Production of reactive oxygen metabolites induced by asbestos fibres in human polymorphonuclear leucocytes

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SUMMARY The ability of quartz and various asbestos fibres to induce the production of reactive oxygen metabolites in human polymorphonuclear leucocytes was assessed. A chemiluminescence assay showed that the activation of polymorphonuclear leucocytes was induced in the following order of effect: quartz; chrysotile A; crocidolite; chrysotile B; amosite; and anthophyllite. Only slight chemiluminescence was produced by cells exposed to wollastonites and titanium dioxide. A positive correlation was seen between production of chemiluminescence and red cell haemolysis. Our results suggest that the potential of various environmental particles and mineral fibres to induce inflammation, fibrosis, and cancer of the lung could be related to their ability to induce inflammatory cells to produce reactive oxygen metabolites.

Asbestosis is characterised by a chronic inflammatory reaction of the lung that has been attributed to the interplay of several biological processes at the level of alveoli and interstitium.¹⁻³ The occurrence of asbestos related disease is influenced by the type of mineral and the dimensions of the fibres, as well as by the concentration of fibres and the duration of exposure.⁴ The pathogenesis of the disease at the cellular level is largely unknown, although studies have been done on the epidemiology of disease induced by asbestos,^{5,6} the physico-chemical structure and aerodynamic properties of the fibres,⁷ their deposition and clearance in the respiratory tract,⁸ the in vitro toxicity, and the cellular biological effects of asbestos fibres.⁹⁻¹¹

Lung injury caused by respirable particles may result either from the direct effects of the pathological agent or as a consequence of an inflammatory cell influx initiated by dust particles. People exposed to asbestos show pronounced neutrophilic and macrophagic alveolitis in the early as well as chronic stages of asbestosis.² When a phagocytic stimulus is recognised, these inflammatory cells undergo metabolic activation which includes the secretion of reactive oxygen radicals.¹² The products of activated phagocytes (hydrogen peroxide, superoxide, hydroxyl radical, and singlet oxygen) have been implicated in considerable tissue damage such as degradation

of DNA,¹³ cytogenetic damage,¹⁴ degradation of hyaluronic acid,¹⁵ inactivation of α -1-antiprotease enzyme binding capacity,¹⁶ autoactivation of latent collagenase,¹⁷ destruction of endothelial cells,¹⁸ and bioactivation of chemical carcinogens.¹⁹

Reactive oxygen products generated during the respiratory burst associated with phagocytosis yield light which can be measured with chemiluminescence enhanced by luminol.²⁰ Using a chemiluminescence assay we studied the ability of quartz and various mineral fibres to induce generation of reactive oxygen metabolites in human polymorphonuclear leucocytes in vitro.

Material and methods

Polymorphonuclear leucocytes were isolated from heparinised human venous blood samples obtained from apparently healthy adults by Ficoll (Ficoll-Paque, Sweden) density centrifugation.²¹ Sedimented cells were treated with NH_4Cl for lysis of erythrocytes, washed twice, and suspended in Dulbecco's phosphate buffered saline.²² Viability of separated cells assessed by trypan blue exclusion was above 90%.

Chemiluminescence was measured with an automated microcomputer controlled luminometer (LKB-Wallac 1251, Sweden) at 37°C. The reaction mixture (final total volume 1.0 ml) consisted of 50 to 100 μl of cell suspension (stock concentration

5×10^6 cells/ml); $700 \mu\text{l}$ 10^{-4} M luminol (5-amino,2,3,-dihydro, 1,4,-phtalazinedione, LKB-Wallac) in Dulbecco's PBS; and 5 to $200 \mu\text{l}$ of mineral fibres suspended in Dulbecco's phosphate buffered saline (stock concentration 1 mg/ml). For standard activators of polymorphonuclear leucocytes, we used phorbol myristate acetate (Sigma Chemicals, USA, P-8139) of a final concentration $0.5 \mu\text{g/ml}$, and 5 to $25 \mu\text{l}$ serum opsonised zymosan (*Sacharomyces cerevisiae*, Sigma) stock concentration 10 mg/ml (about 64×10^6 particles/ml). Chemiluminescence was followed for 40 to 60 minutes after particles had been added to the cells. All particles and fibres were tested in parallel within the same experiment, and each experiment was repeated at least three times.

Four UICC (Union International Contre le Cancer) standard reference asbestos samples—chrysotile A, chrysotile B, amosite and crocidolite—were used. The Finnish anthophyllite (PT 311) and wollastonite (FW 325) samples were from Partek Co, Helsinki, Finland; the United States wollastonite (Wilsboro, USA) and the fractionated Fyle quartz sample (85% of particles less than $5 \mu\text{m}$) were gifts.²³ Titanium dioxide (Titan (IV) oxid) was purchased from the Baker Company, Holland (0343).

To test the haemolytic activity of asbestos a 1.5% (v/v) erythrocyte suspension was prepared in 0.01 M Tris-hydrochloric acid, 0.15 M sodium chloride buffer, pH 7.36, and incubated with mineral fibres (final concentration 2.0 mg/ml for two hours at 37°C).²⁴ The optical density of the lysate was measured at $540 \mu\text{m}$. Complete haemolysis was achieved by Triton X-100 to give an optical density of 1.55 for the lysate.

