Relationship between Surface Properties and Cellular Responses to Crystalline Silica: Studies with Heat-Treated Cristobalite

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A fibrogenic sample of cristobalite dust, CRIS (crystalline silica of mineral origin), was heated to 1300 °C (CRIS-1300) to relate induced physicochemical modifications to cytotoxicity. Heating did not affect dust micromorphology and crystallinity, except for limited sintering and decreased surface area of CRIS-1300. Thermal treatments deeply affected surface properties. Electron paramagnetic resonance showed surface radicals progressively annealed by heating, mostly disappearing at ≥800 °C. Surface hydrophilicity or hydrophobicity, evaluated with water vapor adsorption, still showed some hydrophilic patches in CRIS-800, but CRIS-1300 was fully hydrophobic. Heating modified the biological activity of cristobalite. Cytotoxicity, tested on proliferating cells of the mouse monocyte macrophage cell line J774, showed that CRIS was cytotoxic and CRIS-800 was still cytotoxic, but CRIS-1300 was substantially inert. Cytotoxicity of CRIS to the rat lung alveolar epithelial cell line, AE6, as measured by colony forming efficiency, was greatly reduced for CRIS-800 and eliminated for CRIS-1300. The rate of lactate dehydrogenase release by rat alveolar macrophages was lowered for CRIS-800, and release was completely inactivated for CRIS-1300. The absence of surface radicals and the onset of hydrophobicity may both account for the loss of cytotoxicity upon heating. Differences observed between CRIS-800 and CRIS-1300, both fully deprived of surface radicals, indicate that hydrophobicity is at least one of the surface properties determining the cytotoxic potential of a dust.

Introduction

The crystalline silica polymorphs, quartz, cristobalite, and tridymite, are highly pathogenic mineral dusts. Prolonged inhalation of crystalline silica particles causes lung inflammation and development of the acute and chronic granulomatous and fibrogenic lung disease, silicosis. Silica is also associated with the development of lung cancer. The International Agency for Research on Cancer has classified crystalline silica inhaled in the form of quartz and cristobalite from occupational sources as carcinogenic to humans (*1*). It had previously recognized it as carcinogenic in animals (*2*). Despite extensive work in past and recent years (*3*), the molecular mechanisms whereby silica induces its various pathogenic effects have not yet been fully clarified.

The biological response to silica differs from that to soluble or liquid compounds because solid insoluble particles undergo phagocytosis in alveolar macrophages and interact with other cells and several biological compartments. Silica-originated diseases are thus the consequence of multistep cellular and tissue responses to the crystalline surface of the dust particles. Moreover, as happens with most solid xenobiotics, more than one surface functionality may be implicated in the pathogenic mechanism. This complexity (*4*) may provide an explanation for the fact that not all crystalline silica samples are equally pathogenic. The overall evaluation reported by the IARC was preceded by the statement "...carcinogenicity in humans was not detected in all industrial circumstances studied. Carcinogenicity may be dependent on inherent characteristics of the crystalline silica or on external factors affecting its biological activity or distribution of its polymorphs" (*1*).

Different preparations of crystalline silica, used for in vivo and in vitro studies of silica toxicity and carcinogenicity, were found to vary widely in their order of activity, when evaluated in different assays (*5*). Clearly, the biological response to silica is modulated by differences

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in the state of the surface of the particles, depending on the origin and history of the dust (*6*). These differences may help in the understanding of the molecular mechanisms of toxicity.

Attempts were made to correlate physicochemical properties of silica with toxicity (*6*), but no single defined property, present only on fibrogenic crystalline silica and absent in nonfibrogenic minerals, has been identified (*8*).

The association of a given surface chemistry property with a defined cellular response may be studied in two ways: (a) by identifying physicochemical differences among samples exhibiting different biological responses (*5*) and (b) by preparing samples in which a single physicochemical property is modified at a time to determine if this modification corresponds to changes in the cellular response.

In this paper, we report the results obtained by studying a mineral sample of cristobalite, which was found to be fibrogenic to rats (*6*), before and after heating it at different temperatures up to 1300 °C. Cristobalite, being the high-temperature stable polymorph, does not undergo crystal lattice transformation upon heating. The surface, however, is deeply affected by heating. Thermal treatments of silica cause annealing of the surface radicals originated by grinding and transform silanols (Si−OH, hydrophilic functionalities) into siloxanes (Si−O−Si, hydrophobic functionalities) (*8*, *9*). As surface hydrophobicity determines the extent of protein adsorption and cell adhesion to surfaces (*10*), it is important to investigate whether modifications of the hydrophilicity of the surface relate to the biological response.

The effects of chemical modifications at the silica surface have been previously investigated by several authors by binding polymers to the surface (*11*−*13*). In all cases, the surface reactivity of silica was decreased, indicating that binding hindered or eliminated some surface active sites. The binding process, however, is a complex one, depending on the chain length and terminal functionalities of the chain, and hydrogen bonding to silanols may occur, with consequent nonhomogeneity of the modified surface (*14*).

Heating modifies the state of the silica surface and progressively decreases the membranolytic activity of silica dusts toward erythrocytes (*9*, *15*, *16*, *39*), by eliminating from the surface the sites responsible for the interaction with cell membrane components.

The cellular and tissue responses to heated crystalline silica dust need to be studied so that we may better understand the relationship between the state of the surface and related biological effects. We have therefore studied the physicochemical modifications that occur when a cristobalite dust was heated at 800 °C, a treatment which appears to affect transport and retention, or at 1300 °C, which eliminated all toxic responses to the dust. The effect of the original and of the two thermally modified cristobalite dusts was investigated with two separate cell lines: (1) J774, a mouse monocyte macrophage tumor cell line, in stationary and in exponential growth phases (*17*); and (2) AE6, a rat lung alveolar epithelial cell line immortalized by transfection with the adenovirus-2 E1A gene (see the Supporting Information). The same samples were also tested on rat alveolar macrophages for lactate dehydrogenase (LDH)[1] release. The choice of cell lines was based on the crucial role played by macrophages in the proposed mechanisms of fibrosis and lung cancer (*1*, *4*, *6*) and on the possible direct effects of particles on target cells.

