Short-term Inhalation and in Vitro Tests as Predictors of Fiber Pathogenicity

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A wide range of fiber types was tested in two in vitro assays: toxicity to A549 epithelial cells, as detachment from substrate, and the production of the proinflammatory cytokine tumor necrosis factor (TNF) by rat alveolar macrophages. Three of the fibers were also studied in vivo, using short-term inhalation followed by a) bronchoalveolar lavage to assess the inflammatory response and b) measurement of cell proliferation in terminal bronchioles and alveolar ducts, using incorporation of bromodeoxyuridine (BrdU). The amount of TNF produced by macrophages in vitro depended on the fiber type, with the man-made vitreous fibers, and refractory ceramic fibers being least stimulatory and silicon carbide (SiC) whiskers providing the greatest stimulation. In the epithelial detachment assay there were dose-dependent differences in the toxicity of the various fibers, with long amosite being the most toxic. However, there was no clear relationship to known chronic pathogenicity. Fibers studied by short-term inhalation produced some inflammation, but there was no clear discrimination between the responses to code 100/475 glass fibers and the more pathogenic amosite and SiC. However, measurements of BrdU uptake into lung cells showed that amosite and SiC produced a greater reaction than code 100/475, which itself caused no more proliferation than that seen in untreated lungs. These results mirror the pathogenicity ranking of the fibers in long-term experiments. In conclusion, the only test to show potential as a predictive measure of pathogenicity was that of cell proliferation in lungs after brief inhalation exposure (BrdU assay). We believe that this assay should be validated with a wider range of fibers, doses, and time points. — Environmental Health Perspectives 105(Suppl 5):1235-1240 (1997)

Key words: fibers, cell proliferation, TNF, short-term tests, epithelial toxicity, BrdU

Introduction

The association between exposure to asbestos and development of lung disease is well documented, and the cellular mechanisms whereby asbestos causes these effects are partially understood (1,2). There is concern that other industrial materials, such as refractory fibers, vitreous fibers, and insulation wools, which generate respirable airborne fibers may also be harmful. The relative potency of these fibers to cause lung disease in comparison with asbestos has been the subject of much research and continues to be controversial (3–5). Long-term animal inhalation studies probably provide the most reliable prediction of the hazards of a particular fiber type. However, such tests are time consuming and expensive; therefore, alternative shorter testing regimes have been studied in the hope that they may predict long-term pathogenicity. We have examined other shorter assays to determine if they can discriminate between fibers of differing pathogenicity. We present here the results from two in vitro assays and from a short-term in vivo inhalation study.

A wide range of fiber types was assessed in two in vitro assays: production of the proinflammatory cytokine tumor necrosis factor alpha (TNF-α) and injury to cells of the alveolar epithelial cell line A549. We selected TNF-α for measurement as it is important in initiating a range of proinflammatory processes, and because it is important in the development of pathology in lungs exposed to asbestos or quartz (6,7). Alveolar epithelial cells are primary targets for toxic effects of fibers deposited in the lung, and loss of epithelial integrity is an important event leading to inflammation. Accordingly, we assessed the effects of fibers on epithelial cell adherence in vitro.

For the in vivo study we used bronchoalveolar lavage (BAL) and a measure of lung cell proliferation to assess the lung response to short inhalations of three selected fiber types. Recruited inflammatory cells, measurable in lavage, are able to release mitogenic cytokines and cause injury to the epithelium, which eventually could lead to epithelial hyperplasia, a starting point for pathological change. Measurement of protein in lavage fluid provides an index of epithelial integrity (8). Proliferation is important in the normal maintenance of tissue structure, but increased cell proliferation is key to the development of fibrosis and the clonal expansion of mutated cells leading to cancer. Increases in the proliferative activity of cells in the lungs of rats exposed to chrysotile asbestos fibers have been described in detail by Brody and his associates (9,10).

The results of this study are discussed in terms of the pathogenicities of the fibers, known from published long-term animal studies (3,11–14).

