Toxicity of Lunar and Martian Dust Simulants to Alveolar Macrophages Isolated from Human Volunteers

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NASA is planning to build a habitat on the Moon and use the Moon as a stepping stone to Mars. JSC-1, an Arizona volcanic ash that has mineral properties similar to lunar soil, is used to produce lunar environments for instrument and equipment testing. NASA is concerned about potential health risks to workers exposed to these fine dusts in test facilities. The potential toxicity of JSC-1 and a Martian soil simulant (JSC-Mars-1, a Hawaiian volcanic ash) was evaluated using human alveolar macrophages (HAM) isolated from volunteers; titanium dioxide and quartz were used as reference dusts. This investigation is a prerequisite to studies of actual lunar dust. HAM were treated in vitro with these test dusts for 24 h; assays of cell viability and apoptosis showed that JSC-1 and TiO_2 were comparable, and more toxic than saline control, but less toxic than quartz. HAM treated with JSC-1 or JSC-Mars 1 showed a dose-dependent increase in cytotoxicity. To elucidate the mechanism by which these dusts induce apoptosis, we investigated the involvement of the scavenger receptor (SR). Pretreatment of cells with polyinosinic acid, an SR blocker, significantly inhibited both apoptosis and necrosis. These results suggest HAM cytotoxicity may be initiated by interaction of the dust particles with SR. Besides being cytotoxic, silica is known to induce shifting of HAM phenotypes to an immune active status. The immunomodulatory effect of the simulants was investigated. Treatment of

HAM with either simulant caused preferential damage to the suppressor macrophage subpopulation, leading to a net increase in the ratio of activator ($RFD1^+$) to suppressor ($RFD1^+7^+$) macrophages, a result similar to treatment with silica. It is recommended that appropriate precautions be used to minimize exposure to these fine dusts in large-scale engineering applications.

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INTRODUCTION

NASA has begun preparing to return to the Moon for permanent occupancy and research. Successful lunar habitation will be used as a stepping stone to a Mars mission. The projected lunar tour of duty will be 180 days, during which NASA will use the lunar soil in situ for various purposes, including extracting life-essential resources such as oxygen and fuel. JSC-1, an Arizona volcanic ash that has mineral properties similar to those of lunar soil (McKay et al., 1994), is used for various ground-based experiments on lunar soil utilization and for simulating lunar environments in which to test instruments and other hardware. This research includes environmental dust control, spacesuit durability, agriculture, and oxygen-production (Wagner, 2004). During these activities, workers at NASA and lunar project scientists and engineers at academic institutions could be exposed to this lunar soil simulant about which there is little toxicity data.

JSC-1 is a glass-rich basaltic ash derived from the San Francisco volcanic field near Flagstaff, Arizona (Glaser, 1992). It is very similar to lunar mare soil with respect to chemical composition (rich in silica and low in titanium), mineralogy (plagioclase, pyroxene, and olivine), and mechanical properties (specific gravity, angle of internal friction, and cohesion). These similarities have made it ideal for large-scale soil utilization studies involving agriculture and oxygen extraction for life support. Both lunar soil and the simulant, JSC-1, contain about 50%

SiO_2; other common soil oxides (Al_2O_3, FeO, MgO, and CaO) account for another 42-45%. No trace or heavy metals were found in either lunar soil or JSC-1 (McKay et al., 1994).

The lunar soil simulant, like lunar soil collected on the Apollo missions, contains a fraction of fine dust particles in the respirable size range. NASA is concerned about the potential health risk to workers who are exposed to this dust in Earth-based test facilities. In the study reported here, the interaction of lunar dust simulant with alveolar macrophages obtained from human volunteers and toxicity of the stimulant to the macrophages were assessed; titanium dioxide (TiO_2), a low-toxicity dust, and quartz dust (SiO_2), which is fibrogenic in the lungs, were included as reference dusts. A Martian soil simulant was also included in this study. JSC-Mars 1 is a silica-containing volcanic ash derived from a cinder cone, Mauna Kea, in Hawaii. It is analogous to the bright regions of Mars based on reflectance spectral data obtained by Viking I and II and Pathfinder Landers. The simulant is comparable to Martian soil in mineralogy; both are rich in feldspar and Ti-magnetite [15-22% TiO_2)], with minor olivine and pyroxene in a glassy matrix. Furthermore, JSC-Mars 1 is high in ferric oxide, as is Martian soil (Allen et al., 1998).

Both lunar and Martian soil simulants contain silica. Silica, especially in the crystalline form, causes lung inflammation and fibrosis on prolonged exposure. Macrophages are the first cell type to come into contact with dust in the lung. Silica and silicates in the lung cause macrophage injury leading to necrosis or apoptosis (Iyer et al., 1996). The alveolar macrophage is important in nonspecific host defense of the lung and is a central cell in regulating the inflammatory response in the lung. Necrosis involves rupture of the cell membrane with spillage of

intracellular contents. Apoptosis, which can be followed by necrosis, is a "programmed" cell death that does not involve rupture of cell membranes, but does involve an orderly series of morphological and biochemical changes in the cell cytoplasm, nucleus, and membrane. Changes that can be visualized by light microscopy include cytoplasmic shrinkage, chromatin clumping, membrane blebbing, and apoptotic body formation (Kimbell, 2007).

The mechanisms, by which particulates such as silica initiate alveolar macrophage injury have not been fully elucidated, but seem to involve scavenger receptors (SR). Scavenger receptors Class A I/II (SRA) and macrophage receptor with collagenous structure (MARCO) have been implicated in the mechanism of silica-induced toxicity to the alveolar macrophage (Hamilton et al., 2000; Hamilton et al., 2006). Silica-induced apoptosis has been blocked by pretreatment of the cells with polyinosinic acid, an SRA inhibitor (Iyer et al., 1996; Hamilton et al, 2000), and the requirement for SRA has been confirmed using Chinese hamster ovary cells transfected with the murine SRA (Hamilton et al, 2000). Here we report the results of a similar study with the lunar and Martian dust simulants.

