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Influence of Surfactant Components and Exposure Geometry on the Effects of Quartz and Asbestos on Alveolar Macrophages

by Jutta Schimmelpfeng,[1] Elisabeth Drosselmeyer,[2] Vera Hofheinz,[2] and Arnulf Seidel[2]

Bovine (BAM) and rat (RAM) alveolar macrophages were incubated *in vitro* with DQ12 quartz or UICC chrysotile asbestos either alone or in the presence of dipalmitoyl lecithin (DPL). The reaction of the cells of both species to the untreated dust particles was similar qualitatively and quantitatively, with a loss of viability and release of lactate dehydrogenase and *N*-acetyl-β-glucosaminidase after 20 hr of incubation. In the presence of DPL, the toxicity of quartz to BAM disappeared completely, whereas the protective influence of the phospholipid was distinctly diminished in the case of RAM. The presence of lavage fluid was less effective than that of pure DPL. There was no protective influence of DPL with asbestos either for BAM or for RAM. The effects of phagocytizable, suspended quartz particles were compared with the effects of the same type of particles fixed on a glass surface to exclude the possibility of phagocytosis. The effect of the suspended particles on the viability and release of enzymes was more pronounced than that of the fixed particles. On the other hand, superoxide anion production was stimulated to a much higher degree by the fixed quartz particles. This could be explained by the continuing contact of the outer cell membrane with the silica surfaces, whereas free particles were rapidly phagocytized. The release of lysosomal enzymes induced by fixed quartz particles was a secondary phenomenon following cell death.

Introduction

The inhalation of quartz and asbestos results in pathological changes in the lung. Alveolar macrophages play a central role in lung defense mechanisms and in the development of lung fibrosis. Investigations concerning the cytotoxic effects of quartz, asbestos, and other materials have been performed *in vitro* with macrophages from different species (*1–8*).

The purpose of our studies was to compare the cytotoxic effects of native and surface-modified DQ12 quartz and chrysotile asbestos on alveolar macrophages of the rat (RAM) and cattle (BAM). *In vitro* studies with native dusts may not always be realistic, as dust particles inhaled *in vivo* become coated with protective lung surfactant. Surfactant can be isolated from lavage fluid (LF), which is composed of proteins and lipids, mainly L-α-dipalmitoyl lecithin (DPL). The incubation of cells with dust in the presence of either lavage fluid or DPL represents a more realistic test system. To compare the protective effects of BAM and RAM, a well-defined concentration of DPL was used instead of lavage fluids from the rat or cattle with their different compositions. Wallace et al. (*4*), using short-term cultures, showed that DPL-coated quartz particles are nontoxic to RAM. We have extended their studies by examining the influence of DPL on the cytotoxicity of DQ12 quartz and chrysotile asbestos with alveolar macrophages of two different species over longer periods of time.

The second part of our studies dealt with the site of action of the quartz particles. There is no doubt that quartz is membranolytic and can damage the pericellular membranes, e.g., of erythrocytes. On the other hand, the particles are normally phagocytized rapidly and included in lysosomes; the damage of lysosomes is attributed to the cytotoxic effects of silica (*1,5*). One of the arguments proposed for this hypothesis is the release of lysosomal enzymes after incubation of macrophages with quartz. Another possibly important reaction of the macrophages with quartz is the production and release of reactive oxygen species (*6,7*). In the second part of this paper, the effects of quartz on the pericellular membrane are distinguished from those on intracellular sites, i.e., lysosomes. For this purpose, we developed an exposure system in which alveolar macrophages are in direct contact with silica particles but are unable to phagocytize them. In other experiments, the same type of particles were given to the cells in suspension to allow rapid phagocytosis. We also hoped

[1]Kernforschungszentrum Karlsruhe, Hauptabteilung Sicherheit/Biophysik, Postfach 3640, D-7500 Karlsruhe, Federal Republic of Germany.
[2]Kernforschungszentrum Karlsruhe, Institut für Genetik und Toxikologie, Postfach 3640, D-7500 Karlsruhe, Federal Republic of Germany.
Address reprint requests to A. Seidel, Institut für Genetik und Toxikologie, Kernforschungszentrum Karlsruhe, Postfach 3640, D-7500 Karlsruhe 1, Federal Republic of Germany.

to clarify to what extent the release of lysosomal enzymes indicates either cell damage in general or damage to lysosomes in particular.

Materials and Methods

Alveolar Macrophages

Alveolar macrophages of rats and cattle were obtained by postmortem lavage (*9,10*). Cultures of 1–3 × 10^6 viable cells were maintained in 35-mm plastic dishes (multiwell plates, Falcon) in 1 mL RPMI 1640 medium (Gibco, Paisley, UK) with 100 U/mL penicillin and 100 μg/mL streptomycin. After a 2-hr incubation period (37°C, 5% CO_2, 95% humidified air), the nonadherent cells were removed by a change of medium.

