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THE CYTOTOXIC EFFECTS OF ASBESTOS AND OTHER MINERAL DUST IN TISSUE CULTURE CELL LINES

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Summary.—The cytotoxic effects of 15 dusts have been tested in 2 cell lines. Experimental animal data are available for 11 of the 15 dusts. There is a correlation between the cytotoxic activities of the dusts and the ability to induce mesothelial tumours following intrapleural injection of the dusts. Some preliminary observations on the nature of interaction between the dusts, medium components and the cells are reported. It is suggested that the study of the interactions between cells and dusts in culture may lead to an understanding of the pathogenesis of these dusts in man and animals.

MINERAL dusts which cause diseases in man have been shown to produce several biological effects *in vitro*. Those effects most commonly studied have been the lysis of erythrocytes and cytotoxicity in short-term macrophage cultures. Other tissue culture systems have been used and this subject has been reviewed extensively by Harington (1976) and Harington, Allison and Badami (1975).

There is good correlation between the haemolytic potential, the effect on macrophages and the fibrogenic activity of a series of mineral dusts, including silica and asbestos (Allison, Clark and Davies, 1977). However, there is no correlation between these biological effects and the carcinogenic activities of the mineral dusts. Sincock and Seabright (1975) have reported the induction of chromosome changes in cells which had been exposed to carcinogenic asbestos fibres *in vitro*. Non-carcinogenic fibres did not induce such chromosome changes.

In this present paper we report the cytotoxic effects of a series of mineral dusts in tissue culture cell lines. Fifteen dusts have been tested *in vitro*; experimental animal data are available for 11 of the 15 (Wagner, Berry and Timbrell, 1973; Wagner *et al.*, 1977).

MATERIALS AND METHODS

Cell lines.—(a). V79-4 Chinese hamster lung cells were obtained from Dr C. Arlett, MRC Cell Mutation Unit, Brighton (Chu and Malling, 1968).

(b). Human alveolar Type II lung cells (A549), a continuous cell line with the characteristics of Type II alveolar epithelial cells (Lieber *et al.*, 1976), were presented to Dr M. M. F. Wagner by Dr G. Todaro, National Cancer Institute, Bethesda, Maryland, U.S.A.

Cell cultures.—(a). V79-4 cells were grown in minimal essential medium (MEM—Gibco-Biocult) supplemented with 15% foetal calf serum and antibiotics.

(b). A549 cells were grown in Dulbecco's modification of MEM (Gibco-Biocult) supplemented with 10% heat-inactivated foetal calf serum and antibiotics.

Cells were routinely passaged in 75 cm^2 plastic flasks (Corning).

Mineral dusts.—All the asbestos samples used were from the UICC standard reference series (Timbrell and Rendall, 1971) except SFA chrysotile which has been described previously (Wagner *et al.*, 1973). UICC crocidolite, modified by milling in a ceramic ball-mill, was provided by Dr V. Timbrell. Glass fibre Codes 100 and 110 (Johns Manville), prepared by chopping and grinding (Timbrell, 1976), were provided by Mr J. W. Skidmore. The glass powder was a respirable fraction obtained from a crushed commercial borosilicate glass cullet (Wagner *et al.*, 1973). Min-u-sil (5 μm) was obtained from the Pennsylvania Silica Sand Co., Pa., U.S.A. South African silica (Dawson & Dobson superfine silica) was obtained from Mr N. Z. Goldblatt.

Italian talc was a respirable fraction prepared from a commercial cosmetic grade. Canadian chrysotile, which had been leached by hydrochloric acid to remove all the magnesium, was obtained from Mr A. Morgan, A.E.R.E., Harwell (Morgan *et al.*, 1973). Artificial fluoramphibole "B" was obtained from the same source (Morgan *et al.*, 1975). Brucite, (Nemalite) was obtained from a Canadian mine (Wagner *et al.*, 1973).

