Environmental Health Perspectives
Vol. 51, pp. 173-179, 1983

Effects of Nonfibrous Minerals in the V79-4 Cytotoxicity Test

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The use of the V79-4 system as a primary screen for fiber carcinogenicity is dependent on the observed correlation between cytotoxicity of the test material in the system and mesothelioma following intrapleural injection in animals. This correlation has been established for relatively pure samples of fibrous minerals. The wider application of the system as a screen for industrial dusts may depend on the ability of the system to show an unequivocal response to small yet significant percentages of fibrous dust in the presence of a large excess of nonfibrous material. Some materials tested, including platy minerals, show a response in the test which, while clearly less than that from UICC asbestos samples, could be construed as a positive result. Some, though not all, of these minerals show a nonlinear dose response in the test. This anomalous dose response may aid in the identification of some false positive results but the variety of responses to nonfibrous minerals places a limit on the predictive value of the test mixtures. Results obtained from mixtures of "standard" dust samples suggest this limit is reached at or above 10% fibrous content. Such levels would be significant in terms of human exposure. Extension of the concentration range tested does not improve the predictive value of the test.

Introduction

Epidemiological studies have established that exposure to certain fibrous minerals of natural occurrence is associated with an increased incidence of malignant mesothelioma of the pleura or peritoneum. Mesothelioma has also been induced in experimental animals by injection or implantation of a wider spectrum of mineral dusts. These experiments have been reviewed by Pott (*1*) and have lead various researchers to postulate that the physical dimensions of fibers may be an important factor in the induction of these tumors (*1-3*).

Such experiments require a long time-span for expression of carcinogenic activity and a significant commitment of resources in their execution and in this respect are suitable for neither the screening of large numbers of materials nor the investigation of response to experimentally produced fractions of fiber (when only very small quantities are available). The demonstration that a simple assessment of cytotoxicity to V79-4 Chinese hamster lung cells in culture correlates well with the results of injection or implantation tests (*4*) suggested that this may form a suitable primary screen for potential carcinogenic activity of mineral dusts. Subsequent investigations (*5*) confirmed the predictive value of the system for "pure" fibrous materials and its specificity in terms of fiber size (*16*)

A possible limitation of the system was demonstrated by Chamberlain et al. (*7*), in that dust from the Karain district of Turkey showed only a marginal cytotoxicity to V79-4 cells. This dust contained the fibrous mineral erionite which has been implicated in the high incidence of mesothelioma in the population of Karain district (*8, 9*). The implication of this result is that the other constituents present diluted the effect of the erionite fibers. This may indicate a limitation on the more general use of the test for mixed dusts where ideally a primary screen would be capable of an unequivocal response to small percentages of fibers in the significant size range in the presence of a large excess of (often nonfibrous) diluent. We have, therefore, assessed a number of mineral dusts in the system both alone and in combination with various percentages of UICC crocidolite asbestos in an attempt to establish limits for the predictive value of this test.

Materials and Methods

Dusts

A standard reference sample of UICC crocidolite asbestos (*10*) was supplied by the MRC Pneumo-

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coniosis Research Unit, Llandough Hospital, Penarth, South Glamorgan, UK. Samples of α quartz (Min-U-Sil and a sample of South African origin) were obtained from the same source.

Biotite, muscovite, phlogopite and hydrophlogopite (platy minerals of the mica series) were donated by Dr. S. J. Harris, ICI PLC, Mond Division, Runcorn Heath, Cheshire UK. Cristobalite was a laboratory reference sample believed to originate from the British Ceramic Research Association Stoke-on-Trent, Staffs, UK.

Calcium carbonate (marble chippings), talc and amorphous (precipitated) silica were standard laboratory reagents from various suppliers. The mica minerals and the marble chippings were reduced to powder with a mortar and pestle and then further reduced in a McCrone micronizing mill until most particles (as assessed with the light microscope) were <20 μm (with the majority <5 μm).

Stock suspensions (20 mg/mL) in physiological saline were prepared by ultrasonic dispersion and further dilutions of these suspensions (resuspended by ultrasonication as necessary) were prepared as required. Where mixtures of dusts were to be tested, the stock suspensions were mixed in the appropriate proportions to give a uniform total dust concentration throughout the series. Thorough mixing was ensured by sonication and the mixtures were then tested as required in an identical manner to the "pure" mineral samples.

