Dissociation of Intracellular Lysosomal Rupture from the Cell Death Caused by Silica

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ABSTRACT
The relationship between intracellular lysosomal rupture and cell death caused by silica was studied in P388D1 macrophages. After 3 h of exposure to 150 μg silica in medium containing 1.8 mM Ca²⁺, 60% of the cells were unable to exclude trypan blue. In the absence of extracellular Ca²⁺, however, all of the cells remained viable. Phagocytosis of silica particles occurred to the same extent in the presence or absence of Ca²⁺. The percentage of P388D1 cells killed by silica depended on the dose and the concentration of Ca²⁺ in the medium. Intracellular lysosomal rupture after exposure to silica was measured by acridine orange fluorescence or histochemical assay of horseradish peroxidase. With either assay, 60% of the cells exposed to 150 μg silica for 3 h in the presence or absence of Ca²⁺ showed intracellular lysosomal rupture, whereas cell death occurred only in the presence of Ca²⁺. Intracellular lysosomal rupture was not associated with measurable degradation of total DNA, RNA, protein, or phospholipid or accelerated turnover of exogenous horseradish peroxidase. Pretreatment with promethazine (20 μg/ml) protected 80% of P388D1 macrophages against silica toxicity although lysosomal rupture occurred in 60-70% of the cells. Intracellular lysosomal rupture was prevented in 80% of the cells by pretreatment with indomethacin (5 x 10⁻⁵ M), yet 40-50% of the cells died after 3 h of exposure to 150 μg silica in 1.8 mM extracellular Ca²⁺. The calcium ionophore A23187 also caused intracellular lysosomal rupture in 90-98% of the cells treated for 1 h in either the presence or absence of extracellular Ca²⁺. With the addition of 1.8 mM Ca²⁺, 80% of the cells was killed after 3 h, whereas all of the cells remained viable in the absence of Ca²⁺. These experiments suggest that intracellular lysosomal rupture is not causally related to the cell death caused by silica or A23187. Cell death is dependent on extracellular Ca²⁺ and may be mediated by an influx of these ions across the plasma membrane permeability barrier damaged directly by exposure to these toxins.
sitzing dyes (3), viruses (2), and myocardial ischemia (12).

Although intracellular lysosomal rupture has been documented in these systems, the evidence that it causes cell death is only circumstantial. Two conditions must be met before a causal relationship between lysosomal rupture and cell death can be inferred. Lysosomal enzyme release must occur before cell injury becomes irreversible, and the mechanism whereby such release leads to irreversible injury must be identified. Rupture of lysosomes occurs after irreversible injury has developed in the hepatotoxicity produced by carbon tetrachloride, thioacetamide, dimethylnitrosamine, and sporidesmin (35). In these cases, the late release of lysosomal enzymes simply accompanies lysis or enzymatic removal of dead cells. Numerous other examples of such a scavenging function of lysosomal enzyme release have been reviewed by Van Lanker (40). Lysosomal rupture is temporally related to cell death in the toxicity of silica to macrophages (1, 27). However, in this case the biochemical and functional consequences of intracellular lysosomal rupture have not been identified. Even when a specific biochemical change has been described, e.g., an increased level of lysolecithin (26), it is again not clear whether this is responsible for the irreversible injury or whether it follows the cell death.

We have developed a new approach to the analysis of this problem (23, 32). By removing Ca\(^{2+}\) ions from the culture medium, it was possible to dissociate the interaction of several quite distinct toxins with cultured hepatocytes from the cell death produced by these agents. The killing of these hepatocytes could be separated into, at least, two distinct steps. The first represents plasma membrane damage, which disrupts the normal permeability barrier to Ca\(^{2+}\) ions. The second is a lethal influx of these ions into the cell across the damaged plasma membrane and down a steep electrochemical gradient. In the present paper, we have used such a system to assess the role of intracellular lysosomal rupture in cell death. In cultures of mouse P388D, macrophages, intracellular lysosomal rupture occurs after exposure to either silica particles or the calcium ionophore A23187 in the presence or absence of extracellular Ca\(^{2+}\) ions; however, cell death occurs only in their presence. In addition, biochemical consequences of the release of lysosomal enzymes in the presence or absence of extracellular Ca\(^{2+}\) could not be identified. It is concluded that intracellular lysosomal rupture is neither necessary nor sufficient for the cell death produced by these toxins.

MATERIALS AND METHODS

Cell Cultures

The P388D, mouse macrophage cell line was a gift from Dr. Peter Ralph at the Sloan-Kettering Institute, New York. A subclone was isolated and used for all of the experiments described below. Stock cultures were checked for mycoplasma contamination twice monthly using Hoechst 33258 (8). P388D, macrophages were routinely grown in monolayer in Dulbecco's high glucose modification of Eagle's minimal essential medium (Grand Island Biological Co., Grand Island, N.Y.) containing 5% heat-inactivated fetal calf serum (Flow Laboratories, Inc., Rockville, Md.) at 37°C in an humidified atmosphere of 5% CO\(_2\)-95% air. Cultures were routinely grown in monolayer in Dulbecco's high glucose modification of Eagle's minimal essential medium (Grand Island Biological Co., Grand Island, N.Y.) containing 5% heat-inactivated fetal calf serum (Flow Laboratories, Inc., Rockville, Md.) at 37°C in an humidified atmosphere of 5% CO\(_2\)-95% air.

As an extension of this approach, we have examined the effects of calcium on the survival of macrophages incubated in vitro with silica or the calcium ionophore A23187.

