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Fibrogenic Potential of Intratracheally Instilled Quartz, Ferric Oxide, Fibrous Glass, and Hydrated Alumina in Hamsters*

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ABSTRACT

As a first step in the development of an animal model for determining the role of pulmonary fibrosis in the etiology and pathogenesis of lung cancer, the fibrogenic potential of quartz, quartz and ferric oxide administered together, fibrous glass, and hydrated alumina were studied by multiple intratracheal instillation in groups of male Lak:LVG Syrian golden hamsters. Dose-related decreases in survival were evident for the groups instilled with the two highest doses of quartz or quartz and ferric oxide. Instillation of quartz or quartz and ferric oxide induced the greatest pulmonary fibrosis in response to the materials tested. However, the dense fibrous tissue present in the lungs in classical human silicosis and in experimental silicosis of rats was not observed in this study. The results of this study indicate that the Syrian golden hamster is not a suitable species for studying the role of quartz-induced pulmonary fibrosis in pulmonary carcinogenesis.

INTRODUCTION

Epidemiologic studies have suggested that inhalation of mineral dusts, and the resultant pulmonary fibrosis, may contribute to the development of lung cancer (2, 3, 7, 15–17, 34). Particulate materials have also played a key role in the experimental induction of pulmonary tumors. The success of the intratracheal instillation model of Saffiotti et al (27) was attributed in part to the role of ferric oxide carrier dust in transporting benzo(a)pyrene across cell membranes. Considerable research has been done to determine the role of carrier dust in the production of respiratory tract tumors using Saffiotti's method (4, 11, 23, 31). However, the role of the carrier dust in the induction of pulmonary neoplasia remains unclear.

One proposed mechanism by which ferric oxide exerts its cocarcinogenic effect is stimulation of cellular proliferation (4). If this proposed mechanism is correct, it seems logical that particulate materials such as quartz, which stimulates a pronounced proliferative response in the lung (29), may possess much greater cocarcinogenic potential than ferric oxide. Pylev (25) reported that pulmonary tumors were induced in rats administered quartz intratracheally with benzo(a)pyrene, whereas no tumors were induced with benzo(a)pyrene or quartz alone.

A logical step in determining the role of pulmonary fibrosis in pulmonary cancer is development of an animal model in which intense pulmonary fibrosis is induced by particulate material, without reducing the life

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span. The animal model should also have a predictable neoplastic response to intratracheally instilled carcinogens such as benzo(a)pyrene. If the particulate materials induce pulmonary fibrosis in conjunction with the presence of a known carcinogen such as benzo(a)pyrene, then the role of these particulate materials as potential pulmonary cocarcinogens may be assessed. The objective of this study was to evaluate potential animal models by inducing varying degrees of pulmonary fibrosis using different doses of four particulate materials.

Methods

Min-U-Sil quartz was obtained from Pennsylvania Glass Sand Corp., Pittsburgh, PA. Certified grade red anhydrous ferric oxide (lot no. 785156) and certified grade hydrated alumina (lot no. 784879) were obtained from Fisher Scientific Co., Fair Lawn, NJ. The fibrous glass sample was Tempstran code 100/475, obtained from the Manville Corporation, Denver, CO.

Prior to particle size determinations, samples of each test material were prepared from the bulk powders by dispersing a small quantity of each powder in a 0.05% solution of Aerosol OT (American Cyanamid Co., Princeton, NJ) in deionized water. The samples were then sonicated for 10 min in a Mettler model ME 4.6 ultrasonic bath to disrupt aggregates. The fibrous glass sample was ground for 3.5 min in a planetary mill, suspended in water, filtered, and sieved to break up agglomerates prior to sonication. After sonication, an aliquot of each suspension was filtered through a 0.1 μm pore size Nuclepore filter (General Electric Co., Cleveland, OH). The particulates on each filter were then analyzed for particle size. Particle size and fiber dimension data were generated for samples of each test material (Table I) using a scanning electron microscope (JEOL, Model JXA-50A) equipped with an energy-dispersive x-ray spectrometer system (EG&G, Ortec Model EEDS II) and an image analysis system (LeMont Scientific, Model B-10) using a backscattered electron image. Suspensions of each material in sterile saline were prepared immediately prior to each weekly instillation. To insure adequate dispersal of the material in the saline solution, the suspension was sonicated for 15 min at 200 watts and stirred on a magnetic stirrer prior to instillation.