Lunar soil particles are powdery and have a great tendency to adhere to surfaces, including spacesuits. When these particles were transported on spacesuits into the cabin of Apollo spacecraft after extravehicular activities, they caused visibility problems on resumption of weightlessness. Some mechanical systems aboard the spacecraft were damaged due to dust contamination. Furthermore, astronauts inhaled the dust particles and reported nasal and eye irritation (Wagner, 2004). At least one Apollo astronaut reported symptoms resembling "hay fever" when exposed to lunar dust. Spectral data obtained from robotic Martian missions

indicate that Martian surface soil is both oxidative and reactive. Exposure to reactive Martian dust will create an even greater concern for the astronauts' health and the reliability of mechanical systems (Wagner, 2004).

Silica is associated with immunological impairment in exposed workers (ICMR, 2001; Steenland et al., 2001). The immunomodulatory effects of silica have been previously reported. Crystalline silica induces a shift of human alveolar macrophage (HAM) phenotypes to an immune active status; the treatment led to preferential damage to the suppressor ($RFD1^{+}7^{+}$) macrophage subpopulation, resulting in a net increase in the ratio of activator ($RFD1^{+}$) to suppressor ($RFD1^{+}7^{+}$) macrophages (Holian et al., 1997). We hypothesize that the lunar and Martian soil simulants, both silicates, affect macrophage subpopulations similarly. Such a shift toward a more activated immune status could result in lung inflammation and increased risk for pulmonary and cardiovascular disease (Holian et al., 1998).

The pulmonary toxicity of lunar dust simulant was previously investigated in mice exposed to the dust by intratracheal instillation; the results showed that the dust simulant is relatively low in acute toxic toxicity (Lam et al., 2002a), but high dose (1 mg/mouse) and prolong exposure (90-d) can cause fibrosis (Lam et al., 2002b). The present study with alveolar macrophages isolated from human volunteers complements the animal studies in revealing the toxicity of the lunar soil simulant and its mechanism of toxicity. NASA is planning to conduct toxicity studies with actual lunar dust. One goal of the present study, like that of the animal studies, is to establish experiment protocols that could be applied to the study of lunar dust. NASA requires that investigators conduct successful studies using the lunar dust simulant before they can receive

precious lunar soil for proposed studies, and our HAM study using the lunar soil simulant serves as a prerequisite to a toxicity study of real lunar dust as well as an investigation of the effect of the lunar and Martian dust simulants on human macrophages.

METHODS

Test Dusts

Lunar and Martian Dust Simulants. Samples of raw Arizona and Hawaii volcanic ashes [designated as JSC-1 (McKay, 1994) and JSC-Mars-1 (Allen et al., 1998)], respectively, were provided by the NASA Astromaterials Curator; the soil samples were provided to Lovelace Respiratory Research Institute for size fractionation. The fine particles from the second (50% cutoff aerodynamic diameter 1.95 μm) and third stages (0.28 μm) of the cascade impactors, and the backup filter were collected and pooled for the present study. Thus, the mass median aerodynamic diameter (MMAD) of both dusts was expected to be less than 5 μm. Analysis by Microtrac Inc. (Montgomeryville, PA) showed that the respirable fraction of the lunar dust simulant had a mass median diameter (MMD) of 3 μm or less and contained 5% large particles with an MMD of 81 μm; on ultrasonication, all of the large particles disappeared and the resulting suspension showed a bimodal distribution, with MMDs of 1.05 μm (44%) and 2.99 μm (56%). The Martian dust simulant, which was recently analyzed by Particle Technology Labs (Downers Grove, IL) using TSI Aerosizer LD 8050 (TSI Incorporated, Shoreview, MN), showed the aerodynamic MMD of 0.93 μm and particles with sizes >1.8 μm accounting for 5% mass.

Titanium Dioxide and Crystalline Silica. The titanium dioxide sample, a product of Particle Information Services (Kingston, MA), had an average particle diameter of 0.45 μm. Crystalline silica (quartz) (acid-washed Min-U-Sil-5), which has a mass median diameter of 1.7 μm and in which 97% of the particles are less than 5 μm was obtained from Pennsylvania Glass and Sand (Pittsburgh, PA). These particle-size specifications were from the respective vendors.

Isolation of Alveolar Macrophages from Human Volunteers

HAM were obtained by bronchoalveolar lavage of healthy adult volunteers of both sexes as described previously (Dauber et al., 1979). This protocol was approved by the University of Texas Committee for the Protection of Human Subjects. Briefly, for each volunteer subject, the lung was washed 2 or 3 times with sterile saline (each instillation contained 240 to 300 ml) to collect about 200 to 600 ml of lavage fluid; the fluid was stored at 4°C until cells were isolated by centrifugation at 1500 revolutions/minute (rpm). The saline supernatant was aspirated and discarded, and cells were resuspended in a small volume (1 to 5 ml) of N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid-buffered medium 199 (GIBCO-BRL, Bethesda, MD) with 10% heat-inactivated fetal calf serum (Sigma, St. Louis, MO) and antibiotics (50 U/ml penicillin, 50 μg/ml gentamicin, and 50 μg/ml streptomycin [GIBCO-BRL, Bethesda, MD]). Cells were counted using a ZBI Coulter Counter (Coulter Electronics, Hialeah, FL). Lavages yielded an average of 2×10^7 cells that were > 92% HAM, as verified by Leukostat staining (Fisher Scientific, Houston, TX). Viability was > 90% as determined by trypan blue exclusion.

Culture of Human Alveolar Macrophages

Stock suspensions of test dusts were freshly prepared in buffered saline followed by brief sonication in a water bath just before they were added to HAM culture. Unless otherwise indicated, cells were cultured at 1×10^6 cells/ml in the presence or absence of a test dust at a designated concentration for 24 h at 37°C and 5% CO_2 in a water-jacketed incubator (Queue, Parkersburg, WV). Cell suspension was maintained by end-over-end tumbling (Labquake Shakers, Labindustries, Berkley, CA) in sterile polypropylene tubes. Samples were not pooled, so that each dose response was conducted on cells derived from an individual volunteer.

