

Effect of the shape of mica particles on the production of tumor necrosis factor alpha in mouse macrophages

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Objectives Micas are thin, sheetlike minerals that are abundant in rocks and sand. They are common components of resuspended road dust in spring in Finland. The proinflammatory potential of mica particles was studied.

Methods Respirable-size particles were prepared from phlogopite mica either by milling (milled phlogopite) or water elutriation (elutriated phlogopite). The cations were extracted with acids from one phlogopite sample (acid-treated phlogopite). Minusil (α -quartz) and titanium dioxide were used as positive and negative controls, respectively. Cultured mouse macrophage cells (RAW264.7) were exposed for 24 hours to the mineral particles at concentrations between 10 and 500 $\mu\text{g}/\text{ml}$. The mineral particle concentration of 100 $\mu\text{g}/\text{ml}$ and the time range from 3 to 48 hours were used to study the time-dependency of the responses. Cell viability and the production of nitric oxide and proinflammatory cytokines [interleukin-6 (IL-6), tumor necrosis factor alpha (TNF- α)] were studied in the macrophages.

Results Elutriated phlogopite particles were larger, and they had a smaller surface area and a more regular, plate-like shape than milled phlogopite. The mineral particles showed the following order of potency to induce TNF- α production in macrophages: elutriated phlogopite > Minusil > acid-treated phlogopite > milled phlogopite > titanium dioxide. Only elutriated phlogopite induced a small, dose-dependent IL-6 response. The mineral particle samples did not induce nitric oxide production in the macrophages.

Conclusions Phlogopite mica stimulates macrophages to produce proinflammatory cytokines. The platelike shape of mica particles rather than surface area seems to be important for cytokine production.

Key terms cytokines, inflammation, interleukin-6, mineral dust, nitric oxide, particle shape, phlogopite, silicates, urban air.

Exposure to urban-air particles is associated with increased cardiorespiratory mortality and hospital admissions (1, 2). Particles originating from combustion processes are often regarded as the most harmful. However, the concentration of mineral dust particles in urban air can be high on some occasions, especially in Finland during early spring. Large amounts of sand and crushed rock are spread on the streets during slippery conditions in winter. Cars and trucks crush the pebbles on the street surfaces and generate smaller particles. The rocks contain different minerals, but particulate matter with an aerodynamic diameter of $<10\ \mu\text{m}$ (PM_{10}) usually consists of one particular mineral. Feldspars, quartz, micas, amphiboles, and pyroxenes are typical groups of minerals in Finnish rocks and sands (3).

Micas are aluminosilicates that belong to the phyllosilicate (layersilicate) group. Muscovite, biotite, and phlogopite are the most common micas, comprising about 5% of the earth's crustal volume. Aluminum-rich muscovite and magnesium-rich phlogopite are used industrially for many purposes. The most striking characteristic of all micas is the highly laminated, sheetlike form of the mineral (4).

Environmental exposure to micas is common. Therefore, micas are minerals that have often been found in autopsy samples of human lung tissue (5–7). A pulmonary mineral analysis of 11 unselected autopsy lungs from Finnish adult men showed the following mineral contents: 22% micas, 15% quartz, 13% microcline, 10% kaolinite, 8% plagioclase, and 4% talc (8). The ability

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of mica dusts to induce inflammation or pneumoconiosis in the lungs is poorly understood. However, animal and human studies have suggested that occupational exposure to mica dust could cause pneumoconiosis (9).

Alveolar macrophages exposed to quartz or certain silicates are activated, and they release proinflammatory cytokines such as tumor necrosis factor alpha (TNF- α) and interleukin-6 (IL-6) (10, 11). TNF- α is a protein that can be produced by a number of cells, including macrophages, monocytes and polymorphonuclear leukocytes. The release of TNF- α from activated macrophages stimulates recruitment and subsequent activation of other inflammatory cells. Furthermore, in vitro and in vivo studies have shown a correlation between the ability of mineral particles to produce inflammation in the lung and their capacity to activate macrophage TNF- α production (12). Elevated TNF- α production has been detected in macrophages from asbestosis or idiopathic pulmonary fibrosis patients (13).