[1] Abbreviations: LDHL, actate dehydrogenase; CRIS, cristobalite; CFE, colony forming efficiency.

Experimental Procedures

Materials. Cristobalite dust, prepared by crushing the mineral (C. E. Mineral Co., King of Prussia, PA), was kindly supplied by D. Hemenway (University of Vermont, Burlington, VT). The sample obtained was fibrogenic to rats (*7*). This sample was heated to 150, 500, or 800 °C in a quartz container and at 1300 °C in an alumina crucible. The samples were coded as CRIS for the unheated sample and CRIS-150, CRIS-500, CRIS-800, and CRIS-1300 for those heated at the respective temperatures.

Water vapor used in adsorption experiments was distilled several times in a vacuum and rendered gas free by several "freeze−pump−thaw" cycles.

Methods. (1) Physicochemical Characterization. (i) X-ray Diffraction (XRD). Spectra of the powders were recorded using a Philips diffractometer with the conventional Bragg−Brentano geometry (CoKα incident radiation).

(ii) Electron Microscopy. High-resolution transmission electron microscopy (HRTEM) micrographs were obtained with an electron microscope JEOL JEM 2000 EX instrument (200 kV acceleration), equipped with a top-entry stage. The samples were dispersed in *n*-heptane and then deposited on Cu grids coated with a porous carbon film.

(iii) Surface Area and Particle Size Distribution. The surface area of the samples that were employed was measured with the BET method (nitrogen adsorption at −196 °C, "Quantasorb", Quantacrome). The particle size distribution was kindly determined by scanning electron microscopy by W. E. Wallace, M. J. Keane, and J. C. Anderson (Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Morgantown, WV).

(iv) Electron Paramagnetic Resonance (EPR). The EPR spectra of the heated dust were recorded on a Varian E 109 spectrometer operating at X-band. The spectra were recorded in vacuo at 77 K using a technique described in previous papers (*18*, *19*). An appropriate EPR cell, which allowed heating of the sample in vacuo and recording of the spectra without exposure to the atmosphere, was employed. The samples were heated for 2 h at 250, 350, 500, 800, and 1300 °C.

(v) Heat of Adsorption of Water. Heats have been determined by means of a Tian-Calvet microcalorimeter (Setaram) connected to a volumetric apparatus which allowed simultaneous measurement of adsorbed amount (uptake, n_a), heat released (Q), and equilibrium pressure (p) for small increments of water vapor dosed to the silica sample. Doses of the adsorptive were subsequently admitted onto the sample, the pressure being continuously monitored by means of a 0−100 Torr (1 Torr = 101325/760 Pa) transducer gauge (Baratron MKS). The procedure has been previously described in detail (*20*, *21*). The extent of adsorption of water has been measured on cristobalite heated at 150, 500, 800, and 1300 °C. The samples were outgassed in the calorimetric cells for 2 h at the adopted temperature and subsequently placed in the calorimetric vessel without exposure to the atmosphere. For the sample heated at 1300 °C, the procedure was slightly different because the thermal treatment could not be performed in the calorimetric cell. The sample was heated in a furnace in flowing inert gas, then cooled, and immediately transferred into the calorimetric cell (*21*). The sample was then heated again up to 800 °C to remove any adsorbate which could have deposited on the surface from the air during the above procedure.

The temperature of the calorimeter was maintained at 303 K throughout the adsorption experiment. A typical adsorption sequence comprised two runs, with the following procedure: (i) dosing successive amounts of water vapor to the sample up to a pressure of typically 5−10 Torr (Ads I), (ii) desorption at 303

K under vacuum, and (iii) readsorption of similar doses for evaluating the reversible adsorption (Ads II).

(2) Cellular Studies. (i) Tests on J774 Cells. The J774 cells, a mouse monocyte macrophage tumor cell line (*17*), were a kind gift of A. Corsini (Istituto di Scienze Farmacologiche, University of Milan, Milan, Italy). This cell line was chosen as representative of macrophages and their response to inhaled particles in the lung. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose and 1 mM sodium pyruvate, supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and antibiotics (50 units/mL penicillin and 50 μg/mL streptomycin) at 37 °C under a humidified atmosphere of 5% CO_2. Routinely, J774 cells, grown as a monolayer in 75 cm^2 flasks (Nunc, Roskilde, Denmark), were removed from the substratum with the aid of a cell scraper, and they were split at a 1:2 or 1:3 ratio every 2 or 3 days.

(ii) Cell Proliferation Test on Exponentially Growing Cultures. The J774 cells were plated at a density of 1.5×10^5 cells per well (2 cm^2 surface area) in 24-well plates (Nunc) in 1 mL of growth medium in the presence or absence of each cristobalite sample at 5 and 50 μg/mL. For each concentration, two wells were set up to measure the extent of inhibition of cell proliferation 24, 48, and 72 h after the beginning of exposure. Growth effects were determined by counting the viable cells in a haemocytometer. The cell viability was estimated by Trypan blue exclusion.

(iii) Cytotoxicity As Measured by the Extent of LDH Release. The extent of LDH release was determined both on confluent and on exponentially growing cultures. The LDH activity was measured under the same culture condition described above, both in the culture medium and in the cellular monolayer, after solubilization with 0.1% Triton X-100. The sum of the units of LDH found in the culture medium and in solubilized cells was assumed to be the total LDH activity (*22*). The extent of enzyme leakage was estimated from the amount found in the culture medium expressed as a percentage of the total activity. The amount of LDH was determined every 24 h during culture for 3 days.