Cytotoxicity assays.—(a). Survival of V79-4 cells in the absence and presence of the mineral dusts was determined by cloning efficiency from a single cell suspension. Approximately 200 cells were seeded into 60 mm diameter Petri dishes (Corning) along with the dust. The surviving cells were allowed to grow and form colonies for 6–7 days, when they were fixed and stained with 10% formol saline containing 1% methylene blue. The number of colonies on each Petri dish was counted using a low power binocular microscope (a colony being defined as containing more than 50 cells).

In some experiments the cells were allowed to attach to the Petri dish for 2½ h before the addition of the dust.

(b). The growth of A549 cells in the absence and presence of the mineral dusts was determined by seeding about 10^5 cells in 25 cm^2 tissue culture flasks (Corning) or 5×10^4 cells in each well of 8-well multiplates (Lux). The number of viable cells was determined by removing the cells with trypsin–EDTA and producing a single cell suspension in phosphate-buffered saline containing 5 μg/ml fluorescein diacetate. The number of fluorochromatic cells was determined using a haemocytometer under a fluorescence microscope.

Dose response curves were obtained by a similar method, enumerating the number of cells after 5 days in quadruplicate cultures. The diameters of the cells were measured using the Timbrell/Coulter double image analyser on photographs of a fresh single-cell suspension in a haemocytometer. At least 500 cells were measured from each sample.

RESULTS

1. *Cytotoxicity assays using V79-4 cells*

Seven of the dusts were not toxic at 50 μg/ml. These were the 2 silica samples, Italian talc, glass powder, glass fibre Code 110, leached chrysotile B and 8h-milled-crocidolite. All the remaining 8 dusts were cytotoxic to a greater or lesser extent (Table). Representative survival curves showing results for 4 of the dusts are presented in Fig. 1.

Consistent differences were found between the colonies on control dishes and

TABLE.—*Cytotoxicity of the Dusts Examined*

Dust	LD_{50} for V79-4 cells, μg/ml (Range; No. of determinations)	ED_{50} for A549 growth inhibition (μg/ml)	% of rats developing mesotheliomata after intrapleural inoculation of 20 mg
SFA Chrysotile	26 (11–33; 3)		66
UICC Crocidolite	9 (6–12; 7)	35	61
8 h milled Crocidolite	> 50 (----; 2)		
UICC Crocidolite in heat inactivated serum	30 (----; 1)		
Brucite	12 (----; 1)		56
UICC Amosite	4·5 (4–5; 2)		36
UICC Anthophyllite	20 (---; 1)		34
UICC Chrysotile B	17 (5–34; 6)	5	30
Leached Chrysotile B	> 50 (----; 1)	> 320	
UICC Chrysotile B in heat inactivated serum	35 (----; 1)		
Glass Fibre Code 100	9 (5–13; 2)	24	12
Glass Powder	> 50 (----; 1)		3
Glass Fibre Code 110	> 50 (----; 2)	130	0
Italian Talc	> 50 (----; 1)		0
Min-U-Sil	> 50 (----; 2)	> 320	0
South African Silica	> 50 (----; 1)	> 320	

The concentration of dust causing a 50% reduction in the cloning efficiency of V79-4 cells or a 50% inhibition of growth of A549 cells was determined as described in the text. The figures for the incidence of mesotheliomata are from Wagner *et al.* (1973, 1977). Spaces indicate that the dust has not been tested or that a test would be inapplicable. It should be noted that intrapleural injection of silica causes the formation of malignant histiocytic lymphomata rather than mesotheliomata (Wagner, 1976). The South African silica has the correct mineralogical properties to behave similarly to Min-u-sil *in vivo* (Wagner, personal communication).

those on dishes which had received a cytotoxic dust. Colonies which had developed in the presence of the cytotoxic dusts were smaller and contained cells which were larger, less densely stained and often multinucleate (Fig. 2).

The cytotoxic effects of UICC crocidolite were reduced if the cells were allowed to attach to the plastic for 2½ h before the addition of the dust (Fig. 3A). However, this effect was not observed with UICC chrysotile B (Fig. 3B).

