Black Spots Concentrate Oncogenic Asbestos Fibers in the Parietal Pleura

Thoracoscopic and Mineralogic Study

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Epidemiologic and pathologic data demonstrate that malignant mesothelioma occurs preferentially after exposure to long amphibole asbestos fibers. However, mineralogic studies have rarely detected such fibers in the parietal pleura. We hypothesized that the distribution of asbestos fibers in the pleura was heterogeneous and that they might concentrate in certain areas, as does coal dust in patients showing anthracotic "black spots" of the parietal pleura during thoracoscopy. We collected thoracoscopic biopsy samples from these black spots and from normal areas of the parietal pleura and lung from 14 subjects (eight with and six without asbestos exposure). Asbestos content was determined by transmission electron microscopy. In exposed subjects, mean fiber concentrations were $12.4 \pm 9.8 \times 10^6$ fibers/g of dry tissue in lung, 4.1 ± 1.9 in black spots, and 0.5 ± 0.2 in normal pleura. In unexposed patients, concentrations were 0, 0.3 ± 0.1, and 0, respectively. Amphiboles outnumbered chrysotile in all samples. A total of 22.5% of fibers were ≥ 5 μm in length in black spots. A histologic similarity of these black spots with milky spots is suggested by conventional and electron microscopy. We conclude that the distribution of asbestos fibers is heterogeneous in the parietal pleura. Indeed, the fibers concentrate in black spots, where they can reach high concentrations. These findings could explain why the parietal pleura is the target organ for mesothelioma and plaques. **Boutin C, Dumortier P, Rey F, Viallat JR, De Vuyst P. Black spots concentrate oncogenic asbestos fibers in the parietal pleura: thoracoscopic and mineralogic study.** AM J RESPIR CRIT CARE MED 1996;153:444–9.

Asbestos fibers that reach the respiratory bronchioles and alveoli are subject to different fates. Chrysotile fibers can undergo fragmentation, lixiviation, and progressive clearance, whereas amphibole fibers may remain unchanged for decades (1, 2). If concentrations are sufficiently high in the lung, asbestosis (3) and eventually bronchial carcinoma (4) may develop. In patients with these conditions, asbestos bodies, mostly formed on amphiboles, are usually found in lung sections and bronchoalveolar lavage fluid (5). Fibers may also migrate towards the periphery of the lung (6), especially the lower lobes (7), into mediastinal lymph nodes (8, 9) and the pleura.

Benign pleural plaques and malignant mesothelioma are the main manifestations of occupational or environmental exposure to asbestos fibers (10). Plaques usually develop on the parietal pleura or on the diaphragm. Malignant pleural mesothelioma also originates from the parietal pleural (11). Up to now, however, only few studies have detected significant amounts of asbestos fibers in the parietal pleura.

Human lung tissue analyses have demonstrated that the risk for mesothelioma is related to the concentration of long (> 8 μm) amphibole fibers (12, 13). Animal experiments using intracavitary injections of asbestos confirmed that the most carcinogenic fibers were those measuring ≥ 8 μm in length and < 0.25 μm in diameter (14, 15). In sharp contrast with these data is the fact that all previous mineralogic studies have shown that short chrysotile fibers are the most common type of asbestos present in the parietal pleura (2, 16–19). Amphiboles fibers meeting Stanton's dimensional criteria for carcinogenicity (14) are uncommon or absent.

A possible explanation for this paradox could be that fibers are heterogeneously distributed in the parietal pleura. This would explain why random sampling in unselected areas yields poor concentrations. Indeed, when samples were taken from pathologic zones such as pleural plaques (18) or tumors (19), more fibers were found.

Several findings led us to speculate that asbestos fibers could accumulate in certain areas of the parietal pleura. In mice, subcutaneously injected fibers are known to concentrate in the milky spots of the parietal pleura (20). In humans, milky spots are quite invisible in the healthy pleura. However, thoracoscopy sometimes visualizes foci of parietal anthracosis near lymphatic vessels of the parietal pleura (11), which we have called "black spots." Indeed, these black spots could correspond to the milky spots described by Kanazawa and colleagues in mice (20) and be rendered visible by trapped coal dust.

