Comparison of Pleural Responses of Rats and Hamsters to Subchronic Inhalation of Refractory Ceramic Fibers

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In the present subchronic study, we compared pleural inflammation, visceral pleural collagen deposition, and visceral and parietal pleural mesothelial cell proliferation in rats and hamsters identically exposed to a kaolin-based refractory ceramic fiber, (RCF)-1 by nose-only inhalation exposure, and correlated the results to translocation of fibers to the pleural cavity. Fischer 344 rats and Syrian golden hamsters were exposed to 650 fibers/cc of RCF-1, for 4 hr/day, 5 days/week for 12 weeks. Following 4 and 12 weeks of exposure, and after a 12-week recovery period, pleural lavage fluid was analyzed for cytokotic and biochemical evidence of inflammation. Visceral and parietal pleural mesothelial cell proliferation was assessed by immunocytochemical detection of bromodeoxyuridine incorporation. Pleural collagen was quantitated using morphometric analysis of lung sections stained with Sirius Red. Fiber-exposed rats and hamsters had qualitatively similar pleural inflammation at each time point. Mesothelial cell proliferation was more pronounced in hamsters than in rats at each time point and at each site. In both species, the mesothelial cell labeling index was highest in the parietal pleural mesothelial cells lining the surface of the diaphragm at each time point. Hamsters but not rats had significantly elevated collagen in the visceral pleura at the 12-week postexposure time point. Fibers were found in the pleural cavities of both species at each time point. These fibers were generally short and thin. These results suggest that mesothelial cell proliferation and fibroproliferative changes in the pleura of rodents following short-term inhalation exposure are associated with fiber translocation to the pleura and may be predictive of chronic pleural disease outcomes following long-term exposure. — Environ Health Perspect 105(Suppl 5):1209–1213 (1997)

Key words: fiber, inhalation, pleura, hamster, cell proliferation

Long-term fiber inhalation studies in rats and Syrian golden hamsters have demonstrated that there are significant interspecies differences in fiber-induced pleural disease (1). While both Fischer 344 rats and Syrian golden hamsters developed significant pulmonary inflammation and interstitial fibrosis following chronic inhalation of a kaolin-based refractory ceramic fiber (RCF)-1, there were significant interspecies differences in tumor induction and the development of pleural fibrosis. F344 rats developed lung tumors but relatively few malignant mesotheliomas. In contrast, Syrian golden hamsters exposed simultaneously to the same fibrous aerosols had no parenchymal lung tumors but developed a high incidence of pleural malignant mesothelioma and pleural fibrosis.

The objectives of the present studies were 2-fold: a) to determine if there were differences in pleural responses to short-term RCF-1 exposure in rats and hamsters that would correlate to long-term disease outcomes; and b) to determine if pleural responses were associated with fiber translocation. The purpose of these studies was to test the hypothesis that interspecies differences in pleural response to RCF-1 exposure were associated with differences in fiber translocation to the pleural space.

Methods

Animals

Male CDF (F344)/CrI BR Fischer rats, 13 weeks of age and weighing 250 to 275 g, were obtained from Charles River Breeding Laboratories (Raleigh, NC). Male LkItVg(SyR)BR Syrian golden hamsters, 13 weeks of age and weighing 140 to 150 g, were obtained from Charles River Breeding Laboratories (Montreal, Canada). All animals were quarantined for a minimum of 10 days prior to exposure and remained free from antibodies to common murine mycoplasmal and viral pathogens throughout the course of the experiment. While not on exposure towers, rats were housed in polycarbonate cages on direct-contact cellulose bedding (ALPHA-dri, Shepard Specialty Papers, Kalamazoo, MI) and supplied NIH-07 cereal-based diet (Ziegler Brothers, Gardner, PA) and water ad libitum. Room temperature was maintained at 60 to 65°C and humidity at 40 to 60% throughout the exposure and postexposure periods.

Exposures

Rats and hamsters were exposed to an RCF-1 aerosol by nose-only inhalation using previously described methods (2). Animals were exposed for 4 hr/day, 5 days/week. Various groups were removed from the exposure regimen at 0, 6, and 12 weeks or were held for an additional 12-week recovery period following the final exposure (week 12). During exposures, light scatter (RAM, Monitoring Instruments for the Environment, Billerica, MA) was used to continuously monitor fiber concentrations. Fiber mass (45.6 ± 10 mg/m3) was determined from samples captured on open-faced, 0.2 μm polycarbonate filters (Gelman Sciences, Ann Arbor, MI) and assayed as previously described (2). The aerosol contained 650 total fibers/cc (length/diameter [L/D] > 3), of which 300 fibers/cc were classified as World Health Organization fibers (L/D > 3, L < 5 μm) (3) (Table 1).

