Cellular Effects of Asbestos and Other Fibers: Correlations With *In Vivo* Induction of Pleural Sarcoma

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The phenomenon of fiber-induced cytotoxicity to P388D1 macrophagelike cells has been demonstrated to parallel (thus far without exception) the probability that the fiber will induce a pleural sarcoma (mesothelioma) in rats. This startling parallel in both cases seems to be essentially independent of the chemical nature of the fiber and correlates best with the presence of fibers greater than 8 μm in length and fibers with diameters in the range 0.5 to 1.0 μm (Stanton Hypothesis). In both systems evidence has been produced which cast strong doubts on any role played by adsorbed (or adherent) impurities. The existence of multiple physical forms of the same chemical moiety (aluminum oxide, dihydroxy-sodium aluminum carbonate, borosilicate glass, etc.,) provides additional test material for the chemical independence corollary. The similar, cytotoxic or sarcomatogenous behavior of chemically different materials (e.g. amosite, chrysotile, aluminum oxide) exhibits the necessary converse argument.

As long as the fiber size-shape dependency effect was limited to whole animal phenomena, such as tumor induction, one could make implicitly what were essentially statistical or probabilistic inferences involving transport and/or distribution of fibers to account for the physical effect. The demonstration of strict parallelism at the cellular level *in vitro* suggests the possibility that in the case of durable fiber toxicology we are dealing with a form of cell-solid interaction in which physical properties for which we have as yet no known receptors play a prominent role.

Introduction

This paper establishes several new links in the chain of evidence supporting what has come to be called the Stanton Hypothesis (*1*). Simply put, this hypothesis holds that the mesothelioma-inducing — or more conservatively, the pleural sarcoma-inducing — behavior of various durable fibers is a function primarily of fiber length and diameter, and that within the range of materials examined is seemingly independent of their chemical nature. The hypothesis, until recently, has rested on a series of implantation experiments mostly in rats, the majority of which have been performed by Stanton and his co-workers (*2-5*). The phenomenon of durable fiber induction of mesothelioma has been confirmed by many others (*6-10*), but experimental conditions have not always been such as to allow direct testing

of the Stanton Hypothesis. The hypothesis explicitly is limited to pleural tumors and at this stage is not asserted for pulmonary parenchymal or bronchial neoplasms.

In Stanton's experiments a fixed amount (lately 40 mg) of the durable fibrous material for test is suspended and gelatinized to adhere to a coarse glass fiber pledget. This is surgically implanted in apposition to the visceral pleura and the rat maintained until death or sacrifice at age 2.4 years. The large body of experience with the strain of experimental animals provides control incidence data ancillary to sham-operated and blank preparations for neoplasms which in the natural course of events are extremely rare.

Crucial to the analysis of such experiments are the laborious and painstaking measurements of the dimensions of the mineral samples, employing both light and electron microscopic manual techniques. Samples comprising as many as 18,000 (and never less than 1000) individual fibers characterized by

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width and length measures are the bases for the correlations of physical properties and sarcomatogenic effect. Stanton and Layard (5), in the latest report of the series, establish statistically the dependence of sarcomatogenicity on fiber size and aspect ratio over a wide range of chemically diverse materials, including various forms of asbestos.

The new links in the evidentiary chain supporting the Stanton Hypothesis are a series of in vitro experiments, largely involving an established cell line—P388D1 macrophage-like cells—which we have previously shown (11, 12) to manifest a dose-dependent cytotoxic response to various forms of asbestos fibers, amphiboles and Chrysotile. Reported here in summary and detailed elsewhere (13, 14) are experiments involving samples of durable, nonasbestos fibers taken from lots identical to those employed by Stanton in his animal experiments, and on which he developed the voluminous body of length-diameter data.

Materials and Methods

All results on nonasbestos fibrous materials reported in this paper were obtained by employing samples from the same lots used by Stanton and Layard (5) in generating the table of probabilities of pleural sarcoma development. Dr. Stanton has also generously made available quantities of UICC amosite, chrysotile, and crocidolite employed in these and previous studies both for reference and as primary standards for establishing the nature of the dose response system.

The original culture of P388D1 cells was provided by Dr. Hillel Koren, then of the Immunology Branch, NCI. These cells have been well characterized (15-17). The line is maintained in continuous culture, in stationary Falcon flasks containing a basic medium which consists of 9 parts by volume of Fisher's medium for leukemic cells of mice and 1 part mycoplasma and virus screened fetal calf serum. The same basic medium is used in all experimental and control flasks. Incubation for maintenance and experiment is at 37°C in an atmosphere of 5% CO_2 in air. No antibiotics are used during maintenance or in the experiments.

Test and control flasks for a given experiment are started essentially simultaneously. Approximately 4×10^5 cells are added to Falcon flasks containing 10 cm³ of the basic medium. Five locations on the bottom of each flask are defined by attaching paper computer tape, each containing a single 1-mm diameter preformed circular hole. All cell counts are made from Polaroid photographs of the phase microscopic fields defined by these randomly-placed "windows." This use of set windows significantly

and favorably reduced the temporal sampling variation for a given flask and set of conditions.

After 24 hr of incubation, each window in each flask is photographed, thus effectively determining a "zero time" cell number. Actual counts are performed subsequently and at leisure from the complete set of coded photographs by a single individual for the entire experiment. Immediately following photography, the medium is removed and the young cell sheet is rinsed once with 10 cm³ of phosphate buffered saline (PBS). The medium is replaced by 10 cm³ of basic medium to which has been added the fibrous material under test in concentrations of 10, 50, or 100 micrograms/cm³ or in the case of the cell control, simple unaltered medium. In all non-asbestos tests, a positive control flask containing 100 μg/cm³ of UICC amosite is incubated and counted along with the test and cell control flasks.

Photography was done at times 0, 24, 48, and 72 hr. Immediately following the photography at 48 hr after challenge, the medium is removed, the cell sheet rinsed with 10 cm³ of PBS, and 10 cm³ of basic medium added to all flasks, control and test.

Cytotoxicity is evaluated in terms of the effect of the added fiber on cell number at various times and concentrations, especially with reference to the expected number as indicated by the cell control. More efficient and sensitive statistical measures taking into account all curves and intervals in a given experiment are being developed in a collaboration with Dr. Barry Margolin of NIEHS. These will be reported elsewhere.