(iv) Tests on Rat Alveolar Macrophages. Twelve Sprague-Dawley rats were euthanasied with an intraperitoneal injection of pentobarbital, and a bronchoalveolar lavage was conducted according to the procedure described previously (*23*). Four successive lavages were performed with 8 mL of phosphate-buffered saline (37 °C). All the lavage fluids were pooled, centrifuged (2000 rpm for 10 min at 4 °C), and the cell pellet was recovered in DMEM supplemented with antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin) and 0.1% lactalbumin hydrolysate (DMEM-H). A total of 25×10^6 macrophages was obtained by this procedure. The cells were seeded in 24-well culture plates in DMEM-H (0.5×10^6 cells/well) and incubated overnight in a water-saturated atmosphere (5% CO_2).

(v) Cytotoxicity As Measured by the Extent of Lactic Dehydrogenase Release. The cytotoxicity of the three dust samples was assessed by measuring the extent of LDH release from rat alveolar macrophages exposed in vitro to increasing gravimetric concentrations of the particles. The different cristobalite samples were heated (200 °C for 4 h) to remove any possible trace of endotoxin contamination and suspended in DMEM-H (4 mg/mL) immediately before use. On the second day of culture, the cells were rinsed and exposed to increasing concentrations of the three cristobalite samples (0, 10, 20, 50, 100, and 200 μg/mL DMEM-H, 1 mL/well). For each concentration, three replicate culture wells were examined. After culture for 18 h in contact with the particles, the culture media were transferred into a microcentrifuge tube and centrifuged (2000 rpm for 10 min) to pellet nonadherent cells, and LDH activity was measured in the supernatant by monitoring spectrophotometrically the reduction of NAD^+ in the presence of lactate. The extent of LDH release during the exposure period was expressed in the percentage of total cellular enzyme content determined in parallel culture after disruption in 0.1% Triton X-100.

Table 1. Particle Size Distribution (%)

size (μm)	CRIS	CRIS-800	CRIS-1300
<1	21.0	17.3	21.1
1−2	29.8	36.9	33.1
2−3	21.9	24.3	17.6
3−4	12.5	12.6	9.1
4−5	6.2	3.5	8.0
5−6	3.4	1.5	2.9
6−7	2.0	1.3	0.8
7−10	2.3	1.2	3.2
10−20	0.5	1.3	3.2
>20	0.2	−	0.6

(vi) Tests on AE6 Cells. The AE6 cell line was established from lung alveolar epitehlial cells, isolated from 7-week-old F344 female rats according to the method of Dobbs et al. (*22*), with some modifications, and immortalized by transfection with the plasmid adenovirus-2 pE1A Sac1. One of the clonal colonies, with regular epithelial pavement morphology, was the origin of the AE6 cell line (U. Saffiotti, 1996, details given in the Supporting Information). This cell line was chosen as a representative of rat alveolar type II epithelia, which are the principal target of silica-induced carcinogenesis in vivo (*25*) and also contribute to the mechanisms of inflammation and fibrogenesis through their release of cytokines and growth factors (*26*).

(vii) Cytotoxicity Assays with Colony Forming Efficiency (CFE). AE6 cells, frozen at passage 6, were thawed and expanded in a T75 flask in LEP-RTE-1 with 2% FBS; 4 days later, the cells were plated for the clonal growth assay in 50 mm (20 cm^2) plastic dishes, at 200 cells/dish, 4 dishes/variable, with 4 mL/dish of the same medium, and 48 h later, the medium was changed to medium containing the tested dust at doses up to 50 μg/cm^2. Six days after treatment, the dishes were fixed in methanol and stained with Giemsa. The CFE was determined as a measure of cell survival by counting the number of colonies with 20 or more cells.

Results

Physicochemical Characterization. The cristobalite sample did not change its crystalline structure following thermal treatments. The X-ray diffraction pattern, not reported for brevity, was identical for all the samples that were employed. The diffractograms exhibit sharp lines and no diffuse broad line, indicating high crystallinity and the absence of amorphous material in the samples. Table 1 shows the particle size distribution as evaluated by electron microscopy; the distribution of the finer particles was similar for the three samples, but CRIS-1300 exhibited a higher percentage of particles in the 10−20 μm range. The shape of the individual particles, reported in the TEM pictures in Figure 1A for CRIS and Figure 1B for CRIS-1300, reveals the typical sharp edges and acute spikes of crushed crystalline silicas (*6*, *20*). While no morphological differences between CRIS and CRIS-800 could be detected, CRIS-1300 exhibited some smoothing up on the edges together with an increase in the average dimensions of the particles. This observation is consistent with the measured surface area. The specific surface area of CRIS was 6 m^2/g; it did not vary when the sample was heated to 800 °C, but it decreased to 1.2 m^2/g upon heating at 1300 °C. At this temperature, although still well below the melting temperature, some sintering clearly takes place, with smaller particles merging into bigger ones. The TEM images, however, do not reveal differences in particle size sufficient to justify the observed difference in BET surface area between CRIS-800 and CRIS-1300. We infer therefore that heating at 1300 °C mostly caused sintering of