Cell Lines

V79-4 chinese hamster lung cells were obtained from Dr. M. Chamberlain, MRC, Pneumoconiosis Research Unit, Penarth, and were grown in minimal essential medium (MEM, Gibco Biocult or Flow Laboratories) supplemented with 15% fetal bovine serum and antibiotics. Cells were routinely passaged in 75 cm^2 plastic flasks (NUNC, Gibco Europe)

Cytotoxicity Assay

This assay was performed essentially as described by Chamberlain and Brown (4). Survival of cells was determined by cloning efficiency from a single cell suspension. Cell suspension (20 mL) was added to the appropriate quantity of dust (in 1 ML of physiological saline) in a sterile McCartney bottle. Aliquots (5 mL) of this suspension were seeded into 60 mm diameter Petri dishes (NUNC) and the surviving cells allowed to grow and form colonies for 5-6 days. Colonies were then washed with physiological saline and fixed in 10% buffered formol saline prior to staining with 1% methylene blue. The number of colonies on each dish was counted with Artek Counter Model 880, (Artek Systems Corp, Farmingdale, NY 11735). The detection limit was such that colonies with more than 40-50 cells were counted.

In common with other laboratories using this technique (M. Chamberlain and I. P. Gormley, personal communications), we observed that the cloning efficiency was dependent on the batch of fetal bovine serum used. We therefore preselected batches of serum which gave a high efficiency with untreated cells and used the minimum practical number of such batches in the course of these experiments. A "positive" and "negative" (on the basis of previous experience) control dust was included in each experiment. The following (arbitrary) rules were then adopted to identify atypical experiments. Experiments were rejected where either (a) the values for control (untreated) plates showed a standard deviation in excess of 20% or (b) the CD_{50} (concentration of dust causing a 50% reduction in plating efficiency) was outside specified limits for either positive or negative control materials. This resulted in the rejection of only a

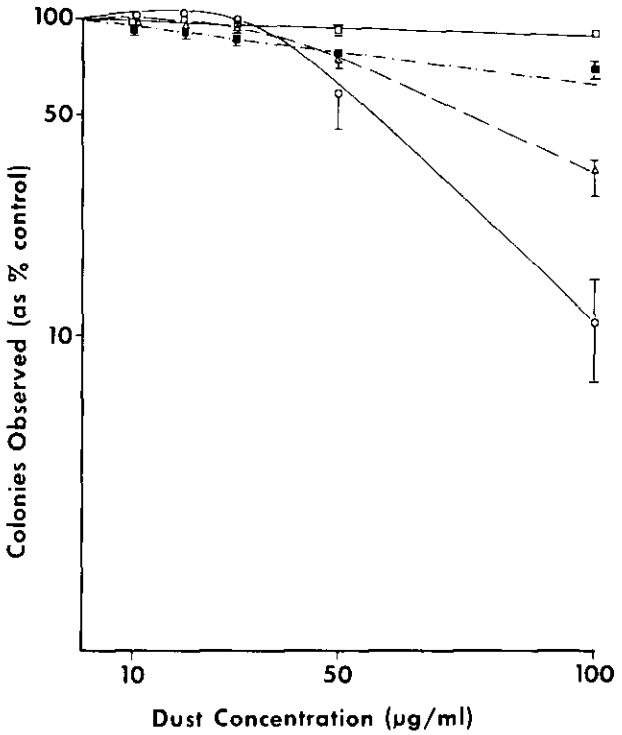

FIGURE 1. Effects of various physical forms of silica in the V79-4 cytotoxicity assay. The dust concentration given represents the quantity present in the cell suspension before plating. Colonies were allowed to develop for 5-6 days before counting. The number of determinations (*n*) is indicated in parentheses for each material (□) cristobalite (2); (△) α-quartz (Min-U-Sil) (9); (■) α-quartz (South African) (5); (○) amorphous (precipitated) silica (4). The standard errors are shown except where overlap would occur or errors are within the symbol.

very few experiments, and in all such cases there was some indication of an underlying contamination of the cultures.

