but no cells was incubated to use as a control.

Estimation of Intracellular Particle Dissolution (*IPD*)

At 6 time intervals over a 2-week period, measurements of the dissolved and particulate ^{57}Co fraction were made. This followed the techniques described by Kreyling in which the medium from 6 wells was carefully removed from the experimental plate and filtered with vacuum through a 0.22-μm cellulose acetate filter (Millipore Ltd, Watford, UK) [6]. The

adherent cells were then lysed by addition to each well of 200 μL Triton X (BDH Ltd, Poole, UK) and incubated at 37°C for 10 min. The subsequent suspension was passed through a further filter. Finally, the wells were thoroughly wiped with a cotton-tipped applicator stick. The filtrates of these samples contained the dissolved ^{57}Co, and the particulate fraction was found on the filters and applicator sticks. At the same time points, 6 wells from the control plate were also filtered. The samples were counted in a 3 in. well type gamma counter (LKB Wallac 1282 CompuGamma). Dissolution rates were calculated by determining the total fraction of ^{57}Co dissolved (in medium and in cells) at the various time points. Subtracting the fraction dissolved in the medium with no cells allowed a net (i.e., cell-dependent) dissolved fraction to be estimated.

Determination of Cell Survival and Viability

To monitor cell survival, the total number of adherent cells in a representative well was estimated over the 14 days using an inverted light microscope (Olympus, London, UK). To assess cell viability over the 14-day period, dye exclusion tests using propidium iodide and lysosomal staining with acridine orange were performed. The uptake 1.75-μm monodisperse fluorescent latex beads at a particle to cell ratio of 10:1 (Park Scientific Ltd, Northampton, UK) was used to estimate the fraction of macrophages that were phagocytic. Due to the slow uptake of these fluorescent beads in the F-344 rat macrophages, the percentage of macrophages containing 2 or more particles was not determined until 48 h after their addition. This cell function test was carried out 4 times during the 2 weeks of incubation. To confirm that the beads were inside the cells and not simply settling on the surface, macrophages were cultured in special dishes and observed with a confocal microscope system (Biorad-Lasersharp MRC-500) [11]. Optical sections (1 μm thick) were imaged through the cell to determine the location of the beads.

Electron micrographs of macrophages cultured over the time course of the study were also examined. Using the technique of Albertine and his coworkers, macrophages cultured without $^{57}Co_3O_4$ particles were fixed within the well of the 96-well plate [12]. Briefly, the medium was removed and 200 μL of 2.5% glutaraldehyde was added per well. After 1 h the cells were rinsed twice with sodium cacodylate prior to postfixing with 100 μL OsO_4 in sodium cacodylate for a further 1 h. After rinsing again, the cells were stained with 4% uranyl acetate in water for 1 h prior to washing in 50% ethanol for 30 min. The cells were then progressively dehydrated before being embedded in Poly/Bed 812 (Park Scientific Ltd, Northampton, UK) in a protective PTFE sleeve within the well.

Observations under electron microscopy (EM) were complemented with confocal images at various time points throughout the 14 days. Techniques used for lipid staining with nile red are well documented [13].

RESULTS

Bronchoalveolar Lavage

Of the 64 mL instilled during lavage, 61.2 ± 0.4 mL (mean ± SEM), equivalent to 95.8 ± 0.6%, was recovered. An average of $6.9 \pm 0.8 \times 10^6$ cells were lavaged, 88.2 ± 2.1% of which were shown by differential counting to be macrophages, 5.4 ± 1.5% red blood cells and platelets, and 7.1 ± 1.4% other types comprising mainly epithelial cells. Neutrophils and eosinophils were rarely seen and never accounted for more than 0.5 and 0.2% respectively.

Survival and Viability Studies

Preliminary studies had shown that the culture conditions were critical for the survival of F-344 rat macrophages. The eventual selection of heat-treated autologous serum for the incubation medium was one item of particular importance.

