THE PULMONARY TOXICITY OF MIXED DUST IS NOT ONLY RELATED TO ITS MINERALOGICAL COMPOSITION

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INTRODUCTION

Studying the relationships between the physico-chemical characteristics of some dust particles and the activity that these particles may exhibit when in contact with various biological systems is a fascinating area of research. Our understanding of the question, however, is quite limited. We know that exposure to asbestos dust may lead to asbestosis, free silica to silicosis and coal mine dust to coal worker pneumoconiosis. But we do not know which parameter at the level of the particles will trigger the relevant biological mechanisms. Several hypotheses have been made, but no satisfactory theory has emerged from the many studies on the subject.

The problem is even more complicated when dealing with mixed dust, such as coal mine dust. In addition to coal from various rank, coal mine dust generally contains free silica and clay minerals. Each component may play a role in the pathogenesis of the disease. For example, studies in rats by inhalation and intratracheal injection revealed that quartz in coal mine dust exhibited less activity than expected.[8] This phenomenon was attributed to the release of aluminum from clay minerals present, especially from illite.[6] It led to the hypothesis that the biological activity of quartz in mixed dust was depending to the ability for accompanying minerals to mask the potential toxicity of quartz.[6,7,10] The toxicity of coal mine dust would more depend on the overall mineralogical composition rather than on the quartz content alone.[12,9]

In order to explore this hypothesis, we tested in the rat two samples of coal dust having the same bulk mineralogical composition in terms of coal, quartz, illite and kaolin. They did not exhibit the same pulmonary activity.

METHODS

Sample #1 was obtained by finely grinding some coal materials extracted from the Aumance coal mine in France. The final product contained 35% coal, 17% quartz, 31% illite and 17% kaolin. Mineralogical determinations were made using a combination of X-ray diffraction and infrared spectroscopy. Sample #2 was a reconstituted mixture of fine particles of "pure" coal and minerals from other origin (illite from Le Puy, kaolin from Cornwall and quartz from Madagascar). The two samples had the same final mineralogical composition by weight. Particles in each sample were examined by Analytical Transmission Electron Microscopy (ATEM) and their number size distributions were established.

Three groups of 40 female Wistar rats were used for this study. Each animal in the exposed groups received a single intratracheal injection of 60 mg of fine particles suspended in 1 ml of saline. Some animals were killed 12 and 24 months later.

The lungs and the tracheobronchial lymph nodes were removed and weighed. Left lobes were used for histopathological examination and ATEM analysis of retained dust particles. Left lobes were perfused under 25 cm H_2O pressure and fixed in 10% neutral buffered formalin. Sections stained by hematoxylin eosine and Picrosirius were examined at three different locations under crossed polaroid filters.[5] The intensity and profusion of the lesions were scored, each on a 0–4 scale. Criteria used for intensity grading are indicated in Table I. A final histopathological score was obtained by multiplying the intensity score and the profusion score.[1] Lung tissue was then extracted from the remaining block by dewaxing in hot toluene. After digestion of the tissue in sodium hypochlorite, retained particles were concentrated by filtration on Polycarbonate membrane and analysed by ATEM.

For each group, right lungs and remaining tissue fragments not used for histology were dried and pooled. The pool was analysed for collagen by the method of Stegeman,[13] for total dust by the formamide method of Thomas,[14] for quartz by X-ray diffraction and for total Al in dust by X-ray fluorescence.

Similar methods were used to prepare and analyse lymph nodes.

RESULTS

For exposed animals, mean weight of fresh lung and mean collagen content of the lung were both above corresponding control values (Table II). The highest figures were measured at month 24 in the group of animals injected with the reconstituted mixture. In particular, the collagen content of the lung was more than three times higher with the reconstituted mixture than with the Aumance coal dust.

No histological changes were noticed in the lung of control animals, but lesions were present in the lung of exposed animals. Histopathological scores are presented in Figure 2.

Table I

Criteria Used for Scoring Intensity of Lung Lesions

Grade 0	Normal histology.
Grade 1	Focal accumulation of dust-laden macrophages without any fibrotic organisation.
Grade 2	Early fibrotic organisation with few thin collagen III fibers peripheraly to the granulomas, or intersperced throughout (green color with Picrosirius stain under cross polaroid filters).
Grade 3	Fibrotic organisation of the granulomas, with thick bundles of collagen I, in addition to collagen III (yellow orange or red color with Picrosirius stain).
Grade 4	Massive fibrotic reaction located around the main bronchus and vessels.

Table II

Weight of Fresh Lung, Weight of Lymph Nodes and Pulmonary Collagen

Month 12			Month 24		
Controls	Aumance	Mixture	Controls	Aumance	Mixture
Mean weight of fresh lungs (g/rat)					
1.3	1.8	1.8	1.3	2.1	3.6
Mean weight of lymph nodes (mg)					
--	0.08	0.28	--	0.36	1.1
Mean collagen content of the lung (mg/rat)					
29.4	47.5	52.8	31.3	52.5	167.3

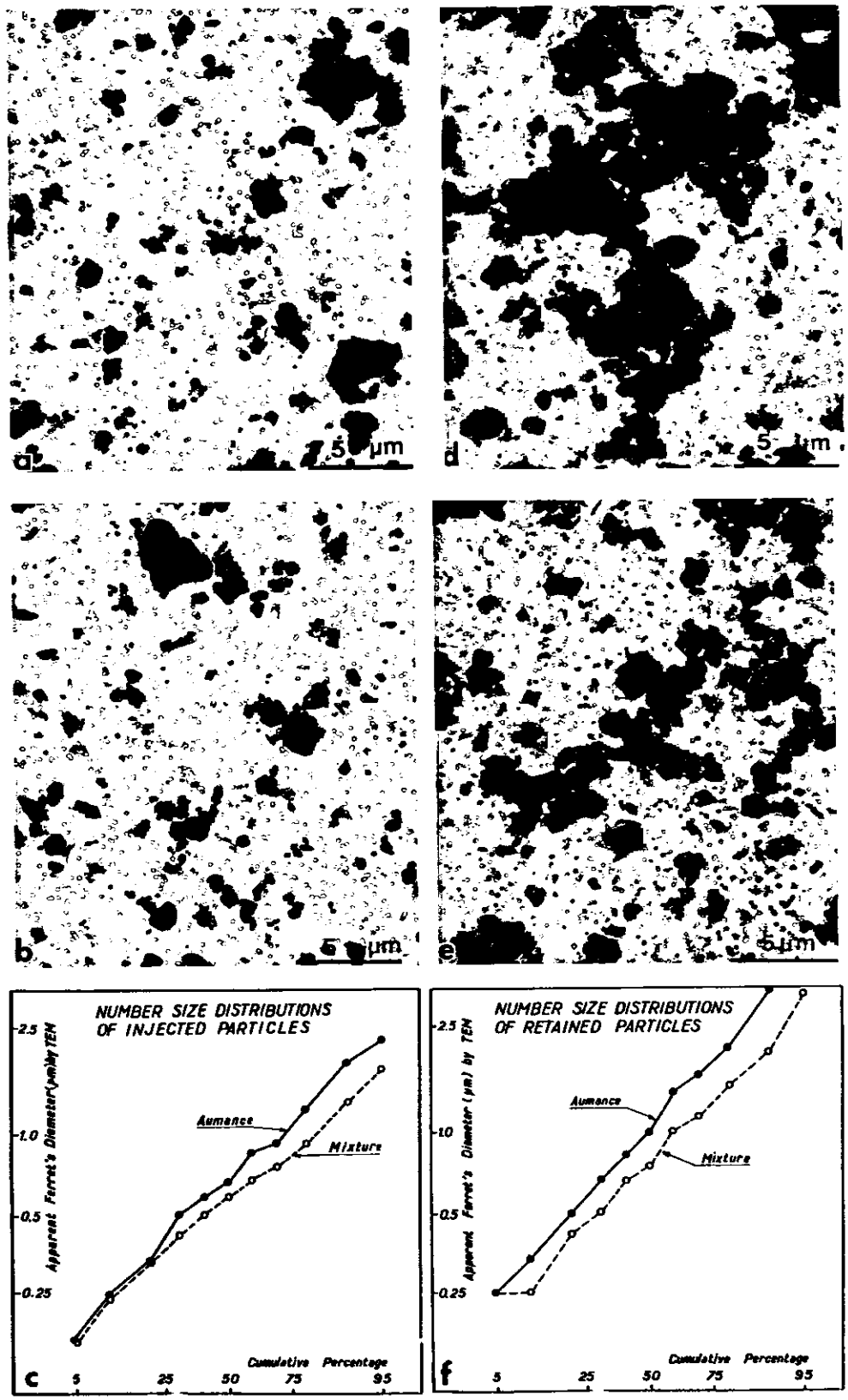

Figure 1. Analysis by transmission electron microscopy of dust particles injected, and extracted from the lung at month 24.

a. Aumance, particles injected
b. Mixture, particles injected
c. Number size distribution of injected particles
d. Aumance, particles retained in the lung
e. Mixture, particles retained in the lung
f. Number size distribution of particles retained in the lungs

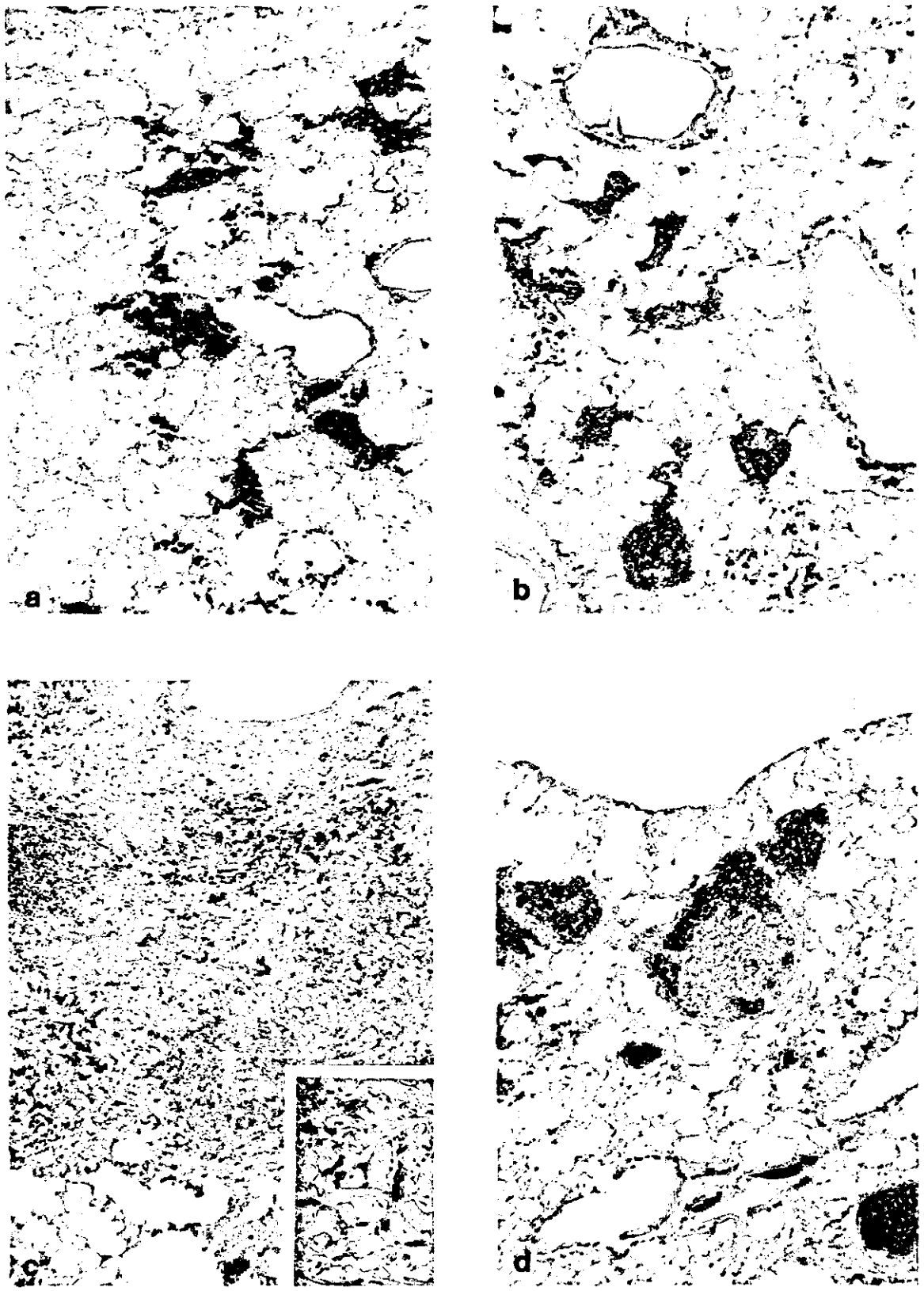

Figure 2. Histological changes of the lungs (HEX50). Note the slight progressive fibrotic reaction between 12 (a) and 24 months (b) for Aumance. Exposure to reconstituted mixture yielded at month 24 fibrotic nodules sparse in the lung (c) and massive fibrotic reaction (see collagen bundles in insert) around the main bronchus and vessels (d).

In each exposed group, the mean score was significantly higher at month 24. At this date, histopathological changes were significantly more pronounced with the reconstituted mixture. At month 12, pulmonary lesions were quite similar in the two groups but the fibrotic reaction of the tracheobronchial lymph nodes was much more intense with the mixture.

Mineral contents of the pooled lung tissue and lymph nodes are reported in Table III. A very high proportion of the injected dust was still present in the lung at month 12 and at month 24. Figures for total dust, quartz and Aluminum were systematically higher in the Aumance group. Quartz accounted for 17% of the injected dust, but overall, the proportions of quartz in the lung dust were less. At month 12, proportions of quartz and proportions of Aluminum in the lung dusts did not differ between the two exposure groups. The lung dusts were richer in quartz at month 24. The quartz contents of tracheobronchial lymph nodes were quite similar in both groups (Table III).

ATEM analysis of dust used for injection indicated the presence of very fine particles in both samples. Most of the particles observed by ATEM were less than 2.5 μm in apparent Ferret's diameter. The number size distributions were similar in the two samples, although the particles were somewhat finer in the mixture (Figure 1). Particles extracted from the lung at month 24 were either isolated or grouped into large agglomerates. Such agglomerates were not detected in the injected dust.

Detailed mineralogical analysis of the lung dust by ATEM is still in progress. Preliminary observations suggest that the clay contents of the lung were different for the two groups, with apparently more illite retained at month 24 by animals exposed to the Aumance dust.

DISCUSSION

The model used was able to produce a fibrotic reaction progressing over the two years of the experiment, and to document different activities of the two dust samples tested.

These experiments illustrate once more how complex are the mechanisms of biological action of mixed dust. The two mixed dust samples with the same bulk mineralogical composition yielded different fibrotic pulmonary responses.

Table III

Mineral Content of the Lung and of the Tracheobronchial Lymph Nodes

Month 12		Month 24	
Aumance	Mixture	Aumance	Mixture
Total mineral dust in the lung (mg/rat)			
47.8	35.5	41.7	35.5
Quartz in the lung (mg/rat)			
5.6	4.2	7.4	4.9
Percentage of quartz in lung dust			
11.7	11.8	17.7	13.8
Aluminum in lung dust (mg/rat)			
1.8	1.3	2.5	1.1
Percentage of Aluminum in lung dust			
3.7	3.7	5.9	3.1
Quartz in the lymph nodes (mg)			
1.0	1.4	1.5	1.5

The bulk analysis of lung dust did not provide any convincing explanation for this difference. May be that the microscopical analysis in progress will bring interesting information. It is conceivable that the particles be differently assembled in the natural and in the reconstituted dust. Preliminary observations of clays in lung dust do support this hypothesis. Surface analysis of injected and retained dust may also be informative.[3]

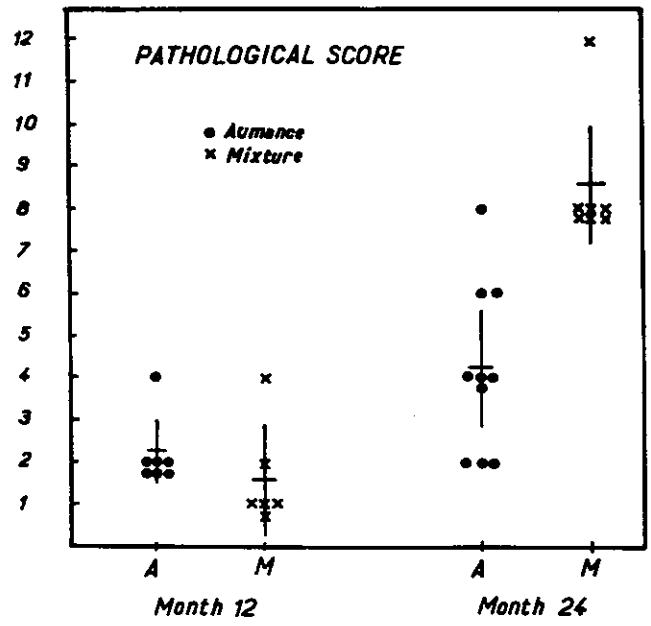

Figure 3. Pathological scores (see text for explanation) at month 12 and month 24 for the two groups of exposed animals.

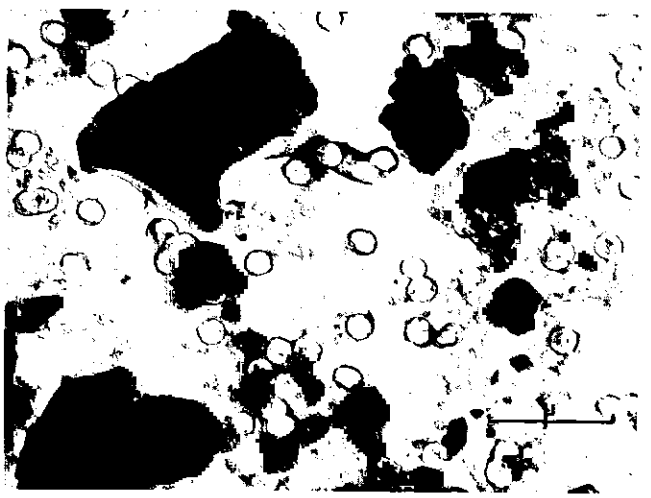

Figure 4. Morphological features of dust extracted from animals exposed to the Aumance coal dust. Note the presence of disolving illite particles.

Whatever the explanation may be, it is clear from these and other data,[2,4,9,11] that the toxicity of coal mine dust and probably of other mixed dust is not only related to its mineralogical composition (as usually determined). More subtle properties of the dust particles may also play a role. This put into question the usefulness of incorporating expensive mineralogical analyses in routine dust measuring programs.

REFERENCES

1. Bégin, R., Massé, S., Rola-Pleszczinski, M., Martel, M., Des Marais, Y., Geoffroy, M., Le Bouffant, L., Daniel, H., Martin, J.: Aluminum lactate treatment alters the lung biological activity of quartz. *Experimental lung research* 10:383-399 (1986).
2. Bruch, J., Rosmanith, J. *In vitro effects of mineral dusts*, pp. 433-440. Third International Workshop Springler-Verlag Berlin-Heidelberg, New York, Tokyo (1985).
3. Fubini, B., Bolis, V., Giamello, E.: The surface chemistry of crushed quartz dust in relation to its pathogenicity. *Inorganica Chimica Acta* 138:193-197 (1987).
4. *IARC Monographs on the evaluation of the carcinogenic risk of chemicals to humans. Silica and some silicates.* Vol 42. IARC Lyon France (1987).
5. Junqueira, L.L.U., Cossermelli, W., Brentani, R.: Differential staining of collagens type I, II and III by Sirius Red and polarisation microscopy. *Arch. Histol. Jap.* 41:267-274 (1978).
6. Le Bouffant, L., Daniel, H., Martin, J.C., Bruyère, S.: Effect of impurities and associated minerals on quartz toxicity. *Ann. Occup. Hyg.* 26:625-634 (1988).
7. Le Bouffant, L., Daniel, H., Martin, J.C., Aubin, C., Lehuede, P.: Recherche communautaire sur le rôle du quartz dans la pneumoconiose des mineurs de charbon et sur l'influence des mineraux d'accompagnement. Rapport CECA. Convention no° 7256.32.018/3. Luxembourg (1983).
8. Le Bouffant, L., Daniel, H., Martin, J.C.: *Inhaled Particules IV*, pp. 389-399. Pergamon Press, Oxford and New York (1977).
9. Le Bouffant, L., Martin, J.C., Daniel-Moussard, H.: Proceedings of the conference on technical measures of dust prevention and suppression in mines. *Commission of the European Communities.* Luxembourg—Document EUR 4937:127-138 (1973).
10. Martin, J.C., Daniel, H., Le Bouffant, L.: *Inhaled Particules IV*, pp. 361-371. Pergamon Press, Oxford and New York (1977).
11. Ray, S.C., King, E.J., Harrisson, C.V.: The action of small amount of quartz on the lung of rats. The action of small amounts of quartz and larger amounts of coal and graphite on the lung of rats. The action of anthracite and bituminous coal dust mixed with quartz on the lung of rats. *Brit. J. Ind. Med.* 8:62-76 (1951).
12. Reisher, M.T.R., Bruch, J., Hilscher, W., Prajsnar, D., Robock, K., Rosmanith, J., Scharfman, A., Schlipkoter, H.W., Strubel, G., Weller, W.: Specific harmfulness of respirable dusts from West-German coalmines. Comparison of experimental and epidemiological results. *Ann Occup. Hyg.* 26:527-539 (1982).
13. Stegeman, H.: Mikrobestimung von hydroxyprolin mit chloramint und p-dimethylaminobenzaldehyd. *Hoppe-Seyler's Zeitschrift für Physiologiscge Chemie* 311:41-45 (1958).
14. Thomas, N., Stegeman, H.: Darstellung der fremsdstaübe aus Lungen und ihre ergenschaften. *Beitrage Zur Silikose forschung.* Herausgeber Bergbau-Forschunginstitut Bochum. 28:1-29 (1954).