Preliminary processing of the fiber size and shape data provided by Stanton has been initiated by Earl Smith of our laboratory utilizing several features of the interactive computer display system available on the NIH PDP10.

Results

Early experiments (11, 12) with UICC samples of asbestos of various types have been repeated on several occasions. For reference purposes one such set of experimental results is summarized in Figure 1. The dependence of cell number in a flask at 72 hr after challenge by fibrous material, in this case UICC amosite, is for the most part, dependent upon the concentration of the material, but also as would be expected on the starting cell number (time 0). Some morphologic changes as distinct from changes in simple cell number will be illustrated and discussed below. The only criterion for cytotoxicity in this report is change in cell number, though it is obvious that reproducible morphologic changes parallel the cytotoxic effect so measured.

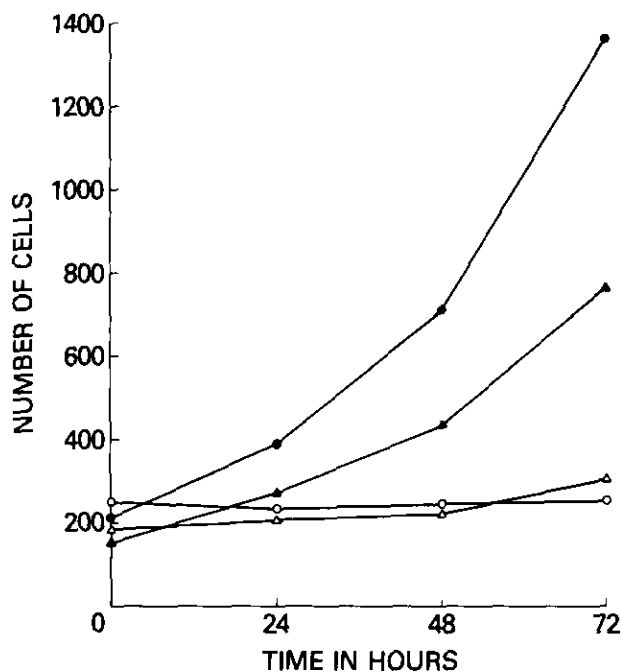Results of a similar experiment involving UICC

FIGURE 1. Effect of UICC amosite on cell number in cultures of P388D1 cells: (●) P388D1 cell control; (▲) 10 μg/cm³ UICC amorite; (Δ) 50 μg/cm³ UICC amosite; (O) 100 μg/cm³ amosite. Innocula in experiments such as this are adjusted to bring initial counts to the neighborhood of 200 cells. The strongly adverse effect of the amosite on cell number, especially when in higher concentration, is evident.

FIGURE 2. Effect of UICC chrysotile B on cell number of P388D1 cells: (▲) no added chrysotile; (Δ) 10 μg/cm³ chrysotile; (O) 50 μg/cm³ chrysotile; (O) 100 μg/cm³. The higher concentrations of chrysotile evidently not only inhibit the increase in cell numbers but result in some cell death, since the number of cells countable at 72 hr is less than the starting number in both the 50 and 100 μg/cm³ concentrations.

chrysotile asbestos is shown in Figure 2. The more marked cytotoxic effect is perhaps related to the differences in fiber size parameter distributions characterizing the two standards. Within the chrysotile system, however, the monotonicity of the dose response function is evident as is the close approximation of the 50 μg to the 100 μg concentration effect, thus paralleling the amosite result.

The results of our cytotoxicity experiments involving a wide variety of chemically distinct durable fibers in which a given chemical moiety (in the case of aluminum oxide and dawsonite) was represented by several different physical forms are summarized in Table 1. Because of the common origin of the samples involved it is appropriate to juxtapose the Stanton and Layard tumor probabilities as published (5).

Aluminum Oxide

Figure 3 summarizes the numerical results of cytotoxicity experiment on four of the defined aluminum oxide samples. The samples are chemically identical but differ widely in physical size and shape of their constituent particles. Details of fiber size and more detailed curves for the entire
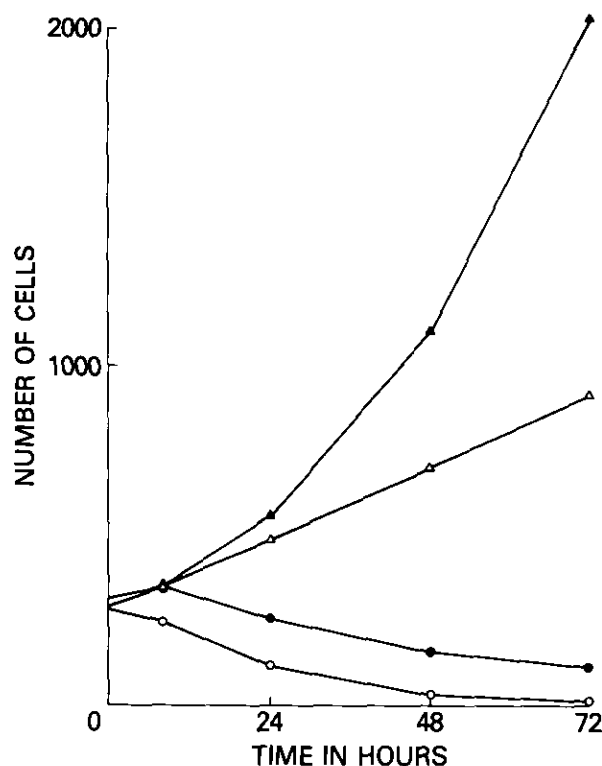
Table 1.

