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Cytotoxic Action of Mineral Dusts on CHV 79 Cells *in Vitro:* Factors Affecting Toxicity

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The cytotoxic action of a series of mineral dusts has been studied using Chinese hamster V79-4 cells obtained from three separate laboratories in the UK. The dusts which have been studied include samples of asbestos from the Union Internationale Contre le Cancer reference series and an internationally available sample of quartz (DQ12). Besides comparing results obtained with the three different cells, two alternative methods of exposing the cells to the dusts were also compared.

The toxicity assay employed uses the inhibition of colony formation from a single cell suspension as a measure of toxicity. The numbers of colonies which grow in the presence of the various concentrations of the dusts are expressed as percentages of the colonies which grow in the untreated control cultures. The absolute toxicity of a dust was found to depend principally on the method of exposing the cells to the dust. However, differences in the relative toxicities of the dusts depended on the source of the cells.

The implications of these results for experiments in which Chinese hamster V79-4 cells are used in *in vitro* test systems to assess the potential pathogenicity of mineral dusts are discussed.

Introduction

Chinese hamster V79-4 cells have been used in *in vitro* studies to assess the cytotoxic potentials of mineral dusts. Fibrous dusts like asbestos, which induce pleural tumors in experimental animals, are relatively toxic to V79-4 cells, whereas nonfibrous dusts like silica, which do not induce pleural tumors, are relatively nontoxic (1).

The interactions between dust particles and cells which may lead to cytotoxicity occur in several stages and include: attachment to the cell membrane, entry into the cell, movement within the cell and interaction with, or effect on, sensitive target(s). Any one of these stages may be a rate-limiting interaction. Differences in the cytotoxicity of any one dust towards two different kinds of cells may be the result of changes in one or more of the above interactions. Variations in culture conditions and in the method of exposing V79-4 cells to dusts have been shown to alter significantly the toxicities of some dusts but not others; this has the effect of altering their relative toxicities (*1*, *2*). For example, heat inactivation of the fetal calf serum used in the culture medium reduces the toxicity of crocidolite but not chrysotile. Individual culture conditions and variations in technique can potentially lead to different results obtained in independent laboratories. In this paper we report three variables which can lead to discrepancies in assessing both the relative and absolute toxicities of mineral dusts.

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Materials and Methods

Cells

Chinese hamster cells derived from the V79-4 line originally isolated (*3*) by Chu, Biology Division, Oak Ridge National Laboratory, TN, were obtained from three different laboratories in the United Kingdom: (i) Dr. M. Fox, Paterson Laboratories, Christie Hospital and Holt Radium Institute, Manchester (V79-U cells); (ii) Dr. M. Chamberlain, previously at MRC Pneumoconiosis Unit, Llandough Hospital, Penarth, South Glamorgan (V79-P cells); (iii) Dr. J. Thacker, MRC Radiobiology Unit, Harwell (V79-H cells).

Media

All cells were routinely grown in Eagles Minimal Essential Medium (MEM) supplemented with 10% fetal calf serum, 2 *mM* L-glutamine and antibiotics. This medium was used for toxicity assay method A (*vide infra*), but for method B Dulbecco's modification of MEM supplemented with 10% fetal calf serum, 2 *mM* L-glutamine and antibiotics were used. used.

Fetal calf serum

Batches of fetal calf serum were obtained from Flow Laboratories (UK) Ltd., Irvine, Scotland and Gibco Europe Ltd., Paisley, Scotland.

Preservation of Cells

Cells were preserved by storage in liquid nitrogen at the first passage to minimize the chance of cross-contamination using growth medium containing 10% dimethyl sulfoxide (DMSO).