The experiments concerning superoxide anion production were performed with the BAM cultured in rectangular plastic dishes (26 × 33 mm, Flow), into which normal or pretreated cover slips were placed (24 × 32 mm). Viable cells, 6 × 10^6, in 2 mL of medium were put into the dishes and the nonadherent cells were removed after 30 min, as described above.

Mineral Dusts

The international standard quartz, DQ12 (*11*), was received from N. H. Seemayer (Medizinisches Institut für Umwelthygiene, Düsseldorf, FRG). The mean geometric diameter of the particles was determined as 0.4 ± 0.2 μm by image analysis of scanning electron microscopic pictures. This corresponds to an aerodynamic diameter (AMAD) of about 0.7 μm.

Canadian UICC chrysotile A asbestos was supplied by K. Spurny (Fraunhofer-Institut, Schmallenberg-Grafschaft, FRG). Approximately 95% of the fibers had a geometric diameter of less than 0.5 μm, while 97% were shorter than 5 μm. For the second type of experiments with fixed or suspended quartz and superoxide anion production, Sikron F 600 quartz (PALAS, Karlsruhe, FRG) was used. The median diameters were 0.1–0.3 μm
(± 0.2 μm) for the particles in suspension culture and 0.2–0.5 μm (± 0.2 μm) for the fixed particles, as described above. The particles used in these two exposure geometries were similar in size (AMAD = 0.2–0.8 μm). Sikron F 600 has the same qualitative effects on alveolar macrophages *in vitro* as DQ12 quartz or min-U-sil (*12*). The quartz particles were suspended in medium and the suspensions sonicated before use.

Fixing Sikron F 600 Quartz on Glass Cover Slips

To fix quartz on cover slips, a resin (Eukitt, Kindler, Freiburg, FRG) was used that is a solution of an acrylic in xylene. This solution was further diluted with xylene 1:2 and then applied uniformly to cover slips to obtain a very fine film. The coated cover slips were then pressed into a quartz dust layer. Thereafter, the cover slips were dried overnight at 60°C. The loosely bound particles were removed by compressed air and the amount of bound quartz was determined by weight analysis. To compensate for particle sedimentation, the reaction period for superoxide anion release was 90 min in the case of suspended quartz as compared to 60 min only for the incubation with fixed particles.

Exposure of Cultures

To determine the cytotoxic effects of DQ12 quartz and chrysotile asbestos, the cells were incubated with 250 μg dust/mL, corresponding to 26 μg/cm², for 20 hr. Separate experients were performed in the presence of L-α-dipalmitoyl lecithin (333 μg/mL), bovine serum albumin (BSA; 5 mg/mL), or lavage fluid. DPL, BSA, and LF alone had no influence on the viability of cells. The three solutions were sonicated on ice before use. LF contained about 14 μg/mL of disaturated phosphatidylcholines, as determined by W. Bernhard (Poliklinik, Philipps-Universität, Marburg, FRG). The final DPL concentration *in vitro* was 7 μg/mL and that of total protein about 4 μg/mL.

The effects of Sikron F 600 quartz on viability and enzyme release into the medium were tested after 20 hr of incubation, either with quartz added as a suspension (500 μg/mL, corresponding to 90 μg/cm²) or after plating the cells on the quartz fixed on cover slips.

Cell Viability and Enzyme Release

After 20 hr of incubation, cell viability was tested by the trypan blue dye exclusion test (0.25% in physiological saline, 3 min). For biochemical analysis (measurement of LDH, *N*-acetyl-β-glucosaminidase [NAG] and total protein), the cell cultures were