The objective of our study was to investigate the production of proinflammatory cytokines and nitric oxide (NO) induced by differently processed phlogopite mica in a standard macrophage cell line. Special emphasis was placed on examining the role of the platelike shape and metal content of mica in these responses.

Material and methods

Mineral dust samples

Finnish phlogopite (tetraferriphlogopite) samples were received from Kemira Oyj, Finland. Respirable-size phlogopite samples were prepared either by grinding with a jet mill (milled phlogopite) or water elutriation (elutriated phlogopite). In elutriation, the phlogopite-water suspension was allowed to rise 0.02 mm per second in a container, and the overflow from the container was collected. One phlogopite sample (acid-treated phlogopite) was treated with sulfuric and nitric acids to extract cations. This sample was then washed several times with water and ground with a jet mill. Alpha quartz was used as a positive control dust, whereas titanium dioxide acted as a negative control dust. Quartz (Minusil, particle diameter <5 μ m, Pennsylvania Sand and Glass Corporation, Pittsburgh, PA, USA) was obtained from the National Institute for Occupational Safety and Health (Morgantown, WV, USA). Titanium dioxide (anatase) was obtained from Kemira Oyj, Finland.

The size distribution, mean size, and specific surface area of the phlogopite particles were analyzed with an optical particle size analyzer (Malvern, Master Sizer, Malvern, UK). The specific surface area of the mineral dusts was analyzed also with a method based

on nitrogen sorption (Micrometric Flow Sorb II 2300, Micrometrics, Norcross, USA). Scanning electron microscopic (SEM), secondary electron image (SEM/SEI; JeoL JSM 5900LV) analysis of the particles was carried out at the Geological Survey of Finland (Espoo, Finland). The chemical composition of the mica samples was analyzed with an atomic absorption spectrometer (Perkin Elmer, MA, USA). The endotoxin content of the dust samples was analyzed with a kinetic Bio Whittaker-QCL method (Walkersville, MD, USA) which is based on the use of limulus amoebocyte lysate (LAL) assay (14). Gram-negative bacterial lipopolysaccharide (LPS) (*Escherichia coli*, serotype 0111:B4, Sigma Chemical Co, St Louis, MO, USA) was used as the reference agent for the biological responses at 10 μ g/ml.

Exposures of mouse macrophage RAW 264.7 cells

The mouse macrophage cell line RAW264.7 was obtained from the American Type Tissue Collection (Rockville, MD, USA). The cells were grown at 37°C, 5% carbon dioxide, in RPMI (Roswell Park Memorial Institute) medium 1640 (Gibco Laboratory, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (HyClone Laboratory, Logan, UT, USA), 1% L-glutamine, and 1% penicillin-streptomycin antibiotic mixture (Gibco Laboratory). The cells were dispensed to 6-well plates (5 $\times$ 10⁵ cells/ml, 2 ml/well), and they were allowed to adhere for 24 hours before fresh complete medium was added. In concentration-response studies, the macrophages were exposed to five concentrations (10, 50 100, 200 or 500 μ g/ml) of each type of mineral particles for 24 hours. In the time-course studies, the macrophages were exposed for 3, 6, 12, 24, or 48 hours to a single concentration (100 μ g/ml) of each type of mineral particles. After the exposure, the adherent cells were resuspended in the culture media, and the cell suspension was centrifuged (5 minutes, 8000 revolutions/minute). NO was analyzed in an aliquot of the supernatant, and the remaining supernatant was stored at -80°C for the analyses of cytokines. An aliquot of cells was used for determining cell viability. The remaining cells were frozen immediately in dry ice and stored at -80°C. The experiments were repeated three times in duplicate.

