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INFLUENCE OF PARTICLE DOSE ON THE CYTOTOXICITY OF HAMSTER AND RAT PULMONARY ALVEOLAR MACROPHAGE IN VITRO

D. Warshawsky, R. Reilman, J. Cheu, M. Radike, C. Rice

Department of Environmental Health, University of Cincinnati, Cincinnati, Ohio, USA

Silica and ferric oxide are common industrial exposures. Studies have indicated that all commonly occurring forms of crystalline silica can cause fibrotic lung disease. There is evidence to indicate that crystalline silica is carcinogenic in humans who have not developed silicosis, while amorphous silica is not carcinogenic in humans. An important biological response to particles deposited deep in the lung is their engulfment by pulmonary alveolar macrophages (AM). To assess the role of AM in silica-induced lung disease, particle size distribution and surface area of crystalline, gelled, precipitated, and fumed silica, ferric oxide, and aluminum oxide were characterized; the cytotoxicity of the particles to hamster and rat AM in vitro was measured at 0.0–0.5 mg/l × 10^6 cells at 24 and 48 h using dye exclusion procedures. The count medium diameter for aluminum oxide, ferric oxide, and amorphous silica was equal to or less than 0.38 μm, while for crystalline silica the value was 0.83 μm. The surface areas for the amorphous silicas and the aluminum oxide ranged from 253 to 125 m^2/g with gelled silica having the highest value; the values for crystalline silica and ferric oxide were 4.3 and 10.8 m^2/g, respectively. Crystalline silica (1.6%) was detected in the fumed silica, while none was detected in precipitated or gelled silica. With gelled silica, based on the dose of the particle, the viability of the hamster AM decreased to 27% at 0.05 mg and to zero at 0.1 mg at 24 h. At doses of 0.05 and 0.1 mg of crystalline, precipitated, or fumed silica, the percent viability decreased significantly to 76–67% and 51–42%, respectively, and to zero at 0.5 mg. Macrophages viable at 24 h decreased further at 48 h compared with the control culture. The ferric oxide and the aluminum oxide showed minimal to no changes in viability. Similar results for the particles were obtained with rat AM. The results indicate that precipitated and fumed amorphous silica tested at equivalent doses are equally as toxic to AM lavaged from two species of rodents as crystalline silica; gelled silica is more toxic than crystalline. Ferric oxide and aluminum oxide are noncytotoxic in this system. The results of this study indicate that the dose as well as the surface area and surface characterization are important determinants in the cytotoxicity of hamster and rat AM to these particles.

Exposure to silica is common in the industrial workplace. It has been estimated that approximately 250,000 workers in 114 occupations are exposed to silica flour alone (NIOSH, 1980); over 500,000 workers are exposed to synthetic amorphous silica (Groth et al., 1981), and 1.25 million workers may be exposed to crystalline silica (Peters, 1986). There have been

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Address correspondence to D. Warshawsky, Department of Environmental Health, University of Cincinnati, 3223 Eden Avenue, Cincinnati, OH 45267-0056, USA.

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epidemiologic and experimental studies relating silica exposure and the development of fibrotic lung disease (Theriault et al., 1974; Rice et al., 1986; Groth et al., 1981, 1986; Holland et al., 1986). These studies have indicated that commonly occurring forms of crystalline silica can cause fibrotic lung disease; for any quartz polymorph the severity of the disease appears to be related to dose. Coating silica particles with Al_2O_3 has an inhibitory effect on the development of fibrotic reactions (Schlipköter, 1963; Webster, 1970). There is evidence to indicate that crystalline silica is carcinogenic in humans, while amorphous silica is not carcinogenic in humans (IARC, 1987).

Ferric oxide is another common industrial exposure. It has been estimated that approximately 455,000 workers are exposed to ferric oxide in 149 occupations (NIOSH, 1980). Numerous epidemiologic studies have examined the effect of exposure to ferric oxide in the workplace. Workers exposed to only ferric oxide had no increased incidence of any form of cancer when compared to control populations (Axelson & Sjoberg, 1979; Tecuwescu & Albu, 1973).