The number of adherent cells surviving at various time points throughout the 14 days, expressed as a percentage of numbers calculated for day 0, is given in Figure 1. During the frequent observations by light microscopy throughout the culture period, no dividing cells were observed. Although the cell numbers were considerably reduced, it was evident from viability studies that almost all of the cells remaining were viable (Table

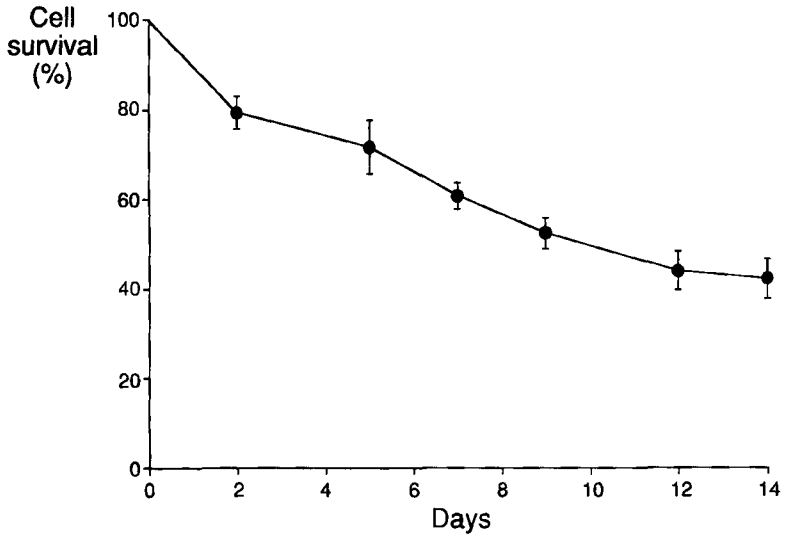

Figure 1 Survival of adherent cells over study period as a percentage of the initial cell number. Means (± SEM) for 9 experiments.

1). Staining with acridine orange showed an abundance of primary lysosomes in almost all macrophages throughout the time of incubation.

The uptake of fluorescent particles at different time points showed the ability of the macrophages to phagocytose particles. Figure 2 is a confocal image of an alveolar macrophage that has taken up fluorescent particles. Serial 1-μm optical sections through the cell showed the particles appearing and disappearing, demonstrating that they are within the cell cytoplasm.

At no time during incubation was a significant amount of cell debris observed, indicating that any dead cells had been phagocytosed by surviving macrophages. In a preliminary study, when latex particles were added at the same concentration as the $^{57}Co_3O_4$ particles, i.e., approximately 1 particle per cell, no free particles were observed between 2 and 14 days.

EM and Confocal Imaging over 14 Days

Electron micrographs studied over the 14-day period gave a detailed picture of the general condition of the cell and an indication of functional capacity. Figure 3 shows an alveolar macrophage 24 h after plating out. The cytoplasm was of an even density and contained all organelles seen in a typical macrophage. By day 7 (Figure 4), the number of cytoplasmic inclusions had increased, especially the lipid droplets. The lipid inclusions varied in density. Apart from them, the cell organelles appeared to be normal.

Table 1 Viability of alveolar macrophages determined over 14-day study period

Day	Propidium iodide exclusion (n = 9)	Acridine orange uptake[a] (n = 4)	Phagocytic fraction at 48 h[b] (n = 9)
0	79.6 ± 3.6	88.0 ± 3.8	86.5 ± 1.8
2	99.9 ± 0.3	93.3 ± 2.0	
5	99.1 ± 0.3	94.5 ± 0.9	91.4 ± 0.8
7	98.8 ± 0.4	96.3 ± 0.8	93.0 ± 0.9
9	98.7 ± 0.2	95.3 ± 1.6	
12	98.8 ± 0.4	97.3 ± 1.5	88.4 ± 1.9
14	99.0 ± 0.3	97.0 ± 1.4	

Note. Number of viable cells expressed as a percentage (mean ± SEM) of the total number at each time point. The phagocytic fraction was determined by the number of cells containing 2 or more particles as a percentage of the total number of cells.

[a]Acridine orange studies were carried out only on the last 4 studies.

[b]Fluorescent latex particles were added on days 0, 5, 7, and 12 and counted on days 2, 7, 9, and 14.