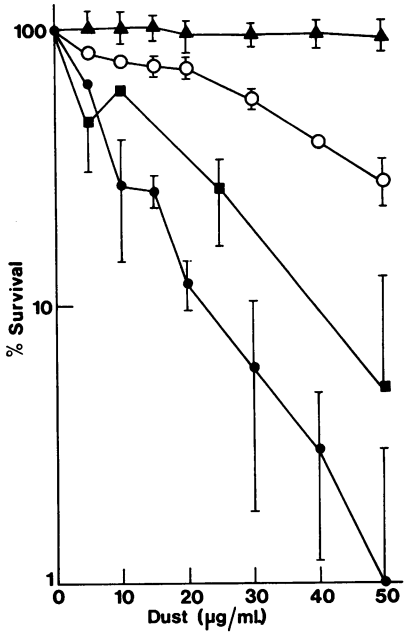

FIG. 1.—The cloning efficiency of V79-4 cells was determined as described in the text. Survival curves for the following dusts are shown; ▲ Glass fibre Code 110, ○ SFA chrysotile, ■ UICC chrysotile B and ● UICC crocidolite. The 95% confidence limits, calculated by the method of Duncan and Brookes (1973), are shown but have been omitted where overlap would occur or the errors are within the symbol.

It was also shown that UICC crocidolite, but not UICC chrysotile B, was less cytotoxic in medium containing heat-inactivated foetal calf serum (Table).

2. *Inhibition of growth of A549 cells*

Three of the dusts, leached chrysotile B and the 2 silica samples, did not inhibit the growth of A549 cells, even at concentrations of the dust as high as 320 µg/ml (Table). Glass fibre Code 110 was weakly inhibitory but glass fibre Code 100 was very inhibitory, as were UICC crocidolite and chrysotile B.

The growth curves tended to plateau from Day 4 onwards both for control cells and those in the presence of the dusts (Fig. 4). There was a consistent dose-related reduction in the number of cells present in the cultures on Day 5. A comparison of the inhibitory effects of the 7 dusts can be made using the number of cells present on Day 5 in the various cultures (Fig. 5 and Table).

3. *Sizes of A549 cells in the absence and presence of glass fibres*

A549 cultures also contained "giant cells" when treated with dusts which inhibited growth. The size distributions of the cells have been measured as described above. Fig. 6 shows the distribution of cell diameters in control and 2 treated populations.

DISCUSSION

Previous work, reviewed by Harington (1976) and Harington *et al.* (1975), has shown that several mineral dusts are toxic for macrophages *in vitro*, both in the absence and presence of serum, although the details of the interaction between dusts and cells are different in these 2 conditions. There is general agreement that silica is the most cytotoxic, followed in order by chrysotile asbestos and crocidolite asbestos (Bey and Harington, 1971). Allison *et al.* (1977) have shown that the effect on macrophages in these *in vitro* tests and in diffusion chambers implanted in the peritoneal cavities of mice are correlated with the fibrogenic potentials of the dusts. These observations may be put

