Mineral Particles of Varying Composition Induce Differential Chemokine Release from Epithelial Lung Cells: Importance of Physico-chemical Characteristics

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Presently, little is known about the potential health effects of mineral particles other than asbestos and quartz. In this study, a human epithelial lung cell line (A549), primary human small airway epithelial cells (SAECs) and primary rat type 2 (T2) cells were exposed to stone quarry particles of two size fractions (<10 and <2.5 μm) from nine different rock samples. The ability to induce the release of chemokines from lung cells was investigated and compared with the particles’ mineral and element composition and the amount of soluble elements. The stone particles induced the release of only low levels of interleukin (IL)-8 from A549 cells. In contrast, some of the other particles induced the release of high levels of macrophage inflammatory protein (MIP)-2 from T2 cells, and high levels of IL-8 from SAECs. Differences in particle surface area could account for differences in activity between the <10 and <2.5 μm fractions of six out of the nine rock samples. For two samples the <2.5 μm fraction was most active and for one sample the <10 μm fraction was most active. Content of the mineral plagioclase displayed a strong, negative correlation with the potential to induce MIP-2, whereas the mineral pyroxene was positively correlated with MIP-2 induction. However, neither plagioclase nor pyroxene content was sufficient to explain differences in bioactivity between the particles. No statistically significant correlation was found between the amounts of total or soluble elements and MIP-2 release. In conclusion, the results suggest that mineral particles with a high content of plagioclase have a low potential to induce a pro-inflammatory response. However, a particular mineral or element responsible for eliciting strong increases in chemokine release could not be identified. Thus, at present it appears that analysing mineral and element content is insufficient to predict stone particle bioactivity, and that biological testing is a necessity.

Keywords: interleukin-8; macrophage inflammatory protein-2; metals; minerals; quartz; size; stone quarry particles; type 2 cells

INTRODUCTION

Mineral particle exposure is an occupational health problem for workers in industries such as mining, sandblasting, stone quarrying and construction. However, sandstorms, volcano eruptions, earth erosions and road abrasion generated by cars with studded tyres (used in the winter season in several northern countries) may also give rise to considerable amounts of mineral particles in ambient air. Presently, knowledge about health effects associated with mineral particle exposure is mostly limited to studies on quartz and asbestos. A recent study on Norwegian mining and industry workers found an association between mineral particle exposure other than to quartz and asbestos and lung and heart diseases (Myran and...
Furuseth, unpublished results). However, the minerals involved in these effects have not yet been identified.

Stones often consist of different minerals with widely varying characteristics with respect to crystalline structure, amounts of different metal elements in the structure and solubility of components. Quartz, which is among the minerals known to have an effect on health in occupational settings, consists almost exclusively of pure SiO₂. Other mineral groups are more complex, such as the pyroxenes with the general formula (Fe,Mg)SiO₃. The most common group of minerals are the feldspars, XAlₙ(1–2)Si₃–ₙO₈ (X = Ca, K and/or Na), which make up almost half the earth’s crust. Potassium-feldspar (K-feldspar) and plagioclase are the main members of this group. Minerals such as epidote and chlorite may contain substantial amounts of heavy metals.

Reactive quartz is known for its silanol (SiOH) groups. When quartz is fractured, cleavage of silicon–oxygen bonds in the crystal structure occurs and reactive groups such as SiO₂(s) and SiO₃(s) may be generated on the particle surface. These reactive groups are thought to be central to the biological reactivity (bioactivity) of quartz (see Shi et al., 2001; Fubini and Hubbard, 2003 for review). Similarly, the surface reactivity of asbestos fibres seems to be the key to explaining the bioactivity of the various types of asbestos (see Kamp and Weitzman, 1999; Shukla et al., 2003 for review). The particle surface of both quartz and asbestos may generate reactive oxygen species (ROS) acting on the exposed cells (Kamp and Weitzman, 1999; Shi et al., 2001; Fubini and Hubbard, 2003; Shukla et al., 2003). Transition metals have been shown to contribute to this ROS generation through the Fenton reaction, thus enhancing particle toxicity (Kamp and Weitzman, 1999; Fubini and Hubbard, 2003; Shukla et al., 2003). In addition, quartz has been shown to mediate effects through interaction with cell surface receptors (Iyer et al., 1996; Stringer et al., 1996; Palecanda et al., 1999; Hamilton et al., 2000), although the mechanisms for these responses are not clear. However, there is little information about the reactivity of the surface of other crystalline silicates and the possible importance of soluble or insoluble metals in these minerals.