Pleural Fiber Burdens

Groups of six animals were sampled at 0, 4, 12, and 24 weeks and used exclusively to determine pleural fiber burdens. As
exposures were for 5 consecutive days per week, Monday through Friday, animals were euthanatized immediately after the termination of Friday exposures. Characterization of pleural fiber burdens was as previously described (2) using an agarose casting method (4) to recover pleural fibers.

Cell Proliferation Studies

Animals used for pathobiology studies (six/group) were implanted with bromodeoxyuridine (BrdU)-filled miniosmotic pumps to quantify DNA synthesis. Three days after the final fiber exposure, miniosmotic pumps (Alza, Palo Alto, CA) were surgically implanted in the dorsal submucosal skin folds of fiber-exposed and control animals. Miniosmotic pumps were filled with a 2-ml sterile saline solution of BrdU at a concentration of 10 mg/ml, with a discharge rate of 5 µl/hr. After 3 days of labeling, animals were euthanatized and used for lavage and histopathology studies.

Pleural Lavage Fluid

At each time point, six animals from each group were anesthetized with pentobarbital, exsanguinated, and both the lungs and pleural cavity were lavaged with sterile calcium-free, magnesium-free, and phenol red-free Hanks balanced salt solution (HBSS) (Gibco Laboratories, Grand Island, NY) as previously described (5). Rat and hamster plural spaces were lavaged twice with 4 and 3 ml HBSS, respectively. Pleural lavage samples were pooled and centrifuged at 200 × g for 10 min at 4°C. The cell pellets were stored on ice while the lavage supernatant was retained for biochemical analyses. Cell pellets were resuspended in RPMI 1640 media (Gibco Laboratories) containing 10% fetal bovine serum (HyClone Laboratories, Logan, UT). Cell numbers were determined with a Coulter Counter (ZM model, Coulter Electronics, Marietta, GA). Cell differentials were determined from cytospin slides stained with Wright Giemsa Diff-Quik (Leukostat, Fisher Diagnostics, Pittsburgh, PA) as described by Gelzleichter et al. (2). The cell-free lavage supernatants were immediately analyzed for lactate dehydrogenase (LDH) and N-acetylglucosaminidase (NAG) using a COBAS FARA II autoanalyzer (Roche Diagnostic Systems, Montclair, NJ). Protein content was assayed using a commercially available kit (Wako Pure Chemical Industries, Osaka, Japan). Fibronectin content was determined by enzyme-linked immunosorbent assay as previously described (6). For cellular and biochemical assays, all results are expressed as mean values ± 1 SD. Significant differences between groups were determined by an analysis of variance with Fisher protected least significant difference as a posthoc test (p < 0.05).

Plural Collagen Determination

Morphometric collagen measurement in the visceral pleura was obtained from Sirius Red-stained paraffin sections [modified from Malkusch et al. (7)] viewed under polarized transmission illumination (20× objective). Black and white video images were acquired and saved using an image analysis system (Image-i/AT, Universal Imaging, West Chester, PA). Regions evaluated were marked manually and the parameters of area and length were measured. The pleural collagen content was measured as the area of polarizable tissue per unit length of the visceral pleural surface, and the results from fiber-exposed animals were expressed as a percentage increase above control values.

Results

Qualitatively similar patterns of inflammatory cell infiltrate were found in the pleural spaces of both rats and hamsters, with increased numbers of pleural macrophages, eosinophils, neutrophils, and lymphocytes in the pleural lavage fluid (PLF) (Figure 1). In addition to cytologic changes, both hamsters and rats had alterations in PLF biochemical profiles indicative of inflammation (Table 2), including increased total protein, lactate dehydrogenase, N-acetylglucosamine, and fibronectin. Hamsters had a greater inflammatory response than did rats, particularly at the 12-week postexposure time point.

Table 1. Bimodal, bivariate lognormal size distribution of fiber and particle aerosol.

| Aerosol parameters | Fibers, L/D ≥ 3 | Particles, L/D < 3 |
|--------------------|----------------|------------------|
| Median length (µm)| 5.3            | 2.41             |
| Median diameter (µm)| 0.63          | 1.20             |
| GSD (length)       | 2.5            | 1.58             |
| GSD (diameter)     | 1.8            | 1.62             |
| Tau                | 0.54           | 0.80             |
| Percentage aerosol | 65             | 35               |

Abbreviations: GSD, geometric SD; Tau, correlation between the natural logarithm length and diameter.