Cell Injury Protocols

After 15-20 h of culture, the P388D, macrophages were rinsed four times with prewarmed Hanks' balanced salt solution minus calcium (Flow Laboratories, Inc.) and placed in Williams' medium E without CaCl\(_2\) (made under special order by Flow Laboratories, Inc.) or serum. The concentration of calcium in this medium was less than 20 nM as measured by atomic absorption spectroscopy. Where indicated, CaCl\(_2\) was added to give a final concentration of 1.8 mM. Untreated cultures in the absence of calcium showed no change in viability compared with cells in the presence of calcium for up to 3 d. The cells were treated with particles of silica or titanium dioxide or the calcium ionophore A23187 (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.) or crystalline silica (quartz) or titanium dioxide (TiO\(_2\)) prepared according to the specifications of the Union Internationale Contre Le Cancer (38), were obtained from the Pneumoconiosis Research Unit, Johannesburg, Union of South Africa. Samples were weighed, suspended in calcium-free phosphate-buffered saline at a concentration of 1-2 mg/ml, sonicated, and autoclaved before use. A23187 was dissolved in dimethylsulfoxide (DMSO) at 1 mg/ml and stored in the dark at -20°C. It was added to cultures at a final concentration of 10 \(\mu\)g/ml. Where indicated, cells were pretreated for 1 h at 37°C with indomethacin (5 \(\times\) 10^{-3} M) or promethazine (20 \(\mu\)g/ml) and washed two times with Hanks' balanced salt solution before exposure to either silica or A23187. Indomethacin (Sigma Chemical Co., St. Louis, Mo.) was dissolved in DMSO at a concentration of 30 mg/ml and stored at -20°C. Promethazine (Wyeth Company, Philadelphia, Pa.) was stored as a stock solution at 25 mg/ml of Hanks' balanced salt solution minus calcium until use.

Viability Assays

Three assays of cell viability were used. Routinely, cells attached to 12-mm cover slips in 1.6-cm multwells were examined under an inverted phase-contrast microscope within 10 min of exposure to trypan blue (0.01%). The number of attached cells that excluded trypan blue was counted using a 10-mm eyepiece grid at \(\times\) 100 or \(\times\) 200 magnification. Trypan blue exclusion was expressed as the percentage of the number of unstained cells in untreated cultures. Parallel cover slips were treated with fluorescein diacetate and examined immediately for fluorescence as described by Rotman and Papamaster (31). At least three separate cultures were assayed for each treatment, and three to five fields per culture counted for viability. All data are expressed as the mean \(\pm\) SD of three separate cultures. To assay for colony formation, cells were scraped with a rubber policeman and plated in 10-cm plastic petri dishes. The number of colonies (>20 cells) per dish was counted after 14 d.

Assays of Intracellular Lysosomal Rupture

P388D, macrophages plated on 12-mm glass cover slips were treated with 10 \(\mu\)g/ml of acridine orange (PolyScience Corp., Niles, Ill.) in Williams' medium E without calcium or serum for 15 min at 37°C. After rinsing four times with Hanks' balanced salt solution minus calcium, the cultures were exposed to silica particles or to A23187 as described above. After treatment, the cover slips were mounted in phosphate-buffered saline and examined with a Zeiss Photomicroscope III at \(\times\) 160 magnification equipped with interference filters BP 436/8 nm and BP 436/17 nm and epifluorescence illumination. Intact lysosomes showed red fluorescence in cytoplasmic granules, whereas intracellular lysosomal rupture caused bright green diffuse fluorescence of both the cytoplasm and nucleus (3). The number of cells with red granular or diffuse green fluorescence was counted in triplicate cultures. The data are expressed as the percentage of cells with red granular fluorescence compared with the total number of cells counted (at least 100 cultures).

Alternatively, P388D, macrophages were preloaded with horseradish peroxidase (Sigma Type II; Sigma Chemical Co.) at 1 mg/ml for 1 h at 37°C in Williams' medium E minus serum. After rinsing three times with Hanks' balanced salt solution minus calcium, cultures were exposed to silica particles or to A23187. After the times indicated in the text, the cultures were fixed in 3% glutaraldehyde-0.1 M cacodylate buffer at pH 7.4 for 6 min at 4°C, rinsed twice with distilled water, and incubated for 90 min at 30°C in the diaminobenzidine tetrahydrochloride medium with 0.01% H\(_2\)O\(_2\) at pH 7.6 described by Graham and Karnovsky (18). Treated or untreated cultures incubated in the absence of diaminobenzidine tetrahydrochloride, without H\(_2\)O\(_2\), or in the presence of 0.01 M KCN were unstained. As shown previously (36) intact lysosomes are indicated by orange-brown granular cytoplasmic deposits as seen at \(\times\) 100 magnification with light microscopy. Intracellular lysosomal rupture is accompanied by diffuse orange staining of the cytoplasm and nucleus. The data are expressed as the percentage of cells with granular staining compared with the total number of cells counted (at least 250) in each of triplicate cultures.
Phagocytosis Assay

P388D, cells plated on 12-mm glass cover slips were exposed to silica particles in the presence or absence of Ca\(^{2+}\) in the culture medium as described above. After fixation in absolute methanol for 20 min, the cover slips were mounted in glycerol, and the number of silica particles ingested per cell was counted under phase-contrast microscopy at ×400 magnification. Cells exposed to silica particles for 30–180 min in either the presence or absence of Ca\(^{2+}\) were surrounded by an area cleared of particles that facilitated counting. Particles were counted as ingested, rather than simply adherent to the surface, if they were in the same plane of focus as the cell nuclei.

