

Effect of Intrabronchially Instilled Amosite on Lavagable Lung and Pleural Cells

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Rats were instilled intrabronchially with 1 mg UICC amosite suspended in 0.2 mL of filtered saline; control animals received the saline instillation only. Five animals from each group were killed on various days after instillation, up to day 128/129. Total retrieved cell counts and differential cell analysis were performed from lung and pleural lavages. In particular, the appearance of peroxidase-positive macrophages (PPM) as indicators of newly arrived macrophages was investigated. Polymorphonuclear cells (PMN) and PPMs in lung lavages increased in number 24 hr after amosite instillation and remained at increased levels until day 62. Alveolar macrophage numbers were significantly decreased after amosite instillation. There was only a very transient increase of PPMs and PMNs in the saline group. The number of PPMs in pleural lavage fluid was already increased 24 hr after amosite instillation. The pleural PPM increase was sustained throughout the study. No pleural reaction was seen in the saline instilled group. The inflammatory reactions indicated by the composition of the lavaged cells of the lung represent the *in vivo* toxicity of intrabronchially instilled amosite. The stimulus for recruitment of PMNs and PPMs is different, since no PMN response was detected in the pleural space. It is suggested that the response of the pleural PPMs is caused by the early arrival of fibers at the pleural sites, which results in the recruitment of PPMs to this space by an unknown mechanism.

Introduction

It is known that prolonged occupational or environmental exposure to asbestos can lead to pathologic changes of the pleura (1,2). At autopsies and in animal experiments, asbestos and other fibers have been found both at parietal and visceral pleural sites, in pleural fluid (3-5) and on rare occasions as asbestos bodies in the pleural space (1). Translocation of fibers from the airways and alveoli to the pleura may involve direct migration through epithelial layers and parenchyma of the lung and/or transport via lymphatic channels to the subpleural lymph vessels (4,6). It is not known to what extent fibers enter the pleural space.

To study the appearance of fibers in the pleural cavity poses difficulties, since the stomata of the parietal pleura represent a readily available clearance pathway from the pleural space (7). The time required for fibers to reach the visceral pleura may be

rather short, less than 5 days as suggested by Bryks and Bertalanffy (8). In their study, a significant rise in thymidine uptake by pleural mesothelial cells 5 days after intratracheal instillation of chrysotile into rats was reported. This observation could suggest mesothelial cell proliferation, triggered by the arrival of asbestos fibers. One could expect that those asbestos fibers will incite a change in the free pleural cell population as well. Therefore, we decided to evaluate and follow the kinetics of free pleural cells from day 1 up to 4 months after intrabronchial instillation of amosite in rats. Additionally, lavage fluid of the pleural cavity was to be investigated for fibers.

Since pulmonary macrophages are thought to play a central role in the pathogenesis of asbestosis through the interaction with fibroblasts (9), we combined the studies of lavagable pleural cells with the investigation of lavagable lung cells after intrabronchial amosite instillation. In particular, we wanted to follow the appearance of peroxidase positive macrophages (PPMs) in pleural and lung lavages. Alve-

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olar macrophages (AMs) that carry this enzyme, myeloperoxidase, in their cytoplasm are believed to be derived from blood monocytes which contain peroxidase positive granules, too. PPMs in the lung lavage fluid then represent young or newly arrived alveolar macrophages (10,11), which enter the air spaces from the circulating blood.

Materials and Methods

Two groups of 34 male Long-Evans rats each were used. The animals of the experimental group, body weight 196.5 ± 12.3 g, received an intrabronchial instillation of 1 mg UICC Amosite, suspended in 0.2 mL of 0.9% filtered NaCl. The animals of the control group, body weight 189.9 ± 11.4 g, were instilled with 0.2 mL of 0.9% filtered NaCl only. The instillation was performed under halothane anesthesia with the use of an 18-gauge needle tipped with a 20-gauge Teflon tube which was advanced close to the carina. Five animals of each group were exsanguinated under pentobarbital anesthesia on days 1; 3; 8; 27/28; 62/63; 128/129 after the instillation. Two of the remaining animals of the amosite group and one control animal died after about 5 months, and the rest—two amosite-exposed, three controls—were killed on day 220/221.

Pleural lavage was performed as follows. The abdomen was opened, an 18-gauge Teflon cannula was inserted through the tendinous part of the diaphragm and 8 mL of 0.9% NaCl was instilled into the thoracic space. The carcass was gently shaken and tilted to ensure adequate lavaging of all spaces. After removal, this procedure was repeated 5 times. Usually the recovered volume was 6-8 mL each

time. For lavaging the lung, 4 mL of 0.9% saline was instilled through an intratracheal cannula and the excised lung was gently massaged for optimum cell retrieval. This was repeated until a total of 12 lavages was reached.