Figure 1. Cell composition of the pleural lavage fluid (A) macrophages; (B) neutrophils; (C) eosinophils; (D) lymphocytes in fiber-exposed and control rats and hamsters. Results are expressed as means ± SD. * significantly different from control values, p < 0.05 (analysis of variance [ANOVA] with Fisher protected least significant difference [PLSD] as a posthoc test).
**Table 2.** Biochemical analysis of pleural lavage fluid. Data expressed as the percent increase over matched controls.

| Biochemical parameters | 4 weeks | 12 weeks | 24 weeks |
|------------------------|---------|----------|----------|
|                       | Rat     | Hamster  | Rat      | Hamster  |
| LDH                   | 5.5±50* | 1.1±32   | 20±49    | 59±56*   | 28±61    | 112±39*   |
| NAG                   | -10±30  | 87±33*   | 8±25     | 35±41    | -12±35   | 78±48     |
| Total protein         | 19±16*  | 46±28*   | 78±28*   | 148±69*  | 44±10*   | 26±43     |
| Fibronectin           | 34±29   | 20±11*   | 153±57*  | 65±18*   | 86±44*   | 45±24*    |

*Each value represents the mean ± SD for data obtained with pleural cell populations lavaged from five to six animals. *p<0.05, as determined by the Fisher PLSD test.

At each time point, labeling indices in mesothelial cells were higher in fiber-exposed hamsters than in rats in both parietal and visceral pleural sites (Figure 2). Mesothelial cell labeling was greater in control hamsters than in rats at all three time points, which indicates a higher basal level of mesothelial cell proliferation in this species. In both species, the labeling indices in parietal mesothelial cells lining the pleural diaphragmatic surface were greater than those of the visceral pleura. The highest labeling indices were found on the diaphragmatic surfaces in fiber-exposed hamsters (Figure 2). Mesothelial cell proliferation remained significantly elevated at both sites in both species at the 12-week postexposure time point.

Visceral pleural collagen was not significantly increased over controls in hamsters or rats at the end of the 12th week of RCF-1 exposure, nor were any pleural lesions noted. In contrast to the findings noted at the end of the 12-week exposure period, collagen was significantly increased in the visceral pleura in hamsters but not rats in the postexposure group at the 24-week time point (Figure 3). This fibrosis was characterized by focal pleural thickenings associated with hypertrophy of visceral pleural mesothelial cells.

In both species, pleural agarose casts had similar numbers of fibers present at each time point, although higher numbers of fibers were found in the casts of rats at each time point compared to hamsters (Table 3). Most of these fibers were very short and thin.

**Discussion**

The presence of significant cytologic and biochemical changes present in the PLF of subchronically exposed hamsters and rats extends our observations made after acute RCF-1 inhalation exposure. In those studies inflammatory changes immediately following a 1-week exposure in rats or hamsters were not noted; although we noted pleural inflammation 1-month postexposure (5,8, Inflammatory changes in the PLF have been reported in asbestos-exposed rodents with similar cytologic profiles (9,10). In this study, inflammatory changes persisted into the postexposure recovery period and became more marked for several of the cytologic and biochemical end points. The exacerbation of pleural inflammation after cessation of fiber exposure suggests a correlation with progressive injury and chronic fibroproliferative disease outcomes. The finding of persistent pleural space inflammation in fiber toxicology studies underscores the value of PLF analysis whenever the pleura is a suspected target for toxicity. Study of PLF is likely to prove useful for examination of changes in the pleural space in a manner analogous to the way bronchoalveolar lavage fluid analysis has advanced knowledge of pulmonary toxicity.