Results

Response to Single Minerals

Silica Samples. The various samples of silica tested showed a spectrum of response which was consistent for a particular sample but varied with sample origin (Fig. 1). In samples where significant cytotoxicity was observed, there was a biphasic response, with little or no effect at dust concentrations up to 30 μg/mL and a progressive response beyond this point. This was most clearly observed with the sample of amorphous silica but was evident to a lesser degree with α quartz (Min-U-Sil). However the second (South African) quartz sample showed a lesser response than Min-U-Sil, while the cristobalite sample was practically inert.

Platy (Mica) Minerals. Phlogopite, hydrophlogopite and biotite all showed a similar minimal response with a CD_{50} value in excess of 100 μg/mL. Talc showed a qualitatively similar response, but was somewhat more cytotoxic. Muscovite produced a slightly different response, resembling that seen with Min-U-Sil, with some indication of a plateau followed by a progressive cytotoxicity (Fig. 2).

Crocidolite. The sample of UICC crocidolite was highly cytotoxic. There were, however, some variations between different stock suspensions prepared from the same sample. Thus, the recorded CD_{50} varied between extremes of 7 μg/mL, in agreement with Chamberlain and Brown (4), and 30 μg/mL (Fig. 3). In all cases the results were reproducible for a given stock suspension.

Calcium Carbonate. The micronized marble chippings were inactive in the test system (Fig. 3).

Response to Mixtures

Three mixtures were examined. These consisted of UICC crocidolite diluted with calcium carbonate, South African quartz or biotite. The stock suspension of crocidolite in the preparation of these mix-

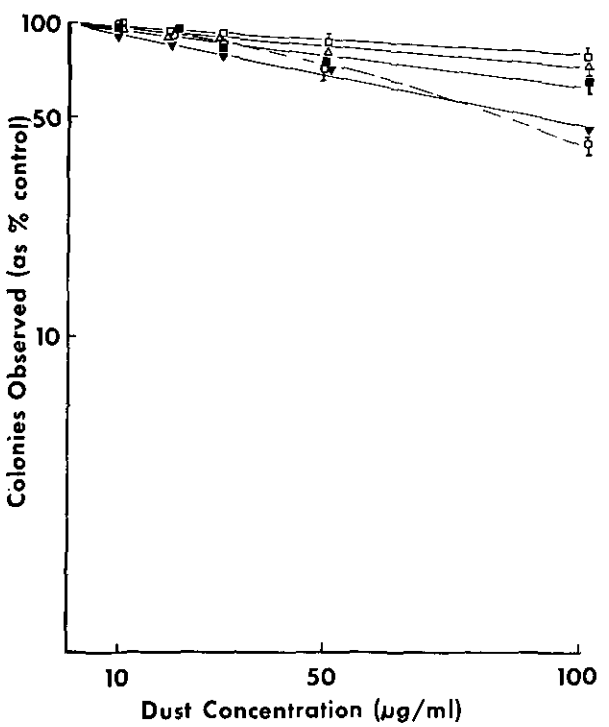

FIGURE 2. Effects of various platy minerals in the V79-4 cytotoxicity assay: The number of determinations (n) is indicated in parentheses for each material: (□) hydrophlogopite (15); (■) phlogopite (9); (▼) talc (1); (△) biotite (12); (○) muscovite (6) (shown with a broken line). The standard errors are shown except when the values fall within the symbol.

