

CYTOTOXIC EFFECTS OF SOME MINERAL DUSTS ON SYRIAN HAMSTER PERITONEAL MACROPHAGES

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The destruction of macrophages by certain phagocytosed mineral dusts is the first step in the sequence of events leading to pulmonary lesions in experimental animals and man (1-3). Later on, secondary interactions between phagocytic cells and other cell types, for example fibroblasts, take place (4) and appear to lead to the ultimate fibrosis. Although the cytotoxicity of various modifications of silica and asbestos fibers has been studied extensively on phagocytic cells in vitro, the time of onset and the extent of damage are still under discussion. While some authors report extensive damage within minutes after the in vitro phagocytosis of certain mineral dusts (principally silica) (5-10), others find that these cells are impaired only when incubated with the dust for long periods or with large amounts of it (11-15).

There appear to be two reasons for this: firstly, the origin, the physicochemical composition, and the pretreatment of the mineral dust samples used in the different studies vary (1, 16-18); secondly, the conditions under which macrophages have been kept in vitro and incubated with dusts have varied considerably from study to study. Generally, macrophages which are brought into contact with dust soon after they have been harvested react more severely to the dust in serum-free medium, as is shown by a decrease of metabolic activity (5-8, 10) and the release of enzymes (9, 19, 20), than cells which have been kept with serum and under culture conditions adequate for long-term maintenance (11-15).

It has been established that phagocytic monocytes are derived from blood monocytes which have migrated into body cavities and tissues (21). The phagocytic monocytes differentiate into mature macrophages without division. Thus, macrophages obtained from various sources, for example from the lung or the peritoneum, do not vary greatly in morphological appearance and phagocytic properties although differences in metabolic pattern and enzyme loads have been described (22). Under appropriate conditions, the maturation of phagocytic monocytes into macrophages also takes place in cell culture where the events mirror those seen in the inflammatory lesion (21).

In the present work a convenient technique for the long-term maintenance of macrophages in culture and the effects of various mineral dusts on these is described. The morphology of individual macrophages after phagocytosis of two different modifications of silica and of three different types of asbestos fibers was examined by photography of fixed and stained cells at various times

after incubation. In addition an attempt was made to determine quantitatively by means of light microscopy the survival rates of macrophages exposed to dusts and the influence of some modifications of these.

Materials and Methods

Media.—Eagle's minimal essential medium (MEM)¹ containing 1.8 mM CaCl₂, 0.1 mM glycine, 0.1 mM serine (23), and 200 units/ml penicillin and 100 units/ml streptomycin was used throughout. Medium A consisted of MEM supplemented with 10–40% heat-inactivated bovine serum, and medium B consisted of medium A supplemented with 10% bovine serum but preconditioned by 24 hr contact with a growing culture of SA7 virus-transformed hamster skin fibroblasts (24). After being decanted from the cells, the conditioned medium was centrifuged for 15 min at 800 g and stored at –20°C.

Harvest of Cells.—Male or female Syrian hamsters about 6 months old were killed by cervical fracture and placed in a sterile cabinet. The abdomen was swabbed thoroughly with 70% alcohol, a skin flap of 2 cm² was carefully removed, and 20 ml MEM without serum but with 5 IU heparin/ml was injected into the peritoneal cavity. In order to avoid fibroblast contamination, the abdomen was not massaged. After 20 min a cell suspension was aspirated from the peritoneal cavity and used for culture.

Cell Culture of Macrophages.—The cell suspension was centrifuged at 800 g for 5 min. For culture purposes, 5 cm diameter Petri dishes containing either one 22 × 40 mm cover slip, or nine round 10 mm diameter cover slips (both from Chance Bros. Ltd., Smethwick, Staffs., England), both pretreated with hot 4% NaOH and washed thoroughly in distilled water, were used. Before sterilization at 160°C in a hot-air oven, the 10 mm cover slips were attached to the floor of the dish with DePeX (George T. Gurr, Ltd., London N. W. 9, England), diluted 5:1 with xylene. Enough DePeX was used to cover the entire floor of the dish with a thin layer of mounting medium. It had been established earlier that DePeX medium is not toxic to cells in culture. The larger cover slips were allowed to remain loose in the dish. Cells obtained from one animal were resuspended in 15 ml of appropriate medium and 5 ml of suspension were inoculated into each of the Petri dishes. These were then placed on specially designed racks and kept at 37°C in desiccators containing 1–2 g dry ice (25).

In order to study the influence of serum and conditioned medium on the maturation of mononuclear phagocytes into macrophages, the harvest of cells from one animal was divided into three equal parts, centrifuged, and resuspended in medium A containing 10, 20, or 40% serum, or in medium A plus B (1:1) containing final amounts of 10, 15, or 25% serum. The cells were planted in three Petri dishes, each containing one large cover slip (22 × 40 mm), and were fixed on the 4th day after planting.

In order to investigate the number and condition of normal untreated macrophages over 10 days, cells were planted in medium A supplemented with 40% serum. The harvest from a single animal was planted into three Petri dishes containing nine 10 mm diameter cover slips. Single cover slips were removed daily for fixation. After 4 days of maturation, the medium was changed by removing 2.5 ml of old medium and replacing it with the same amount of medium A supplemented with 10% serum only. Thereafter, 1 ml of old medium was replaced every day with new MEM supplemented with 10% serum.

For studies of the toxicity of various samples of dust, this same culture procedure was adopted. Each experiment was carried out on the peritoneal harvest of a single animal. On the 5th day after planting, one or more control cover slips were removed from each dish and fixed. 100, 200, or 400 μg of dust, suspended in 0.1, 0.2, or 0.4 ml of tryptose phosphate broth (Difco Laboratories Inc., Detroit, Mich.), respectively, were then added to about 5 × 10⁵

¹ Abbreviation used in this paper: MEM, Eagle's minimal essential medium.

mature macrophages per dish. All volumes were made up to 0.4 ml of tryptose phosphate broth per dish. The contents were swirled by hand to ensure good mixing. Cover slips were fixed at various intervals of time for examination and cell count.

In an attempt to determine whether mitosis of macrophages was occurring to any marked extent, 2 or 4 μg of colchicine (British Drug Houses, Ltd., Poole, Dorset, England)/ml of medium was added to 2- or 3-day old cultures which had previously been given 200 μg of Snowit silica/ 5×10^5 cells. The cells were left in contact with the colchicine for 2.5 or 4 hr, respectively, then fixed, stained, and examined for mitotic figures.

