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DNA damage in lung epithelial cells isolated from rats exposed to quartz: role of surface reactivity and neutrophilic inflammation

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Respirable quartz has been classified as a human lung carcinogen (IARC, 1997). However, the mechanisms involved in quartz-induced carcinogenesis remain unclear. The aim of the present study was to investigate acute DNA damage in epithelial lung cells from rats exposed to quartz. Since surface reactivity is considered to play a crucial role in the toxicity of quartz, the effect of surface modifying agents polyvinylpyridine-*N*-oxide (PVNO) and aluminium lactate (AL) was evaluated. Therefore, rats were instilled with quartz (DQ12, 2 mg/rat) or quartz treated with PVNO or AL. After 3 days animals were killed and brochoalveolar lavage (BAL) was performed to evaluate inflammatory cell influx. BAL-fluid levels of lactate dehydrogenase (LDH), alkaline phosphatase (AP) and total protein were used as lung damage markers. Neutrophil activation was assessed by myeloperoxidase (MPO) measurement, and total antioxidant capacity of the BAL-fluid was determined using the TEAC (trolox equivalent antioxidant capacity) assay. Lung epithelial cells were isolated and DNA strand breakage was determined by single cell gel electrophoresis (comet assay). DNA damage was significantly increased in epithelial cells from rats instilled with DQ12, whereas no enhanced DNA strand breakage was observed when quartz was treated with PVNO or AL. Total protein, LDH and TEAC were increased in rats treated with native quartz, and this was inhibited by both coatings. A significant correlation between neutrophil numbers and MPO levels was observed, indicating neutrophil activation. Inhibition of DNA damage by both coatings was paralleled by a reduction of neutrophil influx as well as MPO activity. In this study we provide evidence that modification of the particle surface prevents DNA strand breakage in epithelial lung cells from quartz-exposed rats. Furthermore, the present data show the feasibility of our *in vivo* model to evaluate the role of inflammation, antioxidant status, and cytotoxicity in particle-induced DNA damage.

Abbreviations: AL, aluminium lactate; AP, alkaline phosphatase; BAL, brochoalveolar lavage; LDH, lactate dehydrogenase; MPO, myeloperoxidase; PVNO, polyvinylpyridine-*N*-oxide; TEAC, trolox equivalent antioxidant capacity.

Introduction

Chronic exposure to quartz (crystalline silica) has been shown to result in tumor formation in the peripheral lung of the rat (1–5). Together with the observation that occupational exposure to quartz is associated with an elevated lung cancer risk in humans this has led to the classification of quartz as a Group 1 carcinogen by the International Agency for Research on Cancer (6). However, at present the exact mechanisms involved in quartz-induced carcinogenicity are still incompletely understood (7).

It is generally accepted that phagocytic cells, and more specifically reactive oxygen species (ROS) released by these cells, are involved in the link between inflammation and cancer (8,9). For particle-induced carcinogenesis for instance, it has been demonstrated that tumor formation in rats was paralleled by the degree of chronic neutrophilic inflammation (10). In considering the possible mechanism of quartz-induced carcinogenicity it is important to note that carcinogenesis is a complex outcome of several consecutive events leading to the formation of a tumor (11). Although *in vivo* mutagenic effects have been demonstrated after quartz exposure (12,13), only few studies focused on more upstream processes, such as acute *in vivo* DNA damage, which is a prerequisite for mutagenicity. Yamano *et al.* found an increased level of the oxidative DNA lesion 8-hydroxydeoxyguanosine (8-OHdG) in whole lung tissue from rats exposed to quartz (14), whereas others demonstrated the induction of 8-OHdG more specifically in the alveolar region (15,16). These studies showed that 8-OHdG induction was associated with a parallel influx of neutrophils into the lung. A possible role of neutrophils has been confirmed by our recent work, showing that isolated neutrophils were able to induce 8-OHdG in lung type II epithelial cells *in vitro* (17).

When considering quartz-induced DNA damage and its possible role in carcinogenicity it should be emphasized that surface reactivity is one of the key factors contributing to the pathogenicity associated with quartz inhalation (7,18,19). For instance, in the past decades various *in vivo* studies demonstrated that quartz-induced silicosis could be inhibited by the administration of compounds such as aluminium salts and polyvinylpyridine-*N*-oxide (PVNO), which are known to modify the surface reactivity of the quartz particles (20–24). In general, the reactivity of the particle surface is closely related to the ability of quartz to generate ROS (19,25,26). Since ROS are implicated in both DNA damage and carcinogenesis (27) we hypothesized that the particle surface is also involved in DNA damage caused by quartz. Indeed, using *in vitro* studies we recently demonstrated that intrinsic ROS generation of quartz was closely related to DNA strand break formation in lung epithelial cells (28). More specifically, we and others showed that quartz-induced genotoxic effects could be reduced by inactivation of its reactive particle surface using surface modifying agents (28,29). However, the implications of such primary genotoxic effects of quartz for lung target cells *in vivo* are still open for research and currently, excessive and persistent

formation of ROS by inflammatory cells is thought to be a key factor in quartz-related genotoxic effects (4,6,10,30). Also in this mechanism of secondary genotoxicity, surface reactivity of quartz might play an important role. For instance, it was demonstrated that the ability of inflammatory cells to release ROS upon quartz exposure *in vitro* could be mediated by using various surface modifying methods such as grinding or coating (31,32). Additionally, we and others found that the influx of inflammatory cells into the rat lung was reduced when quartz was treated with surface modifying agents such as aluminium lactate or PVNO (26,33,34).

Although these studies indicate that the reactivity of the quartz particle surface is implicated in several processes possibly involved in quartz-induced genotoxicity, the role of the particle surface in quartz-induced DNA damage *in vivo* has not yet been investigated. The aim of the present study was to investigate whether quartz (DQ12) could induce acute DNA damage in lung target cells *in vivo* and to elucidate whether this process could be mediated by surface modification of the quartz particles. More specifically, we hypothesized that surface modification of quartz would prevent DNA damage in epithelial lung cells via an inhibition of inflammatory cell influx into the lung. Therefore, rats were exposed to native quartz or quartz treated with either PVNO or aluminium lactate (AL). After three days DNA strand breakage was assessed in epithelial target cells freshly isolated from the lung. Additionally, to characterize the role of inflammatory cells, epithelial DNA damage was related to the presence and activation status of macrophages and neutrophils in the lung.