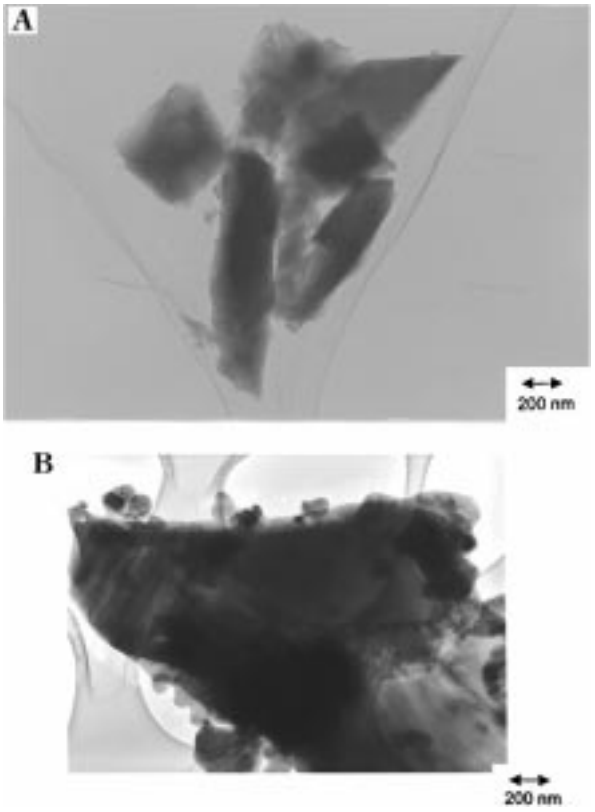


Figure 1. High-magnification TEM images of unheated cristobalite (CRIS) (A) and of cristobalite heated at 1300 °C (CRIS-1300) (B).

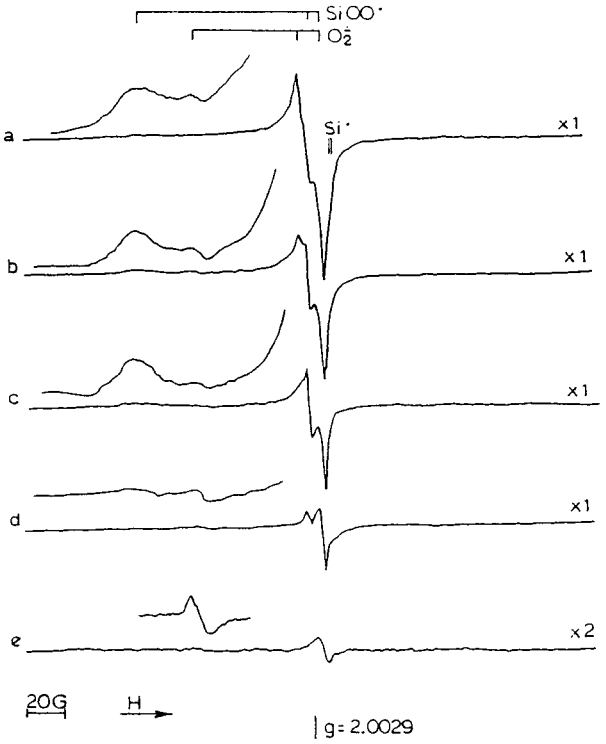


Figure 2. EPR spectra of cristobalite unheated (a) and heated for 2 h at 250 (b), 350 (c), 500 (d), and 800 °C (e). The high-field part of the spectrum is reported at higher magnification to show that the g_{zz} components of $O_2^{-\bullet}$ and SiOO• (*20*, *21*). The spectra were recorded in vacuo at 77 K.

microcracks at the atomic level which contribute to the overall surface area as measured by nitrogen adsorption. Biomolecules or cells do not interact with the surface exposed in microcracks, because only small molecules such as dinitrogen are accessible; therefore, the differences between CRIS-800 and CRIS-1300 as measured by BET may be much less relevant in terms of cytotoxicity. The micromorphology of CRIS-1300 also reveals a relative heterogeneity in size, including both large and small particles. When examined in the cellular assays, particles of CRIS-800 and CRIS-1300 are found to be internalized in comparable patterns, although with different effects as reported below.

The EPR spectrum of the original dust is depicted in Figure 2. It is a structured spectrum with the most intense components close to the free electron value. This kind of spectra, widely discussed in previous papers (*18*, *19*), originates from the dangling bonds created by the homolytic and heterolytic rupture of the silicon−oxygen bonds and their reaction with atmospheric components, mainly oxygen. The major feature of the spectrum is represented by the lines arising from the superposition of the spectrum of the superoxide anion ($O_2^{-\bullet}$) with that of the silylperoxide surface functionality (SiOO•) (*6*, *8*, *18*, *19*). When the dusts are heated, the spectrum undergoes profound modifications. The superoxide anion is destroyed below 350 °C, while the silylperoxide progressively disappears upon heating from 350 to 800 °C. Only minor vestiges of other radicals are present in the sample heated at 800 °C (Figure 2e) which fully disappear on the one heated at 1300 °C (not shown).

The affinity for water, measured by both water uptake and heat released on adsorption of water vapor, decreases upon heating. The total water uptakes, measured under the equilibrium pressure of 5 Torr of water ($p/p^0 \simeq 0.15$), and the related energy of adsorption are reported in Table 2.

Water uptakes progressively decrease upon heating; on CRIS-1300, only 20% of the water adsorbed on the original cristobalite can be adsorbed. The energy of adsorption does not change significantly when CRIS is heated to 500 °C, but decreased down to 44 and 20 kJ on CRIS-800 and CRIS-1300, respectively. The latent heat of liquefaction of water is 44 kJ/mol.

Any surface site adsorbing water with a heat of adsorption above 44 kJ/mol is considered hydrophilic, while those adsorbing water with a heat below 44 kJ/mol are hydrophobic (*20*, *21*). Clearly, the cristobalite surface upon heating switches from being mostly hydrophilic to mostly hydrophobic around 800 °C. The average value of 44 kJ/mol reported for Cris-800, considering the intrinsic heterogeneity in adsorption sites (*21*), indicates the cohesistence at the surface of CRIS-800 of hydrophilic and hydrophobic sites.