Male outbred Syrian golden hamsters (strain Lak:LVG) were purchased at approximately 6 weeks of age from Charles River Breeding Laboratories (Lakeview), Wilmington, MA. Upon arrival, ten hamsters were necropsied, and samples were taken to culture for bacterial pathogens, histopathologic examination, and serologic screening for viral antibody titers. Histopathologic and serologic findings indicated the presence of Sendai virus infection. Because of the unavailability of weanling hamsters free of Sendai virus infection (21), and because the respiratory tract lesions associated with Sendai virus infection in hamsters should not be expected to persist (24), these animals were utilized for the study. At approximately 10 weeks of age, the hamsters were divided into 18 groups of 25 animals each (four dose levels each of four materials plus saline and cage control groups). Hamsters dying within the first 7 weeks of instillation were replaced with animals from the same shipment. During the instillation and postexposure periods, hamsters were individually housed in stainless steel wire cages, fed Wayne Lab Blox (Allied Mills Inc., Chicago, IL), and provided water ad libitum. Intratracheal instillations began at approximately 11 weeks of age, using a modification (1) of the method of Morrow (19). Intraperitoneal injection of 0.42 ml of a 1% solution of methohexital sodium (Brevital sodium; Eli Lilly and Co., Indianapolis, IN) per 100 g body weight was used for anesthesia during instillation (8).

Each group of hamsters received 15 weekly instillations of one of four doses of one of the materials (Table II). Doses of each material were chosen on the basis of information available from the literature (9, 22, 26, 30, 35). Groups of hamsters receiving quartz and ferric oxide were given the same dose range of quartz as those groups receiving quartz alone, plus an equal amount of ferric oxide. This dose range was chosen because the fibrogenic potential of ferric oxide alone was considered to be nearly negligible compared with that of quartz.

Hamsters were observed daily and weighed weekly during instillations, biweekly throughout the postexposure period, and immediately before necropsy. Each group of hamsters was held until survival within the group reached 20%, at which time all remaining animals within the group were killed. All remaining groups were killed when the animals reached 24.5 months of

age. Any moribund animal was killed. Hamsters were killed by intraperitoneal injection of a lethal dose of pentobarbital sodium (Nembutal sodium, Abbott Laboratories, Chicago, IL). Necropsy was performed on all animals. The larynx, trachea, lungs, and associated mediastinal tissues were weighed as a unit. The lungs were inflated with 10% neutral buffered formalin until they reached a visually estimated volume equal to that at full inspiration, then tied off at the trachea, and immersed in formalin. Tissue samples from the heart, stomach, liver, kidney, spleen, and any gross lesions were also fixed in neutral buffered formalin. Following fixation, the lungs were trimmed away from the trachea, and a 5-mm thick section was cut through the widest dimension of each lobe. These tissue sections from each lobe of lung, tracheobronchial lymph node, and trachea were processed, embedded in paraffin, sectioned at 5–6 μm, and stained with hematoxylin and eosin. Representative samples of gross lesions in other tissues were similarly processed and stained, and special stains for collagen (Masson's trichrome stain) and amyloid (Congo red) were made on selected tissues. Histopathologic evaluation of tissues included blind comparisons of lung sections from animals in all tested and control groups to eliminate bias. This was accomplished by first examining the tissues from all groups, with the groups identified, to determine the types of lesions present, then re-evaluating and grading lesions in the target organs without knowing the group from which each animal originated. Pulmonary lesions were classified as to severity (1 = minimal, 5 = severe) and area of involvement (focal, multifocal, diffuse).