Results

Human polymorphonuclear leucocytes respond rapidly after challenge with standard stimulants such as phorbol myristate acetate and opsonised Zymosan by activation of the oxygen metabolism and production of chemiluminescence (fig 1). The maximal chemiluminescence is reached after about 10 to 20 minutes and the chemiluminescence response to these activators is dose dependent (not shown). Polymorphonuclear leucocytes also produced chemiluminescence after the addition of particles of mineral fibres to the cells (figs 2a and b). The peak light intensity was reached in eight to 14 minutes, after which the chemiluminescence decreased. On equal mass basis ($\mu\text{g/ml}$) quartz, mineral fibres, and titanium dioxide induced chemiluminescence (peak light intensity) in the following order of magnitude: quartz; chrysotile A; crocidolite; chrysotile B; anthophyllite; wollastonite SF; wollastonite USA; and titanium dioxide. The chemiluminescence produced

Fig 1 Chemiluminescence response of polymorphonuclear leucocytes induced by phorbol myristate acetate ($0.5 \mu\text{g/ml}$) and opsonised Zymosan ($100 \mu\text{g/ml}$) during 40 minutes of incubation.

by wollastonites and titanium dioxide were only slightly above the control background levels. Fig 3 shows the chemiluminescence intensity correlated linearly with mineral fibre and particle concentration (5 to $100 \mu\text{g/ml}$).

The chemiluminescence response of various fibres and particles correlated positively to their haemolytic effect on erythrocytes (fig 4), except that crocidolite and amosite had a weak haemolytic effect and caused a relatively strong chemiluminescence response. Titanium dioxide had a negligible effect in both the haemolysis and chemiluminescence tests.

Discussion

During phagocytosis of particulate matter, polymorphonuclear leucocytes and macrophages produce electronically excited reactive metabolites (such as superoxide anion radical, hydrogen peroxide, and hydroxyl radical), which can damage tissues.^{12 20 25 26} Although the precise mechanism by which oxygen derived free radicals and their metabolites cause cell injury is not clear, there is indirect evidence that oxygen free radicals may have a role in asbestos induced inflammation, fibrosis, and cancer.²⁷ Intratracheal introduction of specific enzyme substrate systems known to generate oxygen metabolites will cause

Figs 2a and b Chemiluminescence response of polymorphonuclear leucocytes induced by 50 µg/ml of quartz, mineral fibres, and titanium dioxide.

acute lung damage.²⁸ Production of oxygen free radicals by phagocytes does not necessarily require the engulfment and phagosome fusion process, but it too is initiated by cell membrane activating agents such as phorbol myristate acetate, concanavalin A, and formyl peptides.²⁹ The present results suggest that even fibre cell contact will rapidly initiate the generation of reactive oxygen metabolites.

All commercial types of asbestos are associated

with asbestosis, and a dose-response correlation has been well documented.¹¹ Although different fibres produce fibrosis to varying extents, chrysotile, crocidolite, and amosite seem to be more fibrogenic than anthophyllite and wollastonite.^{30, 31} Titanium dioxide dusts are generally regarded as biologically inert. This clinical and experimental experience of fibrogenic po-

Fig 3 Dose response (5, 10, 25, 50, 100 µg/ml) of mineral fibres on chemiluminescence peak light intensity of polymorphonuclear leucocytes.

Fig 4 Relation between red cell haemolysis induced by fibres and oxygen free radical production of polymorphonuclear leucocytes.

tential seems to agree with our data on the ability of mineral fibres to induce polymorphonuclear leucocytes in vitro to generate reactive oxygen products.

All types of asbestos seem to be carcinogenic. Chrysotile, amosite, and anthophyllite are associated with increased risk of lung cancer,¹¹ and epidemiological data suggest that exposure to quartz and crocidolite also carries a risk for lung cancer.^{32,33} Crocidolite has the strongest association with mesothelioma.³⁴⁻³⁷ On the other hand, the tumour rarely, if ever, occurs in workers exposed to anthophyllite.⁴ Breakdown of DNA,¹³ lipid peroxidation,³⁸ and bioactivation of chemical carcinogens¹⁹ and cytogenetic changes¹⁴ occur after exposure of various cell types to generators of oxygen free radicals. The carcinogenic potential of fibres seems to be related to their ability to initiate the production of reactive oxygen metabolites in polymorphonuclear leucocytes.

A variety of biochemical, morphological, and in vitro techniques have been used to document events that might be associated with asbestos toxicity at the cellular level. In vitro haemolysis of erythrocytes has been suggested as a model for the biological activity of dusts.³⁹ A correlation seems to exist between haemolytic activity and cytotoxicity,⁹ and it has been suggested that haemolytic activity correlates with fibrogenicity.⁴⁰ The different charge properties of mineral fibres have been suggested to mediate the different haemolytic and cytotoxic effects of mineral fibres.^{41,42} The relatively negligible effect of crocidolite on cytotoxicity and haemolysis, despite its obvious fibrogenic and carcinogenic potential, has been little studied. Our study shows, however, that crocidolite initiates as strong a reactive oxygen metabolite response as chrysotile, weight for weight.

Although we cannot directly relate our findings on oxygen metabolites, derived from polymorphonuclear leucocytes to red cell haemolysis and cytotoxicity, the chemiluminescence assay for in vitro testing of mineral dust polymorphonuclear leucocytes interactions may predict the inflammatory and fibrogenic potentials of mineral fibres, including the man-made mineral fibres.

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