Dusts

The following samples of asbestos were from the Union Internationale Contre le Cancer standard reference series (*4*): amosite, crocidolite, anthophyllite, chrysotile B. The international standard quartz DQ12 was obtained from Dr. K. Robock, Asbestos-Institute for Occupational and Environmental Hygiene, Gorlitzer Strasse 1, 4040 Neuss, Federal Republic of Germany. Weighed amounts of each dust were sterilized by autoclaving before suspension in the appropriate sterile tissue culture medium.

Toxicity Assays

Method A. This method has been described previously (*1*). Briefly, a suspension of single cells is dispensed into a series of universal bottles and appropriate volumes of medium or dust suspension added to each. The suspension is mixed by inversion before being distributed into four 6-cm^2 diameter Petri dishes. The colonies are allowed to grow without disturbance for 6 days, washed with phosphate-buffered saline, fixed with methanol and stained with Giemsa.

Method B. This method differs from that above in several important respects. The experiments are performed in Dulbecco's MEM as described previously. Cells and dust are mixed together with the use of an electrical suspension mixing machine (Luckham Ltd, West Sussex, U.K.) prior to plating. Twenty-milliliter volumes containing 2000 cells and the appropriate amount of dust are inoculated into three replicate 80-cm^2 tissue culture flasks. The colonies are allowed to grow for 4 days, washed, fixed and stained as already described.

The colonies which resulted from both methods were counted by using an automatic colony counter (Artek Model 880). The toxicity of each treatment is expressed as the percentage survival relative to the control cultures and the 95% confidence limits calculated as described by Duncan and Brookes (*5*). For comparative purposes, the toxicities of dusts are shown in the results section as the amount of dust required to reduce the survival to 50% (S_{50}) of that of untreated control cultures.