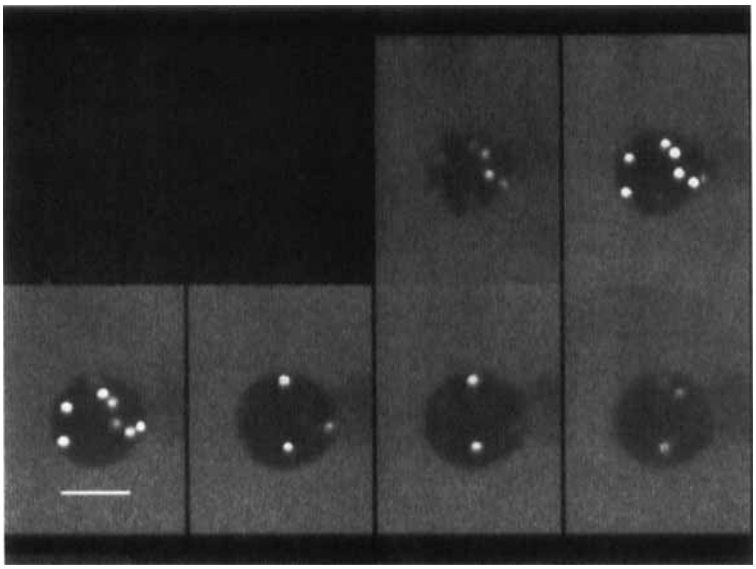

Figure 2 Confocal image through a single macrophage (serial 1-μm optical sections) from below the cell (attached to dish, top left) to the top of the cell (bottom right). The white spheres represent the 1.75-μm fluorescent latex beads. Note that some particles appear in successive optical sections, but none appear at the level of the dish, i.e., outside the cell. Size bar = 10 μm.

At 14 days (Figure 5), the number of lipid inclusions had again increased. There is evidence that some cells had increased in size, mainly due to the concentration of lipid droplets. Confocal imaging of macrophages stained for lipid supported the findings seen under EM (Figure 6). The cell was enlarged but the nucleus was clearly evident within the cytoplasm.

Intracellular Particle Dissolution

In the experimental plates (with cells) examined at different times during incubation, there was very little ^{57}Co on the filters after the initial filtration of the medium. Considerably more ^{57}Co was found in the filtrate. From the second filtration step, after the adherent cells had been lysed, most of the radioactivity was found on the filters. The soluble ^{57}Co in the lysate was frequently below the level of detection. This indicated that the majority of particles remained undissolved and were within the cells, whereas most of the dissolved ^{57}Co had left the cells. These results were also consistent with there being few nonadherent cells containing ^{57}Co and few free particles in the medium.

In Figure 7 the total dissolved fractions of $^{57}Co_3O_4$ obtained from each rat were averaged at each time point and plotted against time. The fraction of ^{57}Co dissolved in the medium alone (without cells) was shown to be very low. After the initial release of a small amount of ^{57}Co, the subse-

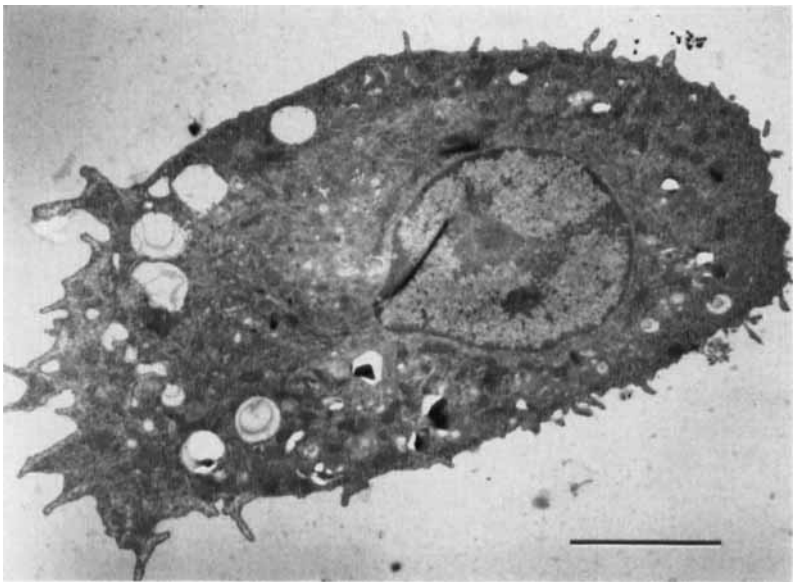

Figure 3 Electron micrograph of macrophage cultured for 24 h in vitro. Size bar = 5 μm.

quent dissolution rate throughout the 14 days of study was only 0.04% per day. However, the amount of ^{57}Co dissolved within the alveolar macrophage increased with time. The difference between the curves indicated the dissolution brought about by the macrophages. This net dissolved fraction increased with time (Figure 8). Following a model developed by