Both the lavaged pleural and lung cells were kept on ice until they were centrifuged at 960g for 15 min. The cell pellet was resuspended in 10 mL of 0.9% NaCl. Total cell counts were determined from these resuspensions in a Coulter counter system and, additionally by a hemocytometer count. Mean volumes and mean diameters of the lung lavage cells were also determined. In addition, two cytocentrifuge preparations of each sample were made and stained with Wright's stain for cell differentiation and with Kaplow's stain (12) for manifestation of peroxidase positivity.

Tissue samples of lung lymph nodes, visceral and parietal pleura and diaphragm were prepared for SEM examination. These results will be reported elsewhere. The remainder of pleural and lung lavage (supernatant and resuspended pellet) were digested with Na hypochlorite and filtered through a 0.2 μ m Nucleopore filter. The filter surface was scanned for the appearance of fibers by using SEM.

For statistical analysis, the Mann-Whitney test was applied.

Results

Body weights increased in both groups in exactly the same way. No differences were found.

The results of the lung lavage cell counts are given in Table 1 (relative numbers) and in Figures 1

Table 1. Total cell count and differential cell count fractions in lung lavages of rats after intrabronchial instillation of amosite in saline.^a

Day	Treatment ^b	Total cell count ($\times 10^{-7}$)	Differential cell counts, %			PPM, % of AM
			PMN	AM	Others	
1	Control (4)	1.28 ± 0.13	5.72 ± 4.35	94.25 ± 4.34	0.04 ± 0.07	4.39 ± 2.31
	Amosite (5)	1.16 ± 0.16	$24.32 \pm 14.16^*$	$75.52 \pm 14.32^*$	0.16 ± 0.26	8.83 ± 4.82
3	Control (5)	1.38 ± 0.56	0.35 ± 0.50	99.65 ± 0.50	0	1.07 ± 0.41
	Amosite (5)	1.05 ± 0.57	$17.53 \pm 10.57^*$	$82.31 \pm 10.59^*$	0.17 ± 0.37	$6.93 \pm 2.80^*$
8	Control (5)	2.41 ± 0.66	0.49 ± 0.49	99.44 ± 0.46	0.07 ± 0.16	0.81 ± 0.56
	Amosite (4)	1.78 ± 0.23	$3.70 \pm 1.64^*$	$96.30 \pm 1.69^*$	0	$2.92 \pm 0.94^*$
27/28	Control (5)	3.62 ± 0.43	0.32 ± 0.20	99.66 ± 0.20	0	0.75 ± 0.44
	Amosite (5)	$2.78 \pm 0.26^*$	$2.82 \pm 1.96^*$	$94.14 \pm 1.93^*$	0.03 ± 0.08	$1.93 \pm 1.00^*$
62/63	Control (4)	2.81 ± 0.17	0.21 ± 0.15	99.74 ± 0.07	0.05 ± 0.10	0.58 ± 0.20
	Amosite (5)	3.05 ± 0.65	$0.84 \pm 0.22^*$	$99.12 \pm 0.23^*$	0.04 ± 0.10	$2.43 \pm 1.25^*$
128/129	Control (5)	2.33 ± 0.56	0.42 ± 0.68	99.58 ± 0.68	0	0.61 ± 0.58
	Amosite (4)	3.17 ± 2.35	0.68 ± 0.91	99.28 ± 0.88	0.05 ± 0.09	1.12 ± 0.37

* Significantly different from controls, $p < 0.05$ (Mann-Whitney Test).

^a Intrabronchial instillation of 1 mg amosite in 0.2 mL saline; controls received 0.2 mL saline only intrabronchially.

^b In parentheses: numbers of animals used.