Cell Morphological Study

Immediately after cell culture, 3×10^4 cells were incubated with phosphate-buffered saline (PBS, pH 7.2) and centrifuged for 5 minutes at 1,500 rpm onto positively charged glass slides (Probe On Plus, Fisher Scientific, Houston, TX) using a Shandon Cytospin 2 (Shandon Upshaw, Pittsburgh, PA). Slides were kept at 25°C until Leukostat fixation and staining were performed. After they were fixed in methanol for 90 seconds, the cells on the slides were stained in Leukostat eosin stain (Fisher Scientific, Houston, TX) for 90 seconds, and then in Leukostat methylene blue (Hema 3, Fisher Scientific, Houston, TX) for 4 seconds. Slides were air-dried and examined by light microscopy with a 600× dry objective (Zeiss Axioskop, Thornwood, NY).

Cell Viability Assay (Trypan Blue Exclusion)

To determine viability, cells were mixed with trypan blue dye (0.04% in PBS, Sigma, St. Louis, MO), placed on a hemocytometer, and examined under light microscopy at 600× dry objective (Zeiss Axioskop, Thornwood, NY). Viable cells exclude this dye and remain unstained.

Apoptosis Assay (Cell Death Enzyme-Linked Immunosorbent Assay)

Intra-nucleosomal DNA cleavage that occurs late in the process of apoptosis can be assessed by cell death enzyme-linked immunosorbent assay (ELISA), which determines cytosolic histone-bound DNA fragments. For this assay of apoptosis, cells from control and dust treatments were processed and analyzed for cytosolic histone-bound DNA fragments determined using the Cell Death Detection ELISA™ kit (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's protocol. The reaction of the Boehringer reagents with histone-bound DNA fragments released into the cytosol produced a colored product that can be spectrometrically measured at 405 nm.

Assessment of the Interaction of Test Dusts with Scavenger Receptors

To determine if scavenger receptors were involved in cytotoxicity, HAM were preincubated with or without polyinosinic acid (100 μg/ml, Sigma, St. Louis, MO) for 15 minutes at room temperature. Cells were cultured at 1×10^6 cells/ml in the presence or absence

of lunar or Martian dust simulant (250 μg/ml) under conditions described above. The upper limit of the suspension concentration was determined by the relative cytotoxicity of these dusts in a 24-h cell culture; at concentrations > 250 μg/ml, both types of particles significantly decreased cell viability. Cells were assayed for apoptosis by Cell Death Detection ELISA™ and morphology and for necrosis by trypan blue exclusion, as described above.

Immunomodulation Assay of Dust-Treated HAM

RFD1 and RFD7 surface markers on HAM subpopulations were analyzed by flow cytometry after the procedures described previously (Holian et al., 1997, 1998) were performed. Briefly, at the termination of the culture, cells were centrifuged, medium was aspirated, and the cell pellet (1×10^6 cells) was resuspended in 500 μl PBS with 3.5% bovine serum albumin (BSA, Sigma, St. Louis, MO). Monoclonal antibodies to $RFD1^+$ (murine IgM) and $RFD7^+$ (murine IgG1) surface antigens (Serotec, Kidington, Oxford, England) were both added at a 1:200 dilution (2.5 μg in 500 μl). The mixture was incubated 30 minutes at room temperature, and the reaction was then terminated by centrifugation and aspiration. The cell pellet was washed 3 times with PBS and resuspended in PBS/BSA, and fluorescein anti-mouse IgM (Vector Labs, Burlingame, CA) and R-phycoerythrin anti-mouse IgG (Vector Labs, Burlingame, CA) were added concurrently at a 1:100 dilution (5 μg in 500 μl) and incubated 30 minutes at room temperature. The incubation was terminated as described above and washed 3 times in PBS. The cells were then suspended in 1% formaldehyde (phosphate buffered) and stored at 4°C before flow cytometric analysis. Flow cytometry was performed on a Coulter EPICS Elite flow cytometer (Coulter, Miami, FL) using the Elite software. Using forward and side scatter of the

total cell population; gates were drawn to include macrophages on the basis of the size and granularity of the cells. The instrument was calibrated with beads coated with fluorescein isothiocyanate and phycoerythrin to compensate for any overlap within the green and red wavelengths. Cells stained without the inclusion of primary antibodies had no significant staining. Controls included unstained cells, cells stained with secondary antibodies only (negative control), and cells stained for only one of these surface markers (positive control).

Statistical Analysis

The experiment results were statistically analyzed by analysis of variance (ANOVA) followed by a Student-Newman-Keuls or Tukey's multiple comparison test. The number "N" in the figure legends denotes the number of HAM cultures given the treatment shown.

RESULTS

Comparison of the Toxicity of Lunar and Martian Dust Simulants with Titanium Dioxide and Crystalline Silica in HAM

HAM in cultures were treated with lunar and Martian dust simulants, TiO_2, or quartz (SiO_2) at a concentration of 100 μg/ml for 24 h; cell viability was determined by trypan blue exclusion. In the apoptosis assay, HAM were treated similarly and the extent of apoptosis was determined by assessing spectrometrically the amount of histone-bound DNA fragments released into the cytosol. The results of cell viability and apoptotic index analyses are shown in Figures 1A and 1B. Statistical tests were performed to compare the simulants with TiO_2 or quartz, and the results show the lunar dust and TiO_2 were comparable, and more toxic than saline control, but less toxic than quartz (Tables 1A and 1B).