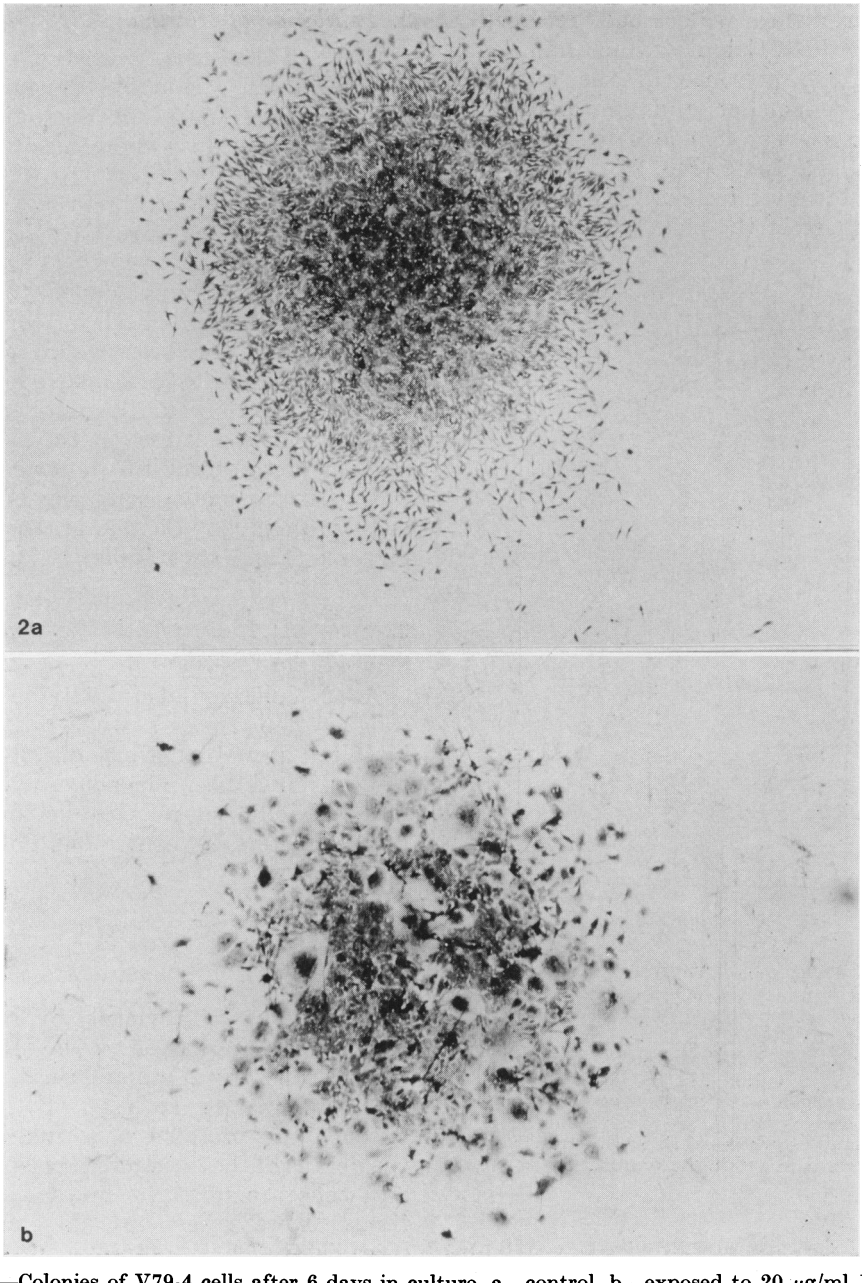

FIG. 2.—Colonies of V79-4 cells after 6 days in culture. a—control, b—exposed to 20 μg/ml UICC crocidolite. The colony which has developed in the presence of crocidolite is of smaller diameter and there are several "giant" cells present.

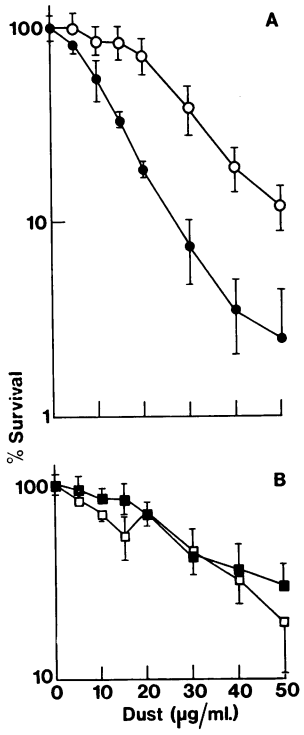

FIG. 3.—The cloning efficiency of V79-4 cells was determined as described in the text. The cells were exposed to; A—UICC crocidolite, B—UICC chrysotile B. The solid symbols are the results with the dust and cells mixed in suspension before plating; the hollow symbols are where the cells were allowed to attach to the plastic for 2½ h before the addition of the dust.