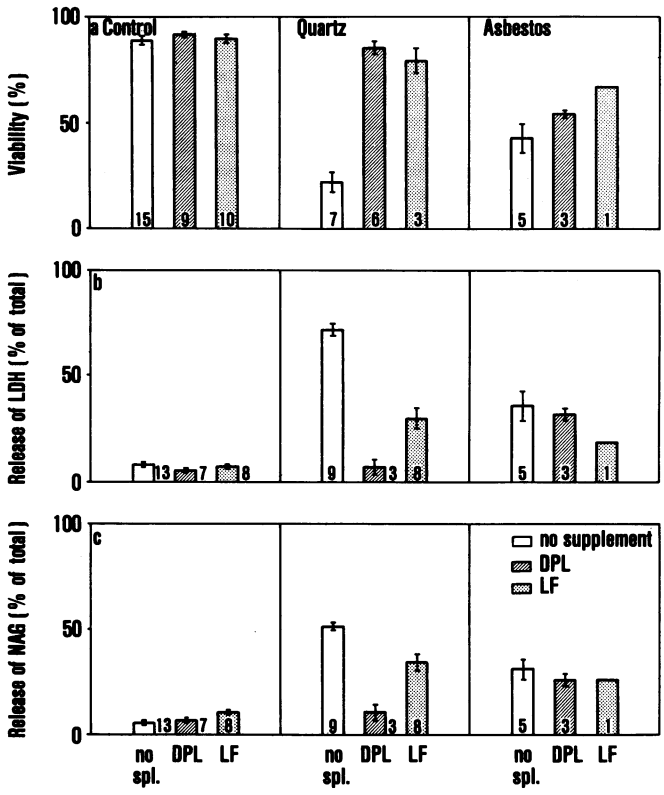


FIGURE 1. Cytotoxic effects of native and surface-modified DQ12 quartz and chrysotile asbestos (250 μg/mL) after 20 hr of exposure to bovine alveolar macrophages. (*a*) Viability, (*b*) release of lactate dehydrogenase (LDH), and (*c*) *N*-acetyl-β-glucosaminidase (NAG) are shown as arithmetic means ± SE. The figures at the base of each bar represent the numbers of individuals tested. No spl., no supplement during incubation; DPL, LF, dipalmitoyl lecithin or lavage fluid, respectively, present during incubation.

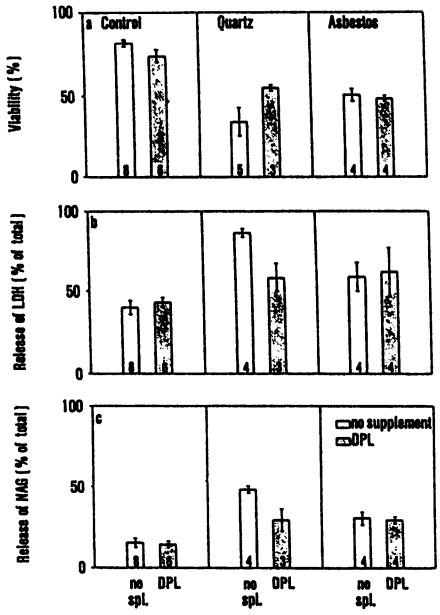


FIGURE 2. Cytotoxic effects of native and dipalmitoyl lecithin (DPL)-coated DQ12 quartz and chrysotile asbestos (250 μg/mL) after 20 hr of exposure to rat alveolar macrophages. (*a*) Viability, (*b*) release of lactate dehydrogenase (LDH) and (*c*) *N*-acetyl-β-glucosaminidase (NAG). For further explanation, see Figure 1.

treated as follows. The culture medium was centrifuged to remove nonadherent cells and cellular debris from the supernatant to form a pellet. The adherent cells were removed by scraping. The three fractions (culture medium, pellet, and cells) were sonicated before measurement of LDH (LDH-Monotest, Boehringer, Mannheim, FRG) NAG (*13*), and total protein (Bio-Rad Assay, Bio-Rad Laboratories, Munich, FRG). The release of enzyme into the supernatant (Figs. 1 and 2) is presented as a function of the total enzyme content measured in the three fractions.

Superoxide Anion Production

For the experiments concerning superoxide anion release, the cells were kept on ice for 90 min before being plated into the culture dishes (6×10^6 cells per dish). After 30 min at 37°C, the nonadherent cells were removed, and the reaction mixtures were added. We assayed the superoxide anion production by the cytochrome C reduction according to Johnston (*14*), with slight modifications. The quartz particles were suspended in the reaction mixtures. Opsonized zymosan (Los Alamos Diagnostics, Los Alamos, NM) was used as a positive control stimulus. After 60 or 90 min, the reaction mixtures were removed and centrifuged at 1000g for 5 min before photometry. For scanning electron microscopy, the cells were fixed in 25% glutaraldehyd/4% osmium tetroxide in cacodylate buffer, dehydrated in ethanol, and then treated with hexamethyldisilazane (*15*).

Results

Influence of Surfactant Components

After 20 hr of incubation *in vitro*, unexposed control macrophages from rats and cattle were similar, except that rat cells released larger amounts of intracellular LDH (Figs. 1*b* and 2*b*) into the culture medium. This phenomenon reduces the differences in this parameter existing between untreated control cells and macrophages treated with dust.

The reaction of BAM and RAM to the toxic dusts was the same, both qualitatively and quantitatively (Figs. 1 and 2). After 20 hr of incubation with DQ12 quartz (250 μg/mL), nearly all cells were damaged as shown by the decrease of cell viability and by the release of cytoplasmic LDH and lysosomal NAG into the culture medium. After the addition of 250 μg chrysotile asbestos/mL, the release of LDH (Figs. 1*b* and 2*b*) and NAG (Figs. 1*c* and 2*c*) and the decrease in cell viability (Figs. 1*a* and 2*a*) were less pronounced.