Degradation of Cellular Macromolecules

P388D, macrophages were plated in 10-cm plastic petri dishes (Falcon Labware; 1.3 × 10\(^5\) cells/cm\(^2\)) and prelabeled overnight with one of the following precursors from New England Nuclear (Boston, Mass.): [methyl-\(^{14}\)C]-thymidine (0.1 μCi/ml; sp act 6.7 Ci/mmol), [5-H]-uridine (1.0 μCi/ml; sp act >25 Ci/mmol), or [1,2-\(^3\)H]-ethanolamine hydrochloride (0.2 μCi/ml; 5 mCi/nmol). After rinsing three times with Hank's balanced salt solution minus calcium, the cultures were exposed to silicaparticles (350 pg) or A23187 (10 pg/ml) in 2.5 ml of medium minus Ca\(^{2+}\). Portions of this suspension were used to determine the amount of nucleic acids (33) or phosphorus in extracted phospholipids.

Assay of Horseradish Peroxidase Activity

P388D, macrophages were plated in 3.5-cm plastic multwells (Costar, Data Packaging; 4 × 10\(^3\) cells/ml) and preloaded with 250 μg/ml horseradish peroxidase (Sigma Type II) in Williams' medium E minus serum for 1 h at 37°C. After rinsing three times with Hank's balanced salt solution minus calcium, cultures were exposed to silica particles (350 μg) or A23187 (10 μg/ml) in 2.5 ml of medium. After 3 h, the monolayers were fixed in 3% glutaraldehyde-0.1 M cacodylate buffer, pH 7.6 for 6 min at 4°C. The activity of ingested horseradish peroxidase was assayed as described by Birnberg et al. (39) using 1.5 ml of 4-aminoantipyrine (Sigma Chemical Co)-phenol reagent (0.5 mg/ml plus 1.6% phenol in phosphate-buffered saline) with 1.5 ml of 0.006% H\(_2\)O\(_2\). After incubation for 30 min at 20°C, absorbance was read at 510 nm. Activity is expressed as absorbance of 510 nm/mg cellular protein/30 min. Cell protein was measured as described above (25). This reaction was linear with increasing cell number. Both 4-aminoantipyrine and H\(_2\)O\(_2\) were at a saturating concentration. Nonspecific adsorption of horseradish peroxidase to the cell monolayer at 4°C caused less than 5% of the activity after incubation for 1 h at 37°C. Cells not exposed to exogenous horseradish peroxidase contained 5% of the activity when assayed with these substrates. No difference in endogenous activity was found after cells were treated with particles or A23187. Less than 0.5% of total activity was detected of H\(_2\)O\(_2\) or 4-aminoantipyrine was omitted from the reaction mixture. Lyases from cells after treatment with particles or A23187 for 3 h did not affect the activity of horseradish peroxidase in this assay.

RESULTS

Silica Toxicity Depends on Extracellular Calcium

We have shown previously that the killing of rat hepatocytes by silica particles is dependent upon extracellular Ca\(^{2+}\) ions (32). A similar result was obtained with a macrophage-like cell line. P388D, mouse macrophages were exposed to silica particles and assayed for viability by trypan blue exclusion. After 3 h with 150 μg silica in the culture medium containing 1.8 mM Ca\(^{2+}\), 60% of the cells did not exclude the dye. In contrast, in the absence of extracellular Ca\(^{2+}\), all of the cells were still viable (Table I, line 2). This result cannot be explained simply by the failure of dead cells to take up trypan blue in medium without Ca\(^{2+}\) ions. P388D, cells were exposed to 150 μg silica for 3 h in medium containing 1.8 mM Ca\(^{2+}\), then placed in medium minus Ca\(^{2+}\) to assay for trypan blue exclusion. Whether assayed for viability in the presence or absence of Ca\(^{2+}\), 60% of the cells did not exclude the dye. Untreated P388D, cells maintained in the presence or absence of extracellular Ca\(^{2+}\) showed no loss of viability for at least 72 h. Particles of titanium dioxide are the same size as those of silica, but did not cause cell death (Table I, line 3). Similarly, macrophages exposed to digestible particles such as zymosan or Escherichia coli, showed no loss of viability (data not shown).

The extent of the killing of P388D, macrophages is dependent on both the dose of silica and the concentration of Ca\(^{2+}\) ions in the medium. Fig. 1 shows that more cells were killed with increasing amounts of silica in the presence of 1.8 mM Ca\(^{2+}\) (open circles), whereas there was no loss of viability with the same amounts of silica in the absence of extracellular Ca\(^{2+}\). Fig. 2 shows the dependency on the extracellular Ca\(^{2+}\) concentration of the cell death produced by 100 μg silica.

These results are based on the use of trypan blue to assess cell viability. Table II correlates the extent of killing of P388D, macrophages by silica in the presence of Ca\(^{2+}\) as assayed by trypan blue with two other methods of determining the viability of cultured cells: the hydrolysis of fluorescein diacetate with retention of free fluorescein in the cytoplasm of viable cells and colony formation. All three assays gave very similar values for the extent of cell killing by silica particles in the presence of extracellular Ca\(^{2+}\). This confirms the close correlation between these three viability assays reported previously (31). In the absence of extracellular Ca\(^{2+}\), the viability of treated or untreated cells as assessed by fluorescein diacetate was the same as that shown in Table I by trypan blue exclusion.