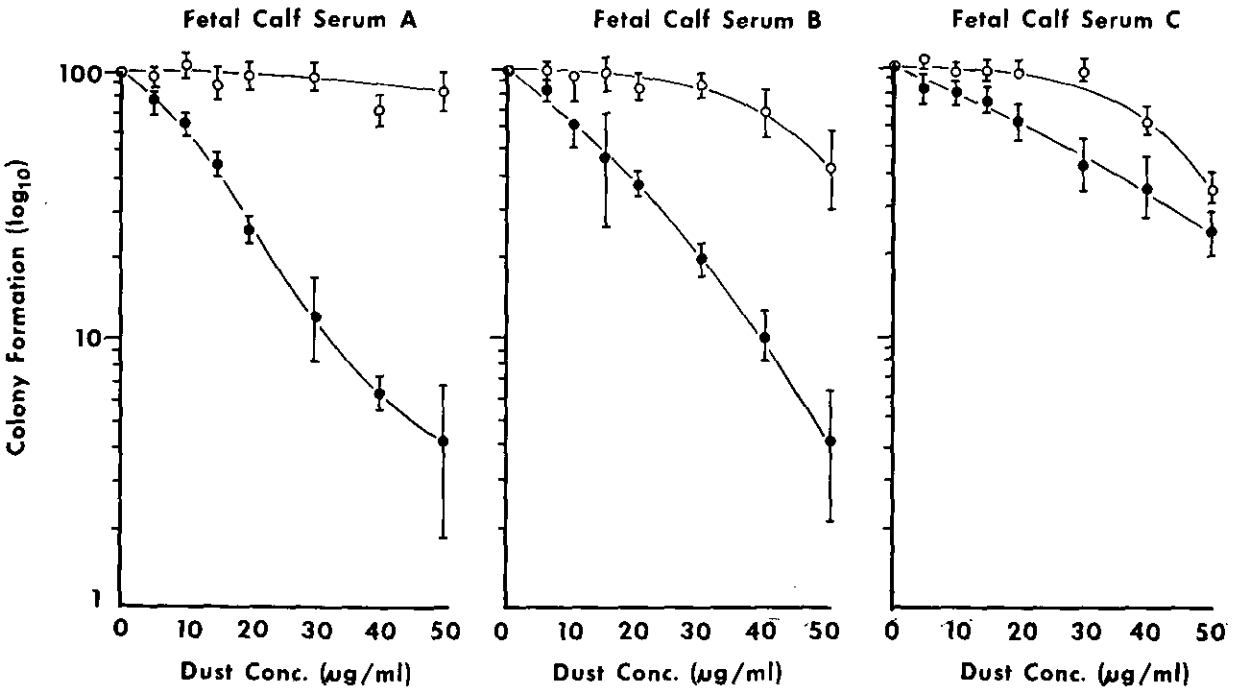

FIGURE 1. Cytoxicity of (○) quartz DQ_{12} and (●) crocidolite to Chinese hamster V79-P cells in different batches of fetal calf serum. Method A.

Results

Comparison of Different Batches of Fetal Calf Serum

The results obtained from assaying crocidolite and DQ12 by use of three different batches of fetal calf serum are shown in Figure 1. The absolute toxicities of the two dusts varied from batch to batch and this also affected the relative toxicities of the dusts. It is concluded that some sera allow better discrimination between the fibrous dusts, like crocidolite, and the nonfibrous dusts, like quartz.

Comparison of Methods

Dusts generally exhibited enhanced toxicities if tested by using assay method B rather than method A, but the magnitude of increase varied for different dusts. This is illustrated in Figures 2 and 3 which show results for anthophyllite and chrysotile assayed in V79-U and V79-P cells by both methods. With method B, both dusts were more toxic to the cells but the increase in toxicity of chrysotile was more marked than with anthophyllite. The overall results are presented in Figure 4, in which the S_{50} of each dust by method B is plotted on a scale on which the S_{50} derived by using method A is expressed as 1.0. Generally method B ranked the dusts as more toxic, but the increased toxicity was particularly marked with chrysotile.

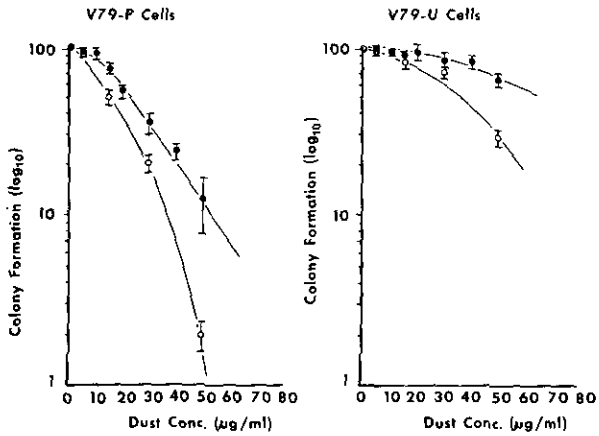

FIGURE 2. Cytotoxicity of anthophyllite to Chinese hamster V79 cells: (●) method A; (○) method B.

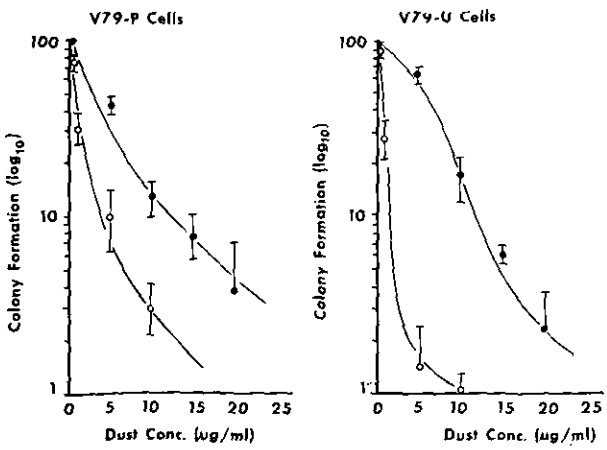

FIGURE 3. Cytotoxicity of chrysotile to Chinese hamster V79 cells: (●) method A; (○) method B.