Cellular Studies. (1) Cytotoxicity toward J774 Cells. The cytotoxic effect of the dusts was determined on exponentially growing cultures. Following exposure to each of the three samples at concentrations of 5 and 50 μg/mL, the cell number was determined every 24 h during culture for 3 days. Figure 3A shows cell numbers as a function of time for the two concentrations of CRIS. A reduction of about 25% in the extent of cell proliferation was evident with 5 μg/mL at 48 and 72 h. A drastic inhibition was observed at 50 μg/mL even after only 24 h. The effects caused by CRIS-800 (Figure 3B) showed no significant difference with respect to those of CRIS at 5 μg/mL, whereas at 50 μg/mL, CRIS-800 was consid-

Table 2. Adsorption of Water Vapor on Cristobalite Heated at Various Temperatures

	CRIS	CRIS-150	CRIS-500	CRIS-800	CRIS-1300
rate of water uptake (μmol/m^2)	9.70	9.45	9.00	6.45	2.00
energy of adsorption (kJ/mol)	51	54	52	44	20

Values measured under an equilibrium pressure of 5 Torr.

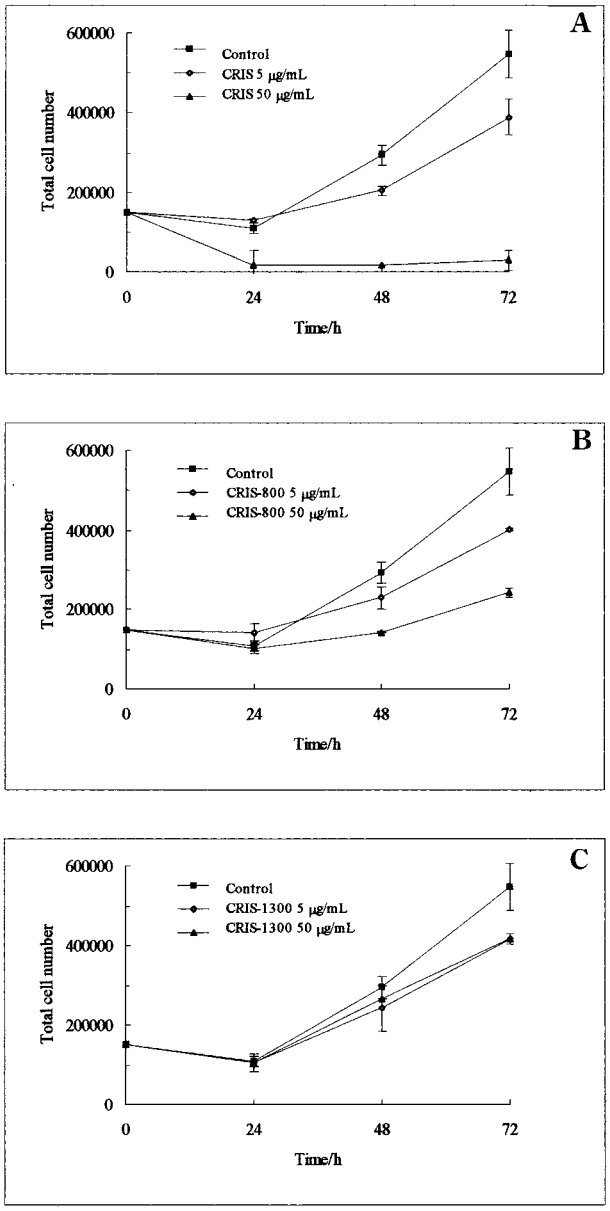


Figure 3. Effect of cristobalite before and after heat treatment on cell proliferation, assessed as the number of cells per dish. J774 cells were cultured for 72 h in the presence of the indicated concentrations of CRIS (A), CRIS-800 (B), and CRIS-1300 (C). A representative experiment out of four is shown.

erably less effective in inhibiting cell proliferation than CRIS. CRIS-1300 showed no significant effect at the two doses of 5 and 50 μg/mL up to 48 h, and only a moderate inhibition of cell proliferation at 72 h (Figure 3C).

The integrity of cell membrane was measured by the extent of leakage of LDH. On confluent cultures, incubated for 24 h with the dusts, only CRIS at concentrations of 5 and 50 μg/mL, induced an increased level of loss of the enzyme, 12.6 and 27.5%, respectively. With

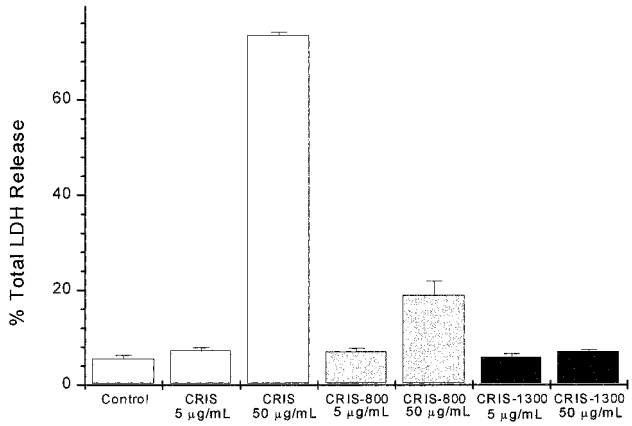


Figure 4. LDH determination on J774 cells cultured for 72 h in the presence of the indicated concentrations of the cristobalite dust. The extent of enzyme leakage is expressed as a percentage of the total activity.

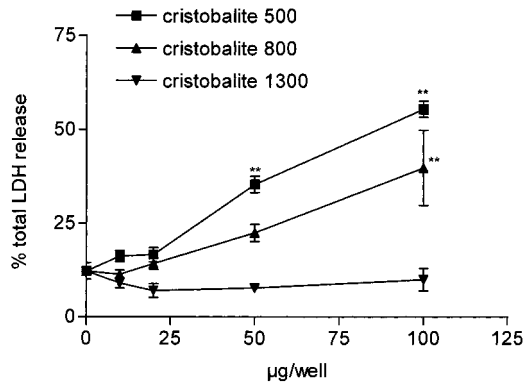


Figure 5. Cytotoxicity of CRIS-500, CRIS-800, and CRIS-1300 on rat alveolar macrophages, as measured by the extent of LDH release (mean ± SD of three separate determinations).