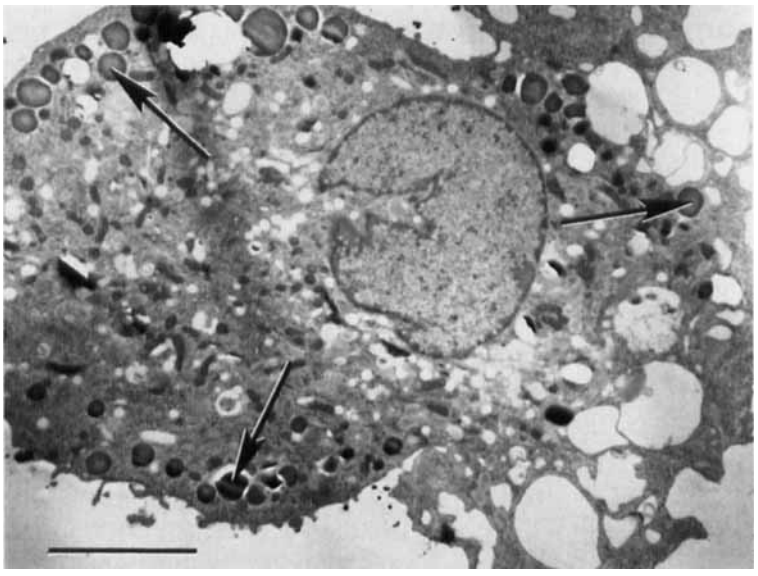

Figure 4 Electron micrograph of macrophage cultured for 7 days in vitro. Note lipid droplets (arrows). Size bar = 5 μm.

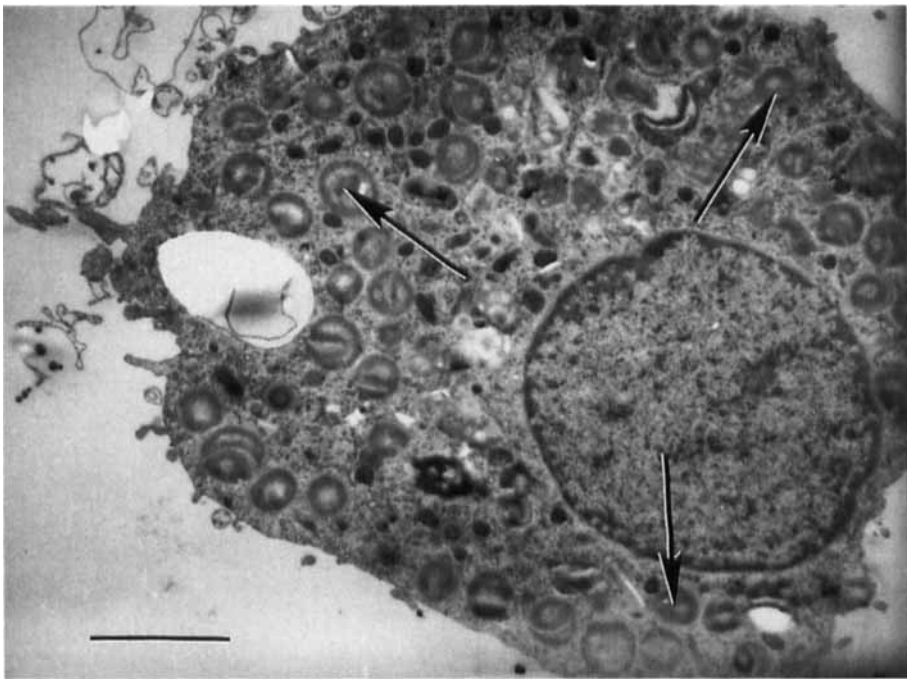

Figure 5 Electron micrograph of macrophage cultured for 14 days in vitro. There are numerous lipid droplets in the cell (arrows). Size bar = 5 μm.

Kreyling and coworkers for this type of porous particle [6], an expression was fitted to the net dissolved fraction (f):

$$f = 1 - e^{-kt}$$

where k is a constant and t is time (days). This provided a reasonable fit to the observed IPD. For the pooled data from 9 experiments k was determined as 0.0036 ± 0.0002 (mean ± SEM), i.e., the rate of intracellular dissolution in the macrophages was 0.36 ± 0.02% per day.