EFFECT OF INTRABRONCHIALY INSTILLED AMOSITE

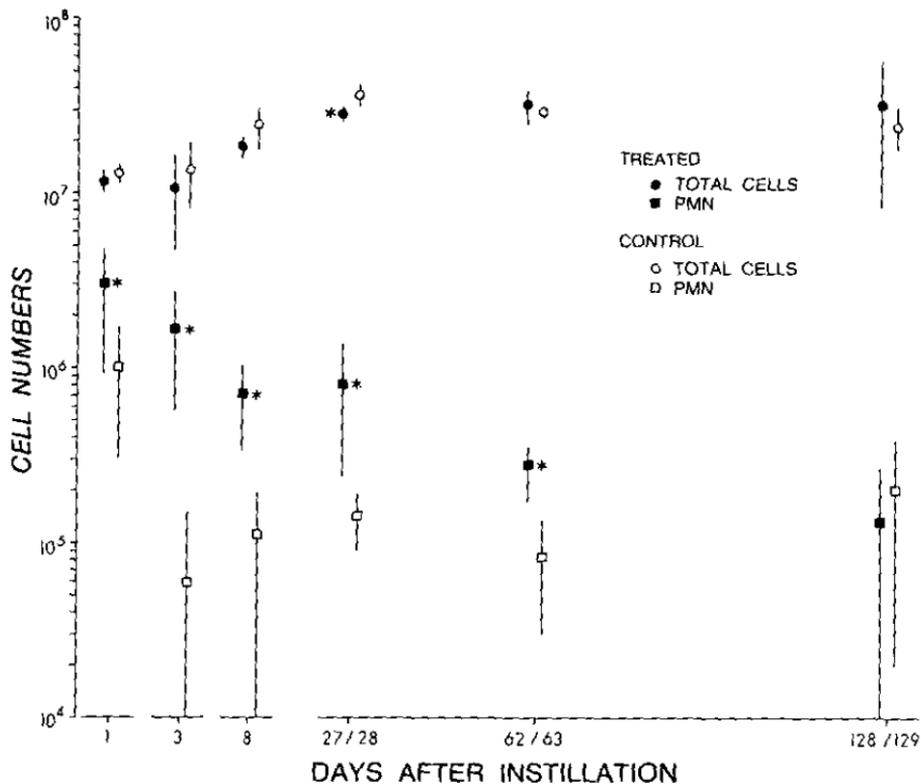


FIGURE 1. Cell kinetics of lung lavage fluid after intrabronchial instillation of 1 mg amosite or 0.2 mL saline on day 0. Total cells and polymorphonuclear cells (PMN). The asterisk denotes significantly different, $p < 0.05$, two-tailed Mann-Whitney test.

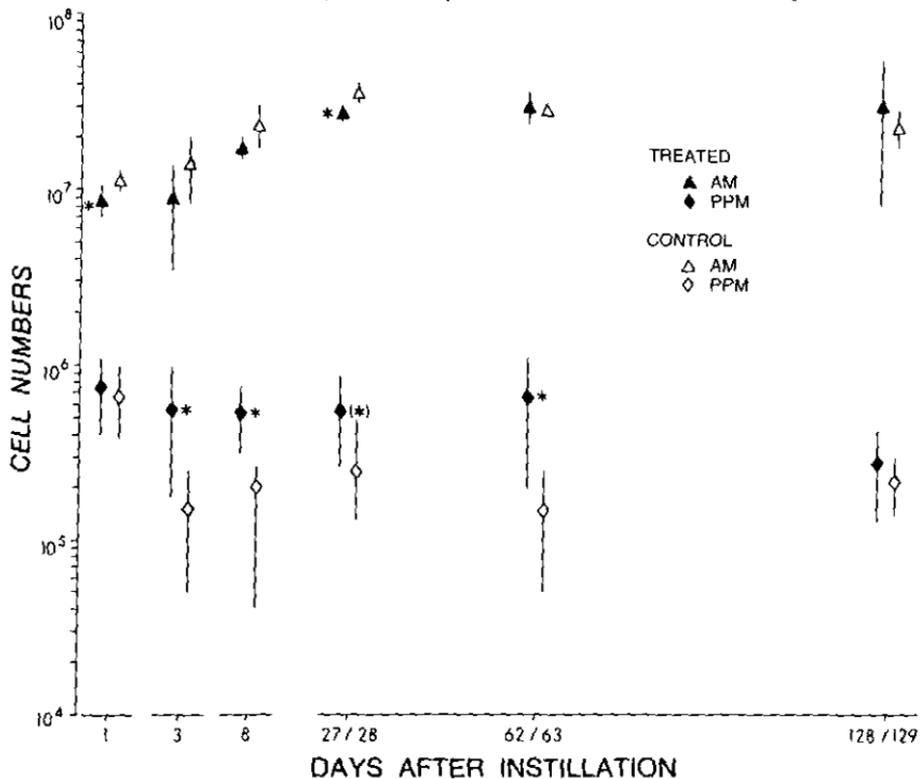


FIGURE 2. Cell kinetics of lung lavage fluid after intrabronchial instillation of 1 mg amosite or 0.2 mL saline on day 0. Alveolar macrophages (AM) and peroxidase positive macrophages (PPM). The asterisk denotes significantly different, $p < 0.05$, two-tailed Mann-Whitney test; (*) denotes $p < 0.05$, one-tailed Mann-Whitney test.

and 2 (absolute numbers). With the exception of day 1, more than 99% of the recovered cells were AMs on all days in control animals. On day 1, an increase in the PMN fraction had occurred. Among the cells designated as others, mainly lymphocytes and cells that could not clearly be classified as PMNs and AMs are listed.