Materials and methods

Chemicals

2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulphonate) (ABTS), dimethyl sulphoxide (DMSO), Dulbecco's modified Eagle Medium (DMEM), ethidium bromide, L-glutamine, Hank's balanced salt solution (HBSS), Ham's F12 medium, HEPES buffer, fetal calf serum (FCS), lucigenin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), penicillin/streptavidin solution, percoll, phosphate buffered saline (PBS), trypsin and Trypsin/EDTA solution, were all obtained from Sigma (St Louis, MO). Agarose, low melting point agarose, horseradish peroxidase (HRPO), guaiacol, phorbol-12-myristate-13-acetate (PMA) were also purchased from Sigma. ABAP (2,2′-azobis-(2-amidinopropane)HCl was from Polysciences, Warrington, USA. DNAse I was purchased from Roche, Mannheim, Germany. All other chemicals were from Merck (Germany) and were of highest purity.

Surface treatment of quartz

Quartz (DQ12, batch 6, IUF, Düsseldorf) was baked at 215°C for 14 h to inactivate endotoxin. Surface modification was performed by suspending the baked DQ12 at 5 mg/ml in a 1% dilution of either PVNO (23) or AL (Sigma-Aldrich, Germany), dissolved in distilled deionized sterile water, based on protocols originally described by Gabor *et al.*, (35) and Bégin *et al.*, (33), with minor modifications. Uncoated DQ12 was suspended in water without any additions. Preparations were sonicated for 5 min (Sonorex TK52 waterbath; 60 W, 35 kHz) and subsequently agitated for 5.5 h at room temperature, spun at 1000 *g*, and washed three times with distilled sterile water by centrifugation (1000 *g*). The washed particles were finally resuspended in sterile water at a concentration of 5 mg/ml, and aliquots of 1 ml in sterile glass tubes were allowed to evaporate under a laminar flow chamber. All quartz processing was performed under sterile conditions. A single batch of quartz and coated quartz was prepared and used for the whole study to avoid effects induced by a possible variable coating efficiency. Transmission electron microscopy was used to determine whether treatment with aluminium lactate or PVNO caused changes in particle size distribution or aggregation of the DQ12, but no differences were found (data submitted elsewhere). The efficiency of the AL lactate coating procedure, which is defined as the amount of adsorbed aluminium per mg quartz, was investigated by atomic absorption spectrometry analysis following 3 h treatment of the coated quartz in 1 M HNO_3 at 80°C. The coating efficiency of PVNO was measured by spectrophotometric determination (260 nm) of the desorbed PVNO upon 3 h treatment in 1 M NaOH at room temperature against a PVNO standard. The estimated adsorbed amounts on the quartz were found to be 11 μg PVNO/mg quartz and 1.6 μg aluminium/mg quartz. For intratracheal instillation, the dried quartz preparations were resuspended in 1 ml of PBS (without Mg^{++} and Ca^{++}) and sonicated in a sonicating waterbath (Sonorex TK52; 60 W, 35 kHz, 5 min). For *in vitro* experiments, the dried dusts were resuspended in HBSS or Ham's F12 medium.

Lucigenin-enhanced chemiluminescence

Human neutrophils were exposed to native DQ12 quartz or DQ12 treated with PVNO or AL and superoxide release was assessed using lucigenin-enhanced chemiluminescence. Therefore, human peripheral blood neutrophils were isolated as described previously (36) and suspended in HBSS at 2×10^6 cells/ml. From this suspension, 50 μl was added to a white maxisorp 96-well plate (Nunc, Germany) and mixed with 50 μl of a quartz suspension (1 or 0.2 mg/ml). Finally 100 μl lucigenin (5×10^{-4} M) was added. PMA (100 ng/ml), was used as a positive control. Lucigenin-enhanced chemiluminescence was then recorded using a luminometer (Multi-Bioluminat, Berthold, Germany), at 37°C. Chemiluminescence output was recorded for 40 min and was expressed as area under the curve. All stimulatory agents and appropriate controls were tested in parallel and the experiment was repeated three times.

In vitro cytotoxicity in RLE cells using MTT-assay

Immortalized rat lung epithelial type II cells (RLE) (37) were kindly provided by Dr K.Driscoll. Cells were cultured in Ham's F12 medium supplemented with 1% 1 M HEPES buffer, 1% penicillin/streptomycin solution (Sigma), 1% L-glutamine solution (200 mM), and 5% heat-inactivated foetal calf serum (FCS) at 37°C and 5% CO_2 (17). Cells were routinely grown in 75 cm^2 cell culture flasks and passaged twice a week. Experiments were performed between passage numbers 40–50. Cytotoxicity of both native and coated quartz was evaluated using the MTT-colorimetric assay according to Mosmann *et al.* (38), which was described previously (36). Briefly, cells were seeded in 96 well plates at 20 000 cells/200 μl in each well. At confluency, quartz preparations were suspended in F12 medium (100 μl) without FCS and penicillin/streptomicin, added to the cells and incubated (37°C, 5% CO_2). After 4 h, 25 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium (MTT, 2 mg/ml) dissolved in PBS, was added and cells were incubated for another 3 h. Finally, solutions were removed, formazan crystals were dissolved in DMSO (200 μl) and absorption was measured using a microplate reader (Labsystems) at 540 nm. Toxicity was calculated as % of control from three individual experiments.

Quartz instillation and bronchoalveolar lavage

Specific pathogen free female Wistar rats were used for the investigations (Janvier, Le Genest St Isle, France). Rats were maintained under controlled environmental conditions with a 12 h dark/light cycle. Food and water were available ad libitum. When 8 weeks old (weighing 200–250 g), animals were lightly anaesthetized with isoflurane and intratracheal instillation was performed using a laryngoscope. From the quartz or coated-quartz suspensions (5 mg/ml in PBS) 400 μl was instilled giving a final dose of 2 mg per rat ($n = 5$ per treatment). Control rats were instilled with only PBS. After 3 days animals were killed by a single i.p. injection of Na-pentobarbital and subsequent exsanguination via the abdominal aorta. Then, the lung was cannulated via the trachea and bronchoalveolar lavage (BAL) was performed. Epithelial cells were isolated from the same lung as described below. BAL was performed *in situ* by infusing the lungs with 5 ml aliquots of PBS. The BAL fluid (BALf) was drained passively by gravity and the procedure was repeated four times, giving a total BAL volume of 20 ml. Total cell number in the BALf was analyzed using a hemocytometer chamber (Neubauer) and viability was assessed by trypan blue dye exclusion. BAL-cell differential was determined on cytospin preparations stained with May-Grünwald/Giemsa (MGG). The BALf was centrifuged twice (300 *g* to collect cells, followed by 1000 *g* to obtain BALf), and the acellular supernatant was analyzed for lung injury parameters (total protein, alkaline phosphatase, lactate dehydrogenase), and myeloperoxidase (MPO).