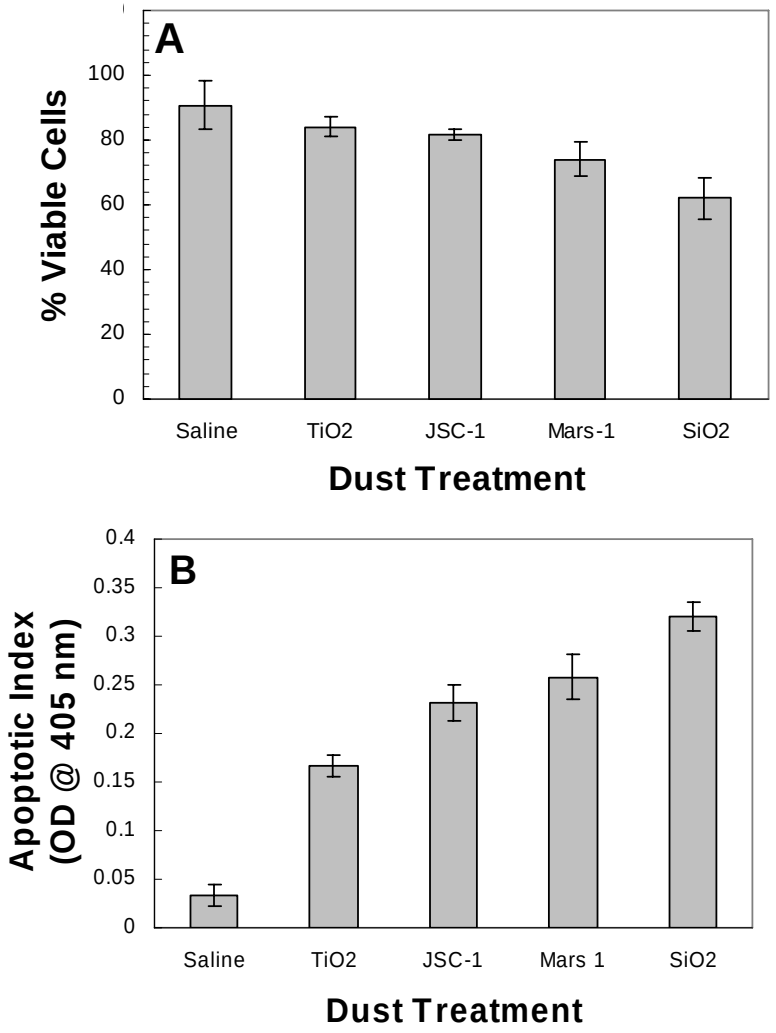

FIG. 1. A: Viability of human alveolar macrophages (HAM) in cultures treated with test dusts (100μg/mL) for 24 h. Each bar illustrates the mean ± SEM percent viable cells. B: HAM in cultures were treated with test dusts for 24 h and then assessed for apoptosis by spectrometrically measuring the release of cytoplasmic histone-bound DNA fragments into the cytosol. Each bar on the graph illustrates the mean ± SEM value of optical density at 405 nm. Statistical tests were performed on these data, and the results are shown in Tables 1A and 1B (N= 6).

TABLE 1 A

Results of cell viability measurements in HAM cultures treated with test dusts for 24 h

Test dust	% viable cells	Effect due to treatment[1]	Test vs. saline	Test vs. TiO_2	Test vs. SiO_2
Saline	91	---	---	$\rho = 0.23$	$*\rho < 0.001$
Titanium dioxide	84	7.7	$\rho = 0.23$	---	$*\rho = 0.04$
Lunar dust simulant	82	9.9	$\rho = 0.11$	$\rho = 0.12$	$*\rho = 0.03$
Martian dust simulant	74	18.7	$*\rho = 0.04$	$*\rho = 0.01$	$\rho = 0.16$
Crystalline silica	62	32.9	$*\rho = 0.01$	$*\rho = 0.04$	---

* Indicates significance at $p < 0.05$ compared with the control by ANOVA, followed by Tukey's procedure.

[1]The difference obtained by minus the result of dust-treated cultures from that of saline controls.

TABLE 1 B

Results of the apoptotic assays of HAM cultures treated with test dusts for 24 h

Test dust	Apoptotic index	Effect due to treatment[1]	Test vs. saline	Test vs. TiO_2	Test vs. SiO_2
Saline	0.033	---	---	$*\rho < 0.001$	$*\rho < 0.001$
Titanium dioxide	0.167	0.134	$*\rho < 0.001$	---	$*\rho < 0.001$
Lunar dust simulant	0.231	0.198	$*\rho < 0.001$	$\rho = 0.10$	$* \rho = 0.005$
Martian dust simulant	0.258	0.225	$*\rho < 0.001$	$*\rho = 0.04$	$*\rho = 0.03$
Crystalline silica	0.320	0.287	$*\rho < 0.001$	$*\rho < 0.001$	---

* Indicates significance at $p < 0.05$ compared with the control by ANOVA, followed by Tukey's procedure.

[1]The difference obtained by minus the result of dust-treated cultures from that of saline controls.

Effects of Lunar and Martian Dust Simulants on HAM Viability

To determine the dose response of cytotoxicity to HAM in cultures treated with lunar and Martian dust simulants, the cultures were treated with simulants at 0, 100, 250, or 500 μg/ml for 24 h and cell viability was measured. The results (Figure 2) indicated that both simulants induced a dose-dependent decrease in viability.