The observed potential for disease in human populations exposed to these common industrial exposures would be strengthened by a better understanding of the mechanism of injury. Similarly, such information would facilitate design of controls to prevent exposure. Silica, administered either by inhalation or intratracheal (IT) instillation, has been shown to elicit a carcinogenic response in rats of two strains and both sexes (Groth et al., 1986; Dagle et al., 1986; Holland et al., 1986), and it has been hypothesized that silica either directly induces lung cancer or causes fibrosis leading to cancer (Goldsmith et al., 1982). In hamsters, however, silica did not produce a carcinogenic response and is usually not associated with fibrosis (Holland et al., 1986; Niemeier et al., 1986; Renne et al., 1985). In the hamster and rat, ferric oxide (Niemeier et al., 1986; Steinhoff et al., 1991) did not produce tumors or only a few tumors in the lung following IT instillation; aluminum oxide did not produce any tumors in the hamster (Stenback et al., 1976).

An important biological response to particles deposited deep in the lung is their engulfment by pulmonary alveolar macrophages (AM). The AM as a result of phagocytosis have been shown to release cytokines, growth factors, and arachadonic acid metabolites. These potential biomarkers are indicators of pulmonary toxicity as well as enzyme levels that are present in bronchoalveolar lavage (Warheit et al., 1991a, 1991b; Lindenschmidt et al., 1990; Driscoll & Maurer, 1991; Driscoll et al., 1990; Koren et al., 1992). The AM cells at noncytotoxic doses are also competent for several metabolic systems, and undergo metabolic and physiologic alterations during in vivo recruitment and activation (Bowden & Adamson, 1978; Kavet et al., 1978; White & Garg, 1981; Henderson et al., 1982). To assess the role of AM in silica-induced lung disease, a number of investigators have studied the cytotoxicity of silica to AM in vitro (Kessel et al., 1963; Bey & Harington, 1971; Allison et al., 1966; Davies, 1981; Vallyathan et al., 1991). Macrophages

studied were obtained from various sources including the peritoneum of mouse, hamster, and guinea pig; several studies have included lung macrophages. The effects of both crystalline and amorphous silica were studied but limited particle size information was reported. The data from some reports indicated that crystalline silica was cytotoxic while others reported no cytotoxic effects; these contradictory findings are likely due to differences in experimental design. Similar inconsistent results were obtained with amorphous silica.

There have not been any investigations reported on cytotoxicity studies that were conducted for well-characterized crystalline and amorphous silica, ferric oxide, and aluminum oxide under the same experimental conditions. Therefore, the objective was to undertake a study of selected silica particles in which particle size distribution and surface area data were developed uniformly and the cytotoxicity of the particles to AM was determined using at least four doses. These AM do not differ greatly in morphological appearance and phagocytic properties from peritoneal macrophages, but do differ in metabolic patterns and enzyme capacities (Pearsall & Weiser, 1970). The results from this study indicate that the dose, the surface area, and surface characteristics are important determinants in the cytotoxicity of hamster and rat AM to these particles.

MATERIALS AND METHODS

Materials

Crystalline, precipitated, and gelled silica and ferric oxide were supplied by NIOSH, Cincinnati. The crystalline silica, 5-μm Min-U-Sil, obtained from Pennsylvania Glass and Sand Corporation, and the positive control ferric oxide (certified grade, red anhydrous, lot 78156, Fisher Scientific, Cincinnati, Ohio) were from the same lot used in the hamster study (Niemeier et al., 1986), and the precipitated and gelled silicas were from the same lot used in chronic studies with monkeys (Groth et al., 1981). Fumed silica was purchased from Cabot Corporation (Tuscola, Ill., Cab-o-sil grade M-5, lot 1D309). Aluminum oxide (Fisher Scientific Co., Fairlawn, N.J., alumina, adsorption lot 760031, 80–200 mesh) was used as a negative control (Stenback et al., 1976). Gelled silica was prepared by slowly reacting sodium silicate in solution with a practical-grade hydrochloric acid (forming silicic acid) so that acidic conditions were always present when forming silica gel. After gelling, the silica material was dried. If the same starting materials are used and the acid is added to the sodium silicate solution and prevented from gelling by stirring, the result is precipitated silica, an aggregation of clusters of particles. The silica gel is a highly porous material, while the precipitated silica has pores between particles and virtually no pores in the particles (Davis et al., 1981). Fumed silica was prepared by vapor-phase hydrolysis of silicon tetrachloride (Stettler et al., 1981). The specific gravities (g/L) for crys-