Incubation of BAM with quartz in the presence of DPL eliminated its cytotoxic effect altogether (Fig. 1). The degree of the protective action of DPL varied between the species. RAM were protected to a lesser degree (Fig. 2). On the other hand, simultaneous incubation of cells with DPL and chrysotile asbestos did not reduce fiber cytotoxicity in either species (Figs. 1 and 2), not even in the presence of higher DPL concentrations (data not shown).

The protective effects of LF and BSA were only tested with bovine cells. Lavage fluid also reduced the cytotoxicity of quartz (Fig. 1), but this effect was reversible, in contrast to that of DPL (Fig. 3). BSA had no influence on the cytotoxicity of quartz and asbestos (*10*).

Influence of Exposure Geometry

The plating efficiency did not depend on the nature of the surface on which the cells were put, amounting to 90% for both untreated resin-coated and resin-plus-quartz-coated cover slips. The morphological appearance of the surface and the cells can be seen in Figure 4. Figure 4*A* shows the quartz particles fixed on the cover slips and they can also be discerned between the cells in Figure 4*B* (BAM on fixed particles). The cytotoxic effects of suspended quartz on the cells are clearly visible in Figure 4*C*. On the other hand, the macrophages plated on the surface with fixed quartz look quite normal, even after 20 hr (Fig. 4*B*). The viability results are in agreement with these morphological observations (Fig. 5). Coating the glass surface of the cover slip with the resin in itself has no cytotoxic effect (Fig. 5). The viability of the cells on fixed quartz is still twice as high as after phagocytosis of the particles (note that similar dust surface densities

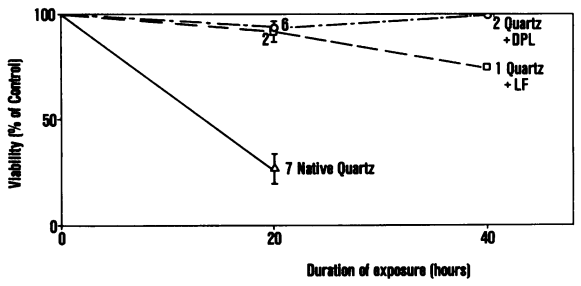
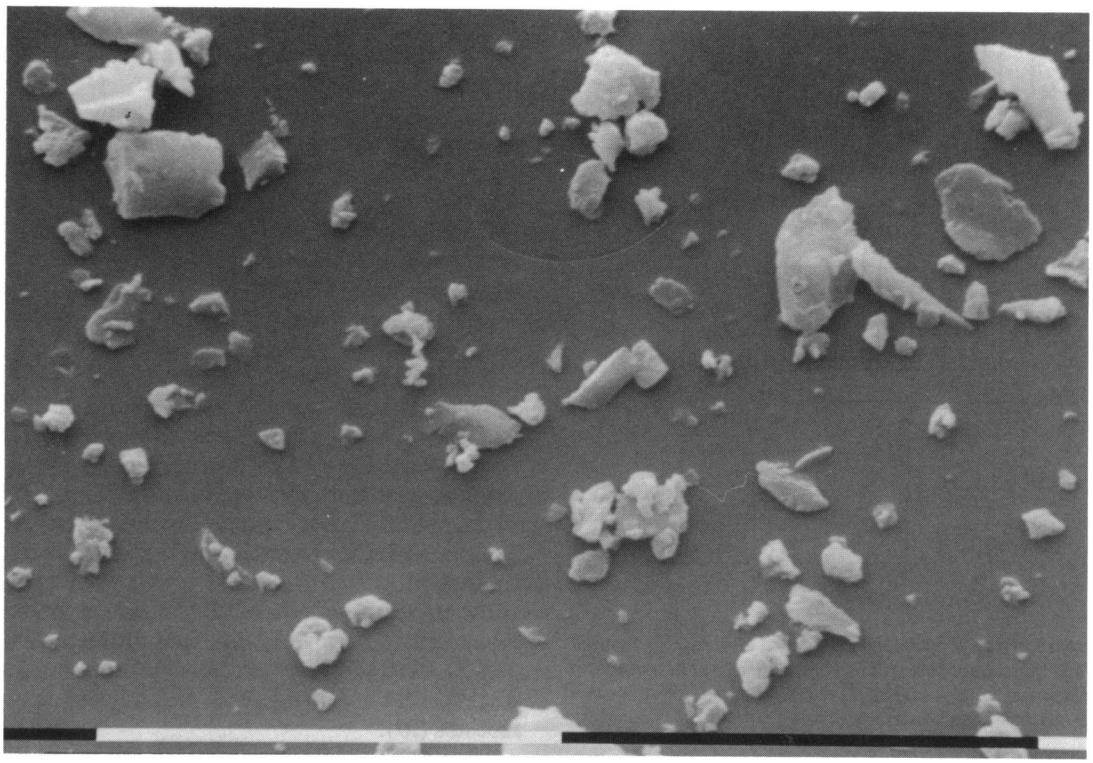


FIGURE 3. Viability of bovine alveolar macrophages after 20 hr of incubation with DQ12 quartz (250 μg/mL) in the presence or absence of lavage fluid (LF) or dipalmitoyl lecithin (DPL). Arithmetic means ± SE and the number of individuals tested are shown. The 40-hr value for quartz plus LF has been confirmed by additional results with different quartz concentrations (*10*).