Materials and Methods

Test Fibers

The fibers used in vitro were a) amosite and crocidolite asbestos; b) special purpose code 100/475 and code 104E glass fibers; c) two types of silicon carbide whiskers, SiC1 (Advanced Composite Materials Corporation, Green, SC) and SiC2 (Third Millennium Technologies, Malibu, CA); d) refractory ceramic fibers (RCF 1, 2, 3, and 4); and e) man-made vitreous fibers (MMVF) 10 and 11 (glass insulation wools), 21 (stone wool), and 22 (slag wool). The amosite was from a batch of South African amosite previously characterized and studied by the authors (13) that upon dust generation gives an aerosol containing a significant proportion of fibers greater than 20 μm in length. The
crocidolite and RCF and MMVF were obtained from the Thermal Insulation Manufacturers Association (TIMA; Denver, CO) repository of size-selected fibers. For the inhalation assay, SiC1 whiskers, code 100/475 glass fiber, and long-fiber amosite were used. Fiber length distributions were determined by phase contrast optical microscopy.

**Detachment of A549 Epithelial Cells from Culture Wells**

Cells of the alveolar epithelial A549 line were labeled with radioactive chromium ($^{51}$Cr) and seeded in 96-well plates at $5 \times 10^4$/well. Fibers in suspension at concentrations of 10, 25, 50, and 100 $\mu$g/ml were added to wells in triplicate. Wells containing medium and cells alone were used as a negative control. After a 4-hr incubation, the amount of cell detachment from the plastic wells was determined by measuring free and cell-associated radioactivity in the supernatants of each well as described (15). Detachment is expressed as the geometric mean of cell-associated radioactive counts per minute for three wells.

**Tumor Necrosis Factor Production by Alveolar Macrophages**

Alveolar macrophages were harvested from control rat lungs by BAL and cultured at $1 \times 10^4$/well in 24-well plates with $8.2 \times 10^6$ fibers (> 5 $\mu$m in length) per well of each test fiber. After 24 hr, supernatants were harvested, centrifuged free of cells and fibers, and stored at $-70^\circ$C until required for assay. TNF-α was measured in the supernatants using the L929 cell bioassay (16). This assay gave results similar to an enzyme-linked immunosorbent assay technique when measuring endotoxin-stimulated rat alveolar macrophage TNF-α (RT Cullen, unpublished observations). Treatment of supernatants with a polyclonal neutralizing anti-human TNF-α antibody (Genzyme, Cambridge, MA), active against rat TNF-α, confirmed that the cytotoxic effect on L929 cells was due to TNF-α.

**Inhalation Exposure**

Rats were exposed by whole body inhalation to the fiber types at a concentration of 1000 (World Health Organization) fibers/ml for 7 hr/day. Untreated control animals were kept in normal room air. Four dosing regimens were used: 1, 3, 8, and 14 days of actual exposure over a 3-week calendar period. The 8- and 14-day regimens included one and two noninhalation weekend breaks, respectively. Six rats per time point were used for the SiC1 whiskers group, and between 6 and 12 rats were used per time point for the amosite and code 100/475 groups. Animals were sacrificed with a single ip injection of Nembutal 18 hr after completion of the final day's exposure.

**Bronchoalveolar Lavage**

After sacrifice, the thoracic cavity was opened, the trachea was cannulated, and the lungs were removed and sequentially lavaged with four 8-ml aliquots of saline at 37$^\circ$C. Cells were recovered from lavage fluid by centrifugation, pooled from the four aliquots, and resuspended in F-10 medium (Gibco, Paisley, UK), containing 0.2% bovine serum albumin (BSA) (Sigma Chemical Co., Poole, Dorset, UK). Total cell counts were made, and cyt centrifuge smears were prepared and stained with Diff-quick (Merz Dade, Dudingten, Switzerland) to obtain differential cell counts.