talline silica and for ferric oxide were 2.66 and 5.24 (Niemeier et al., 1982); for aluminum oxide and amorphous silica the specific gravities were between 2.0 and 3.0, respectively (McCrone & Dally, 1973).

Particle sizing performed by NIOSH on these materials has been described in detail (Stettler et al., 1981) and is reviewed here. An appropriate mass of particles was added to 50–100 ml double-deionized water, sonicated for 10 min, and then diluted to 1 L. The suspension was stirred with a magnetic stir bar for 10 min and an aliquot was poured into a filtering apparatus, where the particles were collected on a 0.1-μm Nuclepore filter. For the aluminum oxide and the fumed silica, a 0.45-μm Millipore filter was used as a backup filter. The Nuclepore filter was then mounted on a carbon planchet and coated with colloidal graphite. Crystalline, gelled, and precipitated silica were analyzed with a scanning electron microscope (JOEL, model JXA-50A) equipped with an energy-dispersive x-ray spectrometer system (EG&G Ortec, model EEDS II) and an image analysis system (LeMont Scientific, model B-10). Fumed silica and aluminum oxide were analyzed with a Hitachi S-570 scanning electron microscope equipped with a Kevex 7000 energy-dispersive elemental x-ray analyzer and a LeMont DA-10 image analyzer. The analyses were performed at a magnification of 1000× to 2000× except in the case of fumed silica where the magnification was between 10,000× and 200,000×. The particle size data were based on the circular area equivalent diameters as determined by the image analysis system. Both electron and light microscopy were used to determine the particle size distribution for ferric oxide.

Surface area determinations were performed by nitrogen absorption using an automatic surface area analyzer (Micromeritics, model ASAP2400, Norcross, Ga.) using ASTM methods D3663 (ASTM, 1985) and C1069 (ASTM, 1986). The degassing temperature and time were 100°C and 960 min, respectively. X-ray diffraction methods were used to determine the percent of crystalline silica in the amorphous silicas. The x-ray diffraction analyses for precipitated and gelled silica were reported previously (Groth et al., 1981); the x-ray diffraction analysis for fumed silica was performed by DataChem (Salt Lake City, Utah) using NIOSH method 7500 (NIOSH, 1989). The latter method was used with two modifications: (1) Filters were dissolved in tetrahydrofuran rather than being ashed in a furnace. (2) Standards and samples were run concurrently and an external calibration curve was prepared from the integrated intensity rather than using the suggested normalization procedure. Both optical and x-ray diffraction were used to confirm the results.

AM Cultures

Male golden Syrian hamsters (Sasco, Omaha, Neb.) and Sprague-Dawley rats (Harlan, Indianapolis, Ind.), 8–10 wk old, 120–150 g body weight, were used in these studies. Animals were housed in a light- and temperature-controlled environment (lights on 0500–1900 h, 22–24°C) and supplied with