Analysis of nitric oxide production

NO was assayed in the culture medium by the Griess reaction, as the stable NO-oxidation product nitrite (15). Briefly, 50 μ l of cell culture medium and 50 μ l of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2% phosphoric acid, all from Sigma) were added to each well of a 96-well plate. The plate was left for 10 minutes at room temperature while

being shaken. The optical density at 540 nm was measured using a microplate reader (iEMS Reader MF, Lab-systems, Finland). The nitrite concentrations were calculated against the standard solutions of sodium nitrite produced in culture medium.

Analysis of cytokines

TNF- α and IL-6 were analyzed by enzyme immunoassay (ELISA) using commercial ELISA kits (Pharmingen, San Diego, CA, USA) as described earlier (16). The samples were analyzed with an ELISA reader (iEMS Reader MF, Lab-systems) at 405 nm.

Cell viability

The viability of the cultured macrophages after the treatments was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide (MTT) test (Sigma) that is based on the MTT reaction with functioning mitochondria to produce formazan. Formazan was quantified spectrophotometrically using the automated colorimetric procedure. The viability of the control cells was measured by calculating the percentage of macrophages alive after treatment with Trypan Blue solution.

Statistics

The means, medians, and standard deviations of the data were calculated. An analysis of variance (ANOVA) and the Mann-Whitney test were used to test statistical significances for the cytokine data. The analyses were made using the SAS statistical package (SAS Inc, Cary, NC, USA).

Results

Physicochemical characteristics of the mineral dusts

The mean geometric diameters and surface areas of the different phlogopite particles are shown in figures 1 and 2 and table 1. The chemical compositions of the samples are given in table 2. The elutriated phlogopite particles were larger and had a smaller surface area per weight unit than the milled phlogopite (figure 1, table 1). Elutriated phlogopite was more regular in shape and platelike in comparison with the milled and acid-treated phlogopite (figure 2). The chemical analysis of the phlogopite samples revealed that the content of cations, especially aluminum, potassium, magnesium, and iron, was very low in the acid-treated phlogopite (table 2). This finding indicates that the cations had not only been extracted by acid-treatment from the surface but also from the interior of the particles, leaving intact only the

porous silica structure of phlogopite. Consequently, the surface area of the acid-treated phlogopite was almost 100 times larger with the nitrogen adsorption method than with the optical method (table 1). The endotoxin contents of the dust samples were low: 0.6 EU/mg of dust for Minusil, <0.01 EU/mg of dust for titanium dioxide, 0.1 EU/mg of dust for elutriated phlogopite, 0.08 EU/mg of dust for milled phlogopite, and <0.01 EU/mg of dust for acid-treated phlogopite.

Cytotoxicity and cytokine production in the dust-exposed macrophages

The concentration dependency of the cytotoxicity induced by mineral dust after 24-hour exposures of cultured mouse macrophages is shown in figure 3. Minusil and all the phlogopite samples were about equally potent inducers of cell death, while titanium dioxide was clearly less potent. The cytotoxicity to 100 μ g/ml of titanium dioxide decreased in a time-dependent