**Protein Measurement in Bronchoalveolar Lavage Fluid**

The cell-free fluid from the first 8-ml lavage aliquot from each animal was used to determine protein levels in lavage using a standard spectrophotometric assay (Biorad Laboratories, Munich, Germany). BSA was used to provide a standard curve. Standards and test samples were measured in triplicate at 595 nm. Protein concentrations in the samples were calculated from the standard curve by regression and expressed in milligrams per liter.

**Cell Proliferation Using BrdU DNA Labeling**

Additional groups of three rats (four for long amosite) were exposed by inhalation for 1 day and were given an ip injection of 5'-bromo-2'-deoxyuridine (BrdU) (20 mg/kg) 2 hr before sacrifice. BrdU is incorporated into the nuclei of dividing cells and can be detected by immunohistochemical staining in 5-μm sections cut from six levels through the lung (apex to base) (17). Results were determined with an image analysis system and expressed as the number of positive staining cells per millimeter length of terminal bronchiolar/alveolar duct perimeter (17).

**Statistical Analyses**

The TNF-α data were analyzed by analysis of variance on the log scale, to investigate the significance of fiber effects and to estimate means and standard errors. Data from all the other assays required some adaptation of this approach—to deal with imbalance through unequal replication (epithelial cell detachment), to adjust for random fluctuations between experimental runs (BAL parameters), or to allow for Poisson variation in counts (BrdU) or binomial variation in proportions (% granulocytes). Since individual means were often based on few replications, standard errors have been calculated from pooled estimates of residual variation. The doses for epithelial cell detachment were not equal in fiber number, and an additional analysis estimated adjusted means for the fiber types by considering fiber number as a continuous covariate. All analyses were carried out in Genstat (18).

**Results**

**Size Characterization of Fiber Types**

A summary of the size characteristics of the airborne fibers obtained in concurrent chronic inhalation experiments, reported elsewhere (14), is given in Table 1. This earlier publication provides more detailed information on size distributions and phase contrast microscopy counting procedures (14). On average, the total numbers of fibers greater than 5 $\mu$m in length was close to the target concentration of 1000 fibers/ml. There were similar numbers of long fibers (> 15 $\mu$m) in each of the dust aerosols.

Size characteristics of the fibers used in the in vitro experiments were determined by scanning electron microscopy. Fibers were defined as having a length to diameter (aspect) ratio greater than 3:1 and a length greater than 0.4 $\mu$m. Table 2 shows the total numbers of fibers (millions) and long fibers (> 15 $\mu$m), MMVF10 had the fewest fibers and code 100/475 the greatest per unit mass. Amosite and code 100/475 had the greatest number of both short fibers (< 5 $\mu$m) and long fibers (> 20 $\mu$m). The table also shows the geometric means and geometric standard deviations of diameter measurements of the fiber types. The amosite and code 100/475 contained

**Table 1. Numbers of fibers (> 5 μm) per milliliter of air in airborne dust clouds in the inhalation study, as determined by phase contrast microscopy.**

| Fiber type | All > 5 μm | 15–20 μm | > 20 μm |
|------------|------------|-----------|----------|
| Code 100/475 | 1119 | 89 | 137 |
| Long amosite | 981 | 100 | 102 |
| SiC1 | 984 | 122 | 91 |
Table 2. Fiber size characteristics determined by scanning electron microscopy.

