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COMPARATIVE *IN VITRO* CYTOTOXICITY AND RELATIVE PATHOGENICITY OF MINERAL DUSTS

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Abstract—*In vitro* bioassays of mineral dust cytotoxicity were compared with *in vivo* pulmonary responses in an attempt to determine the value of bioassays as predictors of fibrogenicity. Release of alveolar macrophage enzyme and sheep red cell haemolysis were monitored as indicators of cytotoxicity for eight well characterized minerals. The pathological reaction induced by intratracheal instillation of 5 mg of three minerals (bentonite, silica and kaolin) into Fischer 344 rats were evaluated at intervals of 1, 7, 90, and 180 days. Bentonite and kaolin were found to be more haemolytic than silica. Kaolin induced greater release of macrophage cytosolic and lysosomal enzymes than silica whereas bentonite appeared to inhibit the activity of alveolar macrophage enzymes. All three minerals, silica, bentonite and kaolin, induced an acute inflammatory cellular response *in vivo*. However, only silica induced a progressive granulomatous and fibrotic response. The pulmonary response to bentonite and kaolin was temporary and had completely resolved six months post exposure. Although only three of the eight minerals have been assayed *in vivo*, these studies indicate that *in vitro* bioassays correlate with the acute *in vivo* reponses to mineral dusts but are not good predictors of fibrogenicity. It would appear that the chronic pulmonary response in animals is still the most reliable predictor of human pulmonary fibrogenic potential.

INTRODUCTION

A PREDICTIVE *in vitro* bioassay for mineral dusts would have a distinct advantage over an *in vivo* bioassay due to its relative simplicity, low cost and timeliness. However, obvious discrepancies exist between the cytotoxicity of various mineral dusts as measured by *in vitro* bioassays and *in vivo* responses to these dusts based on animal and epidemiological data. These discrepancies probably result from differences in complexity between the relatively simple *in vitro* bioassays involving the interaction of a single cell type with a dust and the relatively complex *in vivo* situation in which interactions between cells occur. In order to determine the predictive value of *in vitro* cytotoxicity assays we have compared the *in vitro* and *in vivo* toxicities of a few well characterized mineral dusts.

Alveolar macrophages are thought to play a pivotal role in the cellular reactions initiated by phagocytosis of a dust which lead to inflammation and fibrosis (BRAIN, 1980). Exposure of alveolar macrophages to silica has been shown to result in secretion of factors which stimulate fibroblast proliferation and collagen synthesis (HEPPLESTON and STYLES, 1967 BITTERMAN, *et al.*, 1982, 1983; HEPPLESTON, *et al.*, 1984). We attempted to stimulate the early biochemical changes of these pathogenic events using isolated pulmonary macrophages and monitored the selective release of two lysosomal enzymes, β-glucuronidase (β-GLUC) and β-N-acetylglucosaminidase (β-NAG), and a cytosolic enzyme lactate dehydrogenase (LDH) as indicators of

cytotoxicity. In addition erythrocyte membrane integrity, which is believed to be an indicator of cytotoxic potential, was measured by sheep blood haemolysis. To correlate *in vitro* cytotoxicity with *in vivo* fibrogenicity, rats were instilled intratracheally with aliquots of the dusts and the histopathologic changes studied.

MATERIALS AND METHODS

Min-U-Sil (crystalline silica) was obtained from Pennsylvania Sand and Glass Corporation, Pittsburgh, PA. The other minerals were obtained in native form from primary producers. All the minerals were size fractionated to $< 5\ \mu m$ in a Donaldson particle classifier. Scanning electron microscopic (SEM) analysis in concert with automated image analysis (LeMont) and X-ray spectrometry (XES) analysis of samples prepared on Nucleopore filters were made at a magnification of 1000 × and a minimum of 1000 particles were analysed for elemental composition and circular area equivalent diameters. In addition, the minerals were classified with an aerodynamic particle sizer (TSI Model APS 3300). Quantitative chemical analyses were carried out by proton-induced X-ray emission analysis (PIXE) or by atomic absorption spectrometry using the method of BARTSCH, *et al.* (1982). Surface area measurements were made by the nitrogen adsorption technique (BRUNAUER, *et al.*, 1938).