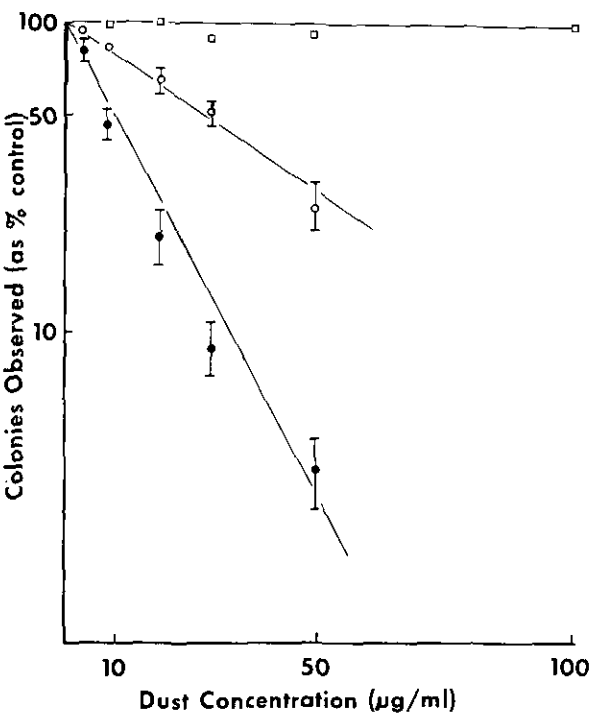

FIGURE 3. Illustration of the range of cytotoxicity encountered with preparations of UICC crocidolite: (□) calcium carbonate (3); (○) UICC crocidolite, sample with low toxicity (9); (●) UICC crocidolite, sample of high toxicity (21). Results shown represent the observed extremes with samples from the same batch of UICC crocidolite. Each preparation gave consistent results when repeated (the number of determinations for each sample is indicated in parentheses). An example of the response to the noncytotoxic material (calcium carbonate) is shown for comparison. Standard errors are indicated by bars except when they fall within the symbol.

tures was of relatively low cytotoxicity (corresponding to the middle line in Fig. 3.) As expected, the cytotoxic effects of the mixtures were dominated by the crocidolite content in all cases. Total cytotoxicity varied depending on the nature of the second component. The value approached that of the diluent at an inclusion level of 10% crocidolite in each case. Mixtures with calcium carbonate as the diluent showed the clearest and most reproducible response (Fig. 4). Where biotite (Fig. 5) or α-quartz (Fig. 6) formed the major component, the results were more variable. There was no evidence of either enhancement or suppression of the cytotoxic effect of crocidolite in any of the mixtures tested. If the results (corrected for cytotoxicity attributable to the diluent when appropriate) are plotted in terms of the crocidolite concentration, the resulting curve is essentially the same as that due to crocidolite. An example of such a curve (for calcium carbonate/crocidolite mixtures) is shown in Figure 7.

Having established the specificity of response in the system, we then attempted to enhance the detection limit for crocidolite in these mixtures by an extension of the range of concentrations tested to a maximum of 1000 μg/mL. In the first instance, the "pure" diluent materials were tested in this range and the results are shown in Figure 8. Four points emerge. First, all dusts show some cytotoxicity at the extreme concentrations. Second, the divergence between calcium carbonate and biotite is considerable, though both of these dusts are regarded as relatively inert up to 100 μg mL. Third, a nonlinear response from South African quartz was evident at these higher concentrations. Fourth, repeat experiments produce less consistent results than in the lower concentration ranges. The wide range of cytotoxicity shown by the diluent dusts precludes any prediction for blind testing of mixtures. However, in the case of calcium carbonate/crocidolite mixtures it was possible to detect a 5% crocidolite inclusion even with the crocidolite stock suspension of relatively low initial activity although this is much clearer with a high activity inclusion (Fig. 9). Careful scrutiny of the figures

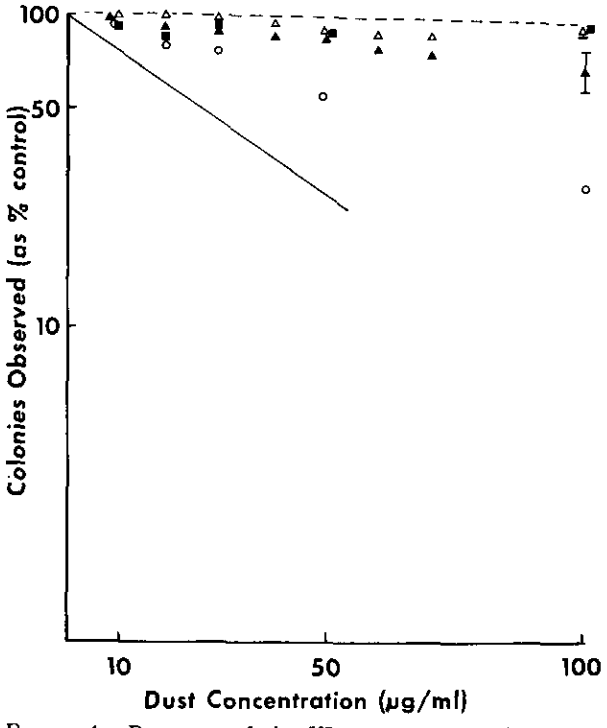

FIGURE 4. Response of the V79-4 system to mixed dusts. Mixtures of calcium carbonate and UICC crocidolite in the proportions indicated were assessed in the test: (——) typical response of the sample of UICC crocidolite used in the mixtures; (— —) response to calcium carbonate in this system. For clarity the data points for these lines have been omitted. Mixtures tested were at various ratios of calcium carbonate:crocidolite: (■) 95:5; (△) 90:10; (▲) 75:25; (○) 50:50. Errors are shown for the top concentration (except when these are within the symbol) but have been omitted from intermediate results for clairity.