A simple interpretation of the Ca\(^{2+}\) dependency of silica toxicity is that calcium is required for silica to interact with the macrophages. However, the rate and extent of phagocytosis of silica particles by P388D, cells are the same in the presence or absence of extracellular Ca\(^{2+}\). The number of silica particles ingested by these cells can be counted under phase microscopy. The number of particles ingested is directly related to the dose of silica: 5.38 ± 1.74 (mean ± SD) particles were ingested after 3 h of exposure to 50 μg silica, 9.38 ± 2.44 with 100 μg silica, and 13.4 ± 3.31 with 150 μg silica. The number of particles ingested per cell at these doses was the same in the presence or absence of extracellular Ca\(^{2+}\). After 30 min of exposure to 100 μg silica in the presence or absence of Ca\(^{2+}\), the cells contained

<table>
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<th>Treatment</th>
<th>Medium plus Ca(^{2+})</th>
<th>Medium minus Ca(^{2+})</th>
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<tr>
<td>None</td>
<td>100 ± 13.5</td>
<td>98.0 ± 5.9</td>
</tr>
<tr>
<td>SiO(_2)</td>
<td>39.3 ± 5.1</td>
<td>105 ± 3.0</td>
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<tr>
<td>TiO(_2)</td>
<td>102 ± 11.7</td>
<td>94.1 ± 7.9</td>
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P388D, macrophages were plated onto 12-mm glass cover slips (1–2 × 10\(^5\) cells/cm\(^2\)) and exposed to 150 μg SiO\(_2\) or TiO\(_2\) in 2 ml Williams' medium without serum in the presence or absence of calcium. After 3 h, viability was assayed by trypan blue exclusion as described in Materials and Methods.
FIGURE 1 Dose response of silica toxicity. P388D1 macrophages were prepared as in Table I. Cultures were exposed to 50-200 μg of SiO2 for 3 h in the presence (open circles) or absence (closed circles) of Ca2+ in the culture medium and then assayed for viability by trypan blue exclusion. The vertical bars indicate the standard deviation of the mean viability determined from triplicate cultures.

FIGURE 2 Dependence of silica toxicity on the concentration of extracellular Ca2+. P388D1 macrophages were prepared as in Table I. Cultures were exposed to 100 μg SiO2 for 2 h in HEPES-buffered saline minus calcium; then CaCl2 was added to give the final calcium concentrations shown on the abscissa. Trypan blue exclusion was measured after incubation for an additional 1 h. The vertical bars indicate the standard deviation of the mean viability determined from triplicate cultures.

5.02 ± 2.78 particles. A maximum of 9.40 particles/cells was reached after 90 min. The frequency distribution of the number of particles/cell remained constant from 90 min to 3 h and is the same whether phagocytosis occurred in the presence or absence of Ca2+ (Fig. 3).

In summary, on the basis of the above data, it can be concluded that the cell death after exposure to silica is dependent upon extracellular Ca2+ ions. This dependency cannot be explained by a failure of the particles to interact with the cells in the absence of Ca2+ because phagocytosis of silica occurred with the same kinetics in the presence or absence of calcium. This system allows, therefore, the interaction of silica with cells to be dissociated from their killing. This permitted assessment of whether cell death caused by silica correlates with intracellular lysosomal rupture.

Intracellular Lysosomal Rupture in the Presence or Absence of Ca2+

Intracellular lysosomal rupture can be detected in intact cells by a variety of sensitive fluorescence or histochemical assays. Acridine orange is selectively concentrated by intact lysosomes, resulting in red, granular fluorescence. P388D1 macrophages were prelabeled for 15 min with acridine orange. As observed at ×160 magnification, these cells contained an average of 20.2 ± 6.1 red granules in the cytoplasm. After a 3-h exposure of the acridine orange-labeled cells to 150 μg silica in medium containing 1.8 mM Ca2+, 60% of the cells were unable to

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<th>Treatment</th>
<th>Trypan blue exclusion</th>
<th>Fluorescein diacetate fluorescence</th>
<th>Colony formation</th>
</tr>
</thead>
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<tr>
<td>None</td>
<td>100 ± 1.2</td>
<td>100 ± 2.7</td>
<td>100 ± 9.6</td>
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<tr>
<td>SiO2 (75 μg)</td>
<td>69.9 ± 2.7</td>
<td>65.4 ± 5.8</td>
<td>58.4 ± 6.7</td>
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<tr>
<td>SiO2 (150 μg)</td>
<td>31.5 ± 1.7</td>
<td>32.2 ± 5.1</td>
<td>29.1 ± 7.4</td>
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</table>

P388D1 macrophages were prepared as described in Table I. After 3 h of exposure to 25 or 150 μg SiO2, trypan blue exclusion, fluorescence with fluorescein diacetate, or ability to form colonies was assayed on separate cultures as described in Materials and Methods. Each number is the mean ± SD of viability assays or colonies formed from triplicate cultures expressed as the percentage of untreated cultures, which is 100%. Untreated P388D1 macrophages have a plating efficiency of 90-90%.