Measurement of BAL parameters

Total protein was analyzed according to the method described by Lowry. Alkaline phosphatase and lactate dehydrogenase were assayed using diagnostic kits from Merck (Germany). MPO activity in the BALf was assayed according to Klebanoff *et al.* (39). Briefly, 200 μl of unconcentrated cell-free BALf was mixed with 800 μl MPO assay solution. This was prepared freshly before use, containing 107.6 ml H_2O, 12 ml 0.1 M sodium phosphate buffer, 0.192 ml Guaiacol, 0.4 ml 0.1 M H_2O_2. The generation of tetra-guaiacol was measured spectrophotometrically (Beckman) at 470 nm and the change of optical density per minute was calculated from the initial rate. The MPO activity was then calculated from the formula: U/ml = ΔOD/min × 0.752 and expressed as mU/ml. One unit of the enzyme is defined as the amount that consumes 1 μmol H_2O_2 per minute.

Trolox equivalent antioxidant capacity assay

The TEAC (trolox equivalent antioxidant capacity) assay was performed according to Van den Berg *et al.* (40) with minor modifications. An ABTS radical solution was prepared by mixing 2.5 mM ABAP with 20 mM ABTS solution in 150 mM phosphate buffer (pH 7.4) containing 150 mM NaCl. The solution was heated for 10 min at 70°C and, if necessary, diluted to obtain a solution with an absorbance at 734 nm between 0.68 and 0.72. For measuring antioxidant capacity 100 µl of the cell-free BALf was mixed with 900 µl of the ABTS radical solution. Both native BALf and deproteinized fluid (10% TCA) were tested. The decrease in absorbance at 734 nm 5 minutes after addition of the sample was used for calculating the TEAC. Trolox was used as reference compound. The TEAC of the sample is given as the concentration of a trolox solution that gives a similar reduction of the absorbance at 734 nm.

Epithelial cell isolation

Lung epithelial cells were isolated according to the method developed by Richards *et al.* (41) with modifications. After BAL, lungs were removed from the rat and pre-washed with 5 ml trypsin via the cannula (37°C, 2.5 mg/ml dissolved in PBS with Ca^{++} and Mg^{++}). The cannulated lung was subsequently attached to a syringe in a retort stand and filled with trypsin solution. The lungs were left for 30 min at 37°C, during which the level of trypsin is constantly 'topped up'. After trypsination the lungs were transferred into a sterile petri dish and the trachea and the main bronchi were dissected free. The remaining lung parenchymal tissue was chopped with scissors into small pieces (~1 mm) and FCS (5 ml) was added to inactivate the trypsin. In all subsequent steps lung preparations and solutions were kept at 4°C to avoid post-experimental DNA repair. The volume of the lung homogenates was adjusted to 20 ml with PBS and DNAse I (500 µl of 4 mg/ml solution) was added followed by shaking (5 min) to prevent cell-clotting. The lung tissue homogenate was subsequently filtered through gauze followed by filtration through nylon filters (150 and 30 µm respectively), and the final filtrate (adjusted to 20 ml with PBS) was layered onto a discontinued Percol gradient of heavy density (1 ml 10× concentrated PBS, 2.5 ml distilled water, 6.5 ml Percoll – 1.13 g/ml) and light density (1 ml 10× concentrated PBS, 6.3 ml distilled water, 2.7 ml Percoll). Preparations were centrifuged for 20 min at 300 *g* (4°C). After separation, the layer containing the epithelial cells (4th from top) was carefully removed, washed with PBS and resuspended in DMEM medium supplemented with 10% FCS, 1% penicillin/streptavidin solution (Sigma) and 1% L-glutamine (200 mM). The cells were then plated into 100 mm Petri dishes to further enrich the epithelial cell fraction by allowing contaminating fibroblasts, macrophages and neutrophils to attach to the surface. After 1 h incubation at 37°C and 5% CO_2, the supernatant was removed and centrifuged (300 *g*) to collect non-attached epithelial cells. Centrifuged cells were resuspended in 500 µl DMEM (cold) and were counted using a Neubauer chamber, while cell viability was determined by trypan blue dye exclusion.

Characterization of isolated epithelial cells

The percentage of epithelial cells present in the isolated fractions was determined using three different methods. First, the proportion of epithelial cells, alveolar macrophages, neutrophils and possible other cells was investigated using MGG staining of cytospin preparations and subsequent differential counting by an experienced observer. Secondly, identification of type II epithelial cells specifically was done by staining for alkaline phosphatase activity, as described by Bingle *et al.* (42). Therefore, cytospin preparations were dried on air and then stained with naphthol (10 mg, dissolved in 40 µl DMSO), added to 0.125 M 2-amino-2-methyl-1-propanol buffer (pH 9–10). Directly before use 10 mg fast red was added and cells were incubated for 15 min at room temperature to the filtered solution (0.4 µm). Slides where washed with distilled water, cells were counterstained with haematoxylin and type II cells were counted using light microscopy. However, definite characterization of the different isolated epithelial cells is only possible using electron microscopy. Therefore, epithelial cells from a PBS-instilled animal were isolated as described previously and centrifuged cell pellets were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 1 h (4°C). After washing with 0.1 M cacodylate buffer and postfixation with 2% OsO_4, cell preparations were encapsulated in agar (2% in PBS). The ice cooled agar blocks were cut in cubes and dehydrated in graded ethanol series. Poststaining en bloc was accomplished with 1.5% uranylacetate and phosphoric tungsten in 70% ethanol. Absolute ethanol was replaced with propylene oxide and the agar cubes were embedded in epon (Serva, Heidelberg) (43), thin-sectioned (Reichert, Ultracut) and examined using a transmission electron microscope (TEM, Philips, CM12) equipped with a digital imaging system (SIS, Münster, Germany).