The present study was carried out to ascertain whether asbestos fibers do concentrate in these black spots. Samples were collected from (*1*) black spots, (*2*) adjacent, macroscopically normal pleura, and (*3*) peripheral lung, in a series of 14 consecutive patients in whom anthracotic spots were detected on parietal pleura during thoracoscopy. The results of mineralogic analysis were compared.

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METHODS

In our department, we perform more than 200 diagnostic video-thoracoscopies a year for pleurisy, spontaneous pneumothorax, and diffuse interstitial lung fibrosis. Thoracoscopic and sampling techniques have been previously described (21, 22). From January 1993 through February 1994, we collected samples from patients in whom visual inspection of the parietal pleura revealed anthracotic black spots with or without anthracotic lymphatic vessels. In addition to the anthracotic parietal samples, specimens were taken from an area of macroscopically normal pleura at a distance of 2 cm ("nonanthracotic parietal samples") and from the peripheral lung opposite the black zone ("lung samples").

Lung biopsy samples were prepared by sodium hypochlorite digestion (5). Because bleach digestion of parietal pleura samples does not provide good preparations, the latter samples were dehydrated, defatted in alcohol and xylene, dried, and ashed in a low-temperature asher. The resulting particles were collected on 0.45-μm-porosity cellulose filters (Millipore®; Millipore Corp., Bedford, MA). After fusion by acetone vapors, pieces of filter were carbon-coated and transferred to transmission electron microscopy (TEM) grids. Grids were studied in a Philips EM400T analytical electron microscope equipped with an EDAX PV9900 energy dispersive X-ray analyzer. A magnification of ×22,000 was used to detect all fibers longer or equal to 1 μm, while a ×4,600 magnification detected only fibers equal or longer than 5 μm. Theoretical detection limits were around 200,000 fibers/g of dry tissue (f/g) for fibers longer than 1 μm and 100,000 f/g for fibers longer than 5 μm. These values were statistically fitted for each sample, depending on the actual concentration of fibers (*see* Table 3). Fibers were identified on the basis of morphology, chemical composition (EDS spectrum), and, in some cases, electron diffraction pattern.

Since data did not show a normal statistical distribution, the Wilcoxon and Mann-Whitney nonparametric tests were used to compare the mean fiber concentration and size values. Because particle size distributions are usually log normal, statistical comparison between lengths and widths were also performed on geometric size data.

RESULTS

Patients

Samples were obtained from 14 subjects. The characteristics of these patients are given in Table 1. Mean age was 60.3 ± 11.2 yr. Eight patients had a history of asbestos exposure: occupational in six patients, environmental in one patient who lived in Corsica (23), and both occupational and environmental in one patient. Mesotheliomas were confirmed by the panel of pathologists of the French Mesothelioma Register.

Thoracoscopic features of parietal pleural anthracosis were observed in all 14 patients. In 12 patients, the lesions consisted of black spots 2 to 4 mm in diameter. In two of these 12 patients, the spots were isolated and, in nine patients, clusters of three to four spots were observed on the lower, posterolateral part of the costo-vertebral gutter (Figure 1). The remaining patient was a former coal miner who had many black spots scattered from the bottom to the top of the pleural cavity. In two patients, anthracotic lesions were located along lymphatic vessels that were very superficially located and clearly distinguishable from intercostal veins. Both black spots and black lymphatic vessels were observed in intercostal spaces near intercostal veins and arteries and were never found on ribs.