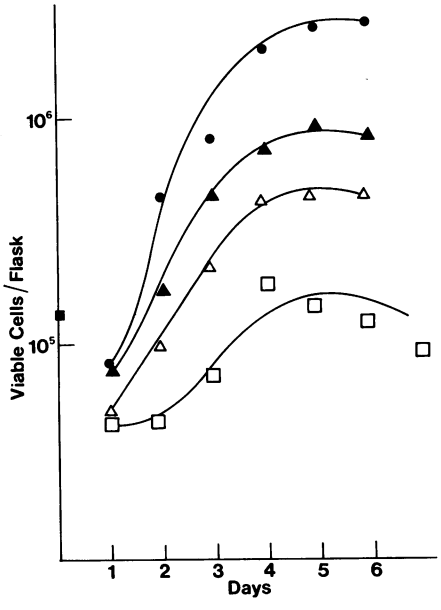

FIG. 4.—Growth curves using A549 cells were determined as described in the text, ● Control, ▲ Glass fibre Code 110 at 300 μg/ml, △ Glass fibre Code 100 at 100 μg/ml, □ Glass fibre Code 100 at 300 μg/ml.

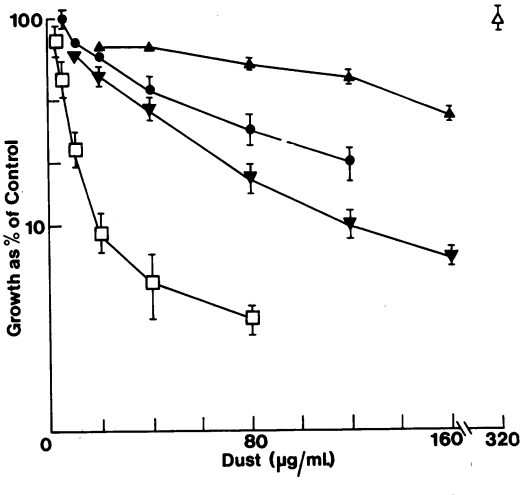

FIG. 5.—The growth of A549 cells in the absence and presence of a series of dusts. The number of cells present in A549 cultures after 5 days exposure to a series of concentrations of dust is expressed as a % of the number of cells in control cultures, △ Min-u-sil, South African silica, leached chrysotile B, ▲ Glass fibre Code 110, ● UICC crocidolite, ▼ Glass fibre Code 100, □ UICC chrysotile B. The 95% confidence limits are indicated by error bars.

forward as a basis for the development of a rapid *in vitro/in vivo* screening test for the detection of fibrogenic dust (Davies *et al.*, 1974; Allison, personal communication).

The cytotoxic effects of mineral dusts on V79-4 and A549 cells differ markedly from those in macrophage cultures in that silica has little effect and the UICC asbestos dusts are very cytotoxic. This suggests that our observations have little relevance to the fibrogenic potential of the dusts. The dusts which are active in our tests are those which cause mesothelial tumours when inoculated into the pleural cavities of rats (Table). Attention is drawn to the fact that the more cytotoxic dusts are not necessarily the most carcinogenic (see