Single cell gel electrophoresis (comet assay)

DNA strand breakage in epithelial cell preparations was assayed immediately after cell-isolation by the comet assay (44,45), according to the guidelines recently proposed by an expert panel (46). Fully frosted slides were covered with a layer of 100 µl 0.65% agarose using a coverslip and stored overnight at 4°C. Epithelial lung cells were isolated as described above and 25 µl cell suspension (2×10^6 cells/ml) was mixed with 75 µl 0.5% low melting point agarose, and added to the slides, on top of the first agarose layer using a coverglass. Slides were stored at least 45 min at 4°C to allow solidification, and then covered with another layer of the low melting point agarose (100 µl). Following solidification for another 45 min at 4°C, coverglasses were removed, slides were immersed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-base, 1% sodium lauryl sarcosinate, pH 10; 10% DMSO and 1% Triton X-100 added just before use) and stored overnight at 4°C. The following day, slides were rinsed with distilled water and placed in an electrophoresis tank filled with ice-cold electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 13) for 30 min. Electrophoresis was conducted at 300 mA and 25 V for 15 min. Slides were neutralized 3 × 10 min using neutralization buffer (0.4 M Tris, pH 7.5). All steps after cell lysis were performed in the dark or under dimmed red light to prevent additional DNA damage. Finally, slides were stained with ethidium bromide (20 µg/ml in H_2O) and comet appearances were analyzed using an Olympus BX60 fluorescence microscope at 1000× magnification. For each individual animal, three slides were prepared and on every single slide 50 cells were analyzed randomly, and classified into one out of five categories according to tail length (I, II, III, IV, and V, in which I = undamaged cells without comet tail) (47). For final analysis the percentage of cells exhibiting comet tails (class II–V cells) was used.

Statistical analysis

Data are expressed as mean ± SEM, unless stated otherwise. Statistical analysis was performed using SPSS v. 10 for Windows. Student *t*-test was applied to test differences between exposed and unexposed groups. Correlations between DNA damage and neutrophilic inflammation or lung toxicity markers were tested using Pearson. Differences were considered to be statistically significant when $P < 0.05$.

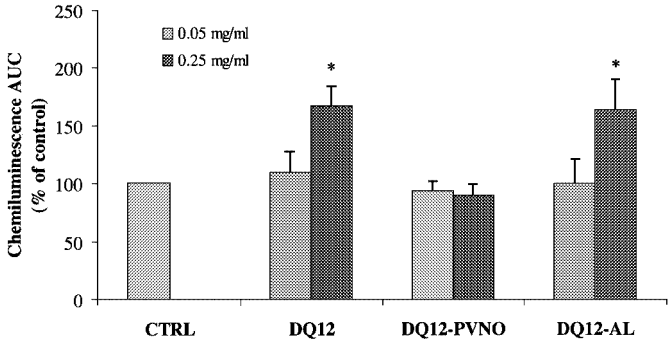

Fig. 1. Lucigenin-enhanced chemiluminescence of human blood neutrophils exposed to DQ12 or DQ12 coated with PVNO or AL. Chemiluminescence was recorded for 40 min and expressed as area under the curve (AUC). Data are shown as mean ± SD of three different experiments. *$P < 0.01$ vs. control (= CTRL).

Results

In vitro studies with native and coated quartz

Before starting with the animal studies we tested the efficacy of the surface treated quartzes in several *in vitro* systems. Isolated human neutrophils were exposed to quartz suspensions and lucigenin-enhanced chemiluminescence was applied to detect superoxide release ($O_2^{\bullet}$). Data are shown in Figure 1. Generation of superoxide was enhanced by DQ12. At 0.25 mg/ml DQ12 quartz, chemiluminescence was increased by ~170% compared with control cells. This effect was inhibited when DQ12 was coated with PVNO, but not with AL. When PMA was used as a positive control, $O_2^{\bullet}$ release was increased by 230% (not shown). The effect of both surface coatings on cytotoxicity induced by quartz was tested using the MTT assay. RLE cells were exposed for 4 h to DQ12 or coated quartz preparations at 0–400 µg/cm^2. As shown in Figure 2, native quartz dose dependently reduced cell viability,

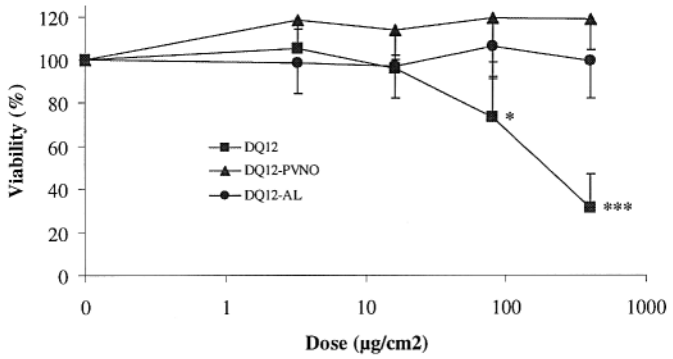

Fig. 2. Viability of RLE cells exposed to native or coated DQ12. Viability was assessed using the MTT-assay and was expressed as % of control. Data shown represent mean ± SD from three experiments. $*P < 0.05$, $***P < 0.001$ vs. control (0 µg/cm^2).

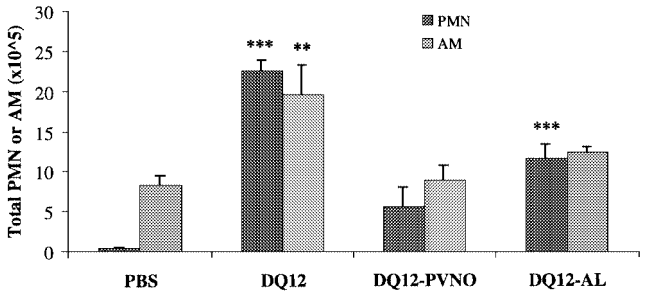

Fig. 3. Total numbers of neutrophils (PMN) and macrophages (AM) in brochoalveolar lavage of rats 3 days after intratracheal instillation of 2 mg of DQ12 or DQ12 coated with AL or PVNO. Data are presented as mean ± SEM ($n = 5$). $***P < 0.001$, $**P < 0.01$ vs. PBS.

whereas this toxicity was significantly inhibited upon treatment of DQ12 with AL or PVNO.

Inflammogenic and cytotoxic effects detected in broncho-alveolar lavage

Bronchoalveolar lavage was used to assess both inflammogenic and toxic effects after quartz exposure. Separate animals were also treated with 22 µg PVNO and 35 µg aluminium lactate, amounts calculated from the coating efficiency of both substances. However, these treatments did not have an effect on any of the studied BAL parameters (34). A significant increase of neutrophil numbers was observed after quartz exposure ($P < 0.001$ vs. control). When DQ12 was coated with PVNO the neutrophil influx was inhibited. Treatment with AL also reduced neutrophil influx, although this effect was less pronounced than in PVNO-coated DQ12 (Figure 3). The number of alveolar macrophages was increased only after instillation with native quartz ($P < 0.05$ vs. control). Further data of relevant BAL parameters are outlined in Table I. To evaluate possible toxic effects of quartz exposure, total protein, alkaline phosphatase and lactate dehydrogenase were analyzed in the BALf. Total protein was only increased by native quartz, whereas LDH levels were significantly increased after exposure to both quartz or AL-treated quartz. No differences were observed for alkaline phosphatase activity in BALf. MPO used as a specific marker of neutrophil activation, was only increased by DQ12. Although neutrophil influx by DQ12-AL was significantly higher than in PBS treated animals (Figure 3), no significant induction of MPO activity levels was seen in these animals. However, on a single animal level, covering all treatments, a highly significant correlation is present between neutrophil numbers and MPO activity ($r = 0.88$, $P < 0.001$) (Figure 4). Interestingly, when MPO activity was adjusted on neutrophil numbers present in the BALf, the activity of MPO per neutrophil was about 8.5 times lower after DQ12-AL compared with native DQ12 (resp. 93.6 ± 72.3 and 796.5 ± 772.0, $P < 0.01$).