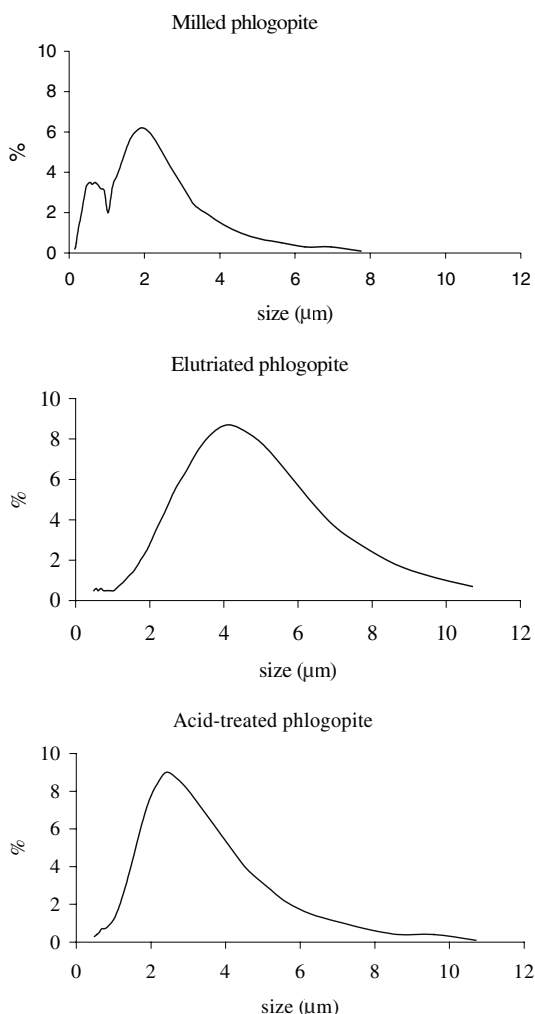


Figure 1. Particle size distribution (%) in three different phlogopite samples.