Accumulating evidence suggests that inflammation is crucial to the development of adverse health effects associated with particle exposure. A key event in the inflammatory processes is the production and release of pro-inflammatory cytokines such as interleukin (IL)-1α and (IL)-1β, tumor necrosis factor (TNF)-α, IL-6, IL-8 and macrophage inflammatory protein (MIP)-2 (Bagnoli, 2001; Gangur et al., 2002). In humans, evidence suggests that the CXC-chemokine IL-8 plays a pivotal role in acute inflammatory diseases (Harada et al., 1996; Gangur et al., 2002). IL-8 is known to trigger chemotaxis in neutrophils and other leukocytes, and inhibition of the biological activity of this chemokine markedly reduces inflammation (Harada et al., 1996). In rodents MIP-2, an analogue to human IL-8, attracts neutrophils to the site of inflammation. Inhibition of MIP-2 has been shown to attenuate neutrophil recruitment in the lungs of quartz-exposed rats (Driscoll, 2000).

Several studies have shown that epithelial lung cells release increased levels of important pro-inflammatory cytokines and chemokines upon stimulation with quartz (Stringer et al., 1996; Hetland et al., 2001b), asbestos (Rosenthal et al., 1994; Stringer et al., 1996) and diesel exhaust particles (DEPs) (Takizawa et al., 1999; Boland et al., 2000). Recent studies by Hetland and Becher (Hetland et al., 2000, 2001a; Becher et al., 2001) have shown that stone particles (<10 μm) containing low amounts of quartz may also induce high levels of TNF-α, IL-6, IL-8 and MIP-2. These stone particles differed in mineral composition and varied considerably in their ability to induce cytokine release in cells and influx of neutrophils in rat lungs (Hetland et al., 2000; Becher et al., 2001). Although the mechanisms remained unknown, mineral and/or metal composition were suggested to be critical determinants for the observed activity differences (Hetland et al., 2000).

In the present study we have exposed lung epithelial cells to stone particle fractions of two size fractions (<10 and <2.5 μm) generated from nine rock samples. The particles were analysed for size, surface area, mineral and element composition and element leaching. The aim was to investigate the potential of stone particles to induce chemokine release from lung epithelial cells and to analyse how the various physico-chemical properties were related to chemokine release. The study also aimed to examine whether information on physico-chemical characteristics could be used to predict the biological effects of stone particle fractions.

**MATERIALS AND METHODS**

**Reagents**

Culture medium, Nutrition Mixture F-12 HAM Kaighn’s modification (HAM’S F-12K), was obtained from Sigma Chemical Company (St. Louis, MO, USA), Bronchial/Tracheal Epithelial Cell growth Medium BulletKit® (BEGM® BulletKit®) was from Bio Whittaker (Walkersville, MD, USA) and William’s medium E was from Bio Whittaker Europe (Verviers, Belgium). Fetal bovine serum (FBS) was from Gibco BRL (Paisley, Scotland). Ampicillin and fungizone were from Bristol-Myer Squibb (Bromma, Sweden), and penicillin/streptomycin was from Bio Whittaker. Cytokine ELISA assays for IL-8 and MIP-2 were purchased from Biosource International.
Mineral particles

Mineral particles were prepared by SINTEF (Trondheim, Norway). Nine different species of rock obtained from eight different Norwegian stone quarries were crushed using the standardized Los Angeles method (Draft European Standard prEN 1097-2). This method of particle preparation was chosen because it yielded particles with a size distribution roughly similar to that found in chipping cuttings (drill dusts). The <10 and <2.5 µm fractions were selected by wet sifting particles through a nylon square sieve (with 10/2.5 µm light opening). All particle preparations consisted of different types of crystalline silicates with varying mineral composition. The commercially available crystalline silica particle MIN-U-SIL® 5 Ground Silica (U.S. Silica Company, Berkley Springs, WV, USA), used as a reference particle, was kindly provided by Dr Paul Borm, Medical Institute of Environmental Hygiene, Düsseldorf, Germany. MIN-U-SIL® 5 is a high-purity, natural crystalline silica of the quartz type. According to the manufacturer, this ground silica is at least 98% SiO₂, with a size distribution of typically 96% passing through 5 µm and a median diameter of 1.6 µm. Before cell culture exposure, the particles were prepared as previously described by Hetland et al. (2000). The range of particle concentrations used in the study was determined after initial screenings on A549 cells and T2 cells. The criteria were relatively linear concentration–responses for particle-induced cytokine release.

Analysis of particle characteristics

The size distribution within the <10 and <2.5 µm fractions was determined by laser diffraction using a Coulter LS particle size analyser. The method measures the dispersion of the laser beam, which increases with decreasing particle size. The samples were dispersed in 10 ml calgon (0.5%) dispersant and sonicated for 10 min prior to analysis. Specific particle surface areas were determined by BET-analysis, using a Flowsorb II 2300. The method measures a quantitative amount of gases adsorbed as a mono-molecular layer on the particle surface. Semi-quantitative mineral distribution of the samples was determined using X-ray diffraction (XRD) analysis (Philips PW 1830). Element analysis was based on inductive coupled plasma atomic emission spectroscopy, after extraction in Aqua Regia for 1 h at 90°C. Element leaching was determined by suspending particles in PBS (1 mM phosphate, 0.9% NaCl, pH 7.4) for 24 h, under constant rotation. The particles were removed by centrifugation, and element concentrations in leachates were determined using a Perkin-Elmer 5100 atomic absorption spectrometer.