| Fiber	Cytotoxicity	Probability of sarcoma induction
Aluminum oxide HC	+++	0.70
Aluminum oxide # 3	++	0.44
Aluminum oxide # 4a	+	0.41
Aluminum oxide # 2	+	0.22
Aluminum oxide # 5	0	0.05
Aluminum oxide LC	0	0.03
Dawsonite V	+++	1.00
Dawsonite I	+++	0.95
Dawsonite VII	++	0.68
Dawsonite IV	++	0.66
Dawsonite III	+	0.44
Dawsonite VI	0	0.13
Dawsonite II	0	0.12
Potassium ocatitanate	+++	1.00
Silicon carbide	+++	1.00
Borosilicate glass MOL	+++	0.85
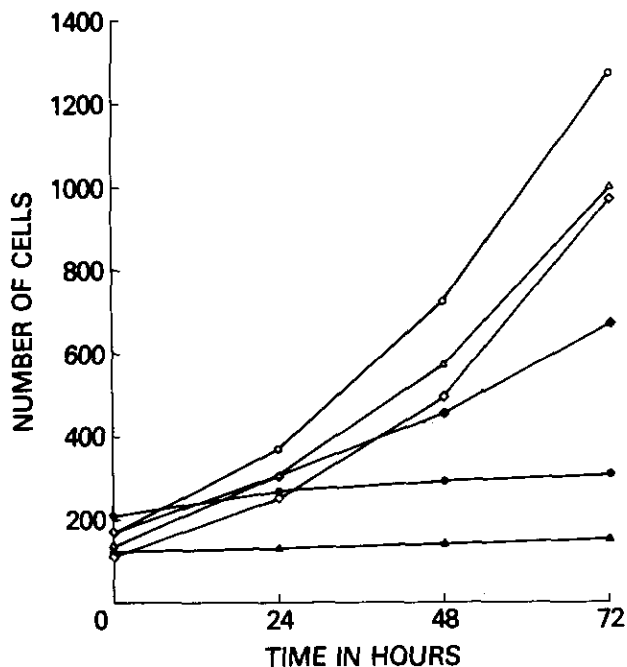
FIGURE 3. Effect of various physical forms of aluminum oxide on P388D1 cells: (●) 100 μg/cm³ UICC amosite; (▲) 100 μg/cm³ dl₂O₃ HC, diameter 0.5-1 μm; (◆) 100 μg/cm³ Al₂O₃ #3, diameter 1-3 μm; (△) 100 μg/cm³ Al₂O₃ #2, diameter 5-10 μm; (◇) 100 μg/cm³ Al₂O₃ #5, diameter 18 μm; (○) P388D1 cell control. Partially successful normalization in terms of starting cell number (0 time) shows that not only does the HC type of fiber (▲) parallel the same concentration of amosite (●) but quantitatively it is similar.

aluminum oxide group will be reported elsewhere (13).

The HC fiber type of aluminum oxide found by Stanton and Layard to the form within the group most likely to produce pleural sarcomas, is the most cytotoxic in terms of number of cells countable at 72 hr. Except for a difference in starting cell number, the curve for the HC type of fiber is almost identical to the positive control, UICC amosite. The least cytotoxic fiber in this aluminum oxide subgroup is that designated by Stanton and Layard as No. 5, which had fiber diameters in the 18 μm range, was also the least likely of this subgroup to produce pleural sarcomas. The results indicate that the ordering of cytotoxicity in this group is HC > No. 3 > No. 2 > No. 5, which is the same ordering as the probability of pleural sarcoma production.

The morphologic changes which take place in P388D1 macrophage-like cells during the standard 72 hr test period are complex and thus far they can be expressed only in qualitative or at best semiquantitative terms. A series of figures, (Figs. 4-13) illustrate changes occurring in three forms of aluminum oxides fiber tests as well as controls. The illustrations are typical window photographs the same as

those employed in cell counts. All figures, then, represent Polaroid photographs of phase microscopic images.

Unchallenged P388D1 cells 24 hr after the beginning of an experiment are moderately well-scattered within the field (Fig 4). Many, perhaps 80% of the cells are roughly "bipolar" showing two predominant processes, one at each end of the somewhat spindle shaped cell. These, as most of the other cells, are adherent to the plastic floor of the flask. Some few "multipolar" cells are encountered. These are larger, with more numerous but more delicate processes. Occasional mitoses are seen in floating cells and late anaphase/telophase cells are seen to be reattaching before cell separation is complete. Under favorable conditions, moderate cytoplasmic activity in the form of extension and retraction of one or more lamellipodia may be seen. A complete extension-retraction cycle may take as little as 15 sec, but usually longer.

After an additional 48 hr, many more cells are in evidence (Fig. 5). Two to three cell cycles have taken place and the cell sheet is much more confluent. Relative to the whole, the proportion of multipolar cells has increased, although the bipolar type has increased absolutely in number. A rare multipolar cell shows a relative increase in perinuclear cytoplasmic granules and vacuoles and an accompanying increase in the complexity of processes, changes which when taken together are at least analogous to the early "activation" ascribed to some macrophages. Such cells are considerably less than 1% of the total.

At 24 hr after challenge by the HC fibers, the changes are both qualitatively and quantitatively striking. The reduction in cell number (Fig. 6) is
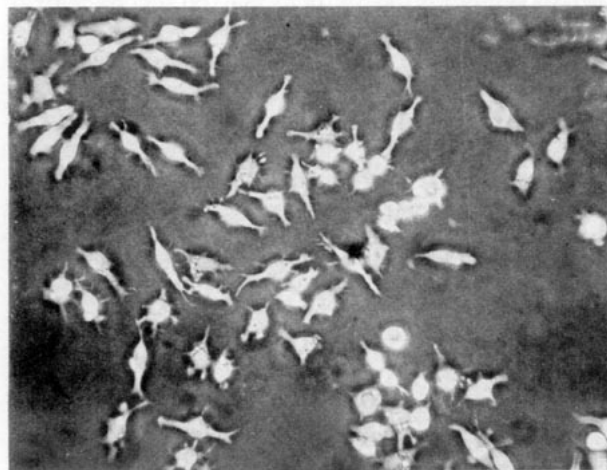