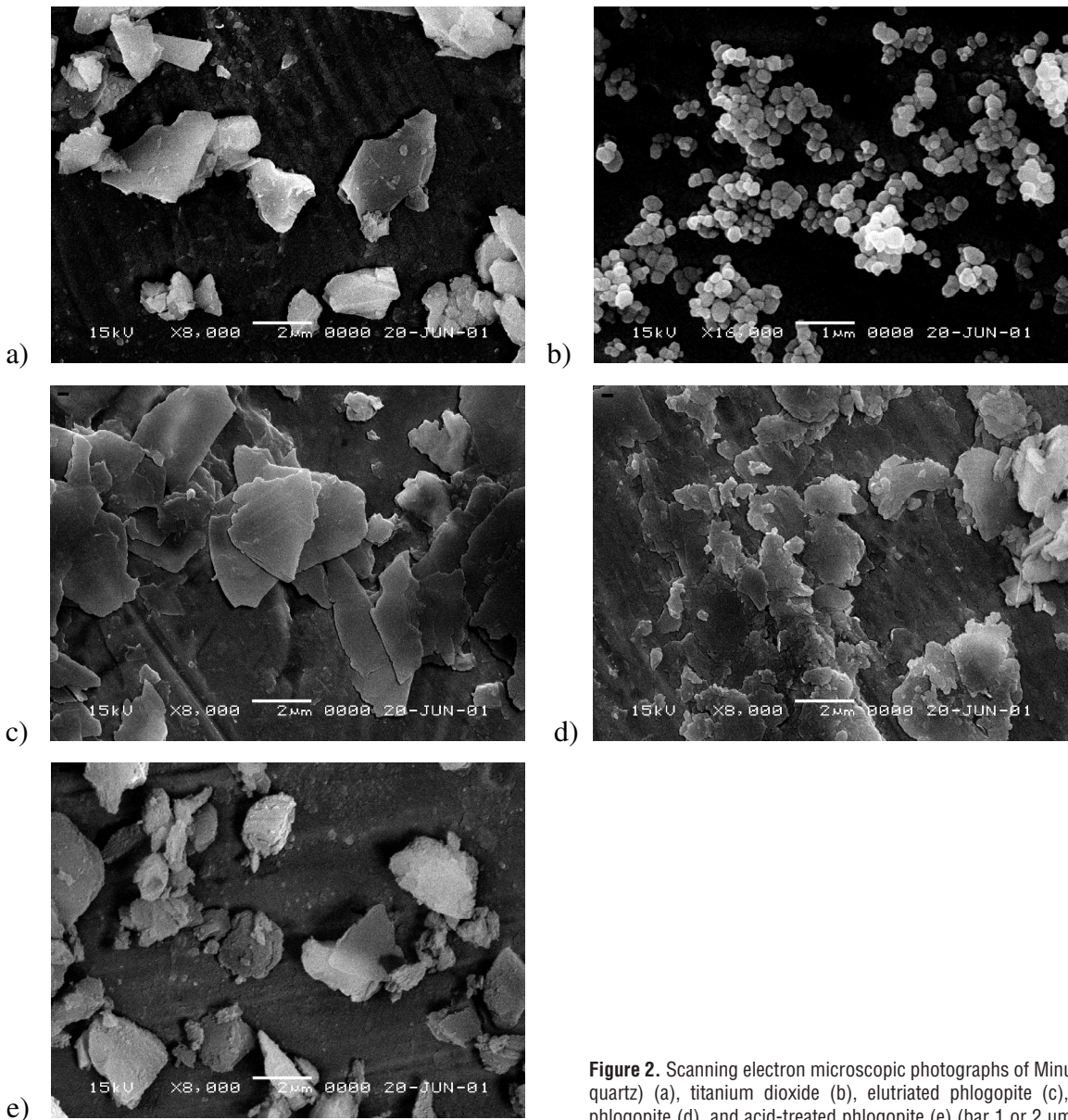


Table 1. Mean geometric diameter (μm) and specific surface area (m^2/g) of milled, acid-treated and elutriated phlogopite, Minusil and titanium dioxide as measured with two different methods.

	Mean particle size (μm)	Specific surface area ^a (m^2/g)	Specific surface area ^b (m^2/g)
Milled phlogopite	1.28	7.71	11.7
Acid-treated phlogopite	2.65	2.78	225.7
Elutriated phlogopite	3.98	2.62	7.6
Minusil	1.24	..	3.1
Titanium dioxide	0.42	8.9	7.4

^a Optical particle size analyzer.

^b Nitrogen sorption method.

manner after 3 hours of exposure (data not shown). The same trend was seen to a less extent with Minusil, but not with the phlogopite samples. LPS

Figure 2. Scanning electron microscopic photographs of Minusil (α -quartz) (a), titanium dioxide (b), elutriated phlogopite (c), milled phlogopite (d), and acid-treated phlogopite (e) (bar 1 or 2 μm).

Table 2. Chemical composition (%) of the milled and elutriated phlogopite (combined) and acid-treated phlogopite samples.

Element/salt	Milled and elutriated phlogopite	Acid-treated phlogopite
Silicon oxide	42.0	94.0
Aluminum oxide	11.0	0.5
Potassium oxide	11.0	0.3
Magnesium oxide	23.0	0.8
Iron	6.4	0.4
Titanium oxide	0.2	0.1
Sodium	0.07	0.09
Tin	0.02	0.04
Manganese	0.02	0.001
Zinc	0.006	0.001
Fluoride	1.1	0.12
Carbon	0.25	0.03

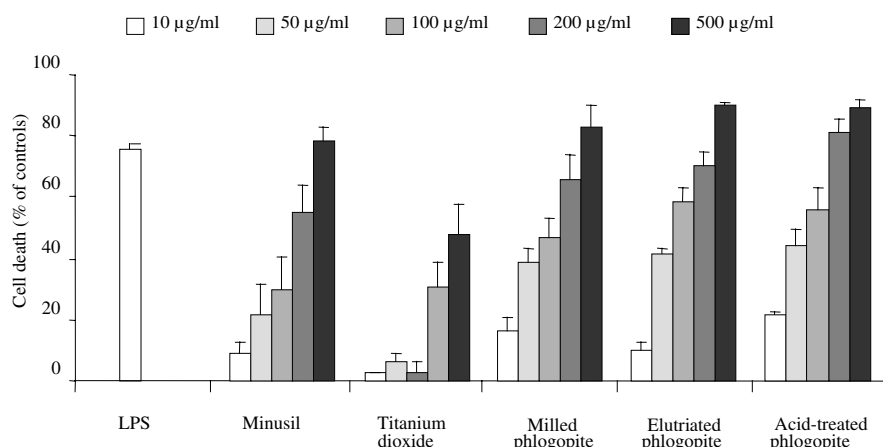


Figure 3. Concentration dependency of cell death (mean and SD, N=6) induced by the different mineral dusts (10, 50, 100, 200, and 500 µg/ml) in 24-hour exposures of RAW264.7 cells. The concentration of lipopolysaccharide (LPS) was 10 µg/ml.

