STUDY ON SUPPRESSION OF SOLUBLE ALUMINUM AEROSOLS ON QUARTZ-INDUCED CYTOTOXICITY
—Combined Effects of Both Aerosols in an Artificial Dust Atmosphere

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INTRODUCTION

Since 1930's it has been noted that aluminum (Al) and its compounds could prevent and treat silicosis. A number of studies have shown that Al could resist the cytotoxicity of SiO_2 particles on macrophages and erythrocytes, and suppress or lessen quartz-induced pulmonary fibrosis.[1-4,6,9,12,18] More recently, it has been found that the mechanism of the effects of Al was the combination of it with SiO_2 particles, which altered some properties of the particle surface, so leading to the decrease of pathogenic activity of SiO_2.[4,18] And the charges on SiO_2 particles were greatly reduced after the mineral particles were combined with Al, which was quite valuable to aggregation and dropping SiO_2 dusts. Consequently, if Al is used in dust workplace, such as spraying aqueous solution of aluminum compound or adding soluble aluminum into the water for dropping dusts from which the soluble aluminum aerosols are generated and probably combined with the SiO_2 dusts in air and/or inside lungs before or after the dusts are inspired into the worker's respiratory passage, it will give play to the dual effects on both resistance against the pathogenicity of SiO_2 and dripping the SiO_2 dusts in working atmosphere. It is possible to explore a new way to treat dusts in our industries and mines to prevent the development of silicosis.

The present study made an attempt to demonstrate whether or not the soluble aluminum aerosols could combine with SiO_2 particles in the atmosphere, and if they could suppress or lessen the cytotoxicity of the minerals on guinea pig alveolar macrophages and rat erythrocytes.

MATERIALS AND METHODS

Development of a Dynamic Inhalation Exposure System and Collection of SiO_2 Particles Combine with AlAs or DWAs

A dynamic inhalation exposure system was designed and developed to simulate an atmosphere interacting of both aerosols—SiO_2 particles and soluble aluminum aerosols (AlAs) or deionized water aerosols (DWAs). SiO_2 dusts generated by a dust generator (modified F-710 Electromagnetic feeder made in China Jiujian) were conveyed into the exposure chamber (made of polymethyl methacrylate, 0.8M^3 in volume) of the system to form the average concentration of 200mg SiO_2/M^3. AlAs or DWAs, on the other hand, nebulized by an ultrasonic nebulizer (JWC-2A Transistor Ultrasonic Nebulizer made in China Anshan, 0.5–10μm in aerosol diameter) were sprayed into the chamber from another inlet. In the exposure chamber both aerosols interacted on each other, and SiO_2 particles combined with and/or without AlAs or DWAs were sampled on a microfilter (0.45 μm in pore size, made in China Beijing) by WY-1 cascade impactor (7 catch stages, made in Chinese Academy of Preventive Medicine),[11] on the basis of which following experiments were done.

Measurement of Aluminum Combined on SiO_2 Particles

The contents of Al and SiO_2 in the sample on the microfilter were directly analyzed and measured by thin film X-ray fluorescence (XRF), based on modification of method of Cui et al.,[7] with PW-1400 X-Ray Fluorescence Meter (made in Philip Inc.) from which the amounts of Al combined on SiO_2 particles were calculated and expressed in μg Al per cm^2 specific area of SiO_2, i.e., μgAl/cm^2 SiO_2. The specific area was calculated from the diameter and density of SiO_2 particles.[16]

Cytotoxicity Examination of the SiO_2 Particles Combined with AlAs

Using hemolysis assay and macrophage viability test monitored in vitro the cytotoxicity of AlAs—or DWAs-combined SiO_2 particle samples collected from the 5th stage microfilter of the Impactor, represented by antihemolysis rate (AHR) and macrophage viability index (MVI). The former was based on the hemolysis assay system previously described by Hefner et al.,[10] Briefly, whole blood was taken from rat aorta abdominalis, washed 3 times in normal salt (NS) by centrifugation at 1500 rpm for 10 min., and the pellets, all of which are almost erythrocytes (RBC), were resuspended in NS to 20% (v/v). 1 mg SiO_2 or SiO_2-Al sample was added to 3 ml or the 2% RBC suspension, and the mixture incubated in 37°C water bath for 30 min and gently shook every 5 min. At the end of incubation, with centrifugating the mixtures the supernatants was measured for optical density (OD) at 420nm wavelength. Antihemolysis rate (AHR) was calculated from below formula:

$$\frac{(\text{HR of } SiO_2\text{-}H_2O) - (\text{HR of } SiO_2\text{-Al})}{\text{HR of } SiO_2\text{-}H_2O} \times 100\%$$

where HR (hemolysis rate)

$$= \frac{\text{OD of } SiO_2\text{-Al group}}{\text{OD of whole hemolysis group}} \times 100\%$$

MVI is complex indicators of damage effects of SiO_2 particles on macrophages, including rate of macrophage viability (RMV), intracellular K^+ contents and lactate dehydrogenase (LDH) activity in the conditioned medium of macrophages cultured with the SiO_2 samples. Alveolar macrophages (7×10^6/ml) harvested from male guinea pig lungs with bronchopulmonary lavage as described earlier[9] were incubated with 150µg/mg SiO_2 (SiO_2-Al or SiO_2-H_2O samples) or normal salt (NS, as negative control) in medium RPMI1640 at 37°C in 5% CO_2-95% air for 4 hours, RMV(%) was assayed by trypan blue exclusion test, intracellular K^+ contents of macrophages by Flame Atomic Absorption Spectroscopy and LDH activity in the conditioned medium by a colorimetric process.[14,18] According to these parameters above, MVI was obtained from the formula:

$$\left(\frac{\text{RMV in } SiO_2\text{-Al group}}{\text{RMV in NS group}} + \frac{K^+ \text{ in } SiO_2\text{-Al group}}{K^+ \text{ in NS group}} \frac{\text{LDH in NS group}}{\text{LDH in } SiO_2\text{-Al group}}\right) \times 300\%$$

and the rate of AlAs-suppressed toxicity of SiO_2 dusts to macrophages (RAST) was calculated from:

$$\frac{(\text{MVI in } SiO_2\text{-Al}) - (\text{MVI in } SiO_2\text{-}H_2O)}{(\text{MVI in NS}) - (\text{MVI in } SiO_2\text{-}H_2O)} \times 100\%.$$

RESULTS AND DISCUSSIONS

Combination of Soluble Aluminum Aerosols with SiO_2 Particles

1) *The amounts of Al combined on SiO_2 particles at nebulization with various aluminum compounds.* Four soluble aluminum compounds, Al-I, Al-II, Al-III and Al-IV (synthesized and supplied by the Beijing Medical University School of Pharmacy) were prepared to 0.5mgAl/mg solution in deionized water. Experiments were respectively done with each of them under the consistent conditions such as nebulizing, sampling and measuring described above. As shown in Figure 1, all but Al-I aqueous solution were found to significantly increase the amounts of Al combined on

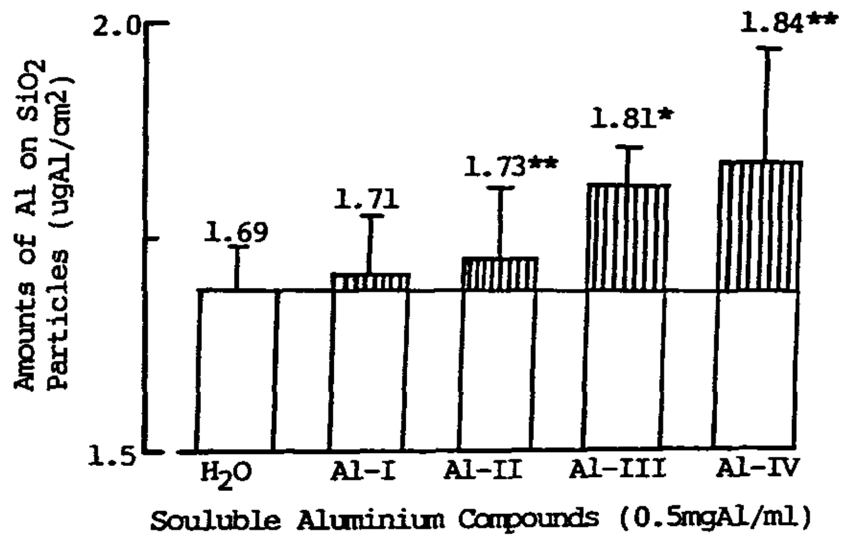

Figure 1. The amounts of Al combined on SiO_2 particles at nebulization using four soluble aluminum compounds (Al-I, Al-II, Al-III and Al-IV aqueous solution) and deionized water (H_2O). Mass Mean Aerodynamic Diameter (MMAD) of SiO_2 particles in these samples was 5.14µm. *$P<0.01$; **$P<0.05$ t-test with H_2O group; expresses the net amounts of Al combined on SiO_2 particles.

SiO_2 particles as compared with the DWAs-sprayed SiO_2 samples. The net amounts of Al on SiO_2 particles ranged from 0.09 to 0.15μgAl/cm^2 SiO_2. Although the amounts of Al at the Al-III-nebulized aerosols group were situated between Al-II and Al-IV, considering that Al-III was in great resource and easy to be produced in our country, our investigations were mainly done on the interaction of Al-III aqueous solution aerosols with SiO_2 dusts as follows.

2) *Combination of SiO_2 particles with the AlAs nebulized with the various concentrations of Al-III aqueous solution.* Al-III aqueous solution was diluted into 0.1, 0.3, 0.5 and 1.0 mgAl/ml with deionized water, and respectively nebulized into the exposure chamber with SiO_2 dusts at same conditions. Significant linearity between the Al-III aqueous solution concentrations and the amounts of Al combined on SiO_2 particles was observed in Figure 2, i.e., the amounts of Al on SiO_2 particles increased with increasing the concentrations of Al-III solution. It was noted that in the presence of nebulization by the lower concentrations of Al-III solution the amounts of Al on SiO_2 particles was 0.16 ± 0.03 μgAl/cm^2 SiO_2 and reached an effective level reducing pathogenicity of the mineral dusts, as the previous experiment in which the interaction of Al with SiO_2 was in test tube had suggested that while the amounts of Al on SiO_2 were 0.15μgAl/cm^2 or so, the cytotoxicity of SiO_2 was obviously suppressed. When the SiO_2 samples combined with AlAs or DWAs were washed 4 to 5 times in deionized water by centrifugation at 4000 rpm for 20 min., respectively resuspended and filtered on microfilter by suction for XRF analysis, it was found that amounts of Al on the post-washed SiO_2 particles were yet more enough (data not shown) to lessen the SiO_2-induced cytotoxicity on the basis 4 of preliminary experiments in test tube.[4] This suggested that the combination of AlAs with SiO_2 particles was quite firm and relatively stable.

3) *Combination of various SiO_2 dusts in diameter with AlAs.* With nebulizing 0.5mgAl/ml of Al-III aqueous solution into the SiO_2 dust atmosphere of the exposure chamber, the SiO_2 samples were collected on four stage microfilters from 2.54 to 6.97 μm MMAD (2.54, 3.68, 5.14 and 6.97 μm MMAD, respectively) by the cascade impactor (10L/min sampling flow rate), and measured by XRF to calculate the amounts of Al combined on various SiO_2 particles in diameter. The results shown that the higher the SiO_2 particle-size distribution was, i.e. the smaller SiO_2 particles, the more AlAs could be combined with them (Figure 3). The net amounts of Al on the 2.54 μm SiO_2 MMAD were 9 times as many as that on the 6.97 μm SiO_2. In accordance with the deposition curve of SiO_2 particles in lungs, the 2 to 3 μm MMAD SiO_2 particles were of maximum deposition in lungs.[17] Therefore, no doubt will the AlAs in dusty workplace be much more significant as a preventive measure to silicosis.

Anti-cytotoxic Effect of Soluble Aluminum Aerosols on SiO_2 Dusts

To estimate the preventive effects of AlAs on silicosis, the changes in cytotoxic effects (AHR and MVI) of the SiO_2

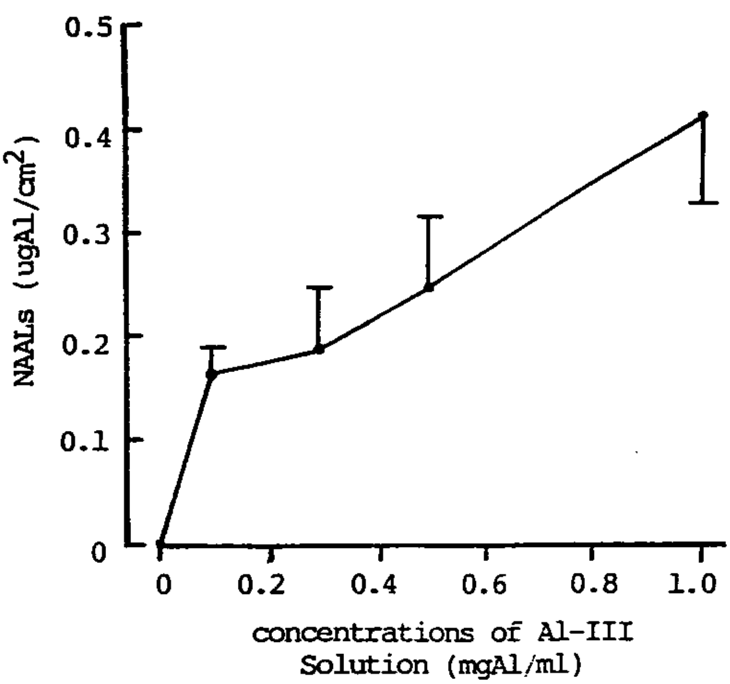

Figure 2. The combination of SiO_2 dusts with AlAs nebulized by various concentrations (0.1, 0.3, 0.5 and 1.0mgAl/ml) of Al-III aqueous solution. NAALs represents the net amounts of Al combined on SiO_2 particles, i.e., the amounts of Al on AlAs-treated SiO_2 minus the ones on DWAs-treated SiO_2. The number of samples was 4 per group.

particles combined with aerosols of Al-III aqueous solution of various concentrations on guinea pig alveolar macrophages and rat erythrocytes were examined, which is rapid, sensitive and common marks to test cytotoxicity of mineral dusts and also an important evidence to reflect pathogenicity of them.[5] The greater AHR and MVI are, the lower the cytotoxicity of SiO_2 particles is and the more effectively the AlAs could resist SiO_2-induced pulmonary damage.