Lung weight data were analyzed statistically using analysis of covariance, with dose of instilled material as a main effect and body weight and survival time as covariates. Differences in survival time were examined statistically using the BMDP life-table program P1L (6). The Kaplan-Meier estimate of the survival function was used (14). The prevalence and grade of pulmonary fibrosis were analyzed using two-way analyses of variance with factors of survival time and dose. Snell's (28) technique for scaling ordered categorical data was applied to equalize the variance in each cell of the two-way classification. The scaling extended the grade scale, so that scaled grades above 5 were possible. For all of the analyses, the data were divided into three survival time strata defined by $t \leq 440$ days, 440 days $< t \leq 600$ days, and $t > 600$ days, where t is the time on study.

RESULTS

Table I gives results of the physical characterization of the four instilled materials. The ferric oxide sample was highly aggregated; the ultimate particle size appeared to be 0.02 mm. The large mass median and aerodynamic diameters for the hydrated alumina sample indicate the presence of a few very large particles in the sample, which on a weight basis were a considerable part of the sample. Forty per cent of the particles characterized from the fibrous glass sample were fibers (aspect ratios greater than 3:1); 15% of

TABLE I—Physical Characterization of Quartz, Ferric Oxide, Alumina, and Fibrous Glass Materials Used in Their Study[a]

Material	No. of Particles	Particle Diameter			
		Median	Average	Mass Median	Mass Aerodynamic
Quartz (6 samples)	1048 ±70	0.84 ±0.07	1.06 ±0.07	3.14 ±0.24	5.13 ±0.40
Ferric oxide (1 sample)	1754	0.27	0.29	0.60	1.37
Alumina (1 sample)	1002	0.50	0.81	6.31	9.81
		Median Diameter	Median Length		
Fibrous glass	1017	1.88	2.97		
Fibrous glass[b]	407	0.75	4.30		

[a] Quartz measurements from six samples; all other materials from one sample. Measurements in micrometers ± SD.
[b] That portion of the fibrous glass sample classifiable as fibers with a length/diameter ratio >3:1.

the fibers characterized were greater than 10 mm in length. The median length of the fibers was 4.30 mm with a median diameter of 0.75 mm.

Survival, compared with the saline control group, was significantly decreased in the groups instilled with the two highest doses of quartz or quartz and ferric oxide ($p < 0.005$), and in the groups instilled with the two lowest doses of hydrated alumina ($p < 0.05$). This decrease appeared to be dose-related in the quartz and quartz plus ferric oxide groups (Table II). Analysis of covariance indicated significant ($p < 0.0001$) dose-related increases in mean lung weight of groups instilled with quartz or quartz and ferric oxide.

At necropsy, patchy discoloration of the lungs and enlargement of the tracheobronchial lymph nodes were observed in a high percentage of animals exposed to the higher two doses of quartz or quartz and ferric oxide, and in lower percentages of other groups, including controls. Generalized edema was noted frequently in both exposed and control groups; however, there was a slightly higher incidence in the group instilled with the highest dose of quartz. Dilation of the right ventricle, mottling of the left atrium, hydrothorax, and pale, roughened kidneys were noted with about equal frequency in exposed and control groups.

Upon histopathologic examination, the instilled particulate materials were distributed throughout all lobes of the lungs; within individual lobes there was often a patchy distribution of material. The instilled materials were visualized within alveolar macrophages and free in the alveolar lumen. The amount of material observed free in alveoli was greatest in groups instilled with the highest doses, and gradually decreased with time after exposure. Materials were also observed free in

TABLE II—Survival, Lung Weights, and Pulmonary Fibrosis Data in Hamsters Instilled with Particulate Materials in Saline