Comparison of Cells

All five dusts were assayed by using the three types of V79-4 cells and the two methods. The results, summarized in Tables 1 and 2, are presented as the S_{50} of each dust. These results are presented in an alternative form in Figure 5, in which the S_{50} obtained with the V79-H and V79-P cells for each dust are plotted on a scale on which the S_{50} obtained with the V79-U cells equals 1.0. This method of displaying the results clearly shows that all the cells are equally sensitive to chrysotile but that V79-P cells are more sensitive to the remaining dusts by factors ranging from 2 to 8 times.

Discussion

The toxicity of a dust towards Chinese hamster V79-4 cells depends on several factors, some of which can be identified as affecting some dusts but

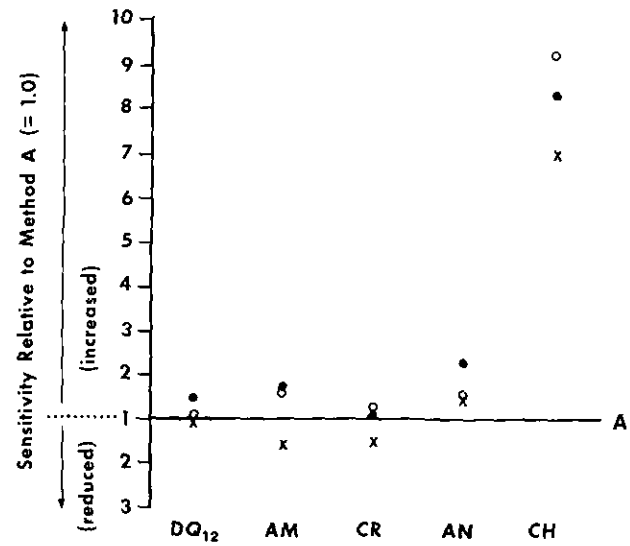

FIGURE 4. Influence of the method of exposing cells to dust on cytotoxic testing. The response of Chinese hamster V79-U (○), V79-H (•) and V79-P (×) cells exposed to dust by method B is plotted relative to the response of the same dusts by method A. Dusts as in Figure 5.

Table 1. Cytotoxic action of mineral dusts to Chinese hamster V79-4 cells: Method A.

Dust	S_{50} (dust concentration of 50% survival), μg/mL		
	V79-P	V79-U	V79-H
Chrysotile	5.0	6.0	6.0
Amosite	5.0	42.0	59.0
Crocidolite	14.0	43.0	45.0
Anthophyllite	23.0	59.0	100.0
Quartz (DQ12)	78.0	200.0	200.0

Table 2. Cytotoxic action of mineral dusts to Chinese hamster V79-4 cells: Method B.

Dust	S_{50} (dust concentration of 50% survival), μg/mL		
	V79-P	V79-U	V79-H
Chrysotile	0.72	0.66	0.60
Amosite	8.0	28.7	36.5
Crocidolite	21.0	35.7	40.0
Anthophyllite	15.1	40.2	42.7
Quartz (DQ12)	90.0	180.0	140.0