CRIS-800 and CRIS-1300, the enzyme leakage was similar to that found in the control cells (data not shown).

On exponentially growing cultures, the amount of LDH was determined every 24 h during culture for 3 days. The rate of enzyme leakage did not change during culture for up to 3 days in the control cells. Only CRIS at a concentration of 50 μg/mL induced a marked release of LDH, revealed after treatment for 24 h. In the following 2 days, the percentage of enzyme release (70−75%) was similar to that found at day 1 (Figure 4). CRIS-800, at a concentration of 50 μg/mL, induced enzyme leakage (20−25%), but to a much lesser extent than CRIS. In contrast, CRIS-1300 did not cause any LDH leakage above the control value. Similar results were obtained in all 3 days of culture (Figure 4).

(2) Cytotoxicity Tests with Rat Alveolar Macrophages. The cytotoxic activity of the three samples, assessed by measuring the extent of LDH release which reflects a loss of membrane integrity, is summarized in Figure 5. A dose-dependent cytotoxic effect was observed with CRIS-500 and CRIS-800, the latter sample being

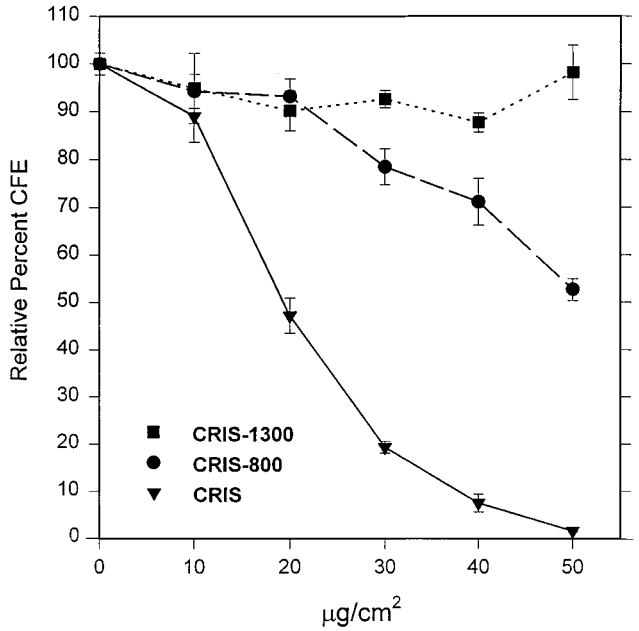


Figure 6. Cytotoxicity in AE6 cells, as measured by relative colony forming efficiency (CFE) as a function of dose (micrograms of dust per square centimeter of culture dish area). Values are means ± SEM. A Student's *t* test showed significant differences between test results ($n = 4$) and controls ($n = 12$) for CRIS at 20−50 μg/cm^2 ($p < 0.01$) and for CRIS-800 at 30−50 μg/cm^2 ($p < 0.01$) but no difference between tests and the control for CRIS-1300 at any dose except 40 μg/cm^2 (which was sligtly lower, $p < 0.05$).

less effective (pc 0.01, ANOVA + Turkey test for multiple comparison). No cytotoxic effect of CRIS-1300 could be evidenced up to a dose of 100 μg/mL.

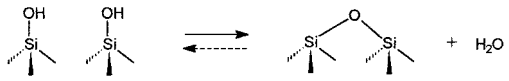
(3) Cytotoxicity for AE6 Cells (CFE). Active uptake and internalization of particles by the AE6 cells were observed with all three tested dust samples. Cells treated with unheated CRIS and, to a lesser extent, CRIS-800 showed morphological evidence of cell killing and accumulation of cell debris, whereas those treated with CRIS-1300 did not.

The level of survival of colony-forming cells was progressively reduced by exposures to concentrations of 10, 20, 30, 40, and 50 μg/cm^2 of the unheated CRIS, with survival at the dose of 50 μg/cm^2 reaching only 1.6 ± 0.2% of those of untreated controls. CRIS-800 induced moderate toxicity, reaching 52.7 ± 2.3% of those of untreated controls at the dose of 50 μg/cm^2. In contrast to both other samples, CRIS-1300 exhibited no significant toxicity, with relative CFEs of 87.7 ± 2.0% at a dose of 40 μg/cm^2 and 98.2 ± 5.7% at a dose of 50 μg/cm^2 (Figure 6).

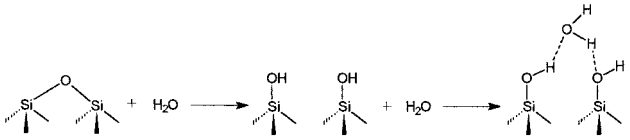
Discussion

Thermal treatment of the original cristobalite sample up to 1300 °C did not alter its crystalline structure, as shown by X-ray diffraction, but simply caused on CRIS-1300 some sintering of particles, smoothing up of the surface, and elimination of microcracks. All these factors contribute to the reduction of the specific surface area. The surface, in contrast, was dramatically altered as surface radicals were removed and silanols were converted into siloxane bridges. Surface radicals were found to play a crucial role in silica pathogenicity (*8*, *18*, *19*, *27*, *28*). When the dusts were heated, the radicals were progressively annealed so that no appreciable amounts of surface radical species were present on CRIS-800 and CRIS-1300. Any biological effect directly linked to the presence of surface radicals should thus be eliminated on samples heated to ≥800 °C.

