A spin label study of the effects of asbestos, quartz, and titanium dioxide dusts on the bovine erythrocyte membrane

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ABSTRACT The effects of five UICC asbestos samples, titanium dioxide, and quartz on the bovine red cell membrane have been studied in erythrocyte ghosts by the spin labelling technique. Analysis of the electron paramagnetic resonance (EPR) spectra of two sulphhydryl reactive spin labels and one fatty acid spin label in red cell ghosts showed modifications in membrane protein after asbestos treatment but no alterations in membrane lipids. In experiments with quartz no membrane changes were noted but titanium dioxide altered the proteins bound with the protein reactive spin label used in the present study. The possible mechanism for these effects is discussed.

Current usage of the term "asbestos" is restricted to six naturally occurring fibrous silicates which differ in chemical composition and physical properties. They nevertheless produce the same diseases when inhaled: diffuse pulmonary fibrosis, malignant mesotheliomas of the pleural and peritoneal cavities, bronchogenic carcinoma, cancers of the digestive system and other organs, and pleural plaques.

Stanton et al found that in the rat the probability of pleural sarcoma correlates with the number of fibres that measure \(<0.25 \mu m\) in diameter and \(>8 \mu m\) in length. The results showed that, in addition to asbestos, a wide variety of other mineral fibres that seem to have only dimension and durability in common are carcinogenic. Some minerals, however, seem to deviate from this correlation, which suggests that the properties of the other fibres are not unimportant for their pathogenicity.

Biological action of asbestos has been analysed both in vitro and in vivo. It has been found that all cytotoxic mineral types are fibrogenic but that not all fibrogenic types are cytotoxic to macrophages. Although haemolysis of asbestos plays no part in the pathogenesis of dust diseases, the technique is often used to study the effects of fibres on biological membranes and correlation between the haemolytic and cytotoxic activity has been found.

Harington et al have shown that UICC chrysotiles are several times more haemolytic than other UICC asbestos types, but animal experiments have not indicated such big differences in their carcinogenic or fibrogenic potencies.

The data reported above may suggest that:

1. The mechanism of the pathogenic action of different asbestos types is not the same (different agents produce the same effect).
2. Carcinogenic and fibrogenic reactions are accompanied by neutral reactions (not all cytotoxic reactions are pathogenic).
3. Mineral fibres do not always induce pathogenic damage to cells.

This study was prepared in the belief that new aspects of red cell membrane alterations induced by asbestos may make it possible, in future, to answer which red cell membrane-asbestos interactions are typical for other cell membranes and thus are important in the cytogenicity and pathogenicity induced by mineral fibres.

We present here some new information on changes in the proteins and lipids of bovine erythrocyte membranes exposed to mineral dust.

Material and methods

Experiments were carried out with: UICC asbestos samples (chrysotile A, chrysotile B, crocidolite, amosite, anthophyllite), quartz DQ-120, and...
titanium dioxide. Quartz and titanium dioxide were milled (particles measured ≤2 μm in length). Bovine blood was taken into citrate in an abattoir and erythrocyte membranes were prepared according to the method of Dodge et al.7

The two protein reactive spin labels 4-maleimido — 2, 2, 6, 6, — tetramethylpiperidino-oxyl (MSL) and 4 — (2-iodoacetamido) — 2, 2, 6, 6-tetramethylpiperidino-oxyl (ISL) and the one lipid label, 5-doxyl stearic acid (5-NS) were synthetised and provided by Dr K Gwoździnski from the Department of Biophysics, Institute of Biochemistry and Biophysics, University of Łódź.

For spin labelling, solutions of the appropriate labels in ethanol were added to membrane suspensions so that the final label concentrations were 5 × 10⁻⁴ M and the final ethanol concentrations were lower than, or equal to, 0.5% (v/v). In the case of protein reactive spin labels the membranes were incubated with the label for 12–14 hours at a temperature of 4°C and the unbound label was removed by three to four washings with phosphate buffer. In the case of lipid spin label the membranes were shaken for 30 minutes at room temperature.