Human lung epithelial cells (A549 and SAEC)

The human alveolar epithelial cell line A549 from American Tissue Type Culture Collection (ATCC, Rockville, MD, USA) was cultured in HAM’s F-12K medium, supplemented with ampicillin (100 µg/ml), penicillin/streptomycin (100 µg/ml), fungizone (0.25 µg/ml) and 10% heat-inactivated FBS. The A549 cells were plated in 35 mm 6-well culture dishes (2 × 10⁴ cells/well) and grown to confluence at 37°C in a humidified atmosphere of 5% CO₂ in air, prior to exposure. The human Small Airway Epithelial Cells (SAECs) from Bio Whittaker were cultured in BEGM® BulletKit® medium. The SAECs were plated in 35 mm 6-well culture dishes and cultured at 37°C in a humidified atmosphere of 5% CO₂ in air, according to the supplier’s protocol. Cell passages between 8 and 12 were used in the study.

Primary T2 cells from rat lungs

T2 cells were isolated from rat lungs using the method described by Lag et al. (1996). In short, lungs were removed from six anaesthetized male, inbred Wistar Kyoto (Wky/NHds) rats between 6 and 8 weeks old. Cells were isolated by sequential use of enzymatic digestion, centrifugal elutriation and differential attachment. The isolated T2 cells were cultured in William’s medium E supplemented with insulin (5 µg/ml), hydrocortisone (0.087 µg/ml), transferrin (5µg/ml), EGF (10 ng/ml), sodium selenite (6.2 ng/ml), ascorbic acid (5 µg/ml), glutathione (5 µg/ml), ampicillin (100 µg/ml), penicillin/streptomycin (100 µg/ml), fungizone (0.25 µg/ml), Hepes (15 mM) and 5% heat-inactivated FBS. The T2 cells were plated in 35 mm 6-well culture dishes at a density of 4 × 10⁵/cm² and grown for 2 days at 37°C in a humidified atmosphere of 5% CO₂ in air, prior to exposure.

IL-8 and MIP-2 assays

Cells were exposed to particles and incubated at 37°C for 24 h. Subsequently, supernatants were removed and centrifuged in two steps, first at 250 × g to remove cells, then at 2500 × g to remove the remaining particles. The final supernatants were stored at −70°C. IL-8 and MIP-2 protein levels were determined using enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s guidelines. Absorbance was measured and quantified using a plate reader (TECAN Sunrise) complete with software (Magellan V 1.10).

Statistical calculations

In this study the correlation between specific minerals and elements in the stone particles and
the particles’ ability to induce cytokine release from lung cells were analyzed. When testing multiple null hypotheses the overall chance of type-I errors increases. To maintain the chance of type-I errors at 5%, a sufficiently stricter threshold ($\alpha$) was estimated from the equation

$$\alpha = 1.00 - 0.95^{(1/N)}.$$ 

$N$ is number of minerals or elements in the analysis. Pearson correlation and linear regression analysis were performed using GraphPad Prism software.

**RESULTS**

**Particle characteristics**

In this study lung cells were exposed to <10 and <2.5 μm fractions of nine different stone particles. An overview of the particles, including information on identification number, size fraction, average particle size, surface area and rock species is given in Table 1. The average size within the <10 and <2.5 μm fractions varied by a factor of 1.3 and 1.9, and particle surface area varied by a factor of 1.8 and 1.6, respectively.

All the rock samples in the study, with the exception of sample IX, were pure crystalline rock species with a negligible amount of non-crystalline components (Tom Myran, personal communication). Sample IX was jasper, which has more complicated characteristics, consisting of crystalline, crypto-crystalline and amorphous minerals. Previous experience with jasper from the same has shown that the crypto-crystalline and amorphous minerals may constitute as much as 50% or more of the total mass (Tom Myran, personal communication). This is important to consider since the obtained values from the mineral analysis are related to the total content of crystalline minerals. The analysis showed that no crystalline mineral occurred in high amounts in all particles (Table 2). However, some minerals seemed to dominate. These were first of all the two feldspar members plagioclase and K-feldspar, and then quartz and also to a certain extent pyroxene. As seen in Table 2, there is a difference in mineral composition between the two size fractions. This may be due to variation in the hardness of different minerals, which causes some minerals to break into finer particle than others during crushing. The element analysis revealed that all particles contained high amounts of Al, Fe, Ti, K, Mg, Mn and P, whereas the remaining elements occurred at considerably lower levels (Tables 3 and 4). We did not have enough of particle IX2.5 to perform the mineral and metal analysis.