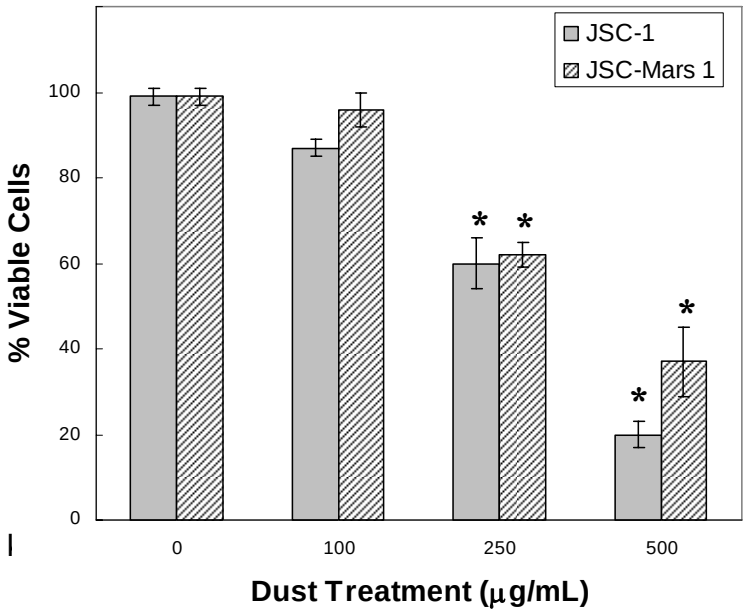

FIG. 2. Viability of human alveolar macrophages in cultures treated with lunar or Martian dust simulant at 0, 100, 250, and 500 μg/ml for 24 h. Each bar represents the mean ± SEM percent viable cells. * indicates significance at $p < 0.05$ compared with the control (0 μg/ml) by the Student-Newman-Keuls procedure (N = 6).

Apoptosis in HAM Induced by Lunar and Martian Dust Simulants

The ability of the lunar and Martian dust simulants to induce HAM to undergo apoptosis in cultures treated with the simulants at 0, 100, 250, or 500 μg/ml for 24 h was determined. The results of the apoptosis assay (Figure 3) show that dust treatment produced a general dose-dependent increase in the apoptotic index. However, at the highest dose (500 μg/ml), the apoptosis induced by lunar dust simulant was less than that produced by the second highest dose (250 μg/ml); the apoptosis induced by the highest dose of Martian dust simulant did not increase over that produced by the second highest dose. It is noteworthy that the highest dose of either test dust produced the highest percent of cell death (Figure 2); it may be that at the highest doses, a greater proportion of the cells undergo necrosis, which cannot be detected by the apoptotic assay (Figure 3)

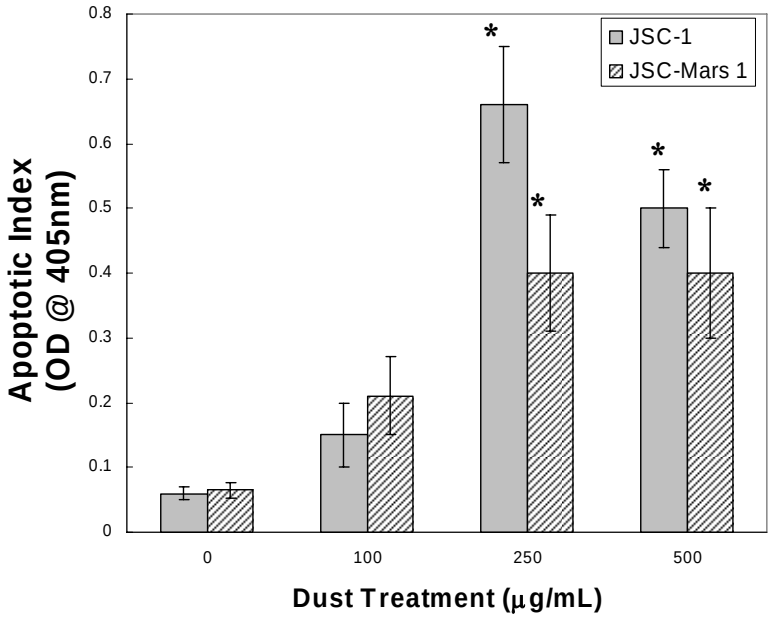

FIG. 3. Human alveolar macrophages in cultures treated with lunar or Martian dust simulant for 24 h were assessed for apoptosis by spectrometrically measuring the release of cytoplasmic histone-bound DNA fragments into the cytosol. Each bar illustrates the mean ± SEM value of optical density measured at 405 nm. * indicates significance at $p < 0.05$ compared with the control (0 μg/ml) by the Student-Newman-Keuls procedure (N = 6).

Morphology of HAM Treated with Martian or Lunar Dust Simulants

To confirm that these dust simulants induce apoptosis in HAM, cells were cultured with or without a 15-minute pretreatment with polyinosinic acid (poly I), exposed to lunar or Martian dust simulant (250 μg/ml), and stained for morphology assessment. Untreated HAM are intact cells with large, distinct nuclei (Figure 4A). Poly I had no effect on control cells (Figure 4B). Cells exposed to lunar dust (Figure 4C) had dark, shrunken, sometimes segmented nuclei characteristic of nuclear condensation common in apoptosis. Pretreatment with poly I protected the cells against apoptosis induced by the dust (Figure 4D). Nuclear disintegration, another feature of apoptosis, was evident in some of the treated cells. Cells treated with Martian dust simulant also had dark, shrunken nuclei (Figure 4E), and pretreatment with poly I also protected them against apoptosis (Figure 4F).