At present, there is a limited study database that correlates pleural inflammatory changes with fiber-induced and nonfibrous particulate-induced pulmonary toxicity. For this reason, there is little present understanding of which, if any, pleural inflammatory parameters or changes are fiber-specific or which correlate with long-term fibroproliferative pleural disease. Complicating the picture is the finding that pulmonary
Table 3. Fiber size characteristics of pleural fiber burden.

| Week | Length  | Diameter  | Tau  | Fiber number, ×1000 |
|------|---------|-----------|------|---------------------|
|      | GML, μm | GSD       | GMD, μm | GSD                      |                      |
| Hamster |         |           |       |                      |                      |
| 4    | 1.4     | 1.7       | 0.10  | 1.6                   | 0.42                 | 17.5 ± 8.3           |
| 12   | 2.1     | 2.3       | 0.14  | 1.8                   | 0.66                 | 14.8 ± 11            |
| 24   | 2.1     | 2.5       | 0.12  | 1.8                   | 0.54                 | 15.6 ± 5.4           |
| Rat  |         |           |       |                      |                      |                      |
| 4    | 1.7     | 2.0       | 0.11  | 1.6                   | 0.28                 | 42.1 ± 35            |
| 12   | 1.5     | 1.7       | 0.09  | 1.5                   | -0.01                | 41.4 ± 9.4           |
| 24   | 1.6     | 1.8       | 0.10  | 1.6                   | 0.21                 | 40.3 ± 10            |

All GML, GSD, and Tau values are averages for animal groups. Abbreviations: GMD, geometric mean diameter; GML, geometric mean length.

parenchymal inflammation itself causes cytologic and cytokine alterations in pleural cell populations (11).

An early mesothelial cell proliferative response has been reported in mice following inhalation or instillation of long crocidolite asbestos fibers under conditions that resulted in pulmonary fibrosis (12). Subsequent study revealed that this proliferation could result as a nonspecific pleural change following fibrogenic insult to the lung (13). Several authors have suggested that this asbestos-induced visceral pleural mesothelial cell proliferation does not represent a direct effect of fibers in contact with mesothelial cells but may be due to fiber-induced release of reactive oxygen species, cytokines, or growth factors that stimulate cell proliferation in these cells (13,14). These conclusions were made because light microscopic examination of lung and pleura sections found no fibers. The present study using RCF-1 strongly suggests that fiber-induced mesothelial cell proliferation is associated with fibers reaching pleural target sites.

At each of the time points in the present study, fibers were recovered in pleural casts, even though no fibers were found by examination of the visceral pleura using light microscopy. Lack of pleural fiber detection in some previous studies is probably due to the methodology employed. Many of the fibers detected in the present study are of a size that requires the use of electron microscopy for detection. In addition, the use of casts of the pleural space is believed to be a more efficient method of fiber recovery than examination of histologic sections. Relatively rapid translocation of short, thin fibers has been previously reported with chrysotile asbestos and thus is not limited to the present study (15). The recovery of fibers from the pleural space, in conjunction with the finding of a high labeling index in the mesothelial lining of the central diaphragm, makes it highly unlikely that local cytokines or elaborated factors in the parenchymal lung were responsible for the mesothelial cell proliferation noted. The pathways through which fibers reach the pleura, and the populations and sizes of fibers that are responsible for this proliferation, are presently unknown. Our finding of site-specific mesothelial cell proliferation in the rodent parietal pleura strongly supports the recent observation of Boutin and colleagues (16), who found that asbestos fibers accumulate in specific sites in the human parietal pleura associated with lymphatic drainage.

Results of the present study show that the Syrian golden hamster develops more severe fibrotic change and mesothelial proliferation in the pleura than does the F344 rat following inhalation of RCF-1. This strongly suggests that hamster pleura is a more sensitive target organ for fiber-induced disease than is the pleura of the rat. Additional studies of the size distributions of pleural fiber burdens are needed to determine whether differences in retained fiber populations contribute to interspecies differences. There is some evidence, as noted by markedly higher mesothelial cell labeling indices in control and fiber-exposed hamsters, that there are inherent tissue susceptibility differences that can explain interspecies differences in disease outcomes. This finding agrees with previous studies in our laboratory, where we used a fiber instillation model to demonstrate that hamsters respond with higher mesothelial cell proliferation to fiber exposure than do F344 rats (17,18). It is premature, however, to speculate on which rodent species, if any, is a more appropriate model for fiber-induced pleural disease in humans.

In summary, the present experiments demonstrate that pleural inflammation and fibroproliferative changes follow subchronic RCF-1 inhalation exposure in rats and hamsters, and correlate with pleural findings reported in long-term rodent inhalation bioassays. The more severe pleural changes noted in hamsters did not correlate with differences in the number of total fibers that translocated to the pleural space. The correlation of findings between subchronic rodent fiber inhalation exposures and long-term inhalation bioassays will allow the development of short-term rodent models useful for predicting fibrogenic and oncogenic pleural disease following chronic fiber exposure.