DISCUSSION

This study has shown that alveolar macrophages obtained from F-344 rats by bronchoalveolar lavage can be maintained as a nondividing population for 14 days and do dissolve cobalt oxide particles at a measurable rate. Rather more cells were lost over this period than had been observed with dog macrophages [6], reflecting the considerable difficulties experienced in culturing rodent alveolar macrophages. However, at the end of this period, the rat macrophages were still viable (propidium iodide exclusion), phagocytic (uptake of fluorescent particles), and showed an abundance of primary lysosomes (stained by acridine orange).

The lipid inclusions seen under EM appear as either pale or to varying

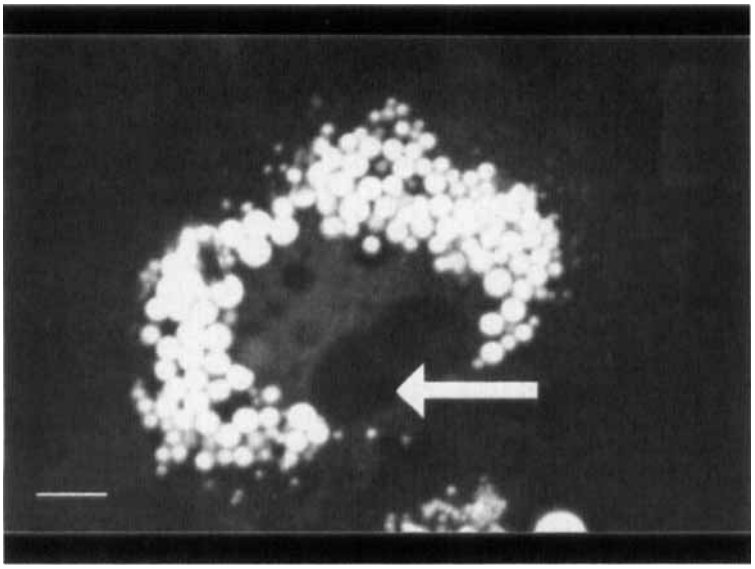

Figure 6 Confocal image of an enlarged macrophage at 14 days. The lipid is clearly visible (stained with nile red). The nucleus is also evident within the cytoplasm (arrow). Size bar = 10 μm.

degrees electron-dense. The density reflects the proportion of unsaturated fatty acids, which preferentially bind with the osmic acid [14]. The accumulation may be the result of the viable macrophages taking up the products of neighboring effete cells, or may reflect changes in the macrophages during culture in vitro, e.g., affecting arachidonic acid metabolism (cf. [15]). Note, however, that by all other criteria used, the surviving cells

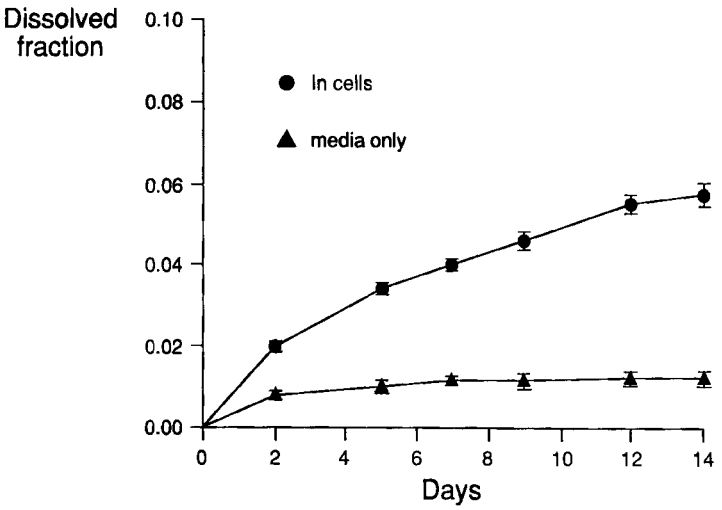

Figure 7 Mean (± SEM) of the dissolved fraction of $^{57}Co_3O_4$ obtained at each time point plotted against time. Mean of 9 experiments.