Material and Weekly Dose	Survival Time[a]	Lung Weight[b]	Alveolar Septal Fibrosis		
			Incidence	Severity[c]	Time-adjusted Grades[d]
Quartz					
6.0 mg	348 ± 26[e]	3.45 ± 0.21[f]	17/25[g]	3.3	4.86
3.3 mg	383 ± 31[e]	2.82 ± 0.84[f]	19/26	2.6	3.53
0.33 mg	506 ± 41	2.04 ± 0.73[f]	11/27	2.1	1.41
0.03 mg	498 ± 44	1.82 ± 0.74[f]	9/25	2.1	1.27
Quartz and ferric oxide					
6.0 mg each	335 ± 32[e]	3.74 ± 0.58[f]	22/26	3.2	5.50
3.3 mg each	379 ± 37[e]	3.00 ± 0.68[f]	21/28	2.8	4.19
0.33 mg each	578 ± 28	2.07 ± 0.80[f]	16/25	1.9	2.00
0.03 mg each	558 ± 32	1.81 ± 0.69[f]	14/24	1.9	1.77
Fibrous glass					
10.0 mg	555 ± 35	2.24 ± 0.71	24/25	2.2	3.18
1.0 mg	517 ± 35	1.97 ± 0.62	10/25	2.1	1.28
0.5 mg	538 ± 34	2.16 ± 1.08	15/25	1.7	1.83
0.05 mg	475 ± 41	1.70 ± 0.69	7/26	1.4	0.62
Hydrated alumina					
20.0 mg	500 ± 37	2.01 ± 0.71	21/25	2.2	3.23
5.0 mg	481 ± 41	2.28 ± 1.00	14/25	1.3	1.14
2.0 mg	440 ± 34[h]	2.04 ± 1.36	8/25	1.6	1.12
0.2 mg	444 ± 34[h]	1.79 ± 0.79	8/25	2.0	1.30
Saline controls	534 ± 35	1.72 ± 0.58	7/27	1.7	0.57
Cage controls	595 ± 14	2.27 ± 1.06	2/25	1.0	—

[a] Number of days ± SE, measured from first instillation to death, including spontaneous deaths, moribund killings, and terminal killings.

[b] Lung weights in grams ± SD.

[c] Total of all grades of pulmonary fibrosis/number of animals in which pulmonary fibrosis was diagnosed.

[d] Scaled according to Snell (28).

[e] Statistically significant (<0.005) when compared with saline control group.

[f] Statistically significant (<0.0001) using analysis of covariance (see text).

[g] Number of animals in each group in which tissues were examined microscopically, including replacement animals.

[h] Statistically significant (<0.05) when compared with saline control group.

the sinuses of tracheobronchial lymph nodes. Smaller amounts of fibrous glass were visible in the lung than the other materials, even in those animals receiving the highest dose or dying relatively early in the study.

Grading the severity of pulmonary fibrosis was complicated by the fact that two principal causes of fibrosis were evident in animals on this study. The most frequent and important cause, since it is the lesion upon which this entire study was based, was the instilled material. The fibrosis induced by the instilled materials was characterized by increased but variable thickness of alveolar septa (Fig. 1) and by thin strands of fibrous tissue surrounding the accumulated instilled material (Fig. 2). Masson's trichrome stains on selected sections confirmed the presence of bands of collagen in these areas (Fig. 2). Grading of severity of fibrosis was based on the most severely affected areas of the lung.

The second cause of pulmonary fibrosis observed in this study was the presence of chronic pulmonary congestion and edema associated with chronic heart disease (18), with or without concomitant severe chronic renal disease secondary to renal amyloidosis (10). The septal fibrosis related to chronic pulmonary congestion (Fig. 3) was recognizable in control animals or in animals exposed to lower doses of materials because of its diffuse, mild nature, associated cardiovascular lesions (atrial thrombosis, cardiac dilation, pulmonary congestion/edema, edema of other tissues), and the lack of fibrosis with visibly instilled material.

The incidence and severity of septal fibrosis correlated well with the dose of instilled quartz or quartz and ferric oxide (Table II). Incidence and severity of septal fibrosis in the groups exposed to fibrous glass or alumina were not clearly dose-related. Although the highest incidence of fibrosis occurred in the group instilled with the highest dose of fibrous glass, the pulmonary fibrotic response to fibrous glass or alumina was clearly less striking than the response to quartz and ferric oxide. The presence of septal fibrosis in saline or cage control groups appeared in most cases to be associated with chronic pulmonary congestion secondary to cardiovascular or renal disease. However, the incidence and severity of fibrosis was slightly higher in the saline control group than in the untreated controls.