Haemolytic activity was determined in a sheep erythrocyte preparation according to the method of HARINGTON, *et al.* (1971) with minor modifications.

Rat alveolar macrophages collected by bronchopulmonary lavages were used in the enzyme release assays for lactate dehydrogenase (LDH), β-GLUC and β-NAG according to the methods of REEVES and FIMIGNARI (1960), LOCKARD and KENNEDY (1976), and SELLINGER, *et al.* (1960); respectively. Pulmonary lavages were made by repeated instillation and aspiration of 5 ml of ice cold calcium and magnesium free Hanks' balanced salt solution until a total volume of 80 ml of lavage fluid was collected. The cells were pelleted by centrifugation at 500 × g for 10 minutes and washed with ice cold HEPES buffer containing 140 mM NaCl, 5 mM KCl, 10 mM HEPES (N-2-hydroxyethyl piperazone N-2-ethane sulphonic acid) and 5 mM glucose. The washed cells were pooled and resuspended in HEPES medium. Cell counts were made using a grid haemocytometer. The purity of macrophage preparations obtained by this technique was between 90–95%.

Aliquots of alveolar macrophage suspensions in HEPES (2×10^6 cells/ml) were incubated with mineral dusts (1 mg/ml) for 2 hours at 37°C in a rotary shaker water bath. At the end of incubation, the cells and dusts were sedimented by centrifugation and the supernatant fluid was assayed for enzyme activity. Total enzyme activities were determined in macrophages incubated for 2 hours without dust and lysed with Triton-X-100 at the termination of incubation.

The optimal dust concentrations required *in vitro* to release detectable activities of all three enzymes were determined from a series of preliminary experiments using silica and barite. Minerals were tested in concentrations ranging from 0.1 to 2.5 mg/ml. A concentration of 1 mg/ml was selected as an adequate concentration to induce detectable enzyme release even for relatively inert minerals such as barite but not great enough to cause significant cell death using the more toxic dusts.

Prior to *in vivo* experiments using intratracheal instillation, each dust was supended in normal saline and mixed vigorously in a vortex for 10 minutes. Dust suspensions (0.5

ml), containing 5 mg mineral, were instilled aseptically into the tracheas of Fischer 344 SPF rats. Animals were anaesthetised lightly with sodium pentobarbital (.05 mg/100 gm body weight) and positioned on a slant board. Upper teeth were hooked onto a wire and the larynx was visualized with transillumination. A blunted 20 gauge 4 inch needle with 1.5 mm rounded tip was inserted about 1–2 cm beyond the larynx into the trachea and instillations were made in phase with respiration to facilitate deposition. Control animals received 0.5 ml saline. Each group, 10 experimental animals and 10 controls, was sacrified at 1 day, 3 day, 7 day, 3 month, and 6 month intervals by an overdose of pentobarbital and exsanguination. Body and lung weights were recorded and lungs were cannulated and instilled with buffered formalin. Tissues were processed for histology; sagittal 5 μm lung sections were cut, processed and stained with haematoxylin-eosin.

RESULTS

Physico-chemical characteristics of the dusts

The maximal mass median aerodynamic diameter (MMAD) of minerals used in this study rarely exceeded 7 μm. Although the size distributions of each mineral differed, approximately 98% of particles were less than 5 μm in diameter (Table 1). Aerodynamic size classification was independently verified by scanning electron microscopy combined with image analysis. PIXE and atomic absorption spectrometry showed the minerals used in this study to be relatively pure and minimally contaminated with crystalline silica (Table 1). Surface area measurements by nitrogen adsorption showed wide variability between the minerals reflecting the size and shape distributions of the minerals (Table 1).

TABLE 1. CHARACTERISTICS OF MINERALS

Mineral	MMAD$^{\Delta}$ μm	% $<5\mu$m	% $<1\mu$m	% by number*	% Si by number**	Surface area M^2/g
Barite	2.5	99.7	23.6	88	3.1	5.8
Bentonite	2.7	96.0	17.6	90	0.8	9.8
Coal	3.4	98.9	17.9	94	2.3	7.4
Feldspar	3.6	98.1	6.9	84	15.2	2.2
Kaolin	2.1	99.6	28.8	95	0	12.5
Silica	3.5	98.7	11.3	98.5	98.5	4.7
Talc	3.8	98.3	18.5	74	3.7	9.3
Vermiculite	3.5	98.6	10.8	78	1.8	9.5

$^{\Delta}$ MMAD—Mass median aerodynamic diameter

* Percent concentration of minerals by number as determined by X-ray spectrometric analysis of 1000 or more particles.

