Comparison of Pulmonary and Pleural Responses of Rats and Hamsters to Inhaled Refractory Ceramic Fibers

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The present study was designed to determine whether pleural fiber burdens or subchronic pleural fibroproliferative and inflammatory changes can help explain the marked interspecies differences in pleural fibrosis and mesothelioma that are observed following long-term inhalation of RCF-1 ceramic fibers by rats and hamsters. Fischer 344 rats and Syrian golden hamsters were exposed to RCF-1 for 4 h per day, 5 days per week, for 12 consecutive weeks. Lung and pleural fiber burdens were characterized during and after exposure. For all time points, approximately 67% of fibers associated with lung tissues from both rats and hamsters were longer than 5 μm in length. In comparison, fibers longer than 5 μm recovered from the pleural compartment, following a 12-week exposure and 12 weeks of recovery, accounted for 13% (hamsters) and 4% (rats) of the distribution. In the 12 weeks after cessation of exposure, the number of fibers longer than 5 μm in length remained constant in the hamster at approximately 150 fibers per cm² pleura. This was 2 to 3 times the corresponding fiber surface density in the rat. Significant pulmonary and pleural inflammation was detected at all time points and for both species. DNA synthesis by pleural mesothelial cells was quantified by bromodeoxyuridine uptake following 3 days of labeling. Labeling indices were higher in hamsters than in rats, both for RCF-1-exposed and filtered air-control animals and was highest for the parietal surface of the pleura. Significantly greater collagen deposition was measured in the visceral pleura of hamsters 12 weeks post-exposure but was not significantly elevated in rats. These findings demonstrate that subchronic inhalation exposure to RCF-1 induces pleural inflammation, mesothelial-cell turnover, pleural fibrosis, and an accumulation of fibers with a length greater than 5 μm in the hamster. The accumulation of long fibers in the pleural space may contribute to the pathology observed in the hamster following chronic inhalation of RCF-1, whereas the presence of short, thin fibers may play a role in the acute-phase biological response seen in both species.

Key Words: refractory ceramic fiber-1 (RCF-1); fiber translocation; pleural mesothelioma; pleural fibrosis; hamster; rat.

Chronic inhalation of RCF-1, a kaolin-based refractory ceramic fiber, is known to induce cancer in both rats and hamsters (Mast et al., 1995a,b; McConnell et al., 1995). As was reported in these studies, long-term exposure to RCF-1 resulted in a 42% incidence of pleural mesothelioma in hamsters, with no significant increase in pulmonary tumors. In rats, however, this same exposure resulted in a significant incidence of pulmonary tumors but a low incidence of pleural mesothelioma. In addition to mesotheliomas, hamsters had more extensive pleural fibrosis than did rats. The reason for this heightened sensitivity of the hamster pleura to RCF-1 is unknown. The development of malignant mesothelioma has been hypothesized to result from the accumulation of carcinogenic fibers in or near pleural tissues (Hillerdal, 1980; Morgan et al., 1977; Suzuki and Kohyama, 1991; Timbrell et al., 1971). Inhaled fibers are capable of migration and accumulation over time to pleural and subpleural regions (Morgan et al., 1977; Sebastien et al., 1980; Suzuki and Kohyama, 1991). In addition, fibers that are not carcinogenic when inhaled (e.g., fiberglass) are known to induce malignant mesothelioma when instilled directly into the pleural cavity of rats (Hesterberg et al., 1993; World Health Organization, 1988). These studies suggest that physical contact of carcinogenic fibers with pleural tissues may play an important role in the pathogenesis of this disease.

Fiber translocation studies have indicated that the fibers found in pleural tissues often differ in size and chemical composition from those found in the lung (LeBoffvant, et al., 1973; Sebastien et al., 1980; Suzuki and Kohyama, 1991). Suzuki and Kohyama (1991) reported that pleural fiber burdens from North American insulation workers contained predominantly short, thin, chrysotile fibers along with relatively small amounts of amosite fibers. In contrast, pulmonary fiber lung samples contain predominantly amphiboles such as crocidolite, anthophyllite, tremolite, and actinolite. Dodson et al. (1991) have speculated that such differences may be driven, to a larger extent, by fiber size differences, rather than chemical differences, and that fiber-size dimensions may be a better predictor of fiber translocation to the pleura.