Total antioxidant capacity in brochoalveolar lavage

Trolox equivalent antioxidant capacity assay (TEAC-assay) was used to evaluate changes in the total antioxidant capacity in the BALf. Data are shown in Figure 5. Compared with control animals instilled with PBS, an increased antioxidant capacity was observed after all quartz treatments, although effects of quartz treated with either PVNO or AL were less pronounced. No changes in antioxidant capacity was detected when the BALf was deproteinized prior to use in the TEAC assay (levels were close to detection limit). TEAC in the native, unprocessed BALf from all treatments correlated with total number of neutrophils ($r = 0.81$, $P < 0.001$), with macrophages present in the BALf ($r = 0.65$, $P < 0.01$), with total protein levels ($r = 0.75$, $P < 0.001$) and MPO activity ($r = 0.74$, $P < 0.001$).

Characterization of epithelial cell isolates

After BAL, lungs were used to isolate epithelial cells. Yield of epithelial cell isolates is shown in Table II. Using MGG-staining total differential counting was performed. The percentage of recovered epithelial cells appeared to be slightly lower for DQ12 treated rats compared with the other treatments, however this difference was not statistically significant. Mean percentage of type II pneumocytes was 38.6 (± 6.5) in control rats, and this was reduced in isolates from rats exposed to either native DQ12 or coated DQ12. In Figure 6 representative electron microscopic pictures of different cells found in the isolates are shown. Differential countings in this preparation indicated that at least 65% of the cells appeared to be of an epithelial origin (13% type II cells, 52% bronchial epithelial cells), which is in line with data obtained after analysis of the MGG-stainings. Other cells appeared to be mainly macrophages.

DNA damage in lung epithelial cell isolates

Single cell gel electrophoresis was used to detect DNA strand break formation in the epithelial cell isolates. Data are shown in Figure 7. Quartz exposure caused a significant increase in DNA strand breakage in the isolated cells. Importantly, when quartz was treated with PVNO or AL no enhanced levels of DNA damage compared to PBS-treated rats were observed (Figure 7A). In Figure 7B the distribution of cells over the various comet classes (II to V) is shown. The increased level of DNA damage by native quartz appears to be present in all comet classes. However, no differences were found in class V, representing the highest damaged cells. No relation was observed between DNA strand breakage and cell isolation characteristics such as the percentage of epithelial cells, type II cells, or neutrophils present in isolates. Viability analysis of the isolated cells, using trypan blue dye exclusion, showed that viability of cells used in the comet assay was always >95%, and no differences between exposure groups were observed (data not shown). No correlation was found between *in vivo* DNA damage and general lung toxicity reflected in total protein levels, alkaline phosphatase or lactate dehydrogenese activity in BALf. Moreover, no relation between antioxidant capacity (TEAC) and DNA strand breakage was found on a

Table I. Inflammatory and cytotoxicity markers analysed in BAL 3 days after intratracheal instillation of DQ12 or DQ12 coated with PVNO or AL (mean ± SD, $n = 5$)

	PBS	DQ12	DQ12-PVNO	DQ12-AL
Total cells ($\times 10^5$)	8.9 (2.5)	48.0 (9.2)***	16.1 (12.2)	27.72 (4.9)**
Neutrophils (%)	3.3 (4.9)	47.6 (8.7)***	31.0 (10.0)***	41.3 (11.7)***
Macrophages (%)	93.0 (7.42)	38.9 (11.34)***	60.8 (14.9)**	44.2 (3.4)***
Total protein (μg/ml)	13.2 (3.3)	46.9 (21.4)*	32.7 (37.1)	30.6 (18.2)
Alkaline phosphatase (U/ml)	9.4 (2.5)	12.3 (2.7)	9.7 (2.6)	12.2 (2.9)
LDH (U/ml)	25.7 (7.8)	120.4 (38.1)***	34.0 (7.2)	59.8 (11.5)*
MPO (mU/ml)	0.027 (0.02)	99.3 (100.6)**	0.54 (0.59)	5.3 (4.1)
MPO/neutrophils (mU/1 $\times$ 10^6 neutrophils)	23.2 (35.0)	796.5 (772.0)**	23.3 (30.5)	93.6 (72.3)

LDH: lactate dehydrogenase; MPO: myeloperoxidase.
***$P < 0.001$, **$P > 0.01$, *$P < 0.05$ vs. PBS.

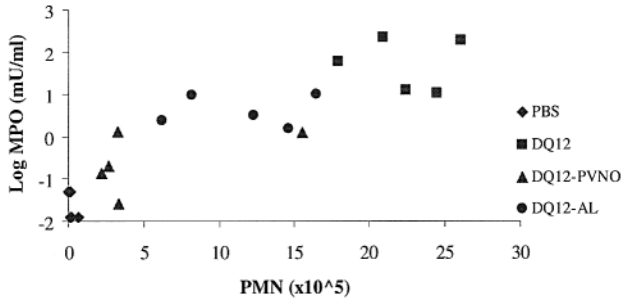

Fig. 4. Correlation between total neutrophil numbers (PMN) and MPO in BALf from rats 3 days after intratracheal instillation of 2 mg DQ12 or DQ12 coated with AL or PVNO. Each point represents a single animal. Pearson correlation = 0.88, $P < 0.001$.