Dusts.—The two forms of silica, kindly provided by the Safety in Mines Research Establishment, Sheffield, England, were: (a) reground and freshly alkali-etched crystalline silica Snowit (X9947) of size range 15% 2.5 μ , 49% 2.5–5 μ , 30% 5.0–7.5 μ , 5% 7.5–10 μ , and of specific surface area 6.79 m^2/g ; and (b) alkali-etched amorphous silica Fransil (X5843 from X5663) of size range 0.1–0.5 μ and of specific surface area 32.82 m^2/g .

The asbestos dusts were standard, finely ground UICC reference samples (26) kindly provided by the Pneumoconiosis Research Unit of the South African Medical Research Council, Johannesburg. The following specific surface areas were obtained: crocidolite 8.88 m^2/g ; amosite 5.55 m^2/g , and after elimination of large fibers by flotation and sedimentation 12.39 m^2/g ; chrysotile 23.54 m^2/g , and after elimination of large fibers by flotation and sedimentation 34.18 m^2/g . Surface areas of all dusts were determined by krypton adsorption.

Dusts were prepared freshly before each experiment by suspending 10 mg in 10 ml of tryptose phosphate broth.

For certain experiments, suspensions of 10 mg of Fransil silica or 10 mg of chrysotile in 8 ml of tryptose phosphate broth were prepared. The suspensions were boiled in a water bath for 15 min and then left at 37°C overnight. This procedure was repeated two to three times after which the suspensions were made up to 10 ml with tryptose phosphate broth and kept at –20°C until used.

Staining Procedure.—Cover slips were washed briefly in 0.95% NaCl, fixed in 1% glutaraldehyde diluted with 0.95% NaCl, stained with Ehrlich's hematoxylin (Gurr) for 20 min, and mounted in DePeX (Gurr).

Cell Counts.—Fixed and stained cultures were used to evaluate the influence of the amount of serum and conditioned medium on the spreading ability and maturation of macrophages, and also to determine the survival rates over 72 hr of macrophages incubated with various amounts and different preparations of silica and asbestos dusts. The average number per cm^2 of cells which had spread out, that is cells with ruffled membranes and extended pseudopodia, was obtained by making counts under a 40 \times objective in 20 areas equally distributed over the entire cover slip. This gave a total of 600–800 cells per control count which was then taken as the 100% value for that particular experiment. Cells without any pseudopodia or ruffled membranes were considered pyknotic or dead and were not counted. The effective amount of dust was calculated as 36% of the amount added because the nine glass cover slips only covered 36% of the area of the dish.

Photography.—Photography under the light microscope (Zeiss Photomicroscope II) of fixed and stained preparations was carried out with pan F film (Ilford Ltd., Ilford, Essex, England).

RESULTS

Macrophage Culture Conditions.—Throughout the study cells were obtained without prior stimulation of the animal with glycogen. In order to find the best possible culture conditions for establishing hamster peritoneal macrophages in

vitro, cells were planted in medium supplemented with varying amounts of inactivated bovine serum. A second medium was made up of 50% of the above medium and 50% of conditioned medium (the supernatant of tumor cell cultures).

After inoculation of the peritoneal harvest into the dish, the phagocytic monocytes became attached to the glass, established themselves there, and matured into true macrophages with ruffled membranes and pseudopodia within 4 days. This took place most effectively and in greatest number in either me-

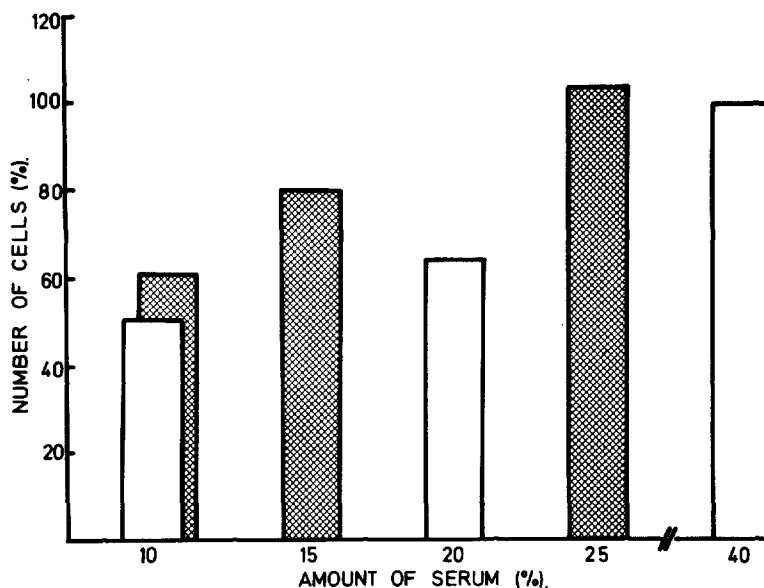


FIG. 1. Number of macrophages (as %) after 4 days of culture in MEM supplemented with various amounts of serum (open histograms), and in MEM supplemented with the same amounts of serum but containing 50% conditioned medium (cross-hatched histograms).

dium with a high serum content or in medium with a high serum content and also containing 50% conditioned medium (Fig. 1).

The average number of macrophages/cm² on the 4th day under these conditions was $7-8 \times 10^4$ cells/cm² when cells from the peritoneum of a single hamster were planted into three 5 cm diameter Petri dishes, each containing one large glass cover slip (22 × 40 mm). Cells kept in medium with a low serum content and no conditioned medium were few in number and very often pyknotic.

Hamster peritoneal macrophages were maintained in culture for at least 10 days without any gross change of morphology or marked decrease in number. Fig. 2 shows the number of cells per cm² from the 1st to the 10th day after planting. The time in which cytotoxicity tests were carried out is indicated.

It can be seen that the period between the 5th and 8th days is clearly the most suitable for such tests.