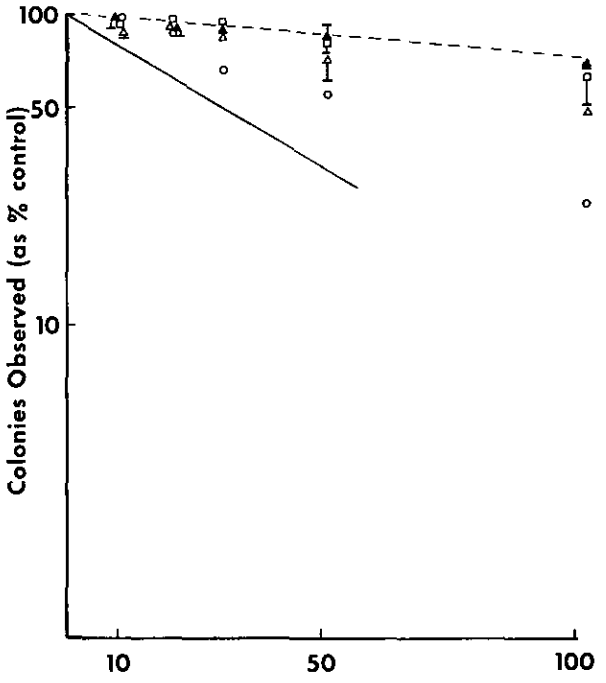

FIGURE 5. Response of V79-4 system to mixed dusts for mixtures of biotite and UICC crocidolite: (——) typical response of the sample of UICC crocidolite used in the mixtures; (— —) response to biotite. For clarity the data points for these lines has been omitted. Mixtures were at various ratios of biotite:crocidolite: (▲) 95:5; (□) 90:10; (△) 75:25; (○) 50:50. Standard errors are shown except where overlap would occur or errors are within the symbol.

indicates that there is some dilution of the effect which might be expected from the crocidolite alone at very high levels of total dust.

Discussion

These experiments confirm the earlier findings of Chamberlain et al. (*5*) and Brown et al. (*6*) in that nonfibrous dusts showed low cytotoxicity in the test system. The response to some forms of silica and to muscovite mica was, however, rather different in character from that of the other dusts tested and in terms of CD_{50} they showed greater cytotoxicity than is usually associated with materials classified as inactive by intrapleural injection into animals. However, in these cases, the dose response observed deviated from that usually observed with some suggestion of a threshold effect. The reason for this has not been ascertained but the relative activities of the various forms of silica tested suggest that crystallinity may be an important factor. Amorphous (precipitated) silica showed the highest level of cytotoxicity. The ultimate particles in this material are of the order of 100 nm, and hence this preparation would be expected to show some colloidal properties in aqueous media whereas the crystalline forms would not. Amorphous silica is also cytotoxic in other cell systems (*10, 11*) and is hemolytic. This latter effect is thought to be related to the interaction of colloidal silica with the erythrocyte membrane (*12*) and may be indicative of a general property of hydrated amorphous silicas to disrupt membrane function of cells in culture. The effect is probably not significant in terms of human exposure by inhalation, and amorphous silica is generally accepted as less hazardous than the crystalline forms. Whatever the reason for the observed response, the shape of the curve allows materials of this type to be differentiated from the cytotoxic fibers which induce mesothelioma in experimental animals.

With the exception of the anomalous response to the above minerals, our experiments show that cytotoxicity to the V79-4 cell line is highly specific. In the concentration range up to 100 μg/mL the observed cytotoxicity of mixed dusts was predominantly attributable to the crocidolite content. While this does not hold at higher dust levels, the response to inert materials at these very high concentrations was considerable and thus precludes this extension of the test system except in carefully defined circumstances.