| Fiber type | Geometric mean Length | Geometric SD | Geometric mean Diameter | Geometric SD | Fibers, ×10⁶/mg All 0.4 µm > 20 µm |
|------------|------------------------|--------------|-------------------------|--------------|----------------------------------|
| Long amosite | 3.03                  | 2.86         | 0.26                    | 1.75         | 295.0                            | 11.6 |
| Crocidolite | 4.96                  | 2.57         | 0.15                    | 1.53         | 753.3                            | 42.4 |
| C100/475    | 2.88                  | 2.62         | 0.22                    | 1.85         | 1331.0                           | 11.9 |
| 194E        | 3.50                  | 2.17         | 0.25                    | 1.6          | 975.6                            | 16.5 |
| SiC1        | 8.73                  | 2.25         | 0.47                    | 1.39         | 58.1                             | 8.6  |
| SiC2        | Not done              |              |                         |              |                                  |      |
| MMVF10      | 23.91                 | 2.39         | 1.13                    | 1.90         | 7.2                              | 3.8  |
| MMVF11      | 14.21                 | 2.64         | 0.57                    | 2.01         | 21.6                             | 6.6  |
| MMVF21      | 15.66                 | 2.76         | 0.81                    | 1.76         | 11.2                             | 3.7  |
| MMVF22      | 13.67                 | 2.34         | 0.89                    | 1.78         | 11.3                             | 2.7  |
| RCF1        | 10.42                 | 2.86         | 0.79                    | 2.07         | 10.0                             | 2.0  |
| RCF2        | 12.43                 | 2.66         | 0.84                    | 2.01         | 8.1                              | 1.8  |
| RCF3        | 14.99                 | 2.64         | 0.71                    | 2.12         | 9.6                              | 2.8  |
| RCF4        | 6.82                  | 2.00         | 0.94                    | 1.71         | 13.2                             | 0.5  |

Table 3. A549 epithelial cell detachment after in vitro exposure to fibers at mass doses.a

| Fiber type | No fiber | 10 | 25 | 50 | 100 | Counts per minute |
|------------|----------|----|----|----|-----|-------------------|
| No fiber   | 2414     | 625|     |    |     |                   |
| RCF4       | 2727     | 706| 2879|745 |3064 |790 |3194 |624 |
| MMVF11     | 2609     | 727| 3030|784 |3066 |796 |3460 |865 |
| MMVF21     | 3097     | 801| 2788|721 |3622 |937 |4198 |1084 |
| RCF2       | 3052     | 790| 2716|703 |3711 |960 |4409 |1141 |
| RCF1       | 3083     | 798| 3467|897 |3528 |913 |3897 |1009 |
| RCF3       | 3132     | 810| 3392|876 |3536 |915 |4012 |1038 |
| MMVF22     | 3254     | 852| 3499|906 |3908 |1011 |4131 |1089 |
| Crocidolite(TIMA) | 3346 | 877| 3401|892 |4445 |1150 |3815 |987 |
| MMVF21     | 3054     | 790| 3342|865 |4606 |1192 |5194 |1344 |
| Long amosite | 4131   | 1138| 4387|1209|5311 |1475 |5129 |1413 |

Table 4. A549 epithelial cell detachment: effect of adjustment for number of fibers in mass-derived dose.a

| Fiber type | Unadjusted | Adjusted | Rank after adjustment |
|------------|------------|----------|-----------------------|
| RCF4       | 2956       | 730      | 3038                  | 750 | 2       |
| MMVF11     | 3088       | 762      | 3081                  | 760 | 3       |
| MMVF10     | 3383       | 825      | 3276                  | 808 | 6       |
| RCF2       | 3413       | 842      | 3817                  | 945 | 8       |
| RCF1       | 3482       | 859      | 3482                  | 905 | 5       |
| RCF3       | 3566       | 865      | 3710                  | 916 | 7       |
| MMVF22     | 3693       | 912      | 3966                  | 978 | 9       |
| Crocidolite(TIMA) | 3726 | 921| 2513                  | 842 | 1       |
| MMVF21     | 3953       | 976      | 4406                  | 1039 |10      |
| Long amosite | 4596   | 1182     | 3540                  | 905  |

*Means of counts per minute are shown (before and after adjustment by regression on fiber number), and rank order after adjustment. Estimated standard errors are in italics.

greater proportions of fine fibers than the other types. Further details of the counting rules and other size categories are given elsewhere (79).