The two-way analysis of variance on the mean scaled grades showed significant dose and time effects and nonsignificant interactions in every case (Table II). Both the quartz- and quartz plus ferric oxide-instilled groups

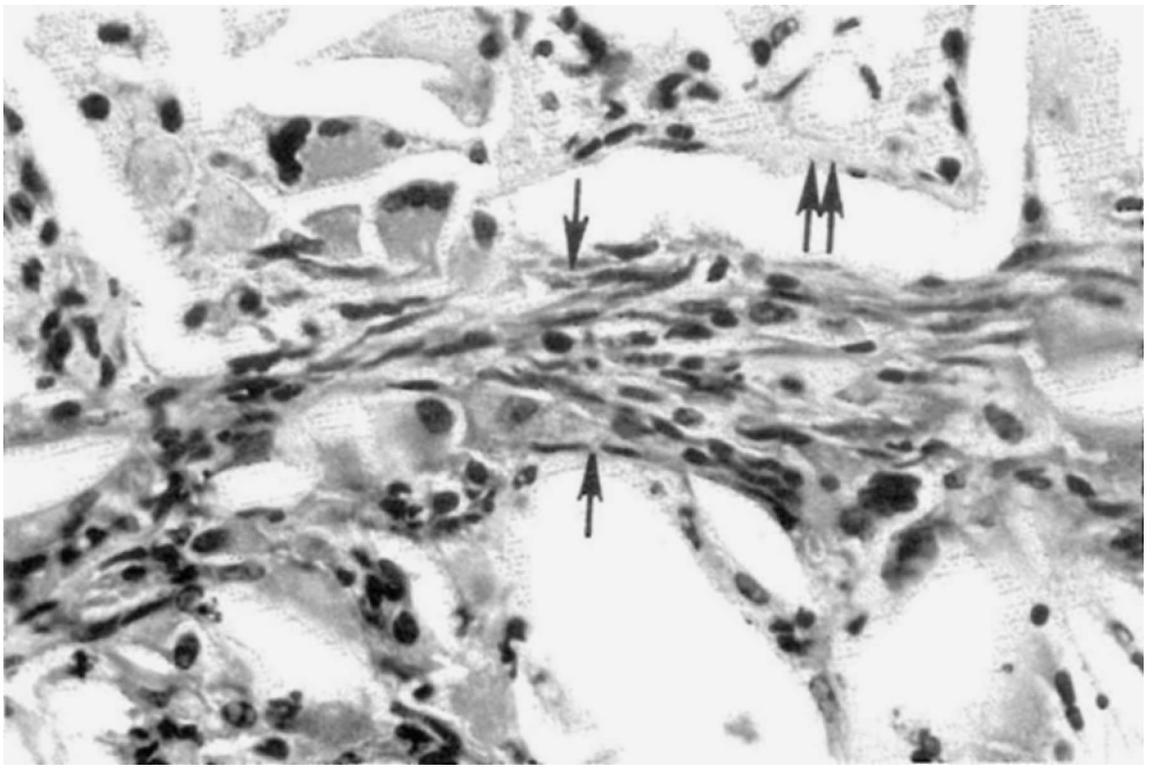

FIG. 1—Grade 5 alveolar septal fibrosis (single arrows) and alveolar lipoproteinosis (double arrows) in a hamster instilled tracheally with quartz. Hematoxylin and eosin. 400 ×.