food and water ad libitum. Primary AM were isolated by tracheal lavage. Animals were anesthetized with sodium pentobarbital ip (0.5 ml 1% solution), exsanguinated by cutting the abdominal aorta and the trachea cannulated with a blunt 18-gauge needle attached to a 3-way stopcock. The lungs were lavaged 10 times in situ with calcium- and magnesium-free cold phosphate-buffered saline (PBS, pH 7.2) at a volume of 3–4 ml. The lavaged fluid was placed in a 50-ml plastic centrifugation tube and centrifuged at a rate of 400 × g for 10 min at 4°C. After centrifugation, the supernatant was discarded and the pellet was resuspended with RPMI-1640 (Sigma, St. Louis, Mo.) medium containing 0.1% gentamicin, 25 m*M* L-glutamine, 0.2% sodium bicarbonate, and 2 mg/ml bovine serum albumin (St. Louis, Mo, pH 7.2). An aliquot of the cell suspension was removed to determine AM numbers using a hemocytometer and selected for AM based on size and morphology and AM viability by dye exclusion using erythrosin B (Sigma, St. Louis, Mo.) (Phillips, 1973). The AM viability was based on 400–500 attached cells. The average viability for each isolation was on the order of 95–98%; 2.5–3 million AM were obtained per animal. Another aliquot of the cell suspension was used to identify the purity of the AM cellular differential staining using Diff-Quik stain set (Scientific Product Inc., Columbus, Ohio). One million cells were plated on a 35-mm plastic petri dish (Corning Glass Works, Corning, N.Y.) at 37°C in a humidified atmosphere with 5% CO_2. After 1.5 h the AM were washed twice with RPMI-1640 medium to remove unattached cells. Two and a half milliliters of fresh RPMI-1640 containing different concentrations of particles was administered to the AM. Two time points (24 and 48 h) were assayed for cytotoxicity. At each time point, the culture medium was removed and the AM were washed with fresh RPMI-1640. Cytotoxicity or viability of the cells was determined by dye exclusion as described earlier as the preferred method. Over 95% of the cells remained attached throughout the exposure period. Other investigators have used lactate dehydrogenase and β-glucuronidase enzymes released into the general bronchoalveolar lavage, as well as cytokines, growth factors, and arachidonic acid metabolites, as indicators of pulmonary toxicity (Warheit et al., 1991a, 1991b; Lindenschmidt et al., 1990; Driscoll & Maurer, 1991; Driscoll et al., 1990; Koren et al., 1992). However, we are interested in the noncytotoxic levels of particles coated with organics in terms of the effects on viability and eventually metabolism and DNA binding.

One of the major objectives of this work was to culture AM without fetal calf serum, since it was thought that the serum would interfere with planned metabolic studies by removing free metabolites from the media. Therefore, the hamster AM were initially cultured without serum. However, for the time frame we were analyzing, the viability dropped from 80.9 ± 3.0% (SE) at 24 h (n = 6) to 61.5 ± 4.4% at 48 h (n = 6). By adding back minimal bovine serum albumin (BSA) at a concentration of 2 mg/ml to the petri dish the viability stabilized at 86.5% ± 0.9% at 24 h (n = 6) to 77.0 ± 1.0% at 48 h (n = 4). Adding more BSA only slightly increased the viability, and therefore we used 2 mg/ml throughout the studies.

RESULTS

Physical Characteristics of Particles

The physical characteristics of the particles are reported in Table 1 and represent values that are typical of occupational settings (Rice et al., 1986). All of the particles were of respirable size with at least 98% less than 5 μm. The count median diameters for aluminum oxide and the amorphous silicas were equal to or less than 0.38 μm; the count median diameter for crystalline silica was 0.83 μm and ferric oxide was 0.32 μm. The larger surface areas for the aluminum oxide and the amorphous silicas were generally consistent with their smaller median diameters, with the values ranging from 124.8 to 253.1 m^2/g. For crystalline silica, with larger count median diameter, the surface area was 4.3 m^2/g. Neither precipitated nor gelled amorphous silicas contained any detectable crystalline silica; fumed silica contained 1.6% crystalline silica. Crystalline silica had a smaller surface area and a larger count median diameter than any of the amorphous silicas. Photomicrographs of fumed silica showed marked clumping of the individual submicrometer particles; this possibly contributes to the lower than expected surface area determination for a submicrometer particle.