The results also show that the system does not by itself offer a satisfactory primary screen for

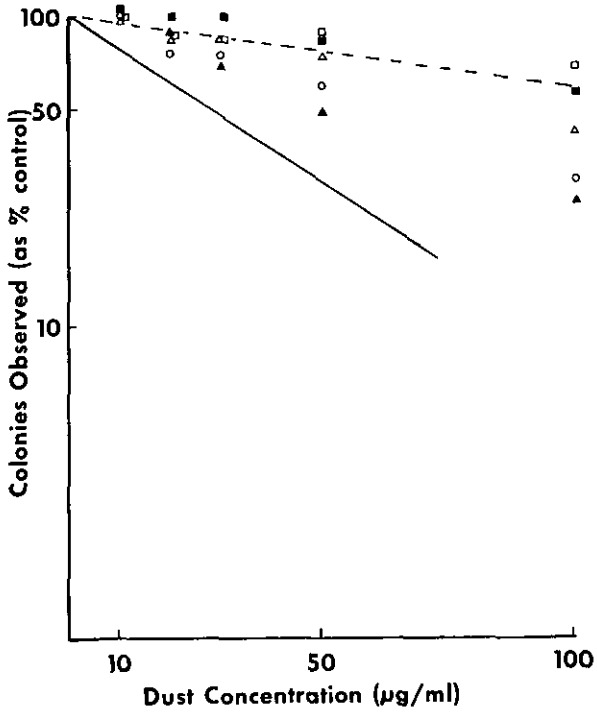

FIGURE 6. Response of V79-4 system to mixed dusts for mixtures of α quartz (South African sample) and UICC crocidolite: (——) the typical response of the sample of UICC crocidolite used in the mixtures; (– –) response of α-quartz (South African). For clarity the data points for those lines have been omitted. Mixtures tested were at various ratios of α-quartz: crocidolite: (□) 95:5; (■) 90:10; (△) 80:20; (▲) 70:30; (○) 50:50. Bars indicate standard error but are omitted when overlap would occur or errors are within the symbol.

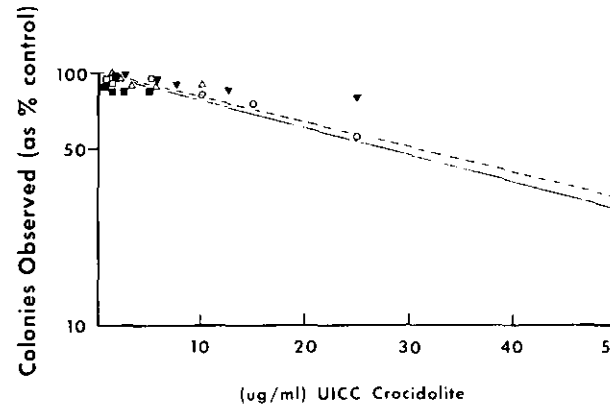

FIGURE 7. Response of mixtures of calcium carbonate and crocidolite expressed solely in terms of crocidolite content: (——) the typical response from the sample of UICC crocidolite used in the mixtures. Data points for this line have been omitted for clarity. Points shown are derived from those in Figure 4 and represent a full spectrum of the ratios tested. Points were derived for a mixture of calcium carbonate:crocidolite in the ratios: (□) 99:1; (■) 95:5; (△) 90:10; (▼) 75:25; (○) 50:50. The broken line (– –) is a linear regression calculated from the above volumes.

mixed dusts. A detection limit of about 10% asbestos in a mixture would not be satisfactory in these terms. Where the components of a mixture are known, the test may be more sensitive. Thus, where a highly cytotoxic fiber is present in an essentially inert diluent, a detection limit of 5% or less is readily achieved (Fig. 9). Under such conditions the system offers considerable advantages as a simple, rapid assay. The test is also of potential value in classification of relatively large numbers of samples of fibrous materials, particularly where there are similar chemical impurities. In such cases it may be possible to use the test to identify particular samples or groups of samples which show a variation in activity and thus provide a rational basis for selection of representative materials for further testing.