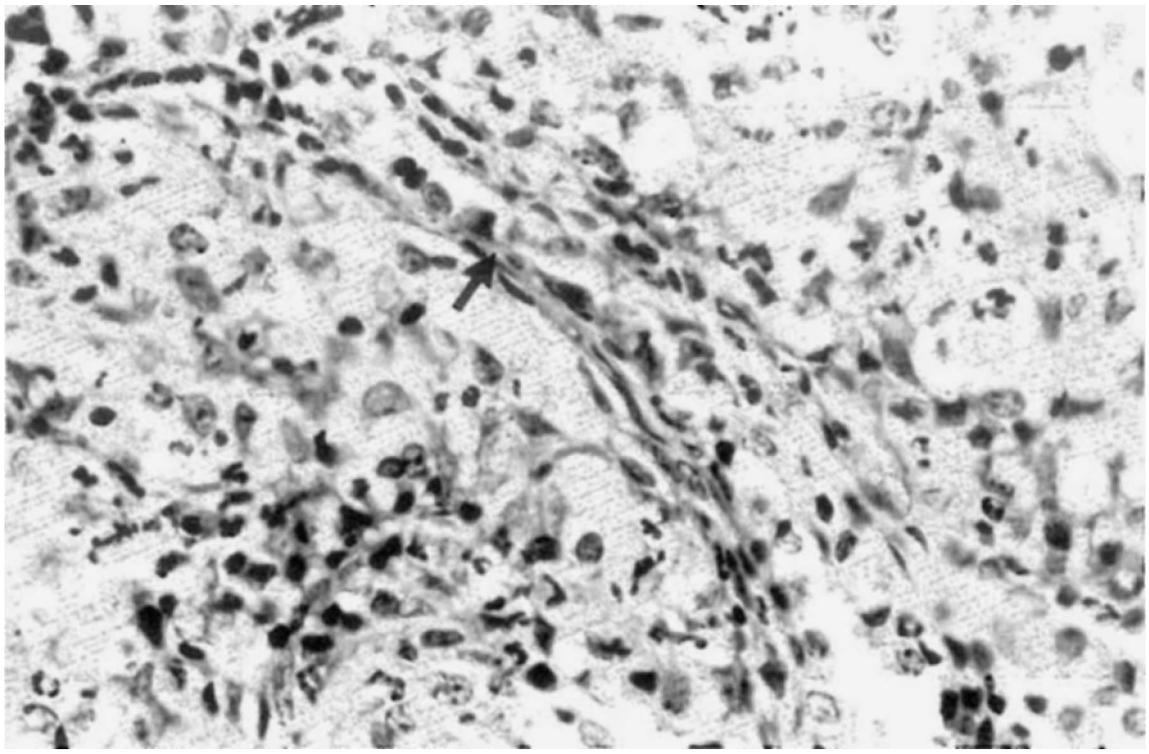

FIG. 2—Strands of mature collagen (arrow) in area of pulmonary fibrosis and granulomatous inflammation in hamster instilled with quartz. Masson's trichrome stain, 400 ×.