EFFECTS OF ANTIOXIDANTS ON EXPERIMENTAL SILICOSIS

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Our previous studies,[15,16,17,18] documented also by other authors,[4,5,9,21,23,34] provided support for the assumption that lipid peroxidation may be one of quartz dust cytotoxic mechanisms in cells and lung tissue. Also, a continuously growing body of evidence indicate that antioxidants as selenium, zinc, vitamins A, E, and C are either incorporated in the biological membranes or/and influence their stability through the antioxidative systems, and, thus can provide line of defense against peroxidative damage.[6,7,11,24,25,30,33] The involvement of lipid peroxidation in lung tissue and macrophage damaging processes promoted by quartz dust justify the use of various antioxidants and free radical scavengers.[2,19,20,22,27]

The present study was conducted to gain some insight into the protective effects of antioxidant agents on experimentally induced by quartz lung changes.

MATERIAL AND METHODS

Male rats with body weights about 180–200 g were used in three experimental series.

Experiment 1. Rats were divided into the following six groups: 1) Control, intratracheally instilled with 1 ml saline; 2) Silicotic rats (DQ12), intratracheally instilled with a single dose of 30 mg DQ12 standard quartz dust, particle size 5 μ (kindly supplied by Prof. K. Robock, Bergbau-Forschung, GmbH in Essen, West Germany); 3) Selenium-supplemented (Se) 1 ppm; 4) Selenium-supplemented (Se) 4 ppm; 5) Se 1 ppm + DQ12; 6) Se 4 ppm + DQ12.

Experiment 2. Designed to investigate the effects of adding Vitamin A (20 mg/kg b.w.), or Vitamin E (40 mg/kg b.w.) to 1 ppm selenium, was performed on the following animal groups: 1) Control; 2) DQ12; 3) Se + Vitamin A; 4) Se + Vitamin A + DQ12; 5) Se + Vitamin E; 6) Se + Vitamin E + DQ12.

Experiment 3. Aimed at evaluating the effectiveness of zinc supplementation (18.5 ppm), and of the concurrent administration of zinc and selenium (1 ppm), used also six animal groups; 1) Control; 2) DQ12; 3) Zn; 4) Zn + Se; 5) Zn + DQ12; 6) Zn + Se + DQ12. The antioxidants were given orally in the drinking water. Rats were maintained on antioxidant supplement for 1 month prior to the dust instillation and 2 months before sacrifice. By the 2 months all the animal groups were killed. The lungs and the tracheal lymph nodes were removed and weighed. The lungs were examined for fibrogenesis development and for peroxidative damage (experiments 2 and 3). In order to evaluate the severity of fibrogenesis the following biochemical parameters were used: lipid,[13,28] phospholipid,[31] and hydroxyproline[29] content of the lungs. The degree of lung peroxidative damage was evaluated by measurements of malondialdehyde formation, as an index of lipid peroxidation release, with thiobarbituric acid (TBA)-test,[32] glutathione peroxidase, GSH-Px,[14] and glucoso-6-phosphate dehydrogenase, G6P-DH,[3] activities.

The data are presented as percent of control and of DQ12-instilled rats. Statistical intergroup significance were performed by using Student's t-test.

RESULTS AND DISCUSSION

Experiment 1. Figure 1 shows that under 1 ppm and 4 ppm selenium treatment no additional benefit was found in the increased by 2 months final lung and lymph node weights of silicotic rats. No significant differences were observed between the DQ12-instilled and supplemented with selenium, and the non-supplemented silicotic groups, except for 1 ppm selenium which diminished lymph node weights, but only at marginal statistical significance ($p < 0.05$).

In contrast, both selenium doses markedly reduced ($p < 0.001$) lung lipids and phospholipids induced by quartz (Figure 2). Selenium supplements exerted the same decrease rate ($p < 0.001$) of lung hydroxyproline in silicotic rats (Figure 3).

Experiment 2. Feeding selenium 1 ppm in combination with vitamin A and E did not modify the increased lung weights induced by quartz, but significantly reduced ($p < 0.01$) the lymph node weights compared to non-supplemented silicotic rats (Figure 4).

Co-administration of selenium with both vitamins was equally effective in decreasing biochemical parameters of lung fibrosis,—lipid, phospholipid and hydroxyproline content (Figures 5 and 6). However, none of the used antioxidant supplements returned the observed changes to control values.

The TBA levels reported in Figure 7 show a significant higher lipid peroxidation in the DQ12-instilled rats. Supplemental to selenium vitamin A and E tended to reduce lipid peroxide release, but the decrease was not significant as against the silicotic rats.

GSH-Px activity depicted in the Figure 8 was found to be increased in silicotic rat lungs, but no significant response was observed compared to control group. The enhanced activity suggests an adaptative reaction and may indicate an

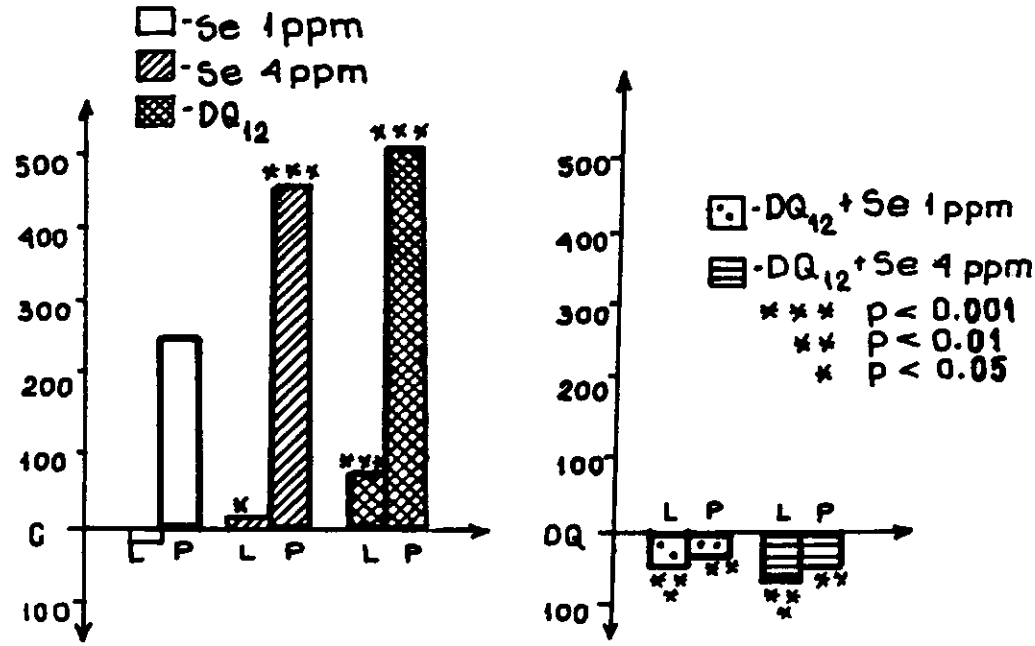

Figure 1. Lung and tracheal lymph node weights of quartz-treated and Se-supplemented silicotic rats.

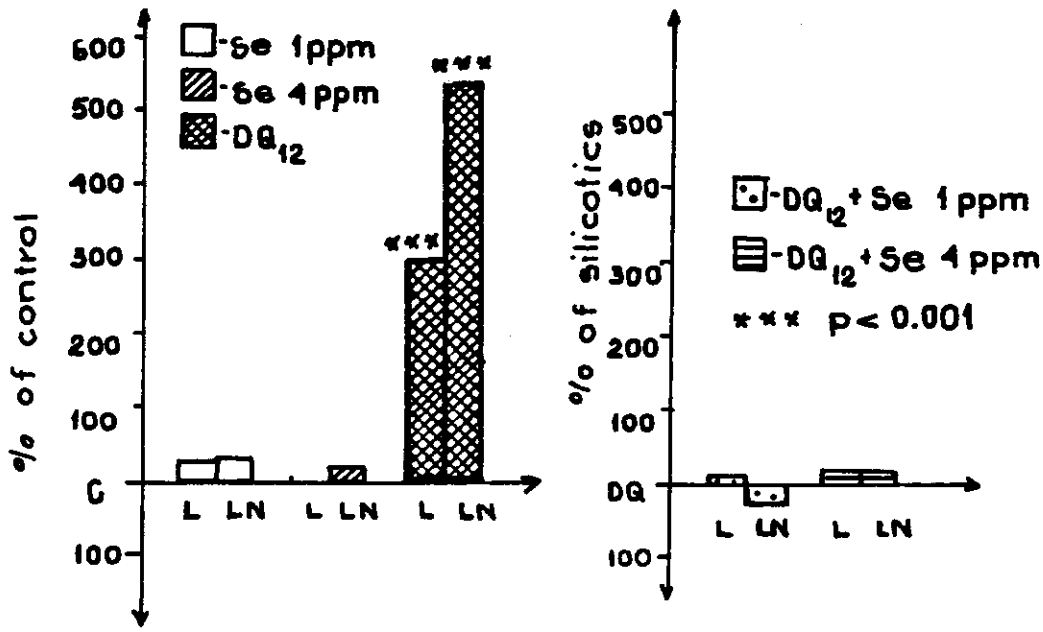

Figure 2. Lung lipids and phospholipids of quartz-treated and Se-supplemented silicotic rats.

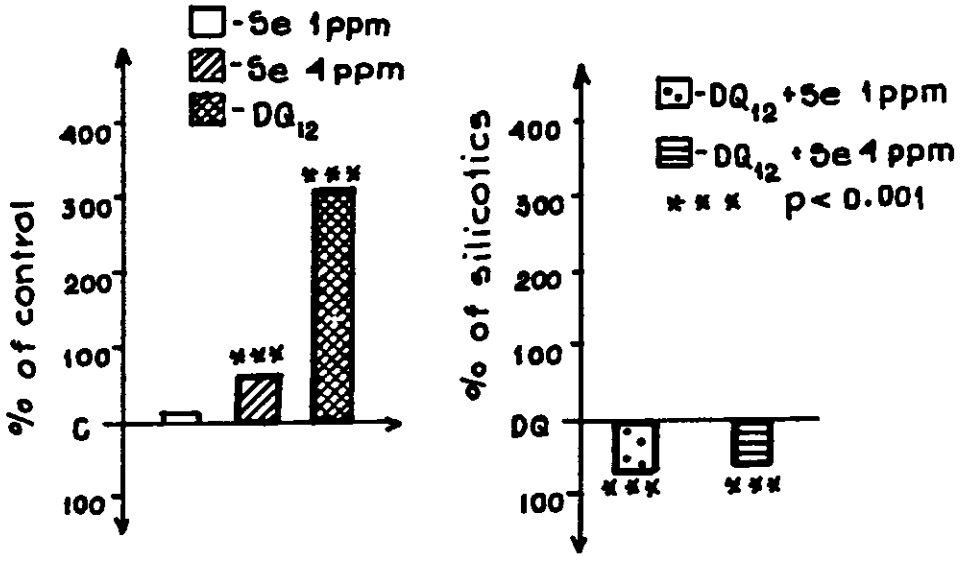

Figure 3. Lung HYPRO of quartz-treated and Se-supplemented silicotic rats.

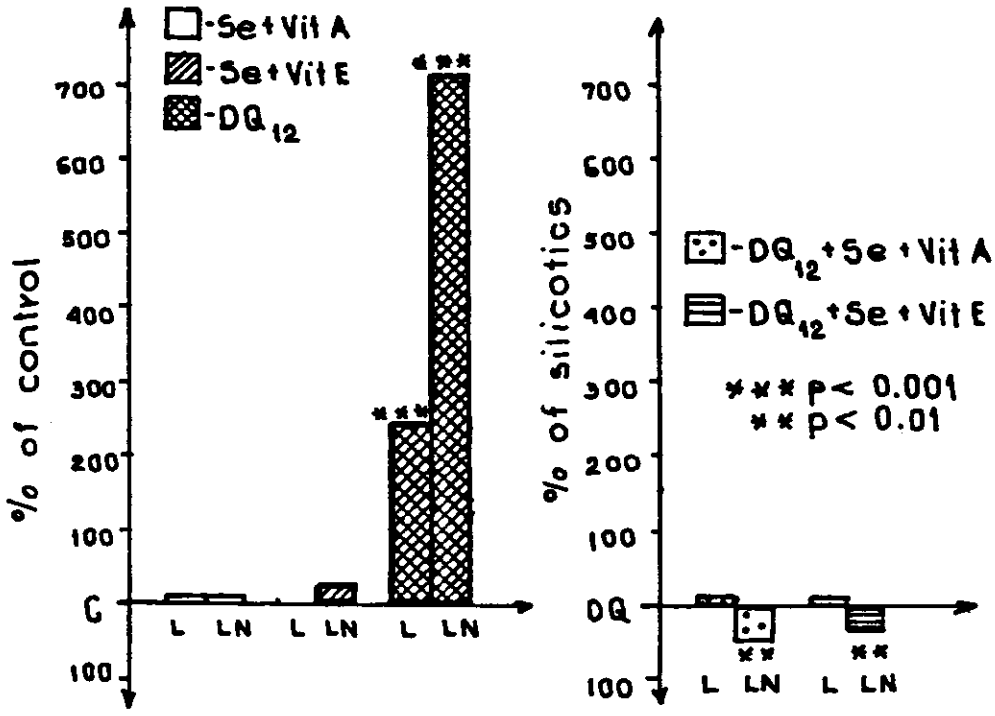

Figure 4. Lung and tracheal lymph node weights of quartz-treated, Se+vitamin A, and Se+vitamin E-supplemented silicotic rats.

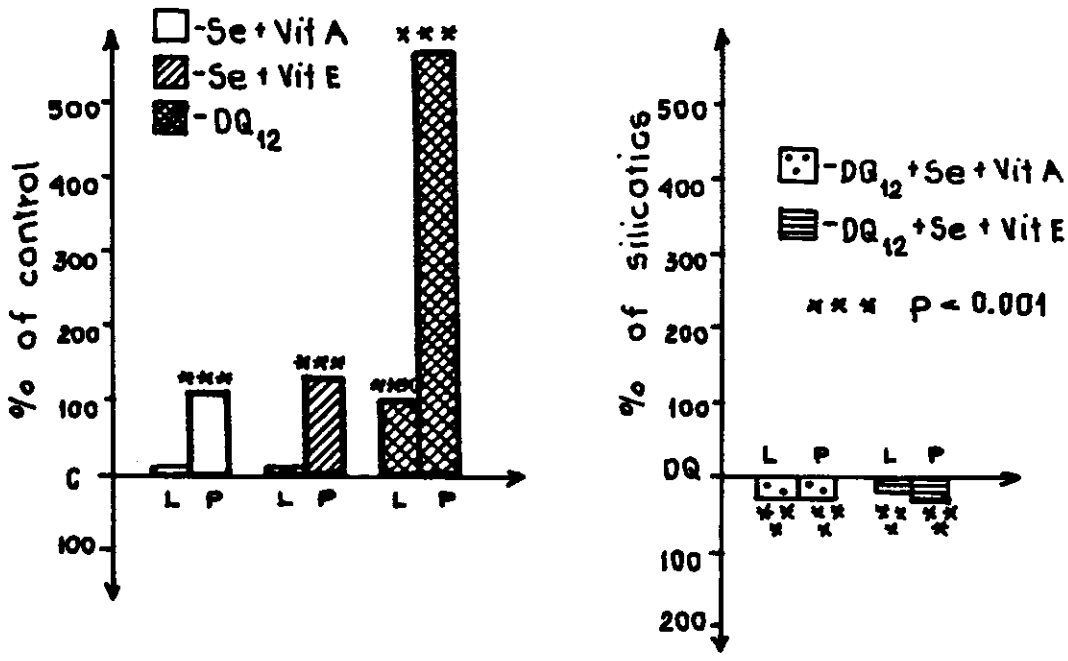

Figure 5. Lung lipids and phospholipids of quartz-treated, Se+vitamin A, and Se+vitamin E-supplemented silicotic rats.

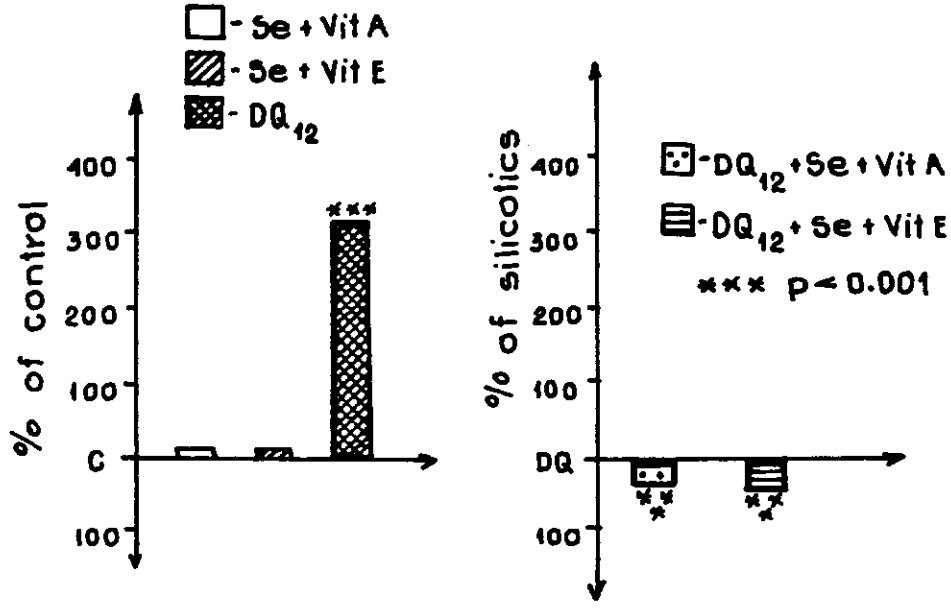

Figure 6. Lung HYPRO of quartz-treated, Se+vitamin A and Se+vitamin E-supplemented silicotic rats.

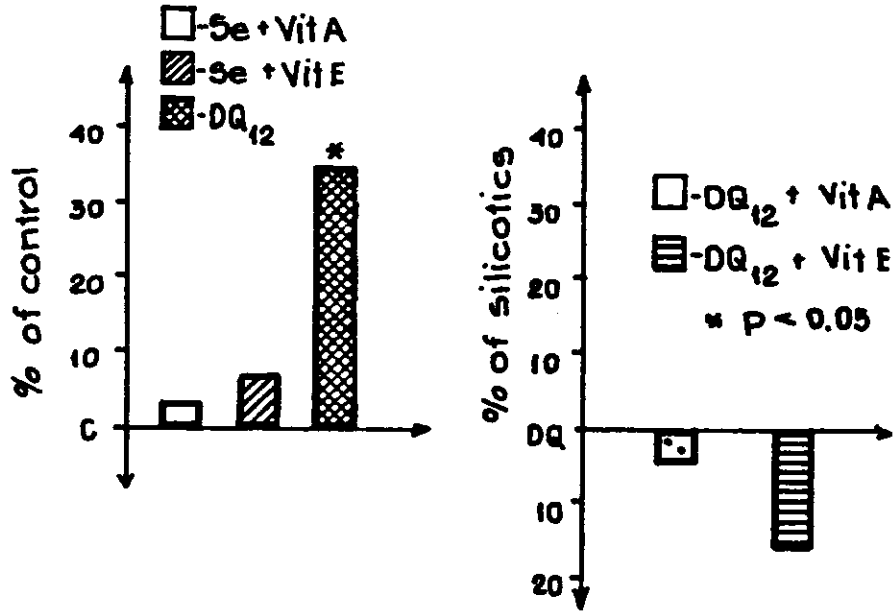

Figure 7. Lung LIPID PEROXIDES of quartz-treated, Se+vitamin A, and Se+vitamin E-supplemented silicotic rats.