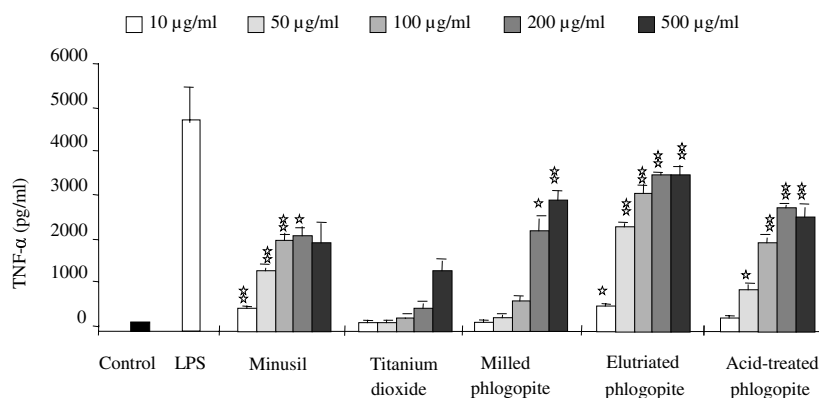


Figure 4. Concentration dependency of the tumor necrosis factor alpha (TNF-α) production (mean and SD, N=6) induced by the different mineral dusts (10, 50, 100, 200, and 500 µg/ml) in 24-hour exposures of RAW264.7 cells. The concentration of lipopolysaccharide (LPS) was 10 µg/ml. Significantly different from control: * P<0.005, ** P<0.0005.

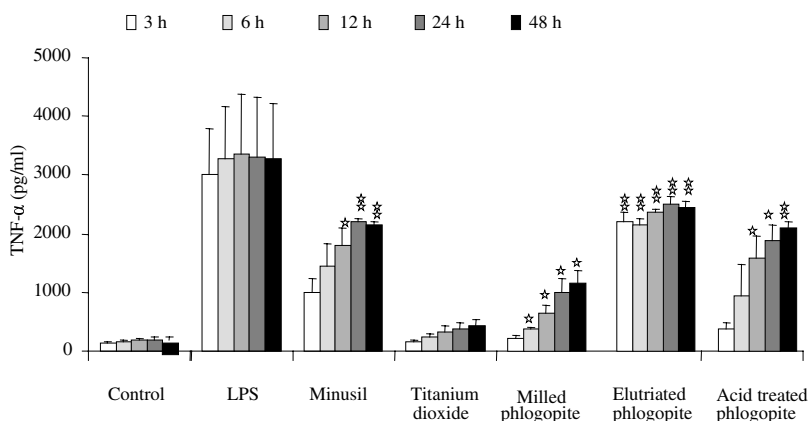


Figure 5. Time dependency of the tumor necrosis factor alpha (TNF-α) production induced by mineral dust (mean and SD, N=6) in RAW264.7 cells. The duration of exposures to a 100 µg/ml dose of the different dust particles varied between 3 and 48 hours. The concentration of lipopolysaccharide (LPS) was 10 µg/ml. Significantly different from control: * P<0.005, ** P<0.0005.

caused a time-dependent increase in cytotoxicity (data not shown).

The concentration dependency of TNF-α production induced by mineral dust in mouse macrophages is shown in figure 4. The order of potency of the dust samples in these responses was elutriated phlogopite > Minusil > acid-treated phlogopite > milled phlogopite > titanium dioxide. Statistically significant TNF-α responses (P<0.005) occurred with elutriated phlogopite and Minusil even at the lowest concentration (10 µg/ml). Elutriated phlogopite caused significantly larger responses (P<0.005) than milled phlogopite at concentrations of

10–100 µg/ml. Titanium dioxide stimulated TNF-α production in macrophages only at the highest concentration (500 µg/ml).