FIGURE 4. Cell control window at 24 hr. Several late telophase cells are evident. Figures 4-13 are all phase contrast images taken at a primary magnification of 170×.
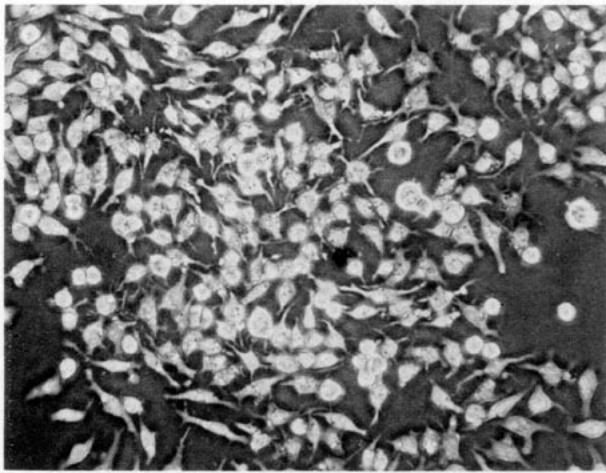
FIGURE 5. Cell control window at 72 hr. This is the same window as in Fig. 4 but 48 hr later. The cells do not show very much more morphologic evidence of "activation" than the previous figure, though a rare cell does exhibit moderately increased size, number and complexity of processes.
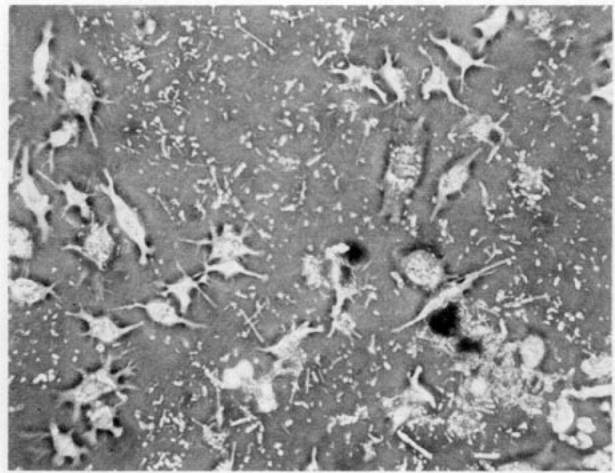

FIGURE 6. Aluminum oxide, Type HC, 24 hr. Some cell loss is evident compared to the equivalent control window (Fig. 4). There is striking increase in cell size, nuclear size in some instances, perinuclear granules, and vacuoles, as well as increased number and complexity of processes on the majority of cells. It is obvious in comparing this figure with Fig. 4 that the morphologic changes are more marked than the cell number differences at this early stage of the experiment. This is another indication that properly quantitated, the morphologic differences form an earlier and more sensitive measure.

obvious, especially when compared with Figure 4. The proportion of multipolar cells is very much greater than encountered in any of the cell control stages. Granulation and vacuolization, particularly perinuclear, is now in marked evidence. The amount and distribution of cell debris suggests the occurrence of repeated cell death. The relatively few biopolar cells seen are larger, flatter, and less cytoplasmically active than the corresponding cell control cells. Lamellipod extension and extrusion though not absent, is sparse, sluggish and is observed at any given time in fewer cells. Corresponding coverslip preparations which allow the use of differential interference microscopy show that every cell has at least one or more visible fibers associated with it if not within it, while some cells may contain literally dozens of thin fibers. Multinucleated cells—two, three, or four in number—are not rare, and like the population as a whole, appear flattened and minimally active cytoplasmically.

At the 72 hr terminus of the assay, the HC fibers have induced a moderate to marked progression of the changes observed at 24 hr (see Fig. 7). If anything, cell number seems further reduced. Despite the initial impression of hypertrophy in the presence of devastation, cell replication is taking place as evidenced by mitoses in various stages, encountered in almost every window.

Morphologic changes in the positive control (100 μg/cm^3 UICC amosite) (Figs. 8 and 9) are quite comparable if not categorically identical to that seen with the HC fibers. Because of the more frequent occurrence of thicker fibers in this sample, the characteristic roughly paranuclear pattern of shorter phago-

cytosed fibers is evident in the cellular debris and occasionally is the only indication that a cell once was there. The attempted phagocytosis of a single
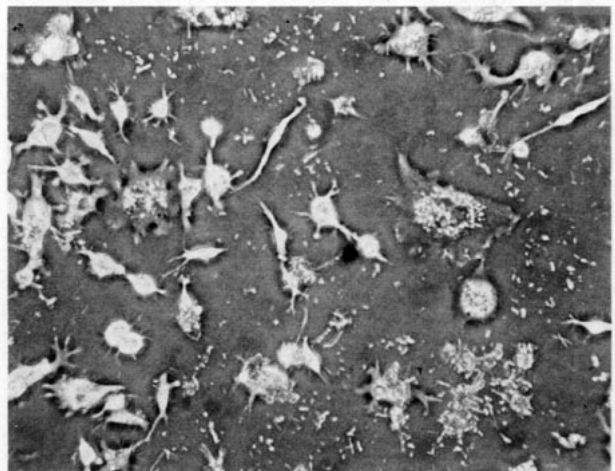

FIGURE 7. Aluminum oxide, type HC, 72 hr. This is the same window as in Fig. 6. Comparison with the 72 hr cell control (Fig. 5) can leave little doubt that cell number is significantly reduced. The morphologic changes have proceeded, but are at best moderately advanced, over those observed at 24 hr. Despite the diminished number of cells, cell replication is taking place as indicated by the telophase figure seen in the lower lefthand quadrant of the window. Stanton (5) noted this sample had a probability of 0.7 of inducing pleural sarcoma in rats. The probability figure for the amosite control was placed at 0.68.
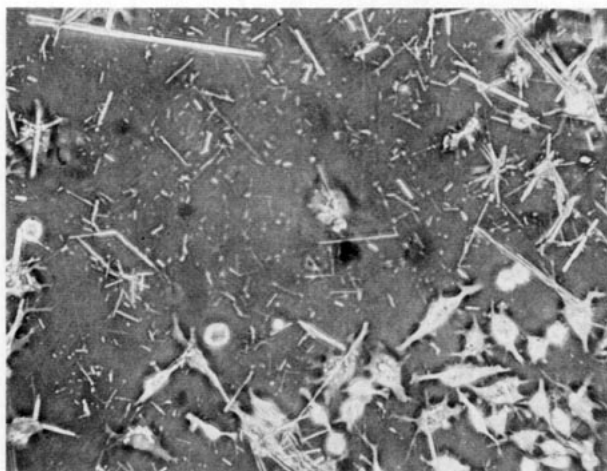
FIGURE 8. UICC amosite positive control, 24 hr. Considerable morphologic change, commonly associated with "activation" of macrophages is evident, roughly of the same order of magnitude as shown by the HC fibers this far along (see Fig. 6). Mitoses are in evidence. The range of fiber size distribution in this UICC sample is obviously much wider than that of the HC aluminum oxide.

FIGURE 10. Aluminum oxide #3, 24 hr. The sample of aluminum oxide added to this culture, although showing large numbers of very long fibers (most visible ones are longer than 20 μm) consists of fibers which are considerably thicker than the HC type. The lessened cytotoxicity of this sample as compared to both the HC type (Fig. 6) and the positive control (Fig. 8) is obvious in the number of cells seen in this window. The appearance of these cells is less "activated" than those resulting from exposure to the HC type.

fiber by two or more cells is also more commonly seen for similar reasons. With progression to the end of the 72-hr test (Fig. 9), the amosite control appearance becomes even more like that of the HC aluminum oxide.