Effects of Standard Dusts on Macrophages.—Macrophages on the 5th day of culture (Fig. 3 A) were incubated with moderate amounts of crystalline silica Snowit, amorphous silica Fransil, and with standard UICC samples of asbestos. Figs. 3 B–3 F show that these dusts had different effects on the cells. Macrophages which had taken up crystalline silica survived for at least 3 days without

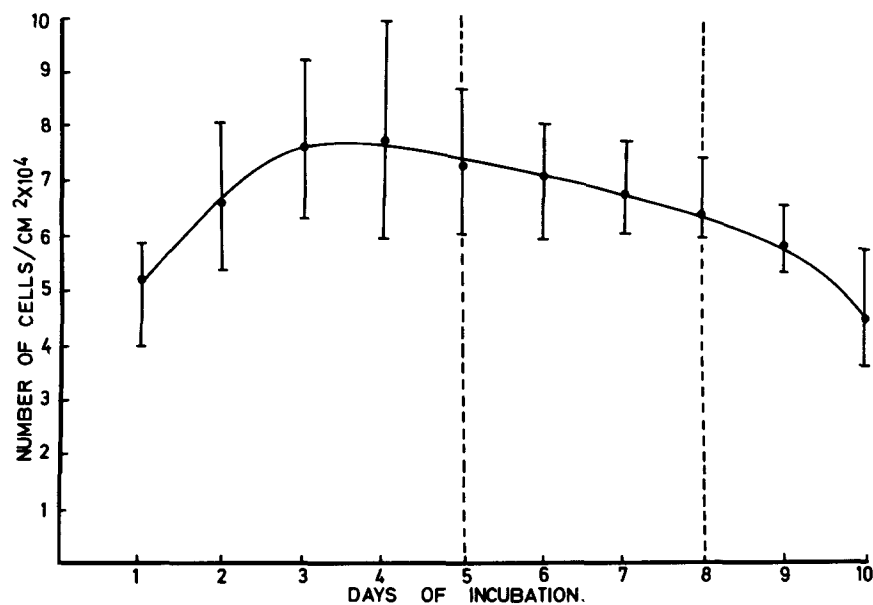


FIG. 2. Number of macrophages/cm² glass cover slip between the 1st and the 10th day after planting in MEM supplemented with 40% serum. Determinations of cytotoxicity of dusts were carried out between days 5 and 8.

showing any cytotoxic effect, whereas amorphous silica, even in small amounts, was toxic within hours of ingestion. Cells which had phagocytosed amosite and crocidolite showed no specifically damaging effect although a certain number of cells were lost from the culture within 3 days due to overingestion of particles. Chrysotile, however, exhibited a typical cytotoxic effect virtually from the start of the experiment.

(a) *Crystalline silica Snowit*: Certain preparations of this type of quartz have been reported to be immediately toxic when phagocytosed by macrophages (5, 6, 9). In the present study however, although particles were actively and uniformly taken up, about 90% of the cells remained morphologically intact insofar as ruffled membranes, extended pseudopodia, and active movement were

concerned (Fig. 4). Hours after the phagocytosis of fairly large amounts of dust, the cells looked almost healthier than the controls (Fig. 3 *B*).

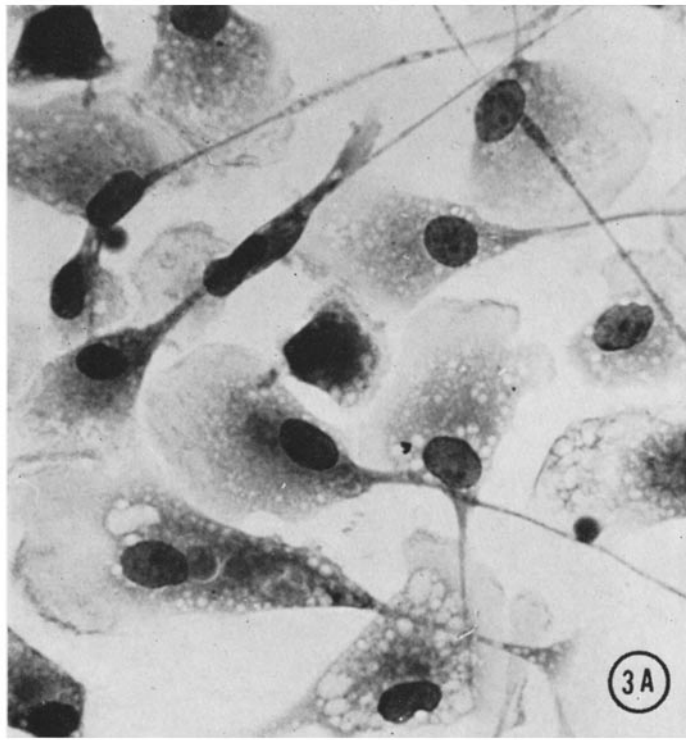
(*b*) *Amorphous silica Fransil*: Unlike the crystalline silica described above, Fransil showed a typical cytotoxic effect within an hour of incubation, even at low concentration (Fig. 3 *C*). This damaging action was first recognized by the foaming appearance of the cytoplasm. Later, the outer membranes appeared to disintegrate and the contents of many of the cells clumped together to form amorphous masses of granular material containing scattered pyknotic nuclei. As Fig. 5 shows, half of the macrophages were killed within 18 or 12 hr, respectively, when fed with 72 or 144 μg of Fransil/ 5×10^5 cells.

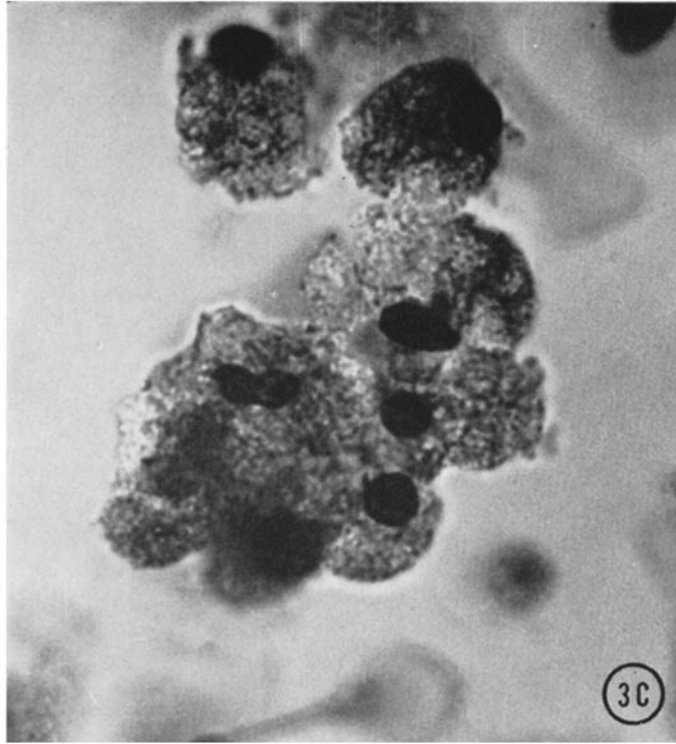
(*c*) *Amosite*: This asbestos dust, even when given to macrophages in amounts which allowed each cell to phagocytose at least several fibers, did not give rise to any typical cytotoxic effect (Fig. 3 *D*). Some loss of cells, however, occurred in the cultures over 3 days as is illustrated in Fig. 6. This is probably due to the fact that some cells had phagocytosed more than others and had rounded up and floated off the cover slips. The remnants of these cells, together with the dust which they had phagocytosed, could be taken up by other macrophages. This recycling of asbestos fibers seems to be independent of the amount of dust given to the culture within ranges of concentration allowing each cell to phagocytose at least several fibers.