As seen in Figure 4, the AlAs-combined SiO_2 particles greatly increased the AHR and MVI with statistical significance as compared to control group. To some extent the increases were correlated with the amounts of Al on SiO_2. When the amount of Al was $0.18 \pm 0.06 \mu gAl/cm^2$ (in the nebulizing group of 0.3mgAl/ml Al-III aqueous solution), AHR and MVI reached maximum level, 66.23% and 64.3%, respectively. At nebulization with the lower concentration

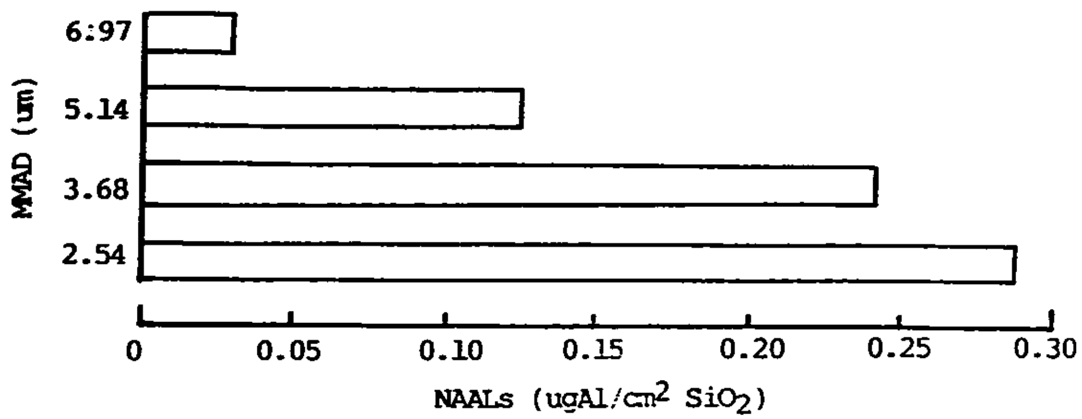

Figure 3. The net amounts of Al (NAALs) combined on the SiO_2 particles of various diameter (MMAD 2.54 to 6.97μm) sampled by cascade impactor (10L/min sampling flow rate).

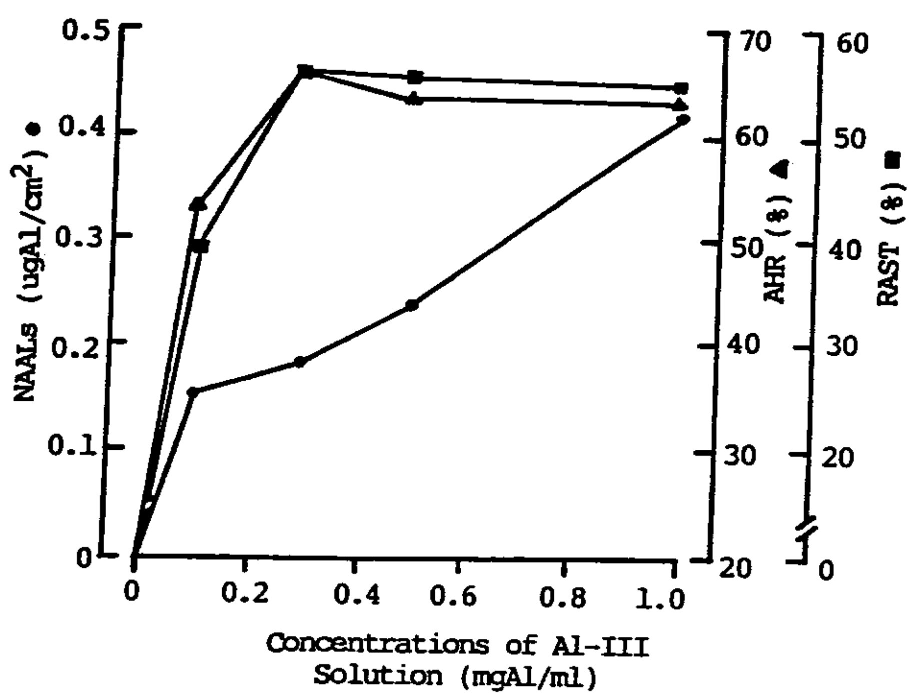

Figure 4. The suppressing effects of soluble aluminum aerosols on SiO_2-induced cytotoxicity to macrophages and erythrocytes. NAALs: net amounts of Al combined on SiO_2 particles; AHR: antihemolysis rate; RAST: rate of AlAs-suppressed toxicity of SiO_2 dusts to macrophages.

of Al-III aqueous solution (0.1mgAl), the cytotoxicity of the SiO_2 particles was also suppressed by more than 40% (AHR by 48.32% and RAST by 52.1%).

CONCLUSIONS

The experimental results indicated that AlAs could not only stably combine with SiO_2 dusts in an atmosphere, but also effectively suppress the cytotoxicity of the minerals of macrophages and erythrocytes, which is consistent with the previous investigations in water system.[4,18] Although the present study did not examine the change of surface charges of SiO_2 particles, according to the preliminary experiments in which the surface charges of SiO_2 particles significantly decreased once the SiO_2 particles were combined with Al, it may be speculated that AlAs could reduce the charges of SiO_2 surface and promote aggregating and dropping of the SiO_2 particles in a dusty atmosphere.

It must be pointed out that in this study the AlAs combined with SiO_2 dusts were only involved, but the other AlAs combined without SiO_2 in the air can be inspired into lungs together with SiO_2 dusts and interact with the minerals in respiratory passage, by which the AlAs could in the same way make resistance against the cytotoxicity of SiO_2 particles. Therefore, though in the nebulizing group with 0.1mgAl/ml Al-III aqueous solution the amounts of Al combined on SiO_2 particles and the suppression of Cytotoxicity were not maximum, the effective amounts of Al will greatly increase if it is considered that the AlAs can combine with SiO_2 dusts in respiratory passage. It is suggested that 0.1mgAl/ml Al-III aqueous solution or lower may be suitable for further experiments in lab and dust working sites. However, the optimal dosage of soluble aluminum compounds used in workplace remain to be further experimented.

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PSEUDO-TUMORAL LUNG FORMATIONS FROM SILICA FREE DUSTS

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INTRODUCTION

In our experience we have encountered some workers who exhibited round shaped opacities in their lung suspected as being neoplastic in nature. In certain cases it was possible to exclude malignancy of such lesions, identifying them on the contrary, as the results of prolonged inhalation of silica free dusts at work.

In this work we report four cases exposed to calcium and magnesium salts (as previously stated) and one case of talc exposure, recently observed, who exhibited these types of lesions.[5]

SUBJECTS AND METHODS

Our study was carried out on five patients: four were magnesium and barium hydroxide production workers and one was a natural rubber processing operator. The first four subjects were allocated in the same industry and had done various tasks in two departments:

1. magnesium production employing heat processing (Pidgeon system), using dolomite, ferrosilicon and calcium fluoride as raw materials;
2. barium hydroxide production employing "Soderbergher" furnaces, by use of barium carbonate.

In the first department the enviromental dust level was between 1.6 and 71.3 mg/m^3 and in the second one from 0.8 to 128 mg/m^3.

The four workers between the ages of 43 and 58, had worked in the two departments for long periods (from 17 to 34 years). Their chest X-ray films were very similar and presented coin shaped lesions measuring 1–2 cm in diameter, single in three cases and multiple in one. Only one case was also a pneumoconiosis suspect with reticular shadows. In two workers the coin shaped lesions were found 3–4 years before.

We had suspected the presence of secondary neoplasm of the lung but as it proved impossible to locate the primary site of neoplasm, in spite of accurate investigations, chest X-ray film follow-ups were suggested.

In three of the four subjects the coin lesions remained unchanged at every follow-up for five years. In the fourth subject, after three years, the two shadows originally identified had increased about 30% in diameter reaching a dimension of 3 and 1.5 cm respectively. We therefore proceeded with a toracotomy which permitted removal of a sub-pleural well encapsulated node.

On cryoscopy it was described as: "fibrous wall node with signs of chronic inflammation and a central area of necrosis." The hystological examination revealed:" well defined node of lung parenchyma consisting essentially of interwoven and whirl-pool-like collagen fibers, free of identifiable cells, and minute calcified particles. The surface area of the node is rich in fibrocytes, newly formed vessels, clusters of lymphoplasmocytic cells and occasional foreign body multinucleated giant cells."

Mineralogical studies on the node employing X-ray diffraction, neutronic activation, and X-ray fluorescence showed scarse presence of silica and no significant concentrations of any specific metals. The Debye X-ray diffraction revealed this node as being rich in calcium and magnesium diphosphate.

The subjects maintained good health and showed no further lesions of the lung whatsoever on radiological investigations.

The fifth worker, 45 years old, had worked in a department for the processing of natural rubber where according to the requirements for production, dust of talc, mica and fecula were alternatively used.

The results of enviromental investigation carried out to determine the type and quality of corpuscolar pollution showed a variation of 0.6 and 1.4 mg/m^3 of dust concentration.

Microscopic examination of the particles removed revealed that the dust was rich of fibers 1–2 μm in diameter, 80% 10–20 μm in length and 20% less than 10 μm long. Fecula consisted of particles less that 5 μm in diameter which under polarized light had the typical "malta cross" appearance. Lastly, mica consisted of lamellae ranging from 20 to 60 μm in size. No asbestos fibers or silica was found.

The patient gave no history of respiratory or other diseases. All at once, a few days before admission, the subject had slight fever, cough and exertional dyspnoea. The chest X-ray film showed presence of an irregular digitated shadow, 3.5 cm in diameter, at the base of the left lung.

All the various investigations carried out were negative. Between thirty and ninety days after, other coin shaped shadows appeared bilaterally, becoming larger and more numerous, tending to be confluent.

The rapid growth of the lesions in the lung compared to the satisfactory clinical state of the patient, led us to proceed with a toracotomy.

The cryoscopic examination of the heaviest mass which was first localized at the base of the left lung, ruled out any presence of neoplasm.

The histological examination reported: "Lung parenchyma greatly altered by wide-spread granulomatose inflammation consisting of epithelioid cells and numerous foreign body giant cells, containing birefrangent needle-like fibers 1-2 μm in length and asteroid bodies."

In conclusion the histological diagnosis was "giant cell talc granulomatosis of the lung."

The results of the study made on the material used by the patient at work confirm that the needle-like formations seen in cytoplasm of the giant cells were talc fibers and one may assume that the asteroid bodies were, on the contrary, fecula granules.

The postoperative course was normal and his overall condition improved. In addition, within the following months a slow but constant regression of the shadows was observed and seven months later only rare radiological irregular opacities were seen. Two years later the chest X-ray film was normal apart from the signs of the toracotomy.

DISCUSSION

The description of pseudo-tumoral lung formations from silica free dusts is rare in literature.[2,4]

Although coin lesions of the lung due to inhalation of calcium and magnesium salts have not been reported, scientific studies have described talc granulomas of the lung and other organs.[3,5,6,7,8]

With regards to the first four cases we have described among the raw materials used at work, dolomite results the most suspected responsible for round shaped opacities of the lung.[1]

The rapid development and progression of talc granulomatosis found in our rubber worker and its disappearance after he stopped work was probably due to reversible flogistic-proliferative tissue reaction, without fibrosis, from talc free of silica or asbestos fibers.

Hence, in the presence of lung lesions, in particular those we have described, it seems beneficial to add to routine clinical and instrumental practice suitably deeper investigation into work history of subjects, possibly including analysis of work environment and raw materials in use.

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MINERAL FIBERS AND DUSTS IN THE LUNGS OF SUBJECTS LIVING IN AN URBAN ENVIRONMENT

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INTRODUCTION

It is well-known that toxic and carcinogenic substances can be present in the breathable airborne particulate of urban areas and the higher incidence of tumors and chronic obstructive lung diseases, documented in these areas as compared with rural ones, is generally considered to be a consequence of the environmental conditions.[3] Recently, the need to better define the carcinogenetic role of atmospheric pollution in comparison with that played by other factors, such as cigarette smoking, has been emphasized.[4]

Here, we have studied mineral particulate from autoptic lungs in a sample of population having lived in Rome area and not occupationally exposed to dusts. Their life-style, in particular their smoke habits, and the quantity and quality of fibers and mineral dusts found in their lungs were correlated.