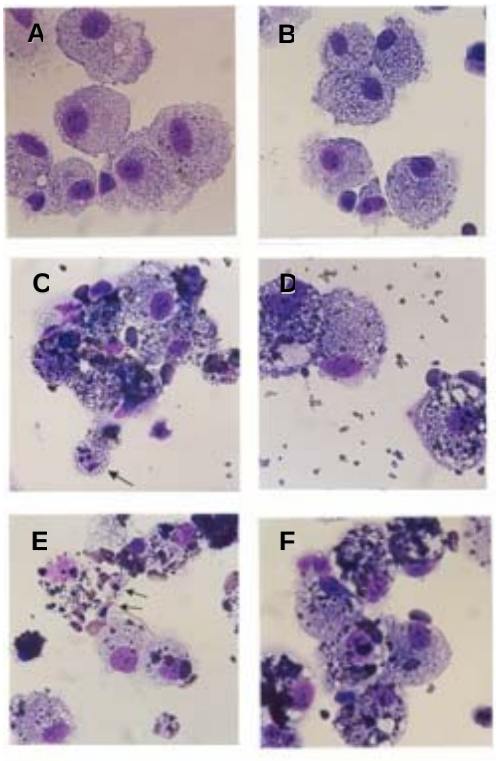

FIG. 4. Morphology of human alveolar macrophages 24 h after exposure to lunar and Martian dust simulants. A, control macrophages cultured for 24 h; (B): Macrophages preincubated with 100 μg/ml polyinosinic acid and cultured for 24 h; (C): Macrophages incubated with lunar dust simulant (250 μg/ml) for 24 h; (D): macrophages incubated with lunar dust simulant (250 μg/ml) for 24 h after a 15-minute preincubation with polyinosinic acid (100 μg/ml); (E): Macrophages incubated with Martian dust simulant (250 μg/ml) for 24 h; (F), macrophages incubated with Martian dust simulant (250 μg/ml) for 24 h after a 15-minute preincubation with polyinosinic acid (100μg/ml). Arrows indicate morphology consistent with macrophage apoptosis (photomicrographs 600X, dry objective).

Assessment of a Mechanism by Which Lunar and Martian Dust Simulants Might Induce Cytotoxicity in HAM

Scavenger receptor class A I/II (SRA) has been implicated in the mechanism of silica-induced toxicity of the alveolar macrophage (Hamilton, 2000). It was speculated that the toxic effects of silica resulted from the interaction between the macrophage surface SRA and silica. Silica-induced apoptosis was blocked by pretreatment of cells with polyinosinic acid (poly I), the SRA-inhibitor (Iyer et al., 1996; Hamilton et al., 2000). To test whether the HAM cytotoxicity induced by the dust simulants involved SRA, cells in culture were preincubated with or without polyinosinic acid, and then exposed to 250 μg/ml lunar or Martian dust simulant for 24 h. Cells were assessed for viability by trypan blue exclusion (Figure 5A) or for apoptosis by cell death ELISA (Figure 5B). These results demonstrate that pretreatment of the cells with polyinosinic acid protected against both dust-induced apoptosis and necrosis of HAM.

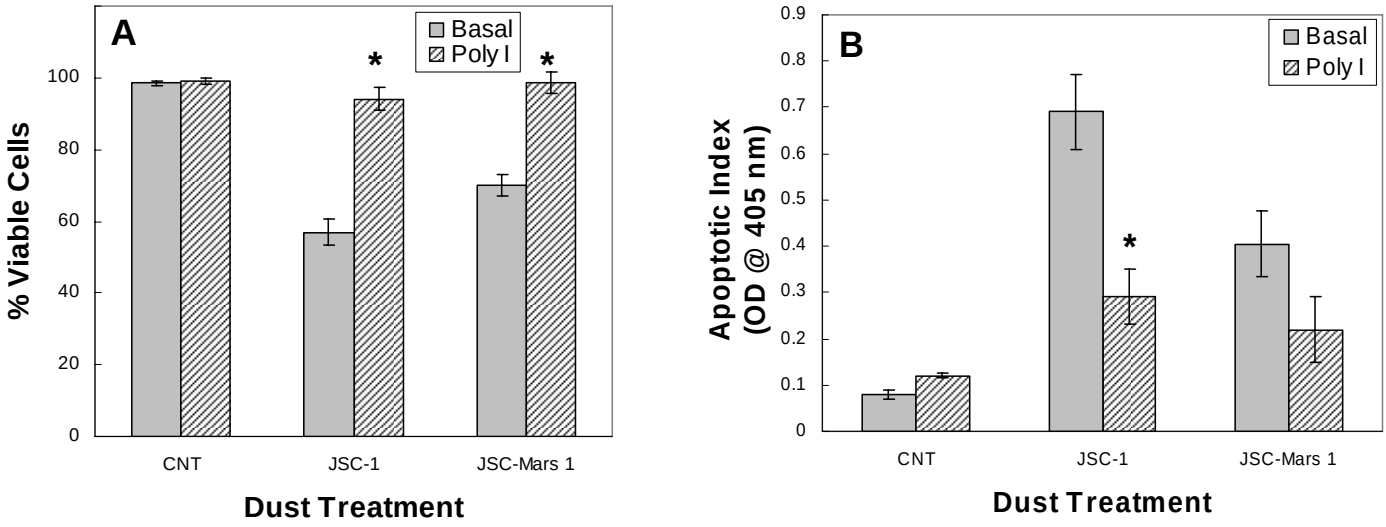

FIG. 5. Effect of the scavenger receptor antagonist, polyinosinic acid, on dust-induced cytotoxicity in human alveolar macrophages. Polyinosinic acid (100 μg/ml) treated or untreated HAM cells were exposed *in vitro* to lunar or Martian dust simulants (250 μg/ml) for 24 h. Cell viability and apoptosis were determined. In (A), bars represent the mean ± SEM percent viable cells; in (B), bars represent the mean ± SEM apoptotic index (optical density at 405 nm). * indicates significance at $p < 0.05$ compared with appropriate control by Student-Newman-Keuls procedure (N = 3).

Immunomodulation of HAM by Lunar and Martian Dust Simulants

To determine if the dust simulants had immunomodulating effects on HAM, the HAM phenotypes were assessed in cultures treated with the lunar dust simulant (0, 100, or 250 μg/ml) or the Martian dust simulant (0, 250, or 500 μg/ml). Both dusts induced a small concentration-dependent increase in the percentage of immune activator ($RFD1^+$) HAM (Figures 6A and 6B). In addition, a concentration-dependent decrease occurred in the percentage of suppressor ($RFD1^+7^+$) HAM (Figures 6A and 6B) compared to controls. These data indicate that a significant shift occurred in HAM subpopulations toward an immune active phenotype on exposure to either dust simulant.