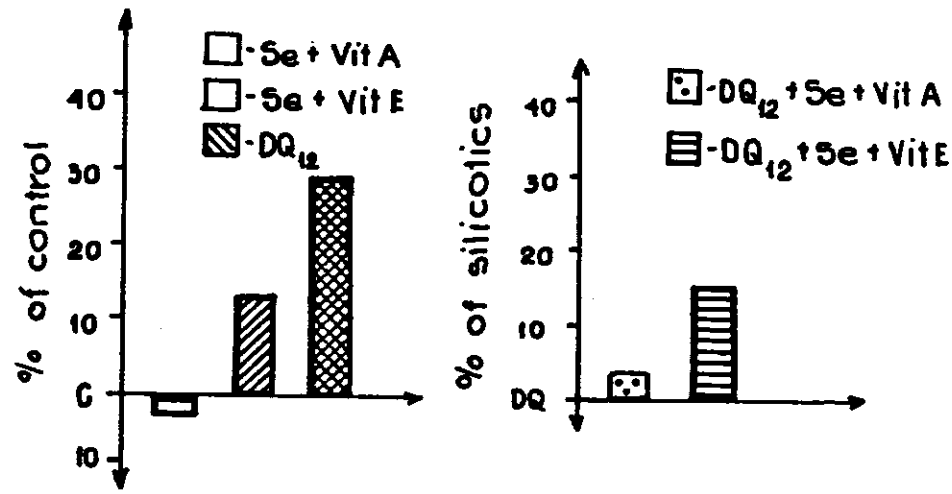

Figure 8. Lung GSH-Px of quartz-treated, Se+vitamin A, and Se+vitamin E-supplemented silicotic rats.

increased demand of the enzymatic activity to cope with tissue peroxidative damage. An inverse relationship between lung GSH-Px activity and lipid peroxidation in selenium + vitamin A and E-supplemented silicotic rats was observed. However, this effect was not significant compared to DQ12-instilled rats. G6P-DH exhibited a significantly higher ($p < 0.001$) activity in the silicotic rat lungs versus of the control group. Addition of both vitamin combinations significantly lowered the enzyme activity by the 2 months when compared to non-supplemented silicotic rats (Figure 9). The reason of the increased enzyme activity in silicosis might be due to the stimulation of the pentoso-phosphate pathway by supplying NADPH for "de novo" lipid biosynthesis and for glutathione redox cycle. Antioxidants exerted a beneficial effect on this metabolic point as demonstrated by the previous Figure 5 showing a reduced rate of lung lipids in antioxidant supplemented silicotic rats.

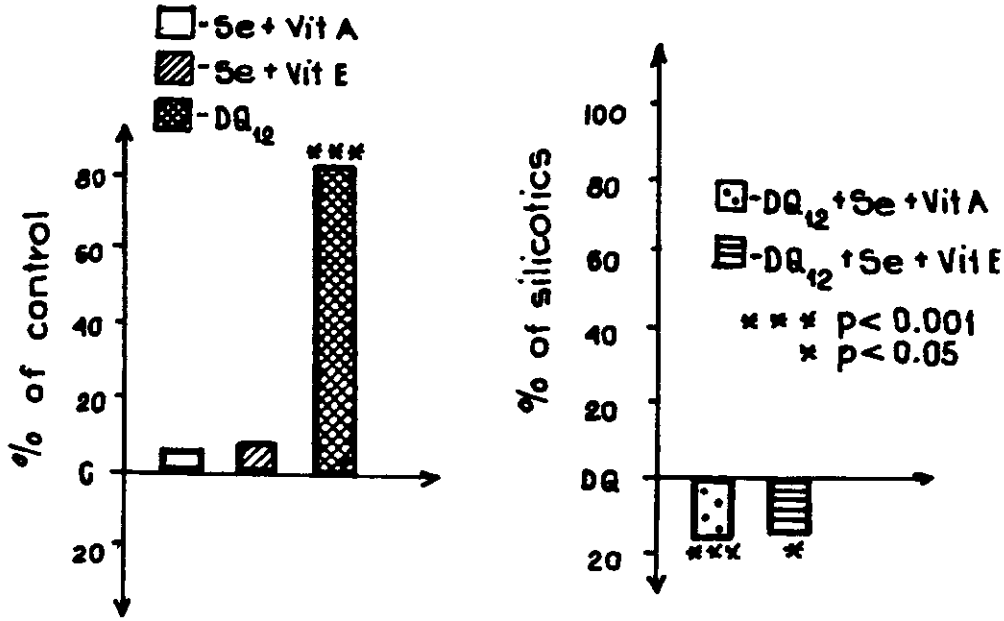

Figure 9. Lung G6P-DH of quartz-treated, Se+vitamin A, and Se+vitamin E-supplemented silicotic rats.

Experiment 3. No response in case of treatment with zinc and zinc + selenium was noted in lung weights of silicotic rats; however, the lymph node weights were found to be increased (Figure 10). In terms of biochemical lesions, zinc supplementation resulted in a significant decrease of lung lipid, phospholipid and hydroxyproline content (Figure 11 and 12). Concurrent administration of zinc and selenium showed the same pattern, except for lung lipids whose values did not differ from those of silicotic non-supplemented group.

Lung lipid peroxides of silicotics were significantly increased ($p < 0.01$) when zinc was supplemented, while zinc + selenium combination alleviated this effect. The failure of zinc to decrease lipid peroxidation induced by quartz dust might be explained by the reported bimodal response of this element in vivo: low doses inhibit, but higher doses enhance lipid peroxidation.[8,26] Most probably, the applied dose of 18.5 ppm zinc, though is considered to be physiological and non-toxic, under our experimental conditions was high enough to increase lipid peroxidation compared to control and silicotic rats.

Lung GSH-Px activity of silicotic rats kept on zinc showed a slight non-significant increment running in parallel with the observed lipid peroxide excess. Adding selenium to zinc resulted in an unexpected decline of this selenium-dependent enzyme at the borderline significance ($p < 0.05$).

Enhanced G6P-DH activity observed in silicotic rats was decreased by zinc supplementation to values significantly lower ($p < 0.001$) when compared to non-treated DQ12-instilled animals. Co-administered selenium to zinc failed to exhibit synergistic effect, the enzyme activity being reduced with a lower significance rate ($p < 0.01$). It is worth mentioning that the less pronounced effect of selenium in the presence of zinc supports the opinion that the biological role of the former might be diminished by its direct binding to the ionized zinc.[12] Consequently, a decreased availability of selenium to antioxidant enzyme systems occurs.

The design of our experiments does not allow a detailed discussion on the mechanisms of the antioxidant effects on silicosis. We can only speculate that selenium, vitamin A and vitamin E as well as their combinations act mainly on the course of inflammatory events preceding fibrosis, by trapping the formed free radicals, and, thus, preventing lipid peroxidation. Zinc, element with a broad range of biologic activity, though is a co-factor of the scavenging free radical metallo-enzyme superoxide dismutase, impairs mainly collagen synthesis and processing.

Our results confirm the reported previously protective effect of zinc with respect to induced by quartz lung collagen accumulation. The beneficial effect of zinc was explained by the interference with macrophage functions, having no direct effect on collagen deposition.[10] Recent evidence, however, indicates that zinc has a direct and selective preventive effect on rat lung collagen accumulation by inhibiting procollagen hydroxylation.[1]

In conclusion, our results give support to the hypothesis that the peroxidative damage plays an important additional role in the fibrotic action of quartz dust. One of the most significant findings in this study is that under antioxidant treatment silicotic fibrosis was diminished. Therefore, given correct concentrations, these antioxidants appear to be beneficial, as prophylactic and therapeutic agents in silicosis. Since the

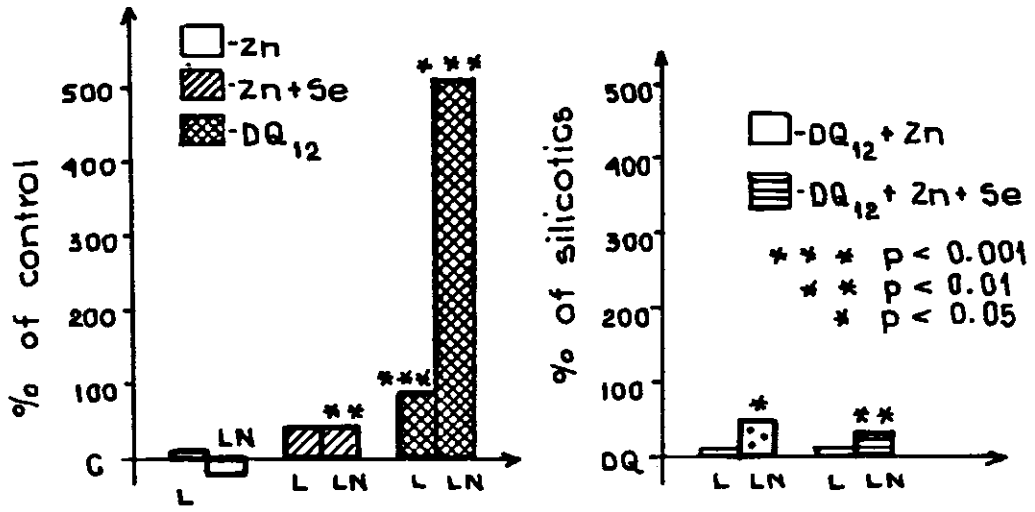

Figure 10. Lung and tracheal lymph node weights of quartz-treated, Zn and Zn+Se-supplemented silicotic rats.

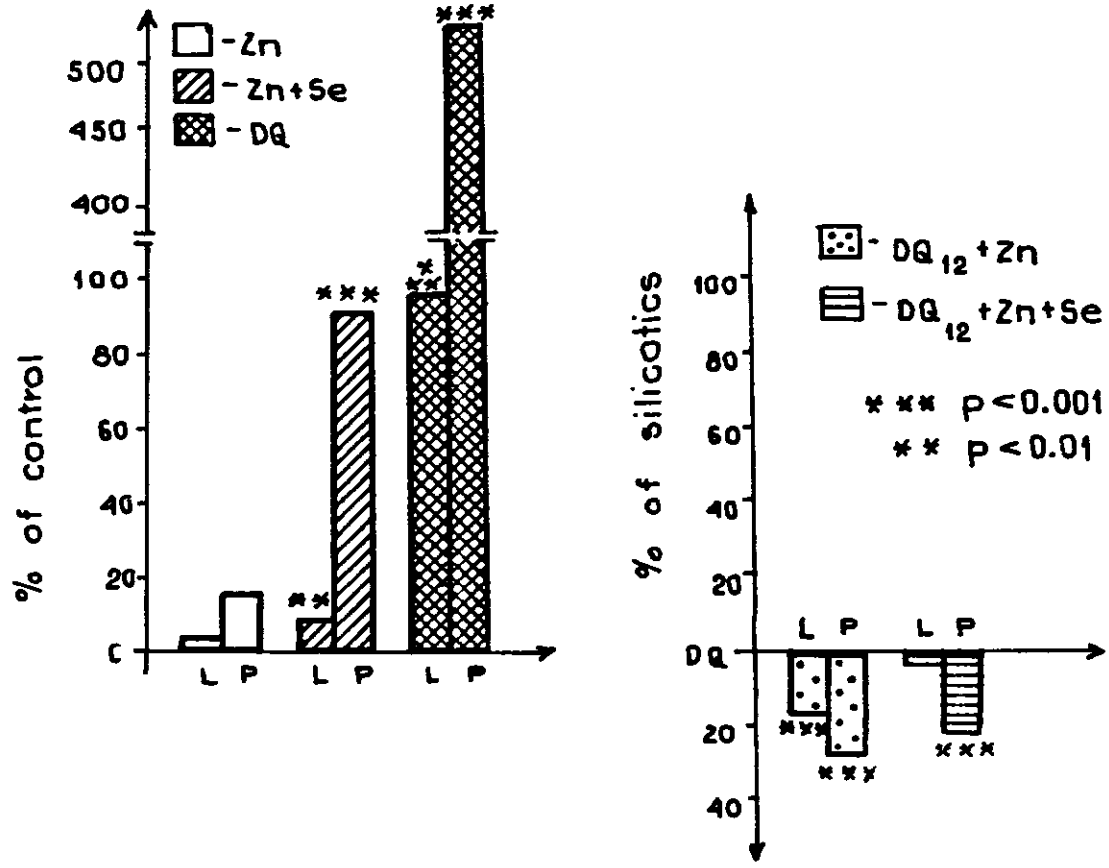

Figure 11. Lung LIPIDS and PHOSPHOLIPIDS of quartz-treated, Zn and Zn+Se-supplemented silicotic rats.

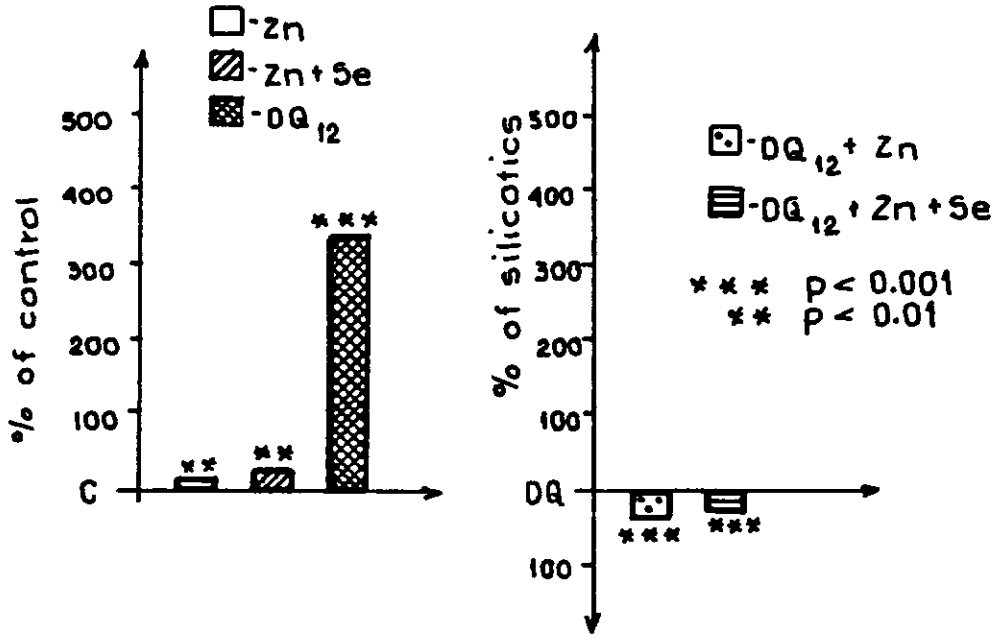

Figure 12. Lung HYPRO of quartz-treated, Zn and Zn+Se-supplemented silicotic rats.

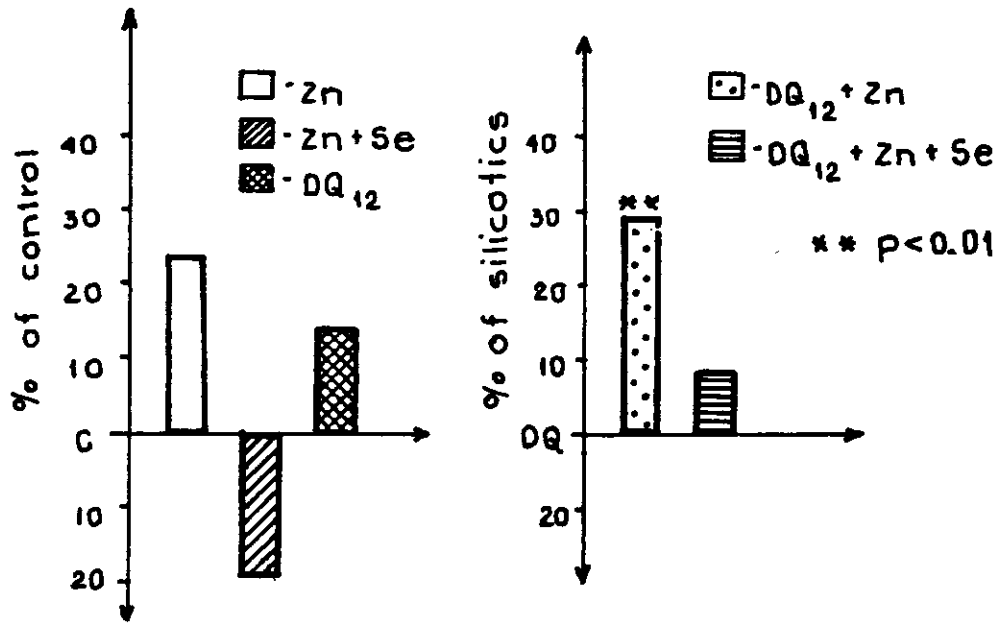

Figure 13. Lung LIPID PEROXIDES of quartz-treated, Zn and Zn+Se-supplemented silicotic rats.

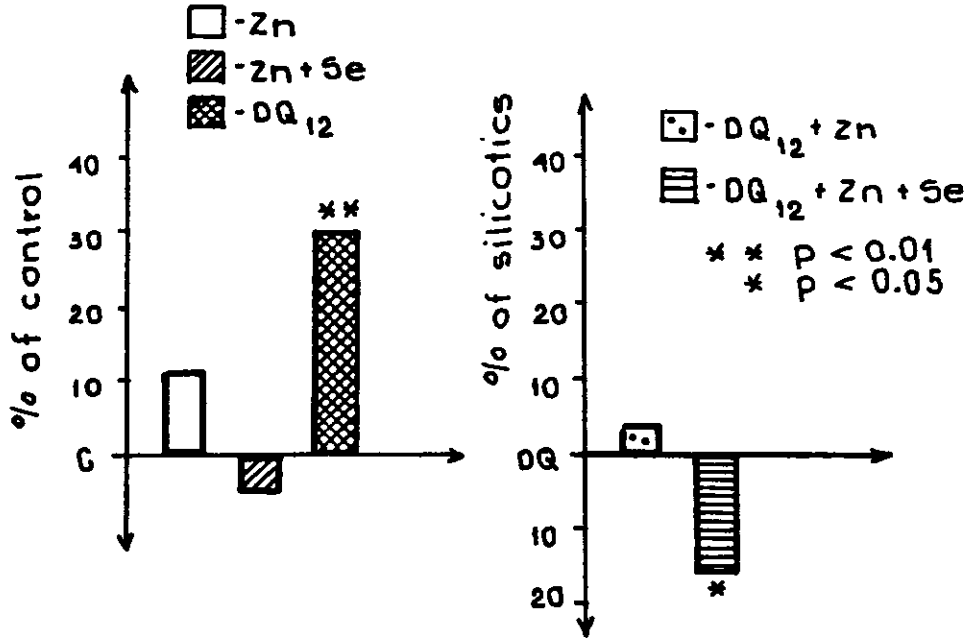

Figure 14. Lung GSH-Px of quartz-treated, Zn and Zn+Se-supplemented silicotic rats.