Element leaching was measured from selected particles. We found considerable variation in the amounts of soluble elements among the analysed particles. However, K, Ca and Si dominated in the leachates, whereas Al, Mg, Fe, B, Sr and Ti occurred at intermediate levels (data not shown).

**Stone particle-induced IL-8 release from A549 cells**

A549 cells were exposed to various concentrations of the <10 and <2.5 μm stone particle fractions as well as quartz. The results showed little variation among the different samples. All particles induced a low increase in IL-8 release from A549 cells compared with the quartz reference particle (Fig. 1). We observed no effects on cell viability of any of the particles with the exception of the highest quartz concentration (not shown). The effect of the quartz reference particle on A549 cell viability has been published elsewhere (Øvrevik et al., 2004); there was no significant effect of 20 and 40 μg/cm² quartz, but ~30% reduction in viability at 80 μg/cm².

**Stone particle-induced MIP-2 release from primary rat T2 cells**

Previous studies have shown that the primary rat T2 cells are more sensitive to particle exposure than the A549 cell line (Øvrevik et al., 2004). This was confirmed in a pilot study with the stone particles, and

<table>
<thead>
<tr>
<th>Particle</th>
<th>Fraction (μm)</th>
<th>Average size (μm)</th>
<th>Surface area (m²/g)</th>
<th>Rock sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>I10/I2.5</td>
<td>&lt;10/&lt;2.5</td>
<td>7.4/1.5</td>
<td>5.3/12.2</td>
<td>Coarse grain syenite porphy</td>
</tr>
<tr>
<td>II10/II2.5</td>
<td>&lt;10/&lt;2.5</td>
<td>7.0/1.6</td>
<td>6.0/11.9</td>
<td>Basalt</td>
</tr>
<tr>
<td>III10/III2.5</td>
<td>&lt;10/&lt;2.5</td>
<td>7.0/1.5</td>
<td>7.1/12.5</td>
<td>Quartzite</td>
</tr>
<tr>
<td>IV10/IV2.5</td>
<td>&lt;10/&lt;2.5</td>
<td>8.0/1.5</td>
<td>4.2/10.1</td>
<td>Fine grain syenite porphy</td>
</tr>
<tr>
<td>V10/V2.5</td>
<td>&lt;10/&lt;2.5</td>
<td>7.5/1.5</td>
<td>5.1/10.3</td>
<td>Mylonitic quartzdiorite</td>
</tr>
<tr>
<td>VI10/VI2.5</td>
<td>&lt;10/&lt;2.5</td>
<td>7.4/1.3</td>
<td>7.1/13.3</td>
<td>Coarse porphy basalt</td>
</tr>
<tr>
<td>VII10/VII2.5</td>
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<td>6.8/1.2</td>
<td>5.2/9.9</td>
<td>Fine felsitic basalt</td>
</tr>
<tr>
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<td>8.1/2.3</td>
<td>7.0/15.0</td>
<td>Hornfels</td>
</tr>
<tr>
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<td>8.7/1.9</td>
<td>3.9/9.4</td>
<td>Jasper</td>
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</tbody>
</table>

The table presents an overview of particle identification number, size fractions, average particle size and surface area per mass unit, and species of rock.
Table 2. Mineral analysis of the <10 and <2.5 μm fractions

<table>
<thead>
<tr>
<th>Particle samples</th>
<th>Minerals</th>
<th>Plagioclase</th>
<th>K-Feldspar</th>
<th>Quartz</th>
<th>Pyroxene</th>
<th>Chlorite</th>
<th>Epidote</th>
<th>Amphibole</th>
<th>Mica</th>
<th>Calsite</th>
<th>Granat</th>
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<td>59</td>
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<td>1</td>
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<td>&lt;1</td>
<td>—</td>
<td>2</td>
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<tr>
<td>II10</td>
<td>43</td>
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<td>13</td>
<td>20</td>
<td>4</td>
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<td>—</td>
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<tr>
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<td>—</td>
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<td>—</td>
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<tr>
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<td>7</td>
<td>14</td>
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<td>—</td>
<td>&lt;1</td>
<td>—</td>
<td>2</td>
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</tr>
<tr>
<td>V10</td>
<td>45</td>
<td>10</td>
<td>26</td>
<td>—</td>
<td>6</td>
<td>12</td>
<td>1</td>
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<td>17</td>
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<td>2</td>
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<td>3</td>
<td>5</td>
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<tr>
<td>I2.5</td>
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<td>—</td>
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<td>4</td>
<td>7</td>
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</table>

The table presents the particles content of different crystalline minerals. The minerals have been ranked according to their average abundance.