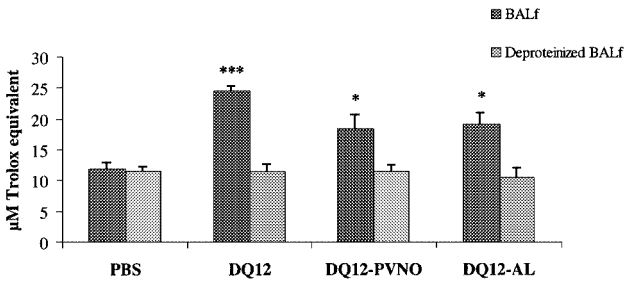

Fig. 5. Trolox equivalent antioxidant capacity (TEAC) in BALf from rats 3 days after intratracheal instillation of DQ12 or DQ12 coated with AL or PVNO. TEAC was measured both in native and deproteinized BALf. Data are presented as mean ± SEM ($n = 5$). ***$P < 0.001$, *$P < 0.05$ vs. PBS.

single animal level, pooling all treatments. Since one of our objectives was to investigate the role of neutrophils in epithelial cell genotoxicity after quartz exposure, we related DNA damage (comet) to both neutrophil numbers and neutrophil activity, reflected in MPO levels in BALf. This relation is given in respectively Figure 8A and 8B, and shows that only at high neutrophil numbers (2×10^6, corresponding with 48% of total BAL cells) or high MPO levels, both corresponding to treatment with native DQ12, epithelial DNA damage was increased. However, no correlation was found between either neutrophil influx or MPO levels and epithelial DNA damage in individual animals, both within different treatment groups ($n = 5$) and in all animals ($n = 20$).

Discussion

Quartz (crystalline silica) has recently been classified as a human lung carcinogen (6). However, the quartz hazard (e.g. its ability to induce cancer) is by no means a constant entity, and its pathological effects may vary depending on its sources or inherent characteristics (7,18,48). One important variable characteristic appears to be the surface reactivity of quartz. The role of particle surface reactivity in quartz-induced cytotoxicity, inflammogenicity and fibrogenicity has previously been demonstrated using various surface modifying procedures, such as coating with PVNO or aluminium salts (18,20,26,31,33–35, 49–51). In early studies, PVNO and AL were administered at high doses to prevent or treat quartz-induced silicosis (21–24). In the present study however, both compounds were used to coat the quartz particles in order to specifically investigate the role of reactive surface functionalities in quartz-induced genotoxicity. We demonstrated that intratracheally instilled quartz (DQ12, 2 mg) causes acute induction of DNA strand breakage in lung epithelial cell isolates *in vivo*. However, this effect was not observed when quartz was treated with either PVNO or AL, suggesting an important role of the reactive particle surface in quartz-induced *in vivo* DNA damage. Since both surface modifications inhibited inflammatory cell influx as well DNA strand breakage, our data provide further support for the possible role of inflammatory cells in quartz-induced genotoxicity (4,6,30).

In quartz-induced carcinogenesis in rats, oxidant release by lung phagocytes, and more specifically neutrophils, is reported to be an important factor (4,10). In the present study we therefore analyzed the role of the reactive quartz surface on inflammatory cell influx as a possible mediator of quartz-

Table II. Differential of epithelial-cell isolates from rat lungs after exposure for 3 days to native DQ12 or DQ12 coated with PVNO or AL. (mean ± SD, $n = 5$)

	PBS	DQ	DQ-PVNO	DQ-AL
Yield (total cells $\times$ 10^5)	34.3 (3.5)	68.5 (20.4)*	39.4 (15.4)	67.5 (28.3)*
% Epithelial cells (MGG)	75.0 (6.4)	62.5 (6.6)	68.3 (14.9)	73.8 (9.4)
% Type II cells (alkaline phosphatase)	38.6 (6.5)	20.7 (14.7)*	22.3 (8.5)*	19.2 (9.0)*
% Neutrophils (MGG)	4.4 (1.9)	17.7 (4.0)**	11.6 (9.0)	11.3 (9.0)
% Macrophages (MGG)	19.6 (6.1)	19.1 (7.1)	18.0 (6.6)	13.2 (2.0)

MGG: staining, evaluated with light microscopy.
**$P > 0.01$, *$P < 0.05$ vs. PBS

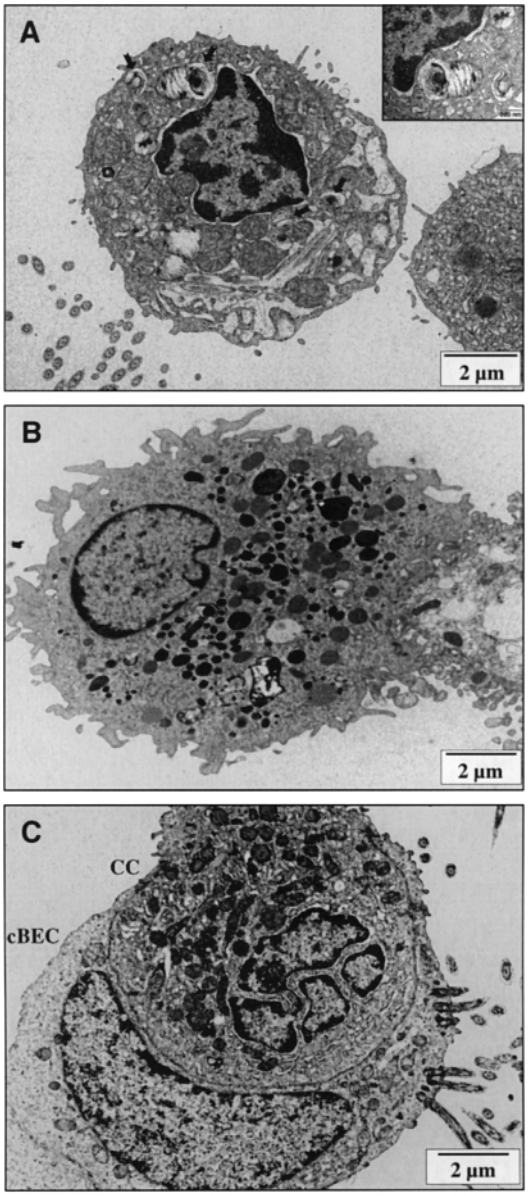

Fig. 6. Representative pictures from electron microscopic analysis of cells found in epithelial cell isolates from rats. (**A**) Type II alveolar epithelial cell. Arrows and insert indicate lamellar bodies. (**B**) Alveolar macrophage. (**C**) Bronchial epithelial cells, two different cells are shown: cBEC, ciliated bronchial epithelial cell; CC, Clara cell.