MATERIALS AND METHODS

Sixty subjects, who had lived in Rome, aging from 15 to 65 years, were selected for this study. Subjects with a history of occupational exposition to mineral dusts or with serious pathologic conditions and drug addicts were excluded. During post-mortem examination, fragments of lung tissue were taken from the upper lobe of right lung.[1,2] They were mineralized in atomic oxygen plasma. Dry weight was also estimated. Inorganic component was resuspended in deionized water and filtered on 0.45 *m cellulose membrane filters. Mineral particles were then transferred on copper grids, which had been coated with carbon films. They were observed under a 430 Philips transmission electron microscope, equipped with an energy-dispersive spectrometer for X-ray. Adjacent fragments of lung tissue were fixed in formalin and embedded in paraffin for light microscope observation.

RESULTS

Concentration and Type of Mineral Particulate in the Lungs

Mineral particle concentrations in the lung parenchima ranged from 0.7×10^5 to 1.7×10^5 particle/mg of dry tissue (Figure 1). Two principal components were found—Silicates and crystalline silica (52%)—Heavy metal oxides and sulfates (48%). The relative percentage of components, however, showed significative differences, since in nearly 12% of subjects the ratio between silicates and metal compounds differed for more than 50% of the average value. Fibrous particles were detected in 16% of subjects. They were generally represented by asbestos fibers (chrysotile and amphiboles), but small amounts of talc, rutile (titanium oxide) and calcium sulfate fibers were also found. Asbestos fiber concentration ranged from 200 to 300 ff/mg of dry tissue and represented 0.5–1% of total particulate.

Seven groups of silicates were detected: micas, clays, talc, chlorites, serpentine and amphiboles. The majority of particles had a diameter ranging from 1 to 5 μm. No particles more than 30 μm in diameter were observed.

Asbestos fibers were represented for more than two thirds by chrysotile and ranged in length from 1 to 8 μm, with a length/width ratio higher than 10 (Figure 2).

Up to sixteen different metallic elements, in the form of oxides and sulfates, were found (Figure 3). Nearly 80% of particles ranged in size from 0.1 to 1 μm and no particles larger than 2 μm were found. Six elements (Al, Ca, Ti, Cr, Fe, Ni) could be identified in more than two thirds of subjects.

Dependence between Mineral Particulate, Age and Life-style of Subjects

The dependence between particle concentrations and the age of subjects is shown in Figure 4. In general, concentration appeared to increase with age. Moreover, observation in light microscopy showed that anthracosis, which was scored from 0 (absent) to 3 (severe) also tended to increase with age (Figure 5). Smoking habit seemed to influence the quantity of mineral particulate deposited in the lungs, since in the same age-groups, a greater amount of particulate was found in the lung parenchyma of smokers than in non-smokers (Figure 6).

CONCLUSIONS

Our results confirm the high degree of dependence between the concentration of mineral particulate in the lung parenchyma and the environmental situation. Particularly, it appears that subjects living in an urban area are exposed to toxic and carcinogenic substances released by motor vehicles (heavy metals, asbestos fibers). Finally, our results confirm the effect of smoking on the quality and quantity of the particulate in the lungs.

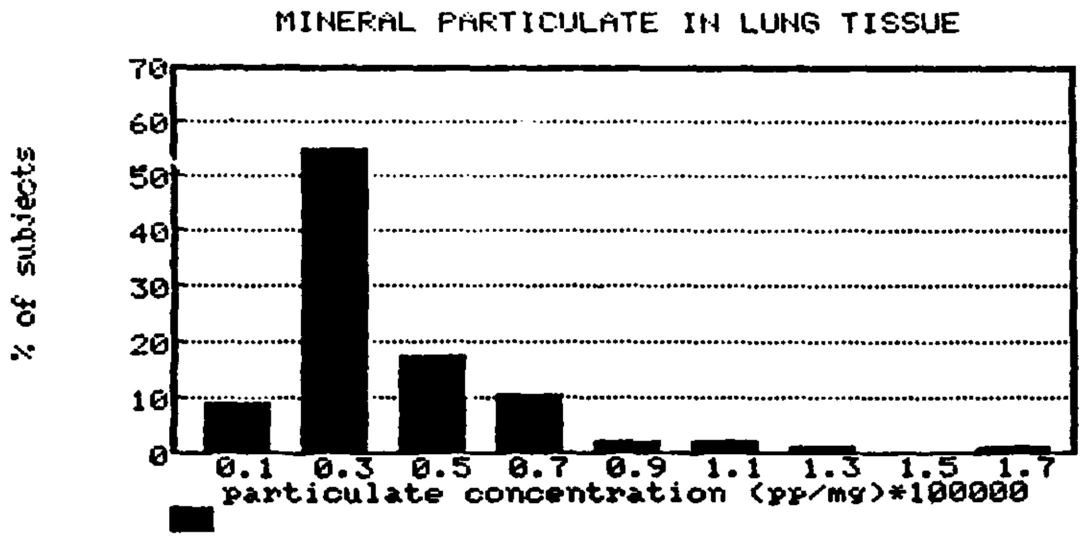

Figure 1. Particulate concentration in the lung parenchyma of sixty subjects.

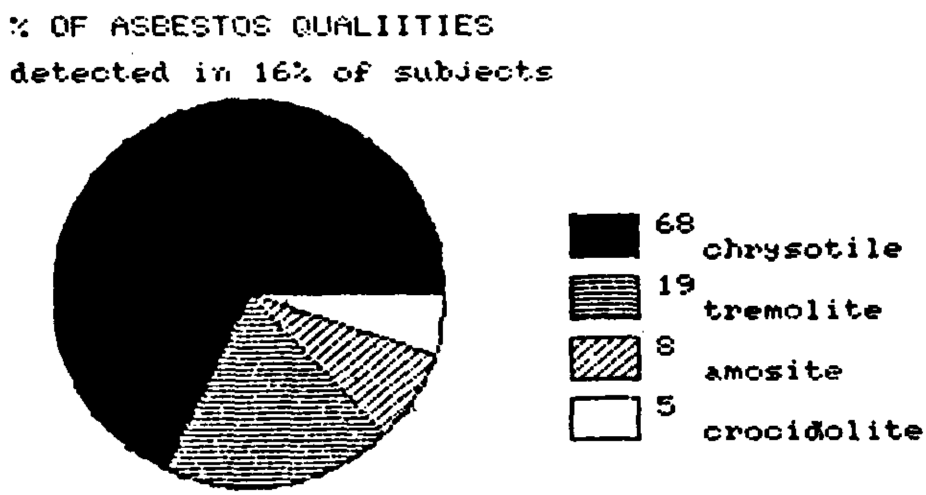

Figure 2. Percentage of asbestos types detected in the lung tissue.

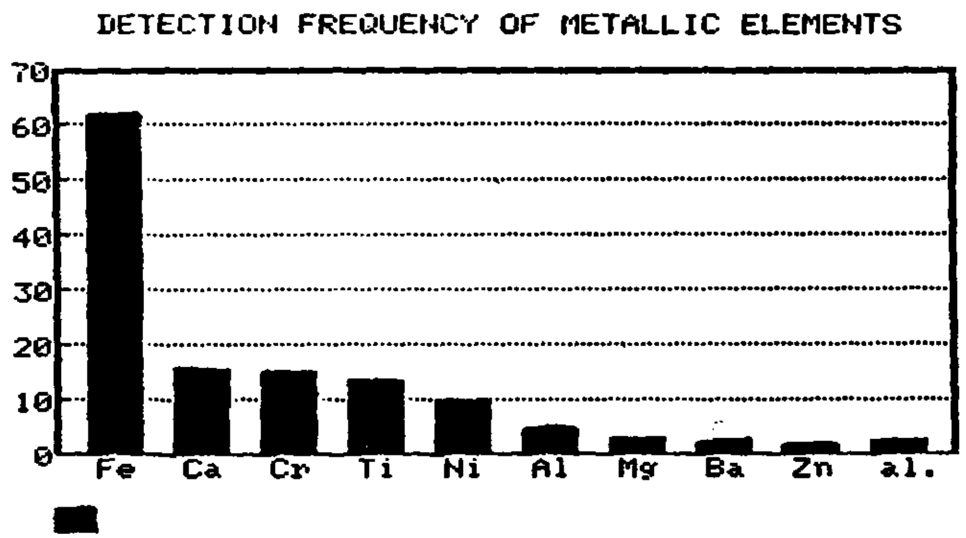

Figure 3. Frequency of the metal elements observed.

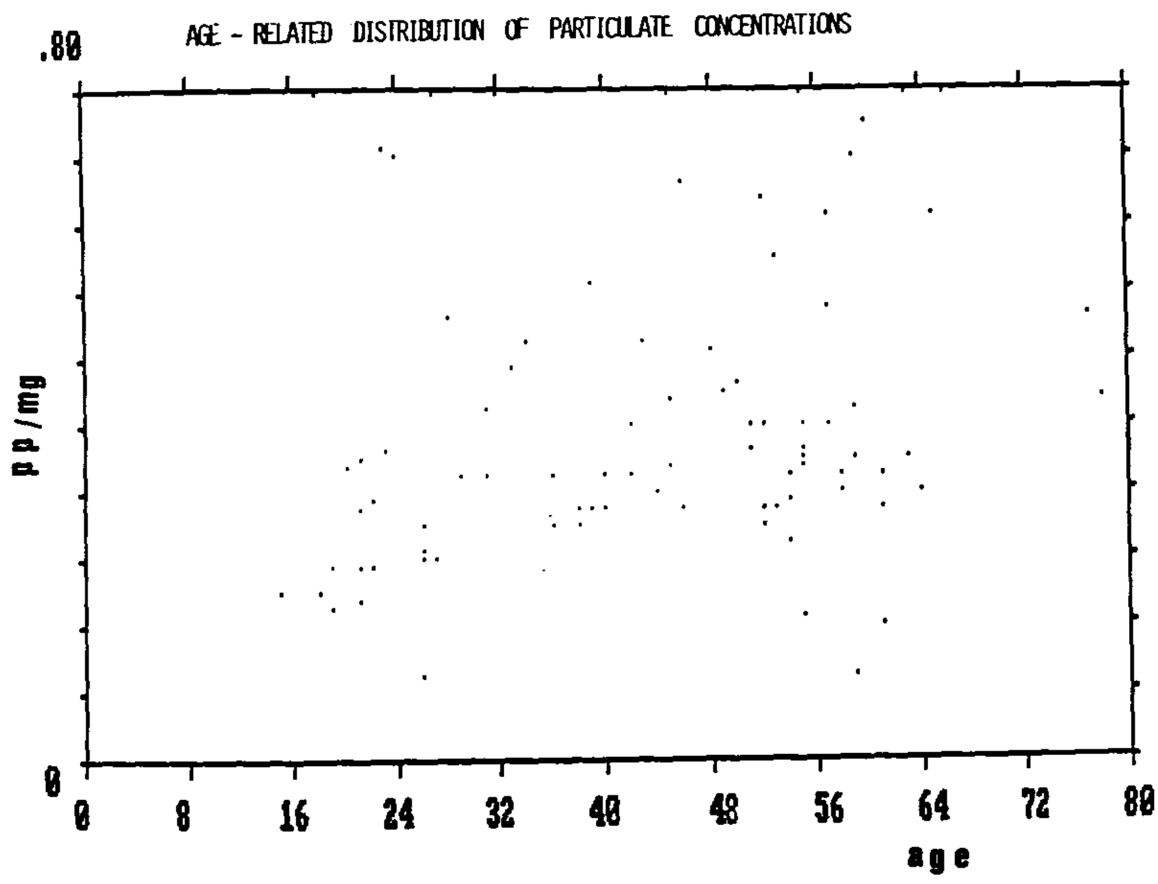

Figure 4. Particulate concentration in relation to the age-groups.

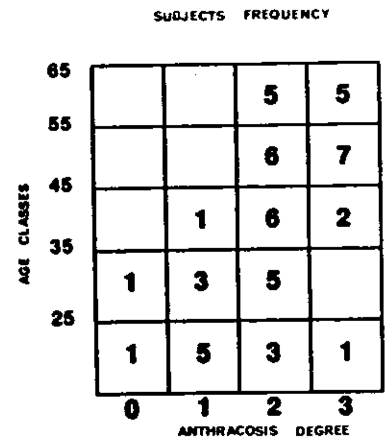

Figure 5. Distribution of anthracosis (scored from 0-absent- to 3-severe) in relation to age-groups in sixty subjects.

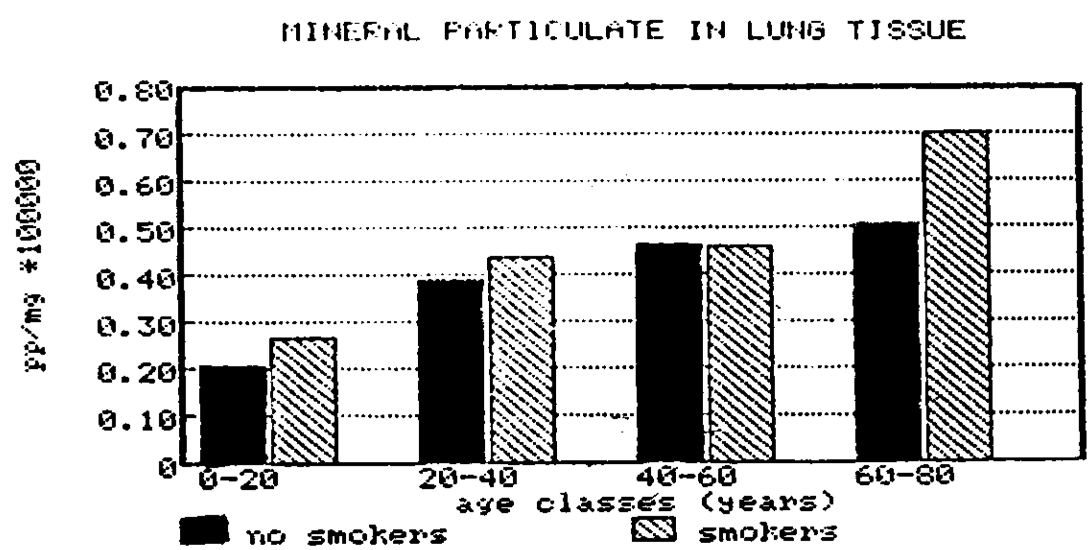

Figure 6. Influence of smoking in particulate concentration in the lung tissue.