FIGURE 3 Particle phagocytosis in the presence or absence of extracellular calcium. P388D1 macrophages were grown on 12-mm glass cover slips. After 3 h of exposure to 100 μg SiO2 in the presence (upper bars) or absence (lower bars) of Ca2+ in the culture medium, the number of silica particles ingested per cell was counted by phase-contrast microscopy at ×400 magnification. The cells were first fixed in methanol and mounted in glycerol. A total of 100 cells/cover slip were counted for each of triplicate cultures.
exclude trypan blue and showed diffuse, green cytoplasmic fluorescence indicating intracellular lysosomal rupture (Table III, line 5). Cells were scored as having ruptured lysosomes if they had none or at most one red, cytoplasmic granule and diffuse green fluorescence of the nucleus and cytoplasm. With 150 µg silica, no cells were observed with an intermediate number of red cytoplasmic granules. The percentage of cells with ruptured lysosomes increased with doses of silica from 50 to 150 µg (Table III, lines 3–5). With each dose, the same percentage of cells was viable as had intact lysosomes. With 50 and 100 µg silica, no cells had an intermediate number of red, cytoplasmic granules. There is, then, correspondence between the amount of silica to which the cells were exposed, the number of particles phagocytized per cell, and the number of cells with either ruptured or intact lysosomes. Increasing doses of silica did not result in a shift in the number of cells with fewer intact lysosomes, but rather resulted in more cells with no intact lysosomes. There is a close correlation between the number of cells with ruptured lysosomes and the loss of viability. In untreated cultures (Table III, line 1) or cultures exposed to nontoxic titanium dioxide particles, zymosan, or E. coli, few, if any, cells showed intracellular rupture.

These results are similar to those of previous experiments (1, 27) with primary macrophage cultures, which led to the hypothesis that intracellular lysosomal rupture by silica particles is responsible for cell death. In the absence of extracellular Ca²⁺ and with each dose of silica, however, the same percentage of cells showed diffuse green fluorescence, although in each case there was no loss of viability (Table III, lines 7–9). In the absence of extracellular Ca²⁺ and with each dose of silica, there were also no cells with an intermediate number of red, cytoplasmic granules. Removing Ca²⁺ from the medium, therefore, had no effect on the number of phagocytized particles or the number of cells with ruptured lysosomes. Loss of viability, however, occurred only when Ca²⁺ ions were present in the medium.

It might be argued that acridine orange interferes with lysosomal function. It has been shown previously that acridine orange does not affect fusion of lysosomes with phagocytic vesicles, subsequent digestion of microorganisms (9), or the activity of several lysosomal enzymes (21). In P388D₃ macrophages exposed to silica, the same percentage of cells showed intracellular lysosomal rupture whether they were labeled with acridine orange before or after treatment (Table IV). In addition, the change in the distribution of acridine orange was correlated with the release of horseradish peroxidase from the lysosomes into the cytoplasm (Table IV). The lysosomal localization of this exogenous enzyme after pinocytosis was shown previously by electron microscopy (36). Table IV indicates that both of these assays gave the same result. The possibility of direct exocytosis of lysosomal enzymes into the medium after exposure to silica was also ruled out. It was not possible to detect any β-glucuronidase activity in the medium after 3-h exposure to silica (data not shown). Similar results have been reported with primary macrophage cultures exposed to silica (11). Media collected from P388D₃ macrophages killed by silica did not affect the viability of untreated cultures.

These results dissociate intracellular lysosomal rupture from the cell death caused by silica. Although phagocytosis and intracellular lysosomal rupture occur to the same extent in the presence or absence of Ca²⁺ ions in the medium, cell death occurs only when extracellular Ca²⁺ is present. Two distinct interpretations of this result are possible. On the one hand, intracellular lysosomal rupture and cell death do indeed occur independently after exposure to silica and are not causally related. On the other hand, it is possible that the functional consequences of lysosomal rupture, which ultimately do cause cell death, are in turn dependent upon calcium. In this case, lysosomal rupture is necessary but not sufficient to kill the cells. In order to investigate this second possibility, effects of intracellular lysosomal rupture on cell structure and function were sought.

### Table IV

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% of control viability</th>
<th>% of cells with intact lysosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control plus Ca²⁺</td>
<td>100 ± 6.4</td>
<td>99.7 ± 0.6</td>
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<tr>
<td>Control minus Ca²⁺</td>
<td>102 ± 6.2</td>
<td>99.3 ± 1.2</td>
</tr>
<tr>
<td>SiO₂ plus Ca²⁺</td>
<td>25.0 ± 4.4</td>
<td>24.3 ± 1.5</td>
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<tr>
<td>SiO₂ minus Ca²⁺</td>
<td>103 ± 3.9</td>
<td>24.3 ± 3.1</td>
</tr>
</tbody>
</table>

P388D₃ macrophages were prepared as described for Table I and exposed to 150 µg SiO₂ in the presence or absence of extracellular calcium for 3 h. Viability and lysosomal assays were carried out as described in Table III. Cultures in the third column were labeled with acridine orange after exposure to particles, whereas cultures in the second column were prelabeled with acridine orange as described in Materials and Methods. AO, acridine orange; HRP, horseradish peroxidase.

### Table III

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% of control viability</th>
<th>% of cells with intact lysosomes</th>
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<tr>
<td>None</td>
<td>100 ± 7.8</td>
<td>99.7 ± 0.6</td>
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<tr>
<td>SiO₂ plus Ca²⁺</td>
<td>71.4 ± 3.2</td>
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<td>50 µg</td>
<td>61.8 ± 1.1</td>
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<td>100 µg</td>
<td>102 ± 4.5</td>
<td>48.0 ± 6.2</td>
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</table>

P388D₃ macrophages were prepared as described for Table I and exposed to SiO₂ in the presence or absence of extracellular calcium. After 3 h, viability was assayed by trypan blue exclusion and intracellular lysosomal rupture by acridine orange fluorescence as described in Materials and Methods.