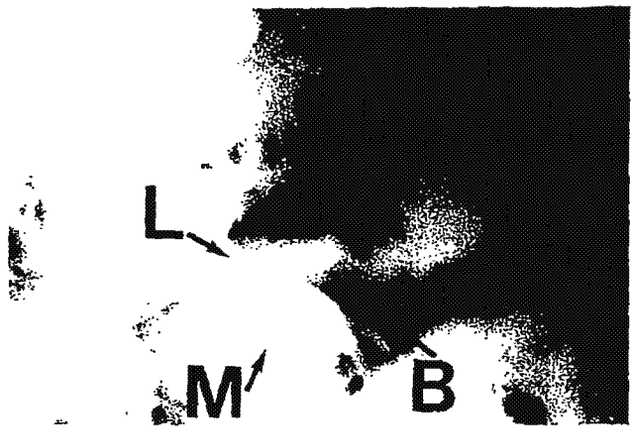

Figure 1. Black spots (B) located in an intercostal space. White lymphatic vessels (L) and malignant mesothelioma nodules (M) are visible in close contact with black spots. The main nodule is about 3 mm in diameter.

In 11 of 14 patients, samples were obtained from the parietal pleura (anthracotic and nonanthracotic) as well as from the lung. In the remaining three patients, lung samples were not available (patients 8, 13, and 14). In patient eight, lung sample was taken but pathologic study revealed only thick visceral pleura tissue without any lung structure. In patient 13 (spontaneous pneumothorax in association with AIDS) and in patient 14 (70 yr old), lung biopsy was not performed to avoid further complications.

Mean sample weight was 4.4 ± 2.9 mg of dry tissue for lung samples, 5.5 ± 3.6 mg for anthracotic parietal samples, and 3.1 ± 2.6 mg for nonanthracotic parietal samples.

TABLE 1

PATIENT CHARACTERISTICS

Patient No.	Sex	Age (yr)	Occupation	Asbestos Exposure	Diagnosis
1	M	58	Insulation worker	+	Asbestos pleural plaques
2	M	55	Tunnel digger	+	Interstitial lung fibrosis
3	M	65	Insulation worker	+	Spontaneous pneumothorax
4	M	63	Shipyard worker	+	Pleural mesothelioma
5	F	65	Seamstress	+	Pleural mesothelioma
6	M	61	Mechanic	+*	Pleural mesothelioma
7	M	68	Metallurgy worker	+	Pleural adenocarcinoma
8	M	59	Railroad worker	+†	Pleural plaques
9	M	45	Hairdresser	–	Lung cancer
10	M	65	Printer	–	Lung cancer
11	M	46	Freight handler	–	Infectious pleural effusion
12	M	83	Coal miner	–	Pleural adenocarcinoma
13	M	40	Restaurant operator	–	Spontaneous pneumothorax‡
14	F	70	Hotel operator	–	Idiopathic pleural effusion

* Environmental and occupational exposure.
† Environmental exposure in Corsica.
‡ AIDS-related.

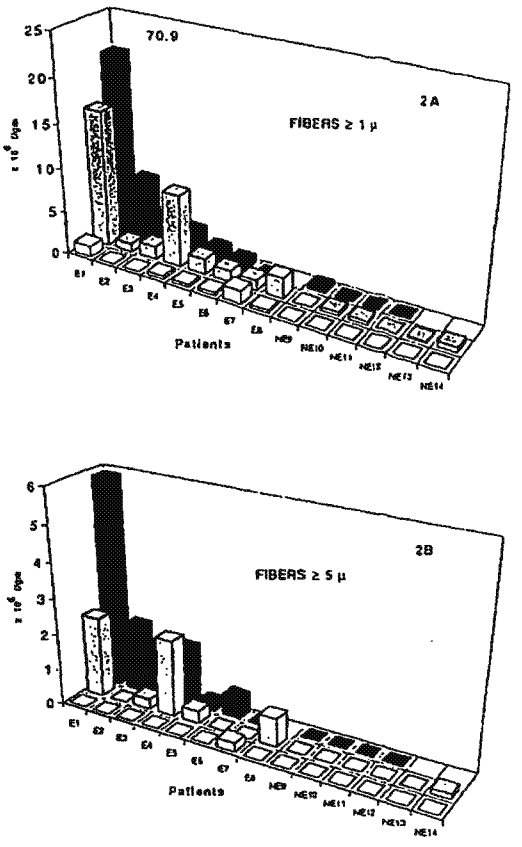

Figure 2. Concentration of asbestos fibers ≥ 1 µm (*A*) and ≥ 5 µm (*B*) as a function of sample types. (*white bars*: normal pleura; *grey bars*: pleural black spots; *black bars*: lung). Results are ranked in order of decreasing fiber concentration in lung biopsy. Patients 1 to 8 were previously exposed to asbestos (E1–E8). Patients 9 to 14 have no known previous exposure (NE9–NE14). Empty square-sample not available.