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PREVALENCE OF PNEUMOCONIOSES AMONG PHOSPHATE ROCK WORKERS IN BRAZIL

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INTRODUCTION

The phosphate rock as a pure chemical compound is defined as a calcium phosphate, $Ca_5(F, C1) (PO_4)_3$. Its use in the fertilizer industry is well known all over the world and has increased since the 2nd world war end period. The industrial manipulation of that compound has brought concern about fluorose hazard by the treatment of the phosphate rock by H_2SO_4 or other strong acids to liberate phosphate rich compounds. Some respiratory illnesses mainly of upper airways were described related to this manipulation.[3,4,6,7,8,9,10] A very poor literature is available concerning pneumoconioses hazard linked with inhalation of the powdered rock.[1,2,6,6,16] In the same way, good reviews about pneumoconioses don't quote any information on phosphate rock pathogenicity.[16]

CRETEANU et al.,[2] in 1969 and PISLARU et al.,[15] in 1969 described 7 (seven) and 6 (six) cases of pneumoconioses in phosphate rock mill and transportation workers. Both of the papers did not tell us anything about the level of free silica in the samples of the inhaled rock. On the other hand, EL GAWABI & IBRAHIM[5] in 1975 described some cases of pneumoconioses in phosphate rock workers, but the analysis of the inhaled dust showed a high percentage of free silica and the lung disease was characterized as silicosis.

MATERIAL AND METHODS

The workers studied in this investigation were exposed to phosphate rock extracted in the states of Goiás and Minas Gerais, Brazil, where the material is crushed and then transported by train reaching Paulinia, state of São Paulo where the compound is stored in underground mills. The underground work and a twelve hours work shift every day, with only one day of rest each fortnight created a condition of very high risk to lung diseases.

Eighty one workers that had some kind of exposition to the rock dust were asked to participate of the study. During the investigation, eight (8) of them were put out of the study because they did not conclude all the proposed examinations. All the remanent 73 were submitted to a) occupational anamnesis; b) detailed respiratory questionnaire; c) physical examination emphasizing respiratory apparatus; d) pulmonary function tests using Collins Maxi Survey Computer Systems analyzing. The parameters analyzed were the CVF%; VEF_1/CVF and MMEF $FEF_{25/75}$;[12,13,14,17] e) thorax X-ray which were read by three readers in a blind schedule using the ILO Classification of Radiographs of pneumoconiosis 1980;[11] f) two workers were submitted to lung biopsies through thoracotomy. Tissue samples were stained by H.E., van Giemsa, Masson and argentic dye to found out fibrosis or even reticulin fibres; g) the quantity of free silica in the airborne samples was measured by colorimetric methods using Physical and Chemical Analysis Branch; h) a semiquantitative analysis was done with the airborne sample using an X-ray spectrometry EG 86 ORTEC.

RESULTS AND DISCUSSION

From the 73 examined workers we found $20^{24.7}$ with pneumoconioses by X-rays characterization.

Trying to determine an average time of exposition for these 20 cases it was found a mean time of 46 months with a range from 12 to 73 months. CRETEANU et al.,[2] and PISLARU et al.,[15] found a mean time for their workers of 24 and 36 months respectively. The differences between ill and not ill workers concerning the smoke habit were not found significant. Relating to past inhalatory risk conditions, only one worker had previously worked in a fertilizer plant for a short period of time. The majority of the 20 cases didn't suffer from any respiratory symptoms (85%). Fourteen cases (70%) showed MEFF $FEF_{25/75}$ alterations; two cases (10%) mild restrictive patterns; three cases (15%) a pure obstructive pattern and five cases (25%) with normal patterns. Data from the study of CRETEANU et al. obstructive pattern in six of the seven cases. No correlation between time of exposition and lung function alterations was found.

Opacities in thorax X-rays were classified as small and round in 17 cases (85%) and small and irregular in 3 cases (15%). No pleura disease or mediastinum alterations were found (see Table I and Figure 1).

Lung biopsies were examined through optical microscopy. What appeared was a very extense deposit of brownish crystal material with focal refringence to polarized light in the perivascular, peribronquic and septa tissues, also occupying intra alveolar spaces. A mild histiocytary inflammatory reaction with alveolar collapses were also seen. Despite the use for special dyes to find out fibrosis or increased reticulin fibres, no significant fibrosis was seen (see Figures 2 and 3).

Diffraction analysis by EG 86 ORTEC showed relative amounts of Ca, P, Fe, Mn, Si, Ti, Ba, Nb and S. Small quantities of Fluoride are not detectable by this method but it is

Table I
Thorax X-rays Alterations in the 20 Cases of Pneumoconioses

radiologic alterations			nº	%
	round	p	10	50
small		q	7	35
opacities	irregular	s	1	5
		t	2	10
	total		20	100

clear this element exists in the composition of that rock. Despite the good sensitivity of the method for free silica, not even traces were found. Colorimetric methods showed free silica in less than 1% of the total inhaled dust.

CONCLUSIONS

From the results one can conclude about the non fibergenicity of this pneumoconioses, at least with the available data. A prospective study must be carried out to assess the former statement. A regular follow-up using lung function tests and thorax X-rays must be included in the routine examinations of the phosphate rock workers. The actual etiology of that pneumoconioses should be studied using electronic microscopy linked with microanalysis by X-rays diffraction.[18] The high prevalence of pneucomonioses found in this study must derive from the specially bad conditions for working people were submitted at this plant.

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Figures not provided.

SIZE CHARACTERIZATION OF INDUSTRIAL PRODUCTS (MMMF) USED IN BUILDINGS AND STRUCTURES AS SUBSTITUTES OF SPRAYED ASBESTOS-CONTAINING MATERIAL

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INTRODUCTION

It is known the fire risk prevention problem has determined a very wide use of insulation materials and mainly of asbestos, as a consequence of its desiderable physical and chemical properties. For these purposes it has been used in many different industrial sectors, and particularly in considerable quantity in the building industry. In this last field insulation products are mainly used as surfacing materials and partitions to provide both protection from fire spread and acoustic insulation.

In the past, asbestos was sprayed extensively on structural steel structures and decks as fireproofing to enhance their fire resistance. Such use was required in Italy by national regulations on fire prevention.

The health hazard due to the inhalation of airborne fibres arises from dust release in air caused by an aging process, bad maintenance or vandalism actions. All those facts have induced many governments to regulate the use of asbestos products and to ban asbestos-sprayed techniques.

Like in some other countries the Italian Health Ministry has taken urgent measures for asbestos-control in schools and hospitals (circ.n.45 on 10.7.86). Such measures aim at identifying the presence of asbestos and to check if it is in good condition or if there is a potential release of asbestos dust into the air. In this case there will be a health risk and asbestos should be removed. These measures have determined indirectly a lot of work in order to remove asbestos materials from buildings, even if they were not in bad conditions.

Fibrous alternative materials (with the same heat and fire resistance) are generally used as substitutes for asbestos. At present, alternative products are available on the market with a silicate like chemical composition and various morphologies, either fibrous or not-fibrous. Most of them have amorphous structures. As a rule, fibres of alternative products have a diameter coarser than asbestos-fibres, although some of them have size similar to asbestos. Generally such man-made mineral fibres (MMMF) are manufactured with different diameters relating to the specific use they are destined. The most important MMMF are grouped in: slag wools, rock wools, glass wools and continuous filament with diameters varying from 1 up to 20 μm, averaging about 8 μm for filament glass fibres 1–5 μm for insulation wools. Often a considerable percentage of these fibres shows diameters in the range from less than 3 μm to 0 2 μm.

In this paper, special attention is given to representative products used in public buildings as banks or offices, as substitutes for asbestos.

These materials have been sent by U.S.L. (Local Sanitary Unit) to our Institute to obtain all information about their chemical composition and size characterization, in order to decide their use as asbestos substitutes.

In a recent summary report of WHO-IARC international symposium,[1] an increased lung cancer risk has been reported among workers exposed to small-diameter fibres since the early days of man-made mineral fibres production. The risk has been greater in the rock or slag wool sector than in the glass wool one. Moreover the IARC has revised of carcinogenicity and it has classified MMMF as possibly carcinogenic to humans (Group 2B).

The cancerogenic activity is generally attributed to the fibre dimensions and to their durability in biological tissues. Today the opinion of the scientific community based on the epidemiological, toxicological and mineralogical studies, indicates that any mineral fibre with specific dimensions and sufficient biological durability must be regarded as possibly cancerogenic.

MATERIALS AND METHODS

All materials studied appear composed of fibrous elements and a low density matrix of rounded aggregates. The color is usually brown to white. Inspection of the bulk materials by a stereomicroscope at about 40 X show a very friable consistency and a largely empty structure. Fibres show a wide range of diameters and often they are in association with unfiberized particles mostly in the form of solidified droplets.

Few milligrams of the bulk materials, taken at random were shaken into a beaker with a "policeman" for a few minutes using H2O2 in order to help particle disgregation and dispersion.

Because of the mechanical treatment, fibre length distribution was not determined in the subsequent analysis. Material was then dispersed in prefiltered distilled water. Aliquots of the suspension were filtered through a polycarbonate filter (NPF), 25 mm diameter, 0.2 μm, by a small funnel (Nuclepore Corp.).

A quarter of each filter was mounted on a carbon stub, 12 mm diameter and coated by a gold layer of approximately 100 nm of thickness.

The samples were analyzed by scanning electron microscope, Cambridge model 200, at 0° tilt angle, 25 KV accelerating voltage. Fibre size was measured directly on the screen, separately recording diameters of 100 fibres.

The airborne samples were collected on 25 mm 0.8 microns cellulosic filters. Half of the filters were cleared and examined by phase contrast optical microscope (PCOM) method. The remaining portion of the filters were mounted on a carbon stub in the same way, but examined by SEM at 15 KV acceleration voltage in order to prevent local overheat.

RESULTS

Table I shows size distributions from bulk samples of materials used as sprayed insulations in buildings.

From diameters distributions it results that in samples 2 and 5 the respirable fraction (diameters less than 3μm) is about 30%, while in the other samples the same fraction is about 60%.

During the removal operation from building structures coated with the material of sample n.1, personal air samples were collected according to the standard method.[2]

Table II shows a consistent size distribution by diameter and by length of airborne respirable fibres from 4 different samples.

Table I

Frequency of Diameter in the Bulk Materials

Sample	D = 1	1 D 3	3 D = 5	D 5
1	29	39	22	10
2	6	27	33	34
3	20	41	26	13
4	25	35	25	15
5	10	19	34	47

Table II
Size Distribution of Airborne Respirable Fraction

A) Distribution by diameter

μm	D = 1	1 D 2	2 D 3	D 5
%	39	44	15	2

B) Distribution by length

μm	5 L 10	10 L 15	15 L 20	20 L 30	30 L 100	L 100
%	41	18	10	15	11	5

In this case the respirable fraction is about 98% showing a clear preferential loss of coarser fibres. About 40% of these fibres are between 5 and 10 microns in length, and the rest is equally distributed among the subsequent length classes.

Chemically fibres consist principally of silicon, calcium and aluminium. Other elements such as iron, megnesium, potassium are also present.

CONCLUSIONS

Recently there has been an increasing demand for insulation products and especially of man-made mineral fibrous materials to replace asbestos-products.

Owing to the possible fibre release from MMMF insulation materials in bad conditions, sometimes they have been removed from structures.

The results from this study, even if limited, indicate that the physical characteristics of MMM products show 50% diameters less than 3 microns, while during the removal about 98% of airborne fibres have diameters less than 3 microns.

Although, at present, investigation[3] carried out in different countries unnecessary exposures to these types of fibres.

Finally, it seems important that clear information be reported on appropriate labels in which should appear not only technical instructions, but also chemical data as well as the diameter range of fibres in order to evaluate the possible risk of people exposed.