Our previous studies in rats (Gelzleichter et al., 1996a,b) demonstrated that short, thin RCF-1 fibers (length < 5 μm,
diameter < 0.4 μm) are capable of very rapid translocation to the pleural space. These studies utilized an agarose-detergent digestion method to capture fibers located within the pleural compartment (Bermudez, 1994). The present study examined the translocation process over a longer period, and compared fiber translocation between the rat and hamster. Exposure to asbestos and other fiber types is known to cause several subchronic conditions including edema, pleural inflammation, cellular necrosis and regeneration, and fibroproliferative disease. Since it is not known whether fiber translocation is necessary to induce subchronic pleural pathological effects, a facet of this study was designed to examine the temporal relationship between fiber translocation and the advent of pleural pathology. Rats and hamsters were exposed to an RCF-1 aerosol for 4 h per day, 5 days per week, for 12 consecutive weeks. The preparation of fibers used in this study was enriched for those fibers likely to penetrate to the deep lung (Hesterberg et al., 1993). Total pulmonary and pleural fiber burdens were quantified at 4 and 12 weeks of exposure and at 12 weeks postexposure. Additional animals at all time points were used to characterize the pathobiological responses in both the lung and pleura.

**MATERIALS AND METHODS**

**Animals.** Male CDF (F344)/CrI BR Fischer rats weighing approximately 250–275 g (Charles River Breeding Laboratories Inc., Raleigh, NC) and male Lk:LVG(SYR) BR Syrian golden hamsters weighing approximately 140–150 g (Charles River Breeding Laboratories Inc., Montreal, Canada) free of antibody to murine mycoplasmal and viral respiratory disease were used for all experiments. Animals were housed in an AAALAC-accredited facility in polystyrene cages on direct-contact cellulose bedding and supplied NIH07 cereal-based diet and water ad libitum. Animals were individually identified by ear tag and were randomly distributed to exposure groups using a computer-generated randomization algorithm. Room temperature was maintained at 60–65°F and humidity at 40–60% throughout the exposure and postexposure periods. All animals were quarantined for a minimum of 10 days prior to exposure.

**Exposures.** Animals were exposed to a nominal concentration of 45 mg/m³ RCF-1 by nose-only inhalation (Cannon et al., 1983) using flow-through exposure tubes (Battelle Memorial Institute, Richland, WA). Fiber aerosol was generated by a previously described method (Gelzleichter et al., 1996a). Animals were placed on the exposure tower and exposed to RCF-1 aerosol for 4 h per day (10 a.m. to 2 p.m.), 5 days per week (Monday through Friday), for 12 consecutive weeks. Various groups were removed from the exposure regimen at 0, 4, and 12 weeks or were held for an additional 12 weeks following the final exposure. During the exposures, fiber concentrations were continuously monitored using light scatter (RAM, Monitoring Instruments for the Environment, Inc., Billerica, MA). Aerosol fiber mass values and size distributions were determined from samples captured on open-faced, 0.2 μm polycarbonate filters (Gelman Sciences, Ann Arbor, MI). Filters used to determine the size distribution were assayed as described below.

**Fiber size distributions.** Tissues were sampled at day 0 (unexposed control group), at weeks 4 or 12 of exposure, and 12 weeks postexposure and used exclusively to characterize pulmonary and pleural fiber burdens. Since exposures were limited to 5 days per week, Monday through Friday, animals were euthanized immediately following the final Friday exposure. Characterization of tissue fiber burdens and fiber aerosols were as previously described (Bermudez, 1994; Gelzleichter et al., 1996a). Following euthanasia and exsanguination, the pleural mesothelium was lysed by intrapleural injection of a 41°C, 2% solution of low melting point agarose (BRL, Gaithersburg, MD) containing 0.1% sodium dodecyl sulfate (SDS). Injection volumes were approximately 8 to 11 ml for rats and 4 to 5 ml for hamsters. This was followed by infusion of the lung with 3 and 2 ml (rat and hamster, respectively) of agarose, after which the animals were placed on ice for 30 min to allow the agarose solutions to harden into a solid cast. After removal from the thoracic cavity, the lung and pleural casts were carefully separated and frozen at −20°C for subsequent analysis of fiber content. Lung and pleural samples were freeze-dried, ashed in an oxygen plasma (Plasma Systems, Inc., model LTAs04), resuspended in distilled water, and filtered onto 0.1 μm polycarbonate filters (Nuclepore, Pleasanton, CA). Filters were gold-coated (Technics, Hummer V, Alexandria, VA) and visualized with a scanning electron microscope (JEOL Model JSM 840A, Tokyo, Japan). Analyses of fiber sizes were conducted as previously described (Gelzleichter et al., 1996a). For aerosol and pulmonary samples, fiber lengths were determined at magnifications of 2000 or 500 and fiber diameters at magnifications of 2000. Pleural fiber lengths and diameters were determined at a magnification of 10,000.