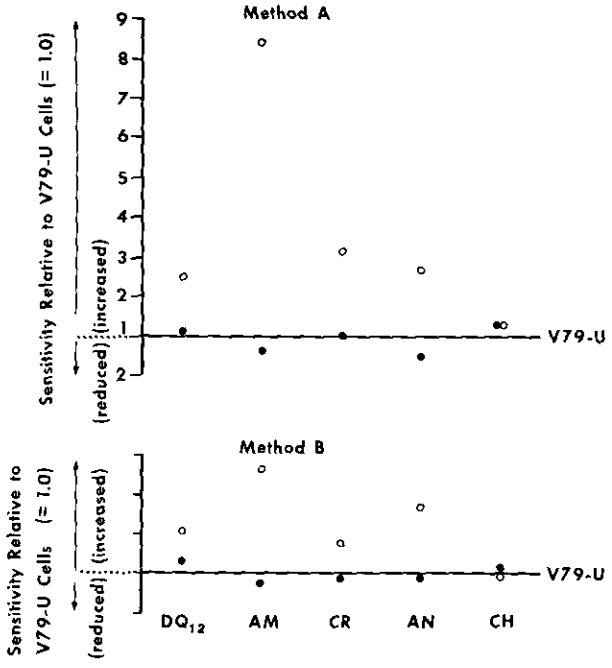

FIGURE 5. Relative sensitivity of Chinese hamster V79-4 cell lines to dust. The response of V79-U cells is plotted as equal to 1.0 in all cases. V79-P (○) and V79-H (•) cells were exposed to quartz (DQ_{12}), amosite (AM), crocidolite (CR), anthophyllite (AN) and chrysotile (CH) by methods A and B.

not others (*1*, *2*). For example, the toxicity of crocidolite, but not chrysotile, depends on whether the serum in the medium has been heat-inactivated. Similarly, V79-4 cells exposed to crocidolite, but not chrysotile, are more sensitive in suspension than cells already attached to a plastic surface.

We report here that dusts exhibit altered levels of toxicity in different batches of fetal calf serum; some sera allow better discrimination between fibrous and nonfibrous dusts than do others. It is therefore wise to test a batch of serum before purchase, not just for cell growth, but also to assay the toxicity of standard dusts.

Relatively minor differences in methodology alter the toxicities of all types of dust tested, in particular, chrysotile. There are several explanations which could account for the enhanced toxicities found by using method B. Cells and dust particles are mixed together by using a suspension mixing device. Exaggerated cell/dust interactions in suspension during the period of mixing (approximately 15 min) may be an important parameter. Chrysotile asbestos is particularly sensitive to changes in particle morphology induced by physical forces. Fibrillation of the chrysotile fibers may account for the seven- to ninefold increase in toxicity found by using method B. There are significant differences in the cell:dust and cell:area ratios resulting from the two methods. The amount of dust for each cell in suspension (expressed as μg/mL) shows a twofold increase in favor of method A. After initial plating, method A provides a growth area of 0.11 cm^2 cell, whereas method B provides only 0.04 cm^2/cell. However, neither of these factors can explain all of the observed differences. Albeit relatively trivial, a difference in methodology should be considered when interpreting results from independent laboratories.

Probably of more significance is the finding that the relative toxicities of dusts varied with the type of V79-4 cells. These results emphasize the need for caution when using V79-4 cells from different sources to establish toxicity assays for predicting the potential pathogenicity of inorganic dusts. Several explanations could account for the different sensitivities exhibited by the three V79-4 lines used here. The cell lines may have beome contaminated during their many years (at least 20) in culture by other cell lines, or the cells obtained from separate sources may be sublines diverging from the original V79-4 line. Experiments are in progress to characterize the trypsin-banded karyotype of each of the V79-4 lines to confirm their species of origin and to determine the nature of any changes. As mentioned in the introduction, there are several possible stages of interaction, some of which will be rate-limiting, between a cell and a dust particle. Let us consider the first of these stages; namely the attach-

ment of a dust particle to the cell membrane. Variations in the sensitivities of two cell types to a dust may be the result of the dust binding to the membrane of one kind of cell more avidly than to the other (assuming that subsequent steps are not rate-limiting). From the initial observations reported in this paper we have constructed a working hypothesis to explain the variations in sensitivity between the V79-4 cell lines and postulate that: (1) there are at least two distinct sites on the membranes of V79-4 cells to which dust particles can bind; (2) the amphibole dusts and chrysotile exhibit different affinities for two (postulated) binding sites; and (3) the two kinds of site occur in dissimilar ratios on the different cell surfaces.

Experiments which measure the rates of binding of chrysotile and the amphibole asbestos dusts to the three V79-4 cell lines are in progress to test this hypothesis.

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