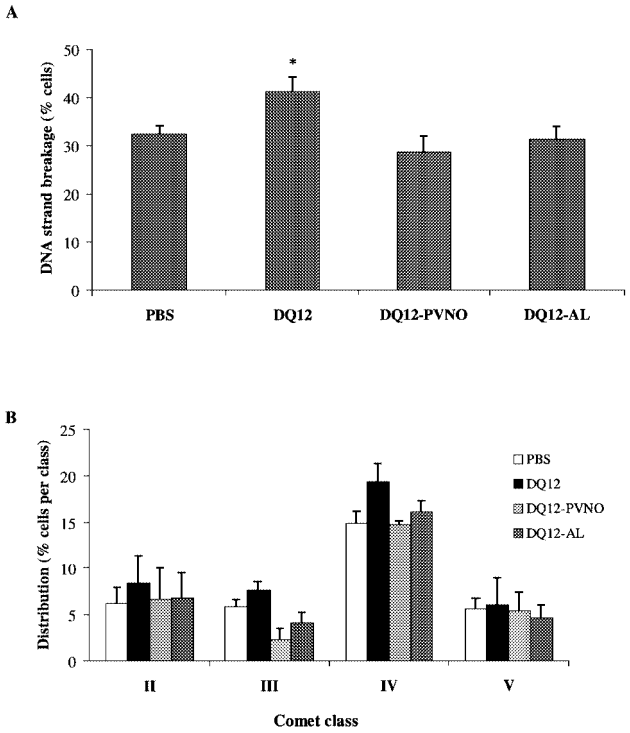

Fig. 7. DNA strand breakage in epithelial cell isolates from rat lungs after intratracheal instillation of DQ12 or DQ12 coated with PVNO or AL. DNA strand break formation was assessed using the comet assay and was expressed as % of cells showing comet tails (**A**). In figure (**B**) the distribution of the damaged cells over the various comet classes (II–IV) is shown. Data are mean ± SEM ($n = 5$). *$P < 0.05$ vs. PBS.

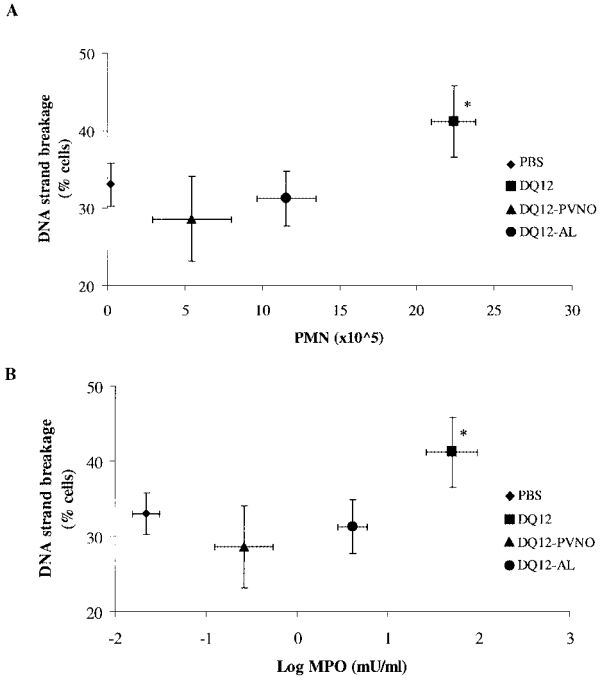

Fig. 8. Relation between (**A**) neutrophil influx (PMN) or (**B**) MPO activity in BALf and DNA strand breakage in epithelial cell isolates from rats 3 days after instillation of DQ12 or DQ12 coated with PVNO or AL. Data are presented as mean ± SEM ($n = 5$). *$P < 0.05$ vs. PBS.

induced DNA damage. We clearly showed that neutrophil influx, induced by quartz was inhibited by both PVNO and, to a lesser extent, also by AL. The inhibitory effects of both coating materials on inflammatory cell influx are in line with observations in earlier studies using aluminium lactate (26,33), and confirm the important role of the reactive particle surface in quartz-induced inflammation. In addition to neutrophil presence we also assessed neutrophil activation by measuring the enzyme myeloperoxidase in BALf, since MPO is a marker of neutrophil activity (52–54). Our data suggest that the quartz-induced influx of neutrophils into the lung is accompanied by a subsequent activation. Both neutrophil influx and MPO activity were inhibited by the coatings, although AL was less effective than PVNO. Interestingly, MPO activity expressed per neutrophil number seemed to be 8-fold higher in DQ12-treated rats than in rats instilled with DQ12-AL. This suggests a possible difference in the 'activation status' of neutrophils between the different treatments. Schmekel *et al.* (52) previously demonstrated that MPO measured in BALf is of local

origin, rather than passively diffused from the blood. However, the increased levels of total protein and LDH activity in BALf from native DQ12-treated rats could imply a loss of membrane integrity, possibly leading to an influx of MPO from the capillary bed.

In addition to MPO release, activation of neutrophils is further characterized by an oxidative burst (53). In line with data from Hedenborg and Klockars (55), we showed that *in vitro* superoxide release by human neutrophils was abrogated when quartz was coated with PVNO. However, AL treatment of quartz did not inhibit superoxide release. These *in vitro* data led us to suggest that we had a feasible model to dissect between primary and inflammatory cell-mediated secondary genotoxicity after *in vivo* quartz exposure, since both coatings had a distinct effect on the neutrophils' oxidative burst. However, *in vivo* the differences between the coatings on inflammatory processes were less pronounced than anticipated, as they both inhibited inflammatory cell influx and activation. It should be emphasised that the interaction of the two coating materials with the quartz surface is very different. PVNO is a polymer which is considered to interact with the quartz surface via H-bonding of its NO groups with silanol groups (18,50,56). On the other hand the mechanism of action of AL is not fully clarified, although aluminium appears to affect the acidity and the solubility of the quartz, and has also been reported to hinder the formation of surface radicals and to block charges caused by grinding (18,20,56). The inhibitory effect of the PVNO coating on *in vitro* ROS release by neutrophils is unlikely to be caused by its proposed antioxidant activity (57), as the adsorbed PVNO content was only 11 μg per mg quartz. Even if released from the particle surface, this amount was found not to have any detectable antioxidant activity as determined by electron spin resonance techniques (data not shown).

DNA damage in the epithelial cell isolates was evaluated using the sensitive comet assay. We found that DQ12 caused an increase in DNA strand break formation in lung epithelial cells. Interestingly however, no induction was observed when quartz was treated with either PVNO or AL, suggesting that, as for inflammogenic effects also DNA damage induced by quartz is related to its surface reactivity. Although both coatings have different capacities to inhibit inflammation, our data show that they have more or less similar capacity to reduce quartz-induced acute genotoxic effects in the rat. Also here, a direct antioxidant action of PVNO (57), and thus a possible effect on ROS-induced DNA strand breakage is negligible, as the absolute dose of PVNO is only 22 μg (~0.09 mg/kg body weight). For comparison, a therapeutic, silicosis-inhibiting effect of PVNO is only reported at seven repeated subcutaneous doses of 40 mg/kg body weight (58).