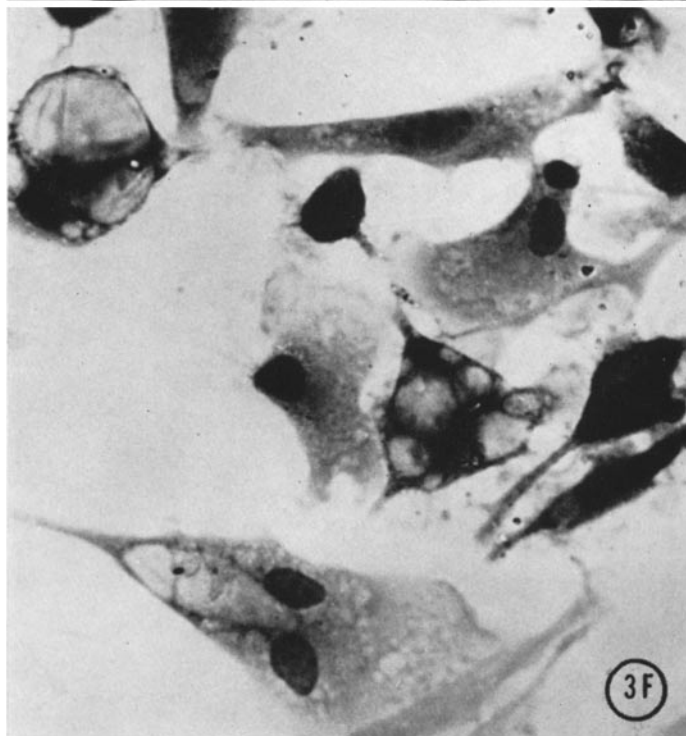
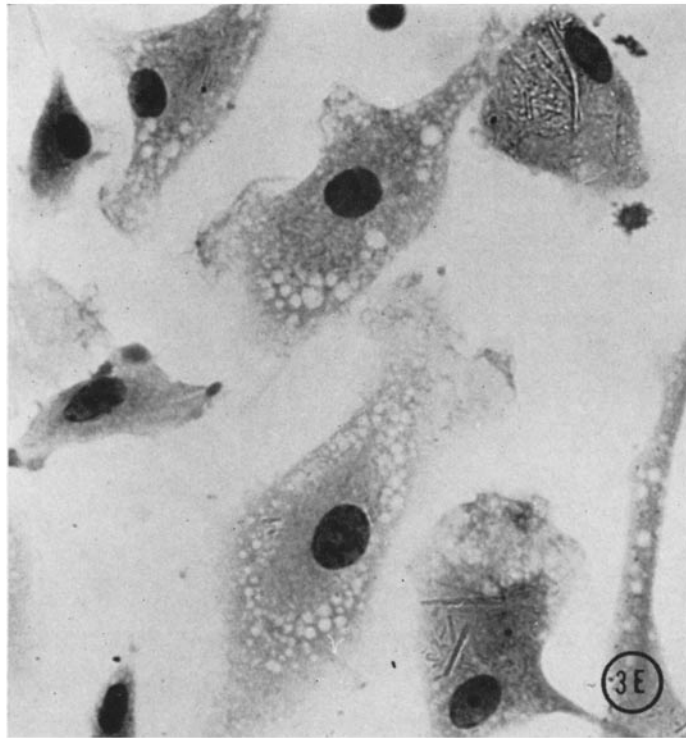
(*d*) *Crocidolite*: This dust had almost the same effect on macrophages as amosite (Fig. 3 *E*). In this case too, cells were reduced in number (Fig. 7). Those remaining on the cover slips had ingested large amounts of fiber, again suggesting a recycling of dust derived from dead cells which had earlier ingested excessive amounts.

(*e*) *Chrysotile*: In contrast to the effects of amosite and crocidolite, chrysotile had a specific cytotoxic effect on hamster peritoneal macrophages. This first became evident in a vacuolization of the cytoplasm (Fig. 3 *F*), the collapse of ruffled membranes, and the appearance of large numbers of pyknotic cells stuck together in lumps. It was found that the effect of chrysotile depends on the amount given and the length of time during which the cells are exposed to the dust (Fig. 8). For example, 72 μg of fiber had killed 50% of the 5×10^5 cells per dish by the end of 36 hr. This decline should be seen against the much

FIG. 3. Hamster peritoneal macrophages fixed and stained with hematoxylin. $\times 480$. All treated cells were given dusts on the 5th day of culture. *A*. Untreated cells on 6th day of culture. *B*. Treated with crystalline silica Snowit (144 $\mu\text{g}/5 \times 10^5$ cells). Photographed 72 hr after administration of dust. *C*. Treated with amorphous silica Fransil (72 $\mu\text{g}/5 \times 10^5$ cells). Photographed 4 hr after administration of dust. Note pronounced cytotoxic effects. *D*. Treated with UICC amosite (144 $\mu\text{g}/5 \times 10^5$ cells). Photographed 72 hr after administration of dust. Note fibroblast at center showing no phagocytosis. *E*. Treated with UICC crocidolite (144 $\mu\text{g}/5 \times 10^5$ cells). Photographed 72 hr after administration of dust. *F*. Treated with UICC chrysotile (72 $\mu\text{g}/5 \times 10^5$ cells). Photographed 4 hr after administration of dust. Note pronounced cytotoxic effects.