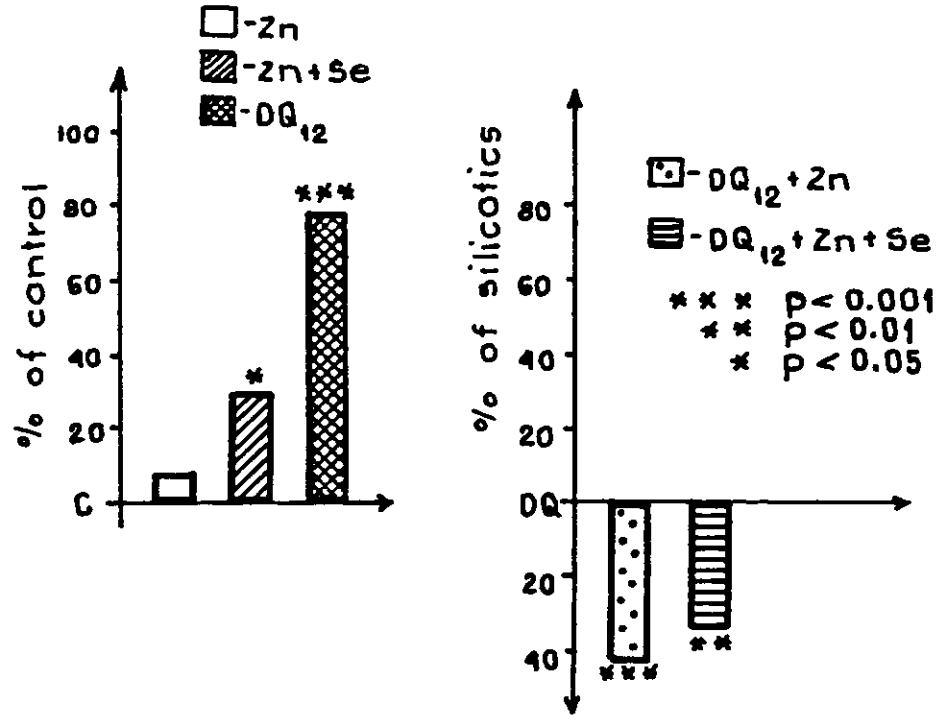

Figure 15. Lung G6P-DH of quartz-treated, Zn and Zn+Se-supplemented silicotic rats.

applicability of our findings to humans can only be speculated upon at this time, it is suggested that a clinical trial with antioxidant supplements to silicotic patients will have a similar effect.

REFERENCES

1. Anttinen, H., Oikarinen, A., Puistola, U., Pääkö, P., Ryhänen, L.: Prevention by Zinc of Rat Lung Collagen Accumulation in Carbon Tetrachloride Injury. *Am. Rev. Respir. Dis.*, 132:536-540 (1985).
2. Babushkina, L.G., Katsnelson, B.A., Kislitsina, N.S.: Effect of Antioxidants on Impairment of Lipid Metabolism and on Fibrogenesis in Lung Tissue under Conditions of Experimental Silicosis. *Biul. Exp. Biol. Med.*, 2:466-469 (1981).
3. Bergmeyer, H.U., Gasvethn, K., Grassl, M.: *Methods of Enzymatic Analysis*, Vol. 1, pp. 425-522, Bergmeyer, H.U. ed. Academic Press, New York (1974).
4. Bezrukavnikova, L.M., Arkhipova, O.G., Voznesenskaya, T.V.: Variations in Free Radical Reactions of Lipid Peroxidation under the Effect of Industrial Aerosols of Different Fibrogenicity. *Ghig. San.*, 3:80-81 (1987).
5. Borm, P.J.A., Bast, A., Wouters, E.F.M., Slangen, J.J., Swaen, G.M.H., deBorder, T.J.: Red Blood Cell-Antioxidant Parameters in Silicosis. *Int. Arch. Occup. Environ. Health*, 58:235-244 (1986).
6. Burk, R.F.: Biological Activity of Selenium. *Ann. Rev. Nutr.*, 3:53-70 (1983).
7. Burton, G.W., Ingold, K.U.: Mechanisms of Antioxidant Action. Studies on Vitamin E and Related Antioxidants in Biological Systems. *Protective Agents in Cancer*, pp. 1-92, Academic Press. London (1983).
8. Chvapil, M., Ryan, J.N., Zukoski, Ch.F.: Effect of Zinc on Lipid Peroxidation in Liver Microsomes and Mitochondria. *Proc. Soc. Exp. Biol. Med.*, 141:150-153 (1972).
9. Chvapil, M., Stankova, S., Malshet, V.: Lipid Peroxidation as one of the Mechanisms of Silica Fibrogenicity. *Environ. Res.*, 11:78-88 (1976).
10. Chvapil, M., Stankova, L., Weldy P.: The role of zinc in Function of some Inflammatory Cells. *Zinc Metabolism: Current Aspects in Health and Disease*, pp. 103-122. Brewer, G.J., Prasad, A.S. eds., Alan R. Liss Inc., New York (1977).
11. Diplock, A.T.: The Biological Function of Vitamin E and the Nature of Interaction of Vitamin with Selenium. *Wld. Rev. Nutr. Diet*, 31:178-183 (1978).
12. Eybl, V., Sykora, J., Mertl, F.: In Vivo Interaction of Selenium with Zinc. *Acta Pharmacol. Toxicol.* Suppl. VII, 59:547-548 (1986).
13. Folch, J., Lees, M., Sloane, Stanley G.H.A.: A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissue. *J. Biol. Chem.*, 226:497-509 (1957).
14. Fukuzawa, K., Tomura, G.: Glutathione Peroxidase Activity in Tissue of Vitamin E-Deficient Mice. *J. Nutr. Sci. Vitaminol.*, 22:405-407 (1976).
15. Gabor, S., Zugravu, E., Frits, T., Anca, Z.: Taux des Lipoperoxides dans le poumons et le myocarde droit dans la silicose experimentale chez le rat. *Arch. Mal. Prof.* 32:553-558 (1971).
16. Gabor, S., Anca, Z.: Effect of Silica on Lipid Peroxidation in Red Cells. *Int. Arch. Arbeitsmed.* 32:327-332 (1974).
17. Gabor, S., Anca, Z., Zugravu, E.: In Vitro Action of Quartz on Alveolar Macrophage Lipid Peroxidation. *Arch. Environ. Health*, 30:499-501 (1975).
18. Gabor, S., Anca, Z., Zugravu, E., Ciugudeanu, M.: In Vitro and In Vivo Quartz-Induced Lipid Peroxidation. *The In Vitro Effects of Mineral Dusts*. pp. 131-137, R. C. Brown, M. Chamberlain, R. Davies, I.P. Gormley eds. Academic Press. London (1980).
19. Gabor, S., Ciugudeanu, M., Surcel, D.: Antioxidants in Relation to Quartz-Induced Peroxidative Damage in Macrophages. *VIth International Pneumoconiosis Conference*, Vol. 1, pp. 503-512. International Labour Organization (ILO), Geneva (1983).
20. Gabor, S., Ciugudeanu, M., Botoc, M., Surcel, D.: Selenium Effects on the Cytotoxicity and Lung Fibrosis Induced by Silica. *Acta Pharmacol. Toxicol.* Suppl. VII 59:191-194 (1986).
21. Gulumian, M., van Wyk, A.: Free Radical Scavenging Properties of Polyvinylpyridin N-Oxide: A Possible Mechanism for its Action in Pneumoconiosis. *Med. Lav.*, 78:124-128 (1987).
22. Gulumian, M., Kilroe-Smith, T.A.: Crocidolite-Induced Lipid Peroxidation II. Role of Antioxidants. *Environ. Res.*, 44:254-259 (1987).
23. Gupta, G.S., Kaw, J.L.: Formation of Lipid Peroxides in the Subcellular Fractions of Silicotic Lungs in Rats. *Eur. J. Respir Dis.* 63:183-187 (1982).
24. Hoekstra, W.G.: Biochemical Functions of Selenium and its Relation to Vitamin E. *Fed. Proc.*, 34:2083-2089 (1975).
25. Kappus, H.: Lipid Peroxidation: Mechanisms, Analysis, Enzymology and Biological Relevance. *Oxidative Stress* pp. 273-310, H. Sies ed. Academic Press Inc., London (1985).
26. Karl, L., Chvapil, M., Zukoski, Ch. F.: Effect of Zinc on the Viability and Phagocytic Capacity of Peritoneal Macrophages. *Proc. Soc. Exp. Biol. Med.*, 172:1123-1127 (1973).
27. Kubow, S., Bray, T.M., Bettger, W.J.: Effects of Dietary Zinc and Copper on Free Radical Production in Rat Lung and Liver. *Can. J. Physiol. Pharmacol.*, 64:1281-1285 (1986).
28. Marsh, J.B., Weinstein, D.B.: Simple Charring Method for Determination of Lipids. *J. Lipid Res.*, 7:574-576 (1966).
29. Neumann, R.E., Logan, M.A.: The Determination of Collagen and Elastin in Tissues. *J. Biol. Chem.*, 186:549-551 (1950).
30. Niki, E.: Antioxidants in Relation to Lipid Peroxidation. *Chem. Phys. Lipids*, 44:227-253 (1987).
31. Parker, F.R., Petersen, N.: Quantitative Analysis of Phospholipids and Phospholipid Fatty Acids from Silica Thin-Layer Chromatogramms. *J. Lipid Res.*, 6:455-462 (1965).
32. Serafini-Cessi, F., Cessi, F.: Thiobarbituric Acid Test as an Index of Phospholipid Peroxidation. *Sperimentale*, 5:371-378 (1968).
33. Schwartz, K.: Essentiality and Metabolic Functions of Selenium. *Med. Clin. North Am.*, 4:745-758 (1976).
34. Zsoldos, T., Tigyi, A., Montsko, T., Puppi, A.: Lipid peroxidation in the Membrane Damaging Effect of Silica-Containing Dust on Rat Lungs. *Exp. Pathol.*, 23:73-77 (1983).

ALTERATIONS IN PULMONARY RESPONSE AND BRONCHOALVEOLAR LAVAGE CONSTITUENTS IN RATS CO-EXPOSED TO QUARTZ AND COAL FLY ASH

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ABSTRACT

Coal fly ash is the major particulate pollutant present in the effluent stream of thermal power stations where coal is burnt for generation of electricity. Co-exposure to fly ash and dusts rich in free silica occur in and around industrial settings, particularly in developing countries, among stone cutters and workers engaged in and around road and building construction.

The effect of coal fly ash on lungs and its potential to modify pathogenesis of pulmonary silicosis was investigated in rats. Exposure to coal fly ash alone resulted in concentration of the particulates within hyperplastic alveolar macrophages, situated in the various compartments of the pulmonary parenchyma and the draining lymph node. In spite of its long residence in lungs, fly ash elicited only a meagre fibrotic reaction. Quartz exposed rats developed nodular silicotic reaction, comprised predominantly of reticulin and collagen fibers. In silicotic animals exposed to fly ash the increase in lung weight, hydroxyproline contents and laying down of collagen was less than in silicotic animals not exposed to fly ash. The elevated levels of soluble proteins, content of lysosomal enzymes and the cellular constituents of the bronchoalveolar lavage were similarly less in fly ash-quartz exposed rats than in those exposed to quartz alone.

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INTERACTION OF MINERAL FIBRES WITH EXTRACELLULAR MATRIX AND MESOTHELIUM AFTER INTRAPERITONEAL INJECTION IN RATS

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INTRODUCTION

Serosal tests have proven as appropriate methods for detecting the neoplastic potency and fibrogenicity of asbestos fibres and man-made mineral fibres.[4,7,15,24,28,29] The results have strongly supported the hypothesis, that besides the elongated shape of the fibrous particles (lengths and diameter) their dose, durability and possibly also their surface properties may be the cause of their pathogenic effects.[5,6,16,22] The correlation between fibre-induced fibrosis and the development of mesotheliomas had been discussed contradictory and not yet been fully understood.[8,9,14,18,19,23,25,26] The mainly commercially used kinds of asbestos crocidolite and chrysotile obviously possess different fibrotic and neoplastic potency in man.[1,2,3,12,13,17,20,21,27] Because of this fact our studies on rat omentum aimed first for the presentation of differences in the composition of the extracellular matrix components collagen types I and III, laminin and fibronectin in crocidolite and chrysotile-induced granulomatous lesions. In addition we have examined in which way these fibrous natural dusts with very different physico-chemical properties lead to malignant transformation of the mesothelium. We have wondered, if these mechanisms were the same, which account for the tumour-inducing effect of some man-made mineral fibres.

Material and Methods

Our investigations were carried out on omentums of altogether 64 female Sprague-Dawley rats which had been sacrificed under narcotization 8 hours to 15 months after intraperitoneal injection of 1.5 or 15 mg crocidolite (South Africa, like UICC reference sample but fibre lengths greater) and chrysotile B (UICC reference sample) either. The omentums were divided into several parts and their preparation and fixation in formaldehyde, cold phosphate-buffered (0,5 M, ph 7,4) 2,25% glutaraldehyde and 1,3% osmium tetroxide for the light microscopical and the electron microscopical examination were done in normal manner. Sections were stained with HE chromo-trope-aniline blue, Prussian blue or Toluidin blue and Uranyl acetate. Cryostat sections for the immunofluorescence microscopical investigations were incubated with specific antibodies against collagens types I and III, the multifunctional glycoprotein fibronectin and the basement membrane glycoprotein laminin or with non-immune-serum as described elsewhere.[11] We further have examined specimens of the omentums from long term carcinogenicity studies on natural and man-made mineral fibres which were already referred in detail.[24] In these intraperitoneal tests very low doses between 0.05 and 0.5 mg asbestos, for example, have led to tumour incidences of about 20 to 80%. Only those animals have been chosen for our light microscopical investigations, however, which had been sacrificed in a bad health condition not earlier than two years after the intraperitoneal injection of different fibrous dusts. They have macroscopically shown no tumour growth. Doses of all intraperitoneally applied dusts and the life-spans of the animals after injection are listed in Table I.

Results

Focal granulomatous lesions of the omentum have been found only in those animals, which had been intraperitoneally injected with more than 1 mg of either natural or man-made mineral fibres in 1 ml physiological saline solution. Already 3 days after crocidolite administration the fibres have accumulated within the area of the "milk spots" of the omentum. These are circumscribed deposits of cells belonging to the monocyte macrophage system especially in the area of vascular branching. The granulomatous foreign body reactions characteristically have had a large number of mononuclear macrophages and multinucleated giant cells not only within the first week but also in the end of the investigation period. The crocidolite fibres are spread over the entire area of the granulomatous lesions and they are deposited especially at the surface of multinucleated giant cells in form of larger accumulations (Figure 1B). Shorter fibres can also be seen under the electron microscope within the cytoplasm of macrophages. New collagen, especially type III, can be demonstrated with the immunofluorescence microscope already after 3 days. At that time you can electron microscopically identify mainly macrophages and some lymphocytes in perivascular and submesothelial position. Fibroblasts and myofibroblasts are nearly absent (Figure 2A). There was a steady increase of fibrillogenesis throughout the 6 months studied immunofluorescence microscopically and during this time the collagen fibres were distributed symmetrically between the macrophages all over the lesions (Figure 1A).

On the other hand in chrysotile-induced lesions collagen types I and III synthesis was limited on the periphery of the granulomas (Figure 1C). Different to earlier reports chrysotile fibre bundles could be shown in the center using light microscopy (phase contrast or differential interference

contrast) even up to six months (Figure 1D).[28] They seem to possess collagenolytic activity. The cellular debris lying between them can only be detected with the transmission electron microscope (Figure 2B).

Table I
Intraperitoneally Applied Natural Mineral Fibres (a) and Man-made Mineral Fibres (b)

	Dosis i.p. (mg)	Microscopically investigated omentums (n)	Life-span after i.p. injection (months)
a)			
Crocidolite (S.Africa)	1-15	32	<1-15
Chrysotile	1-15	32	<1-15
	0,05	7	24-27
Actinolite	0,01	6	24-28
	0,05	8	25-28
	0,25	4	25-28
	0,25 + PVNO	7	24-26
Erionite, Oregon	2,00	3	28
Wollastonite	100	5	26-28
b)			
Glass fibres JM 104/475	5	6	27-28
Polypropylene fibres	50	3	28
Kevlar fibres	20	2	27

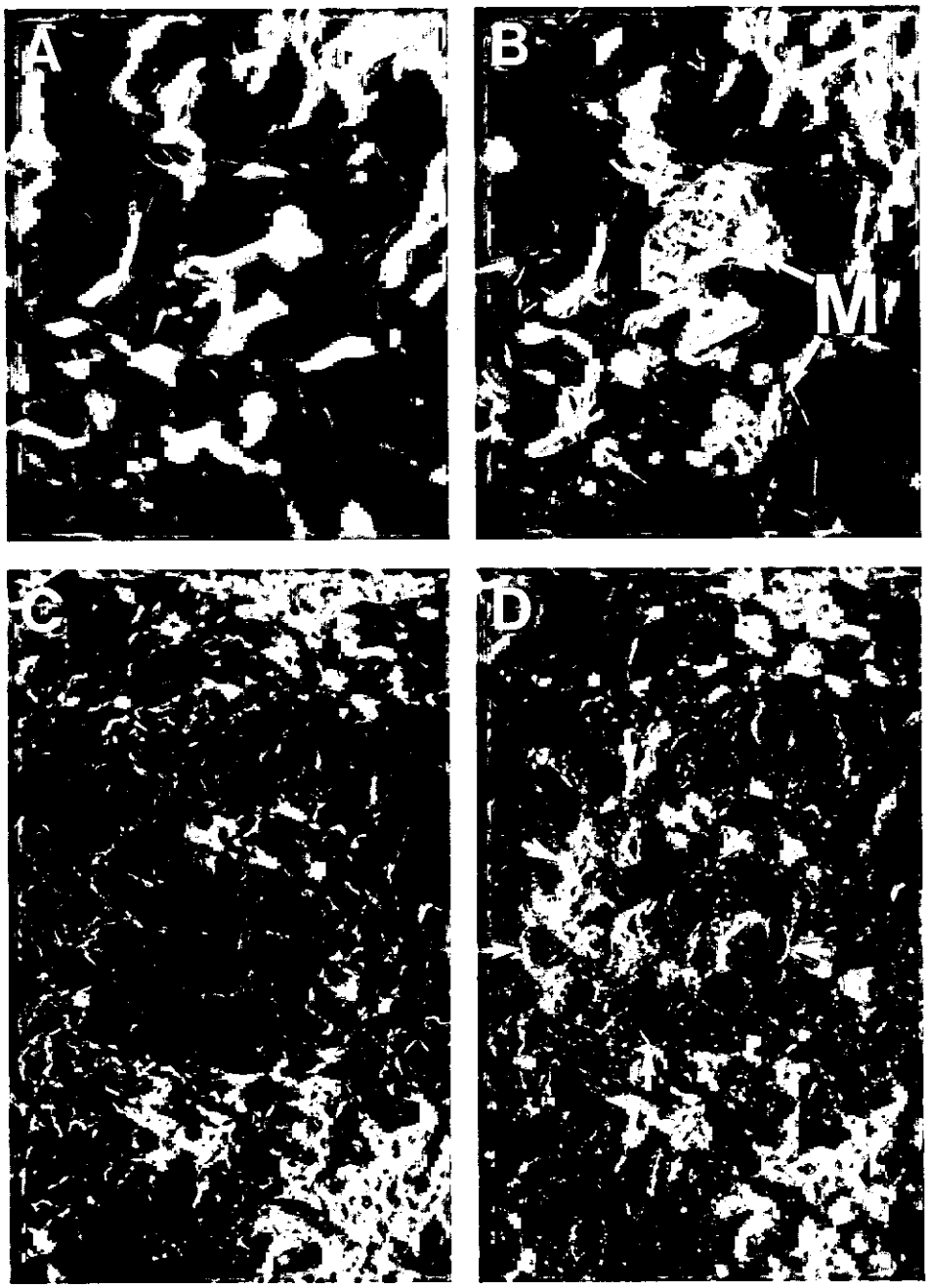

Figure 1. Asbestos induced fibrosis in the rat omentum 1–2 weeks after the i.p. injection of the dusts.
(A) and (B) Intact pericellular network of collagen type III in crocidolite containing granulation tissue.
((A) Immunofluorenscence microscopy 460 X;
(B) The same section as in (A) with light from the bottom and phase-contrast 460 X)
M: Multinuclear giant cells containing crocidolite fibres.
(C) and (D) Dissolution of collagen fibres (type III) and necrotic macrophages in the center of chrysotile containing granulomas.
((C) Immunofluorescence microscopy 290 X;
(D) the same section as in (C) with light from the bottom and phase-contrast 290 X)
➡ Chrysotile fibres