No relation was found between DNA damage and isolated cell differentials, suggesting that differences in DNA damage by quartz and coated quartz are not caused by slight changes in the cell populations analyzed. The background level of DNA damage in the isolated cells appeared to be relatively high, since at least 30% of the isolated cells were found to have comet tails. Background levels of DNA strand breakage in isolated type II epithelial cells were shown to be five times higher than for alveolar macrophages, which appeared not to be caused by trauma induced during isolation procedures (59). Our own data on viability (trypan blue) of the isolated cells did not show any difference between the treatments as this was always >95%. This was further confirmed by the observation that the percentage of comet class V cells (Figure 7B), possibly representing dead cells with extremely fragmented DNA ('clouds') (60), was only ~5% and did not differ between the treatments. Therefore, our results do not reflect DNA strand breakage that occurs secondary as a consequence of cytotoxicity (46). Moreover, no relation was found between DNA damage and BALf markers of lung toxicity (LDH, AP or total protein), implicating that routine lung toxicity markers are not associated with DNA damage in epithelial lung cells in the acute phase of quartz exposure. Additionally, the absence of increased levels of alkaline phosphatase, an enzyme specifically present in type II epithelial cells, suggests that no *in vivo* cytotoxicity is present in this specific target cell. In terms of carcinogenicity these observations are rather important, because initial DNA damage will not evolve into mutagenesis when cytotoxicity prevents the target cells carrying the DNA lesion to proliferate.

Our data on DNA strand breakage are in agreement with earlier observations on the formation of 8-OHdG in the rat lung after quartz exposure (14–16). In these studies, DNA damage was evaluated in total lung tissue homogenates or using immunohistochemical techniques. However, evaluation of the role of DNA damage in particle-induced carcinogenicity is ideally done in cells relevant for neoplastic outcomes (1,30,61). Therefore, in the present study epithelial cells were isolated from the lung prior to quantification of DNA damage. Using this approach we were unable to apply quantitative analysis of 8-OHdG levels as the yield of the cell isolates did not allow analysis using HPLC-ECD. Therefore, we concentrated on detection of DNA strand breakage in single cells, using the comet assay. Considering acute DNA strand breakage as a prerequisite for later mutagenic effects (62), our data also confirm observations on mutagenesis in the HPRT-gene of isolated epithelial cells after sub-chronic quartz exposure (12). They suggested a key role for neutrophils in the mutagenicity observed after quartz exposure. Indeed, numerous *in vitro* studies demonstrated that neutrophils are capable of inducing several genotoxic effects such as 8-OHdG (17), DNA strand breaks (63,64), SCEs (65), mutations (66,67) and promotion of preneoplastic events (67). In the present study, inhibition of DNA strand breakage by both coatings was paralleled by an overall reduction of neutrophil influx as well as of MPO activity. These observations support the current general consensus on the important role of inflammatory cells in quartz-induced genotoxic effects (30). More specifically, data presented in Figure 8 are in line with data from Driscoll *et al.* (12) and Seiler *et al.* (16), suggesting that there is a threshold for *in vivo* genotoxicity in the rat. However, pooling all treatments and doing statistics on a single animal level, we were not able to show a clear correlation between neutrophilic inflammation (neutrophil influx, MPO activity) and epithelial DNA damage. This could be due to the relatively high background and rather low variability in absolute levels of DNA stand breakage, but might possibly also relate to the presence of other, inflammatory cell-independent mechanisms of DNA damage. Using an *in vitro* model of lung epithelial cell lines, allowing to rule out secondary neutrophil effects, we found that quartz-induced cytotoxicity (this study) and DNA damage was clearly

abrogated when the native quartz particles were coated with either PVNO or AL (28). These observations would fit within our present *in vivo* data, suggesting that in addition to secondary, inflammatory cell-mediated genotoxicity, direct effects of quartz may have contributed to acute DNA damage induced in epithelial lung isolates (68).

Apart from oxidants generated during *in vivo* quartz exposure, other factors that determine the overall genotoxic response are the effectiveness of both intra- and extracellular antioxidant defense systems and of DNA repair systems in the target cells exposed to quartz (28,30). Currently, the role of DNA repair mechanisms in genotoxic effects induced by quartz particles is not yet clear, although recent *in vitro* studies showed that DNA repair was rapidly induced in lung epithelial cells upon exposure to mineral fibers (69,70). The relative high levels of endogenous DNA damage in the isolated epithelial cells could imply possible low DNA repair capacities, which would be consistent with their possible role as target cells for quartz-induced carcinogenicity (1,59). However in addition to DNA repair, the respiratory tract contains a vast number of enzymatic and non-enzymatic antioxidant defense systems, present in both extracellular and intracellular compartments (71). We applied the TEAC assay to evaluate changes in the total extracellular antioxidant capacity of the lung upon quartz exposure, and our data indicate that the increase in antioxidant capacity in the lung was most pronounced upon exposure to native quartz, although this was particularly related to the protein fraction of the BALf. These observations complement our previous work demonstrating that both message and protein levels of several antioxidant enzymes, such as MnSOD are increased upon exposure to crystalline silica (72,73). However, the fact that DNA strand breakage was found to be induced in the epithelial cell isolates from rats exposed to native quartz, notwithstanding the increased level of antioxidant activity, confirms earlier reports that suggest that in these animals the antioxidant defense systems are overwhelmed by the level of oxidants generated by inflammatory cells (12,16).

In conclusion, in the present study we found that quartz caused DNA strand breakage in isolated epithelial lung cells. This effect was efficiently reduced after surface modification with PVNO or aluminium lactate. Since we demonstrated that simple coating treatments dramatically change the DNA damaging capacity of quartz, the current data strengthen the body of evidence that quartz is not uniformly carcinogenic across industries where there is quartz exposure (6). Indeed, in collaboration we showed that the reactivity of the DQ12 quartz greatly differed from work place samples, where quartz dust is likely to be mixed with other minerals, such as aluminium salts (48). Moreover, the present data show the feasibility of our *in vivo* model to evaluate the role of inflammation, antioxidant status, and cytotoxicity in particle-induced DNA damage. Since induction of DNA strand breaks is reported to be closely associated with mutagenesis (62), our data might therefore reflect early molecular events in the initiation stage of quartz-induced tumor formation. However, the mechanism by which quartz causes lung tumors is still by no means clear, and currently we are investigating the significance of the observed acute genotoxic events in later stages of the quartz induced-pathogenesis.

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