The variation of water adsorption features on the heated samples is the consequence of the progressive condensation of silanols into siloxanes during the thermal treatment and of the partial reverse reaction upon exposure to water:



The reverse reaction does not fully occur, and the higher the heating temperature the lower the extent of rehydroxylation (*8*, *21*). The data presented here are in agreement with previous studies on quartz which also showed progressive dehydroxylation of the surface (*16*, *39*). The adsorption of water on heated surfaces is thus the superposition of two processes: (1) dissociation of water onto siloxane bridges (Si−O−Si) with formation of two adjacent silanols and (2) adsorption of water via H-bonding onto the surface silanols.



Process 1 is irreversible under the experimental conditions adopted for adsorption; i.e., all siloxanes converted into silanols stay as such if the sample is not heated again. Process 2, in contrast, is fully reversible; i.e., by evacuation of the gas phase, the adsorbed water is desorbed. The technique adopted here, of performing a first adsorption run followed by desorption and subsequent readsorption, allows a precise evaluation of processes 1 and 2. Process 2 is readily achieved by the second adsorption run, while process 1 (irreversible) is achieved by subtracting data of adsorption II from adsorption I. Panels a and b of Figure 7 show, as a function of the temperature of the thermal treatments, the extents of water adsorbed in processes 1 and 2, respectively, always measured under an equilibrium pressure of water vapor of 5 Torr. It can be clearly seen that the extent of reversible adsorption, related to H-bonding of water, decreases upon heating, because silanols are progressively eliminated by the surface. The extent of irreversible adsorption exhibits a maximum for a thermal treatment at 500 °C. At this temperature, a large portion of silanols have been transformed into siloxanes, but this transformation is still largely reversible; i.e., upon contact with the surface, water molecules are dissociated and again form surface silanols. At higher temperatures, more and more silanols are progressively converted into siloxanes, but most of the latter are stabilized and stay as such even upon contact with substantial amounts of water; the surface becomes progressively hydrophobic in this way, as siloxanes are the typical hydrophobic moieties at the silica surface (*8*, *20*). It is important to note that in this respect CRIS-1300 is fully hydrophobic (very low heat of adsorption of water and no surface rehydroxylation) while CRIS-800 has indeed lost about 60% of its original silanols, but still holds some, partly obtained by water dissociation, and therefore, it has to

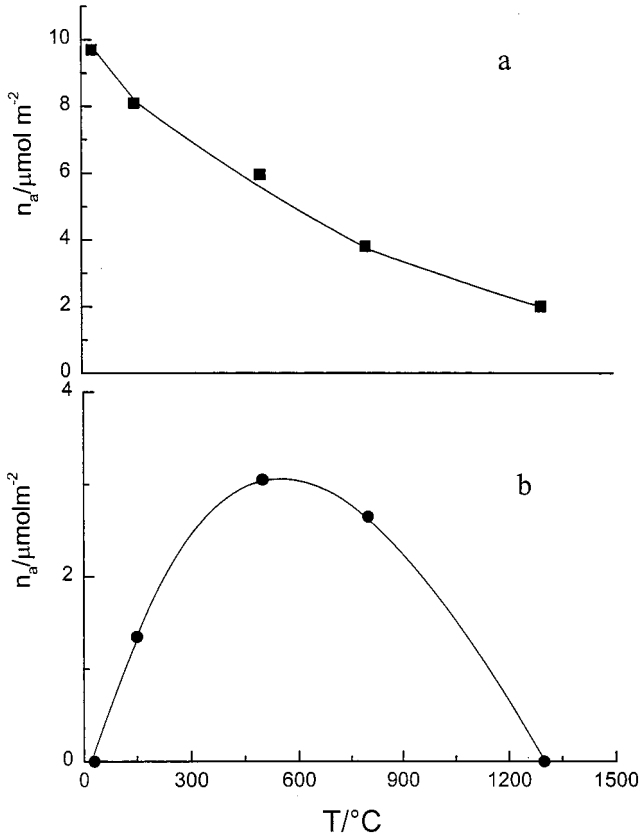


Figure 7. Water adsorbed in reversible (a) and irreversible (b) processes as a function of the temperature of the thermal treatments, measured under an equilibrium pressure of 5 Torr.

be considered bifunctional, i.e., having both hydrophilic and hydrophobic patches, in agreement with the energy of adsorption reported in Table 2.

Cellular studies showed that the original sample of cristobalite was cytotoxic to all the tested cell types and exhibited dose-dependent effects, in agreement with previous findings on the cytotoxicity on cristobalite of mineral origin (*29*). Thermal treatments clearly decreased the cytotoxic activity of the dust, clearly indicating that the surface properties present on cristobalite particles and responsible for the cytotoxic effect are removed by thermal treatment and that inactivation is complete after treatment at 1300 °C.

In apparent contrast, the hydroxyproline content of the lung, an index of fibrogenicity, did not vary significantly in rats examined up to 150 days, following an inhalation period of 6 h/day for 8 days at the mean exposure dose of 10 mg/m^3 of unheated cristobalite or cristobalite heated at 800 °C (*30*). It is noted, however, that the histological evidence reported by the same laboratory (*31*) for lung fibrosis in rats exposed to unheated cristobalite (5 h/day for 8 days) at a higher mean exposure dose (27.5 ± 10 mg/m^3) only showed small intraalveolar granulomas and diffuse interstitial collagen fibers. It is therefore possible that more intense and localized exposures may show a difference in the fibrogenic potential of the heat-treated cristobalite samples.