**Mesothelial cell proliferation.** Three days following cessation of fiber exposure and 3 days prior to euthanasia, rats and hamsters designated for cellular and biochemical analyses were implanted subcutaneously with osmotic pumps (Alzet, 5.0 μl/h, Alza Corporation, Palo Alto, CA) containing 10 mg/ml 5-bromo-2’-deoxyuridine (BrdU, Sigma Chemical Co., St. Louis, MO; CAS No. 59–14–3, > 99% pure). Animals were killed following 4 or 12 weeks of exposure or 12 weeks after the last exposure, by pentobarbital overdose and exsanguination. The thoracic cavities, including the lungs, were fixed in situ with 4% paraformaldehyde. After 24 h, tissues were removed from paraformaldehyde fixative and stored in 70% ethanol until sections of the left lung lobe, intracostal parietal wall, and diaphragm were embedded into paraffin. Paraffin sections were stained for BrdU by established methods (Rutten et al., 1994). Mesothelial cell proliferation at the various sites was determined by assessing the number of BrdU-labeled mesothelial nuclei as a percentage of the total number of mesothelial nuclei counted (i.e., labeling index, LI). Mean LI with 95% confidence interval (CI) was calculated for each group of 6 animals.

**Pulmonary and pleural free cells.** Following 4 or 12 weeks of exposure, or 12 weeks of recovery, 6 animals from each group were anesthetized with pentobarbital, and exsanguinated. 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 (Gelzleichter et al., 1996b). Rat lungs were lavaged in 2 stages with a total of 10 and 30 ml (5 ml per wash). Similarly, hamster lungs were lavaged in 2 stages but with a total of 8 and 16 ml (4 ml per wash). Rat and hamster pleura were also lavaged in 2 stages using 2 washes per stage and 4 and 3 ml per wash. Recovered lavage fluids were centrifuged at 200 g for 10 min at 4°C. The cell pellets from both stages were combined and stored on ice, while the combined lavage supernatant from the first stage was retained for biochemical analyses. Cell pellets were resuspended in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) containing 10% fetal bovine serum (FBS, Hyclone Laboratories, Inc., Logan, UT), 0.25 μg/ml amphotericin B, 100 U/ml penicillin, and 100 μg/ml streptomycin (antifungal and antibacterial agents from Gibco Laboratories, Grand Island, NY). Cell numbers were determined with a Coulter Counter (ZM model, Coulter Electronics, Marietta, GA). Determinations of cell differentials were from cytocentrifuge slides stained with Wright Geimsa Diff-Quik (Leukostat, Fisher Diagnostics) as previously described (Gelzleichter et al., 1996b).

**Biochemical assays.** The cell-free lavage supernatants were immediately analyzed for lactate dehydrogenase (LDH), alkaline phosphatase, and N-acetylgulcosaminidase (NAG) using a COBAS FARA II autoanalyzer (Roche Diagnostic Systems, Inc., Montclair, NJ). Protein content was determined using a commercially available kit (WAKO Pure Chemical Industries, Osaka, Japan). Fibronectin content was determined by ELISA, as previously described.
Pleural collagen quantitation. The lungs of control and exposed animals were infused with 4% paraformaldehyde under 30 cm of hydrostatic pressure to insure proper and uniform lung expansion. Collagen accumulation in the visceral pleura was morphometrically analyzed using paraffin sections of the left lung lobe stained with Sirius Red. Sections were viewed under polarized transmission illumination at a 200¥ magnification. Black and white video images were captured using an image analysis system (Image-1/AT, Universal Imaging Corp., West Chester, PA). Pleural collagen content was measured as the 2-dimensional area of positively stained tissue per unit length of the visceral pleural surface. Results are expressed as percentage increase above average values from filtered air-control animals.