The analysis detected only crystalline minerals. Sample IX also contains a considerable proportion of crypto-crystalline and amorphous minerals, constituting as much as 50% or more of the total mass. For the remaining samples, the proportion of crypto-crystalline and amorphous minerals is negligible.

There was not enough of particle IX 2.5 to perform the mineral analysis.

Table 3. Element analysis of the <10 μm fractions

<table>
<thead>
<tr>
<th>Particle samples</th>
<th>Elements</th>
<th>Fe</th>
<th>Ca</th>
<th>Al</th>
<th>Mg</th>
<th>Ti</th>
<th>K</th>
<th>Na</th>
<th>P</th>
<th>Mn</th>
<th>Ce</th>
<th>Sr</th>
<th>Zn</th>
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<td>23 100</td>
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<td>14 000</td>
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<td>4 010</td>
<td>1 970</td>
<td>2 210</td>
<td>799</td>
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<td>20 100</td>
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<td>4 890</td>
<td>2 470</td>
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<td>1 310</td>
<td>2 151</td>
<td>117</td>
<td>202</td>
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<td>&lt;100</td>
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<td>33 700</td>
<td>33 100</td>
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<td>142</td>
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<td>18 900</td>
<td>35 500</td>
<td>26 700</td>
<td>3 760</td>
<td>1 650</td>
<td>1 130</td>
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<td>29 300</td>
<td>33 300</td>
<td>30 500</td>
<td>9 820</td>
<td>3 590</td>
<td>1 990</td>
<td>2 490</td>
<td>887</td>
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<tr>
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<td>19 000</td>
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<td>7 370</td>
<td>657</td>
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<td>585</td>
<td>480</td>
<td>894</td>
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<td>78</td>
<td>45</td>
<td>&lt;100</td>
<td>71</td>
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</table>

The table presents the particles content of different elements. Values are given as mg/kg. The elements have been ranked according to their average abundance.
the T2 cells were, therefore, exposed to lower particle concentrations. All stone particles induced an increase in MIP-2 release from T2 cells, but the ability to induce MIP-2 release varied considerably within both size fractions (Fig. 2). Of the <10 µm particles, particles I10–V10 and VII10 induced only weak increases in MIP-2, whereas particles VI10, VIII10 and IX10 induced high levels of MIP-2 compared with the quartz reference particle (Fig. 2A). In the <2.5 µm fraction, particles I2.5–IV2.5 and IX2.5 induced less MIP-2 than the <2.5 µm particles. We observed no effects on cell viability of any of the tested particles (not shown).

Stone particle-induced IL-8 release from human SAECs

Exposure of A549 and T2 cells revealed considerable response differences to stone particle exposure, which may be due to species differences, differences between cell lines or primary cells or differences in regulation of IL-8 versus MIP-2. Therefore, particle-induced IL-8 release from human SAECs, which are primary epithelial cells from the small airways, were

Table 4. Element analysis of the <2.5 µm fractions

<table>
<thead>
<tr>
<th>Particle samples</th>
<th>Elements</th>
<th>Zr</th>
<th>La</th>
<th>Cu</th>
<th>Cr</th>
<th>Ni</th>
<th>Li</th>
<th>Co</th>
<th>Y</th>
<th>B</th>
<th>Pb</th>
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<th>Be</th>
<th>Mo</th>
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<td>10</td>
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<td>16</td>
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<tr>
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<td>91</td>
<td>42</td>
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<tr>
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<td>&lt;1</td>
<td>1.05</td>
<td>&lt;1</td>
<td></td>
</tr>
</tbody>
</table>

The table presents the particles content of different elements. Values are given as mg/kg. The elements have been ranked according to their average abundance.

*There was not enough of particle IX2.5 to perform the element analysis.

Fig. 1. IL-8 release from particle-exposed human alveolar epithelial cells (A549). A549 cells were exposed to increasing concentrations of stone particles and incubated for 24 h at 37 °C. IL-8 release was measured as described in ‘Materials and methods’. The figures display the effect of the <10 µm (A) and <2.5 µm (B) particle fractions with quartz (<5 µm) as the reference particle. Particle concentrations are given as mass per surface area of the cell culture dishes (µg/cm²). Each point depicts mean ± SEM of independent experiments (n = 3).
also investigated. We tested particles I₁₀ and I₂.₅, which produced little effect on chemokine release from both A549 and T2 cells, and particles VIII₁₀ and VIII₂.₅, which produced little effect on IL-8 release from A549 cells, but which were potent inducers of MIP-2 from T2 cells (Figs 1 and 2). As in the A549 and T2 experiments, quartz was used as a reference particle. The results show that the order of ability to induce IL-8 from SAECs for the <10 μm particles was VIII₁₀ > quartz > I₁₀, and for <2.₅ μm particles, VIII₂.₅ > quartz > I₂.₅ (Fig. 3). However, compared with A549 and T2 cells, SAECs produced relatively low chemokine levels in response to quartz exposure (Figs 1–3). Moreover, IL-8 release decreased at 40 μg/cm² of particle VIII₂.₅ and quartz, and at 60 μg/cm² of particle I₂.₅, compared with lower doses. This decrease was probably due to particle-induced cell death (not determined).