The protein concentrations of the spin labelled membrane preparations (in 10 mM sodium phosphate buffer, pH 7.4), measured by the method of Lowry et al8 were adjusted to about 3 mg/ml. After incubation for one hour at 37°C the “ghost” suspensions (0.5 ml) were carefully mixed with 10 mg of the dust previously wetted with 0.1 ml of the buffer and incubated for one hour at 37°C. After 14 hours storage of the samples at 4°C the EPR spectra were recorded at room temperature (21–22°C) in a SE/X-28 spectrometer (Wrocław Technical University, Poland), operating at the X band. All the experiments were repeated at least five to seven times on different membrane preparations. Results are presented as means ± SEM. Calculations of statistical significance of differences were made using Student’s t test after checking the data with Q-Dixon’s test. For clarity of comparison, the asbestos induced changes in the EPR spectra parameters are expressed as a percentage of the appropriate control values taken as 100%.

Results

The maleimide spin label binds covalently mainly to the sulphydryl groups in the erythrocyte membrane proteins. The population of membrane -SH groups is heterogenous and, so far as the interactions with MSL are concerned, is composed of two subpopulations, causing weak and strong immobilisation respectively, of the bound spin label residues. The ratio \( H_{W+1}/H_{S+1} \) of the spectral amplitudes of strongly and weakly immobilised residues of MSL attached to ghosts treated and untreated with dust, was compared.

We observed an increase in the \( H_{W+1}/H_{S+1} \) ratio in membranes treated with chrysotiles whereas crocidolite, amosite, anthophyllite, and titanium dioxide decreased this parameter (fig 1).

Changes in the shape of ISL were evaluated by the comparison of the rotational correlation times \( \tau_c \) of the membrane bound spin label residues (fig 2) using the following formula where

\[
\tau_c = k \Delta W \left( \frac{H_w}{H_s} \right)^{-1} > 1
\]

Fig 1 Effect of titanium dioxide, quartz DQ-120, and UICC asbestos samples on the \( H_{W+1}/H_{S+1} \) ratio of MSL bound to erythrocyte membranes. Absolute control value: 3.94 ± 0.19 (=100%). Bars represent ± SE of mean.

Fig 2 Effect of mineral dusts on rotational correlation time of membrane bound ISL. Absolute control value: (9.82 ± 1.17) \( 10^{-10} \) s (=100%). Bars represent ± SE of mean.
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where \( k = \) constant, 
\[ k = 6.5 \times 10^{-10} \text{ smT}^{-1}; \]
\( H_0 \) and \( H_{-1} \) = heights of the midpoint and highfield peaks of the EPR spectrum; and 
\( W = \) width of the midpoint peak.\(^{10}\)

We observed an increase in ISL \( \tau_c \) of asbestos treated membranes whereas neither TiO\(_2\) nor quartz DQ-120 altered this parameter.

The EPR spectra of spin labelled lipid analogues which are incorporated into cell membranes like their natural counterparts give information about the fluidity of the membrane lipid matrix. From such spectra, the order parameter, \( S \), of membrane lipids is usually derived\(^{11}\) where

\[ S = \frac{A_o - A_i}{A_o + 2A_i} \cdot \frac{a_o}{a_i} \cdot \frac{a'_o}{a'i} \cdot \frac{a'_n}{a_n} \]

where \( A_o \) and \( A_i \) refer to the outer and inner hyperfine splitting read from the spectra, while \( A'_o \) and \( A'_i \) refer to completely ordered systems, \( a_n = 1/3 \) (\( A'_o + 2A_i \)), \( a'_n = 1/3 \) (\( A'_i + 2A'_o \)). For control membranes the value of \( S \) was 0.61 ± 0.01.

No alteration of this value was observed after treatment with dusts.

**Discussion**

In our previous study we established the conditions for membrane spin labelling, the dosage of dusts, incubation of the samples, and the recording of EPR spectra.\(^{12}\) In the present paper we have compared the effects induced by seven types of dust on the erythrocyte membrane. There is no other published report of the application of spin labelling technique in experiments on the biological effects of asbestos, quartz, or titanium dioxide.

After chrysotile B treatment in both the previous and the present study we observed an increase in the size of the weakly immobilised population of maleimide spin label bound to membrane proteins. Such alterations are usually assumed to be an effect of some MSL residues coming to the membrane lipid bilayer surface which causes the decrease in the rotational correlation time; this parameter was not calculated for the MSL spectrum in the present study.