The time-course analysis of TNF-α production in mouse macrophages by the mineral dust concentration of 100 µg/ml is shown in figure 5. Elutriated phlogopite caused a significant (P<0.0005) and nearly maximal response at all exposure times. The other phlogopite samples and Minusil induced the highest TNF-α productions at 24 and 48 hours. TNF-α production induced by elutriated phlogopite was significantly larger (P<0.005) at all exposure times than for milled phlogopite.

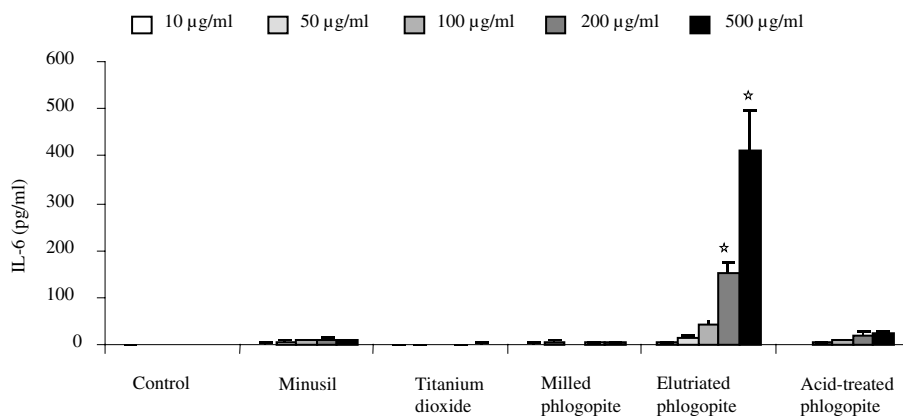


Figure 6. Concentration dependency of interleukin-6 (IL-6) production (mean and SD, N=6) induced by the different mineral dusts (10, 50, 100, 200, and 500 µg/ml) in 24-hour exposures of RAW264.7 cells. Significantly different from control: * $P < 0.01$.

The concentration dependency of IL-6 production induced by mineral dust in macrophages is shown in figure 6. Only elutriated phlogopite induced a clear concentration-dependent IL-6 response, which was statistically significant at doses of 200 and 500 µg/ml ($P < 0.01$). However, the IL-6 response to the positive LPS control was about 30 times greater than the response to elutriated phlogopite at 500 µg/ml.

None of the mineral dust samples induced NO production in macrophages at any time point, whereas LPS evoked clear time-dependent responses (data not shown).

Discussion

In this study, the potency of differently processed phlogopite particles in inducing the production of proinflammatory cytokines and NO were investigated in a mouse macrophage cell line. Elutriated phlogopite was a more potent inducer of TNF- α production than Minusil (α -quartz), and it was the only mineral dust that induced significant IL-6 production. TNF- α is known as an important proinflammatory cytokine that is involved in lung inflammation induced by mineral dust (13, 12).

It is commonly assumed that fine particles (diameter < 2.5 µm) are more harmful than coarse particles (diameter 2.5–10 µm) because they have a larger surface area and they can penetrate to the alveoli. However, in our in vitro study, the proinflammatory potency of phlogopite micas seemed to depend somewhat differently on the size and shape of particles. Elutriated phlogopite had the largest particle size and a relatively small specific surface area (table 1), but it was the most potent stimulator of macrophages.

Phlogopite micas are thin, sheetlike particles that have broken lateral surfaces. The SEM photographs revealed that the elutriated phlogopite particles were thin sheets typical of mica, whereas the forms of milled

particles were more diverse, due to crushing during the jet milling (figure 2). Hence a larger particle size, but not a large specific surface area, combined with a plate-like form appeared to increase the potency of phlogopite to induce the production of proinflammatory cytokines.