** Percent concentration of silica by number as determined by X-ray analysis of 1000 or more particles.

In vitro studies

The haemolytic activity of all minerals showed a linear, dose-dependent, response up to 70% lysis; thereafter it declined (Figs. 1, 2). Dust concentrations required to induce 50% lysis of cells varied markedly (Fig. 3). Bentonite, kaolin, silica and vermiculite

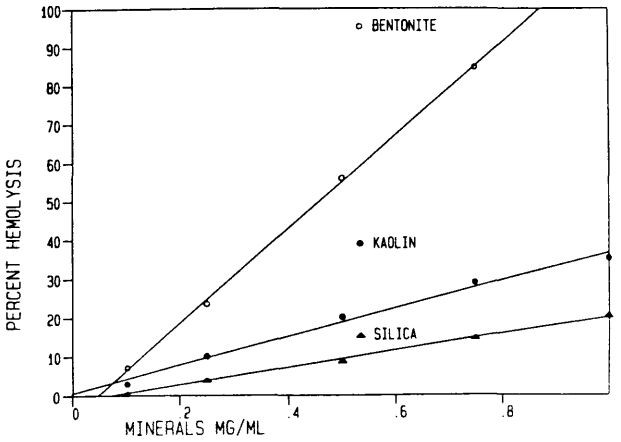


FIG. 1. Haemolytic potential of bentonite, kaolin and silica on a mass basis. The slopes and points indicate the results of a minimum of 10 replicate experiments.

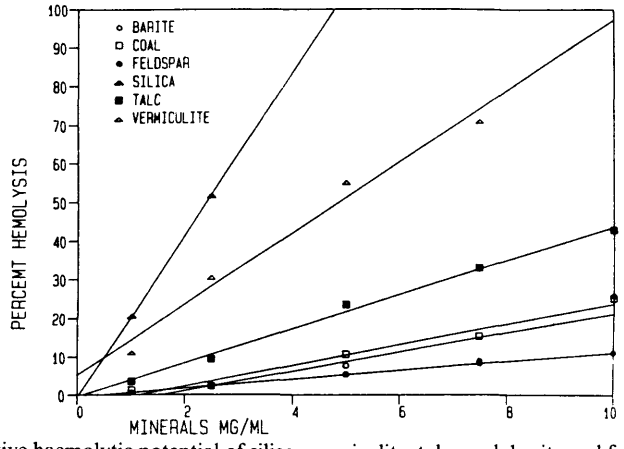


FIG. 2. Comparative haemolytic potential of silica, vermiculite, talc, coal, barite and feldspar on a mass basis. Dose response results indicated are the results of a minimum of 10 replicate experiments.

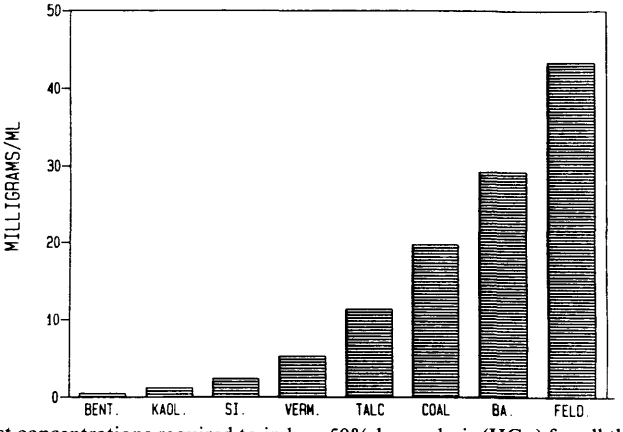


FIG. 3. The dust concentrations required to induce 50% haemolysis (HC_{50}) for all the minerals are illustrated.

showed the greatest haemolysis and talc, coal, barite and feldspar showed the least. Bentonite and kaolin were more haemolytic than silica at all concentrations of dust; however, when the degree of haemolysis was standardized for differences in surface area, bentonite and silica were the most haemolytic, followed by kaolin, vermiculite, talc, feldspar, coal and barite. All the minerals were tested for changes in osmolarity due to leaching of substances into the medium. No significant effects were detected.