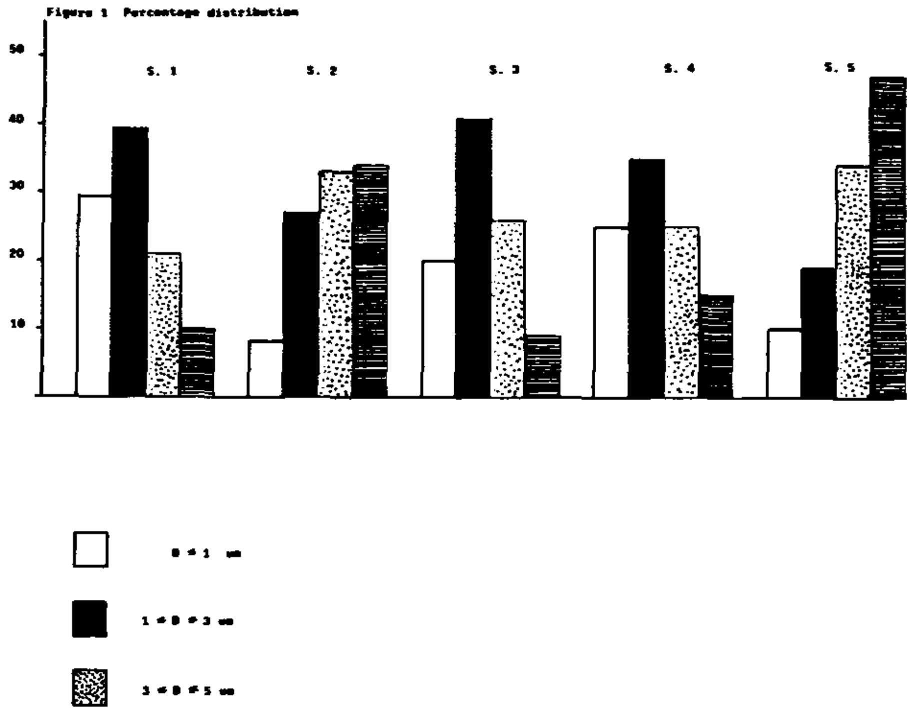

Figure 1. Distribution of fibre diameters of bulk materials.

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SILICOSIS IN PIT DIGGERS IN SERRA DA IBIAPABA, CEARÁ, BRAZIL

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A survey carried out by technicians from FUNDACENTRO, Pernambuco, showed that out of 134 pit excavators, 38 presented silicosis after clinical and laboratorial examinations. Out of these 38, 5 died during this survey. Environmental evaluations in workplaces were also made through dust measurements inside pits being excavated. A conventional water supply is here considered a definite solution to these problems. Specific measures are also proposed as transient solutions.

We participated in the research on silicosis in Serra da Ibiapaba, approx. 350 km from Fortaleza, because of the professional concern and particularly because of the great social vision of the INAMPS pneumologist, Dr. Márcia Alcântara Holanda, who in her ordinary work as the coordinator of the apprenticeship in pneumology at the INAMPS hospital in Messejana, remembered Ramazzini and asked two patients: "What's your occupation?"

Today, only in Tianguá, a town with 40,355 inh. (IBGE 1980), 138 pit diggers have already been clinically and radiologically examined, and a very large number of workers suffering from the disease was found. FUNDACENTRO participated in this work through its Divisions of Occupational Medicine and Hygiene, its Regional Center in Pernambuco, supported by its National Technical Center, making clinical examinations, radiological comparisons based on OIT standards (International Labor Organization) environmental dust analyses and other risk analyses, general guidance in relation to occupational safety, hygiene and medicine, and chiefly in the proposition of solutions compatible with the serious nature of the situation, in order to solve definitely this crucial problem that affects seven towns in Serra da Ibiapaba, comprising approximately 200,000 people. None of these towns has a conventional water supply, and there is no natural water reservoir in the surroundings. The population has to dig water holes manually, one per family, keeping a large number of workers busy.

HAZARDS OF THE ACTIVITY—BACKGROUND

A pit is generally dug by two persons who substitute each other alternatively. While one worker is inside the pit, the other stays on the surface, helping with whatever is necessary. The pits usually have a diameter of 1,50m and they are around 12m deep. The geologic profile of the soil shows a stratum containing 10m of sandstone, which is easily excavated using tools. There is a hard silicified stratum below it that may only be removed with the use of explosives or mechanized tools. A sample of this silicified stratum was taken to be analyzed and it was found to contain 97.44% of SiO_2. After this stratum we reach the saturated zone, where the walls of the pit are then covered with premolded concrete rings. A bucket lifted with ropes through a drum fixed to a wooden girder set at the pit entry is used to remove the material. It can also be used by the worker to go up to the surface or down to the bottom. In February 1987, they used to charge the amount of US $10.00 for each "palm" (approx. 25cm) excavated or US $385.00 for a whole pit of any size.

Operational Hazards

Due to the poor conditions where the work is performed, it is easy to foresee the numerous hazards involved:

- Falls caused by rope rupture, drum break, rupture of drum fastening, rupture of drum supporting girder, slipping.
- Being buried in the earth by caving in of pit walls.
- Accidents caused by tools in the confined workplace.
- Ergonomic risks due to worker's posture because of confined workplace.
- Misuse of explosives.

Here we wish to focus on a number of irregular proceedings, starting with equipment and material purchase, which is traded freely, with no observance of current laws. The transport, storage and handling are also totally inappropriate. Explosives have been seen in worker's houses in places within children's reach. The preparation of explosives for detonation is done in a very primitive way, even the teeth are employed to fix the detonator to the fuse and it is done in the presence of ordinary people (even children) in residential areas, with no measure taken to isolate the area. Thus, besides the danger of an accidental explosion, there is the danger of stones being thrown at considerable distances, often causing physical and material damage.

Another circumstance which deserves attention is that the worker has only 2 minutes and a half to get out of the pit, after lighting the fuse of the explosive charge at the bottom of the pit. It is easy to imagine what may occur in case he falls because of any reason already mentioned. In the survey we found that at least one fatal accident had been caused by this reason.

Environmental Hazards

Undoubtedly, the biggest hazard of this type of work is the dust produced by the excavation. In rainy weather, the aerosol is present almost exclusively in the operation of removing the silicified sandstone stratum through mechanical means or explosions (the usual manner). The situation is aggravated in dry weather when the dust is also present in the strata prior to the silicified sandstone. Therefore, the risk generating factors are:

- Low humidity of the soil above the saturated zone.
- Pit depth, making the dust reduction by means of natural ventilation impossible.
- Lack of any exhaustion device.

The concentration of dust is higher just after the silicified sandstone stratum is exploded and it remains like that for hours. The worker generally goes back to the bottom of the pit to remove fragments 3 hours after the detonation which is not enough time for the dust to have dissipated.

MATERIAL AND PROCEDURE

The measurements of these concentrations were carried out since then. The adopted procedures are described on Table I. The threshold limit values represent dust concentrations in workplaces under which it is believed that most of the workers may be repeatedly exposed to during their work-life with no harm to their health.

Procedures for Evaluation and Analysis

The dust sampling was collected by means of 37mm PVC filters, with 5μm porosity, where low flow rate suction pumps were used (pump BENDIX model BDx 44). A one-inch cyclone was used to select respiratory particles. It allows 90% of particles having a diameter smaller than or equal to 2μm to go through and it detains particles with diameters bigger than 10μm. The determination of dust concentration was made by gravimetry and an analytical balance of 0.000010 precision was used. The percentage determination of crystallized free silica in the dust samples was carried out through X-Ray diffractometry.

Note. The BENDIX sampling kit is an equipment for individual use, fitted out in the worker. The samples collected represent the circumstances of exposure to dust when performing any analyzed activity.

The flow rate used in the collection of total dust is 1.5L/min and in the collection of respirable dust it is 1.7L/min. The period for sampling varied according to environmental characteristics.

Procedure for Assistance

The procedure for assisting exposed workers has been the following:

Table I
Threshold Limit for Crystallized Free Silica Dust

THRESHOLD LIMIT		COLLECTED DUST	SAMPLING METHOD	PROCEDURE FOR ANALYSIS
$\frac{8.5}{\%SiO_2+10}$	MPPCD*	Total	Impinger	Field Count
$\frac{8.0}{\%SiO_2+2}$	Mg/m^3**	Respirable	Gravimetric with Cyclone	Gravimetric (weighing)
$\frac{24}{\%SiO_2+3}$	Mg/m^3**	Total	Gravimetric	Gravimetric (weighing)

(*) MPPCD = Millions of particles per cubic decimeter
(**) Mg/m^3= Milligrammes per cubic meter

1. Simple spirometry, measuring the forced vital capacity, measuring the forced expiratory volume in the first second of time and forced respiratory flow between 25% and 75% of forced vital capacity.
2. Effort tests (with arterial blood gases, exhausted gas sample and realization of ECG).
3. Fiberoptic bronchopy and collection of bronchiole-alveolar lavage (BAL). Patients with changes in effort tests and normal radiological examinations are subject to a transbronchial biopsy of the pulmonary parenchyma.
4. Determination of the immunological profile of each patient, including: Hemogram, plasmatic protein electrophoresis, serum immunogloblins, cutaneous tests for cellular immunity evaluation, rheumatoid factor.
5. Outdoor pulmonary biopsy, where transbronchial biopsy is not enough for a diagnosis.
6. Diagnosed patients are registered in their hometowns for attendance and especially for treatment of the associated diseases mentioned above.

These stages are carried out in Tianguá, by the team of Dr. Marciá Holanda.

MEASUREMENTS

The evaluations were based on Tianguá. The measurements were done in pits located in 3 different regions of the town. Pit No.1 (urban area), Pit No.2 (suburban area) and Pit No. 3 (rural area). The situation of this hard working population is very serious, as can be seen in Table II, The threshold

Table II

Concentration Measurement of Environmental Dust Inside Pits in the Town of Tianguá, Brazil

PLACE	SAMPLE WEIGHT (Mg)	VOLUME OF AIR SAMPLE (m^3)	$\%SiO_2$	CONCENTRATION (mg/m^3)	THRESHOLD LIMIT (mg/m^3)	EXCESS*
Pit no.1 Bairro Sto. Antonio	0,33	0,0066	18,2	55,0 TD	1,13	48
Pit no. 1 Bairro Sto. Antonio	0,96	0,0068	18,8	141,2 TD	0,41	344
Pit no. 1 Bairro Sto. Antonio	0.25	0,0034	16,0	73,5 RD	0,44	167
Pit no. 2 Rua das Almas	0,40	0,0045	10,0	88,9 TD	1,85	48
Pit no. 2 Rua das Almas	0,19	0,0075	10,5	25,3 TD	1,78	14
Pit no. 3 Health Center	0,11	0,0051	9,1	21,6 RD	0,72	30
Pit no. 3 Health Center	0,27	0,0085	3,7	31,8 RD	1,40	23

According to NR 15, Governmental Decree 3214/78 of the Ministry of Labor
TD = Total Dust
RD = Respirable Dust
* = Number of times the Threshold limit was exceeded

limit for silica dust occupational exposure was exceeded up to 344 times. The legislation considers these cases as maximum degree insalubrity. Dust concentration at these levels, inevitably causes silicosis. This was confirmed by a number of diagnosed cases in the region.

In Tiangua, out of the 200 pit diggers estimated, 134 were examined. Out of these, silicosis was confirmed in 38 through clinical and laboratorial examinations, and 5 workers, out of these 38, died within 60 days.

CONCLUSIONS AND RECOMMENDATIONS

As far as the workers already affected by the disease are concerned, consequently with no chance of cure, what could be done to help them is give them a monthly life annuity. Therefore, it is necessary that the workers be examined and considered disabled by means of a medical examination. The disease is known to exist and to be extremely serious, however, because it occurs in a deep poverty region, distant from the big cities, prevention is laid aside by the supervising, educational and medical assistance structures in charge of the matter.

It is common knowledge that silicosis prevention in general includes environmental control measures, such as:

- Product substitution
- Process alteration
- Dust suppression in the source by means of moistening
- Process insulation
- Dust removal through a general scattered ventilation, on/site exhausting ventilation or electrostatic precipitation
- Use of personal protective equipment

We consider a conventional water supply system a definite solution to the problem. A dam has already been built 18km from Tianguá, which is just waiting for a governmental decision so that supplementary works can be built and put in operation (water mains, water-treating plant and water supply system). In the rural area the solution would be the machine sinking of wells, with no direct participation of the worker in the process.

In addition to the benefits that these solutions could bring, they would also create jobs for the local population. However, these are not short term measures, thus while water-holes still need to be built as the unique source of potable water for the people, some specific measures could be carried out:

- For the use of explosives—Careful attention to safety instructions about transportation, storage and handling of these materials.
- Provide appropriate safety conditions in order to avoid falls when going down and up the pit. Provide appropriate dimensions for ropes, drums,sheaves and support beam.
- Keep the excavation site moist.
- Wait for at least 24 hrs after the explosion before entering the pit.
- Standardize a diameter for the water-hole of at least 2,50m so that the worker may have better working conditions.
- Limit the use of personal protective equipment, which should only be used as a transient solution for its several deficiencies and above all, its low efficiency and discomfort for the person who uses it.
- Cover the pit walls with pre-molded concrete rings as the excavation gets deeper.
- Make regularly the previously mentioned examinations in accordance with the current labor legislation at the expense of the Ministry of Social Welfare.

However, a question is to be asked: How are these pieces of information to get to the workers, and principally, how to make them understand how relevant they are? Dr. Márcia Alcântara has taken the first steps in relation to this. In Tianguá, on a daily radio program she talks about the general aspects of the problem attacking the pit diggers of the region. The workers themselves, after the first deaths, have mobilized to fight for better working conditions, but due to the fact that most workers are not educated, and need to support themselves and their family, they do not give attention to the matter. While definitive solutions are not adopted, it is necessary to make the transient measures known to mitigate the disease. For this to occur, we suggest the following measures be taken:

1. Campaign about silicosis prevention in the Tianguá daily radio program. Fundacentro may cooperate recording cassette tapes with this information.
2. Fundacentro can prepare posters and booklets to be distributed to health centers, town halls, church communities and labor unions.
3. Educative lectures held at community centers, health centers, schools and churches, given by technicians from the Regional labor office, Welfare and State Health office.
4. Intensification of supervision by the Regional Labor Office of preventive aspects and of the work entailment between employees and employers. Orientation about the use of explosives by the Army.
5. Facilities for purchase of personal respirators or even their distribution free of charge to workers, by the Ministry of Social Welfare together with the State Health office.