Fiber Distribution

The concentration of fibers observed in different samples is shown in Figure 2. Fibers 1 µm or longer (Figure 2A) were present in seven of 11 lung samples, in 12 of 14 anthracotic parietal samples, and in six of 14 nonanthracotic parietal samples.

Extreme values in lung samples ranged from 0 to 70.9 × 10^6 f/g of dry tissue, with a mean of 7.9 ± 6.3 × 10^6 f/g. In anthracotic parietal samples, fiber concentration ranged from 0 to 15.7 × 10^6 f/g, with a mean of 2.5 ± 3.7 × 10^6 f/g. The two mean values were not statistically different. In six of 11 cases, fiber concentration was higher in anthracotic parietal samples than in lung samples. In nonanthracotic parietal samples, mean fiber concentration was 0.3 ± 0.13 × 10^6 f/g of dry tissue. This is significantly lower than in lung or anthracotic parietal samples ($p < 0.01$). Asbestos fibers were detected in normal pleural tissue only if fiber concentration in anthracotic lesions exceeded 1 × 10^6 f/g of dry tissue.

Visual comparison of TEM photographs from a given patient showed similar particle burden in lung and anthracotic parietal samples and practically no particles in nonanthracotic parietal samples. The weight of tissue per surface unit of filter was roughly the same for the three samples (Figure 3). In addition to coal and amphibole fibers, black spots also contained rutile and nonfibrous particles such as silica, felspar, talc, kaolinite, mica, and metallic oxides (Fe, Al, Ti).

Fiber Concentration and Previous Asbestos Exposure

In asbestos-exposed subjects, mean fiber concentrations were 12.4 ± 9.8 × 10^6 f/g of dry tissue in lung, 4.1 ± 1.9 in black spots, and 0.5 ± 0.2 in normal pleura. In unexposed patients, concentrations were 0, 0.3 ± 0.1, and 0, respectively (Table 2).

Known or probable asbestos exposure was consistently correlated with fiber concentration in the lung ≥ 1 × 10^6 f/g (Figure 2A). However, no fibers were observed in the lung of patient seven, who had both chrysotile and tremolite fibers in anthracotic and nonanthracotic parietal samples. The possibility of inadequate sampling of lung tissue cannot be excluded.

In the three patients with mesothelioma, asbestos burden was greater in anthracotic samples than in lung samples. This difference was not significant, however, probably because of the small number of patients studied.

Types of Asbestos Fibers

Table 3 summarizes the types of asbestos found in the different samples. Chrysotile fibers were found in small amounts in only one lung sample (patient 5) and in two anthracotic and four nonanthracotic pleural samples from exposed patients.

Amphibole fibers accounted for 99.9% of fibers recovered in lung samples, 95.5% of fibers in black spots, and 61.3% of fibers in nonanthracotic samples. Crocidolite and amosite were the most common in subjects with a history of occupational exposure to asbestos. Tremolite, which is characteristic of environmental exposure in Corsica (two patients in this series), was the second most common type.

Fiber Dimensions

Table 4 shows the mean dimensions and length distribution of the fibers obtained from the different samples. There was no significant difference in length between the fibers from lung and anthracotic pleural samples. Conversely, there was a significant difference in diameter, pleural fibers being broader than pulmonary ones ($p < 0.001$).