Aluminum oxide type 3 fibers produce changes in P338D1 cells which may be regarded in sum as a muted version of the violent HC type of reaction (Fig. 10, compare Fig. 8). More cells persist at 24 hr

and at 72 hr (Fig. 11) while morphologic manifestation which could be regarded as "activation" phenomena are considerably less, both within individual cells and in terms of number of cells affected. "Activation" does appear to progress, moderately with time (Fig. 11). Limited observations of cytoplasmic activity show about the same degree of di-

FIGURE 9. UICC amosite positive control, 72 hr. This is the same window as in Fig. 8. The morphologic alterations associated with activation have progressed so that the appearance is quite comparable with cells exposed to the HC fibers for the same amount of time (see Fig. 7). In this particular window cell loss appears less than that apparent in the 72 hr HC window illustrated.

FIGURE 11. Aluminum oxide #3, 72 hr. This is the same window as shown in Fig. 10. Relative deficiency in cell number is small and "activation" moderate. At the upper right hand quadrant a large fiber is seen incompletely engulfed by two distinct cells. This is a frequent occurrence with fibers significantly larger than the long diameter of the cell.
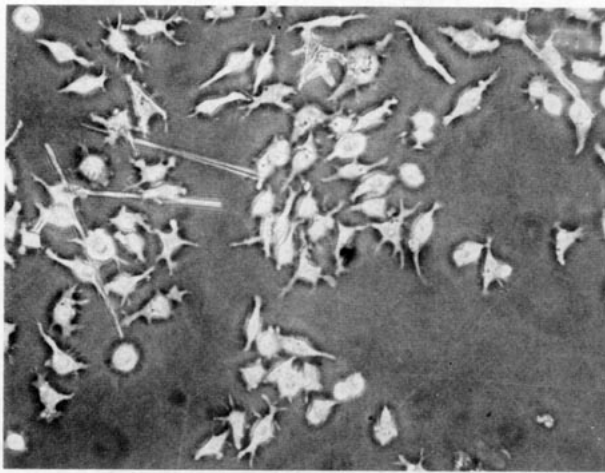
FIGURE 12. Aluminum oxide #5, 24 hr. Two cells show morphologic changes associated with "activation," but otherwise, except for the obvious coarse long fibers, the similarity to the cell control is striking (see Fig. 4).
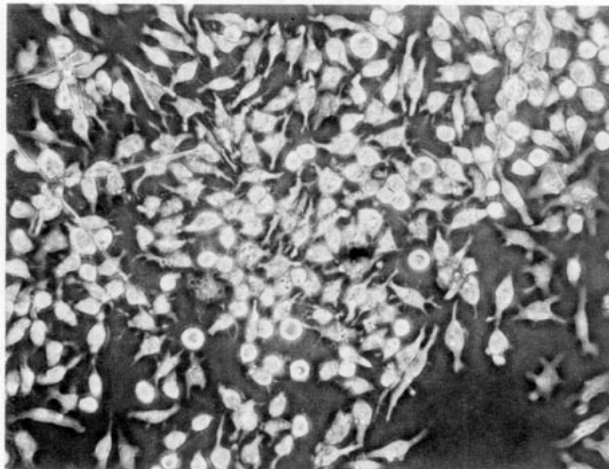

FIGURE 13. Aluminum oxide #5, 72 hr. The difference in cell number between this window and that resulting from HC fibers (see Fig. 7) is as striking as the actual counts exhibited in Fig. 3. The appearance of this window more strongly resembles the cell control in both number and morphology than any of those produced by the more cytotoxic and sarcomatogenic but chemically identical fibers in the aluminum oxide group.

minished lamellipodial activity as seen in the case of the HC fibers.

The least cytotoxic of the subgroup of aluminum oxide fibers, No. 5, produces minimal qualitative and quantitative changes in P388D1 cells. A few more multipolar cells than seen in control cultures at 24 hr are evident (Fig. 12). The "bipolar" cell predominance seen in controls is here obvious, and only a very rare cell shows any significant activation. The relatively uninhibited replication of cells in this sys-

tem results in an overall appearance of a window at 72 hr which is even closer to the unchallenged control (compare Fig. 13 with Fig. 5).

Dawsonite (Dihydroxysodium Aluminum Carbonate)

Figure 14 presents the temporal course of the effect of 3 of the Dawsonite samples tested. A detailed treatment of the whole dawsonite group is in press (14). Equal weight concentrations of Stanton's samples V, VI, and VII show widely different effects on cell number, obvious even at 48 hr. Dawsonite samples V and VII show cytotoxicity approximately comparable to an equal concentration of UICC Amosite, the positive control. These samples in Stanton's hands were highly likely to produce pleural sarcomas in rats. Sample VI shows considerably less cytotoxic effect in terms of cell number and was much less likely according to Stanton and Layard to produce pleural sarcomas.
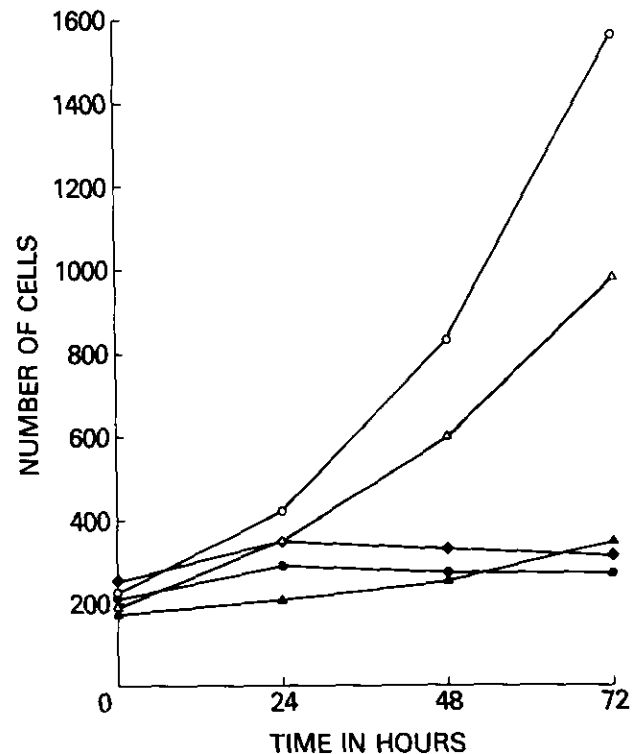
Morphologically the changes seen in the aluminum