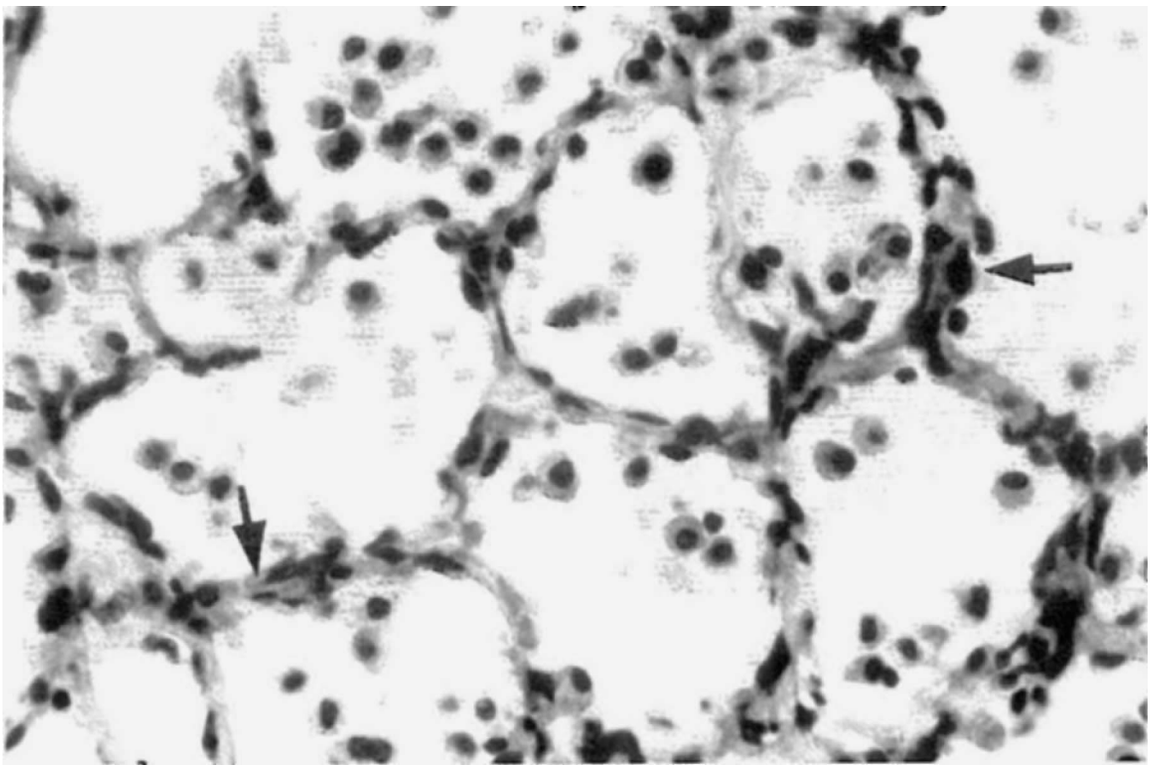

FIG. 3—Grade 2 alveolar septal fibrosis (arrows) and alveolar macrophage aggregates in a hamster with chronic cardiac disease, killed 683 days after instillation of physiologic saline. Hematoxylin and eosin, 400 ×.

exhibited a progressive increase in septal fibrosis with increasing dose. For hydrated alumina and fibrous glass animals, the highest dose groups and the saline controls were the high and low extremes, respectively. However, the graded scores from the intermediate groups did not show dose relationship. These time-adjusted mean values could be used to rank the various treatment groups and no attempt was made to attach statistical significance to differences among groups exposed to different materials.

Granulomatous inflammation (Fig. 2) was the most striking pulmonary lesion observed in the study; and the incidence and severity of lesions were in most cases related to the dose of material received. As in the case of induced pulmonary fibrosis, the granulomatous response was most severe in the groups exposed to quartz or quartz plus ferric oxide. Aggregates of alveolar macrophages were observed in association with chronic pulmonary congestion in both exposed and control groups.

Alveolar lipoproteinosis (Fig. 1), an intraalveolar accumulation of pulmonary surfactant, was observed as part of the response to instilled materials, most noticeably with quartz. This lesion has been observed and well characterized in rats exposed to quartz (12), and represents an abnormal accumulation of phospholipid-protein surfactant material normally secreted by alveolar type II cells. "Bronchiolization" of alveoli (20, 33) was observed in high incidence in both exposed and control groups, more frequently in animals dying late in the study.

Severe chronic glomerulonephritis, observed in virtually all animals in which the kidneys were examined microscopically, appeared to be the cause of death in many cases. Congo red stains of representative kidney sections demonstrated the presence of large amounts of amyloid in glomeruli of these animals. Chronic degenerative myocardial lesions, hypertrophy of myocardium, and degeneration of coronary arteries, accompanied by mural thrombi of the atrium in various stages of organization were observed in heart sections examined microscopically from instilled and control groups.

DISCUSSION

Survival of the quartz- and quartz plus ferric oxide-instilled animals was clearly dose-related. A consistent trend toward shorter survival with increasing dose was measured by the mean survival time for animals exposed to these two compounds (Table II). In interpreting the survival data on these groups of animals, one must consider the possibility that instillation of large doses of quartz and the subsequent response aggravated or shortened the latency period for the cardiovascular and/or renal disease observed in all groups, and that the decreased survival rate observed in these groups may be due to the combined effects of several disease processes.