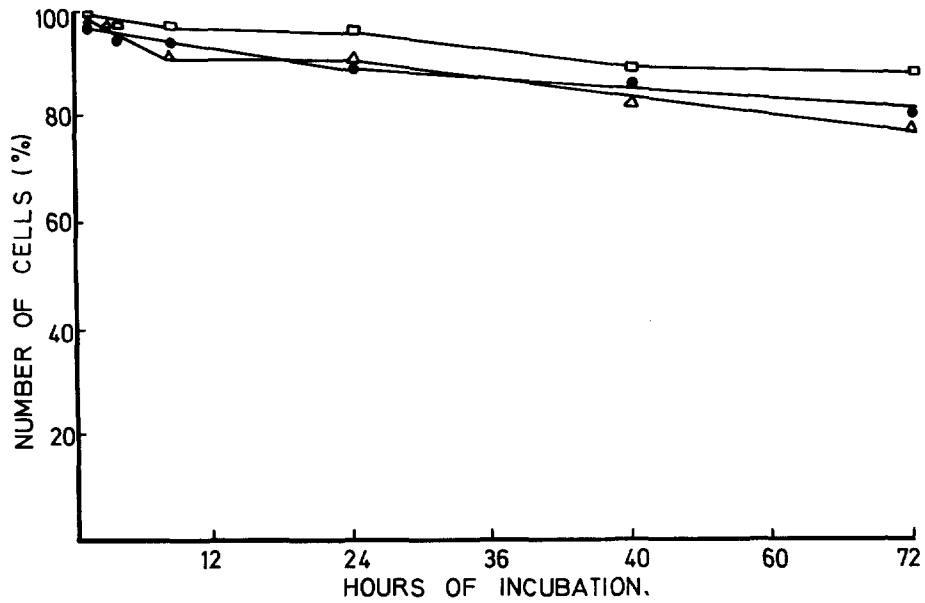


FIG. 4. Number of macrophages (as % of control) treated with crystalline silica Snowit. \square , 36 $\mu\text{g}/5 \times 10^5$ cells; \bullet , 72 $\mu\text{g}/5 \times 10^5$ cells; \triangle , 144 $\mu\text{g}/5 \times 10^5$ cells.

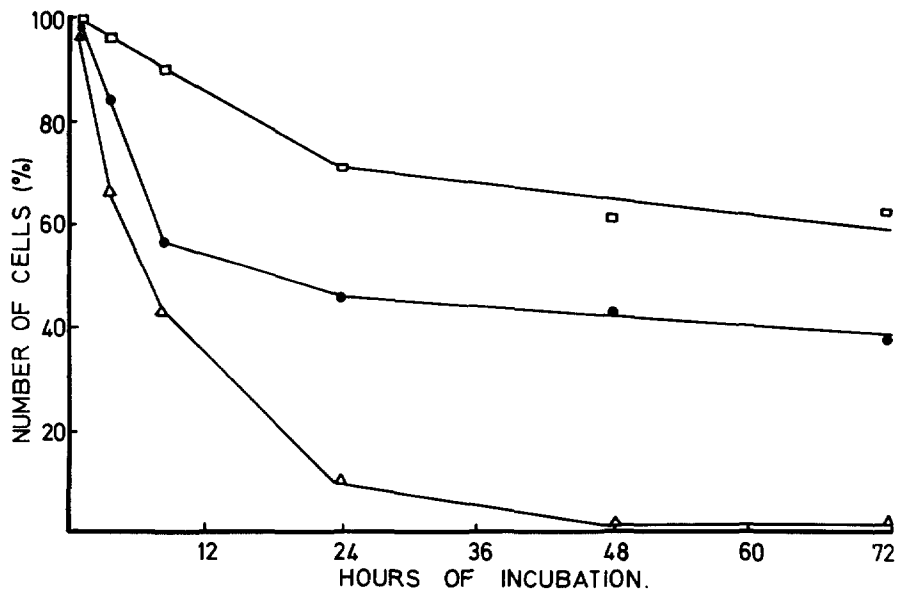


FIG. 5. Number of macrophages (as % of control) treated with amorphous silica Fransil. \square , 36 $\mu\text{g}/5 \times 10^5$ cells; \bullet , 72 $\mu\text{g}/5 \times 10^5$ cells; \triangle , 144 $\mu\text{g}/5 \times 10^5$ cells.

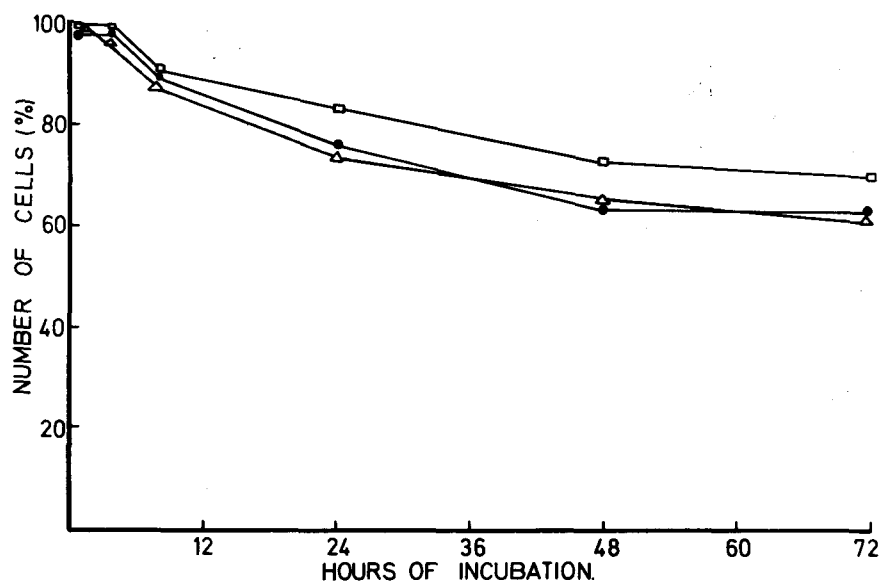


FIG. 6. Number of macrophages (as % of control) treated with UICC amosite. □ 36 μg/5 × 10⁵ cells; ● 72 μg/5 × 10⁵ cells; △ 144 μg/5 × 10⁵ cells.

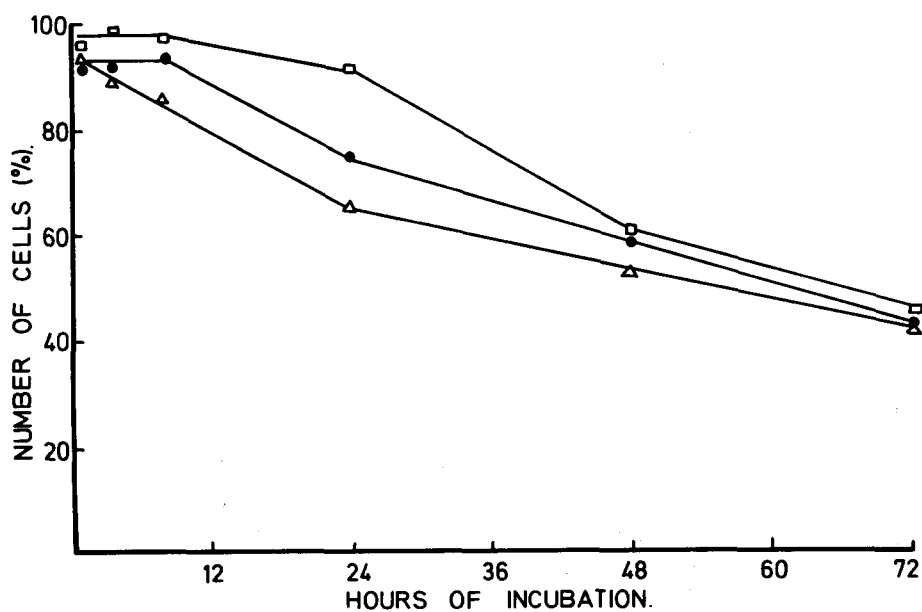


FIG. 7. Number of macrophages (as % of control) treated with UICC crocidolite. □ 36 μg/5 × 10⁵ cells; ● 72 μg/5 × 10⁵ cells; △ 144 μg/5 × 10⁵ cells.

more gradual reduction in number of cells in the control culture between the 5th and 8th days during which the cytotoxicity experiments had been carried out.