FIGURE 14. Effect of various physical forms of dawsonite on P388D1 cells: (O) P388D1 cell control; (Δ) 100 μg/cm³ dawsonite #4; (●) 100 μg/cm³ dawsonite #5; (◆) 100 μg/cm³ dawsonite #7; (▲) 100 μg/cm³ UICC amosite control. With dawsonite (dihydroxysodium aluminum carbonate) as with aluminum oxide, a single chemical moiety evidently produces a wide range of cytotoxic effects. The most cytotoxic samples are just those most likely to produce pleural sarcomas in rats.
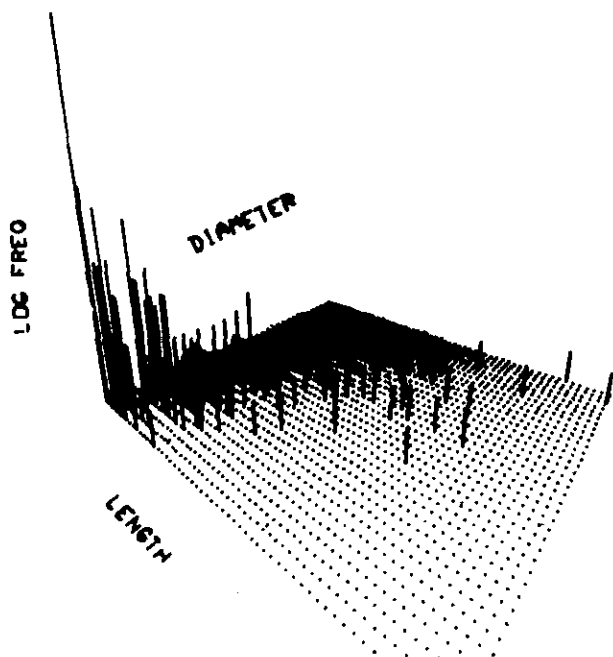
DAWSONITE 6

DAWSONITE 7

FIGURE 15. Dawsonite VI fiber dimension distribution. This is essentially a two-parameter histogram. The length and diameter axes are at intervals of 0.5μm. The vertical axis is the log frequency of fibers within a given 0.5 μm length-diameter element. Although few in number, the fibers which are large and thick account for considerable mass. This underlines the unsuitability of a simple mass measure of dose when dealing with a size-shape limited range effect, where fibers outside the range only serve to dilute the few effective members of the sample.
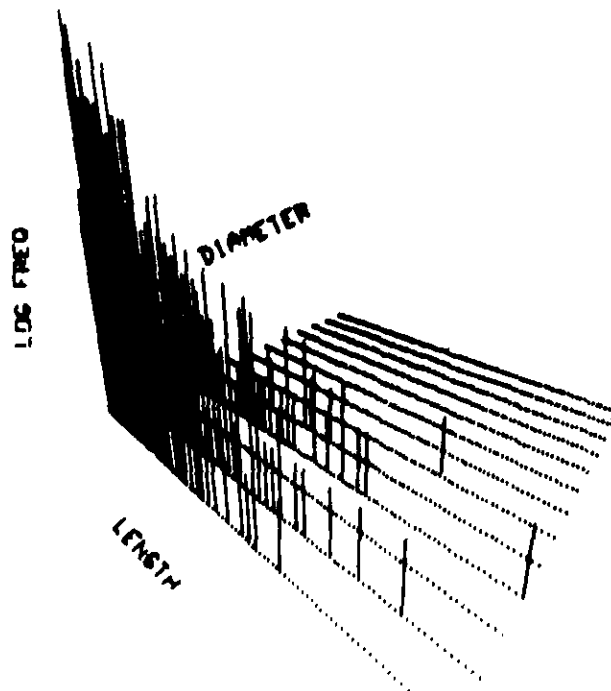
FIGURE 16. Dawsonite VII fiber dimension distribution. The length interval=diameter interval=0.5; Vertical axis is log fiber frequency. There are large numbers of fibers > 8 μg that are also thinner than 1.5μm microns. One would expect that this specimen is both cytotoxic and sarcomagenic. Figure 14 confirms the former, and the latter is indicated by the probability noted in Table 1.

oxide-bearing cultures are also in evidence in the Dawsonite material. Early and marked "activation" changes are seen in those cultures combining fibers which produce a cytotoxic effect in appropriata concentrations, and are less marked in those cultures which show a lesser quantitative effect.

Figures 15 and 16 show two examples of Earl Smith's treatment of the dawsonite fiber measures provided us by Stanton. Each interval between dots represents 0.5 μm both for length and diameter of fibers. The vertical axis is the logarithm of the frequency of fibers of the given dimensional class. Computerized three-dimensional plots such as these allow dynamic interaction and immediate display of comparisons of wholes or selected subsets of fiber distributions. In addition one can, by image subtraction techniques immediately see the number of fibers in a distribution which meet a particular set of dimensional criteria. Thus distribution of physical properties are easily explored once the somewhat laborious data entry is accomplished.

The distributions for dawsonite sample VI show

few if any fibers of sufficient length to be associated with high pleural sarcoma probability according to the Stanton-Layard criteria. Many are thin enough but they are none also long enough. Figure 16 shows this kind of plot for dawsonite sample VII. Here there are many thin and long fibers, a finding that would predict that they are likely to induce pleural sarcomas as experimentally they indeed do. They are also quite markedly cytotoxic, as Figure 14 amply demonstrates.