Statistical methods. Fiber length and diameter were assumed to follow a bivariate lognormal distribution (Cheng, 1986; Moss, et al., 1994; Siegrist and Wylie, 1980). Size distributions were described by the means and variances of the natural logarithms of fiber length and diameter and the correlation between $\ln(L)$ and $\ln(D)$. With the data collected from 6 animals per group, estimates were made of the 5 parameters of the bivariate distribution: the geometric mean length (GML), the geometric standard deviation of length [GSD(L)], the geometric mean diameter (GMD), the geometric standard deviation of length [GSD(D)], and the correlation between $\ln(L)$ and $\ln(D)$ (tau). For completeness, the estimated parameters of the bivariate distributions were recorded for all groups, along with the number of objects sized and the calculated total number of fibers per lung or pleura. However, because of the small number of objects recovered for analysis in some of the animals (as low as 69 in all of the pleura samples from the 4-week-exposed group and 32 in all of the pleura samples from the control group of hamsters), comparisons between species and groups were made using the total estimated number of fibers in each animal fitting in either 5 or 2 length intervals ($L \leq 1, 1 < L \leq 3, 3 < L \leq 5, 5 < L \leq 8, L > 8$ or $L \leq 5, L > 5$). There were insufficient numbers of fibers, particularly from the pleura, to create diameter classes. Standard counting rules (World Health Organization, 1988) were applied for weighting and scaling up the total count represented by each observed fiber. For each length interval, the mean value ± standard error of the mean (SEM) is reported for a pool of 6 animals. The weighted fiber counts were then scaled up on a per-lung or per-pleura basis and normalized by the surface area for the respective species. Hamster and rat lung surface areas of 2800 cm² and 6600 cm², respectively, were obtained from the literature (Sahebjami, 1992; Valberg and Blanchard, 1992). The pleural surface area of the rat and hamster were estimated using the volume of agarose needed to fill the pleural cavity, with the lung partially inflated. Using this volume (5 and 10 ml for the hamster and rat, respectively) the minimum surface area for an equivalent sphere was determined to be 14.1 and 22.4 cm² for hamster and rat, respectively. The calculated rat value showed good agreement with the empirically measured pleural surface area of 24.5 ± 2.2 cm² (personal communication, R. R. Mercer, Duke Center for Extrapolation Modeling, Durham, NC). The calculated value for the hamster and the empirical value for the rat pleural surface area were then used to normalize the burden data. Significant differences between groups were determined by a Student’s t-test; p values of less than 0.05 were considered statistically significant.

For cellular and biochemical assays, all results are expressed as mean values ± 1 standard deviation. Significant differences between groups were determined by a Student’s t-test; p values of less than 0.05 were considered statistically significant.

RESULTS

Characterization of RCF-1 Aerosol

The aerosol mass concentration averaged 46 ± 10 mg/m³. From the bivariate size analysis of $\ln(L)$ and $\ln(D)$, the GML was 6.1 μm with a GSD(L) of 2.8. The GMD was 0.64 μm with a GSD(D) of 2.0. The tau value was 0.57. The average fiber concentration (all lengths) was 647 ± 205 fibers/cc, while the average number of WHO fibers (aspect ratio ≥ 3, length ≥ 5 μm) was 296 ± 126 fibers/cc. About 32% of the total aerosol by number was nonfibrous particles having a ln-normal, bivariate size distribution of 2.2 μm, 1.7, 1.1 μm, 1.7 and 0.87 [GML, GSD(L), GMD, GSD(D), and tau respectively] based on 3788 particles and fibers sized.

Analyses of Pulmonary and Pleural Fiber Burdens

Pulmonary and pleural fiber burdens were characterized from specimens sampled at weeks 0, 4, 12, and 12 weeks after the last exposure. The estimated parameters of the bivariate size distribution of ln(length) and ln(diameter) of fibers in the lungs of rats and hamsters was indistinguishable between pooled lung samples at each time point and between each species. The size distribution obtained by pooling all of the rat and hamster lungs was (8.3 μm, 2.2, 0.62 μm, 1.7, 0.12) and (8.8 μm, 2.3, 0.58 μm, 1.8, 0.18) respectively for 9117 and 6150 objects sized from 18 animals of each species (6 each from weeks 4, 12, and 24). There was little or no correlation between diameter and length in exposed animals compared to a slight correlation (tau = 0.65 and 0.78 for rat and hamster) in the background distribution of non-exposed animals. The background bivariate distribution was very roughly estimated to be (3.1 μm, 1.6, 0.53 μm, 1.8, 0.65) and (2.9 μm, 2.0, 0.52 μm, 1.8, 0.78) respectively for the 77 and 55 objects sized in the lungs of six non-exposed rats and hamsters.