Table 2 and Figure 2B show the concentrations of fibers 5 µm or longer: significant amounts were found only in lung and anthracotic pleural samples, except in patient 7, who also had fibers in nonanthracotic pleural samples. All fibers ≥ 5 µm were amphiboles (crocidolite, amosite, and tremolite) and were associated with previous asbestos exposure, except in patient 14 (in whom amphibole concentration was at the limit of detection). As shown in Table 2, the mean concentration of long fibers was 1.7 × 10^6 f/g in lung samples and 0.7 × 10^6 f/g in anthracotic pleural samples, the difference being nonsignificant. The proportion of long fibers (Table 4) was the same in lung and anthracotic pleural samples (21.7 and 22.5%, respectively).

Microscopy of Black Spots

Light microscopic examination of black spots revealed the presence of anthracosis associated with a concentration of lymphocytes, plasmocytes, and macrophages. Polynuclear neutrophils and eosinophils were scarce. Whenever it could be visualized, the surface of the mesothelial layer was normal. Congestion of capillaries was sometimes observed, and lymphatic vessels were noted in two patients. In one patient, a giant-cell granuloma was observed.

Scanning electron microscopy (Figure 4) confirmed the presence of lymphocytes, red cells, and, most importantly, active macrophages. These findings are in agreement with those reported in milky spots in animals by Kanazawa (24). Pores and crevices

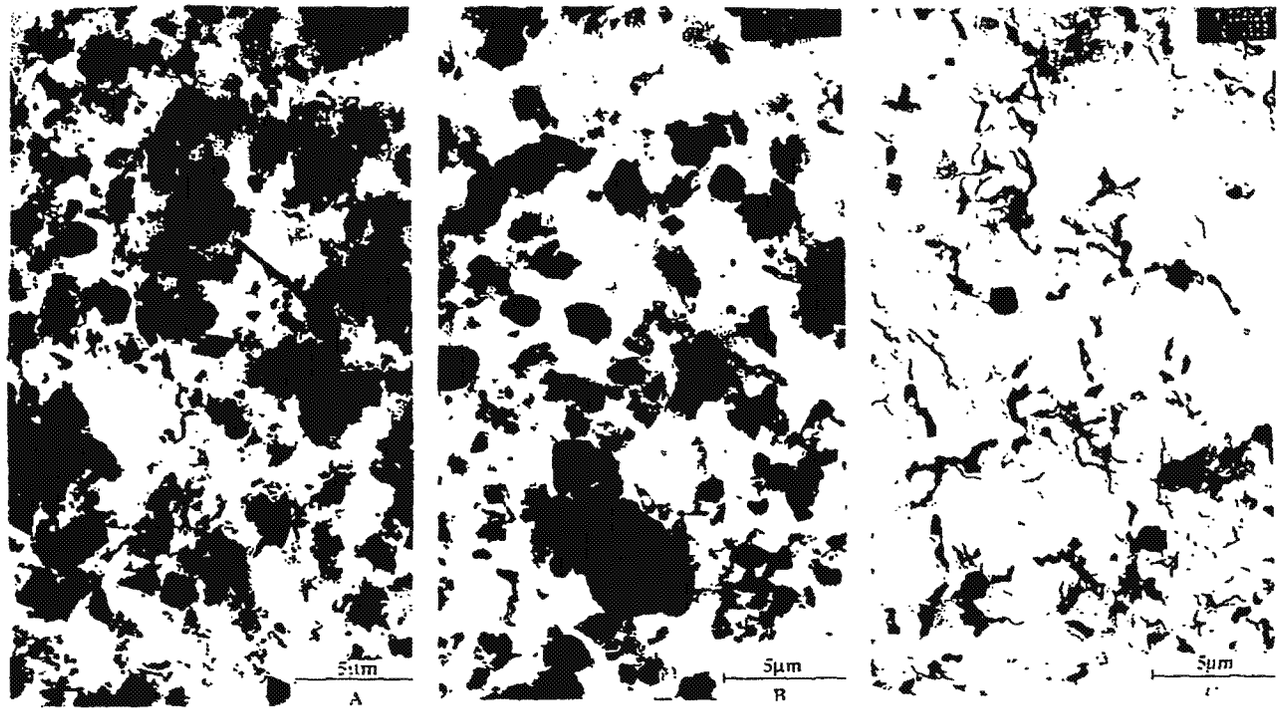

Figure 3. Comparison of particle burden in samples from the same patient (patient 2) (TEM photographs; original magnification: ×3,600). Burden was similar in lung (*A*) and anthracotic parietal (*B*) samples. Particles were virtually absent from nonanthracotic parietal samples (*C*).

resembling Wang stoma (25) and corresponding to pleural-lymphatic communications were also visualized.