Release of LDH, a cytosolic enzyme, β-NAG, and β-GLUC two lysomal enzymes showed significant differences after exposure to the different minerals (Figs 4–6). Among the three enzymes, LDH appeared to be the most sensitive indicator of dust-induced membrane damage since a significant elevation of this enzyme could be observed even with barite, an inert dust (Fig. 4). Although kaolin, silica, and vermiculite caused the most significant elevations in LDH release, coal, feldspar, talc and barite also induced significant elevations compared to controls.

Selective release of the two lysosomal enzymes β-NAG and β-GLUC were significantly higher with kaolin than with the other minerals (Figs. 5, 6). These two lysosomal enzymes were also elevated in response to silica, vermiculite and talc but showed no response to coal, feldspar and barite. Bentonite appeared to inhibit all three enzymes; however, the inhibition was higher with β-NAG than with LDH and β-GLUC.

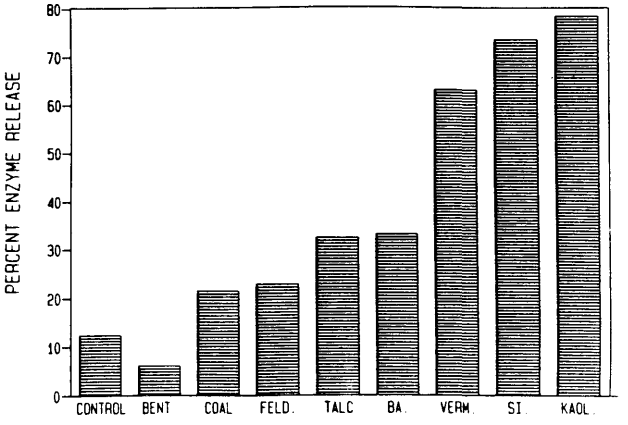


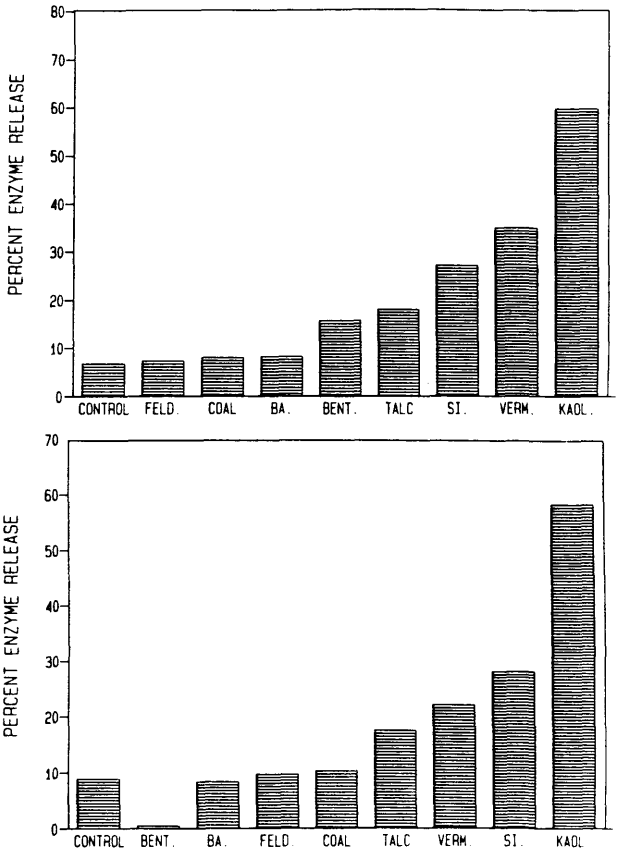
FIG. 4. Comparative effect on the release of lactate dehydrogenase (LDH) from alveolar macrophages. Percent enzyme released from 2×10^6 cells/2 hr on incubation with 1 mg mineral/ml.