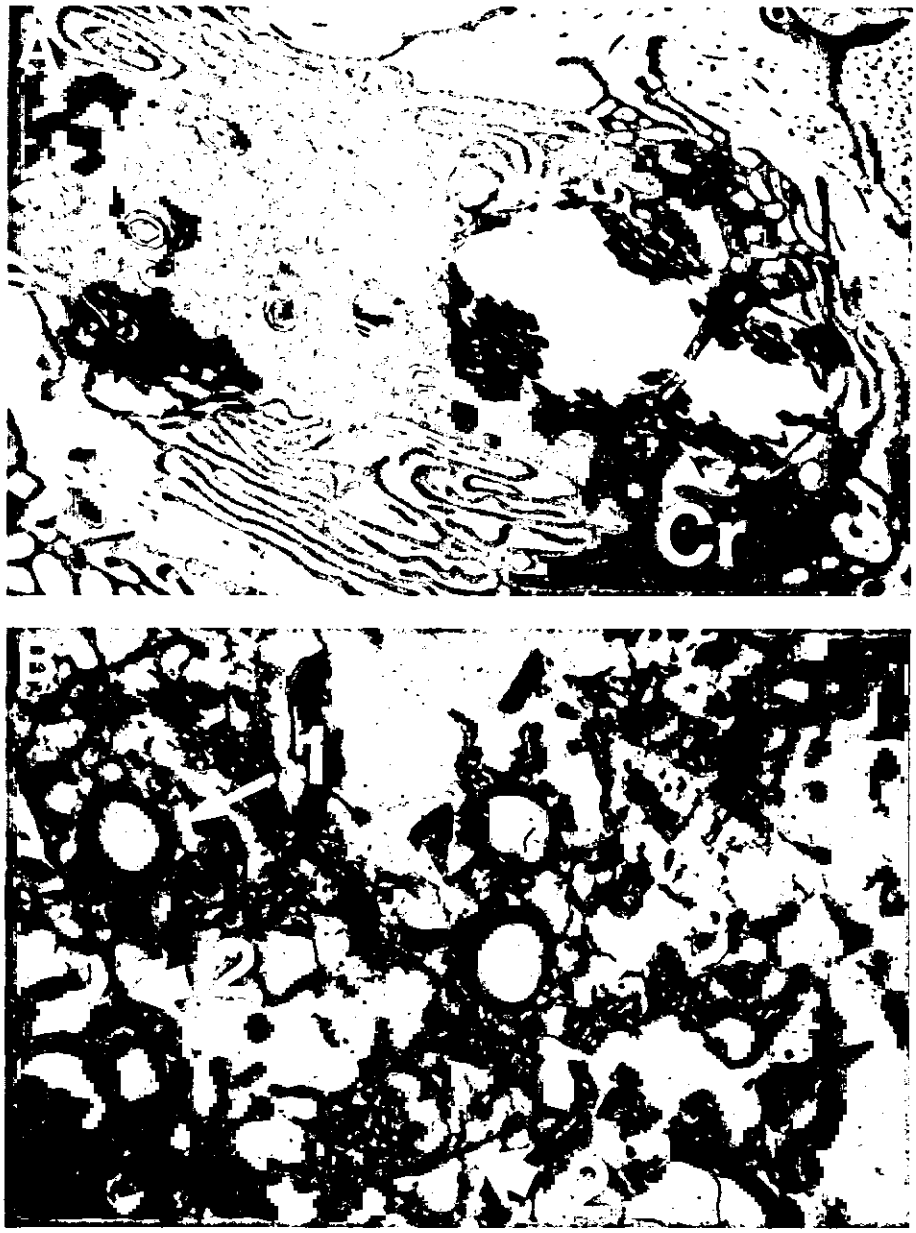

Figure 2. (A) 3–7 days after i.p. injection of crocidolite the granulomatous lesions in the rat omentum are dominated by macrophages. Fibroblasts and myofibroblasts are nearly absent. (EM 55.500X) Cr: Crocidolite.
(B) Cellular debris and chrysotile fibres in the center of granulomas six months after i.p. injection of 15 mg/ml saline solution. (EM 7.000X).
1. Fatvacuoles
2. Chrysotile fibres

As we have reported elsewhere the glycoprotein fibronectin can be found between and at the surface of macrophages in crocidolite induced inflammatory infiltrates and especially accumulates at the surface of very long crocidolite fibre bundles.[10] Therefore we have discussed the importance of its opsonic activity in case of asbestos induced fibrosis. Also, chrysotile fibres and clustered cellular debris were coated with fibronectin (Figure 3). In this way delayed scarring of the inflammatory reactions induced by chrysotile depositions in the fat tissue of the omentum might be promoted too. The scarring has not been finished 6 months after chrysotile application. Until this moment also the remarkable differences in the vascularization of chrysotile and crocidolite induced lesions continue (Figure 4). The surface properties of chryso-

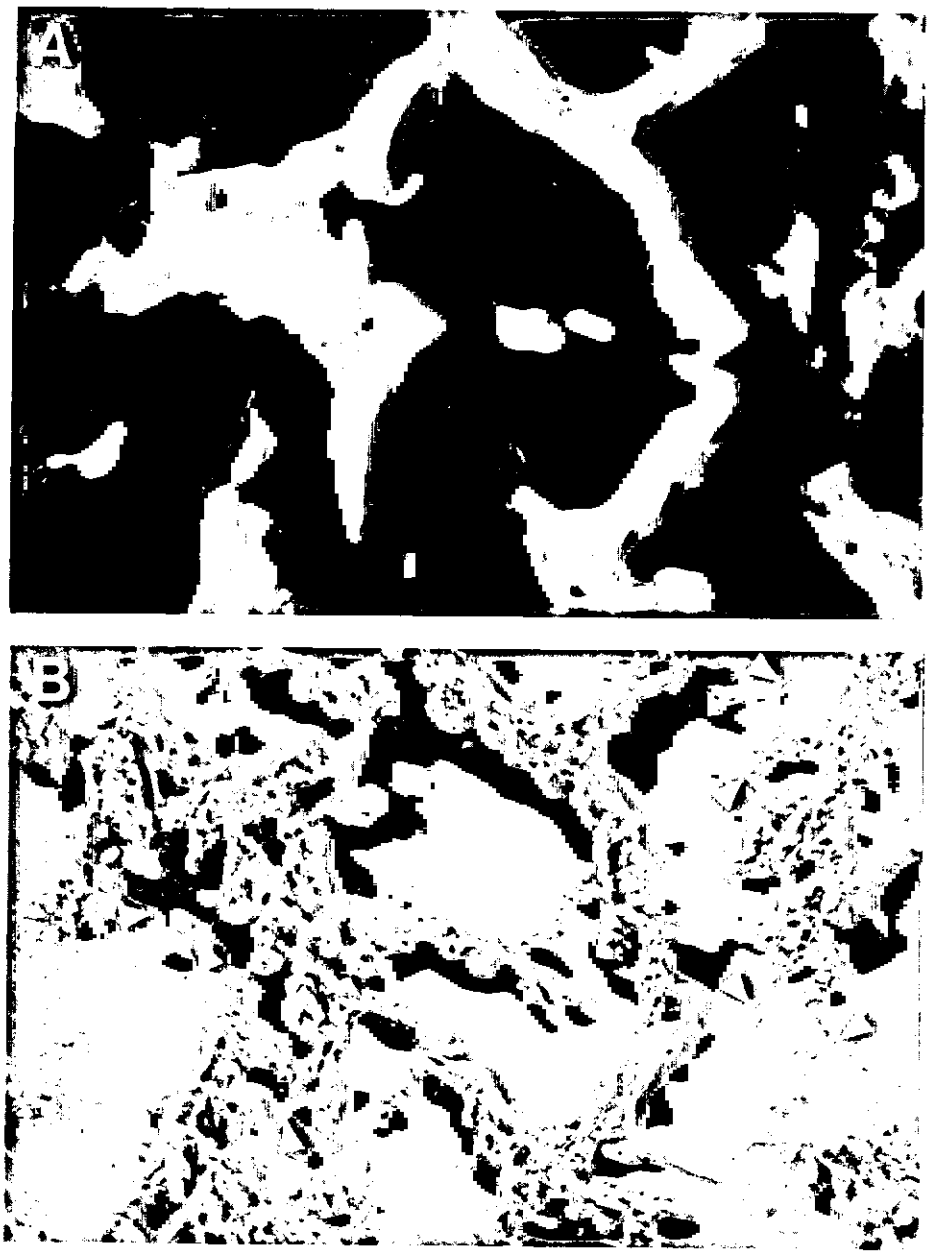

Figure 3. Immunofluoroscence microscopical demonstration of fibronectin (A) at the surface of chrysotile fibres and clustered cell detritus (B) in the center of a foreign-body granuloma in the rat omentum 2 months after i.p. injection of the fibrous dust.
((A) Immunofluorescence microscopy 460X;
(B) The same section as in (A) with light from the bottom, polarization and phase-contrast 460X)
➡ Chrysotile fibres

tile fibres seem not only to counteract the chemotactic and opsonic activity of fibronectin but also a vascular sprouting.

In combination the reaction patterns of extracellular matrix components, crocidolite induces a well vasculated granulation tissue and after 6 months much more fibrosis than chrysotile (Figure 5 left on top). Around the latter foreign-body-granulomas were formed with central necrosis and collagenous connective tissue only in the periphery (Figure 5 at the bottom). Fibres containing granulation tissue and granulomas, both however, are no obligatory conditions for mesothelial proliferation (Figure 5, right).

On the contrary even without contact to the focal dust containing lesions we have found narrow connective tissue

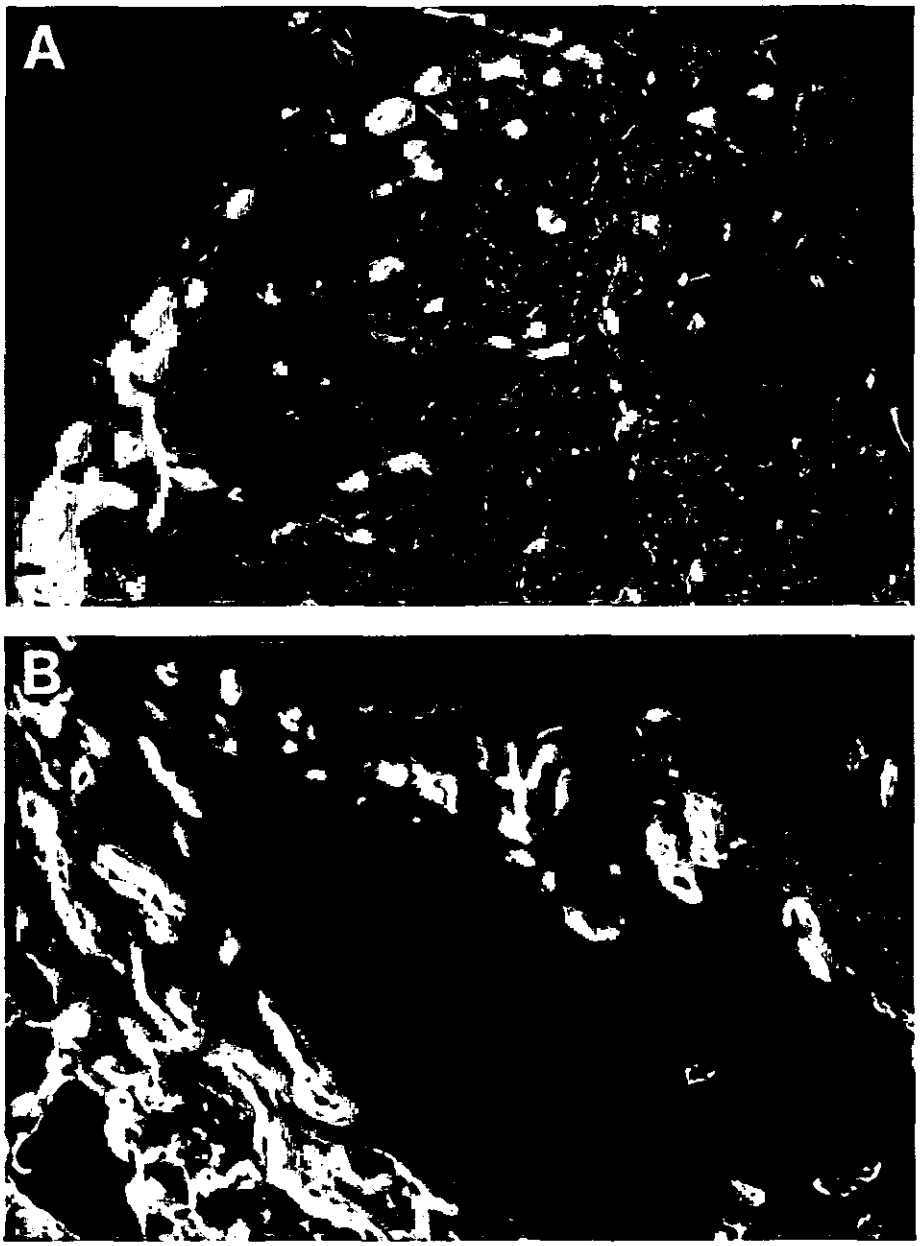

Figure 4. Dense vascularization of the granulation tissue induced by crocidolite fibres (A) and only in the periphery of a chrysotile containing granuloma (B).
((A) and (B) Immunofluorescence microscopy with an antibody against the basement-membrane glycoprotein laminin; (A) 190X, (B) 300X)

strands directly beneath the mesothelium already 7 days after intraperitoneal injection of 15 mg crocidolite, for example (Figure 6A). The covering cells were rounded, enlarged and often multinuclear. They have never stored asbestos fibres. In our opinion these changes can be conceived as a repairing process of the submesothelial mesenchyme. They are not only present in the fat areas of the omentum but also in the normally very thin mesothelial duplicatures spread between them (Figure 6B). The latter are the preferred localization of the generally less intensive chrysotile induced submesothelial fibrosis which also last for months and years after fibre administration. Even 28 months following the intraperitoneal injection of not more than 0.05 mg chrysotile/ml fibrotic thickening of the mesothelial duplicatures of the omentum could be observed.

Although macroscopically the omentums from long term carcinogenicity studies showed no tumour growth we microscopically have often found focal mesothelial proliferations associated with submesothelial fibrosis. In some ani-

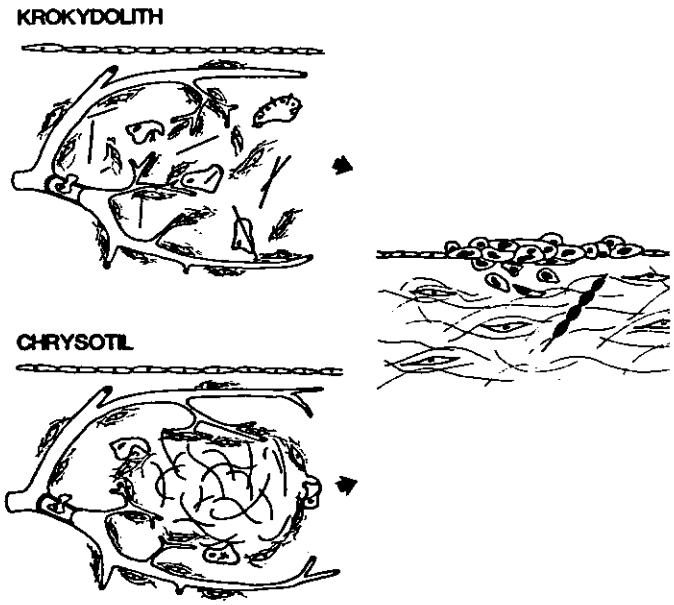

Figure 5. Crocidolite containing granulation tissue (left side on top) and chrysotile-induced granulomas (left side on the bottom) in the rat omentum as non obligatory precondition of mesothelial proliferation (right side).

mals mesothelial proliferation has reached the intensity of early mesotheliomas infiltrating the underlying connective tissue (Figure 7B). The activation of the submesothelial mesenchyme and the mesothelial proliferation, both proved to be a rather unspecific answer to injuries of the mesothelium by fibrous dusts provided that the single particles were only long and fine enough. These changes could be observed in a similar way 28 months after intraperitoneal deposition of the natural mineral fibres, type actinolite, and of man-made mineral fibres as glass microfibres and Kevlar (Figure 7A). They seem to depend in the quantity on fibre type only.

CONCLUSIONS

1. Phagocytosis of asbestos fibres is obviously mediated by fibronectin and in early fibrosis macrophages may be one place of collagen synthesis.
2. There are remarkable differences in the intensity of collagen synthesis in crocidolite induced granulation tissue with a symmetrical fibrillogenesis and in chrysotile induced granulomas with central necrosis and newly formed collagen fibres only in the periphery.
3. Natural mineral fibres and man-made mineral fibres tested intraperitoneally induce lesions of the serosal surfaces which are followed by submesothelial fibrosis and mesothelial proliferations up to the development of malignant mesotheliomas. These changes seem to depend in the quantity on fibre type only.

LITERATURE

1. ACHESON ED, GARDENER MJ, PIPPARD EC, GRIME LP (1982): Mortality of two groups of women who manufactured gas-masks from chrysotile and crocidolite asbestos: a 40 year follow-up. *Br J Ind Med* 39:344-348.
2. ARMSTRONG BK, DE KLERK NH, MUSK AW, HOBBS MST (1988): Mortality in minors and millers of crocidolite in Western Australia. *Br J Ind Med* 45:5-13
3. BECK EG, SCHMIDT P (1985): Epidemiologische Untersuchungen bei verstorbenen Arbeitnehmern der Asbestzement-Industrie in der BRD. *Zbl Bakt Hyg*, I Abt Orig B 181: 207-215
4. BOLTON RE, DAVIS JMG, MILLER B, DONALDSON K, WRIGHT A (1983): *The effect of dose of asbestos on mesothelioma production in the laboratory rat.* VIth Internat. Pneumoconiosis Conference 1983. VI. Internat. Pneumokoniose-Konferenz 1983. Bochum, 20-23., Sept. 1983, vol. 2. Internat Labour Organisation (ILO), Genf 1984: 1028-1046.
5. CHURG A, TRON V, WIGGS B, WRIGHT J (1986): Effect of fiber size on short-term clearance of asbestos from the lungs of smoking and non-smoking guinea pigs. *Am Rev Respir Dis* 135 (Suppl): A 19.
6. DAVIS JMG, ADDISON J, BOLTON RE, DONALDSON K, JONES AD, SMITH T (1986): The pathogenicity of long versus short fibre samples of animosite asbestos, administered to rats by inhalation and intraperitoneal injection. *Br J Exp Path* 67:415-430.
7. EDWARDS RE, WAGNER MMF, MONCRIEFF CB (1984): Cell population and histochemistry of asbestos related lesions of rat pleural cavity after injection of various inorganic dusts. *Br J Ind Med* 41:506-513.
8. FRIEMANN J, BRINKMANN O, POTT F, MÜLLER K-M (1988): Peritoneale Differenzierungsstörungen als Reaktion auf Asbest und Asbestersatzstoffe. Tierexperimentelle Untersuchungen. *Verh Dtsch Ges Path* 72: in press.
9. FRIEMANN J, POTT F, VOSS B, GONZALES S, MÜLLER K-M (1988): Preneoplasia of mesothelium induced by mineral fibres. Experimental results in animals. *J Canc Res Clin Onc:* 114-162.
10. FRIEMANN J, VOSS B, MÜLLER K-M (1987a): Asbestbedingte granulomatös-fibröse Reaktionen im Peritoneal test. Immunhistochemische Untersuchungsbefunde. *Verh Dtsch Ges Path* 71: 371.
11. FRIEMANN J, VOSS B, WELLER W, MÜLLER K-M (1987b): Asbestos induced fibrosis in the omentum of rats. Immunofluorescence microscopical demonstration of collagen types I and III, laminin and fibronectin. *Virchows Arch A* 411: 403-408.
12. HOBBS MST, WOODWARD SD, MURPHY B, MUSK AW, ELDER JE (1980): The incidence of pneumoconiosis, mesothelioma and other respiratory cancer in men engaged in mining and milling crocidolite in western Australia. In: *Biological effects of mineral fibers*, Vol 2. WAGNER JC, (ed). IARC Scientific Publications No. 30, Lyon 1980: 615-625.
13. HUGHES J, WEILL H (1980): Lung cancer risk associated with manufacture of asbestos-cement products. In: *Biological effects of mineral fibres*, Vol 2. WAGNER JC (ed). IARC Scientific Publications No. 30, Lyon 1980: 627-635.
14. HUTH F, POTT F (1979): Ist die Fibrose nach intraperitonealer Applikation faser-förmiger Stäube (Asbest, Glasfasern) ein obligate Praneoplasie? *Verh Dtsch Ges Path* 63:437-439.
15. *IARC Monographs* on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. *Vol. 42, Silica and Some Silicates*, 1987. World Health Organization International Agency for Research on Cancer, Lyon (in press).
16. JAURAND MC, BRODY AR, DAVIS J, FISHER JL, LANGER AM, LE BOUFFANT L, PEZERAT H, ROBOCK K, SCHARMANN A, TILKES F (1985): Consensus panel. Role of various parameters of fibrous dusts (dose, dimension, type, surface properties) in relation to pathogenesis. *In: In vitro effects of mineral dusts.* BECK EG, GIBNON (eds). NATO ASI Series G. Ecological Sciences No. 3. Springer-Verlag, Berlin-Heidelberg-New York-Tokyo: 449-450.