DISCUSSION

Black Spots and Milky Spots

Inhaled coal and black dust that reach the deep lung are engulfed by alveolar macrophages, some of which are drained through lymphatic vessels towards the visceral pleura (6) and mediastinal lymph nodes (8, 9, 18). Thus, anthracosis has been reported to be a good marker of pleural lymphatic vessels (26–28). Anthracotic deposition in the parietal pleura is characterized by a pigmentation of lymphatic vessels along the intercostal spaces and by clusters of "black spots" located in the posterior and lower part of the costo-vertebral gutter and on the diaphragm. Jones (26) also reported in coal miners black spots within lymphatic vessels located in the middle of the diaphragm and along the lower ribs in the back of the thoracic cavity.

TABLE 2

CONCENTRATIONS OF ASBESTOS FIBERS IN TISSUE SAMPLES FROM PATIENTS WITH AND WITHOUT A HISTORY OF ASBESTOS EXPOSURE (× 10^6 f/g OF DRY TISSUE)*

Tissue Sample	Length (µm)	Exposed	Unexposed
Lung	⩾ 1	12.37 ± 9.8^a	–
	⩾ 5	1.67 ± 0.75^b	–
Black spots	⩾ 1	4.13 ± 1.86^{ac}	0.26 ± 0.1^c
	⩾ 5	0.73 ± 0.47^b	–
Nonanthracotic	⩾ 1	0.52 ± 0.2^c	–
Parietal pleura	⩾ 5	–	–

* Values are expressed as mean ± SEM. – = not detected. Statistical analysis: a–a: NS; b–b: NS; c–c $p < 0.01$.

Several findings suggest that these black spots correspond to the milky spots observed in animals. Scanning electron microscopy has confirmed the similarity between these black spots and the milky spots described by Kanazawa (24). Both structures contain inflammatory cells (macrophages and lymphocytes).

Concentration of Asbestos Fibers in the Pleura and Lung

The clear-cut concordance between fiber burden in black spots

TABLE 3

CONCENTRATION OF AMPHIBOLE AND CHRYSOTILE FIBERS (× 10^6 f/g OF DRY TISSUE) IN VARIOUS TISSUE SAMPLES OF 14 PATIENTS

Patient No.	Lung		Black Spots		Nonanthracotic Pleura	
	A	C	A	C	A	C
Exposed						
1	70.91	–	15.66	–	0.83	0.42
2	7.08	–	0.6	0.4	–	–
3	3.59	–	1.46	–	–	–
4	2.61	–	8.28	–	0.37*	
5	1.33	0.063*	1.83	–	–	0.35*
6	1	–	1.28	–	0.33	–
7	–	–	–	1.2	1.03	0.3*
8	ND	ND	2.42	–	–	0.33
Nonexposed						
9	0.65	–	–	–	–	–
10	–	–	0.4	–	–	–
11	–	–	0.39*	–	–	–
12	–	–	–	–	–	–
13	ND	ND	0.17*	–	–	–
14	ND	ND	0.6	–	–	–

Definition of abbreviations: A = amphiboles; C = chrysotile; – = fibers not detected; ND = not done.

* Concentrations within or under detection limit.