### Effect of Intracellular Lysosomal Rupture on Cellular Macromolecules

Macrophages contain a wide variety of lysosomal enzymes capable of degrading macromolecules into acid-soluble products. It might be presumed that their release into the cytoplasm would lead to uncontrolled degradation of cellular constituents. In order to determine whether the intracellular rupture of lysosomes by silica results in such a degradation of macromolecules, P388D₃ macrophages were labeled for 18 h (approximately equal to the length of one cell cycle) with either radioactive thymidine, uridine, leucine, or ethanolamine. After rinsing four times with Hanks' balanced salt solution, the cultures were treated with silica or titanium dioxide particles in medium containing 1.8 mM Ca²⁺. If extensive degradation of cellular macromolecules occurs after release of lysosomal enzymes into the cytoplasm, the total content of radioactive DNA, RNA, protein, and/or phospholipid should decrease.
accompanied by an increase in acid-soluble radioactivity in the cells and/or medium. Such an experiment has detected a 40% increase in proteolysis in fibroblasts placed in nutritionally deficient medium (4). With the prelabeled P388D1 macrophages there was a 60-70% decrease in viability in cultures exposed to silica compared again with no change in viability of untreated cultures or cultures exposed to titanium dioxide. Table V summarizes the total content of DNA, RNA, protein, and phospholipid as well as the total acid-insoluble radioactivity in control cells and cells exposed to either silica or titanium dioxide. There was no change in the content of total DNA, RNA, protein, or phospholipid in P388D1 macrophages after exposure to either silica or titanium dioxide. No increased acid-soluble radioactivity could be detected in the medium or cell monolayer (data not shown). There was also no decrease in the acid-insoluble radioactivity in DNA, RNA, or protein (Table V). There was, however, a significant (P < 0.025) decrease in the specific activity of total cellular phospholipids in cells exposed to both silica and titanium dioxide. This most likely reflects an increased membrane turnover and biosynthesis, which have been described previously in macrophages after phagocytosis of latex beads (44). Consistent with this interpretation, there was increased incorporation of [14C]glycerol into phospholipid extracted from P388D1 macrophages after phagocytosis of silica or titanium dioxide particles (data not shown). Therefore, the changes in cellular phospholipid metabolism measured in these experiments reflect changes in membrane turnover, which accompanies phagocytosis in general, rather than a specific event accompanying intracellular lysosomal rupture and cell death after exposure to silica. On the basis of these studies, it can be concluded that, although intracellular lysosomal rupture occurs in P388D1 macrophages exposed to silica, the release of lysosomal enzymes does not seem to result in any detectable breakdown of cellular constituents. It is still possible, however, that release of lysosomal enzymes causes a very specific change in a few macromolecules that could not be detected by the above method, but which still relates to the cell death produced by silica. To examine this possibility we determined the fate of the activity of one particular protein in P388D1 macrophages exposed to silica.

**Effect of SiO2 and TiO2 on Content and Degradation of Cellular Macromolecules**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DNA, µg</th>
<th>SiO2</th>
<th>TiO2</th>
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<td>AIF, cpn/µg DNA</td>
<td>2,580 ± 121</td>
<td>2,290 ± 26.3</td>
<td>2,460 ± 56.7</td>
</tr>
<tr>
<td>RNA, µg</td>
<td>43.3 ± 9.2</td>
<td>35.5 ± 9.1</td>
<td>49.0 ± 4.5</td>
</tr>
<tr>
<td>AIF, cpn/µg RNA</td>
<td>7.15 ± 1.11</td>
<td>8.17 ± 1.87</td>
<td>7.83 ± 0.70</td>
</tr>
<tr>
<td>Protein, µg</td>
<td>319 ± 13.1</td>
<td>230 ± 8.69</td>
<td>312 ± 12.6</td>
</tr>
<tr>
<td>AIF, cpn/µg protein</td>
<td>27.9 ± 0.70</td>
<td>29.9 ± 2.0</td>
<td>30.3 ± 0.94</td>
</tr>
<tr>
<td>Phospholipid, nmol</td>
<td>410 ± 53</td>
<td>367 ± 29</td>
<td>587 ± 162</td>
</tr>
<tr>
<td>AIF, cpn/nmol phospholipid</td>
<td>296 ± 12</td>
<td>197 ± 46</td>
<td>193 ± 47</td>
</tr>
</tbody>
</table>

**TABLE VI**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% of control viability</th>
<th>% of cells with intact lysosomes</th>
<th>HRP activity units/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100 ± 3.7</td>
<td>99.3 ± 0.6</td>
<td>4.55 ± 0.62</td>
</tr>
<tr>
<td>SiO2</td>
<td>19.2 ± 1.5</td>
<td>19.0 ± 3.0</td>
<td>3.69 ± 0.85</td>
</tr>
<tr>
<td>TiO2</td>
<td>93.8 ± 11.2</td>
<td>83.3 ± 12.2</td>
<td>4.52 ± 0.12</td>
</tr>
</tbody>
</table>

P388D1 macrophages were plated in 3.5-cm plastic multiwells (3.4 x 10⁶ cells/cm²) containing two glass cover slips, preloaded with horseradish peroxidase (HRP) (0.25 mg/ml) for 1 h, rinsed, and treated with 350 µg SiO2 or TiO2 in the presence or absence of extracellular calcium. After 3 h, the glass cover slips were removed and assayed for trypan blue exclusion or labeled with acridine orange for 15 min to determine intracellular lysosomal rupture. The remaining cells were fixed and assayed for horseradish peroxidase activity as described in Materials and Methods.