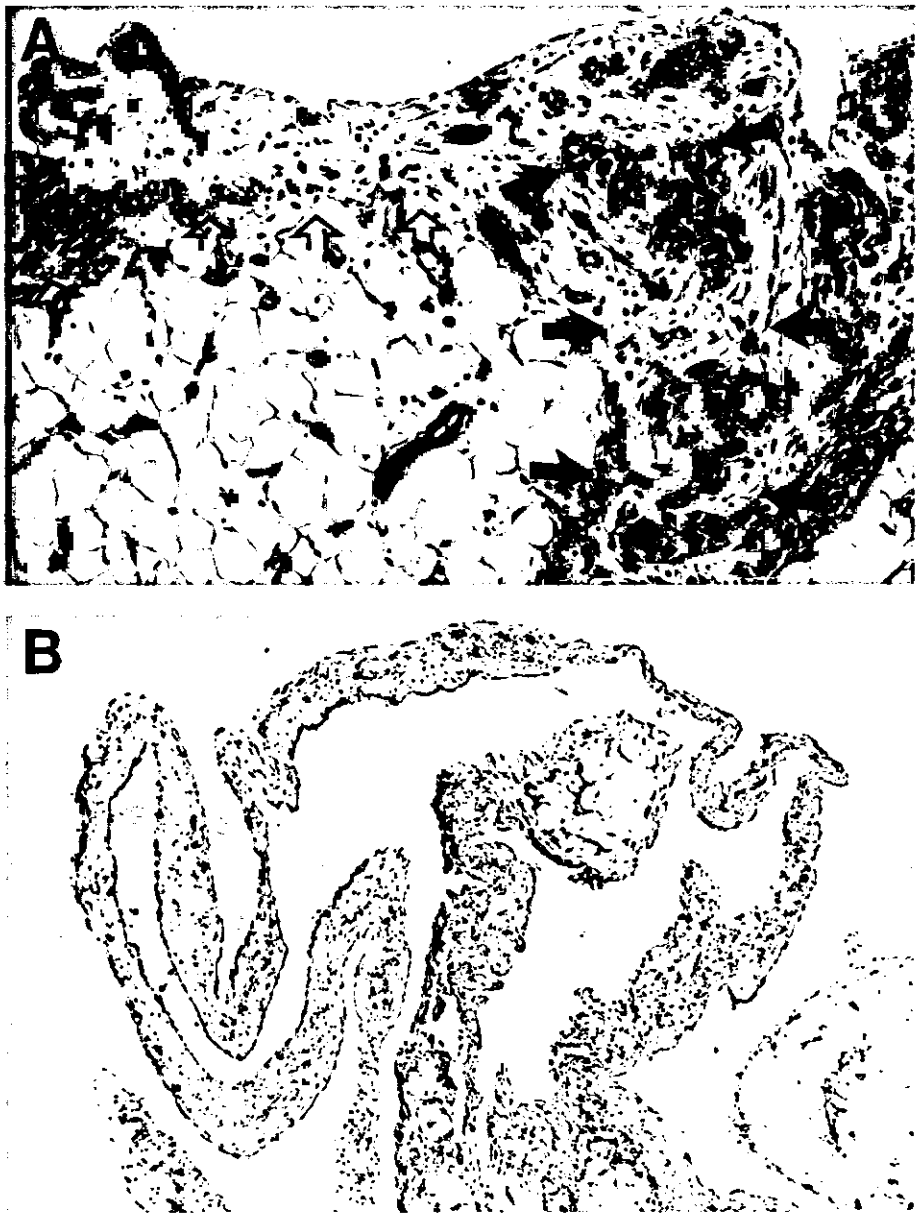

Figure 6. (A) Submesothelial fibrosis without crocidolite fibres visible with the light microscope (➡) and adjacent proliferating mesothelial cells without close connection to the foreign-body granuloma (➡) on the right side of the figure, 7 days following crocidolite administration.
(B) Cross section of mesothelial duplicatures of rat omentum. 3 weeks after crocidolite application the fibrotic thickening is remarkable.
((A) HE 74 X; (B): HE 60 X)

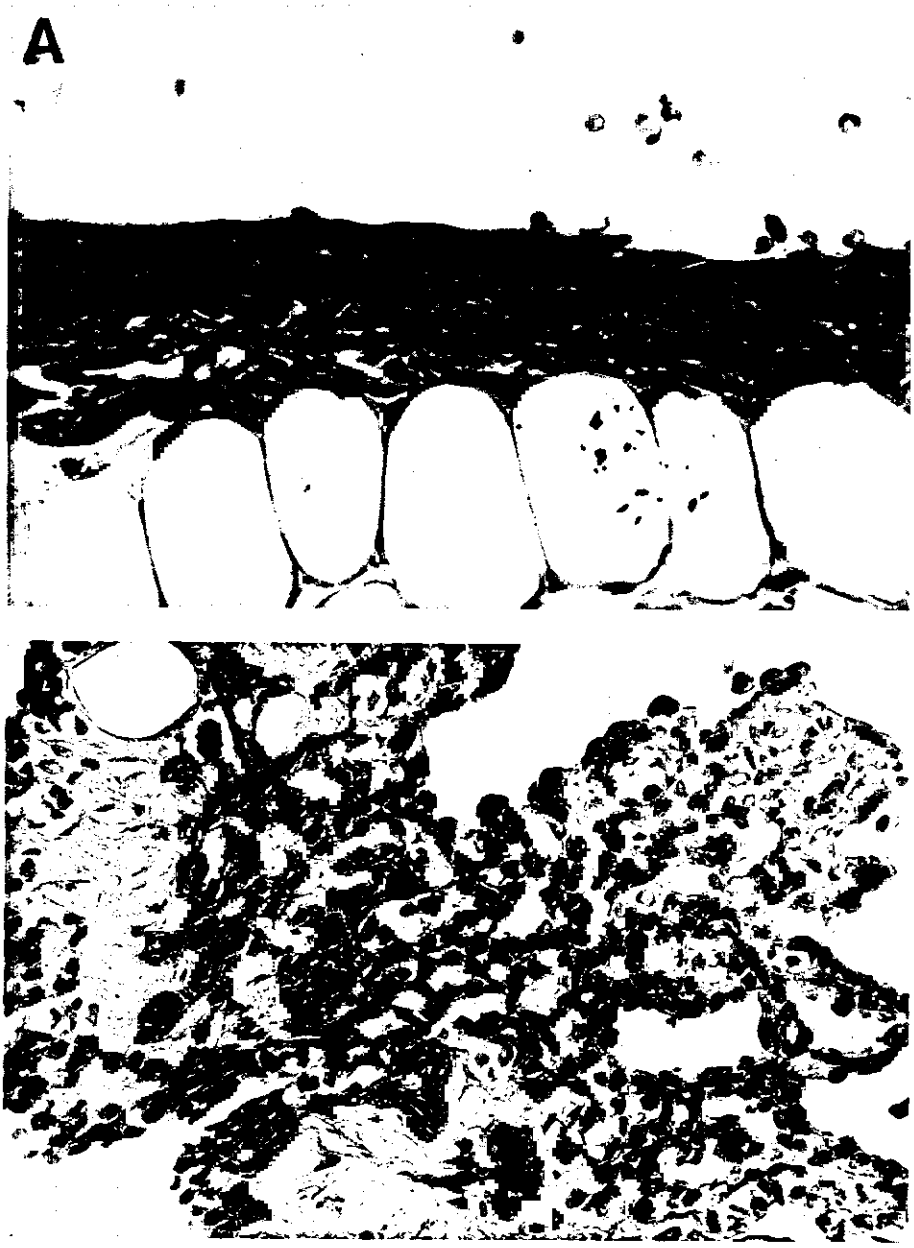

Figure 7. (A) Proliferation of probably preneoplastic cells in the submesothelial tissue 28 months after glass micro-fibre-induced injury of the mesothelial lining. (HE 400 X)
(B) Early mesothelioma of the mesothelial duplicature of rat omentum 21 months after exposure to actinolite. (HE 360 X)

17. JONES JSP, LUND C, PANTEYDT HT (1985): *Colour atlas of mesothelioma*. Commission of the European Communities. Industrial Medicine and Hygiene Division. MTP Press Limited.
18. KUSCHNER M (1987): The effects of MMMF on animal systems: some reflections on their pathogenesis. *Ann Occup Hyg* 31: 791-797.
19. LÖBLICH HJ, BUSCHE TH (1982): Die Entwicklung des Pleuramesothelioms nach Asbestapplikation. *Verh Dtsch Ges Path* 66:593.
20. MC DONALD AD, FRY JS, WOOLEY AJ, Mc DONALD JC (1984): Dust exposure and mortality in an American chrysotile asbestos friction product plant. *Br J Ind Med* 41:151-157.
21. MORGAN A, HOLMES A (1985): The enigmatic asbestos body: its formation and significance in asbestos-related disease. *Environ Res* 38:283-292.
22. POTT F (1987): Die Faser als krebserzeugendes Agens. *Zbl Bakt Hyg B* 184: 1-23.
23. POTT F, ROLLER M, ZIEM U, REIFFER F-J, BELLMANN B, ROSENBRUCH M, HUTH F (1988): Carcinogenicity of studies on natural and man-made fibres with the intraperitoneal test in rats. Mineral fibres in the Non-occupational Environment. *Proceedings of a joint symposium held at IARC, Lyon, France, 8-10 September, 1987*. IARC Scientific Publ. Lyon: Intern. Agency for Research on Cancer (in press).

24. POTT F, ZIEM U, REIFFER F-J, HUTH F, ERNST H, MOHR U (1987): Carcinogenicity studies on fibres, metal compounds, and some other dusts in rats. *Exp Pathol* 32:129-152.
25. SHIN ML, FIRMINGER HI (1973): Acute and chronic effects of intraperitoneal injection of two types of asbestos in rats with a study of the histopathogenesis and ultrastructure of resulting mesotheliomas. *Am J Pathol* 70:291-314.
26. SUZUKI Y, KOHYAMA N (1984): Malignant mesothelioma induced by asbestos and zeolite in the mouse peritoneal cavity. *Environ Res* 35:277-292.
27. SUZUKI Y, SELIKOFF IJ (1986): Pathology of lung cancer among asbestos insulation workers. *Fed Proc* 45:744.
28. WELLER W, KISSLER W, FRIEDRICHS K-H, MORGENROTH K (1984): *Fibrogenität ultramikroskopischer Chrysotil-Asbest-Fasern.* In: VI. Internationale Pneumokoniose-Konferenz, Bochum, 20-23 Sept. 1983, Vol 2. Bergbau-Berufsgenossenschaft (Hrsg). Wirtschaftsverlag NW, Verlag für neue Wissenschaft GmbH, Bremerhaven 1984: 1021-1027.
29. WINKLER GC, RÜTTNER JR (1983): Early fibrogenecity of asbestos fibers in visceral peritoneum. *Expl Cell Biol* 51:1-8.

IN VITRO INJURY TO ELEMENTS OF THE ALVEOLAR SEPTUM CAUSED BY LEUKOCYTES FROM THE BRONCHOALVEOLAR REGION OF RATS EXPOSED TO SILICA

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INTRODUCTION

Quartz exposure is associated with lung fibrosis (silicosis) and Type II alveolar epithelial cell hyperplasia is also commonly present.[1] Bronchoalveolar lavage studies using rats in our own laboratories,[2] and in humans,[3] have demonstrated that there is leukocyte recruitment into the lungs following inhalation exposure to silica. Studies on other fibrotic lung diseases have stressed the importance of the leukocytes of the alveolitis in the progression of disease via release of important mediators.[4] We have therefore set out to examine the ability of bronchoalveolar leukocytes from rats exposed to silica by a single intratracheal instillation, to cause injury to the extracellular matrix and cellular elements of the alveolar septum *in vitro*. Leukocytes from rats exposed to two other inflammogenic particulates—a heat killed bacterial preparation and a yeast cell wall preparation (zymosan)—were similarly assessed, for comparison with quartz.

MATERIALS AND METHODS

Animal Model of Silicosis

Syngeneic PVG rats, SPF bred, were exposed by intratracheal instillation to 1 mg of DQ_{12} standard quartz. As controls, the heat killed bacterial preparation *Corynebacterium parvum* was also injected as was the yeast cell wall preparation zymosan; both of these particulates are known to cause inflammation. Bronchoalveolar leukocytes were obtained by lavage as described in detail elsewhere[5] at various time points after injection. In this model quartz exposure causes fibrosis, Type II epithelial cell hyperplasia and alveolar lipoproteinosis beyond 1 month exposure which are evident in histological sections of exposed lung.

Assay of Leukocyte-Mediated Type II Alveolar Epithelial Cell Injury

This assay is described in detail elsewhere[6] and involves labelling of Type II alveolar cell line (A549) with ^{51}Cr. Bronchoalveolar leukocytes are then added to the labelled cells in microtitre wells and co-cultured for 4 hours; the ability of the leukocytes to cause lysis or detachment of the epithelial cells is assessed.

Assay of Leukocyte-Mediated Proteolysis of Fibronectin

Leukocyte-mediated proteolysis of fibronectin was assessed using a solid phase assay of ^{125}I-labelled fibronectin in microtitre plate wells. This assay has been described in detail elsewhere[7] and measures protease-mediated injury. The leukocyte-mediated proteolyic activity shown here against fibronectin is also active against ^{125}I-labelled collagen and laminin. Leukocytes are cultured on the solid phase of ^{125}I-labelled fibronectin and allowed to degrade the matrix for 4 hours; products of proteolysis of fibronectin are measured as free counts in the supernatant.

Leukocyte Separation

Whole inflammatory bronchoalveolar leukocyte populations from quartz-exposed rats were separated by centrifugation through Sepra-Cell medium into macrophage and neutrophil-enriched fractions.

Statistical Analyses

Results were analysed by analysis of variance and differences in treatments compared for significance using a 't' test.

RESULTS

Inflammation Caused by a Single Injection of Silica, *C. parvum* or zymosan

Figures 1 and 2 show the total number of bronchoalveolar leukocytes and percentage neutrophils lavaged from rats injected intratracheally with quartz, *C. parvum* or zymosan. All three particles caused initial burst of inflammation characterized by recruitment of large numbers of leukocytes containing high proportions of neutrophils. In the case of *C. parvum* and zymosan this initial alveolitis was followed by a return to the normal situation where no neutrophils were present although the numbers of macrophages remained raised indicating a mild macrophage alveolitis. In the case of quartz, however, an intense macrophage/neutrophil alveolitis persisted until at least one month. Previous studies have shown that this alveolitis persists for up to three months.[8]

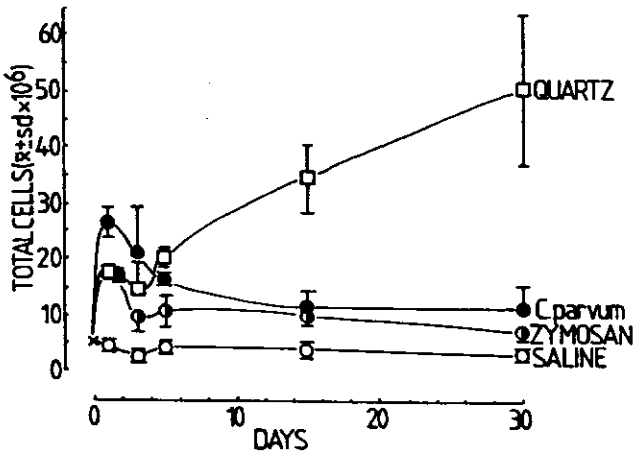

Figure 1. Total leukocytes in bronchoalveolar lavage up to 30 days after instillation of saline, quartz, *C. parvum* or zymosan into the lungs of rats. Data is mean ± standard deviation from 3 rats. Significant ($P<0.01$–0.001) increases with all particulates compared to saline.

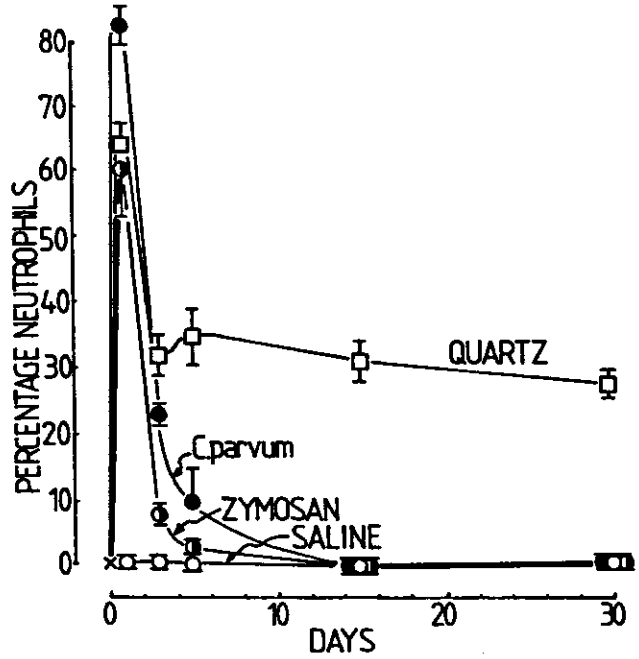

Figure 2. Percentage neutrophils in bronchoalveolar lavage up to 30 days after instillation of saline, quartz *C. parvum* or zymosan. Data derived as in legend to Figure 1. Significant ($P<0.01$–0.001) increases in percentage neutrophils, compared to saline, for quartz at all time points and for *C. parvum* and zymosan at 1, 3 and 5 days.

Activity of Bronchoalveolar Leukocytes in Breaking Down Fibronectin

As shown in Figure 3 the bronchoalveolar leukocytes obtained from the lungs following injection of different particles showed varying abilities to break down fibronectin. During the acute inflammatory phase the leukocytes from lung exposed to all three particulates were capable of breaking down fibronectin. However, only quartz was capable of eliciting a sustained high level of proteolysis, in keeping with the persistence of the inflammation in quartz-exposed lung. It was notable that the ability to break down fibronectin correlated strongly with the presence of neutrophils.

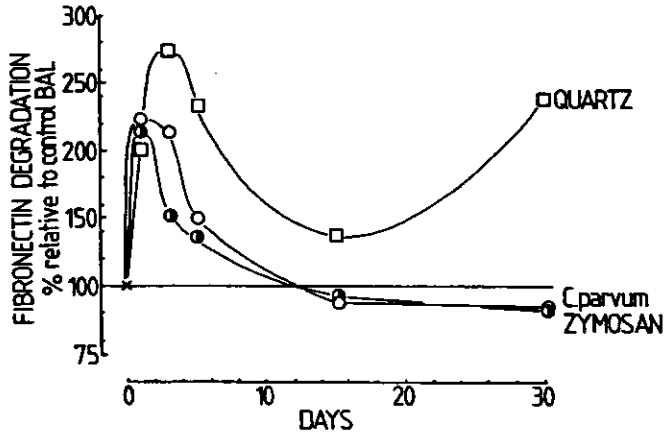

Figure 3. Proteolytic activity against fibronectin shown by bronchoalveolar leukocytes from rats injected with the indicated particulates. Data expressed as a percentage of the activity shown by control bronchoalveolar leukocytes.

Injury to Alveolar Epithelial Cells Caused by Bronchoalveolar Leukocytes

Bronchoalveolar leukocyte populations elicited with quartz or C. parvum were tested for their ability to cause injury to cells of an alveolar epithelial cell line *in vitro*. Both *C. parvum*-elicited bronchoalveolar lavage cells obtained after one day (70–90% PMN) and 5-day quartz leukocytes (50% macrophages/50% neutrophils) were capable of causing the target cells to detach from the sub-stratum (Figure 4). There was no lytic injury to the target cells and the detachment injury could be completely inhibited by protease inhibitors such as alpha 1-protease inhibitor.[6]

We have also examined the ability of leukocytes from the lungs of rats chronically inhaling coalmine dust to mediate injury. This showed that rats exposed, by inhalation, for 48 days to coalmine dust collected from the air of a British colliery[5] also caused epithelial injury and degradation of fibronectin (Figure 5).