TABLE 1. Physical Characteristics of Particles

Particle	Size distribution (μm)	Count median diameter (μm)	Surface area (m^2/g)	X-ray diffraction
Al_2O_3	99%, <5[a] 80%, <1	0.36[a]	198.4[b]	
Fe_2O_3	98.9%, ≤5[b] 91.5%, ≤1	0.32[b]	10.8[b]	
Silica, crystalline	99%, <5[c] 50%, <1	0.83[c]	4.3[b]	
Silica, amorphous				
Gelled	99.8%, <5[d] 93%, <1	0.27[d]	253.1[b]	Crystalline silica not detected[d]
Fumed	99.9%, <5[a] 99.5%, <1	(Submicrometer 15–50 nm)[a]	196.2[b]	1.6% Crystalline silica[e]
Precipitated	98%, <5[d] 85%, <1	0.38[d]	124.8[b]	Crystalline silica not detected[d]

[a]Analyzed by NIOSH, 1992.
[b]Analyzed by Micromeritics, Norcross, Ga., 1992.
[c]Stettler et al. (1981).
[d]Groth et al. (1981).
[e]Analyzed by DataChem, Salt Lake City, 1992.

TABLE 2. Effects of Al_2O_3 and Fe_2O_3 on Hamster AM Viability

	Percent viability of cells	
	24 h[a]	48 h
Al_2O_3 (mg)		
0.5	79.8 ± 5.5	67.1 ± 6.6 (n = 8)
0.1	88.0 ± 2.8	78.1 ± 3.7 (n = 4)
0.0	86.4 ± 4.2 (n = 6)	69.6 ± 8.0 (n = 5)
Fe_2O_3 (mg)		
0.5	81.1 ± 1.3[b]	66.7 ± 3.0[b] (n = 9)
0.1	85.5 ± 1.2[c]	74.5 ± 2.6 (n = 10)
0.05	89.2 ± 0.8	82.2 ± 3.8 (n = 3)
0.0	89.3 ± 0.8	76.7 ± 1.3 (n = 8)

[a]n, Number of plates ± SE where each plate represents an aliquot of pooled cells; if the number is not shown, n is equal to replicates at 48 h.

[b]Significantly different from control at $p \leq .01$ level using Student's t-test.

[c]Significantly different from control at $p \leq .05$ level using Student's t-test.

Phagocytosis and Cytotoxicity

In initial studies, particles were added to hamster AM to insure that AM were able to phagocytize the particles. The AM, 1.8×10^6, were placed onto glass cover slips 0.8 x 2 cm in a 35-mm dish. Ferric oxide and aluminum oxide were added at doses of 52 $\mu g/cm^2$, and all the silicas were added at 5.2 $\mu g/cm^2$ due to cytotoxic responses at the higher doses. The cells were incubated for 24 h and electron micrographs were taken at 6200x. The results indicated that the AM were able to phagocytize the particles under study (data not shown).

Comparative viability studies of the AM in the presence of ferric oxide, aluminum oxide, or the four forms of silica (crystalline, gelled, fumed, precipitated) were undertaken to determine noncytotoxic doses. Doses of particles ranged from 0.0 to 0.5 mg/plate, and viability results are reported in Tables 2–4. The viability of the hamster AM in the presence of aluminum oxide up to the highest dose was similar to controls (Table 2). After 24 and 48 h, the viability of the AM for aluminum oxide was approximately 80% and 70%, respectively. Similar results were obtained for ferric oxide with the exception of an apparent small cytotoxic response ($p \leq .01$) of the AM at the highest dose, 0.5 mg (Table 2). In the presence of silica (Table 3), the viability of the hamster AM was similar to controls up to a dose of 0.01 mg except for precipitated silica, where the viability was 57% at 48 h. The percent viability decreased with increasing dose and time. Zero percent viability occurred at 0.1 mg gelled silica. For precipitated, fumed, and crystalline, zero viability was observed at 0.5 mg. Viability at 48 h was consistently