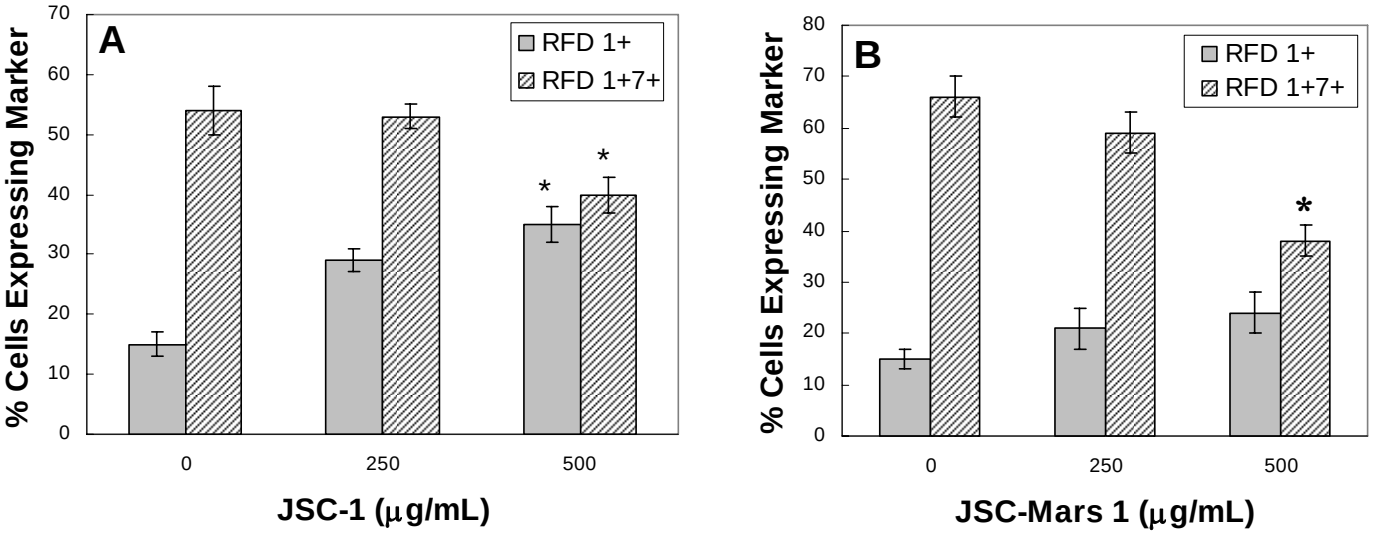

FIG. 6. A: Effects of lunar dust simulant on human alveolar macrophage phenotype distributions. Each bar represents the mean ± SEM percent activator macrophages ($RFD1^+$) and suppressor macrophages ($RFD1^+7^+$) after 24 h of culture with lunar dust simulant (0, 100, and 250 μg/ml). * indicates significance at $p < 0.05$ compared with appropriate control (0 μg/ml) by the Student-Newman-Keuls procedure (N = 3). B: Effects of Martian dust simulant on human alveolar macrophage phenotype distributions. Each bar represents the mean ± SEM percent activator macrophages ($RFD1^+$) and suppressor macrophages ($RFD1^+7^+$) after 24 h of culture with Martian dust simulant (0, 250, and 500 μg/ml). * indicates significance at $p < 0.05$ compared with appropriate control (0 μg/ml) by the Student-Newman-Keuls procedure (N = 3).

DISCUSSION

The present study was conducted to address NASA's concern about its workers being exposed to a lunar dust simulant for which little toxicity information is available. Thus, the potential toxicity and immunomodulatory effects of this simulant (JSC-1) and a Martian regolith simulant (JSC-Mars 1) were evaluated. A previous study from our laboratory showed that silica at a dose of 66 or 133μg/ml in cultures cause apoptosis in HAM, with the results of the high dose approached a plateau value; TiO_2 at 100, or 200 μg/ml produced little apoptosis (Iyer et al., 1996). Based on these findings, a dose of 100 μg/ml was chosen for our present comparative study to examine the apoptosis and cytotoxicity induced by the test dusts in HAM isolated from human volunteers. In this study, the HAM were treated with the test dusts *in vitro* for 24 hours; the cells were examined for viability by trypan blue exclusion and for apoptosis by morphology and Cell Death Detection ELISA™ assay. The findings, which are consistent with the results of our previous study (Lam et al., 2000a) on biomarkers of toxicity in lung alveolar lavage fluid from intratracheally-instilled mice, showed that the lunar dust and TiO_2 were comparable, and more toxic than saline control, but less toxic than quartz. These results showed that both simulants are relatively low in acute toxicity.

Even both dust simulants are relatively low in toxicity when compared with silica, they did cause significant necrosis and apoptosis of HAM in a concentration-dependent manner. Since both simulants consist of more than 40% silica, we had hypothesized that their bioactivity on HAM would be similar to that observed with crystalline silica, but reduced. To elucidate a possible

mechanism by which these fine dust simulants are cytotoxic, we investigated the role of SR. SR has been reported to mediate silica-induced cytotoxicity of HAM (Iyer et al, 1996). The class A type II scavenger receptor was specifically implicated in silica-induced cytotoxicity in studies with a Chinese hamster ovary cell line stably transfected with the murine gene for this receptor (Hamilton et al., 2000). However, recent studies demonstrated that MARCO may be more important in mediating binding and toxicity of silica particles (Hamilton et al., 2006). The results of the present study also implicate scavenger receptors as the mediator of cytotoxicity induced by both lunar and Martian dust simulants, since a 15-minute pretreatment with polyinosinic acid (poly I), a SR antagonist, significantly inhibited both apoptosis and necrosis.