Cellular Origin of Epithelial Cell Detaching Injury in Quartz-Elicited Bronchoalveolar Leukocyte Populations

As shown above, high proportions of neutrophils seem to accompany fibronectin-degrading and epithelial-injuring activity in the inflammatory leukocyte populations which we have examined. To determine whether the macrophages could also be producing proteolyic activity against fibro-

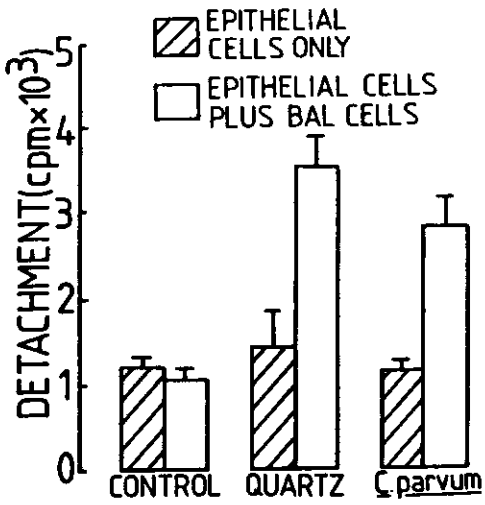

Figure 4. Detachment injury caused to alveolar epithelial cells *in vitro* by co-culture with control, quartz or *C. parvum* elicited bronchoalveolar leukocytes. All data given as mean + SEM of triplicate cells in 3 separate experiments. Significantly increased detachment caused by quartz and *C. parvum* treatment ($p<0.001$).

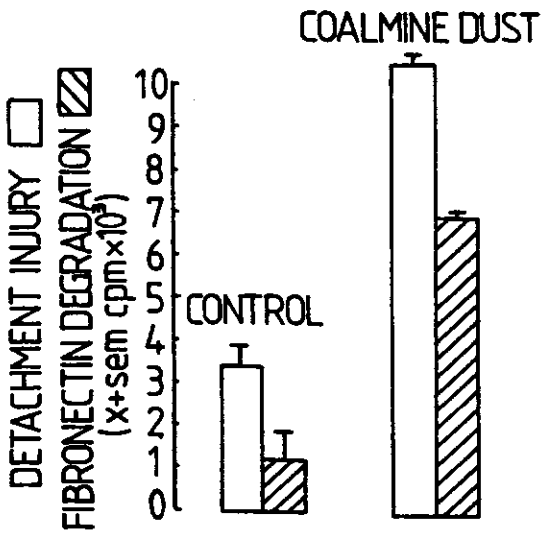

Figure 5. Detachment injury and fibronectin degradation caused by control bronchoalveolar leukocytes and bronchoalveolar leukocytes from rats inhaling coalmine dust for 45 days. Significant increases ($P<0.001$) in both parameters with coalmine dust exposed bronchoalveolar leukocytes compared to controls.

nectin, and causing detachment injury, the 5 day quartz bronchoalveolar leukocytes were separated into macrophage-enriched and neutrophil-enriched populations. These were then tested for their ability to cause epithelial cell detachment injury. Figure 6 demonstrates that separation of the mixed population into the enriched populations resulted in very high levels of epithelial injury being caused by the neutrophil-enriched fraction. However, despite the macrophage-enriched fraction containing only 5% PMNs, this population caused 5-fold more detachment injury than control alveolar macrophages.

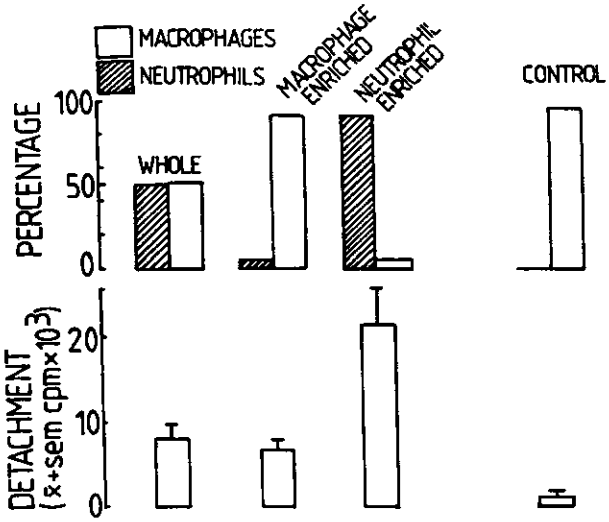

Figure 6. Cellular composition (upper panel) of, and detachment injury (lower panel) caused by, whole quartz-elicited bronchoalveolar leukocytes and both macrophage-enriched and neutrophil-enriched fractions obtained from it. Proportions of neutrophils and macrophages shown as mean percentage. Detachment injury shown as mean + SEM of cpm in detached cells.

DISCUSSION

This study has shown that a single injection of silica into the rat lung causes a long-term alveolitis. The alveolitis is characterized by a 3–12 fold increase in bronchoalveolar leukocytes comprising 30–40% neutrophils. Intratracheal instillation of a heat-killed bacterial preparation (*C. parvum*) or yeast cell walls (zymosan) also caused large scale burst of inflammation immediately following injection but these resolved quickly, returning to near normal levels by 15 days. Thus the initial severity of the alveolitis is not the main factor determining the persistence of silicotic inflammation in the intratracheal model.

The exact events which engender persistent inflammation with silica are speculative but cytoxicity of quartz towards alveolar macrophages might be central. The consequence of silica-induced alveolitis is likely to be fibrosis since the ability of inflammatory leukocytes to mediate further damage and pathological change in the lung is well established for a range of aetiologic agents.[4] In an attempt to understand which leukocyte-derived injurious factors might be important in the development of quartz-related pathology we examined the ability of the leukocytes from quartz-exposed lung to break down fibronectin. During the acute inflammation engendered

by *C. parvum* and zymosan there were high levels of proteolyic activity present; the levels of protease however returned to normal within 15 days. An examination of the proteolytic activity of quartz-elicited leukocytes showed that this proteolytic activity, capable of breaking down fibronectin and other connective tissue elements[7] and so generating chemotaxin[10] and causing epithelial injury and basement membrane damage,[6] was 4, present persistently, and in increased quantities, for up to 1 month following quartz instillation; previous studies suggest that this inflammation and hence the increased protease burden persist for up to 3 months and possibly longer. The total proteolyic burden of the lung is not reflected adequately as the increase, on a per cell basis, in dust-elicited bronchoalveolar leukocytes since the total number of leukocytes is also increased. If the increase in cell numbers is taken into consideration (a 16-fold increase on day 30) this produces a greater than 30-fold increase in the total protease burden of the lung following silica exposure for 30 days. Although the present study has utilized intratracheal instillation we have found that inhalation exposure to a pneumoconiotic dust (coalmine dust containing quartz) also caused an alveolitis producing greatly enhanced lung burdens of fibronectin-degrading activity.[11]

The ability of inflammatory bronchoalveolar lavage leukocytes to injure epithelial cells correlates with proteolyic activity[6] and so we examined this aspect of injury production by quartz bronchoalveolar leukocytes. The quartz bronchoalveolar leukocytes caused detachment injury which appeared to be mediated by both macrophages and neutrophils as shown by separation studies where the different leukocyte types were obtained in enriched form. It is therefore possible to conclude that the bronchoalveolar macrophages from quartz-exposed lung are activated with regard to proteolyic activity. The time course studies with *C. parvum* revealed a modest macrophage alveolitis present beyond 15 days but this population was not activated with regard to protease production. The fact that the cell numbers were increased compared to controls argues for the fact that this did indeed represent an inflammatory population albeit one which was not characterized by increases in neutrophils. It is possible therefore that only inflammatory macrophages from mixed populations, where neutrophils are also present, show increased proteolytic activity. A likely explanation for this is that the alveolar macrophages from such populations have internalized neutrophil elastase as has been previously reported.[12] It was noteable that the neutrophil-enriched fraction had twice the proportion of neutrophils found in the whole population but produced a 3-fold increase in detaching activity. This suggests that either the separation procedure caused activation of neutrophils or that macrophages suppressed neutrophil proteolytic activity in the mixed population.

This study has shown that a single deposition of 1 mg of quartz in the rat lung causes a prolonged and intense alveolitis characterized by increased proteolytic activity of bronchoalveolar leukocytes, capable of causing injury to the epithelial and matrix elements of the alveolar septum. The results strongly suggest that leukocytes from rats exposed by inhalation to pneumoconiosis-producing dust also have these properties and that both macrophages and neutrophils express this injurious proteolytic activity, although in the case of macrophages this may be due to sequestered neutrophil elastase.

REFERENCES

1. Gibbs, A.R., Seal, R.M.E., Wagner, J.C.: Pathological reactions of the lung to dust. *Occupational Lung Diseases* pp. 129-162. W.K.C. Morgan and A. Seaton, Eds. W.B.Saunders Co., Philadelphia (1984).
2. Donaldson, K., Bolton, R.E., Jones, A., Brown, G.M., Robertson, M.D., Slight, J., Cowie, A.H., Davis, J.M.G.: Kinetics of the bronchoalveolar leukocyte response in rats following exposure to equal airborne mass concentration of quartz, chrysotile asbestos and titanium dioxide. *Thorax* 43:525-533 (1988).
3. Begin, R., Bisson, G., Boileau, R., Masse, S.: Assessment of disease activity by Gallium 67 scan and lung lavage in the pneumoconioses. *Sem. Resp. Med.* 7:275-280 (1986).
4. Fantone, J.C., Ward, P.: Mechanisms of lung parenchymal injury. *Am. Rev. Resp. Dis.* 130:484-491 (1984).
5. Donaldson, K., Bolton, R.E., Brown, D.M., Brown, G.M., Cowie, A.H., Jones, A.D., Robertson, M.D., Slight, J., Davis, J.M.G.: Studies on the cellular response in lung tissue to the inhalation of mineral dust. Institute of Occupational Medicine Report No. TM/88/01 (1988).
6. Donaldson, K., Slight, J., Brown, G.M., Bolton, R.E.: The ability of inflammatory bronchoalveolar leukocyte populations elicited with microbes or mineral dust to injure alveolar epithelial cells and degrade extracellular matrix in vitro. *Br. J. Exp. Path.* 69:327-338 (1988).
7. Brown, G.M., Donaldson, K.: The degradation of connective tissue components by lung-derived leukocytes in vitro: the role of proteases and oxidant. *Thorax* 43:132-139 (1988).
8. Brown, G.M., Donaldson, K., Brown, D.M., Seaton, A.: Bronchoalveolar leukocyte response in experimental silicosis and its modulation by a soluble silica compound. *Thorax* 43:262 (1988).
9. Vaes, G.: *Developments in Cell Biology 1 Secretory Processes.* pp. 99-117. R.T. Dean and P. Stahl, Eds. Butterworth, London (1985). Macrophage secretory products and connective tissue remodelling: role of macrophage enzymes and of "matrix regulatory monokines."
10. Kunimoto, M., Jay, M.: Elastin fragment-induced monocyte chemotaxis. The role of desmosines. *Inflammation* 9:183-188 (1985).
11. Brown, G.M., Donaldson, K.: Inflammatory responses in lungs of rats inhaling coalmine dust: enhanced proteolysis of fibronectin by bronchoalveolar leukocytes. (Submitted for publication 1988 to Env. Res.)
12. Campbell, E.J.: Human leukocyte elastase, cathespin G and lactoferrin: family of neutrophil granule glycoproteins that bind to an alveolar macrophage receptor. *Proceedings of the National Academy of Science USA.* 79:6941-6945 (1982).

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THE EFFECT OF TACHYKININ DEPLETION ON HYDROGEN SULPHIDE TOXICITY IN RATS

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INTRODUCTION

Hydrogen sulphide (H_2S) toxicity is one of the leading causes of sudden death in the work place. Hydrogen sulphide occurs naturally in coal, oil and natural gas deposits, and is also produced by anaerobic decomposition of sulphur containing organic matter. it is used extensively in industry and more than 70 occupations are potentially exposed to H_2S.[9] The problem of H_2S toxicity is particularly acute in Alberta; approximately one in six gas wells emit sour (H_2S) gas, and the major emphasis of sulphur containing energy resources in the province has increased the risk of exposure for workers in the petro-chemical industries and the general public.[5]

H_2S is both an irritant and asphyxiant gas which exerts its primary toxic effects on the respiratory and neurologic systems.[4] Fatal cases almost invariably exhibit fulminant hermorrhagic pulmonary edema as well as cerebral edema and severe damage to the conjunctiva, olfactory nasal mucosa and upper respiratory tract.[1,5] Similar findings are observed in experimental animals.[4,14,15,16]

The mechanism for H_2S induced pulmonary edema is not understood. H_2S induced paralysis of the respiratory control centre and/or stimulation of carotid body receptors, may be involved.[4,3,9] There is also evidence that H_2S is directly toxic to the lungs. H_2S is only moderately soluble, and is able to penetrate to the lung periphery. Injury to the alveolar/capillary membrane would result in increased vascular permeability and edema.[10] The high protein and cellular content in the alveolar fluid in experimental H_2S exposure support the latter possibility.[14] A direct toxic effect on the respiratory system is also indicated by the observation that pulmonary edema occurs at exposure levels below those associated with severe central nervous system depression.

A further possible mechanism for H_2S induced pulmonary edema might involve stimulation and release of vasoactive neuropeptides from vagal nerve fibres. Unmyelinated postganglionic nerves of the C-fibre group contained in the vagus nerve, are important mediators of neurogenic inflammatory responses in the lung. This response is mediated by a specific neurotransmitter known as substance P which, in part, is responsible for increased vascular permeability and edema occurring during the acute stages of lung inflammation.[18,19,11,6] In addition to modulating vascular permeability in the respiratory tract, substance P is a potent constrictor of bronchial smooth muscle, stimulates mucociliary activity and promotes mucous secretion in the airways.[23,26,28] Immunohistochemical studies have revealed a rich plexus of substance P containing nerve fibres within and beneath airway epithelium, and around blood vessels and seromucous glands.[20,21] Capsaicin, the main pungent ingredient of hot peppers, is a vanilly/amide derivative that produces selective depletion of tachykinins, including substance P, in C-afferent fibres.[12]

In view of the important role of substance P in airway inflammatory responses, we decided to study the effects of hydrogen sulphide in animals previously depleted of substance P. This report will focus on the pathophysiology of the airway lesions. The vascular and edemogenic component will be published in detail elsewhere. In addition, the histological changes induced in the lungs of animals exposed to H_2S were compared with those observed in the lungs of human cases of fatal hydrogen sulphide exposure.

REVIEW OF WORKPLACE EXPOSURES IN ALBERTA, 1977–1986

One hundred and sixty two lost-time workman's compensation cases were recorded in Alberta in the decade 1977–1986.[2] The majority of these (68%) involved exposures in the oil and gas industry. 79% were aged 34 or less. There were 21 fatalities; most of these occurred in facilities where the dangers were known and protective equipment was available. Eight fatalities were a direct result of failure to follow correct safety practices. 87% of all workers exposed to H_2S developed respiratory system symptoms. In all cases where H_2S intoxication was the primary cause of death, autopsy revealed pulmonary edema. (Figure 1)

ANIMAL STUDIES

Materials and Methods

Thirty-six male, Fischer-344 (CDF24Cr1BR), eight week old rats (Charles River, Inc., Quebec) were obtained for this study and acclimatized for ten days under carefully controlled conditions.[24] The guidelines provided by the Canadian Council of Animal Care, were followed throughout all phases of the study.[7] At the time of exposure, the rats weighed

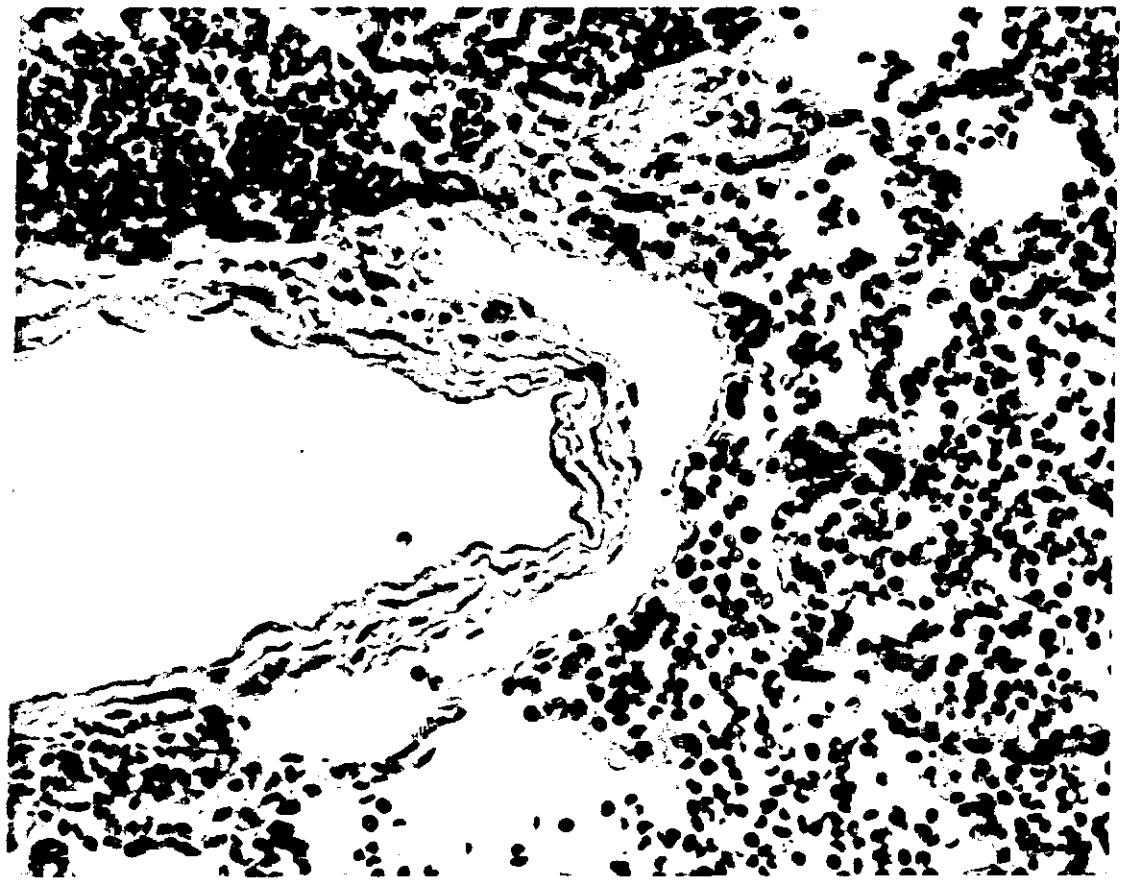


Figure 1. Microscopic appearance of lungs from human fatal case of acute H_2S intoxication. A. The alveoli are flooded with hemorrhagic edema fluid. Polymorphonuclear cells are noted in the alveoli and marginating along vessels in the alveolar interstitium. (Hematoxylin and eosin x 150)

138.4±4.8 gms. Rats were assigned to one of four different groups using a randomized model (Table I). Within one hour of termination of exposure, rats were anesthetized with Halothane (5%) and exsanguinated by incising the abdominal aorta.

Depletion of Substance P

Capsaicin (Rotichrome[R] Carl Roth) was dissolved in 10% alcohol and 10% tween 80. In the treated group, capsaicin was administered to a total dose of 150 mg/kg subcutaneously, in eight divided doses over a period of two days. The acute effects of capsaicin were reduced by pretreatment with aminophylline 10 mg/kg IP. Rats of the control group received physiologic saline and aminophylline.

Hydrogen Sulphide Exposures

Fourteen days after the last injection of capsaicin or saline, rats were divided into four groups and exposed for four consecutive hours to either air or H_2S. The concentration of gas in the two H_2S chambers is shown in Table I. The H_2S exposure system has been described in detail elsewhere.[24] A schematic diagram of the exposure system is shown in Figure 2. The chamber atmosphere was sampled every two minutes and analyzed by gas chromatography (Model 5790A, Hewlett Packard[R]).