In vivo studies

To date *in vivo* responses have only been evaluated for the three dusts silica, bentonite and kaolin. The fibrogenic response to intratracheally instilled dusts was evaluated in groups of 10 animals at 1, 7, 90, and 180 days post-exposure. The lungs of control animals showed no major pathology for any time period. Occasional collections of lymphocytes were seen in the perivascular tissues and foamy macrophages were seen in some alveoli.

Silica

One-day silica exposed animals showed an acute alveolitis centered on the respiratory bronchioles and adjoining alveoli. At seven days, ill defined granulomata



FIGS. 5,6. Comparative effect on the selective release of β-glucuronidase (β- GLUC) and β-N-acetylglucosaminidase (β-NAG) from alveolar macrophages. Percent enzymes released from 2×10^6 cells/2 hr on incubation with 1 mg mineral/ml.

were seen which were predominantly interstitial. The adjoining alveoli showed alveolar lipoproteinosis and type II cell hyperplasia. Dust-containing foamy macrophages predominated over acute inflammatory cells at this stage. By three months, silica-exposed animals showed well circumscribed interstitial granulomas composed of central histiocytic cells with lymphocytic cells at the periphery. The granulomata measured 3–4 mm in size without obvious fibrosis. Alveolar lipoproteinosis was seen in adjacent alveoli. Animals exposed to silica for 6 months developed well circumscribed granulomata composed of histiocytic cells with a mantle of lymphocytes (Fig. 7). The lesions were larger than those seen at three months. Focal areas of alveolar lipoproteinosis with foamy macrophages and type II cell hyperplasia were still present.

Bentonite

The lungs of bentonite-exposed animals showed an acute inflammatory reaction similar to that seen with silica at one day. Acute inflammatory cells were also seen in airways. Dust was present within macrophages. At one week interstitial granulomatous lesions measuring up to 3 mm (Fig. 8) were seen. The lesions were concentrated

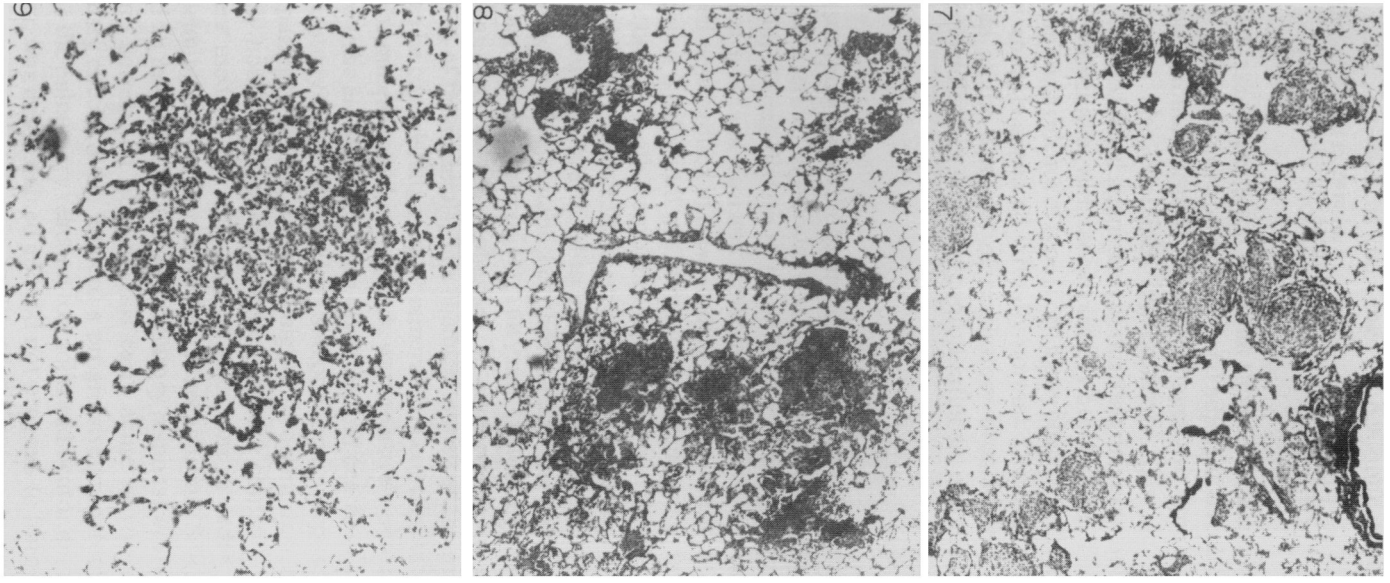


FIG. 7. Photomicrograph of rat lung section showing the interstitial granulomatous lesions at 180 days after intratracheal instillation of 5 mg crystalline silica. The lesions are well circumscribed and contain histiocytes with a mantle of lymphocytes (60 ×).