Nevertheless, we stand upon the point that the eradication of the problem will only occur completely, when there is the engagement of the federal, state and municipal governments in order to provide these populations with what they do not have so far: a potable water supply system.

DNA FLOW CYTOMOETRIC ANALYSIS OF MESOTHELIAL CELLS EXPOSED *IN VIVO* TO ASBESTOS

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ABSTRACT

Epidemiologic and experimental studies have established a causal relationship between malignant mesothelioma and exposure to asbestos and other fibrous minerals (e.g., erionite) which have similar structural but not chemical characteristics. Predicting whether a given fibrous mineral will be a human carcinogen is very difficult in view of the long latency interval (usually in excess of 30 years) seen in human cases of mesothelioma. It has been shown by others that mesothelial cells exposed to fine chrysotile fibres *in vitro* exhibit chromosomal damage associated with fibre penetration of the nucleus. In view of the likelihood of DNA damage induced by asbestos, we studied DNA profiles in mesothelial cells exposed to asbestos *in vivo*. Fischer 344 rats given a single intraperitoneal injection of 50 mg crocidolite are being followed for periods up to 18 months. Cell cycle analysis using DNA flow cytometry showed a rapid increase in DNA synthesis (S-Phase) by mesothelial cells, which peaked at 3 days and remained relatively stable until 28 days. Observations at later times have not yet been completed. The proportion of mesothelial cells in S-phase following asbestos stimulation was approximately 3 times greater than that seen in normal mesothelium. The mesothelial cell proliferative response was accompanied by an inflammatory reaction which peaked at 2 weeks. Light and electron microscopy showed asbestos fibres within mesothelial cells. The evidence so far indicates that asbestos stimulates an early and intense proliferative response in mesothelial cells. Based on DNA flow cytometric analysis of 2 human cases of mesothelioma showimg marked aneuploidy, we expect to observe the development of chromosomal abnormalities in our experimental lesion. We consider that quantitation of these changes using different types of fibrous minerals and fibrous minerals of different dimensions, will ultimately lead to a short-term bioassay of high predictive value.

INTRODUCTION

The association between asbestos exposure and diffuse malignant mesothelioma (DMM) has now been well established by experimental and epidemiological methods.[2,9,13,14] Moreover, other fibrous minerals (e.g., erionite) have also been causally implicated.[15]

The latency interval of this disease is long (usually 15–40 years from initial exposure) and death generally occurs within 12–14 months of diagnosis. DMM is exceedingly difficult to diagnose, no effective treatment exists, and currently there are no screening techniques for high-risk individuals such as asbestos workers.

In recent years, cell proliferation characteristics of normal and malignant cells have gained increasing attention and have been the focus of studies of various human and animal tumors.[6,7,10,1] Cytokineticists have relied heavily on the frequency of DNA synthesizing cells to determine the cell cycle traverse characteristics of normal and malignant cells. Structural chromosomal damage and changes in cellular DNA content have been shown in association with several tumors including mesotheliomas.[3,6,12] Analysis of two cases of human mesothelioma in our laboratory revealed DNA ploidy changes. (Figure 1) Although the mechanism(s) of these karyotypic changes are not entirely understood, it may involve a direct interaction between asbestos fibres and metaphase chromosomes.[16]

Flow cytometry is the latest and in many ways the most practical of the current adjunctive techniques for studying the cytokinetic properties of malignant tumours. However, the cell dynamics of malignant, or indeed of normal mesothelial cells are virtually unknown. To determine the possible value of DNA quantitation by flow cytometry in the evolution of DMM, we studied the cytokinetic properties, specifically the DNA synthesizing phase (S-phase) changes of mesothelial cells exposed to asbestos *in vivo*. Our main purpose was to correlate phase distribution changes of cycling cells with the early histological and cytological alterations induced by asbestos.

MATERIALS AND METHODS

Dust Suspensions

Crocidolite samples were obtained from NIOSH (courtesy Dr. V. Vallyathan). Details of fibre dimensions are shown in Figure 2.

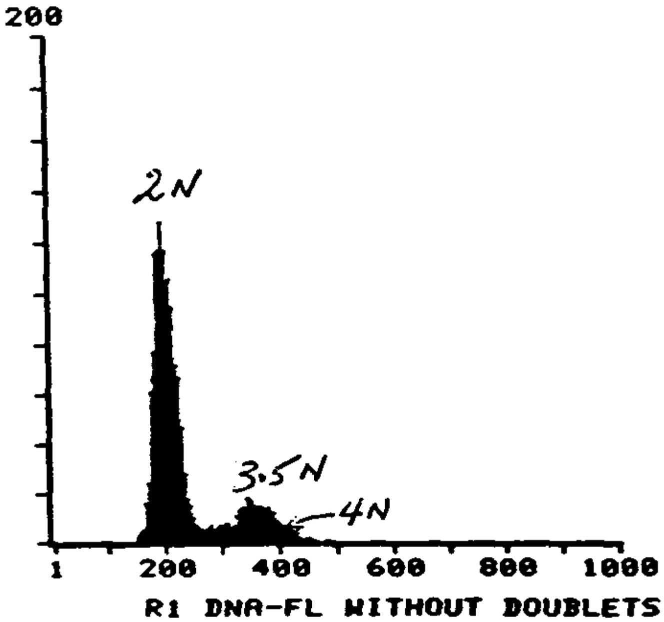

Figure 1. DNA histogram of human mesothelioma showing an aneuploid population at 3.5 N.

Animals

Male Fischer 344 rats were intraperitonially injected with 50 mg crocidolite asbestos in 10 ml of saline or with 10 ml saline alone. Uninoculated animals served as additional baseline controls. Four animals from each treatment group were sacrificed, under halothane anesthetic, at 1, 3, 7, 14 and 28 days post-inoculation.

Cell Isolation and Separation

The peritoneal cavity was lavaged thrice with normal saline to remove the majority of free-floating cells, mostly macrophages. EDTA (20 ml) was then instilled in the peritoneal cavity, which was lavaged 30 minutes later to extract mesothelial cells from the parietal peritomeum and serosal surfaces (Figure 3). The cell sheets were separated by gentle mechanical agitation. In crocidolite treated animals, firm nodules surrounding asbestos fibres were present throughout the omental and serosal surfaces. These were surgically excised. Cells were extracted by mechanical dissociation, and suspended in RPMI 1640 media.

Cytology

Cytological smears from the peritoneal lavage fluids and from single cell suspensions of the reactive modules, were spray-fixed with Cytospray, an alcohol-based fixative, and stained with Papanicalaou. Differential cell counts were performed on 100 cells for each specimen. A hemocytometer was used to obtain total cell counts.

Histology

Tissue blocks obtained from the reactive peritoneal nodules and the parietal and visceral peritoneal surfaces, were fixed in Carnoy's solution (60% ethanol, 30% acetic acid and 10% chloroform), and processed in paraffin. 5 μm tissue sections were stained with hematoxylin and eosin and examined by light microscopy.

Scanning Electron Microscopy

Samples of parietal and visceral peritoneum were immediately excised from animals at termination and immersed in Karnovsky's fixative. Following complete fixation, samples were dehydrated in graded concentrations of ethyl alcohol, critical point dried and coated with gold/palladium. Specimens were examined in a Hitachi S-450 scanning electron microscope.

Flow Cytometry

Single cell suspensions from the above tissues were prepared in RPMI media and fixed in 50% ethanol. The cells were washed twice in PBS and resuspended in 1 ml of PBS, containing 5 μg of Propidium iodide (Sigma #P-4170, Lot 107F-08201). (Figure 4) This suspension was then filtered through a 44 μ nylon mesh to exclude cell clumps. Cell cycle analysis was performed with a FACS Ortho® flow cytometer. During flow cytometry, cells were excited at 488 nm. Red fluorescence from Propidium iodide (PI) was collected through a 600 nm wavelength long pass filter and recorded as a measure of total DNA.

The resulting electrical pulses were stored in the memory unit of a pulse height analyzer and displayed as a histogram. The phase distribution of each cell population was ascertained from the DNA histogram.[8]

RESULTS

Cytology

Analysis of cells isolated from the abdominal cavity by saline wash (free floatimg cells) from all groups, showed that more

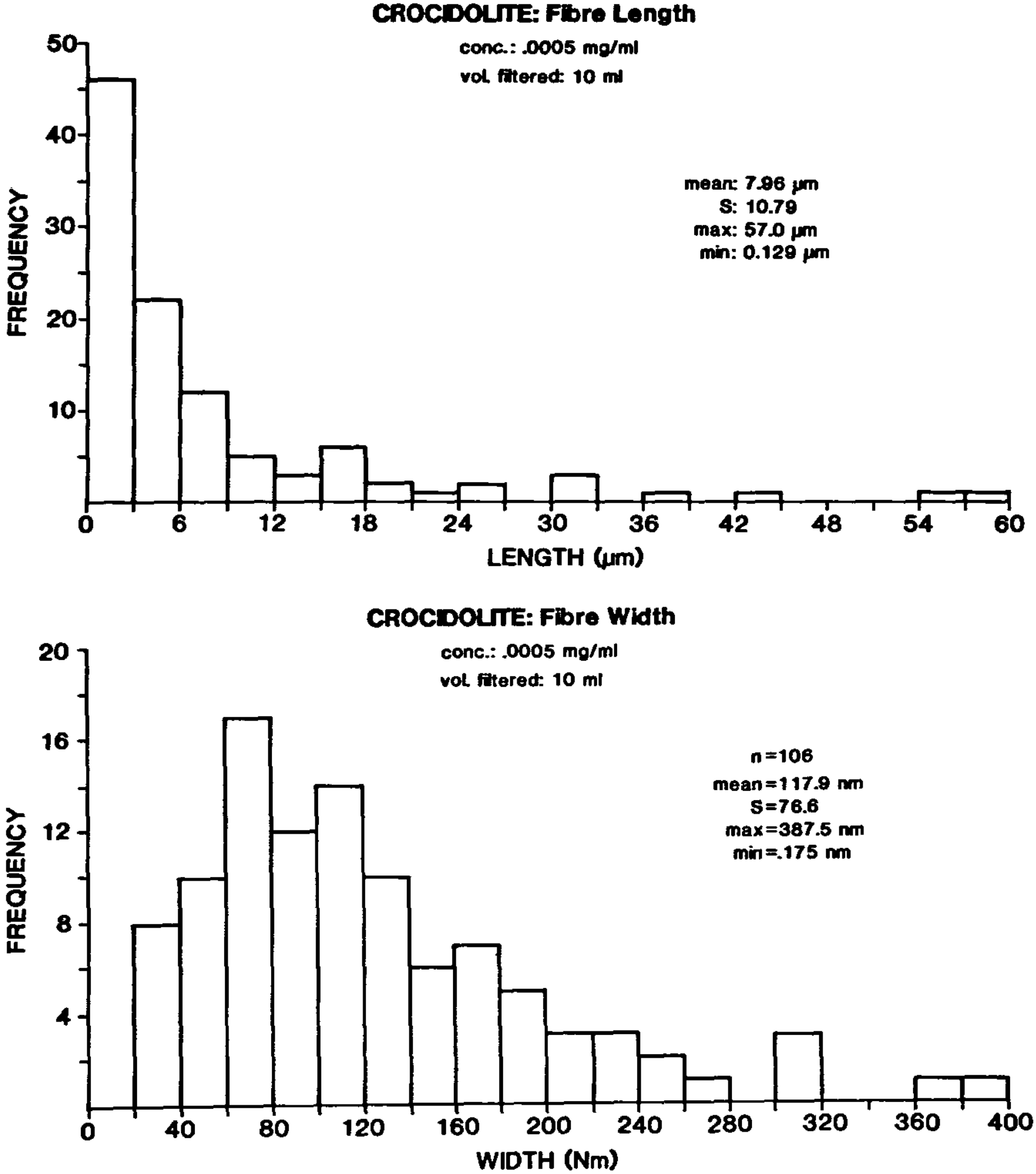

Figure 2. Crocidolite asbestos—fibre dimensions: a) fibre length; b) fibre diameter.

Figure 3. Sheet of normal mesothelial cells obtained by EDTA extraction (Papanicalaou stain X 400).

than 90% were macrophages and polymorphonuclear cells. Rats injected intraperitoneally with saline showed a small increase in inflammatory cells at 24 hrs which returned to normal values by 3 days. (Figure 5) Asbestos injection, on the other hand, produced an intense and sustained inflammatory reaction which peaked at 14 days. Lavageates from asbestos exposed animals also contained large numbers of red blood cells, at 1, 3 and 7 days.

Cytological examination of cells extracted from the mesothelial surfaces of saline injected rats by EDTA, showed that over 90% were of mesothelial type. ETDA extraction of asbestos treated rats produced a more mixed cellular profile, with mesothelial cells constituting 70–90% of the total population. The number of cells retrieved from saline injected rats remained relatively constant at all time periods. (Figure 5) Maximal retrieval of mesothelial cells in the crocidolite treated group occurred at 7 days, reflecting the increased proliferative response (see below).

Histology and Scanning Electron Microscopy

The normal mesothelium consisted of a single layer of flattened mesothelial cells on a basal lamina overlying a thin connective tissue stroma. A distinct submesothelial cell population was not apparent by light microscopy.