Bronchoalveolar Lavage and Protein Determination

The left lung was cannulated and three consecutive bronchoalveolar lavages performed. The protein concentration (g/l) in the lavage fluid supernatant was determined using methods previously reported.[14]

Light and Electron Microscopy

The tracheas of subgroups of rats were cannulated and the lungs inflated with 2.5% glutaraldehyde (320 M Osmol) at a constant pressure of 20 cm of water for 30 minutes in situ. They were then removed from the thoracic cavity and allowed to fix for twenty-four hours. Following fixation, blocks were processed for routine light microscopy and 5 μ sections were mounted on glass slides and stained with hematoxylin-eosin.[17]

For scanning electron microscopy and morphometry, the intrapulmonary portion of the left main bronchus was excised with adjacent lung and dehydrated in graded concentrations

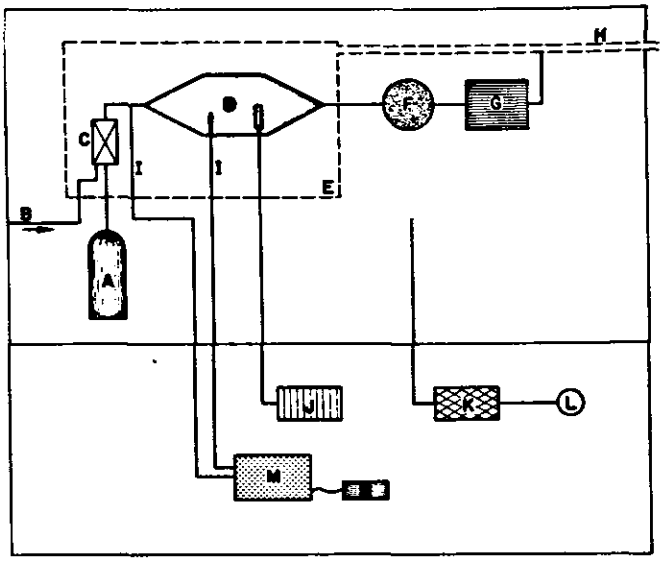
Figure 1. Microscopic appearance of lungs from human fatal case of acute H_2S intoxication. B. Section of main bronchus showing ciliated epithelial cell exfoliation and mucosal edema. (Hematoxylin and eosin x 400)

Table I
Experimental Design

Treatment	Hydrogen sulphide (mg m^3)		
	0	H_2S (1)	H_2S (2)
Capsaicin	6*	6*	6**
Saline	6*	6*	6**

Total number of rats = 36

(1) actual concentration 559 ± 144 mg m^3
(2) actual concentration 525 ± 87 mg m^3
* for bronchoalveolar lavage
** for histopathology



LEGEND

A - GAS CYLINDER
B - AIR
C - FLOW CONTROLLERS
D - EXPOSURE CHAMBER
E - FUME HOOD
F - VACUUM PUMP
G - SCRUBBER
H - EXHAUST TO OUTSIDE
I - SAMPLE LINE
J - RELATIVE HUMIDITY & TEMPERATURE MONITOR
K - H_2S MONITOR
L - ALARM
M - GAS CHROMATOGRAPH
N - COMPUTER

Figure 2. Diagram of the H_2S exposure system.
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of ethyl alcohol, critical point dried and coated with gold/palladium. The specimens were examined in a Hitachi S-450 scanning electron microscope. Five approximately equally spaced photographs were obtained of the proximal 3 mm of the left intrapulmonary bronchus at 1000 x magnification and constant working distance. The density of ciliated and nonciliated epithelial cells, expressed as a percent of the total area, was determined for each photograph using a Dapple[R] image analysis system.

Detection of Substance P

Substance P was detected in tissues using an indirect immunofluoresence technique[27] and examined using a Reichert-Jung Polyvar microscope, equipped with filter system Bl (excitation wavelength 450-495 nm).

Statistical Analysis

The effect of capsaicin and/or H_2S on mortality was tested by the Fischer Exact Test. The effects of capsaicin and H_2S on airway epithelial cells was tested by analysis of variance.[25]

RESULTS

Sections of lung and trachea stained by immunofluorescence for substance P, showed staining of nerve fibres in the mucosa of the trachea and within the walls of small airways and around blood vessels in the lung.The density of fibres was greatest in the trachea and least in the peripheral lung. Animals treated with capsaicin showed almost complete depletion of substance P containing nerve fibres.

Animals pretreated with capsaicin showed normal weight gain and exhibited no signs of toxicity or mortality prior to exposure. Exposure to H_2S for four hours, produced 100% mortality in the capsaicin treated animals and 20% mortality in the saline treated controls. (Table II) At postmortem examination, frothy blood-stained fluid was noted to exit from the mouths and noses of all animals dying from hydrogen sulphide exposure. The lungs of these animals were deeply congested and failed to collapse when the thorax was opened. Histological examination of the affected lungs revealed large quantities of hemorrhagic and highly proteinaceous fluid within the alveolar spaces. Edema fluid was also noted in perivascular and interstitial locations.

Animals exposed to H_2S alone, showed significantly more protein in bronchoalveolar lavage fluid than was seen in air exposed saline or capsaicin pretreated animals. This effect was even greater in animals pretreated with capsaicin and then exposed to H_2S. Animals pretreated with saline and exposed to H_2S, showed a significant increase in lung wet weights. This effect was even greater in animals pretreated with capsaicin.

Table II

Mortality (%), and Substance P in Rats Pretreated with Saline Solution or Capsaicin and Exposed to Hydrogen Sulphide

Variable	Air		H_2S	
	Saline	Capsaicin	Saline	Capsaicin
Substance P	+	-		
Mortality (%)	0	0	20	100*

* $P<0.05$

Examination of large and small conducting airways by light and scanning electron microscopy revealed evidence of severe mucosal damage following H_2S exposure. The changes were most marked proximally and occasional areas of ulceration were noted in the mucosa of the trachea but not in the major bronchi. (Figure 3) The primary finding in the major conducting airways was wide-spread exfoliation of epithelial cells with lateral spreading of basal and intermediate cells. (Figure 4) The bronchioles were relatively spared of toxic effects. The effects noted above were much more severe in animals pretreated with capsaicin and subsequently exposed to H_2S. (Figure 5) The results of morphometric eval-

Figure 3. Scanning electron micrograph of area of ulceration in trachea from saline pretreated rats exposed to H_2S for 4 hours. (x 2000)

uation of the extent of loss of ciliated epithelial cells in the left main bronchus, are shown in Figure 6. There is evidence of an additive effect attributable to capsaicin alone p=0.0003 and an additive effect attributable to hydrogen sulphide p=0.003. There was no evidence that the effect of capsaicin depended on the presence or absence of hydrogen sulphide p=0.61.

Figure 4. Scanning electron micrograph of left main bronchus from saline pretreated rat exposed to 559 mg M^3 H_2S for 4 hours. There is exfoliation of ciliated epithelial cells with lateral spreading of basal and intermediate cells. (x 2000)

DISCUSSION

These experiments confirm the previously reported findings concerning the toxic effects of H_2S on the lungs. Animals exposed to H_2S without capsaicin pretreatment, had a 20% mortality at an average concentration of 542 mg M^3. These results are similar to those previously reported from this laboratory with this strain of rat where the LC50 and LC10 values for four hours of exposure were 701 and 591 mg per m^3, respectively.[24] The exposure concentration was selected such that there would not be significant mortality in normal

Figure 5. Scanning electron micrograph of left main bronchus from: (A) air-exposed, saline pretreated rat. Approximately 50% of the mucosal surface is ciliated.

rats. Animals pretreated with capsaicin and exposed to the same concentration of hydrogen sulphide, showed 100% mortality. In addition, the animals died at an earlier time during exposure than animals pretreated with saline and then exposed to H_2S. Animals pretreated with capsaicin and subsequently exposed to H_2S, also had more severe pulmonary edema with greater concentrations of protein in the lavage fluid and heavier lungs postmortem. Although to

Figure 5. Scanning electron micrograph of left main bronchus from: (B) saline pretreated H_2S exposed rat showing loss of ciliated epithelium.

some extent these changes could reflect transudation of fluid into the lungs postmortem, we consider this unlikely in view of the magnitude of the effect.

The airway lesions noted in the H_2S exposed rats are similar to those reported for sulphur dioxide.[22] The lesions were most severe in the proximal airways with relative sparing

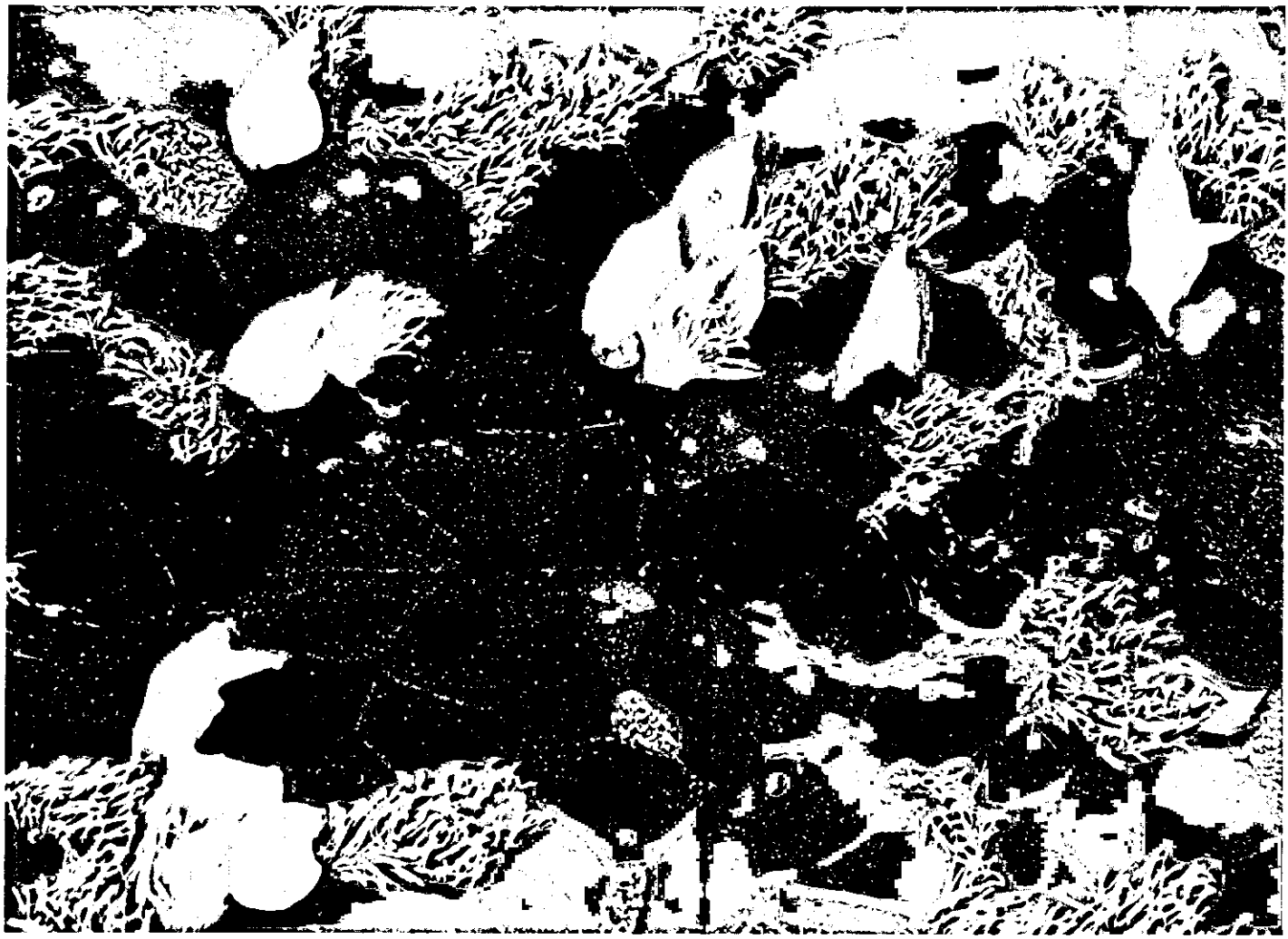

Figure 5. Scanning electron micrograph of left main bronchus from: (C) capsaicin pretreated H_2S exposed rat showing greater loss than is seen in B. (x 1000)

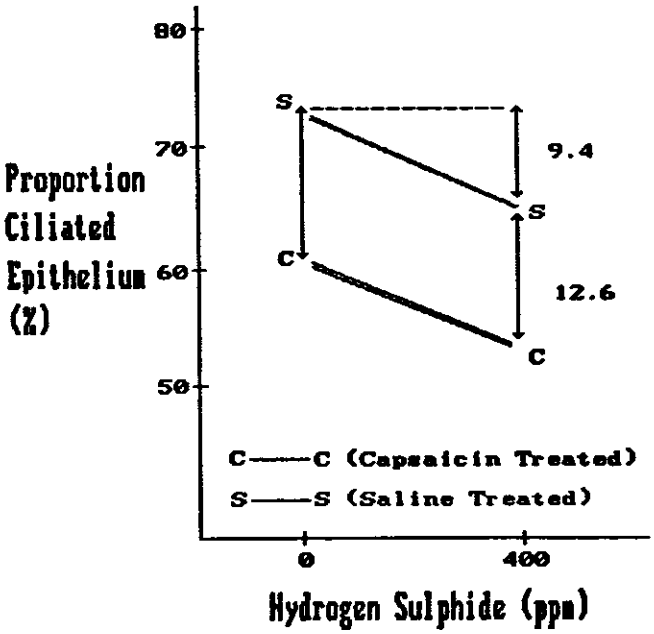

Figure 6. Proportion of airway mucosa occupied by ciliated epithelium by group. H_2S exposure alone and capsaicin pretreatment alone result in loss of ciliated epithelium. Capsaicin plus H_2S has an additive effect.

of the bronchioles and alveolar ducts. Previous studies have shown that the respiratory and olfactory epithelial cells of the nasal mucosa are also very sensitive to H_2S induced injury.[15] This pattern of injury is consistent with the moderate solubility of H_2S in water. Scrubbing of H_2S (as H_2SO_3) in the nasal passages and upper airways should result in maximal concentrations of gas proximally with greatest injury to this site.

The mechanism of irritant and oxidant gas injury of the upper airway has been extensively studied. (reviewed in 22) Ciliated epithelial cells appear to be more susceptible to injury than non-ciliated cells. This is true for SO_2, O_3 and NO_2[22,13] and based on this study, appears to be also true for H_2S induced injury. Lipid peroxidation is probably the primary biochemical mechanism for cell injury due to oxidant gases. The mechanism of cellular toxicity due to H_2S is likely to be different. H_2S is toxic to a number of cellular systems and biochemical pathways,[3,4] however the ability of H_2S to react with metal ion-containing proteins is probably of primary importance. H_2S is able to reduce one of the hemes of the intracellular mitochondrial enzyme cytochrome C oxidase, thus interfering with oxidative metabolism. (reviewed in 3) H_2S is reported to be a more potent inhibitor of cytochrome oxidase than hydrogen cyanide.[8] H_2S also interacts with succinic dehydrogenase, catalase and peroxidase and these interactions may also be important in promoting epithelial injury.

Injury of airway epithelium results in cytoskeletal abnormalities and disruption of the tight junctions between the epithelial cells. This leads to an increase in paracellular permeability and exfoliation of the ciliated cells. (reviewed in 22) The repair process is initiated immediately as the remaining viable non-ciliated epithelial cells spread laterally to maintain the epithelial barrier. The data presented in this paper indicate that a similar sequence of events occurs following H_2S injury.

An unexpected finding in this study was the demonstration of a potentiating effect of neuropeptide depletion on H_2S induced airway injury. Thompson *et al.*[29] have demonstrated that airway responsiveness to toluene diisocyanate in guinea pigs is mediated by capsaicin sensitive afferent nerves. These findings indicate a role for tachykinins in pulmonary defense mechanisms against inhaled toxic agents and in the maintenance of structural integrity of the airway mucosa. Studies are underway to fully characterize this phenomenon and elucidate the mechanisms.

REFERENCES

1. Adelson, L., Sunshine I.: Fatal hydrogen sulfide intoxication. *Arch. Pathol.* 81:375-380 (1966).
2. Alberta Workers' Compensation Board Statistical Master File, 1977-1986.
3. Amman, H.M.: A new look at physiologic respiratory response to H_2S poisoning. *J Hazardous Materials.* 13:369-374 (1986).
4. Beauchamp, R.O., Bus, J.S., Popp, J.A., Boreiko, C.J., Andjelkovich, D.A.: A critical review of the literature in hydrogen sulfide toxicity. *CRC Crit. Rev. Toxicol.* 13:25-97 (1984).
5. Burnett, W.W., King, E.G., Grance, M., Hall, W.F.: Hydrogen sulfide poisoning: A review of 5 years experience. *Can. Med. Assoc. J.* 177:1277-1280 (1977).
6. Bayliss, W.M.: On the origin of the spinal cord of the vaso-dilator fibres of the hind-limb, and on the nature of these fibres. *J. Physiol.* 26:173-209 (1901).
7. Canadian Council of Animal Care (CCAC): *Guide to Care and Use of Experimental Animals.* Ottawa, Ontario, Canada (1980).
8. Chance, B., Schoener, B.: High and low energy states of cytochromes. I In mitochondria. *J. Bio. Chem.* 241:4567-4573 (1966).
9. Frank, R.: Acute and chronic respiratory effects of exposure to inhaled toxic agents. In *Occupational Respiratory Diseases.* Ed. Merchant, J.A. et al. DHHS (NIOSH) Publication No. 86-102, pp 583-585 (1986).
10. Hurley, J.V.: Types of pulmonary microvascular injury. *Ann. NY Acad. Sci.* 384:269-286 (1982).
11. Jancso, N., Jancso-Gabor, A., Szolcsaryi, J.: Direct evidence for neurogenic inflammation and its prevention by denervation and by pretreatment with capsaicin. *Brit. J. Chemother.* 31:138-151 (1967).
12. Jansco, G., Kirally, E., Jansco-Gabor, A.: Pharmacologically induced selective degeneration of chemosensitive primary sensory neurones. *Nature* 170:741-743 (1977).
13. Kleinerman, J.: Effects of nitrogen dioxide on elastin and collagen contents of lung. *Archs. Envir. Hlth.* 34:228-232 (1979).
14. Lopez, A., Prior, M., Yang, S., Albassam, M., Lillie, L.E.: Biochemical and cytologic alterations in the respiratory tract of rats exposed for four hours to hydrogen sulfide. *Fundam. Appl. Toxicol.* 9:753-762 (1987).
15. Lopez, A., Prior, M., Yong, S., Lillie, L., Lefebvre, M.: Nasal lesions in rats exposed to hydrogen sulphide for four hours. *Am. J. Vet. Res.* 49:1107-1111 (1988).
16. Lopez, A., Prior, M., Lillie, L.E., Gulayets, C., Atwal, O.S.: Histologic and ultrastructural alterations in the lungs of rats exposed to sublethal concentrations of hydrogen sulfide. *Vet. Pathol.* 25: (1988). In press.
17. Luna, L.G.: *Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology.* McGraw Hill, New York (1968).

18. Lundberg, J.M., Saria, A., Brodin, E., Rosell, S., Folkers, K.: A substance P antagonist inhibits vagally induced increase in vascular permeability and bronchial smooth muscle contraction in the guinea pig. *Proc. Natl. Acad. Sci.* 80:1120-1124 (1983).
19. Lundberg, J.M., Sara, A.: Capsaicin-induced desensitization of airway mucosa to cigarette smoke, mechanical and chemical irritants. *Nature* 302:251-253 (1983).
20. Lundberg, J.M., Brodin, E., Saria, A.: Effects and distribution of vagal capsaicin-sensitive substance P neurons with special reference to the trachea and lungs. *Acta. Physiol. Scand.* 119:243-252 (1983).
21. Lundberg, J.M., Hokfelt, T., Martling, C.R., Saria, A., Cuello, C.: Substance P-immunoreactive sensory nerves in the lower respiratory tract of various mammals including man. *Cell Tissue Res.* 235:251-256 (1984).
22. Man, S.F.P., Hulbert, W.C.: Airway repair and adaption to inhalation injury. In: *Pathophysiology and Treatment of Inhalation Injuries.* Ed. Loke, J. Marcel Dekker, Inc. New York. pp. 1-47 (1988).
23. McDonald, D.M.: Neurogenic inflammation in the respiratory tract: Actions of sensory nerve mediators on blood vessels and epithelium of the airway mucosa. *Am. Rev. Resp. Dis.* 136:565-571 (1987).
24. Prior, M.G., Yong, S., Sharma, A., Lopez, A.: Concentration-time interactions in hydrogen sulphide toxicity in rats. *Canad. J. Vet. Res.* 54:375-379 (1988).
25. Raktoe, V.L., Hubbert, J.J.: *Basic Applied Statistics.* Dekker, New York (1979).
26. Richardson, P.S. Webber, S.E.: The control of mucous secretion in the airways by peptidergic mechanisms. *Am. Rev. Resp. Dis.* 136:572-576 (1987).
27. Sharkey, K.A., Sobrino, J.A., Cervero, F.: Evidence for a visceral afferent origin of substance P-like immunoreactivity in Lamina V of the rat thoracic spinal cord. *Neuroscience.* 22:1077-1083 (1987).
28. Said, I.: Influence of neuropeptides on airway smooth muscle. *Am. Rev. Res. Dis.* 136:S52-S58 (1987).
29. Thompson, J.E., Scypinski, L.A., Gordon, T., Sheppard, D.: Tachykinins mediate the acute increase in airway responsiveness caused by toluene diisocyanate in guinea pigs. *Am. Rev. Resp. Dis.* 136:43-49 (1987).

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