FIG. 8. Photomicrograph of rat lung section showing the developing granulomatous lesions 7 days after exposure to 5 mg bentonite. Acute inflammatory changes are still present (60 ×).

FIG. 9. Photomicrograph of rat lung sections to show the early acute inflammatory reaction (alveolitis) one day after exposure to 5 mg kaolin (120 ×).

in and around respiratory bronchioles. The adjacent alveoli showed lipoproteinosis with type II cell hyperplasia and contained foamy macrophages. At three months the granulomas had largely resolved, leaving a residuum of foamy macrophages in some alveoli. After six months the lungs of bentonite exposed animals could not be distinguished from controls.

Kaolin

Lungs of animals exposed to kaolin showed an acute inflammatory reaction in the region of the respiratory bronchioles and adjacent alveoli (Fig. 9) at one day. The ratio of polymorphonuclear cells to macrophages was approximately 10: 1. A few of the large airways also contained inflammatory cells. At seven days, kaolin-exposed animals showed small aggregates of dust-bearing macrophages at the junctions of the terminal bronchioles with the respiratory bronchioles. There was slight interstitial cell thickening and evidence of type II cell hyperplasia. By three months, the lungs of kaolin exposed animals appeared virtually normal. Occasional collections of macrophages were seen at the divisions of respiratory bronchioles. Animals exposed to kaolin showed no difference from the controls after six months.

DISCUSSION

This study compares the *in vitro* cellular and *in vivo* pathological reactions of various well characterized minerals that have been implicated in the pathogenesis of occupational lung disease in man. All the minerals investigated induced detectable responses in both *in vitro* and *in vivo* systems. Although particle size and homogeneity were well standardised in these studies, the data should be interpreted with a degree of caution as it is difficult if not impossible to standardise for all particle parameters.

For example, both bentonite and kaolin had smaller MMADs than silica and produced greater haemolysis on a unit mass basis. However, when the degree of haemolysis was standardised to unit surface area, bentonite and silica were the most haemolytic. It is well established that particle size and/or surface area are important determinants of cytotoxicity (OTTERY and GORMLEY, 1978). This study confirms this finding and serves to emphasise the importance of characterising the physical properties of the minerals. The chemical purity of the minerals is also an important factor to be considered, as most minerals are contaminated with silica in their native state. The minerals used in this study were relatively pure and there was no correlation between their *in vivo* and *in vitro* toxicities and degree of silica contamination.

In this study the cellular toxicity and pulmonary responses of minerals were assessed by comparing their reactivities to a highly fibrogenic dust, silica (positive control) and a relatively non-fibrogenic dust, barite (negative control). Only small doses of dust (5 mg) were used for the intratracheal injections in order to reduce the likelihood of overwhelming the pulmonary defence mechanisms. Silica, bentonite, and kaolin all induced an immediate acute inflammatory response *in vivo*. However, there appeared to be no correlation between this early acute *in vivo* response and their chronic pulmonary effects. Silica induced a progressive granulomatous and fibrotic response whereas the pulmonary reaction to kaolin and bentonite, while initially similar to that for silica, was not sustained and did not progress to fibrosis. It would appear that the initial pulmonary response to these minerals correlates well with their *in vitro*

haemolytic activity but that neither response is predictive of the eventual fibrogenic properties of the dust. Similarly, there was a poor correlation between the *in vitro* macrophage responses to dust and their known human pathogenicity; moreover, macrophage response did not correlate well with haemolytic activity. The situation is further confounded by the observation of an apparent inhibition of β-GLUC by bentonite, indicating that other as yet unknown mechanisms need to be taken into account.