**Significance of particle size**

It appears that the <2.₅ μm particles had a greater potential to induce MIP release from T2 cells than the <10 μm particles when the concentrations were calculated as total particle mass (Fig. 2). However, it is believed that total surface area might be a more accurate measure of particle concentration than the mass (Fenoglio *et al*., 2000; Hetland *et al*., 2001b). When cytokine release was related to equal surface areas of the particles, no difference in bioactivity was observed between the two size fractions of samples I–IV, VI and VIII (Fig. 4). Particles VII₂.₅ were more active than particles VIΙ₁₀ and VIΙ₂.₅, respectively, and particle IX₁₀ was more active than particle IX₂.₅.

**Estimation of particle bioactivity**

To get a measure of the particles’ potential to induce chemokine release, we first estimated linear regression curves between chemokine release and particle concentration given as total surface area (Fig. 4). Particle bioactivity was then defined as the slope of the linear regression curves. The estimation of particle bioactivity confirmed the impression from the concentration–effect relationship seen in
Figs 1 and 2. The potential to induce IL-8 release from A549 cells was relatively low and showed little variation (quantification not shown), whereas the potential to induce MIP-2 from T2 cells was much more variable. Particles V 2.5, VI 10, VII 2.5, V10, VIII 2.5 and IX 10 were considerably more active than the others (Fig. 4). Moreover, the effect of stone particles on MIP-2 release seemed to be distributed in two distinct groups, either being very active (slope = 0.13–0.20) or producing little effect (slope = 0.01–0.05).

Correlation between particle composition and bioactivity

Mineral and element contents were compared with particle bioactivity to investigate the relationship between chemokine induction and particle composition. However, because of the unknown level of...
crypto-crystalline and amorphous material in sample IX, this sample was not included in the correlation analysis of mineral content with bioactivity. The potential to induce IL-8 from A549 cells varied only by a factor of 3.5 between the least and most active particles (not shown). No correlation was found between the potential to induce IL-8 and the content of any of the minerals or elements (not shown). However, owing to the lack of variation in IL-8 induction, these data were not well suited to correlation analysis. The ability to induce MIP-2 from T2 cells varied by a factor of 20 between the least and most potent particles (Fig. 4). With the exception of plagioclase and pyroxene content, we did not observe any correlation between the potential to induce MIP-2 and the amount of individual minerals or elements in the particles (not shown). Plagioclase content displayed a relatively strong, significant, negative correlation with particle bioactivity, (Fig. 5A), whereas pyroxene displayed a somewhat weaker, positive correlation with particle bioactivity, with borderline significance (Fig. 5B). However, the plagioclase and pyroxene contents alone were not sufficient to explain all the differences in MIP-2-inducing ability among the tested stone particles.

The connection between element leaching and potential to induce chemokine release of selected particles was also investigated. As with the total element analysis, no statistically significant correlation between soluble elements and bioactivity was found. However, there was a relatively strong correlation between soluble arsenic and particle bioactivity ($r = 0.93, P = 0.0022$), which was close to the threshold for significance—$\alpha = 0.0015$ (data not shown).

As described earlier, the <2.5 μm fraction of samples V and VII was more active than the <10 μm fraction. However, no apparent differences in particle composition or element leaching were found that could explain the discrepancy in the potential to induce chemokine release. Unfortunately, the available amount of particle IX2.5 was too little to perform the composition and leachate analysis. Hence the reasons for the lower activity of IX2.5 than IX10 could not be investigated.

**DISCUSSION**

Previous studies by Hetland and Becher (Hetland et al., 2000, 2001a; Becher et al., 2001) have shown that stone particles (<10 μm) of various compositions have different potentials to induce inflammatory responses. Although mineral or metal composition were suggested to be critical determinants for particle activity, no minerals or metals were identified that explained why some particles were more active than others. In the present study, stone particle size, surface, mineral and element content, element leaching and ability to induce chemokine release were examined in detail to understand better the characteristics that are of importance for the inflammatory potential of a particle.