At intermediate stages of the progression of the disease, nevertheless, the in vivo response to the dust was altered by the thermal process. The cristobalite heated at 800 °C was accumulated at higher levels in the lung during an 8 day exposure period and elicited a postexposure increase in the number of alveolar macrophages. The heated sample had a reduced rate of short-term clearance and enhanced long-term clearance and was transported to the thymus and mediastinal lymph nodes in greater amounts than the unheated sample (*15*). These findings indicate that the surface state of the particle plays a role in all these stages of particle−cell interactions in vivo. The silica content of macrophages from broncoalveolar lavage was much higher in the case of heated than in the case of unheated cristobalite. This may be related to a decreased toxicity to macrophages, with a delay in macrophage death as a consequence of a reduced level of membrane damage. Total lung burden remained higher, which may be correlated with the enhanced long-term recruitment of inflammatory cells observed in heated cristobalite. A poorly fibrogenic quartz sample was transported to the lymphoid tissue only when heated (*30*). Most of these effects may be due to the loss of membranolytic activity, but other factors may also be involved and require further investigations. Intratracheal instillation tests in rats of a dust containing 90% cristobalite, obtained from silicic fire bricks used in Martin furnaces, showed a rapid onset of macrophage toxicity and a high degree of fibrogenic activity (*32*). In this case, however, the dust was generated from mechanical rupture of the bricks exposed to high temperatures; the particle surface therefore exhibited a fresh surface and not a heated one. A cristobalite sample, obtained by heating a pure quartz dust above 1300 °C (*33*), was nonfibrogenic[2] in contrast with the sample heated to 800 °C (*15*, *30*).

It is generally accepted that cytotoxicity may be relevant to macrophage activation and consequent inflammation (*1*, *3*, *4*, *6*, *7*, *29*, *34*). Fibrogenicity, however, is a much complex process which cannot be explained only on the basis of cytotoxicity.

As the particle cristallinity and morphology were not affected by the thermal treatments, except for the reduction in the specific surface area of CRIS-1300, the variations in cytotoxicity have to be ascribed to some modification at the surface of the particles, caused by heating. On both CRIS-800 and CRIS-1300, surface radicals in the form of reactive oxygen species had disappeared. Any cytotoxic effect due to lipid peroxidation caused by active oxygen radicals should therefore be suppressed upon heating at ≥800 °C. This suppression may only partially account for the observed effects since, in most of the reported cellular studies, a clear-cut difference was found between CRIS-800 and CRIS-1300, which may be attributable to the partial hydrophilicity of CRIS 800 as opposed to the full hydrophobicity of CRIS-1300. The role played by the conversion of silanols into siloxanes in the cytotoxicity of silica dust was hypothesized long ago by Robock and Klosterkötter (*34*).

One possible explanation of these observations resides in the degree of hydrophobicity of the surface. Hydrophobic surfaces adsorb plasma proteins more readily and in larger amounts, and these proteins usually form a complete monolayer, hindering the bare surface from contact with cells (*8*). Under these conditions, cytotoxic effects due to the contact of the silica surface with the cell membrane would be suppressed. Furthermore, cell adhesion takes place more extensively and strongly on hydrophilic than on hydrophobic surfaces (*35*). In both

[2] S. Honnons, I. Fenoglio, and B. Fubini, unpublished results.

cases, cytotoxic interactions would be prevented by increasing hydrophobicity.

The interaction of cells with the polished surfaces of large quartz plates (~4 cm^2), cut either parallel or perpendicular to the 001 plane from a single large crystal of quartz, was studied by placing the quartz plates in culture dishes with culture medium; both mesenchymal and epithelial cells (mouse BALB/3T3/A31-1-1 fibroblasts and rat AE6 cells) were found to attach to the surface of the quartz plates and to grow well, forming confluent monolayers with no observable cytotoxic response (U. Saffiotti and G. D. Guthrie, personal communication). While ground quartz dusts are mostly hydrophilic because of the reaction of broken bonds with water (*8*), the polished quartz surface appeared to be unreactive, even when placed in an aqueous medium. The loss of reactivity may depend on how the surface aged following polishing (*36*).

The tests with AE6 cells show that CRIS-800 still induced a marked decrease of colony forming efficiency, but CRIS-1300 did not. For comparison, tests run concurrently on AE6 cells with other mineral dusts[3] showed that two preparations of Min-U-Sil 5 quartz had cytotoxic activities comparable to that of CRIS, whereas dust samples considered nonfibrogenic exhibited percent CFEs closer to that of CRIS-800 (at the 50 $\mu g/cm^5$ dose), 47.1 $\pm$ 2.7 for alumina and 73.5 $\pm$ 1.3 for anatase; a sample of the nonfibrogenic crystalline silica polymorph, stishovite, exhibited no cytotoxic activity (percent CFE at 50 $\mu g/cm^5$ of 99.7 $\pm$ 2.5) and in this respect was comparable to CRIS-1300. It is possible that the moderate cytotoxicity still observed in CRIS-800 may be due to mechanisms it shares with some other nonfibrogenic dusts. The significance of surface area as measured by the extent of nitrogen adsorption in relation to particle size and biological activity remains to be more precisely investigated.

It is not yet clear to what extent the effects reported here may be directly related to silica-induced diseases. Since the silica−macrophage interaction is reported by some authors as the key event for both fibrosis (*37*) and lung cancer (*1*), obviously a modulation of cytotoxicity should reflect also modulations in pathogenicity. Early research on the fibrogenicity of fly ashes claimed that, because of the high temperature that is experienced, these ashes, even containing crystalline silica, were not pathogenic (*38*). Further tests appropriately designed for fibrogenicity and carcinogenicity with the same well-defined samples would be required to relate cellular effects to the various pathogenetic mechanisms.

The results presented here show that changes in surface properties, such as those between CRIS-800 and CRIS-1300, may cause remarkable differences in cell responses to dust, confirming a crucial role played by the hydrophilic patches of silanols on the silica surface in determining the cytotoxic effects of crystalline silica dusts.

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Supporting Information Available: Information about the AE6 cell line. This material is available free of charge via Internet at http//:pubs.acs.org.

[3] U. Saffiotti, unpublished results.

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