TABLE 4

MEAN DIMENSIONS (μm) AND PROPORTIONS OF FIBERS IN LUNG SAMPLES AND PLEURAL BLACK SPOTS

	Lung	Black Spots	p Value
Mean length*	4.45 ± 0.45 (2.9)	3.82 ± 0.22 (3.03)	NS
Mean diameter*	0.13 ± 0.01 (0.10)	0.19 ± 0.01 (0.13)	p < 0.001
Maximum length	90	26	
Length ⩾ 5 μm	21.7%	22.5%	
Length ⩾ 8 μm	11.4%	10%	
Length ⩾ 15 μm	3.9%	2.1%	

* Values are expressed as mean ± SEM; geometric mean is given in parentheses.

and in the lung is remarkable: in most subjects exposed to asbestos, fiber concentrations in lung and anthracotic pleural samples were $> 1 \times 10^6$ f/g, whereas there were practically no fibers in nonanthracotic pleural samples. In five of 11 patients, including all three mesothelioma patients, the concentration of fibers was even higher in black spots than in lung samples. These findings confirm that fibers are not only heterogeneously distributed in the parietal pleura (2, 16, 17) but also concentrate in black spots, probably by an active mechanism, whereas few or no fibers can be detected in normal pleural areas only a few centimeters away.

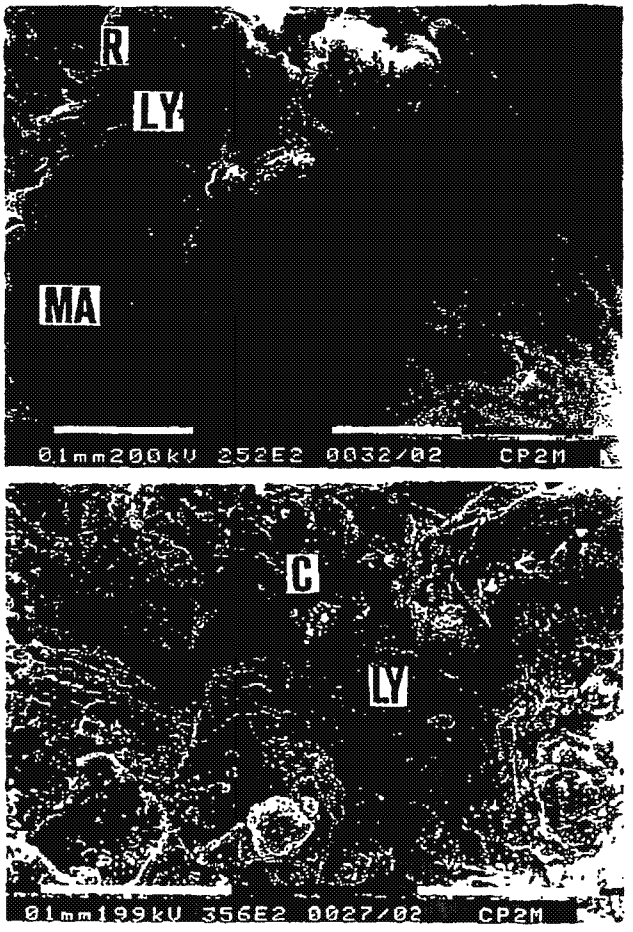

Figure 4. Scanning electron microscopic appearance of a black spot. Macrophages (MA) are clustered along the edge of the lesion. Lymphocytes (LY) and red blood cells (R) can be distinguished (*upper panel;* original magnification: ×252). More centrally, lymphatic crevices (C) and macrophages (MA) can be seen (*lower panel;* original magnification: ×356).

This heterogeneity of fiber distribution in the parietal pleura could explain inconsistencies in previous reports in which tissue sampling was random. Dodson and associates (18) documented higher concentrations of fibers in the pleura than in the lung in five of eight cases, Kohyama and Suzuki (19) in six of 13, and Sebastien and coworkers (29) in only eight of 29. Indeed, Sebastien and coworkers stated that eight previously exposed patients had no fibers in the parietal pleura. Both Dodson and associates (18) and Sebastien and coworkers (29) noted that fiber concentration was higher in costal and diaphragmatic plaques.