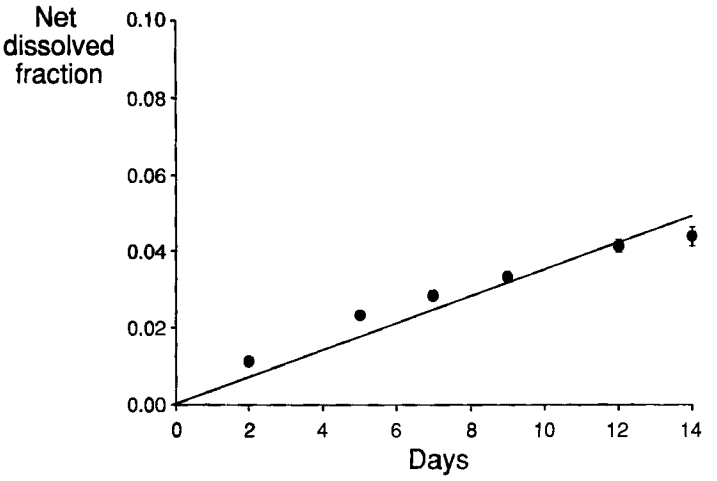

Figure 8 Mean (± SEM) of the net dissolved fraction of $^{57}Co_3O_4$. Data taken from Figure 7 and fitted to an exponential expression (see text).

remained viable throughout the 14-day period. Since they remained capable of phagocytosis, it is unlikely that the accumulation of lipid alone would affect the rate of IPD. This decreased only after day 12, whereas lipid was evident by day 7, but some effect of the lipid cannot be ruled out.

It was not possible to demonstrate by microscopy that 0.5-μm $^{57}Co_3O_4$ particles were quantitatively taken up by the macrophages. It may be concluded that this was the case and also that those $^{57}Co_3O_4$ particles that were released from macrophages that died during the 14 days of incubation were rephagocytosed by surviving macrophages, since

1. The number of cells remaining in the culture did not fall below that necessary to assure complete phagocytosis [6].
2. The studies with fluorescent latex particle uptake, especially using the confocal microscope, established that those particles were intracellular.
3. When latex particles where added to the cultured cells at the same concentration as the $^{57}Co_3O_4$ particles, no free latex particles were observed at any time.
4. No cell debris was observed, indicating that dead cells were also rephagocytosed.
5. When dog macrophages were incubated with larger (1.7-μm) $^{57}Co_3O_4$ particles, complete uptake was observed microscopically; latex particles were also completely phagocytosed [6].

The distribution of ^{57}Co when the medium was filtered and after the cells were lysed indicated that (1) a small fraction of the particles was progressively dissolved intracellularly, and (2) having dissolved the particles, the macrophages released almost all of the soluble ^{57}Co.

As with alveolar macrophages from the dog [6], $^{57}Co_3O_4$ particles

dissolved to a greater extent in macrophages than in culture medium without cells (Figure 7). The net dissolution rate was reasonably well fitted by the simple exponential expression (Figure 8), as shown earlier for dog macrophages by Kreyling [6], although there was some tendency for the rate to decrease more toward the end of the study period than predicted by the expression. The reason for this decrease is not known, but may, for example, reflect a change in phagolysosomal pH.

The estimated dissolution rate of 0.36 ± 0.02% per day is considerably lower than the 1.4 ± 0.05% per day obtained with macrophages from the beagle dog using the same test particles [7]. However, (1) we used slightly different culture conditions to ensure survival of the rat macrophages, (2) the rat macrophages accumulated lipid during the culture period, and (3) the two studies were not simultaneous or carried out in the same laboratory. Therefore, it cannot be concluded unequivocally that there is a difference in vitro between the F-344 rat and the beagle dog. Nevertheless, the experimental protocol was very similar for the two species, and the particles were from the same batch and stored dry before use.

Various factors may affect the rate of particle dissolution. Phagolysosomal pH in alveolar macrophages is approximately 5 [16–19]. It is this low pH that has been suggested as a mechanism by which alveolar macrophages dissolve particles [20–22]. Differences in phagolysosomal pH may be one explanation for the differing abilities to dissolve particles in vivo. Studies in the rabbit, dog, guinea pig, and baboon have failed to show significant differences in pH [23–24], but further studies are in progress; the pH of the Fischer rat macrophage has yet to be determined. Another possible factor could be endogenous chelating agents that bind dissolved cobalt and affect the rate of release from alveolar macrophages [25].

Further work with different species, as used in the comparison carried out in vivo, is under way. This is intended to show to what extent the species differences seen in vivo are entirely reproduced in macrophages cultured in vitro.

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