The results with ISL-bound membranes indicate an increase in \( \tau_c \). The increase in this parameter is a manifestation of stronger immobilisation of -SH groups by MSL in the membranes treated with chrysotiles than in the control membranes.

Fung and Simpson found that 80% of the maleimide spin label intensity in erythrocyte membrane arises from label sites at the spectrin-actin complex.\(^{13}\) Thus MSL may be used as a spectroscopic probe to study the spectrin-actin complex in the red cell membrane. The observed alterations in the spectra of MSL attached to membranes treated with chrysotiles may be ascribed to a spectrin release from the membrane surface.

After treatment with amphiboles we observed an increased immobilisation of protein -SH groups in the membranes; this result was obtained with both MSL and ISL. The submerging of proteins into the lipid bilayer may be the cause of the reduction of protein lateral diffusion.\(^{14}\)

Thus our results suggest that spectrin is released after the membrane is treated with chrysotile. Spectrin-actin binding together condition the native erythrocyte membrane structure and affect its viscoelasticity and the resealing of red cell ghosts.\(^{15}\) The spectrin-actin system also reduces the mobility of the integral membrane proteins\(^{16}\) whereas in presence of bivalent ions (at a concentration of about \( 10^{-3} \) M) and at low pH values (range of 4, 5 for bovine and 5, 1 for human erythrocytes), spectrin is known to aggregate.\(^{17}\) Thus both chrysotile surface Mg\(^{2+}\) and the negative charge of the amphibole fibre surface (producing a decrease in pH values in the neighbourhood of the fibre) could cause the aggregation of spectrin, which might cause an increase in protein translation motility. Nevertheless, all the experiments with different asbestos types suggest an increase in membrane protein immobilisation (an increase in \( \tau_c \) values in ISL experiments).

Chrysotile asbestos may induce the movement of glycoproteins within the red cell membrane to form clusters from which lipid hydrocarbon chains are excluded. These clusters increase cation permeability and the cells undergo osmotic lysis.\(^{4}\) To support this hypothesis the authors\(^{5}\) have quoted Allison's report showing membrane proteins distributed at random in normal erythrocytes and aggregated after various agents treatment.

The release of spectrin after chrysotile treatment and its immobilisation in the red cell membrane after amphibole treatment (MSL experiments) may cause a temporary decrease in the immobilisation of membrane proteins. This phenomenon promotes the movement of proteins in the plane of the erythrocyte membrane and cluster formation. The increased protein -SH groups immobilisation observed in the ISL experiments may be considered the result of a decreased mobility of the -SH groups of the membrane protein aggregates compared with the motility of the thiol groups of control red cell ghost proteins.

Our data could also suggest, however, other hypotheses by which to explain the asbestos induced alterations in the erythrocyte membrane. Possibly...
the protein clusters observed by Allison are spectrin-actin aggregates. Furthermore, the topology of MSL in erythrocyte membranes suggested by Fung and Simpson may not be the same in our experiments since there are some methodological differences between our experiments and those of Fung and Simpson. On the other hand, an asbestos protein interaction does not explain the adsorption of liposomes on the chrysotile fibres studied by Jaurand. The recorded spectra of lipid labels give information of membrane lipid fluidity determined by the lipid order. In our experiments we found no alterations in membrane rigidity at the depth of the fifth carbon. This suggests that phospholipid order was not altered by chrysotiles or by the other dusts studied. Thus it remains unclear how an asbestos-phospholipid interaction may determine an increase in erythrocyte permeability. This phenomenon may depend on alterations at a greater membrane depth than has been studied.

Our experiments were carried out in 10 mM phosphate buffer. It is possible that phosphate ions prevent the asbestos-phospholipid interaction suggested by Jaurand et al., the more so as Harington et al. have observed that phosphate reduces chrysotile induced haemolysis. Nevertheless, our unpublished results on chrysotile induced haemolysis suggest that bovine erythrocytes haemolysed after treatment with doses comparable to those reported by Harington et al. Moreover, Jaurand et al. prevented chrysotile induced haemolysis with glutaraldehyde, whereas Jost et al. observed no changes in membrane lipid, but did note conformational protein alterations after membrane treatment with glutaraldehyde.

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*These aggregates may not be localised inside the membrane lipid bilayer.

References