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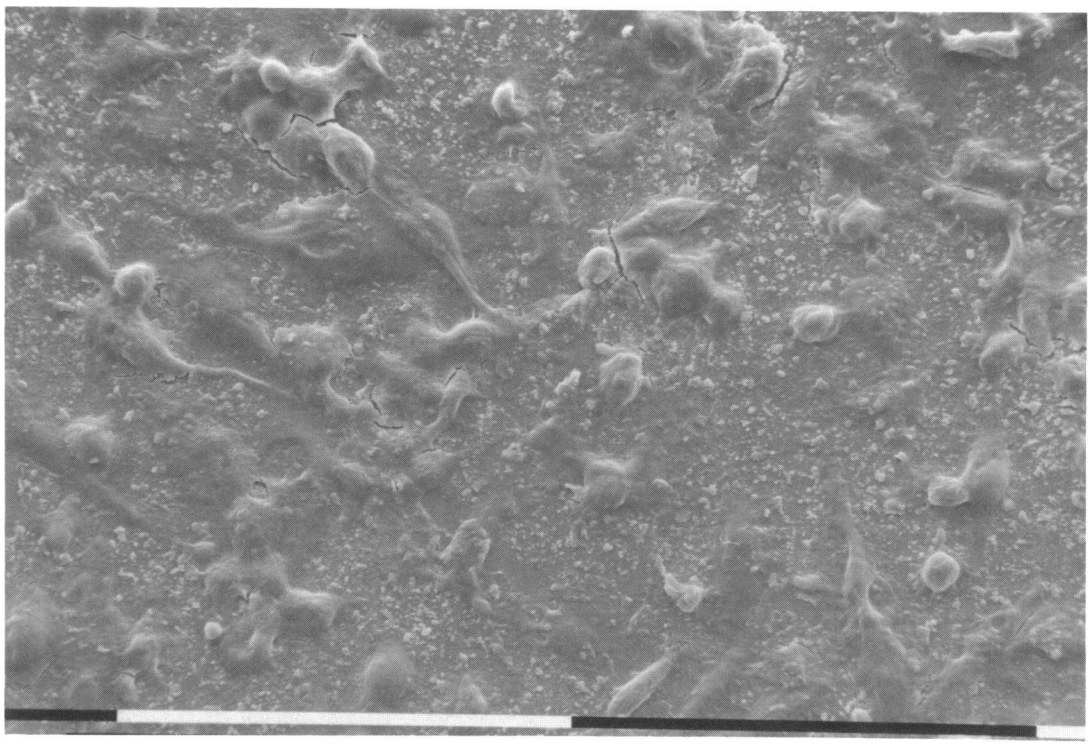
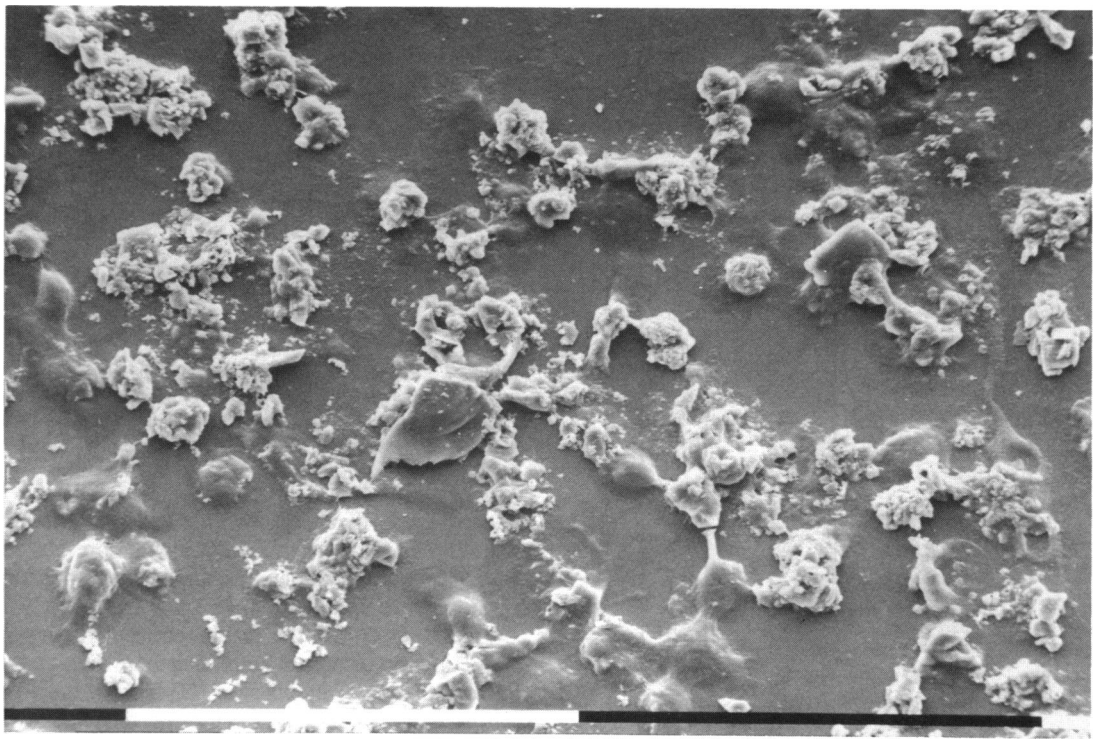