**Effect of Intracellular Lysosomal Rupture on Turnover of Horseradish Peroxidase Activity**

P388D1 macrophages were prelabeled with horseradish peroxidase for 1 h, washed, and exposed to silica or titanium dioxide particles. After 3 h, the cultures were assayed for viability, intracellular lysosomal rupture, and horseradish peroxidase activity. During the prelabeling period, the exogenous enzyme is taken up by pinocytosis and concentrated in lysosomes, where it is gradually inactivated and digested (17). If intracellular rupture results in uncontrolled hydrolysis, an accelerated rate of turnover of horseradish peroxidase activity would be expected. Decreased enzyme activity would be detected even if the enzyme were not completely digested to acid-soluble products. As shown in Table VI, there was no change in horseradish peroxidase activity in cultures exposed to silica or titanium dioxide although 80% of the cells treated with silica showed intracellular lysosomal rupture. Enzyme activity was expressed per milligram of cell protein to correct for any cell detachment after exposure to silica. Over the duration of this experiment, cell number and protein content in untreated cultures increased by less than 10%, so there was no apparent loss of horseradish peroxidase specific activity because of an increase in cell number. No endogenous peroxidase activity could be detected under the assay conditions employed. The t₁/₂ of horseradish peroxidase in P388D1 macrophages in the presence of silica was 5.5 h. This agrees with a t₁/₂ of 7–9 h reported for primary cultures of peritoneal macrophages (17).

The data presented in Tables V and VI indicate that there is no significant degradation of cellular macromolecules or an exogenous protein in P388D1 macrophages exposed to silica, even though intracellular lysosomal rupture occurred. It cannot be argued that the activity of released lysosomal enzymes is dependent on Ca²⁺ ions in the culture medium because the same results were obtained in cells treated with silica in the presence or absence of extracellular Ca²⁺. These results suggest that, in the absence of any evidence that the release of lysosomal enzymes causes a breakdown of cellular macromolecules, it must be considered that intracellular lysosomal rupture is not causally related to the cell death produced by silica. Two additional experiments provide further support for this conclusion.
Protection against Silica Toxicity by Promethazine

It was also possible to dissociate intracellular lysosomal rupture from the cell death produced by silica with the use of two drugs with differing effects on the cells. P388D1 macrophages were treated with silica in the presence or absence of added Ca2+. After 3 h, viability was assayed by trypan blue exclusion and lysosomal rupture determined after prelabeling with acridine orange as described in Materials and Methods.

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<table>
<thead>
<tr>
<th>Treatment</th>
<th>% of control viability</th>
<th>% of cells with intact lysosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100 ± 13.5</td>
<td>99.0 ± 1.0</td>
</tr>
<tr>
<td>SiO2 plus Ca2+</td>
<td>39.3 ± 5.1</td>
<td>38.7 ± 5.5</td>
</tr>
<tr>
<td>SiO2 minus Ca2+</td>
<td>105 ± 3.0</td>
<td>41.0 ± 4.4</td>
</tr>
<tr>
<td>Promethazine pretreatment, SiO2 plus Ca2+</td>
<td>68.6 ± 3.1</td>
<td>33.0 ± 7.9</td>
</tr>
<tr>
<td>Indomethacin pretreatment, SiO2 plus Ca2+</td>
<td>46.5 ± 2.3</td>
<td>80.3 ± 7.4</td>
</tr>
</tbody>
</table>

P388D1 macrophages were prepared as described in Table I, pretreated where indicated for 1 h with promethazine (20 µg/ml) or indomethacin (5 × 10⁻⁵ M), and exposed to 150 µg SiO2 in the presence or absence of extracellular Ca2+. After 3 h, viability was assayed by trypan blue exclusion and lysosomal rupture determined after prelabeling with acridine orange as described in Materials and Methods.

Intracellular Lysosomal Rupture by A23187

In agreement with our previous observations with primary cultures of rat hepatocytes (32), P388D1 macrophages were rapidly killed by A23187 in the presence (Fig. IV, closed squares), but not in the absence of extracellular Ca2+ ions (open squares). Similar to the effect of silica, 90–98% of the same cells treated with A23187 rapidly manifested diffuse, green cytoplasmic fluorescence with acridine orange, which is indicative of intracellular lysosomal rupture in the absence of extracellular Ca2+ (Fig. IV, open circles). When Ca2+ ions were added to the culture medium after 1 h of exposure to A23187 in the absence of Ca2+, there was a rapid loss of viability (Fig. IV, closed squares) without any additional rupture of the lysosomes (Fig. IV, closed circles). P388D1 cells treated with A23187 in the absence of extracellular Ca2+ remained viable for at least 4 h, although in almost 100% of the cells there was intracellular lysosomal rupture. The percentage of cells with intracellular lysosomal rupture after exposure to A23187 in the presence or absence of extracellular Ca2+ was the same whether measured by the acridine orange assay (prelabeled or postlabeled) or by the horseradish peroxidase assay. Similar results with A23187 were obtained with primary cultures of rat hepatocytes (data not shown).

These data show that in cells treated with A23187 intracellular lysosomal rupture is not dependent upon added Ca2+. Although A23187 was shown to alter lipid structure in plasma membrane vesicles or liposomes in the absence of added Ca2+ (24), it is not possible to conclude that the rupture of lysosomes is independent of Ca2+. In intact cells the drug may be causing redistribution of intracellular Ca2+ (30).

Similar to silica, the intracellular release of lysosomal enzymes by A23187 was not accompanied by any evidence of the degradation of DNA, RNA, protein, or phospholipid (data not shown). There was also no inactivation of horseradish peroxidase. As with silica, there was no exocytosis of lysosomal enzymes into the medium with exposure to A23187 for 3 h. With polymorphonuclear leukocytes, in contrast, A23187 caused exocytosis of lysosomal enzymes (22). This effect of
A23187 is distinct from that reported here with P388D1 macrophages. In polymorphonuclear leukocytes, A23187 is most likely inducing a normal secretory reaction dependent upon an elevated cytosolic Ca\(^{2+}\) concentration.