Epithelial Cell Detachment

Results pooled from three experiments are shown in Table 3. Fibers are ranked according to increasing overall detachment. There were dose-dependent differences in the toxicity of the fiber types, with amosite being the most toxic. However, there was no clear relationship to pathogenicity, as crocidolite caused no more detachment than many of the man-made fibers. As the fiber size distributions of the fiber types were different, the numbers of fibers per unit mass (and hence the number of fibers added to wells) for each fiber type would also have been different. Using phase contrast microscopy counts of fibers greater than 5 µm in length, the estimated mean for each fiber type was adjusted by regression to give a common fiber number. Table 4 compares the results before and after adjustment and shows the changes in rank order. The major effect of adjustment was that crocidolite and amosite asbestos were moved much lower in the order of effects.

TGF-α Production

TGF-α production by alveolar macrophages in vitro is shown in Table 5 as the geometric means of data pooled over three experiments. Fibers have been ranked according to increasing TGF-α production. Differences between fibers were highly significant. The SiC1 whiskers were particularly active, even when compared to SiC2 whiskers. The two asbestos samples ranked next in terms of TGF-α stimulation, with the two glass microfibers (code 100/475 and code 104E) showing intermediate activity. The other fibers stimulated no more activity than that seen in control cultures.

Bronchoalveolar Lavage Studies after Short-term Inhalation

Table 6 summarizes the data for total cells, percentage of granulocytes, and protein in lavage fluid recovered after exposure by inhalation for 1, 3, 8, or 14 days. Overall, the total numbers of cells recovered were increased when compared with the unexposed controls, but there were no clear differences between fiber types nor any clear trend with length of inhalation exposure. Cell numbers in the amosite and silicon carbide groups appeared to return to control levels after longer exposures.
Table 6. Results of assessments from bronchoalveolar lavage: total cell numbers, percent granulocytes, and protein concentrations.*

| Fiber inhaled | Length of inhalation, days | Total cells in BAL \( \times 10^6 \) | Percent granulocytes in BAL | Protein in BAL, U/ml |
|---------------|---------------------------|---------------------------------|-----------------------------|---------------------|
|               | 0                         | 1                               | 3                           | 6                   |
| No fiber      | 6.2                       | 0.5                             |                             |                     |
| 100/475       | 7.2                       | 1.4                             | 6.8                         | 1.6                 |
| Sic 1         | 5.9                       | 1.5                             | 9.0                         | 2.0                 |
| Long amosite  | 9.0                       | 1.8                             | 7.4                         | 1.0                 |
|               |                           |                                 |                             |                     |
| No fiber      | 0.4                       | 0.2                             |                             |                     |
| 100/475       | 1.9                       | 1.1                             | 6.3                         | 1.3                 |
| Sic 1         | 5.0                       | 3.3                             | 5.8                         | 3.6                 |
| Long amosite  | 6.5                       | 2.8                             | 4.4                         | 1.5                 |

Table 7. Numbers of BrdU-positive cells at six levels (from apex to base) in the lungs of rats after inhaling fibers.*

| Fiber type | Level in lung (apex = 1, base = 6) | Counts per mm duct |
|------------|------------------------------------|--------------------|
| No fiber   |                                    | 0.61 (0.36)        |
| 100/475    |                                    | 1.47 (0.49)        |
| Sic 1      |                                    | 2.99 (0.73)        |
| Long amosite |                                  | 4.78 (0.98)        |

*Means with estimated standard errors in italics.

Each of the fiber types caused some inflammation, as shown by the significantly increased proportions of granulocytes (principally neutrophils) among the recovered cells. Compared to the code 100/475 microfiber group, animals exposed to long amosite had a greater early response, although Sic produced the greatest response on average. There were no consistent differences between the fibers, nor any convincing trends with length of exposure. To summarize, although changes with time in total cell and granulocyte numbers were not consistent across the three fiber types, brief inhalation exposure to each of the fibers did result in inflammatory lung responses.

A similar lack of pattern was observed in the levels of protein in the lavage fluid. Although exposed animals had higher average levels than the controls, again there was little evidence of trends with length of exposure, nor consistent differences between fiber types.