B

FIGURE 4. (*A*) Scanning electron micrograph of Sikron F 600 quartz particles fixed on a cover slip. Bar = 10 μm. (*B*) Bovine alveolar macrophages after 20 hr of culturing on fixed quartz particles (50 μg/cm²). Bar = 100 μm. (*Continued on next page.*)



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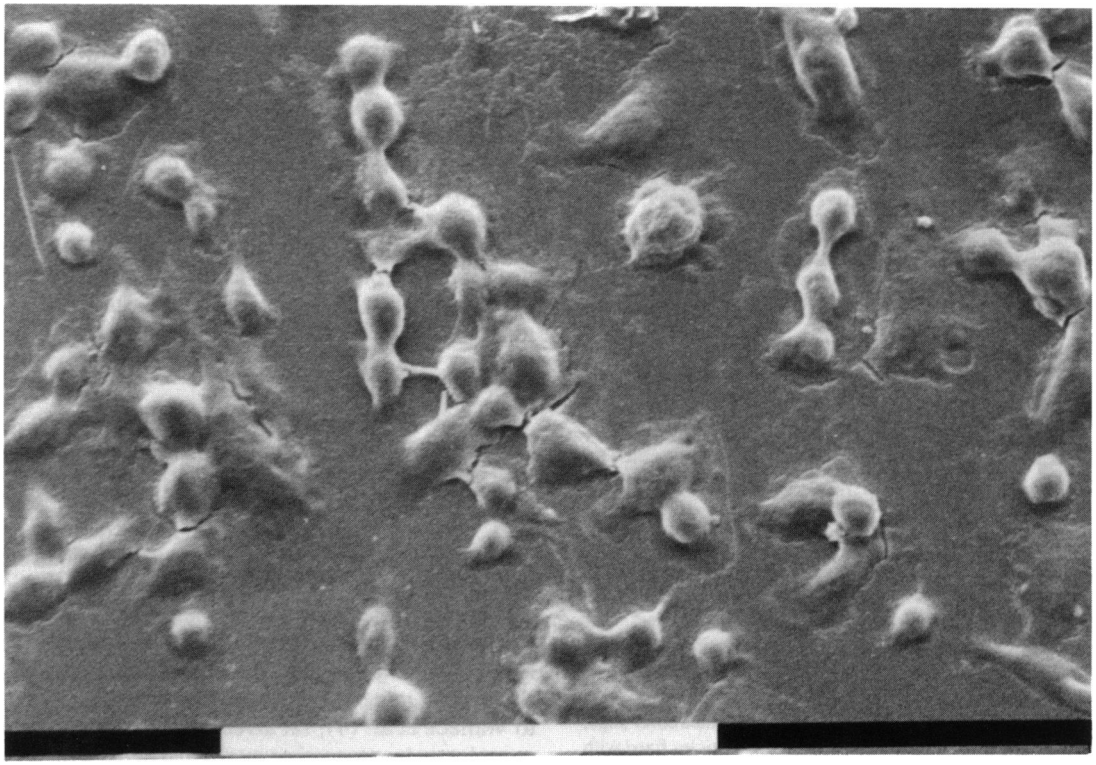

D

FIGURE 4. *Continued.* (*C*) Bovine alveolar macrophages, most of them heavily damaged, after 20 hr of culturing with quartz that had been suspended in culture medium ($\cong$ 90 $\mu g/cm^2$). Bar = 100 μm. (*D*) Controls on resin-coated cover slip. Bar = 100 μm.