Finally, the impact of these dust simulants on macrophage subpopulations was examined using flow cytometry to classify treated HAM as activator ($RFD1^+$) or suppressor ($RFD1^+7^+$) subpopulations based on expression of these surface antigens. Silica was previously shown to be more toxic to suppressor than to activator HAM phenotypes (Spiteri and Poulter, 1991; Holian et al., 1997, 1998). In addition, phenotype shifts were observed in HAM treated with the fibrogenic particles silica or asbestos but not with non-fibrogenic particles, such as titanium dioxide or wollastonite (Hamilton et al., 1996; Iyer et al., 1996; Iyer and Holian, 1997). In the current study, both lunar and Martian dust simulants caused an increase in the ratio of $RFD1^+$ (immune active) to $RFD1^+7^+$ (immune suppressor) HAM. Thus, HAM subpopulations were significantly shifted toward an immune active phenotype on exposure to either of these dust simulants. If similar changes occur *in vivo*, chronic lung inflammation could develop, precipitating fibrosis. In fact, inflammation and fibrosis were observed microscopically in mice 7-d and 90-d after

intratracheally exposed to bolus doses of lunar and Martian dust simulants, but not with TiO_2(Lam et al., 2002b).

The present study with alveolar macrophages isolated from human volunteers complements our previous animal studies in revealing potential toxicity of JSC-1 and JSC-Mars 1. The overall results of these three studies showed that these dusts, especially the JSC-1, are relatively low in toxicity, but are more toxic than titanium dioxide, whose toxicity has been equated with that of nuisance dusts; taken together, the above results indicate that individuals inhaling moderate to large amounts of these dusts for prolong periods could suffer adverse health effects. The results of this study, therefore, are relevant to human health here on Earth, since these soil simulants are used for large-scale engineering applications at NASA. It is reasonable to speculate that lunar dust would be more toxic than its surrogate. Lunar dust resides in near-vacuum conditions and is constantly bombarded by solar winds and cosmic rays (Stubbs et al., 2005); on the Moon, the chemical bonds on the grain surfaces are not pacified by moisture, thus making them very reactive (Stubbs et al., 2007). The results of the present study support NASA's directive that the lunar dust needs to be tested toxicologically.

The current investigation supports NASA's requirement to develop experiment protocols using dust simulants before conducting toxicity studies with extraterrestrial dusts (Wagner, 2004). Furthermore, this study is consistent with the National Research Council's recent recommendation to utilize *in vitro* toxicity testing methods to evaluate toxicological effects on cells, cell lines, or cellular components, preferably of human origin (NAS, 2007). Using *in vitro* methods such as these would allow investigators to conduct toxicology studies of extraterrestrial

dusts with minute quantities of these precious materials. Therefore, the design and methodology of the current study could be adopted for pulmonary and immunotoxicity studies of actual lunar and Martian dusts.

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FIGURE LEGENDS

FIG 1. A: Viability of human alveolar macrophages (HAM) in cultures treated with test dusts (100μg/mL) for 24 h. Each bar illustrates the mean ± SEM percent viable cells. B: HAM in cultures were treated with test dusts for 24 h and then assessed for apoptosis by spectrometrically measuring the release of cytoplasmic histone-bound DNA fragments into the cytosol. Each bar on the graph illustrates the mean ± SEM value of optical density at 405 nm. Statistical tests were performed on these data, and the results are shown in Tables 1A and 1B (N= 6).

FIG. 2. Viability of human alveolar macrophages in cultures treated with lunar or Martian dust simulant at 0, 100, 250, and 500 μg/ml for 24 h. Each bar represents the mean ± SEM percent viable cells. * indicates significance at $p < 0.05$ compared with the control (0 μg/ml) by the Student-Newman-Keuls procedure (N = 6).

FIG. 3. Human alveolar macrophages in cultures treated with lunar or Martian dust simulant for 24 h were then assessed for apoptosis by spectrometrically measuring the release of cytoplasmic histone-bound DNA fragments into the cytosol. Each bar illustrates the mean ± SEM value of optical density measured at 405 nm. * indicates significance at $p < 0.05$ compared with the control (0 μg/ml) by the Student-Newman-Keuls procedure (N = 6).

FIG. 4. Morphology of human alveolar macrophages 24 h after exposure to lunar and Martian dust simulants. A: Control macrophages cultured for 24 h. B: Macrophages preincubated with 100 μg/ml polyinosinic acid and cultured for 24 h. C: Macrophages incubated with lunar dust simulant (250 μg/ml) for 24 h. D: Macrophages incubated with lunar dust simulant (250 μg/ml) for 24 h after a 15-minute preincubation with polyinosinic acid (100 μg/ml). E: Macrophages incubated with Martian dust simulant (250 μg/ml) for 24 h. F: Macrophages incubated with Martian dust simulant (250 μg/ml) for 24 h after a 15-minute preincubation with polyinosinic acid (100μg/ml). Arrows indicate morphology consistent with macrophage apoptosis (photomicrographs 600×, dry objective).

FIG. 5. Effect of the scavenger receptor antagonist, polyinosinic acid, on dust-induced cytotoxicity in human alveolar macrophages. Polyinosinic acid (100 μg/ml) treated or untreated HAM cells were exposed *in vitro* to lunar or Martian dust simulants (250 μg/ml) for 24 h. Cell viability and apoptosis were determined. In (A), bars represent the mean ± SEM percent viable cells; in (B), bars represent the mean ± SEM apoptotic index (optical density at 405 nm). * indicates significance at $p < 0.05$ compared with the appropriate control by Student-Newman-Keuls procedure (N = 3).

FIG. 6. A: Effects of lunar dust simulant on human alveolar macrophage phenotype distributions. Each bar represents the mean ± SEM percent activator macrophages ($RFD1^+$) and suppressor macrophages ($RFD1^+7^+$) after 24 h of culture with lunar dust simulant (0, 100, and 250 μg/ml). * indicates significance at $p < 0.05$ compared with the appropriate control (0 μg/ml) by the Student-Newman-Keuls procedure (N = 3). B: Effects of Martian dust simulant on human alveolar macrophage phenotype distributions. Each bar represents the mean ± SEM percent activator macrophages ($RFD1^+$) and suppressor macrophages ($RFD1^+7^+$) after a 24-h culture with Martian dust simulant (0, 250, and 500 μg/ml). * indicates significance at $p < 0.05$ compared with the appropriate control (0 μg/ml) by the Student-Newman-Keuls procedure (N = 3).