TABLE 3. Effects of Silica on Hamster AM Viability

Silica (mg)	Percent viability of cells	
	24 h[a]	48 h
Crystalline, Min-U-Sil 5		
0.5	0	0 ($n = 2$)
0.1	51.0 ± 8.3	39.4 ± 9.0[b] ($n = 6$)
0.05	76.7 ± 3.3	55.2 ± 6.3 ($n = 6$)
0.01	87.7 ± 1.0	63.6 ± 4.4 ($n = 5$)
0.005	89.4 ± 1.1	66.6 ± 2.1 ($n = 3$)
0.0	85.0 ± 1.7	71.3 ± 3.6 ($n = 6$)
Amorphous		
Gelled		
0.5	0	0 ($n = 3$)
0.1	0	0 ($n = 3$)
0.05	27.7 ± 2.6[b] ($n = 6$)	5.5 ± 3.4[b] ($n = 5$)
0.01	79.3 ± 1.6[c]	75.2 ± 1.0 ($n = 3$)
0.005	87.8 ± 2.1	81.1 ± 1.1 ($n = 3$)
0.0	88.6 ± 1.0 ($n = 6$)	84.0 ± 2.9 ($n = 5$)
Fumed		
0.5	0	0 ($n = 2$)
0.1	42.9 ± 8.9[b] ($n = 9$)	29.6 ± 6.3[b] ($n = 8$)
0.05	67.3 ± 9.2	49.9 ± 7.9[c] ($n = 4$)
0.01	83.5 ± 1.1	66.9 ± 2.2 ($n = 4$)
0.0	87.9 ± 1.2	71.7 ± 2.9 ($n = 8$)
Precipitated		
0.5	0	0 ($n = 3$)
0.1	44.5 ± 2.4[b] ($n = 5$)	33.6 ± 1.8[b] ($n = 2$)
0.05	70.6 ± 2.9[b] ($n = 5$)	52.2 ± 2.2[b] ($n = 3$)
0.01	85.8 ± 2.1 ($n = 5$)	57.0 ± 4.3 ($n = 3$)
0.0	86.8 ± 2.0 ($n = 5$)	68.4 ± 3.1 ($n = 3$)

[a]n, Number of plates ± SE where each plate represents an aliquot of pooled cells; if the number is not shown, n is equal to replicates at 48 h.
[b]Significantly different from controls at $p \leq .01$, see Table 2.
[c]Significantly different from controls at $p \leq .05$, see Table 2.

lower than at 24 h. Each set of experiments for a specific particle had its own set of controls, which varied slightly at 48 h but had consistently higher viability values than the experimental values. Similar results for all the particles were obtained for the Sprague-Dawley rat AM (Table 4).

DISCUSSION

The National Institute for Occupational Safety and Health lists occupational lung disease among the 10 leading occupational diseases or illnesses found in the workplace (Centers for Disease Control, 1983). Numerous epidemiologic and experimental studies have indicated that various types of dusts and/or chemical carcinogens are important in the development of res-

piratory disease in the workplace (Perera, 1981; Perera et al., 1991; Nelson, 1970). An important aspect in the mechanism of dust-induced disease is the potential contribution of cocarcinogenic exposure. This laboratory has an ongoing research effort to assess the effects of particles and chemicals such as benzo[a]pyrene (BaP) on the metabolism and binding of xenobiotics in the lung. It has been reported that ferric oxide, crude air particulate, and fly ash alter the metabolism of BaP in the isolated perfused lung (Warshawsky et al., 1983, 1984; Schoeny & Warshawsky, 1983; Morgan et al., 1984). It was concluded that particles would enhance the metabolic activation of BaP as well as act as a carrier for penetration and retention in the lung.