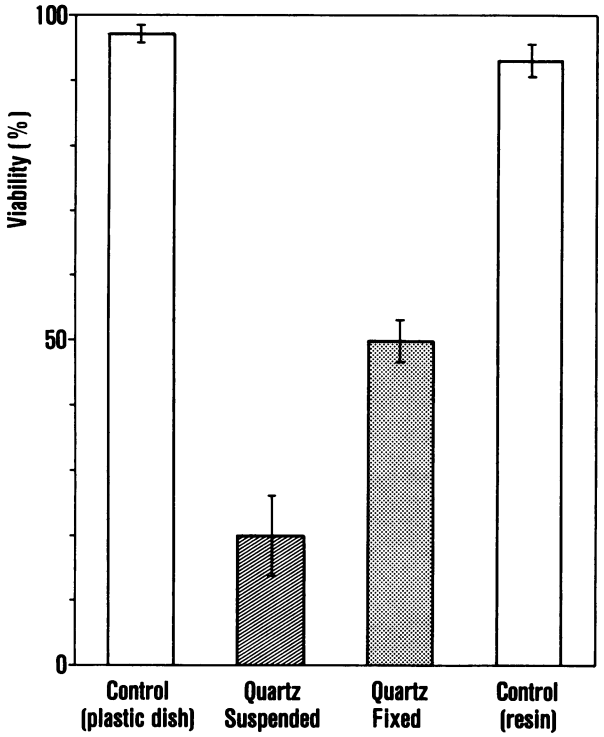

FIGURE 5. Viability of bovine alveolar macrophages after 20 hr of culturing under various conditions. Control (resin): culture on resin-coated cover slip. Quartz experiments with 100 μg quartz/cm^2. Arithmetic means with SEs from three experiments.

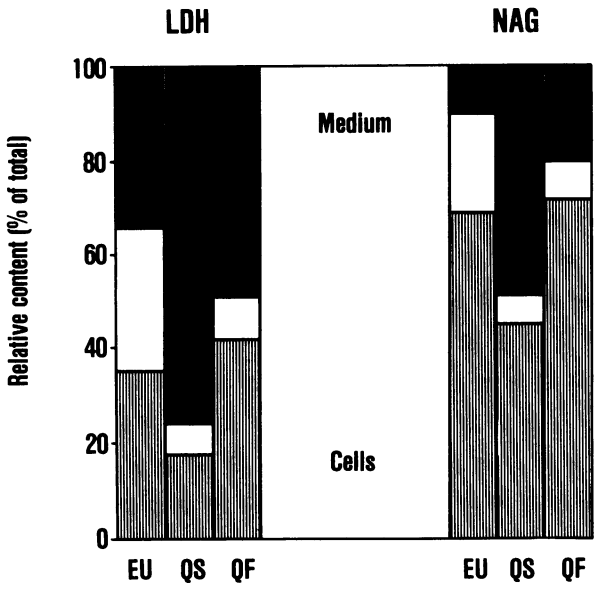

FIGURE 6. Influence of culturing conditions on the distribution of lactate dehydrogenase (LDH) and *N*-acetyl-β-glucosaminidase (NAG) between cells and culture medium after 20 hr of incubation of bovine alveolar macrophages with Sikron F 600 quartz particles. EU, controls on resin-coated cover slips; QS, quartz particles suspended in medium; QF, quartz particles fixed with resin on cover slips. For QS and QF, 90 μg quartz/cm^2. The white area between "cell" and "medium" corresponds to the enzyme content of the 250-g pellet resulting after the supernatant (detached cells and debris) has been centrifuged off the culture. Arithmetic means of three experiments. SE is omitted for greater clarity (~ 6% of the means).

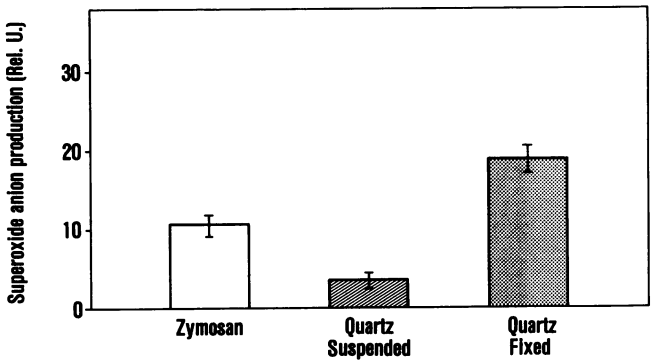

FIGURE 7. Production of superoxide anion by bovine alveolar macrophages after 60 min (zymosan, quartz fixed) or 90 min (quartz suspended) of incubation with particles. "Quartz suspended" corresponds to 90 μg/cm^2; "quartz fixed" corresponds to 50–100 μg/cm^2. Arithmetic means with SE of four experiments.

are compared). Figure 6 shows the release of LDH and NAG into the culture medium after 20 hr of incubation. The results parallel those of the viability assay, suspended quartz particles clearly being more toxic than fixed ones.