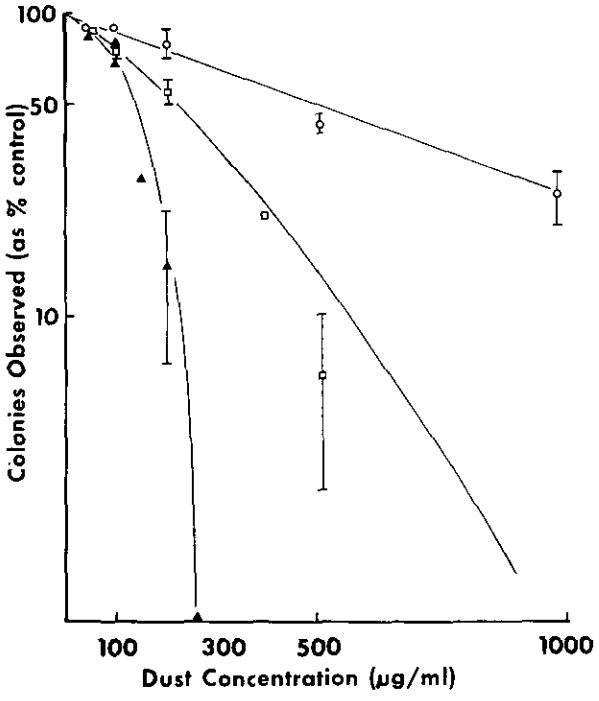

FIGURE 8. Cytotoxicity of various materials at an extended concentration range. The diluent materials used in mixtures were tested at elevated concentrations. The response shown represents the mean value of the number of determinations shown in parentheses: (○) calcium carbonate (2); (□) biotite (2); (▲)α-quartz (South African) (2). Bars indicate standard error (except where these are within the symbol).

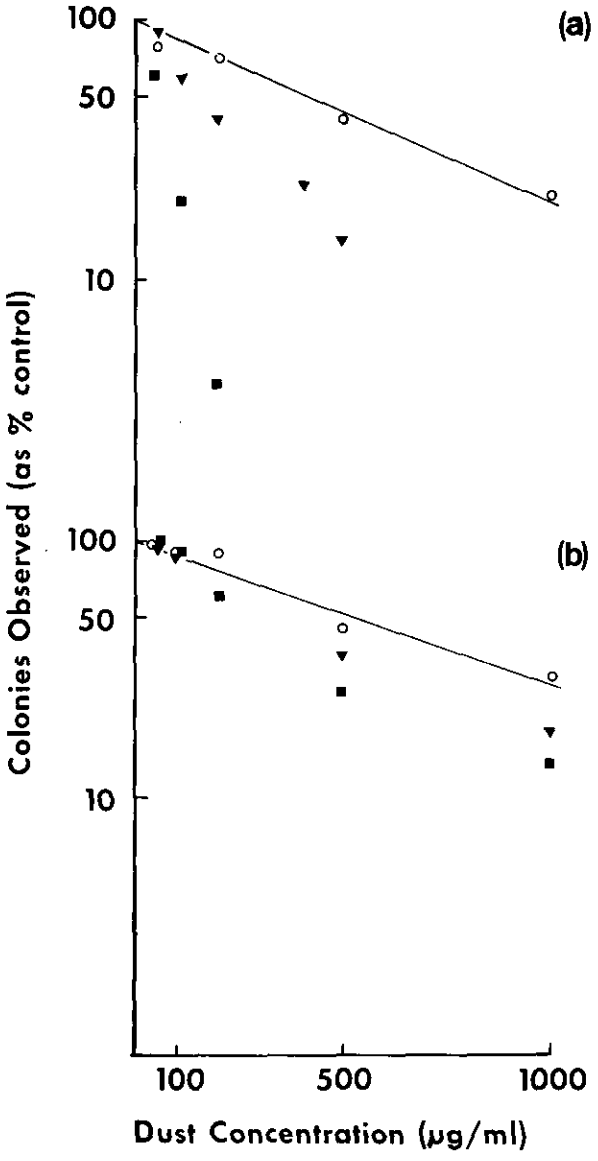

FIGURE 9. Effects of mixtures of calcium carbonate with crocidolite at an extended concentration range: (a) mixtures where a proportion of crocidolite of relatively high cytotoxicity was used; (b) results for mixtures which included a crocidolite preparation of low toxicity (these materials correspond to the two curves for crocidolite shown in Fig. 3). The solid line is the response observed with pure calcium carbonate. In each case the points represent the same ratio of calcium carbonate to crocidolite in (a) and (b): (○) 100% calcium carbonate; (▼) 95:5 calcium carbonate:crocidolite; (■) 90:10 calcium carbonate:crocidolite.

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