TABLE 4. Effects of Fe_2O_3 and Silica on Rat AM Viability

	Percent viability of cells	
	24 h[a]	48 h
Fe_2O_3 (mg)		
0.0	89.6 ± 1.3	83.2 ± 3.9 ($n = 2$)
0.5	85.1 ± 1.8	79.4 ± 1.4 ($n = 3$)
Silica (mg)		
Crystalline, Min-U-Sil 5		
0.5	5.4 ± 1.0[b]	3.7 ± 1.1[b] ($n = 4$)
0.1	63.2 ± 1.3[b] ($n = 6$)	57.7 ± 3.8[b] ($n = 5$)
0.05	70.3 ± 5.1[c]	71.3 ± 3.5[c] ($n = 7$)
0.01	84.1 ± 2.1	81.5 ± 1.8 ($n = 5$)
0.0	86.2 ± 2.0	81.9 ± 1.4 ($n = 8$)
Amorphous		
Gelled		
0.5	5.1 ± 0.3[b] ($n = 4$)	1.6 ± 0.6[b] ($n = 3$)
0.1	10.5 ± 1.3[b]	12.1 ± 2.0[b] ($n = 3$)
0.05	29.1 ± 2.0[b]	23.2 ± 4.2[b] ($n = 4$)
0.01	74.9 ± 4.3[c]	67.1 ± 7.2[c] ($n = 4$)
0.0	87.9 ± 1.4	82.9 ± 1.8 ($n = 6$)
Fumed		
0.5	3.7 ± 1.1[b] ($n = 4$)	2.9 ± 1.2[b] ($n = 3$)
0.1	27.4 ± 3.3[b] ($n = 4$)	10.2 ± 1.1[b] ($n = 3$)
0.05	58.2 ± 3.3[b]	40.8 ± 5.7[b] ($n = 4$)
0.01	81.4 ± 1.1	77.3 ± 2.6 ($n = 4$)
0.0	86.0 ± 2.4	85.3 ± 2.0 ($n = 4$)
Precipitated		
0.5	6.0 ± 1.3[b] ($n = 3$)	3.5 ± 1.1[b]
0.1	75.8 ± 1.9[c]	75.8 ± 3.8 ($n = 3$)
0.05	73.0 ± 3.5[c] ($n = 4$)	70.9 ± 6.9[c] ($n = 3$)
0.01	83.8 ± 2.1	78.8 ± 4.5 ($n = 4$)
0.0	90.4 ± 1.7	88.0 ± 1.5 ($n = 4$)

[a]n, Number of plates ± SE where each plate represents an aliquot of pooled cells; if the number is not shown, n is equal to replicates at 48 h.

[b]Significantly different from controls at $p \leq .01$, see Table 2.

[c]Significantly different from controls at $p \leq .05$, see Table 2.

To assess in greater detail the effects of the deep penetration of ferric oxide into the lung, the effect of coexposure of ferric oxide with BaP to hamster AM in vitro in suspension culture was determined (Greife & Warshawsky, 1993). The results indicated that in the presence of ferric oxide, both dihydrodiols of BaP and superoxide anions were significantly enhanced. The data suggested that localized release of superoxide anions could cause localized damage to the surrounding tissue, predisposing the epithelial tissue to further injury by the increased production of the biologically active BaP metabolites. The results raised questions as to whether these effects in the AM were specific to ferric oxide or to particles in general. Therefore, crystalline silica, a suspect human and confirmed animal lung carcinogen (IARC, 1987), was chosen for study because it also enhanced benzo[a]pyrene carcinogenesis in the lungs of hamsters. The amorphous silicas were also assessed due to their noncrystalline structure, previous study, and occupational use.

An AM plate-assay culture system was developed appropriate for assessing phagocytosis and cytotoxicity as well as metabolic parameters such as DNA binding and the production of mutagenic metabolites. The crystalline silica had a smaller surface area and a larger count medium diameter than any of the amorphous silicas; the gelled silica had the largest surface area of all the particles tested. The cytotoxicity data from the interactions of silica with AM were generally consistent with this physical characteristic in that the particle with the largest surface area was the most cytotoxic; that is, gelled silica was the most cytotoxic, which was consistent with its large surface area.

A possible explanation of the observed effect is the presence of silanol groups on the particle surface. The silanol groups associated with crystalline silica have been hypothesized to play an essential role in its cytotoxicity (Allison et al., 1966). Silanol groups are also found on amorphous silica (Iler, 1979). It should be noted that gelled and precipitated silica in aqueous solution are covered with silanol groups, while pyrogenic silica (fumed) is only partly covered with silanol groups; the remainder is covered with siloxane groups (Iler, 1981). The test materials with the largest surface area present more silanol groups for interactions with phospholipids on the surface of the AM (Langer, 1978). This would explain why ferric oxide and aluminum oxide were not cytotoxic or only weakly cytotoxic at these doses, while all silicas were markedly cytotoxic. At a constant dose of the silica, the surface area available to interact with AM membranes differs, as do the total silanol groups available. Therefore, it should be noted that dose as well as surface area and surface characterization of silica particles are all important in the overall assessment of AM.