The estimated bivariate size distribution of fibers in the pleura cavity of rats and hamsters was indistinguishable between pooled pleura samples at each time point although the geometric mean length in rat pleura was consistently less than the geometric mean length in hamster pleura. The size distribution obtained by pooling all of the rat and hamster pleura was (1.7 μm, 1.9, 0.10 μm, 1.6, 0.20) and (2.0 μm, 2.4, 0.12 μm, 1.9, 0.62) respectively for 594 and 299 objects sized from 18 animals of each species (6 each from weeks 4, 12, and 24). There was little or no correlation between diameter and length in exposed animals compared to a slight correlation (tau = 0.65 and 0.78 for rat and hamster) in the background distribution of non-exposed animals. The background bivariate distribution was very roughly estimated to be (1.6 μm, 1.6, 0.11 μm, 1.6, 0.18) and (2.0 μm, 2.0, 0.12 μm, 1.6, 0.22) respectively for 95LUNG AND PLEURAL RESPONSES TO RCF-1
fibers per lung in hamster following 12 weeks exposure (Table 1). However, when results were normalized to lung surface area, resulting fiber burdens were similar; 13,400 and 9100 fibers per cm² for rats and hamsters, respectively, 12 weeks after the last exposure (Table 1).

Although fibers were capable of rapid translocation to the pleural compartment in both rat and hamster, total pleural fiber burdens were over 3 orders of magnitude less than in lung. Background levels of fibers, most likely due to environmental contamination, were found. As previously reported (Gelzleichter et al., 1996a), the number of these background fibers was small and there were substantially fewer than the number of fibers found in RCF-1-exposed animals. After 12 weeks of exposure, we recovered 4.1 × 10⁵ fibers per pleura in rat and 1.4 × 10⁴ fibers per pleura in hamster. Similar numbers of fibers per pleura (4.0 × 10⁵ and 1.9 × 10⁴) were found 12 weeks after the last exposure. Normalization of total fiber burden to the pleural surface results in a reduction in the species difference (1600 and 1300 fibers per cm² of pleural surface for rats and hamsters, respectively, 12 weeks after the last exposure; Table 1). When examining pleural surface burdens as a function of fiber size, significantly more long fibers (p < 0.05) (with a length exceeding 5 μm) were present in hamster than rat samples after 12 weeks (140 compared with 40 per cm², respectively). At the end of the 12-week recovery period, the difference was 150 compared with 70 per cm², but was not significantly different at the p < 0.05 level. Conversely, pleural burdens of fibers shorter than 5 μm were greater in the rat than in the hamster at all time points (Table 1). These estimates were made by grouping the observed fibers for 6 hamster pleura and 6 rat pleura samples. In the hamster pleura, the surface density of fibers longer than 5 μm in length was based on 11 out of 75 fibers and 8 out of 69 fibers for weeks 12 and 24, respectively. For the rat pleura, the surface density of fibers longer than 5 μm was based on 6 out of 236 fibers and 9 out of 201 fibers for the same time periods. In comparison, for weeks 12 and 24, there was no difference between species in the calculated lung surface density of fibers having length greater than 5 μm (Table 1). These latter calculations were based on grouped data from the 6 hamster and rat lungs. For these 2 time points, the observed number of fibers in this size range was 1403 out of 2222 fibers and 1426 out of 1937 fibers for the hamster lung, and 2421 out of 3615 fibers and 1458 out of 2124 fibers for the rat lung, respectively.

**Pulmonary and Pleural Lavage Fluid Analysis**

In both rat and hamster, a massive influx of neutrophils was observed in BALF, with peak responses occurring at week 12, which remained above filtered air-exposed control values after