The thin and wide shape of mica particles may increase their potency to stimulate macrophages. The effect of the aspect ratio of mineral fibers is well known. It has been shown that long fibers are more potent stimulators of macrophages than short fibers (17, 18). Mica particles resemble fibers when observed from the lateral perspective. Consequently, the aspect ratio may play a role also in the case of sheetlike particles. However, this is only one possible explanation for the higher potency of the larger mica particles in inducing the production of proinflammatory cytokines.

The mineral content of dust could be one critical determinant of the particle-induced production of cytokines from target cells, such as alveolar macrophages and respiratory epithelial cells. In a study by Hetland et al (19), using rock samples from quarries, quartz, amphiboles, chlorite, and epidote induced cytokine production in epithelial cells, whereas plagioclase was less potent. The capacity of these mineral particles to induce the release of proinflammatory cytokines IL-6 and IL-8 was linked to their iron content (19).

Surface chemistry, particularly on the lateral surfaces of mica, may be involved in the stimulation of macrophages. Phlogopite is potassium, magnesium, aluminum silicate, which belongs to the class of trioctahedral micas. The crystal structure of phlogopite consists of a combination of two tetrahedral sheets with one octahedral sheet in between. In trioctahedral micas, all three octahedral centers are occupied with divalent cations, in phlogopite typically with magnesium and iron (20, 21). In most natural phlogopites, iron occupies the octahedra and even tetrahedra, and in the octahedra it generally occurs in its divalent form (22). The lateral surfaces of phlogopite particles consist of broken tetrahedral and octahedral sheets with iron (Fe) at the surface. When Fe^{2+} is present at the surface of the particles

in a biological solution, its reducing properties promote the reduction of molecular oxygen into reactive oxygen species including hydroxyl radicals (23, 24). Freshly ground biotite mica particles have had oxidizing surface properties in association with their increased toxicity (25).

In our study, acid-treated phlogopite, with low iron content, was a more potent stimulator of TNF- α production than the iron-containing milled phlogopite (figures 4 and 5). Consequently, this finding does not support the theory that iron would be a critical constituent for macrophage activation. However, the acid-treated phlogopite particles were larger than the milled phlogopite particles, which might have influenced the responses of the cells.

Acid-treated phlogopite had a very large surface area (225.7 m²/g) as measured with the nitrogen sorption method, but the surface area was small (2.78 m²/g) when studied with the optical method (table 1). The nitrogen sorption method also measured the porous surface inside the particles that had been generated by extraction of the interlayer, tetrahedral and octahedral cations. This porous silica structure of acid-treated phlogopite was a rather weak stimulator of macrophages, a finding indicating that the inner surface of the particles did not play a role in the macrophage-particle interaction. However, acid-treated phlogopite has been shown to be very hemolytic (26), indicating the importance of silanol groups on silica surfaces in evoking the lysis of red cell membranes.

Dust-exposed macrophages produced relatively little IL-6 in comparison with that of the responses to LPS. Only elutriated phlogopite caused a clear concentration-dependent IL-6 response, a finding suggesting that some of its properties (eg, larger particle size and platelike form) may have been important to this selective stimulation of macrophages. It is, however, possible that priming the cultured macrophages before the dust exposures would have increased the IL-6 responses. Stringer & Kobzik (27) have shown that the priming of human lung epithelial cells with TNF- α can increase IL-8 production when compared with that of nonprimed cells. In our study, LPS induced strong IL-6, TNF- α , and NO responses in macrophages and thus confirmed that the low IL-6 and NO responses to the mineral dust samples were true and not methodological artifacts.

In conclusion, our results suggest that, in addition to quartz, phlogopite mica may belong to the mineral components of spring dust that have the capacity to stimulate inflammatory cells. Recently, resuspended road dust from Helsinki has been shown to induce macrophage activation and proinflammatory cytokine production (28). The platelike shape of mica particles, rather than the surface area, seems important to cytokine production.

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