Effect of Modified Dusts on Macrophages.—Three of the dusts described above were modified as follows: (a) a smaller particle size range was obtained for amosite and chrysotile. (b) Fransil silica and chrysotile were boiled several times in tryptose phosphate broth to see if this procedure in any way altered the surface properties of the dusts. (Boiling of dusts in tryptose phosphate broth and subse-

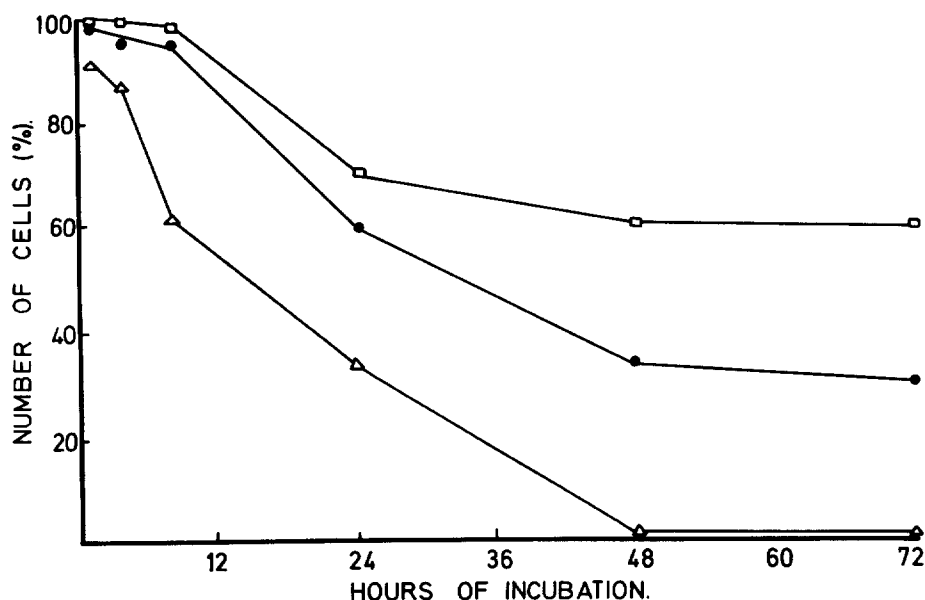


FIG. 8. Number of macrophages (as % of control) treated with UICC chrysotile. \square $36 \mu\text{g}/5 \times 10^5$ cells; \bullet $72 \mu\text{g}/5 \times 10^5$ cells; \triangle $144 \mu\text{g}/5 \times 10^5$ cells.

quent incubation of the suspension at 37°C was originally introduced as a sterilization procedure.)

Particles of small size range: When large fibers of amosite or chrysotile are removed by flotation and sedimentation, the particle:weight and the surface:weight ratios are altered. This could affect the extent to which active surface of the dusts is exposed to the cell. Fig. 9 shows that there is no difference in toxicity between chrysotile with a surface area of $23.54 \text{ m}^2/\text{g}$ and chrysotile with a surface area of $34.18 \text{ m}^2/\text{g}$. Similarly no difference in effect was obtained for two samples of amosite of different surface areas.

Effect of boiling in tryptose phosphate broth: The effect on macrophages incubated for 72 hr with freshly suspended chrysotile in tryptose phosphate broth was compared with that of chrysotile boiled several times. Fig. 10 shows that

the modified form of chrysotile was as toxic as the freshly prepared, nontreated material but that the cytotoxic effect of the former appeared earlier. Fransil silica, after being boiled in tryptose phosphate broth, showed no difference in toxicity compared with untreated Fransil.

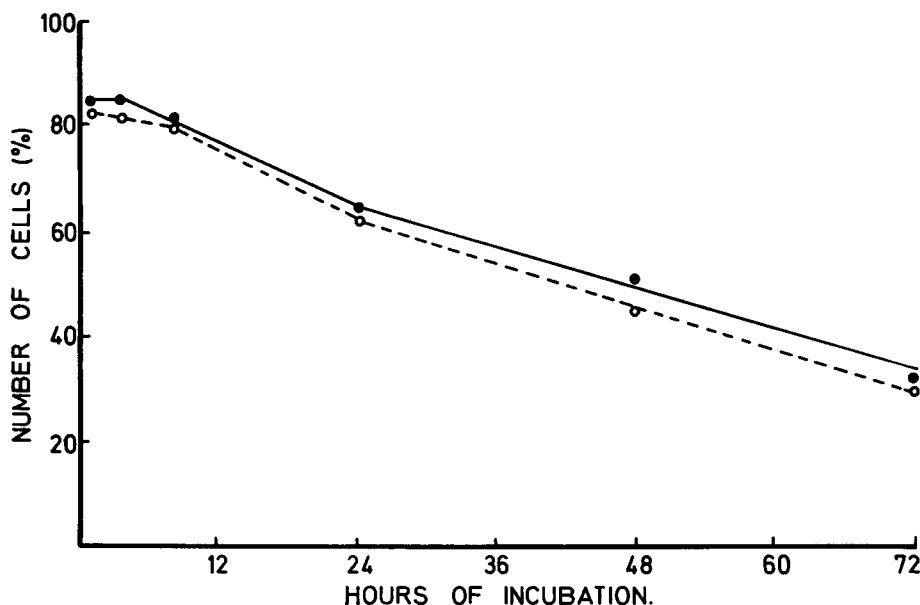


FIG. 9. Number of macrophages (as % of control) treated with standard UICC chrysotile and a modification of this; $72 \mu\text{g}/5 \times 10^5$ cells. ●—● standard chrysotile of specific surface area $23.54 \text{ m}^2/\text{g}$; ○---○ treated by flotation and sedimentation for larger surface area $34.18 \text{ m}^2/\text{g}$.

DISCUSSION

The choice of a meaningful experimental model is of particular importance in silicosis and asbestosis research (17). The use of experimental animals is one possibility, cell culture experiments another. The present work is concerned with the latter.