### Table 1

Categorization of Lung and Pleural Fiber Burdens by Length

<table>
<thead>
<tr>
<th>Week</th>
<th>Length category (μm)</th>
<th>Fibers per cm² lung surface (^a) (10⁻³)</th>
<th>Fibers per cm² pleural surface (^a) (10⁻³)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Hamster</td>
<td>Rat</td>
</tr>
<tr>
<td>0</td>
<td>L ≤ 1</td>
<td>— (^b)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>1 &lt; L ≤ 3</td>
<td>1 ± 0</td>
<td>1 ± 0</td>
</tr>
<tr>
<td></td>
<td>3 &lt; L ≤ 5</td>
<td>0 ± 0(^c)</td>
<td>1 ± 0</td>
</tr>
<tr>
<td></td>
<td>5 &lt; L ≤ 8</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td></td>
<td>8 &lt; L</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>4</td>
<td>L ≤ 1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>1 &lt; L ≤ 3</td>
<td>6 ± 1</td>
<td>4 ± 1</td>
</tr>
<tr>
<td></td>
<td>3 &lt; L ≤ 5</td>
<td>12 ± 1</td>
<td>10 ± 1</td>
</tr>
<tr>
<td></td>
<td>5 &lt; L ≤ 8</td>
<td>10 ± 1</td>
<td>11 ± 2</td>
</tr>
<tr>
<td></td>
<td>8 &lt; L</td>
<td>22 ± 3</td>
<td>18 ± 2</td>
</tr>
<tr>
<td>12</td>
<td>L ≤ 1</td>
<td>—</td>
<td>—</td>
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<tr>
<td></td>
<td>1 &lt; L ≤ 3</td>
<td>14 ± 2</td>
<td>12 ± 1</td>
</tr>
<tr>
<td></td>
<td>3 &lt; L ≤ 5</td>
<td>21 ± 2</td>
<td>25 ± 0</td>
</tr>
<tr>
<td></td>
<td>5 &lt; L ≤ 8</td>
<td>20 ± 2</td>
<td>27 ± 1</td>
</tr>
<tr>
<td></td>
<td>8 &lt; L</td>
<td>41 ± 4</td>
<td>48 ± 3</td>
</tr>
<tr>
<td>24(^d)</td>
<td>L ≤ 1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>1 &lt; L ≤ 3</td>
<td>6 ± 1</td>
<td>12 ± 3</td>
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<td>3 &lt; L ≤ 5</td>
<td>18 ± 2</td>
<td>30 ± 4</td>
</tr>
<tr>
<td></td>
<td>5 &lt; L ≤ 8</td>
<td>21 ± 2</td>
<td>34 ± 4</td>
</tr>
<tr>
<td></td>
<td>8 &lt; L</td>
<td>46 ± 5</td>
<td>58 ± 5</td>
</tr>
</tbody>
</table>

\(^a\) Mean count ± SEM; n, 6.
\(^b\) No fibers found in this class.
\(^c\) 0 ± 0 is a result of rounding.
\(^d\) 12 weeks of exposure followed by 12 weeks of recovery.
12 weeks of recovery (data not shown). The number of BALF lymphocytes progressively increased in both species with no sign of attenuation following recovery (data not shown). In PLF, significant influxes of inflammatory cells, including macrophages, neutrophils, eosinophils, and/or lymphocytes, were evident for both species. In contrast to results from BALF analysis, pleural inflammation contained a strong eosinophilic component (Fig. 1). The magnitude of these responses indicates that pronounced pleural inflammation was present as early as 4 weeks into the RCF-1 exposures and was sustained throughout the exposure and 12-week postexposure periods. Total soluble protein and fibronectin were significantly elevated in both rat and hamster PLF, with peak responses occurring at week 12 in both species. LDH release, indicative of cellular damage, was significantly elevated in hamster PLF samples after 12 weeks of exposure and after 12 weeks of recovery (Table 2).

**BrdU Labeling of Pleural Mesothelium**

BrdU labeling indices are shown in Figure 2 for the visceral and diaphragmatic pleura. Basal levels of cell turnover, as measured in air-exposed control animals, was substantially greater in hamster than rat for both the visceral and parietal pleura. RCF-1 exposure in both rats and hamsters induced significant mesothelial-cell labeling on the visceral pleural surface at 4 and 12 weeks, but had returned to near baseline values by the end of the 12-week recovery period. Although
the magnitude of the increases relative to filtered-air control animals was similar between the two species, the labeling index was substantially higher in the hamster (e.g., 11.2% for hamster and 1.6% for rat at 12 weeks). Cells of the parietal pleura in RCF-1-exposed animals had the greatest amount of BrdU labeling, with labeling indices nearly identical for the costal (data not shown) and diaphragmatic surfaces. Pronounced increases were evident for both species, and, unlike responses on the visceral pleura, were sustained throughout the 24-week examination period. As was seen in the visceral pleura, the magnitude of response in RCF-1 exposed animals was similar in both species relative to filtered-air control values, but in absolute terms the labeling indices were much higher in hamster samples. In the hamster, 42% of all cells were labeled at 12 weeks on both the costal and diaphragmatic pleura, compared with peak labeling of 16% for rat costal and diaphragmatic pleura.

Collagen Accumulation in the Visceral Pleura

The extent of collagen accumulation in the visceral pleura was determined by morphometric analysis of cross sectional areas of the lung paraffin sections. Collagen accumulation was calculated as the summation of cross-sectional areas visualized by polarized light divided by the total length of analyzed pleura. No significant increase in collagen deposition was observed after 12 weeks of exposure in rats (0%) or hamsters (8%). However, 12 weeks after the last exposure, collagen accumulation was elevated in RCF-1 exposed rats (17%) and hamsters (44%), although this was only statistically significant in hamsters.