Two size fractions (<10 and <2.5 μm) of particles from nine different rock samples were tested in this study. To get an impression of the relative ability of the stone particles to induce chemokine release from the various lung cells, a quartz particle was included as a reference. The harmful effects of quartz are well documented through epidemiological and clinical studies (see American Thoracic Society, 1997 for review). Several experimental investigations have shown that quartz induces inflammatory responses in the lungs of exposed animals (Vallyathan et al., 1995; Johnston et al., 2000; Becher et al., 2001; Shukla et al., 2001) as well as increased cytokine

![Fig. 5. Correlation between mineral content and particle bioactivity. Data from the correlation analyses of plagioclase (A) and pyroxene (B) content versus particle bioactivity are given in the figures. The figures also display the linear regression lines for the relationship between the content of plagioclase (A) and pyroxene (B), and particle bioactivity, with 95% confidence intervals. Bioactivity was measured by estimating the slope of the linear regression line of the concentration–effect curves for particle-induced MIP-2 release from T2 cells. Each point depicts the mean of independent experiments ($n = 3$). Threshold for significant correlation: $\alpha = 0.005$.](image-url)
release from various cell systems (Stringer et al., 1996; Barrett et al., 1999; Hetland et al., 2001b). In comparison with quartz, both the <10 and <2.5 μm fractions of the rock samples had little impact on IL-8 release from A549 cells. However, particles V2.5, VI10, VI2.5, VII2.5, VIII10, VIII2.5 and IX10 induced a strong increase in MIP-2 release from T2 cells compared with quartz. Experiments with a limited number of particles on human SAECs showed an order of ability to induce the release of IL-8, which resembled the results from the T2 cells. Thus, it seems reasonable to believe that the stone particles tested may also induce the release of high levels of chemokines from human lung cells, despite the lack of response from the A549 cells.

In response to quartz exposure A549 and T2 cells reacted in a similar manner, whereas only the T2 cells were sensitive to stimulation with the stone particles. This discrepancy suggests not only differences between A549 cells and T2 cells, but also that the active stone particles reacted through mechanisms other than quartz.

Another interesting observation was the lack of MIP-2-inducing stone particles of intermediate bioactivity. When expressing particle concentration in terms of particle surface area, the stone particles seemed to divide into two distinct groups according to their effect on MIP-2 release, either being very potent or producing little effect. This ‘either/or’ effect of stone particles may also be seen in results from a previous study from our laboratory (Hetland et al., 2000). This response pattern could be due to a threshold separating particles with and without the ability to elicit a specific harmful effect on the cells. One explanation might be that some particles have the ability to interact with cell surface receptors such as scavenger receptors (Iyer et al., 1996; Stringer et al., 1996; Hamilton et al., 2000; Chao et al., 2001; Obot et al., 2002). The weak chemokine increase induced by all the stone particles in A549 cells and by the low-potency particles in the T2 cells may suggest that all particles elicit a non-specific effect on the cells.

A primary aim of this study was to investigate the connection between particle characteristics and bioactivity, measured as the ability to induce chemokine release. To analyse this correlation a quantitative measure of particle bioactivity was required. A linear regression of chemokine release versus particle concentration (given in terms of surface area) has been performed, and bioactivity has been defined as the slope of the regression lines. This approach is obviously not ideal, as concentration–effect curves are seldom linear. However, after initial screenings a concentration range with relatively linear curves for all the particles was chosen. The regression line slopes provide a measure of bioactivity which account for differences in particle size and which is based on several particle concentrations, as opposed to choosing a single concentration for comparison of particle bioactivity. Since only the release of one chemokine at one time point has been studied, we cannot exclude the possibility that the relative bioactivities of the particles would have been different had other chemokines been measured or measured at different time points. However, previous studies from our laboratory do not suggest major differences in the patterns of stone particle-induced MIP-2, IL-6 and TNF-α release from T2 cells and rat alveolar macrophages, or IL-8 and IL-6 release from A549 cells (Hetland et al., 2000; Becher et al., 2001). It has also been found that the time-course of chemokine release induced by quartz and different stone particles was relatively similar during the first 48 h of exposure, even though the magnitude of the induction varied (Øvrevik et al., 2002, 2004).

Since the effect of stone particle exposure on IL-8 release from A549 was relatively low and exhibited little variation, these data were not well suited for correlation analysis. The remaining discussion therefore focuses on the results obtained from the T2 experiments.

The importance of size to bioactivity has been frequently discussed in particle toxicity. The results from the T2 experiments suggested that the <2.5 μm fractions were considerably more active than the <10 μm fractions, when compared at equal mass. However, the bioactivity of mineral particles is likely to depend on direct contact between the particle surface and the cells, on the ability of the particle surface to generate ROS or on soluble particle components. All of these factors are more or less connected with particle surface area. Since small particles have higher total surface area to mass ratio than large particles, this may explain the apparently higher activity of the <2.5 μm fraction. Indeed, previous studies with A549 cells have shown that differences in the ability to induce cytokine release between quartz particles of various sizes were minimal if cytokine release was related to total particle surface area and not to mass (Hetland et al., 2001b). Similar findings have also been reported from studies of polystyrene particles of various sizes (Brown et al., 2001). For six out of the nine rock samples in this study, correlation for differences in total surface area eliminated differences in ability to induce MIP-2 release between the two size fractions. For the remaining three rock samples, where differences in particle surface areas could not account for differences in activity, there was no clear trend towards a higher activity, neither for the <10 μm nor for the <2.5 μm fraction. Hence, it is reasonable to believe that the size per se is of little importance to mineral particle bioactivity compared with qualitative particle characteristics such as surface reactivity.