Distribution of Potentially Carcinogenic Fibers (⩾ 5 μm)

According to the criteria proposed by Stanton and colleagues (14) and Pott (15) from animal experiments and confirmed in mesothelial cell cultures (30), asbestos fibers ⩾ 5 μm are the most carcinogenic. In our study, fibers ⩾ 5 μm were detected in the parietal pleura of subjects with a history of asbestos exposure, where in some cases their concentration exceeded 1×10^6 f/g of dry tissue. These potentially carcinogenic fibers were observed almost exclusively in black spots (22.5% of fibers were 5 μm or longer and 10% were 8 μm or longer). The diameter of fibers recovered in black spots was greater than 0.1 μm (mean diameter: 0.19 ± 0.01 μm). Our observation is thus contrary to the conclusion by Lippman (31) that only fibers with diameters ⩽ 0.1 μm reach the pleural space.

The proportion of long pleural fibers has been variable in previous reports. Le Bouffant (16) reported that 7% of amosite fibers and 5% of chrysotile fibers in the parietal pleura were 5 μm or longer. Sebastien and coworkers (2) stated that 24% of amphibole fibers and 14% of chrysotile fibers in the parietal pleura exceeded 4 μm. Dodson and associates (18) found that 10% of amphibole fibers and 3.1% of chrysotile fibers were longer than 5 μm.

Asbestos Type of Pleural Fibers

In our study, the proportion of amphibole fibers was 99% in the lung, 95% in pleural black spots, and 61% in nonanthracotic pleura. This finding contrasts with those of previous studies that had underlined the preponderance of short chrysotile fibers in the pleura (2, 16-19, 29).

There is no clear explanation for the fact that no chrysotile fibers were found in black spots, even when there was a confirmed history of exposure to chrysotile. An explanation would be that chrysotile clearance is stimulated by large (60 to 70 μm in diameter), intensely active macrophages observed in black spots (Figure 4). From a technical point of view, short and thin chrysotile fibers could be less easily detected among a "background" of particles in anthracotic samples.

Migration of Fibers toward the Parietal Pleura

How do pathogenic, carcinogenic, or fibrosing fibers reach the parietal pleura? The answer to this question may involve several potentially interrelated pathways.

Few studies have focused on the passage of fibers into the physiologic pleural fluid. In rats, we (32) observed that after intratracheal instillation crocidolite and chrysotile fibers pass into the pleural space and diaphragm, but recovered fibers were short, i.e., 0.44 to 1.32 μm for chrysotile and 0.47 to 2.2 μm for crocidolite (only 5% of crocidolite fibers were longer than 5 μm). These observations are in accordance with those of Pott and colleagues (33) and Le Bouffant and coworkers (8).

In the present study, the correspondence between the num-

ber, type, and dimensions of fibers in the lung and anthracotic pleura is consistent with direct migration of fibers from the alveoli into the pleural cavity through the visceral pleura and a reabsorption by the parietal pleura through Wang stoma and/or milky spots. According to Hillerdal (34), connections between pulmonary and pleural lymphatics could also account for this migration.

Another possible pathway for rapid dissemination of short fibers is the bloodstream (35). The glomerular vascular structure of milky spots may facilitate the uptake of circulating fibers. Indeed, after subcutaneous or intravenous injection of asbestos fibers in mice, Kanazawa and associates (20) demonstrated that fibers concentrated only in milky spots.

Fibers can also migrate via the lymphatic pathway (28, 34). It is responsible for translocation of inhaled particles such as silica or coal into lymphoid tissue, particularly in regional lymph nodes (8, 9, 18). It is not possible, however, to deduce which of these routes is preferential on the basis of current evidence. Though our results suggest a direct transpleural migration, our study was not designed to assess such a type of translocation.

This study demonstrates that, whatever route they followed, long amphibole fibers concentrate in the same structures of the parietal pleura that trap other particles such as coal dust. The accumulation of potentially carcinogenic fibers in these black spots make them possible starting points for asbestos-induced lesions of the parietal pleura. This mechanism is consistent with the clinical observation that early-stage mesothelioma is observed on the parietal and diaphragmatic pleura rather than on the visceral pleura and with the usual location of pleural plaques.

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