Asbestos injection produced an early and intense acute inflammatory reaction in the mesothelial and submesothelial tissues, consisting of capillary dilatation, edema and macrophage and polymorphonuclear cell infiltration. The serosal surfaces were covered with a fibrinous exudate. (Figure 6) This inflammatory response was observed at all time periods, but by 28 days macrophages predominated in the inflammatory infiltrate.

Initially, the asbestos fibres lay on the peritoneal surfaces and were concentrated on the omentum, upper small intestine, and around the liver, spleen and stomach. Fewer fibres were noted on the parietal surfaces of the peritoneal cavity. At subsequent time intervals, the majority of fibres were actively phagocytosed by reactive mesothelial cells and incorporated into the submesothelial tissues. Fibres also became trapped within the tissues through infolding of omental surfaces (Figure 6). Incorporation of fibres into the peritoneum was associated with the proliferation of both surface and submesothelial cells (Figure 7). The former became multilayered and developed hyperchromatic and irregularly shaped nuclei. Many assumed bizarre multinucleate forms (Figure 6). Scanning electron micrographs revealed these cells to be highly abnormal and many had fibres penetrating their cytoplasm. (Figure 8) Submesothelial cell proliferation was associated with diffuse thickening of the peritoneum and nodule formation. (Figures 9, 10) At 28 days post-injection, collagen was also found within the nodules.

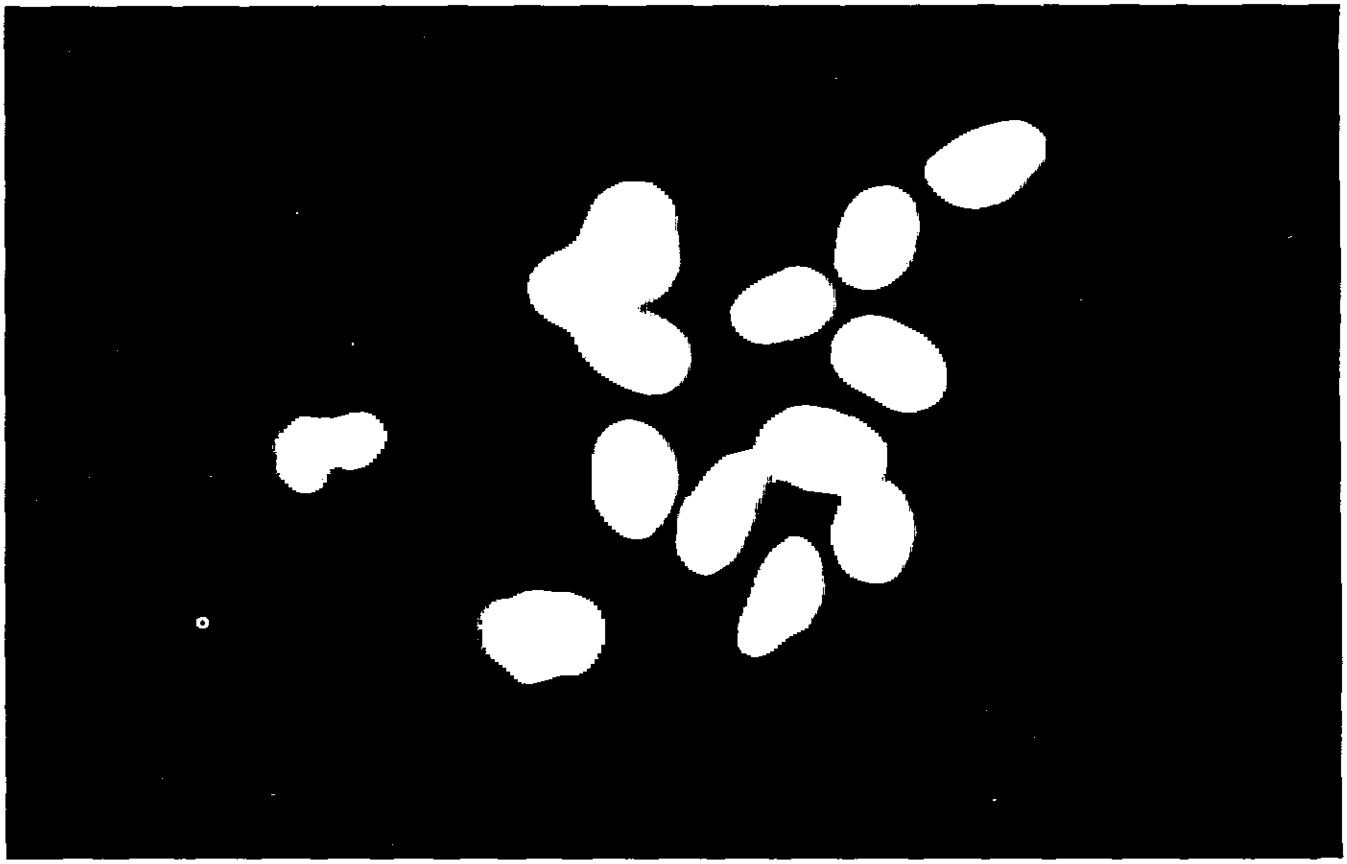

Figure 4. Fluorescent micrograph of normal mesothelial cells stained with Propidium iodide (X 1000).

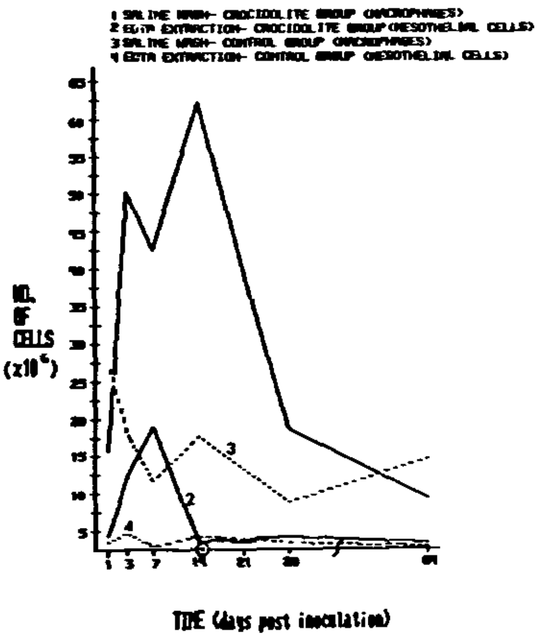

Figure 5. Total and differential cell counts in lavageates from abdominal cavities of rats exposed to crocidolite asbestos.

Flow Cytometry

Cell cycle analysis of inflammatory cells retrieved from the abdomimal cavities, showed similar phase distribution for control and asbestos treated rats. The S-phase values were low and ranged from 0.5–3%. Values pertaining to mesothelial cells differed markedly between the two groups (Figure 11). Approximately 7% of normal mesothelial cells were in the S-phase of the cell cycle at any given time. Saline injection produced a small increase in DNA synthesizing cells (S-phase) at 24 hours, which peaked at 3 days and returned to normal values at 7 days. Crocidolite asbestos, by contrast, produced a rapid increase in the proportion of DNA synthesizing mesothelial cells which reached maximal values at 3 days and was sustained up to 28 days.

Discussion

The inflammatory and proliferative changes associated with intraperitoneal injection of asbestos, have been well documented by others.[3,4,14] In this study, saline injection alone produced a mild inflammatory response and increased proliferation of mesothelial cells. This response was short lived and normal values returned by 7 days.

Cell cycle analysis of inflammatory cells retrieved from the abdominal cavities of both saline and asbestos treated animals, showed similar distribution of cells in S-phase. These values were typically low (1–2%) and indicate that

Figure 6. Section of visceral peritoneum (omentum) from rat dosed with 50 mg crocidolite IP 7 days previously. There is an intense proliferation of surface and subepithelial mesothelial cells associated with a fibrinous and inflammatory response. The surface mesothelial cells also show giant cell formation and nuclear atypia (X 100 hematoxylin and eosin).

inflammatory cells migrating into the abdominal cavity, are terminally differentiated and do not proliferate in response to asbestos stimulation.

Mesothelial cells, on the other hand, developed an early and sustained proliferative response to asbestos with S-phase populations constituting 20–30% of the total mesothelial cell population. The relatively high S-phase value (7%) for normal mesothelial cells indicates that these cells have a fairly rapid turnover rate. Unfortunately, there is very little recent information on cell cycle characteristics of normal mesothelial cells. Experiments have been initiated in our laboratory to determine these parameters, using DNA flow cytometry combined with bromodeoxyuridine (BuDr) incorporation.[5]

At this stage, it is not possible to say whether the changes observed in mesothelial cell populations exposed to asbestos, are specific for this mineral type or whether they progress to neoplasia. Preliminary experimental studies with Fischer rats suggest that intraperitoneal injection of other dusts (silica, iron oxide and wollastonite) induce less proliferative activity of mesothelial cells than crocidolite asbestos. It should be noted that wollastonite is a fibrous silicate mineral that has potential for substitution in asbestos products. If these preliminary experiments are confirmed, they would indicate the potential utility for this approach as a short term bioassay for assessing the carcinogenic potential of fibrous minerals.

Analysis of two human mesothelioma cases showed a definite second population of cells in both instances with DNA con-

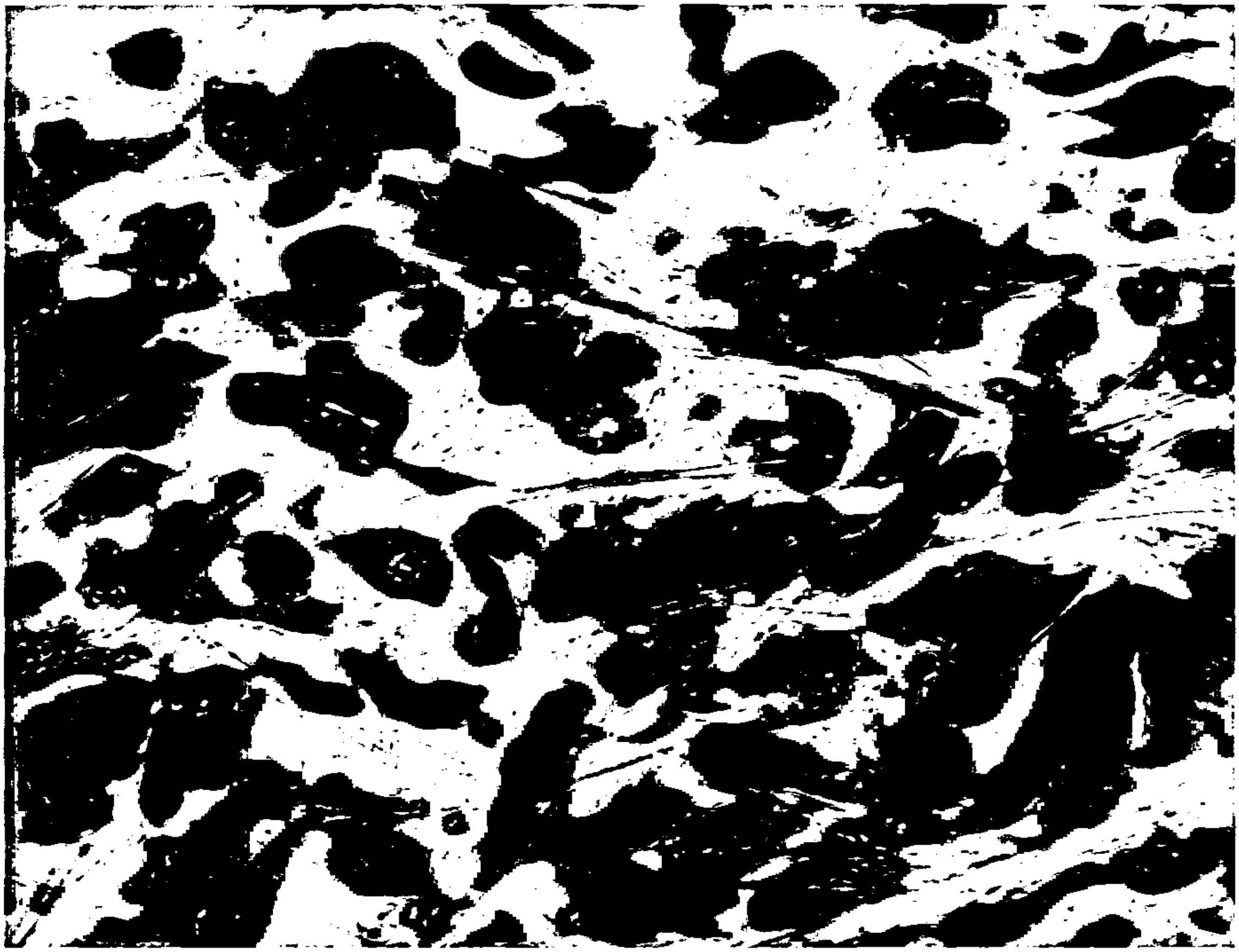

Figure 7. Proliferating mesothelial cells in juxtaposition to asbestos fibres (X 400 hematoxylin and eosin).

tents of approximately 3.5 N (Figure 1). Craighead et al., have reported similar ploidy changes in experimentally induced malignant mesotheliomas in rats.[3] We therefore expect to see the development of malignant change and changes in DNA ploidy in our experimental animals. We are particularly concerned to determine the time of onset of these changes, the clonality of the resulting tumors and the presence or absence of specific cytogenetic abnormalities which might serve as markers of exposure to asbestos or other carcinogenic fibrous minerals.