Comparison of data from the various groups on lung weight indicates that groups exposed to the two highest doses of quartz or quartz and ferric oxide were the most profoundly affected. Lung weight increases in these groups appeared to reflect the intense inflammatory responses in the lungs of these animals. The mass of retained particulate material may also have increased lung weights; however, lung weights were not increased in the group exposed to 15 weekly doses of 20 mg of hydrated alumina.

Statistical analysis of pulmonary fibrosis data indicated that all instilled materials caused significant dose and time effects after analysis of variance and multiple classification analysis. The instillation of quartz or quartz and ferric oxide produced consistent increases in fibrosis with time postinstillation and with increasing dose. For fibrous glass, the response to the 1.0 mg dose level appeared low, but otherwise the trend was present. Instillation of fibrous glass with a greater percentage of fibers over 10 mm in length might have induced a more striking fibrogenic response (22, 35). Although the statistical tests indicated a significant positive dose effect on pulmonary fibrosis for instillation of hydrated alumina, the results were somewhat ambiguous.

Generalized edema and cardiac and renal lesions observed at necropsy reflected the high incidence of serious cardiovascular and renal disease. Most obvious were the cardiac lesions observed grossly and microscopically. Atrial thrombosis and myocardial degeneration frequently occur spontaneously in hamsters and have been well described (18). Severe glomerulonephritis secondary to amyloid deposition in glomeruli, another disease which frequently occurs spontaneously in hamsters (10), was prevalent in animals in this study and may have been responsible for

some of the edema and cardiac dilation through the osmotic effect of protein and electrolyte loss in the urine. Another factor was the potential vascular effect of pulmonary fibrosis, congestion, and edema caused by the instilled materials. The pulmonary fibrosis observed in the animals in this study did not appear to be severe enough to cause problems with pulmonary blood flow. However, the combined fibrosing and inflammatory effects of the instilled materials may have contributed to a decrease in pulmonary vascular flow, which in combination with the other factors mentioned above contributed to death from congestive heart failure. The highest incidence of edema was observed in the groups instilled with the largest doses of quartz or quartz plus ferric oxide.

The results of our study indicate that the most appropriate material to induce pulmonary fibrosis is quartz. Instillation of higher doses of fibrous glass or hydrated alumina seems not to be practical because of the high probability that the fibrogenic response to instilled material would be overshadowed by an even more severe and prolonged granulomatous inflammation. Thus grading of the severity of pulmonary fibrosis would be difficult. Recommending a dose of quartz for use in the hamster is difficult based on our results. We did not succeed in inducing a pronounced fibrosis without decreasing the life span of the animal. However, as mentioned above, there were several factors responsible for the shortened life span of hamsters instilled with the highest doses of quartz or quartz and ferric oxide. In retrospect, if one used an animal species or strain not subject to the renal and cardiac disease as observed in the hamsters in this study, decreased survival might not have been a problem.

Another factor is the question of the degree of severity of fibrosis and the amount of mature collagenous fibrous tissue required to have an effect on chemically induced pulmonary neoplasia. The most severe fibrosis observed in our study was less severe than that induced in rats with quartz (26), and the amount of mature fibrous tissue induced in the instilled hamster lungs was relatively small. No animal in our study had large, discrete foci of dense fibrous tissue containing solid areas of mature collagen, as observed in classical silicosis of man and in experimental silicosis in rats. One must consider the possibility that the Syrian golden hamster, like the mouse or guinea pig (9), is simply less susceptible than the rat to the induction of intense pulmonary fibrosis. Recent reports (5, 13, 32) on the induction of lung tumors in rats by exposure to quartz alone are further evidence that the rat is the preferred species in the development of an animal model to study the pathogenesis of particulate-induced pulmonary fibrosis and its relationship to pulmonary cancer.

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