Fybex II (Potassium Octatitanate)

Brief mention should be made of our repeated measures of Fybex II cytotoxicity. Figure 17 shows the result of one such experiment. The closely similar values produced by 50 and 100 μg/cm³ concentrations are partially explained by the significantly larger starting number of cells for the lower concentration. It would seem however that this is an incomplete explanation for the cytotoxic response though a monotonic function of dose, is certainly not a linear
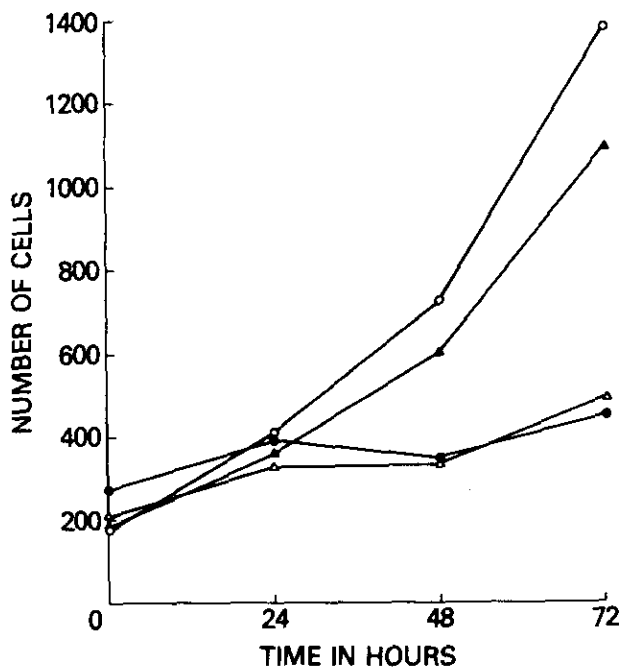
Figure 17. Effect of varying concentrations of potassium octatitanate (Fiber II) on P388D1 cells: (O) P388D1 cell control; (▲) 10 μg/cm³; (Δ) 50 μg/cm³; (●) 100 μg/cm³. A monotonic but nonlinear dose response relationship is suggested by the cell counts obtained at 72 hr. The cytotoxic effects as measured by number at the higher concentrations is obvious. It should be noted that though an amosite positive control was run it was not plotted for reasons of clarity.

Figure 18. Effect of varying concentrations of borosilicate glass MOL on P388D1 cells: (Δ) P388D1 cell control; (●) 10 μg/cm³ glass fiber; (▲) 50 μg/cm³ glass fiber; (O) 100 μg/cm³ glass fiber. The downward displacement of the 50 μg/cm³ concentration curve appears to be due to the relatively small starting number of cells.

one. Because of this and other observations, the need to evaluate the effect of enrichment by Stanton critical size fibers and the self-dilution phenomenon (see below), a revised protocol (Protocol C) is under development in our laboratory. This should significantly increase the sensitivity of the measures and allow better elucidation of the dose response function.

Borosilicate Glass

Another example of this kind of dose response function is seen in Figure 18. This specimen is reported by Stanton and Layard as borosilicate glass MOL and has in their analysis an associated probability of 0.85 of inducing pleural sarcoma.

At 72 hr the smaller number of surviving cells in the 50 μg/cm³ test is certainly attributable in large measure to the purely fortuitous low starting number of cells. The shape of both high concentration curves is identical. Preliminary (and still unsatisfactory) attempts to properly weight curves in terms of initial cell number would again suggest that the dose response function though monotonic is non-linear. Highly cytotoxic materials such as this glass speci-
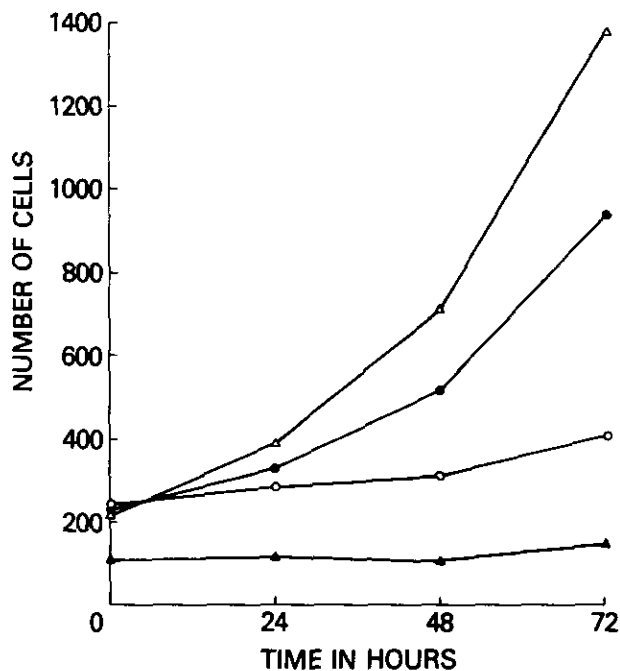
men and the Fybex II sample noted above show as expected a diminished difference between higher concentrations as compared to wider spreads seen in less potent cytotoxic fibers.

Miscellaneous Materials

In addition to those fibers reported in Table 1, we have experimentally demonstrated cytotoxic effects of such asbestos materials as crocidolite and the chrysotile fibers extracted from the serpentine rock of the Rockville quarry (18). Characteristically such nonfibrous materials as talc and celite repeatedly fail to show cytotoxic effects on P388D1 cells.

Discussion

The P388D1 macrophagelike cell line was first reported by Dawe and Potter (15), as derived from an originally lymphocytoid tumor induced by methylcholanthrene in a DBA/2 mouse. The line acquired its macrophage/reticulum cell sarcoma-like properties as a result of repeated mouse passage. More recently Koren et al. (16) have detailed some of the macrophage-like properties of this derived line. In

addition to active phagocytosis of polystyrene spheres and carbon particles, these amoeboid cells have a strong tendency to adhere to glass. In common with normal macrophages they exhibit nonspecific esterase activity, more or less prominent lysosomal bodies, a surface C3 receptor and a receptor for the Fc fragment of immunoglobulin. They do not have surface immunoglobulin nor do they possess surface T-cell markers. Snyderman et al. (17) have emphasized some unusual features of the P388D1 line vis a vis other cell lines and phytohemagglutinin-activated peritoneal macrophages. Although P338D1 cells are phagocytic, they are less so than the J774.1 line or activated peritoneal macrophages. P338D1 cells are distinctly not chemotactic with respect to activated mouse serum or lymphocyte derived chemotactic factor. In general P388D1 cells do not show very much if any at all random migratory activity in contrast to other macrophages and macrophage like cells examined by Snyderman et al.

Some of these unusual "deficiencies" of P388D1 cells make them better subjects for a cytotoxicity assay. For example, the absence of active chemotaxis makes for at least one less class of chemical variables affecting the outcome of a cytotoxicity test. Its fortuitous absence is particularly fortunate when measuring an effect which seems to be independent of the chemical nature of the stimulus. Similarly the strong adherence to glass and the lack of random migratory activity (unanticipated when the assay was being developed) makes for greater reproducibility and hence precision when employing a repeat window technique such as used here for the selection of fields to be counted.