**DISCUSSION**

Previous investigations of silica cytotoxicity led to the suggestion that intracellular lysosomal rupture is responsible for the cell death produced by this as well as other toxins and environmental hazards (16). The present study has subjected this hypothesis to critical evaluation. It was possible to dissociate intracellular lysosomal rupture from subsequent cell death after exposure to either silica particles or the calcium ionophore A23187. Furthermore, it was not possible to demonstrate any changes in the content of cellular macromolecules subsequent to lysosomal rupture even in the presence of extracellular Ca\(^{2+}\). On the basis of these experiments, it is concluded that intracellular rupture probably does not have a causal role in the cell death produced by these agents.

The P388D1 macrophages used in these experiments have the same response to mineral particles as the primary cultures of peritoneal macrophages used previously (1, 27). Silica, but not biologically inert particles of the same size such as titanium dioxide, killed P388D1 cells (Table I) and caused intracellular lysosomal rupture (Table III). This was shown by three different assays of viability (Table II) and two assays of lysosomal integrity (Table IV). In this experimental system, cell death was dissociated from intracellular lysosomal rupture by the omission of Ca\(^{2+}\) ions from the culture medium (Table III) or by pretreatment with promethazine (Table VII). The calcium ionophore also caused intracellular lysosomal rupture and cell death (Fig. 4). As with silica, A23187 produced intracellular lysosomal rupture in the presence or absence of extracellular Ca\(^{2+}\), yet cell death occurred only in the presence of these ions.

What is the calcium-dependent step that correlates with cell death in this system? Calcium ions are not necessary for the interaction of silica particles with the macrophages. Phagocytosis (Fig. 3) and intracellular lysosomal rupture (Table III) occurred to the same extent in the presence or absence of extracellular Ca\(^{2+}\). This is consistent with previous observations that silica causes hemolysis (19) and rupture of liposomes (42). It seems likely, therefore, that extracellular Ca\(^{2+}\) is required at some point subsequent to the interaction of the silica particles with the cells. Calcium ions are acting to couple some consequence of this interaction to the eventual cell death.

This effect of calcium ions was dependent upon their concentration in the culture medium (Fig. 2), and was most likely mediated, therefore, by an influx of these ions into the cell across a damaged membrane permeability barrier. We have suggested previously that such an influx of Ca\(^{2+}\) represents a final common pathway for the cell death produced by a number of different agents (32). All of these agents have in common the ability to interact with cellular membranes. All cells in the body are bathed in a fluid very rich in Ca\(^{2+}\) (10\(^{-3}\) M), whereas intracellular Ca\(^{2+}\) concentrations are much lower, on the order of 10\(^{-7}\)–10\(^{-8}\) M. The electrical potential across the plasma membrane of these cells tends to drive Ca\(^{2+}\) into them. Such a large electrochemical gradient is maintained by the relative impermeability of the plasma membrane to Ca\(^{2+}\) and by active extrusion (29). Damage to the plasma membrane by any one of the toxins studied in this and previous reports (23, 32) would disrupt this permeability barrier with a consequent influx of Ca\(^{2+}\). Calcium ions are biologically very active, being capable of considerable disruption of metabolic order.

If the calcium-dependent step in silica cytotoxicity represents such an influx of Ca\(^{2+}\) ions across a damaged plasma membrane, what is the nature of such damage and how is it produced by silica? The data presented in this report suggest that such a plasma membrane lesion is not the consequence of the intracellular rupture of lysosomes with uncontrolled hydrolysis of cellular constituents. There was no evidence of such an action of the released lysosomal enzymes on either DNA, RNA, protein, or phospholipid (Table V), either in the presence or absence of extracellular Ca\(^{2+}\). This conclusion is limited, of course, by the sensitivity of the measurements of the total quantities of each of these cellular constituents. We cannot eliminate the possibility that a single vital component was attacked by a specific lysosomal enzyme to an extent undetectable by the assays employed. Such a situation, however, is unlikely in light of the observation that indomethacin prevented lysosomal rupture without reducing the cell death produced by silica (Table VII).

It seems more likely that lethal membrane injury is produced by the silica particles directly, rather than through the intermediary action of lysosomal enzymes. A number of previous studies have documented the direct effects of silica particles on natural and artificial membranes. Silica particles lyse erythrocytes (19) and isolated lysosomes as well as alter the permeability of phospholipid liposomes (42). These actions are thought to result from a disruption of lipid structure in membranes by the formation of hydrogen bonds between the surface of the silica particles and phospholipids (28). It is reasonable to expect silica to disrupt similarly the integrity of the plasma membrane of P388D1 macrophages.

These considerations readily allow a consistent interpretation of the major findings presented in this study. We consider the intracellular rupture of lysosomes and the killing of P388D1 macrophages as independent and direct consequences of the interaction between silica particles and cellular membranes. There is presently no evidence that intracellular lysosomal rupture has any relevance to the cell death produced by silica. The killing of the cells is most likely the consequence of the interaction of silica particles with the plasma membrane. The functional effect of this is an alteration in the normal permeability barrier to Ca\(^{2+}\) ions with their resultant influx into the cell. Calcium ions are the ultimate mediator of the metabolic deterioration and death of the cells. This suggests that future studies should concentrate on the mechanism whereby silica particles disorganize membrane structure to produce an increased permeability to calcium.

This work was supported by grants ES02251, CA19154, CA12073, and CA12923 from the National Institutes of Health.

Received for publication 11 June 1980, and in revised form 6 August 1980.

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