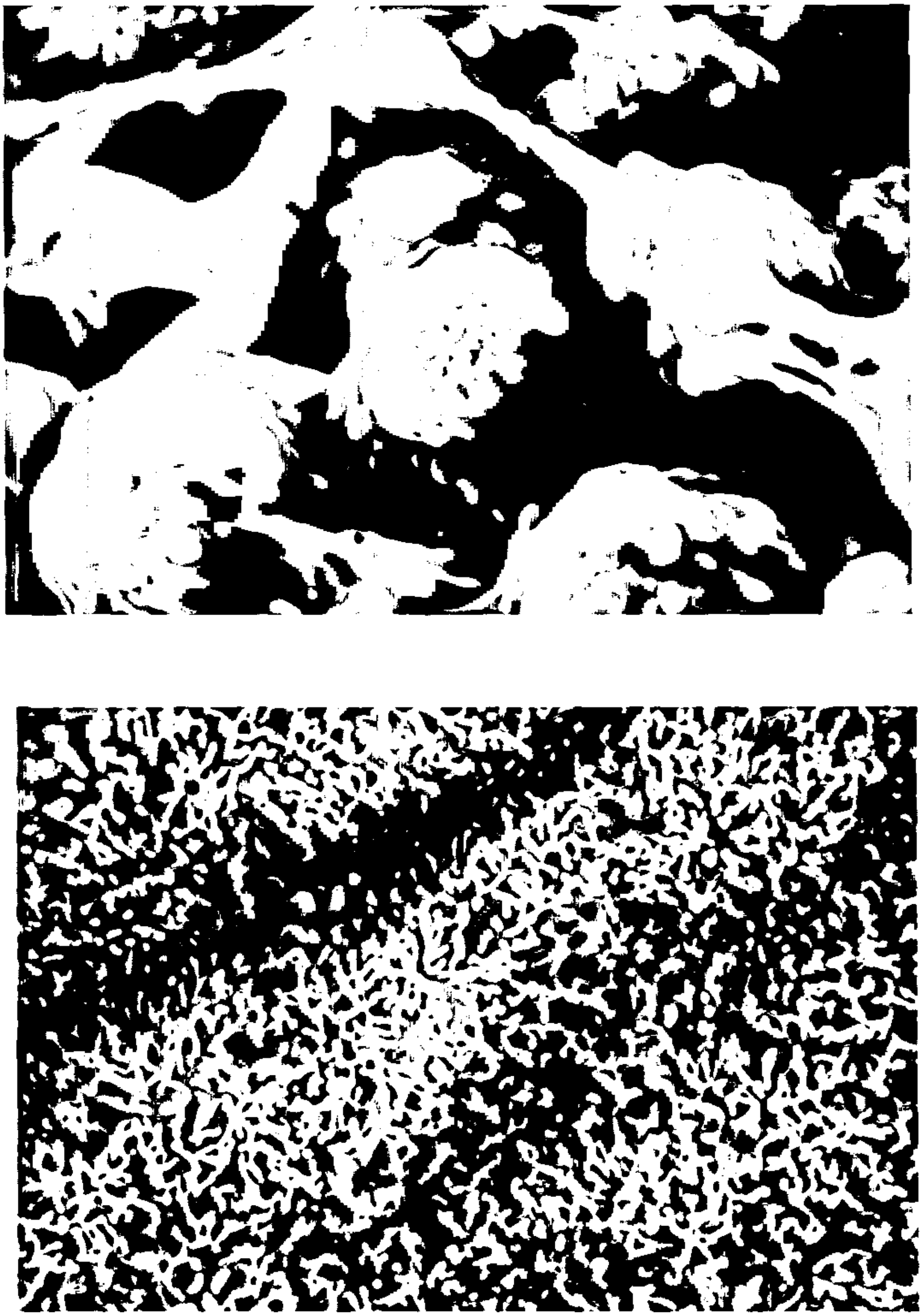

Figure 8. A. Scanning electron micrograph of peritoneal surface of rat two weeks after receiving 50 mg crocidolite asbestos I.P. The mesothelium is hyperplastic and shows incorporation of asbestos fibres into the mesothelial cells.
B. Normal mesothelium (X 2000).

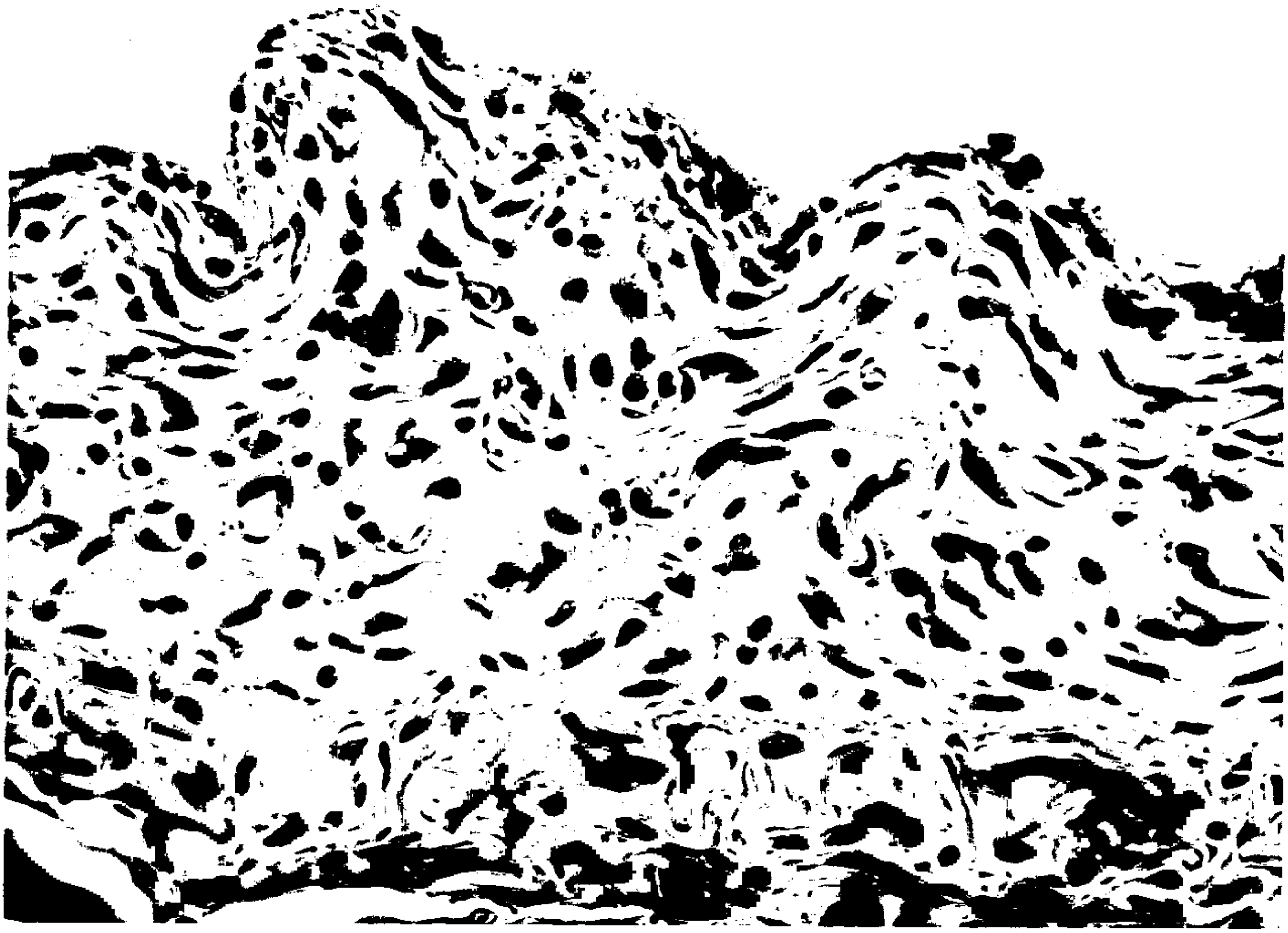

Figure 9. Serosal aspect of abdominal cavity from rat exposed to crocidolite asbestos two weeks previously. There is a marked proliferation of spindle-shaped submesothelial cells (X 250 hematoxylin and eosin).

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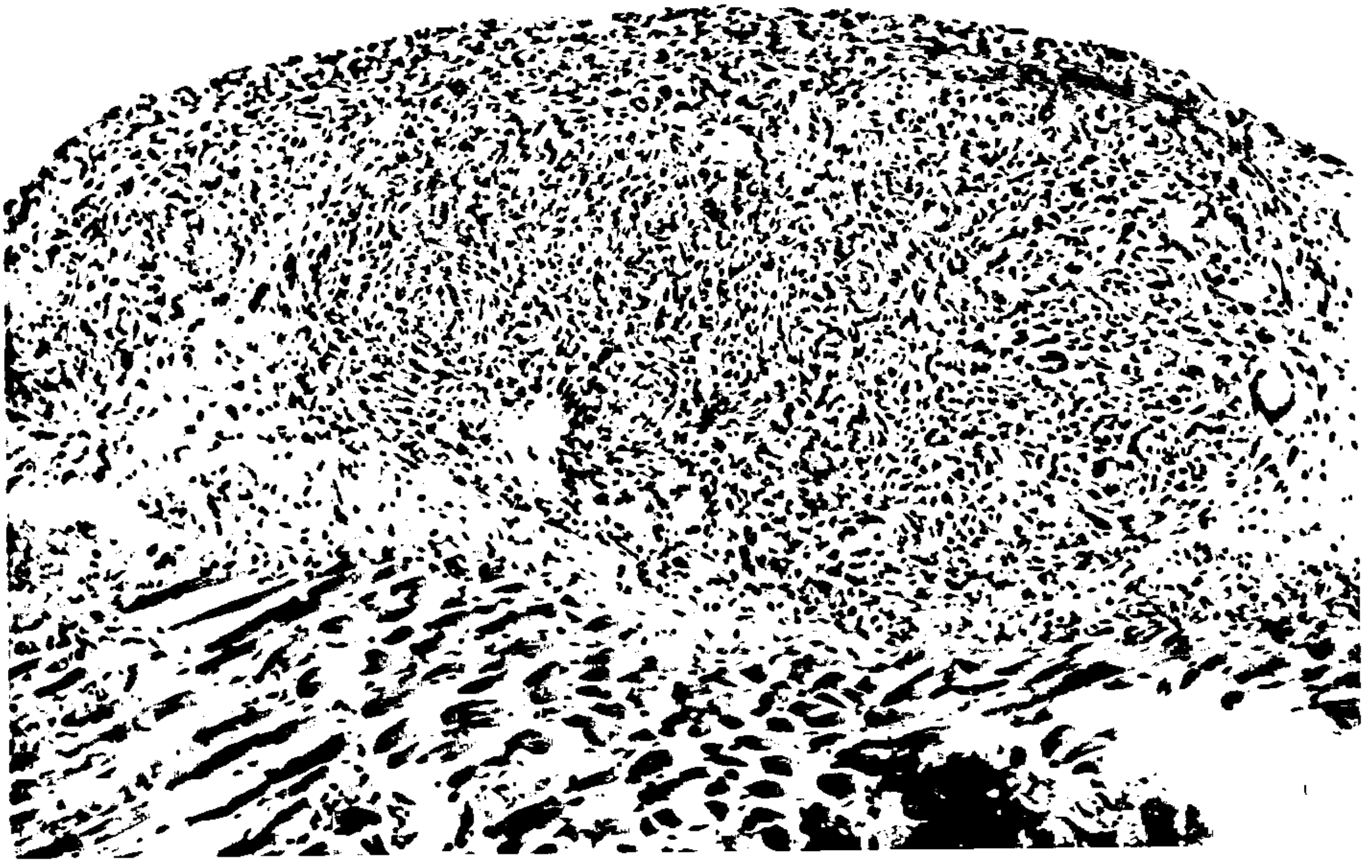

Figure 10. Nodular lesion on parietal aspect of peritoneum of a rat 7 days after receiving 50 mg crocidolite I.P. Giant cells are common in this lesion (X 100 hematoxylin and eosin).

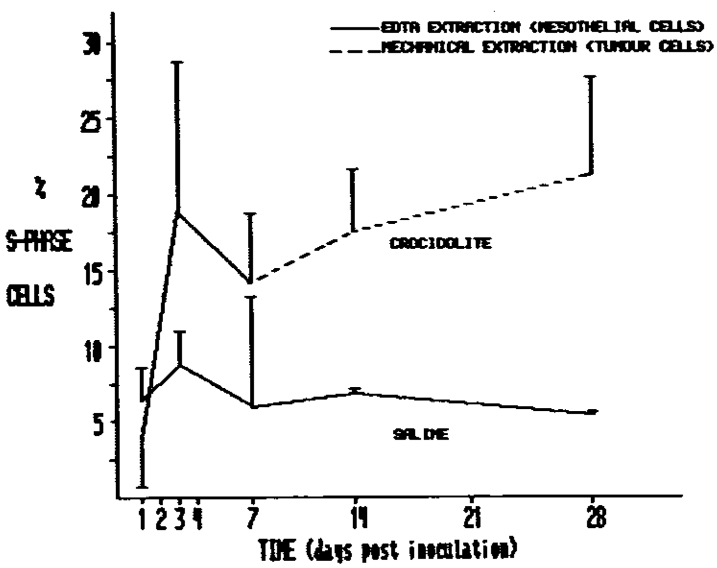

Figure 11. Percentage change in mesothelial cells in S-phase with time in response to intraperitoneal injection of asbestos or normal saline.

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