Cell Proliferation in Terminal Bronchiolar/Alveolar Ducts after Short-term Inhalation

Table 7 summarizes the numbers of cells staining positive for BrdU in the terminal bronchiolar/alveolar ducts. Exposure to amosite and Sic caused an increase in cell proliferation, while the mean for 100/475 microfiber was close to that for controls. The effects were most marked in the highest levels of the lung, decreasing toward the lower levels. There were significant differences between animals treated with the same fibers (data not shown in Table 7, but reflected in the standard errors). Analysis showed that the principal patterns could be adequately summarized as multiplicative effects of the estimated fiber type and lung level means shown in Table 7. This summary is shown graphically in Figure 1, which displays both the fiber type and lung level effects as constant differences on a logarithmic scale.

Discussion

The processes of inflammation and epithelial cell injury with consequential cell proliferation may be important in the development of disease associated with the inhalation of fibers (1,2,6,9,14,20). Evidence is emerging from both in vitro (21,22) and in vivo (14,23) studies that Sic whiskers can be as toxic as asbestos fibers. In this study we examined a number of short-term in vitro and in vivo assays for the biological effects of various fiber types and considered these effects in relation to known pathogenicity. The drawback of in vitro and short-term in vivo studies is that they generally do not consider the long-term changes to fibers within the lung caused by the process of dissolution. One approach is to pretreat fibers in vitro with a solution that causes partial dissolution (24).

It has been suggested that macrophage cytokines might be central to the inflammation produced by inhaled fibers. Increased release of cytokines and factors mitogenic for fibroblasts by BAL macrophages from asbestos-exposed rats have been reported in many short-term studies (20). We have confirmed our earlier finding (25) that Sic whiskers are very active in stimulating TNF-α. It is interesting that the two asbestos fibers are also highly ranked for TNF-α production. This assay may be able to discriminate markedly pathogenic fibers such as Sic and amosite asbestos from the other fibers, but it is not sufficiently sensitive to separate the MMVF from the RCF. No attempt was made to determine whether there was differential phagocytosis of the fiber types by macrophages. This might affect the amounts of TNF-α produced.

The A549 epithelial cell detachment assay demonstrated the in vitro toxic effects of asbestos fibers as shown previously by the authors (26) and others (27) using similar dose ranges. However, the results reported here indicate that in vitro toxicity assays are not appropriate models of fiber pathogenesis, a conclusion reached in another study (28) of a wide range of fiber types.
In the short-term inhalation experiments, all three fiber types caused recruitment of inflammatory cells and increased concentrations of protein in lavage fluid. The granulocyte response, commonly taken as a measure of inflammation, showed a different pattern of dependence on exposure time for each fiber type. Protein in lavage fluid showed no clear differences between fiber types.

Overall, short-term measures of inflammation did not correlate with pathogenicity. Recent work by Warheit and colleagues (29,30) suggests that better discrimination between fibers can be achieved by studying persistence of inflammation at intervals after cessation of exposure. This may be a fruitful area for further investigation.

Bronchiolar/alveolar deposition of fibers results in increased proliferation in the epithelial and interstitial cells in the lung (10,31,32). In our study the proliferative response to code 100/475 was no greater than that in unexposed control animals, whereas amosite and SiC both produced significant increases in proliferation. In a concurrent study (14), we showed that deposition, in terms of the numbers of fibers greater than 10 μm recovered from lungs at the end of a 12-month inhalation at 1000 fibers/ml, was roughly equivalent for the three fiber types. However, the code 100/475 microfiber differed from long amosite and SiC in the very high numbers of fibers that were less than 5 μm in length. It is likely that the deposition patterns would have been similar in the short-term inhalation study. In addition, dust aerosols for the three fiber types contained similar numbers of long fibers (by phase contrast optical microscopy), and thus, cell proliferation in lungs cannot simply be due to differences in deposition or the numbers of long fibers.

In conclusion, the only test to show promise as a predictive measure of pathogenicity was that of cell proliferation in lungs after brief inhalation exposure (BrdU assay). We believe that this assay should be validated with a wider range of fiber types, doses, and time points.

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