Since P388D1 cells show a locally heterogeneous spatial distribution when grown in stationary flasks, sampling errors may be large enough to obscure significant changes in cell number. In order to keep the requisite number of cells to be counted within reason, and consequently increase test sensitivity, some way of maintaining field identity had to be found. The "hole in computer tape defined window" solution, though simple and primitive, is quite effective in reducing variances. In order to reduce temporal effects (counting several hundred cells is not instantaneous) each window is photographed (polaroid) at the appropriate time and counts are performed subsequently. The adoption of a set of standard counting conventions for the photograph completes the relatively simple but effective list of procedures for reduction of non-treatment determined variance in counts.

Operationally, the cytotoxicity assay procedure employed in these studies is quite simple. The underlying complexity of cell-fiber interaction should not on that account be ignored. We have shown (12) that, despite the low motility of P388D1 cells, significant cell-fiber association takes place within five minutes of exposure to, for example, UICC amosite; furthermore, such a brief exposure yields a statistically significant cytotoxic effect. Increasing exposure time up to 12 hr or more increases the cytotoxic effect, presumably due to the (optically observable) increasing cell-fiber association. Although as noted, the typical P388D1 cell does not exhibit very much translational motility, there is very considerable cell surface activity in the form of process and lamellipod extension and retraction. This, combined with the initial mixing occasioned by the addition of fiber containing medium, may be sufficient to account for the initial cell-fiber association which is followed by phagocytosis, accomplished or attempted.

The fact that P388D1 cells in contrast to "normal" macrophages such as peritoneal cells are continuously replicating makes for additional complexity. The cell is capable of replication even while bearing an engulfed or partially engulfed fiber as we have shown by time lapse cinematography (12). Microscopically, individual cell death undoubtedly occurs with liberation and subsequent rephagocytosis of fibers, so that it is likely that some if not many fibers have passed through more than one cell during the 72-hr test period. This is probably more closely analagous to what occurs in the human than with the "normal" macrophage in vitro where (19, 20) the macrophage is the first cell to deal with inhaled fibers. Throughout the course of pneumoconiosis it is the cell most likely to carry fibers. Macrophages in the airways may differentiate from blood monocytes or be derived from dividing tissue precursors. In any case, in vivo, there are facilities for the maintenance or increase in macrophage number in the face of cytotoxic fibers, which is not the case with alveolar or peritoneal normal cells in vitro.

The simple curves such as seen in Figure 1 are thus resultants of a set of complex and in a sense competing processes. A further complication is the strong probability that only a portion of the fibers are effective in producing a cytotoxic effect. If as now seems likely, the length and diameter limits suggested by Stanton apply to the cytotoxicity effect as well as to in vivo pleural sarcoma induction, only a (usually minor) proportion of fibers in a given sample are effective while the remainder are inert. This phenomenon, if confirmed, should be regarded as a self-diluting effect; i.e., one in which fibers of a given chemical moiety because of physical dimensions act to dilute or diminish a biologic effect by other fibers of the same moiety. It is obvious that a set of critical experiments are both possible and urgently needed. A durable fiber sample of narrowly limited and pre-

cisely known length and diameter distribution would be the basis for such a direct test. Less powerful and definitive would be experiments based upon the self-dilution effect where precise and known distributions outside the Stanton limits are used to dilute a known distribution of the same or different moiety. The cytotoxicity assay, requiring about three decimal orders of magnitude less (50 $\mu g/cm^3$ compared to 40 mg) material than for a single animal and about 1/200th the time (72 hr as opposed to > 2 years), would certainly seem to be the most likely first ground for such critical testing. Properly defined fiber samples, in the sense outlined above, are likely to be very expensive tools so that effective pre-screening at the microgram level may be essential as well as expeditious.

In the hope that such well defined specimens will in fact become available, the cytotoxicity assay is being improved to increase its sensitivity by perhaps an order of magnitude so that it will be able to detect differences occasioned by experimental dilution effects, as well as more finely stepped stimuli. It is hoped that these changes coupled with proper and efficient statistical treatment will allow numerical values as outputs of cytotoxicity measures, rather than the rank ordering presented herein.

The P388D1 cell-fiber system may alternatively be regarded as a "partial" model. Insofar as P388D1 cells mimic alveolar macrophages, the model may be thought to mirror the initial cell response to the durable fiber. The implication of long-term pathogenic effects of repeated phagocytosis of the same fiber, a most probable *in vivo* event as it is *in vitro*, are still to be evaluated. For example, it may be that repeated intracellular passage results in membranous lamellar depositions on fibers (without frank ferruginous body formation) with consequent altered cellular recognition properties of the fiber. In any case, the concept of a partial model allows for this type or other varieties of fiber "preprocessing" without any commitment to a carcinogenic mechanism or mechanisms.

Perhaps separate from the partial model concept is the more basic biologic phenomenon of the size-diameter effect on tumorigenesis or cytotoxicity. This is the *in vivo* work of Stanton and others (8), our own *in vitro* work, which recently has been paralleled by that of Chamberlain and Brown (21).

Returning to the question of models, if one is concerned with estimating the risk to populations of a particulate atmospheric pollutant, an animal model involving inhalation loading of the noxa is certainly to be preferred. On the other hand investigation of the biologic results of interaction between noxious agents and target tissues is greatly enhanced by deliberate bypassing of the (in this context) irrelevant

variables involved in nasal and bronchial trapping, suspension stability, the mucocilliary elevator, etc. Intrapleural implantation experiments address central pathogenetic and biologic questions. Implantation experiments are uncomplicated by distributional and delivery considerations which are of additional critical importance to inhalation experiments. The purposefully constrained domain of injection and/or implantation studies should no more be condemned as "unrealistic" (22) than the still more constrained cellular and subcellular studies of fiber effects.

The parallelism to *in vivo* experimental (and human clinical) results developing from cell studies such as these and others (21), suggests that even before the above noted critical experiments are possible, a good deal of perhaps novel durable fiber effect biology is accessible to experimental analysis. Biologic data of this type will be particularly important, not only for their novelty but because of their immediate relevance to a set of serious environmental dangers.

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