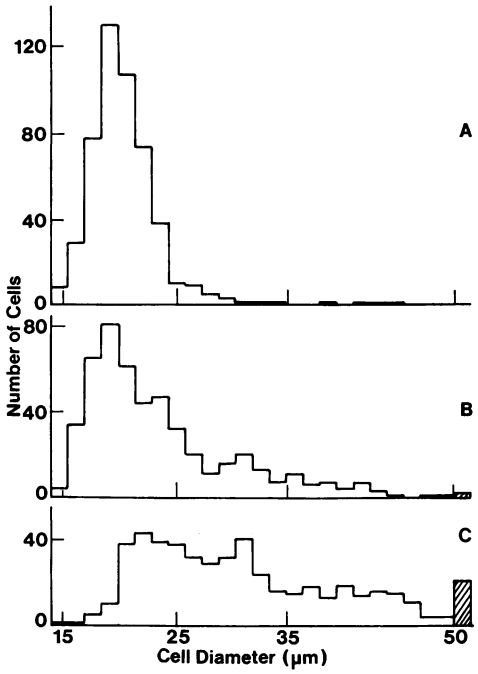

FIG. 6.—The size distributions of A549 cells were determined as described in the text, A—control, B—Glass fibre Code 110 at 500 μg/ml, C—Glass fibre Code 100 at 300 μg/ml. The hatched category includes all cells > 50 μ in diameter.

Table); for example SFA chrysotile, which is the most active dust tested in rats, is not as cytotoxic as the less carcinogenic UICC amosite.

The end points used in these experiments—reduction in cloning efficiency of V79-4 cells and inhibition of growth of A549 cells—may result from several different interactions between the cells, medium and dusts. For example, the involvement of heat-labile serum components in the cytotoxicity of crocidolite but not chrysotile on V79-4 cells is shown in the Table. This may be related to the involvement of complement in the haemolysis caused by amphibole asbestos but not that caused by chrysotile or quartz (Harington, Miller and Macnab, 1971*a*; Harington *et al.*, 1971*b*). Another phenomenon indicating that chrysotile and crocidolite interact with cells by different mechanisms is that attachment of the cells to plastic partially "protects" them from crocidolite but not chrysotile (Fig. 3). Further work on these interactions between dusts and cells *in vitro* may elucidate which of them (if any) are relevant to tumour production. For example, we have demonstrated that milled crocidolite was very much less cytotoxic than the standard UICC crocidolite (Table), despite the fact that an equal mass of the milled sample contains many more particles. This is in contrast to the suggestion by Wade *et al.* (1976) that the number of particles per unit mass was a principle determinant in the cytotoxicity of asbestos to a macrophage cell line (P388D1).

Our observations go some way towards suggesting that the fibrous morphology of the dust is an important determinant of cytotoxicity. This should be compared with the demonstration that the tumourigenicity of 0·5 μm diameter glass fibres increased with increasing fibre length (Stanton *et al.*, 1977). We are extending this work to study the relationship of fibre size and shape to cytotoxicity using different sizes of glass fibres and milled crocidolite samples (to be published elsewhere).

The "giant" cells found in our cultures (Fig. 2, 6) may be similar to those reported by Beck (1976) and parallel the appearance of larger nuclei in the cells of human embryonic lung organ cultures (Rajan and Evans, 1973) and the induction of polyploid cells in CHO-K1 cultures (Sincock and Seabright, 1975). Beck presented evidence that the "giant" cells which he observed arose by cell fusion. The "giant", polyploid and multinucleate cells may arise by the same mechanism and further work is necessary to determine the origin of these cells in our cultures.

Further investigations using the *in vitro* systems discussed in this paper may lead to an understanding of the interactions between mineral dusts and cells. Thus these studies may eventually provide an *in vitro* screening method for the detection of hazardous dusts.

Harington *et al.* (1975) drew attention to the fact that "the important criterion for activity appears to be structural features including a durable fibrous shape and a narrow range of size". Whilst able to detect fibres of the active sizes and shapes, short-term *in vitro* testing may not distinguish between durable fibres and those which would be inactivated *in vivo*. For this reason a close quantitative parallel between the *in vitro* and *in vivo* activities may not be obtainable.

We would like to thank Dr J. C. Wagner for initiating this work and for his advice and suggestions. Our thanks are also due to Mr J. W. Skidmore and Dr V. Timbrell for providing the samples of glass fibre and milled crocidolite respectively. Technical assistance was provided by David Brown, Vanessa Sawicka, Gill Gubbings and Joyce Hoy. Thanks are due to Michael Campbell for statistical advice and to Rosemary Hill and John Park for the preparation of the manuscript.

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