REFERENCES

1. McConnell EE, Mast RW, Hesterberg TW, Chevalier J, Kotin P, Bernstein DM, Thevenaz P, Glass LR, Anderson R. Chronic inhalation toxicity of a kaolin-based ceramic fiber in Syrian golden hamsters. Inhal Toxicol 7:503–532 (1995).
2. Gelzleichter TR, Bermudez E, Mangum JB, Wong BA, Everett JJ, Moss OR. Pulmonary and pleural responses in Fischer 344 rats following short-term inhalation of a synthetic vitreous fiber. I: Quantitation of lung and pleural fiber burdens. Fundam Appl Toxicol 30:31–38 (1996).
3. WHO/Europe Technical Committee for Evaluating MMMF. Reference Methods for Measuring Man-Made Mineral Fibers. Copenhagen: Denmark: World Health Organization, 1985.
4. Bermudez E. Recovery of particles from the pleural cavity using agarose casts: a novel method for the determination of fiber dose to the pleura. Inhal Toxicol 6:115–124 (1994).
5. Gelzleichter TR, Bermudez E, Mangum JB, Wong BA, Everett JJ, Moss OR. Pulmonary and pleural responses in Fischer 344 rats following short-term inhalation of a synthetic vitreous fiber. II: Pathobiologic responses. Fundam Appl Toxicol 30:39–46 (1996).
6. Driscoll KE, Maurer JK, Lindenschmidt RC, Romberger D, Rennard SL, Crosby L. Respiratory tract responses to dust: relationships between dust burden, lung injury, alveolar macrophage fibroblast release, and the development of pulmonary fibrosis. Toxicol Appl Pharmacol 106:88–101 (1990).
7. Malkusch W, Rehn B, Bruch J. Advantages of Sirius Red staining for quantitative morphometric collagen measurements. Exp Lung Res 21:67–77 (1995).
8. Everitt J, Bermudez E, Mangum J, Wong B, Miller F, Moss O. Acute pleural response of hamsters and rats to inhaled ceramic fibers. In: Toxic and Carcinogenic Effects of Solid Particles in the Respiratory Tract (Mohr U, ed). Washington: ILSI Press, 1994;599–602.
9. Oberdoerster G, Ferin J, Marcello NL, Meinhold SH. Effect of intrabronchially instilled amosite on lavageable lung and pleural cells. Environ Health Perspect 51:41–48 (1983).
10. Li XY, Lamb D, Donaldson K. Intratracheal injection of crocidolite asbestos depresses the secretion of tumor necrosis factor by pleural leukocytes in vitro. Exp Lung Res 18:359–372 (1992).
11. Li XY, Brown GM, Lamb D, Donaldson K. Reactive pleural inflammation caused by intratracheal instillation of killed microbes. Eur Respir J 6:27–34 (1993).
12. Adamson IYR, Bakowska J, Bowden DH. Mesothelial cell proliferation after instillation with long or short asbestos fibers into mouse lung. Am J Pathol 142:1209–1216 (1993).
13. Adamson IYR, Bakowska J, Bowden DH. Mesothelial cell proliferation: a nonspecific response to lung injury associated with fibrosis. Am J Respir Cell Mol Biol 10:253–258 (1994).
14. Sekhon H, Wright J, Churg A. Effects of cigarette smoke and asbestos on airway, vascular and mesothelial cell proliferation. Int J Exp Path 76:411–418 (1995).
15. Viallat JR, Raybuad MD, Passarel M, Boutin C. Pleural migration of chrysotile fibers after intratracheal injection in rats. Arch Environ Health 41(51):282–286 (1986).
16. Boutin C, Dumortier P, Rey F, Viallet JR, DeVuyst P. Black spots concentrate oncogenic asbestos fibers in the parietal pleura. Thorascopic and mineralogic study. Am J Respir Crit Care Med 153(1):444–449 (1996).
17. Everitt JI, Bermudez E, Mangum JB, Wong B, Moss OR, Janssen D, Rutten AA. Pleural lesions in Syrian golden hamsters and Fischer rats following instillation of man-made ceramic or glass fibers. Toxicol Pathol 22:229–236 (1994).
18. Rutten AA, Bermudez E, Mangum JB, Wong BA, Moss OR, Everitt JI. Mesothelial cell proliferation induced by intrapleural instillation of man-made fibers in rats and hamsters. Fundam Appl Toxicol 23:107–116 (1994).