With the exception of the previously mentioned studies by Hetland and Becher (Hetland et al.,
Knowledge on the ability of mineral particles to induce inflammatory responses is limited to studies on silica and asbestos. Thus, the existing literature provides little support for a connection between stone particle composition and the ability to induce cytokine release. In the present study, considerable variation in mineral composition among the different particles was found. Although some minerals dominated, such as the feldspars (plagioclase and K-feldspar), quartz and also to a certain extent pyroxene, no mineral occurred at high levels in all particles. With the exception of plagioclase and pyroxene, there was no significant correlation between mineral content and particle bioactivity. The plagioclase content, however, showed a strong, negative correlation with the ability to induce MIP-2 release from T2 cells. Previous studies of stone particle-exposed A549 and T2 cells have shown that the rock species mylonite (38% plagioclase) and gabbro (25% plagioclase) induced high levels of cytokine release, whereas basalt (77% plagioclase) and feldspar (99% plagioclase) had little or no effect (Hetland et al., 2000; Becher et al., 2001). Moreover, these in vitro cytokine responses were shown to correspond well with neutrophil recruitment in the lungs of particle-exposed rats (Becher et al., 2001). Therefore, it seems reasonable to believe that particles with high plagioclase content have relatively low bioactivity. In contrast, pyroxene was positively correlated with stone particle bioactivity, suggesting that particles with a high pyroxene content might represent a potential health hazard. However, neither the plagioclase nor pyroxene content was sufficient to explain the bioactivity of all the tested particles. For instance, particle VII,10 had a relatively high pyroxene content and low plagioclase content but induced little MIP-2 release. Surprisingly, the quartz content did not correlate with particle bioactivity. However, previous studies have shown that the bioactivity of quartz particles of different origin may vary substantially (Donaldson et al., 2001). This may explain the lack of correlation between quartz content and chemokine release, despite the potent effect of the quartz reference particle. It seems likely that the bioactivity of other minerals may also vary in a similar manner, thus hampering the correlation analysis.

It has been suggested that transition metals are important to the toxicity of mineral particles (Fubini and Hubbard, 2003; Shukla et al., 2003), the results of this study suggest that there is no good correlation between particle bioactivity and total or soluble elements. However, it is important to consider that the element analysis measured total particle contents. This may reflect neither the composition of available elements on the particle surface nor the amount of redox active metal ions. Also, if several elements are able to induce a similar cellular response, variations in these elements among the different particles may obscure their individual effects, thus hampering the correlation analysis. This may also explain why only pyroxene was found to be positively correlated with bioactivity, although the distribution of this mineral was insufficient to explain variation in activity for all the particles. Another possible scenario is that it is the total sum of surface characteristics that is crucial to elicit a biological response. For instance
scavenger receptors, which have been suggested as being important to apoptosis induced by quartz and other particulates (Iyer et al., 1996; Stringer et al., 1996; Palecanda et al., 1999; Hamilton et al., 2000; Obot et al., 2002), recognize a broad range of ligands with a certain spatial distribution of negative charges (Pearson et al., 1993). Indeed, a recent study of apoptosis induced by combustion particles suggested that the overall particle matrix is of importance, and not just specific components (Obot et al., 2002). Based on our analysis it seems reasonable that this may be the case for stone particles as well. However, an apparent challenge will be to find out which surface characteristics are causing the observed effects. This will require additional studies both on the surface reactivity of these particles, similar to the studies on quartz by Fubini et al. (2004), as well as studies on the molecular mechanisms involved in the cellular effects.

CONCLUSION

In the present study we have shown that stone particles of various mineral and element composition can induce high levels of chemokine release from epithelial lung cells compared with quartz. Particle size does not seem to be an important factor in the ability to induce chemokines, and one must therefore assume that qualitative particle characteristics are of greater importance. In accordance with studies on quartz and asbestos, the surface reactivity of the stone particles appears to cause the observed effects. The results suggest that stone particles with a high content of plagioclase exhibit a low potential to induce a pro-inflammatory response. However, a particular mineral or element responsible for eliciting strong increases in chemokine release could not be identified. Thus, at present it appears that analysing mineral and element content alone is insufficient to predict stone particle bioactivity, and that biological testing is a necessity.

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REFERENCES


Hetland RB, Myhre O, Lag M et al. (2001a) Importance of soluble metals and reactive oxygen species for cytokine release induced by mineral particles. Toxicol; 165: 133–44.