DISCUSSION

The present study was designed to determine whether pleural fiber burdens or subchronic pleural fibroproliferative and inflammatory changes can help explain the marked interspecies differences in pleural fibrosis and mesothelioma observed following long-term inhalation of RCF-1 ceramic fibers by rats and hamsters (Mast et al., 1995a,b; McConnell et al., 1995). The translocation of fibers to the pleura following subchronic inhalation of RCF-1 fibers was examined. As we had previously demonstrated in rats (Gelzleichter et al., 1996a), small but significant numbers of RCF-1 fibers were capable of translocation to the pleural compartment in both rats and hamsters at all time points in this study. The estimated bivariate size distribution of the fiber lung burden for both species did not appear to shift over time. This is in agreement with previous studies with RCF-1 (Hesterberg et al., 1993). Similarly, the estimated bivariate size distribution of pleural fibers did not dramatically shift over time in hamsters or rats. The lack of

<table>
<thead>
<tr>
<th>Species</th>
<th>Time (wks)</th>
<th>Treatment</th>
<th>Lactate dehydrogenase (μ/l)</th>
<th>Total protein (mg/dl)</th>
<th>Fibronectin (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hamster</td>
<td>4</td>
<td>Filtered air</td>
<td>35.0 ± 13.7*</td>
<td>23.2 ± 7.2</td>
<td>0.53 ± 0.08*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RCF-1</td>
<td>35.4 ± 11.2</td>
<td>34.0 ± 6.5*</td>
<td>0.66 ± 0.05*</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>Filtered air</td>
<td>29.0 ± 5.9</td>
<td>17.8 ± 3.3</td>
<td>0.43 ± 0.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RCF-1</td>
<td>46.2 ± 16.9*</td>
<td>44.2 ± 11.0*</td>
<td>0.68 ± 0.04*</td>
</tr>
<tr>
<td></td>
<td>24†</td>
<td>Filtered air</td>
<td>41.0 ± 14.5</td>
<td>26.2 ± 7.9</td>
<td>0.27 ± 0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RCF-1</td>
<td>86.8 ± 16.1*</td>
<td>32.9 ± 11.4</td>
<td>0.38 ± 0.05*</td>
</tr>
<tr>
<td>Rat</td>
<td>4</td>
<td>Filtered air</td>
<td>50.8 ± 24.5</td>
<td>17.8 ± 1.3</td>
<td>0.87 ± 0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RCF-1</td>
<td>48.0 ± 25.3</td>
<td>21.2 ± 2.9*</td>
<td>1.18 ± 0.23*</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>Filtered air</td>
<td>44.0 ± 10.0</td>
<td>22.2 ± 3.6</td>
<td>1.01 ± 0.14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RCF-1</td>
<td>52.8 ± 21.8</td>
<td>39.4 ± 6.2*</td>
<td>2.46 ± 0.59*</td>
</tr>
<tr>
<td></td>
<td>24†</td>
<td>Filtered air</td>
<td>56.2 ± 37.1</td>
<td>30.8 ± 6.5</td>
<td>1.71 ± 0.31</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RCF-1</td>
<td>72.2 ± 34.0</td>
<td>44.3 ± 3.1*</td>
<td>3.04 ± 0.70*</td>
</tr>
</tbody>
</table>

* Numbers represent mean values ± 1 SD.
† Numbers in parentheses denote percent increases above filtered air control values.
± Relative hamster fibronectin values determined using standard curves for rat fibronectin.
‡ Twelve weeks of exposure followed by 12 weeks of recovery.
* Significantly different from animals exposed to filtered air alone, p ≤ 0.05 (Student’s t-test).

The biochemical analysis of pleural lavage fluid is presented in Table 2.
change in the estimates of the size distributions may be due to the smoothing nature of fitting a bivariate distribution surface to the ln(length)-ln(diameter) frequency data.