In summary our data indicate that chronic *in vivo* animal studies are the best indicators of pulmonary fibrogenic potential in humans, and that the two *in vitro* assays used in this study did not reflect the fibrogenic potential of the mineral dusts. Correlative studies should also be performed for other *in vitro* test systems.

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DISCUSSION

F. H. BAKER: Does the high activity of kaolin, shown in the various slides, suggest an inhibiting ability of this mineral if mixed with silica and other minerals?

V. VALLYATHAN: No. In these studies kaolin is not inhibiting the toxicity of other minerals. We have not evaluated that aspect by mixing the minerals. All the minerals tested were pure and there was no major contamination.

S. R. MOORES: My colleague, Sue Sykes, reported our studies at the last BOHS conference 5 years ago, and it is, therefore, reassuring that you also find that neither in vitro haemolysis nor macrophage assays are accurate determinants of long-term fibrogenic potential in the lung.

However, I would suggest that short term *in vivo* responses can be monitored in the way she described and serve to distinguish, for example, between cytotoxic but non-fibrogenic dusts and fully fibrogenic materials.

V. VALLYATHAN: I agree that short-term *in vivo* bioassays may be better predictors of fibrogenicity. We are currently doing these studies using intratracheal instillation of dust and lung lavages at various time intervals and monitoring the release of enzymes LDH, β-GLUC, β-NAG and protein and albumin. Drs. Beck and Brain may have some information on the validity of these short-term *in vivo* bioassays. However, our preliminary studies on three mineral dusts have not shown any correlation with the fibrogenicity or the initial acute pulmonary response.

R. C. BROWN: Dr. Vallyathan's data demonstrate the danger of using empirical *in vitro* tests for correlation with *in vivo* observations. There is no reason to believe that lysosomal enzyme release or haemolysis is involved in vivo.

V. VALLYATHAN: I agree with Dr. Brown that isolated cellular *in vitro* studies are often misleading. However, an *in vitro* response similar to that occurring in vivo may trigger a chain of biological events leading to fibrosis. So to study the specific events in response to a cellular interaction with dust, monitoring of enzyme release from macrophage and haemolysis resulting from membrane damage are justified.

J. L. ABRAHAM: I was very interested in your mention of a short-lived, but nevertheless recognized, alveolar lipoproteinosis (PAP) reaction acutely following kaolin instillation.

We have recently observed a human case of proteinosis in a china clay worker in whom mineralogical analysis has shown high concentrations of kaolin with negligible silica. Yet reported human and animal studies have not shown PAP associated with kaolin exposure. I would be interested in your comments and those of the other attendees with experience in this area.

V. VALLYATHAN: I would like to point out that alveolar lipoproteinosis (PAP) was minimal and transient in kaolin-exposed animals as compared to silica and bentonite-exposed animals. Bentonite exhibited an initial maximal response which disappeared with time, whereas PAP persisted in silica-exposed animals even at six months. Kaolin-exposed animals showed only occasional alveoli filled with granular lipoproteins.

A. R. GIBBS: I have looked at a number of lungs obtained on autopsy from china clay workers. Alveolar proteinosis was not seen. Is it probably a matter of selection?

V. VALLYATHAN: Probably it is a matter of selection. PAP in the animals were very transient and did not progress and also was minimal.

J. C. WAGNER: In animal inhalation experiments, we have not observed lipoproteinosis with kaolin exposure. We have also noted an early proliferative respiratory reaction similar to your results, but this rapidly regresses even if the exposure to dust is continued up to one year.

V. VALLYATHAN: The initial acute reaction of kaolin is almost similar to silica-induced acute alveolitis. In the case of bentonite and kaolin, these acute reactions did not lead to a chronic fibrogenic response as with silica.

A. G. HEPPLESTON: Perhaps some of the *in vitro—in vivo* disparities might be overcome by adopting a direct test of *in vitro* fibrogenicity (HEPPLESTON *et al., Am. J. Ind. Med.*, 1984; **6**, 373–386), rather than relying on the conventional indirect procedures devoted solely to toxicity against membranes.

V. VALLYATHAN: In such *in vitro* systems, also, we will be dealing with isolated cell lines which will not be comparable to the *in vivo* chain of events leading to fibrosis.