It has been found in many studies that the macrophage is a particularly suitable and obviously relevant cell for studies of the biological effects of mineral dusts. There is general agreement that monocytes migrate from the circulation and mature into typical tissue and exudative macrophages, normally without any further division *in vivo*. Such maturation is accelerated in inflammatory processes. It has been established (21) that such *in vivo* maturation closely resembles those events taking place under *in vitro* culture conditions.

In an investigation of mouse macrophages from the peritoneum, lung, and

other sites, Bennett (27) found slight differences in their ability to spread, to phagocytose, and under appropriate conditions, to divide. According to these properties, macrophages were classified into three groups: peritoneal and peripheral blood macrophages, bone marrow, spleen, and liver macrophages, and lung macrophages. With regard to the effect of silica dust on macrophages in culture, Allison, Harington, and Birbeck (9) found little difference between alveolar or peritoneal cells. In the present work, peritoneal cells were used since these are easier to obtain in large quantities than cells from the lung. The peritoneal

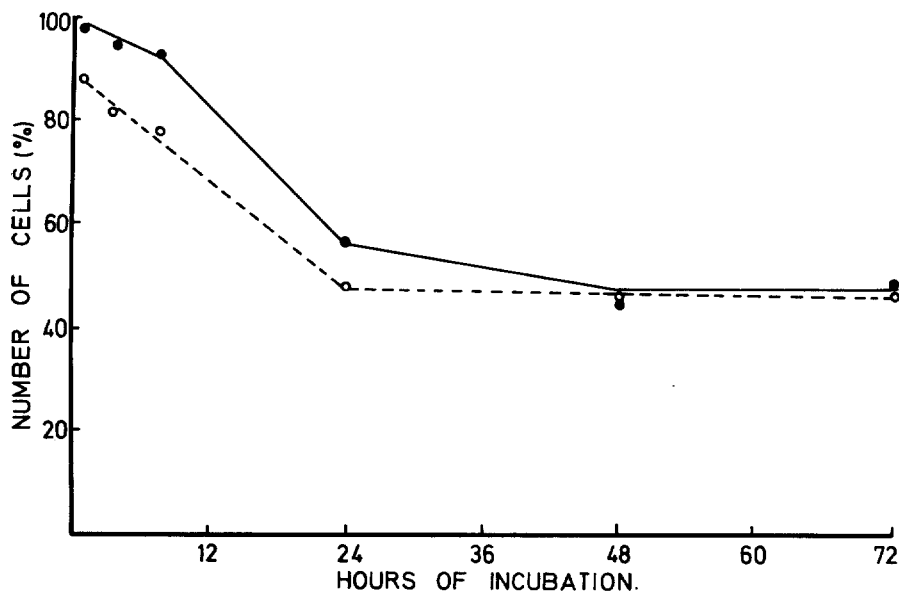


FIG. 10. Number of macrophages (as % of control) treated with standard UICC chrysotile and a modification of this; $72 \mu\text{g}/5 \times 10^6$ cells. ●—● standard chrysotile; ○—○ same chrysotile boiled in tryptose phosphate broth.

exudate of the hamster, without any prior stimulation by glycogen, consists predominantly of mononuclear phagocytes with a small proportion of lymphocytes and fibroblasts (28).

When the cells from a peritoneal exudate are planted *in vitro* under conditions first described by Chang (29), the monocytes and fibroblasts attach firmly to the glass while the lymphocytes do not and are washed away in the course of culture. Monocytes require several days in which to adapt to culture conditions and to mature into macrophages (21). Mature macrophages in culture, as compared to monocytes, are cells which have spread out and which have active pseudopodia limited by ruffled membranes. They also have extended mitochondria and an increased number of lipid-containing organelles and hydrolase-

containing dense granules (30). When conditioned medium (the supernatant from fast-growing cells) is present, mononuclear cells from spleen or bone marrow form colonies in agar (31). Virolainen and Defendi (32) showed that peritoneal macrophages can be stimulated to grow logarithmically 2-3 days after planting over a period of 10 days if growth factor(s) produced by fast growing primary cultures or by established cell lines are continuously supplied. Similarly, in the course of separate experiments carried out in this laboratory, the persistent presence of macrophages in tissue culture explants of SA7 hamster tumors was noticed.

In order to establish optimal culture conditions for macrophages we therefore supplemented the planting medium with 50% conditioned medium obtained from fast-growing SA7 transformed cell cultures. Little difference was seen in the number of cells per cm^2 on the 4th day between cultures planted in medium with a high serum content and cultures planted in medium containing less serum but 50% conditioned medium (Fig. 1). The number of cells, and their ability to spread, that is the degree of maturation, is somewhat greater when conditioned medium is used in conjunction with large amounts of serum. This suggests that there are factors present in the 24 hr supernatant of fast-growing tumor cell cultures which induce proliferation of phagocytes and help them to establish themselves in culture and to mature into true macrophages. All experiments described here, however, were carried out on cells which had been planted in medium containing 40% serum only. This was done because conditioned medium is not universally available for tests of the type described here and because the quality of such medium varies according to the type of cell conditioning it. It was also considered desirable to use a culture medium which would produce stable cultures by not stimulating mitosis.

Fig. 2 shows clearly that the number of macrophages/ cm^2 , planted and maintained under the conditions described above, increases steadily up to the 4th day of culture. Thereafter, the number declines very gradually up to day 8. From day 10 onwards the macrophages degenerate into multinucleated giant cells and fibroblast overgrowth may occur. The increase in the number of macrophages over the first 4 days of culture could be due to mitosis, to cells which had earlier appeared pyknotic and which for this reason had not been accounted for, or to migration of cells from the surrounding areas to the alkali-treated glass cover slips.

Taking each of these possibilities separately, it can be said that a few mitotic figures were seen after colchicine treatment but not in numbers sufficient to suggest active mitosis. From about the 9th day onwards, mitotic figures were seen frequently in clusters of fibroblasts which were beginning to overgrow the culture. The reason for the apparent paucity of mitosis in the macrophage cultures may lie in the fact that after 4 days' adaptation the cultures were kept on medium with 10% serum only, and with only 1 ml of the medium being changed every day.

With regard to pyknosis, about 20% of the increase in the number of cells up to day 4 could be accounted for by the subsequent stretching out of previously pyknotic cells. Finally, there remains the possibility that cells might have migrated from surrounding areas to the cover slips. In our view, this migration accounts for most of the increase in cell number as it has been found that macrophages prefer a glass surface to DePeX.