The data for silica indicated that these particles with count median diameter of 0.38 μm or less could induce AM cytotoxicity. This suggests that a greater amount of the particles of amorphous silicas entered the AM (Karnovsky, 1962). Another possible contribution to the observed results

would be undetected crystalline silica in the amorphous samples. The particle sizes observed are substantially below the optimal range of x-ray diffraction (S. Leslie, DataChem, personal communication, 1992). However, it appears unlikely that crystalline silica could be present in sufficient quantity to result in the observed cytotoxicity and not be detected. The presence of metallic impurities was also considered. In a comparison of gelled and precipitated silica, the metal impurities in the precipitated silica were greater than the gelled but the cytotoxicity was greater in the gelled silica (Groth et al., 1981). The crystalline silica had little in the way of impurities but was not more cytotoxic than precipitated silica (Stettler et al., 1981).

The results support the hypothesis that the silica is taken up into the phagosomes in the cytoplasm (Harington & Allison, 1965). The silica then by surface action through the silanol groups damages the membrane of the phagosomes, resulting in the release of hydrolytic enzymes from lysosomes into the AM cytoplasm. The process then repeats itself as the particles that are released are phagocytized in a continuing cycle. Under this hypothesis, the more silanol groups available, the faster and the more severe the cytotoxic response. In this set of experimental results, gelled silica demonstrates a highly cytotoxic response, which may indicate the presence of silanol groups on the very large surface area; the particles are available inside the phagosome following uptake. In support of this hypothesis, it has been indicated that amorphous silica can denature protein and disrupt cell membranes (Iler, 1981). An alternative explanation could involve the perturbation of intracellular calcium levels. It has been theorized that silica "piggybacks" ionic calcium into AM, where it is released, resulting in cell toxicity by attack on the cell structure and activation of nucleases, which destroys the DNA. Following cell death, the silica becomes available for phagocytosis again by AM (Kane et al., 1980; Van Dyke et al., 1993; Chen et al., 1991). It should be noted that the cytotoxicity of AM by silica can be modified by coating the particles with organosilane material, Al_2O_3, or alkylamine derivatives (Vallyathan et al., 1991; Kessel et al., 1963; Marks, 1957; Brown et al., 1989). This would be important in occupational settings in coating of freshly prepared particles to decrease any cytotoxic effects. Further studies in vivo would be required to answer whether these measures would be effective.

The differences that are present in the rat and hamster with crystalline silica in both the carcinogenic responses and the necrosis of AM in vivo (Saffiotti, 1986) are not seen in the cytotoxic responses to crystalline or amorphous silica in vitro. This may be due in part to fact that the hamster and rat in vivo studies were done independently of each other using different particle size samples and sources. Since many of the cellular reactions involving AM appear dependent on the degree of surface toxicity of the particles (Saffiotti, 1986), it would appear that the characterization of the physical properties of particles used in experimental studies is of paramount importance (Langer & Nolan, 1986; Hemenway et al., 1986). This study represents one in which the response in both species has been evaluated

using material from the same sources under the same environmental conditions, and therefore the results are directly comparable in vitro. It should be noted that freshly fractured crystalline silica is more cytotoxic when compared with aged crystalline silica in vitro in rats AM (Vallyathan et al., 1991). Comparative studies with both rat and hamster need to be carried out in vivo with carefully characterized aerosols. In a long-term chronic study of inhaled amorphous silicas of comparable physical characteristics relative to the study in guinea pigs, rats, and monkeys, only in the monkey did fumed silica induce early nodular fibrosis (Groth et al., 1981). Very little amorphous silica was found in the macrophage of the rat or guinea pig, while large amounts were present in the monkey AM, which would suggest that the small particles were not deposited in the deep lung in the rat.

In summary, the in vitro AM system described can be used to determine the noncytotoxic doses of particles to AM for two species, hamster and rat, and can be easily used in changing variables such as particles versus organic-coated particles. This will be essential information that will be used in arriving at a better understanding of AM exposure to a variety of particles and carcinogen-coated particles.

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