When considering the results concerning superoxide anion production, one must not forget that these data are based on much shorter periods of incubation (60 and 90 min, respectively). Compared to the effects of zymosan, those of quartz particles, which can be phagocytized, are comparatively small (Fig. 7). On the other hand, fixed quartz induces an even greater release of superoxide anions than the standard challenge with zymosan.

Discussion

One of our aims was to compare the sensitivity to quartz and chrysotile asbestos of alveolar macrophages from two different animal species. According to our results, there are no systematic differences between the species tested.

In contrast to untreated bovine cells, control cells from rats released large amounts of LDH into the supernatant, similar to the results of Pasanen and co-workers (*16*), where the release of LDH also did not correlate with any cellular damage. Other groups have reported lower LDH in the culture medium (*17,18*). We suggest that the rat cells synthesized and secreted these amounts of LDH because they definitely contained more LDH and less NAG than alveolar macrophages from other species after 20 hr of incubation (*10*).

The extent of the protective action of DPL varied according to species. Incubation of BAM with DQ12 quartz in the presence of DPL led to the complete disappearance of the cytotoxic quartz effect. No protection was found when BAM were cultured with DPL, followed by incubation with native quartz (*10*). Therefore, the protective effect of DPL must be due to a direct interaction between DPL and the surface of the quartz particles. Our study was primarily descriptive and, therefore, cannot explain the differences in the magnitude of the protective DPL effect observed between the two species. These differences may be due to variations in the rate of digestion by lysosomal enzymes. According to Wallace et al. (*4*), rat alveolar macrophages were protected completely by precoating quartz particles with DPL and short-time exposure to the cells (2 hr). The DPL-coated quartz particles were then retoxified in phagolysosmes of the rat cells (*19*). In contrast, DPL- precoated quartz remained nontoxic in BAM for at least 70 hr (*20*).

The protective effect of lavage fluid decreased after 20 hr for reasons not yet known. One explaination could be that DPL is the most effective protective component, and its concentration in the lavage fluid was too low to protect the cells for more than 20 hr. The magnitude of the protective effect of LF in BAM was similar to the results obtained by Emerson and Davis (*17*) on rat alveolar macrophages. In those studies, the quartz particles were preincubated with concentrated LF before addition to the cells. The mixed composition of the LF may also play may a role in the reduced protection by LF in comparison to pure DPL solutions.

The incubation of macrophages with DPL and chrysotile asbestos had absolutely no protective effect in any species. According to Bignon and Jaurand (*21*), DPL is adsorbed onto the surface of chrysotile asbestos. The toxicity of chrysotile asbestos may thus depend on a parameter other than its surface. BSA was not found to be protective at all, which is similar to the finding of Tilkes and Beck (*22*).

Regarding the second objective of our studies, the question of intracellular versus extracellular sites of quartz-induced cell damage, some conclusions can be drawn. There is no doubt that an exclusively extracellular mode of cytotoxicity exists for macrophages. Such a "primary" or "immediate" reaction of macrophages to quartz has already been postulated by Münch et al. (*23*) and Robock et al. (24). With regard to its influence on viability and enzyme release, fixed quartz is distinctly less toxic than suspended quartz particles. Impaired cell contact with the particles could be a reason, but the plating efficiency was normal on quartz-covered surfaces. The question arises whether the quartz surfaces might have been covered accidentally by the resin. However, DPL pretreatment of the surfaces of fixed quartz, in analogy to the experiments described above, made all cytotoxic effects disappear completely, which indicates that there were indeed free surfaces. More plausibly, the less pronounced effect of fixed quartz on viability and enzyme release could be due to the reduction in the total quartz surface with which the cells could come into contact. It can be estimated that for fixed quartz of the same particle size as in suspension culture, only about half of the surface area can come into contact with the macrophages.

The results regarding superoxide anion production are opposed to those concerning cytotoxic effects, as fixed quartz was much more effective than suspended particles. This result could be explained by assuming that the continuous contact of the quartz surfaces with the outer cell membrane permanently triggers the respective receptor system, whereas free particles are rapidly taken up by phagocytosis. In any case, viability remained at 85% during the 60–90 min of incubation. Thus, cellular death alone cannot explain the low superoxide anion production. We have shown, using latex particles, that phagocytosis as such does not cause superoxide anion production by BAM (unpubished data).

Another conclusion from these results concerns the release of lysosomal enzymes. Though it is obvious that quartz particles were not in contact with lysosomes inside the macrophages, NAG was released into the the medium. In this case, lysosomal leakage is not proof of lysosomal damage as the primary event, but follows cellular death.

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