For the study of toxic effects of mineral dusts on macrophages, the period from the 5th to 8th day after planting was chosen, as by this time cultures are well established and stable (Fig. 2). The use of Petri dishes each containing nine cover slips made it possible to carry out complete investigations over optimal periods of time on any particular cell population obtained from individual animals. In this way, for example, the effects of three concentrations of dust over 3 days could be studied on the harvest of cells taken from a single hamster, thus avoiding biological variation.

Dusts were added to the Petri dishes in such amounts that all cells were able to phagocytose at least several particles. The results show that crystalline silica Snowit had no cytotoxic effect whatsoever on macrophages over 72 hr (Fig. 4), although all particles had been phagocytosed within the 1st hour. The reason for the present divergence of opinion on the toxicity of crystalline silica is most probably due to the difference in conditions under which macrophages are brought into contact with this mineral. The amorphous form of silica Fransil which was used had, on the other hand, a specific toxic effect on hamster peritoneal macrophages, this being dependent on the amount given (Fig. 5). Fransil has already been shown to be taken up into phagosomes of macrophages and to cause disintegration of subcellular organelles, leading to the death of the cells (9).

Standard UICC samples of amosite and crocidolite in amounts of 36, 72, and 144 $\mu\text{g}/5 \times 10^6$ macrophages did not show significant effects on the number of cells/cm² and their ability to remain spread out over long periods of time (Figs. 3 *D* and *E*, 6 and 7). This was evident in spite of the fact that phagocytosis had been completed within the 1st hr of the experiment. When excessive amounts of crocidolite and amosite, for example 200 μg per Petri dish, were given the macrophages phagocytosed continuously until they were virtually filled with fiber, rounded up, and floated off. This appears to be a mechanical effect rather than a direct cytotoxic one, and can be seen when any other apparently "inert" dust is given in sufficient amounts to macrophages. Nevertheless, it is possible that released fibers could be recycled through other healthy cells and in this way lead to a delayed disruptive effect. Chrysotile, if given in the same amounts as amosite and crocidolite, exhibits a typical cytotoxic effect on macrophages, the extent of which again depends on dose (Fig. 8).

The results of the present work indicate that under the experimental conditions described, standard samples of amosite and crocidolite asbestos and a

form of crystalline silica are not obviously toxic to hamster peritoneal macrophages, whereas chrysotile asbestos and a form of amorphous silica are specifically cytotoxic.

There is at present a considerable divergence of opinion concerning the degree of cytotoxicity of various mineral dusts or forms of these (5-15). One reason for this may be the degree of maturation of the cells used. The phagocytic activity of the mononuclear phagocyte is fully expressed only after the cell has established itself as a mature macrophage (21). On these grounds, it is perhaps to be expected that mature phagocytes may behave differently towards particulate materials such as mineral dust than immature phagocytes.

Other factors responsible may lie in the type of animal used. Little is known about the *in vivo* effects of mineral dusts on the hamster. There is evidence that the expression of silicosis in the hamster is atypical compared to that seen in other experimental animals (33). The present study, however, is related to another in which the interrelationship of hamster macrophages and fibroblasts in the presence of mineral dusts is being considered.²

Yet another difficulty concerns the wide variation in type and state of mineral dust used. While standard UICC reference samples of at least four different types of asbestos are available (34, 35), no such facility exists for a supply of silica. A result of this is that many diverse types of this mineral are used, including quartz, tridymite, cristobalite, stishovite, and various forms of amorphous silica. While it is undoubtedly important that the various biological effects of all of these be known, the inclusion of standard reference samples of silica as agreed to for example, at international level, seems to be long overdue (36).

With regard to pretreatment of dust, a smaller particle size-range for amosite and chrysotile was obtained by flotation and sedimentation. Thus, the surface: weight ratios (that is the particle: weight ratios) for both dusts were increased by about 100% for amosite and 50% for chrysotile. Increase in the surface area of amosite was not associated with a decrease in the number of macrophages over the duration of the experiment. This was to be expected because of the particular shape of the dose-response curves for the three increasing concentrations of untreated dust. Each of these offer a 100% increase in the total surface area available to the cells (Fig. 6). In the case of chrysotile, a decrease in the number of macrophages fed with the same amount of dust but with a greater specific area was to be expected according to the data given in Fig. 8. This, however, did not occur (Fig. 9) and might be accounted for by the possibility that the weight of fiber used, i.e. the actual dose given, is more important than the actual surface area. Were this not the case a 50% increase in surface area, for example, should have produced a significant effect. At the same time it

² Harington, J. S., P. C. King, and K. Miller. Manuscript in preparation.

should be mentioned that the peculiar properties of chrysotile fibers do not allow accurate determinations of surface area to be made (37).

It has been postulated by Robock (38) that the damaging effect of various modifications of silica on macrophages depends on the activation energy of their electron-adhesion points, this being explained by catalytic activity according to the electron theory of catalysis (39). We tested this by incubating macrophages with dust suspensions boiled in tryptose phosphate broth, a procedure which might have been expected to influence surface properties. In the case of chrysotile, a slight but significant increase in toxicity was regularly observed in the early stages of the experiments (Fig. 10). Thus, pretreatment of this fiber may have altered the electronic surface structure of the dust (38), perhaps by leaching out of ions (40, 41) or by adsorption of materials from the tryptose phosphate broth on to the surface of the dust. However, there is no difference in the effect of boiled compared to unboiled Fransil on macrophages.

In conclusion, it is felt that, provided a correlation can be established between events *in vivo* and those *in vitro*, experimental procedures of the type described here should prove to have considerable practical advantages over corresponding *in vivo* systems. The experimental model dealt with here not only provides for the quantitative evaluation of the cytotoxicity of various dusts but also makes it possible to test a large number of different samples in a comparatively short time.

SUMMARY

Hamster peritoneal macrophages were grown in cell culture and their response to various conditions was examined. The cultures responded favorably to high concentrations of serum and to medium which had been preconditioned by contact with tumor cells. After 2-3 days of adaptation, they entered into a period of stability which lasted from the 4th to the 9th day. Macrophage cultures in this stable phase were treated with various samples of mineral dusts and their response determined by counting the number of viable macrophages/cm² at intervals over a period of 72 hr. Crystalline silica Snowit was found to be nontoxic. Amorphous silica Fransil caused a characteristic cytotoxic effect and a rapid decline in cell population at doses less than 150 $\mu\text{g}/5 \times 10^5$ cells. Of the three different kinds of asbestos used, chrysotile was toxic and amosite and crocidolite nontoxic at equivalent concentrations. A comparison of two preparations of chrysotile which differed in surface area showed that weight rather than surface area determines toxicity. Pretreatment of chrysotile with tryptose phosphate broth under drastic conditions accelerated but did not increase the final intensity of the cytotoxic effect.

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