The ability of fibers to migrate to pleural tissues is well documented. Several studies have shown that short, thin mineral fibers readily deposit in subpleural regions of the lung. For example, chrysotile-laden macrophages not only aggregate near the pleural surface but are capable of rapid penetration into the pleural compartment (Coin et al., 1992; Holt, 1983; Viallet et al., 1986). How fibers, particularly long fibers, translocate to the pleural space is unknown, but may occur through passive movement through the pleura or may be mediated by the movement of inflammatory cells that have taken up fibers. Sebastien and coworkers (1980) examined parietal pleural fiber burdens in asbestos-exposed workers and found predominantly short, thin fibers (length < 8 μm). Similar findings have been reported from other laboratories (Dodson et al., 1986; Suzuki and Kohyama, 1991), which suggest that the more complex architecture of the human pleura does not alter the sizes of fibers translocating into the pleural space, although it may affect the kinetics of translocation. Long, thin fibers are generally regarded to have the greatest mesotheliomagenic potential. Such a size dependence has been reported for inhalation, intratracheal instillation, and intrapleural and intraperitoneal administration studies using animal models (Davis et al., 1986, 1991; Pott, 1978; Stanton et al., 1977; Stanton and Wrench 1972). In the present study, approximately 67% of pulmonary fibers in both rats and hamsters, at all time points, were longer than 5 μm and approximated the composition of the aerosol. In comparison, 12 and 4% of the pleural burden (12 weeks after the end of exposure) in hamster and rat, respectively, was composed of fibers longer than 5 μm. In the 12 weeks after the cessation of exposure, the number of fibers longer than 5 μm in length remained constant in the hamster at approximately 150 fibers per cm² pleura. This was from 2 to 3 times the pleural surface density of corresponding fibers in the rat. It is possible that under conditions of continuous exposure, the accumulation of these longer fibers will reach a critical fiber burden that leads to the induction of mesothelioma. Species differences in the pleural burden of short fibers (length < 5 μm) were also observed. The pleural burden of short fibers in the rat was approximately 1.5 to 2 times that of the hamster. Although the role of these shorter fibers in the biologic response of these species is not clear, it can be speculated that it involves the induction of the inflammatory response.

There was substantial pleural inflammation and cellular damage as early as 4 weeks into the RCF-1 exposure. Labeling indices of mesothelial cells were dramatically elevated in both rats and hamsters for RCF-1-exposed animals on both the visceral and parietal pleural surfaces. Strikingly, these indices

![Fig. 2. Visceral and diaphragmatic mesothelial cell proliferation in air and RCF-1-exposed rats and hamsters. The time points shown represent 4 and 12 weeks of exposure and 12 weeks of exposure followed by 12 weeks of recovery. Data are expressed as means with 95% confidence intervals. *Significantly different from control values; p < 0.05 (ANOVA).](image-url)
were higher for the parietal than for the visceral pleura and were still prominent on the parietal surface after 12 weeks of recovery in both species. This finding is in agreement with recent studies in asbestos-exposed people that demonstrate more severe accumulation of fibers in the parietal pleura (Boutin et al. 1996). Examination of collagen deposition indicated no evidence of fibrosis after 12 weeks of exposure. After 12 weeks recovery however, collagen accumulation was significantly elevated in RCF-1-exposed hamsters. These results indicate that RCF-1 inhalation led to significant and sustained pleural inflammation and fibroproliferative changes that were more severe in the hamster than in the rat. Although substantial and progressive pleural pathological changes were evident in this study and were accompanied by translocation of fibers to the pleura, it is difficult to conclude a direct causal relationship. Proliferation of pleural or subpleural cells, mainly mesothelial cells and fibroblasts, may be mediated by cytokine release from alveolar macrophages (Adamson, et al., 1991; Bermudez et al., 1998; Schapira et al., 1991). It has been suggested that several non-fiber-related causes of mesothelioma, such as radiation, tuberculosis, or emphysma may be associated with chronic inflammation (Browne, 1991; Pelnar, 1988). The large influx of inflammatory cells, prominent at all time points in this study, indicates that a sustained inflammatory response is present in the pleural compartment of both rats and hamsters exposed to RCF-1 by an inhalation route.

We have demonstrated that subchronic exposure to RCF-1 fibers by an inhalation route is capable of inducing a rapid and sustained pleural inflammatory response in both rats and hamsters that culminates in progressive fibroproliferative serosal pathology. In general, RCF-1-exposed hamsters differed from rats in that they had a more pronounced pleural response and accumulated more fibers per unit pleural surface area, with lengths exceeding 5 µm. The two species had substantial differences in both pleural inflammatory-cell responses and mesothelial cell proliferation. The interspecies differences in mesothelial cell cytokinetics are evident by marked differences in basal levels of pleural mesothelial cell labeling. In summary, the present study demonstrates that rats and hamsters differ in their subchronic responses to inhaled ceramic fibers, and that these differences may be due in part to differences in retained pleural fiber burdens.

ACKNOWLEDGMENTS

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REFERENCES