In both groups, the total cell counts increased initially with age. After about one month, a plateau was reached. Until day 27/28, the amosite-treated animals tended to have a lower cell recovery rate, although this was significant only on day 27/28. The PMNs, on the other hand, consistently represented a significantly greater fraction of the lung lavage cells in the amosite group up to day 62/63 (Table 1). Accordingly, the percent of AMs was significantly lower during this period, whereas the fraction of PPMs was significantly increased starting at day 3. A transient increase only in both PMN and PPM fractions was apparent on day 1 in control animals.

When comparing absolute numbers of lavaged pulmonary cells (Figs. 1 and 2), essentially similar results were seen. Amosite-instilled animals responded with an increase in PMN and PPM numbers which came down to control levels on day 128/129 only. For the saline-instilled group, the mentioned transient increase on day 1 was also noticeable.

AM numbers of the amosite group appeared to be lower than that of the controls until day 27/28

(Fig. 2). Individual AM volume, determined with the Coulter counter, was significantly increased; on day 8 by 21%, on day 27/28 by 20% and on day 62/63 by 11% above controls. Because of the high percentage of PMNs on days 1 and 3, AM volume determinations could not be performed then. However, the cytocentrifuge preparations of day 1 revealed that about 1% of these AMs had phagocytosed as many as 25 red blood cells (usually about 5). Their diameter was greatly enlarged. Some of the vacuoles containing the red blood cells had lost their color and appeared as white vacuoles the size of red cells. At day 3, on average, AM appeared to be larger, also, and there were still AMs with phagocytized red blood cells. These pictures could not be seen in control lavages.

Results of the pleural lavage cell counts are given in Table 2 and Figures 3-5. In both groups, the number of total lavaged pleural cells increased with age, similarly to the total cell count of the lung lavage. However, beginning at sacrifice day 62/63, lavaged cell numbers increased significantly more in the amosite group. In general, about one-third of the cells of the pleural lavage fluid consisted of PMNs in both control and treated animals, and roughly an equal number of mast cells and eosinophils constituted another third of the total cells. The category "others" included mainly mesothelial cells and macrophages, which were not easily distinguishable in the Wright's stain preparation. PPMs,

Table 2. Total cell count and differential cell count fractions in pleural lavages of rats after intrabronchial instillation of amosite in saline.^a

Day	Treatment ^b	Total cell count ($\times 10^{-7}$)	Differential cell counts, %				
			PMN	Mast cells	Eosinophils	Others	PPM
1	Control (5)	0.69 \pm 0.10	35.48 \pm 13.15	16.17 \pm 5.97	14.82 \pm 5.33	33.53 \pm 13.93	2.29 \pm 1.09
	Amosite (5)	0.82 \pm 0.14	34.72 \pm 6.68	9.49 \pm 4.51	19.68 \pm 4.70	36.10 \pm 3.04	8.15 \pm 2.87*
3	Control (5)	0.75 \pm 0.12	26.71 \pm 8.64 (4)	11.11 \pm 3.05 (4)	17.19 \pm 1.93 (4)	44.98 \pm 7.90 (4)	1.17 \pm 0.28 (4)
	Amosite (5)	0.93 \pm 0.17	30.27 \pm 5.06 (4)	14.56 \pm 6.66 (4)	15.99 \pm 5.60 (4)	39.18 \pm 4.66 (4)	3.78 \pm 1.41* (3)
8	Control (5)	0.81 \pm 0.10	28.64 \pm 7.19	14.46 \pm 5.69	12.18 \pm 3.33	44.72 \pm 8.38	2.01 \pm 1.05 (4)
	Amosite (5)	1.04 \pm 0.33	30.06 \pm 7.68	16.37 \pm 9.62	15.95 \pm 8.28	36.81 \pm 10.01	3.07 \pm 1.26
27/28	Control (5)	1.19 \pm 0.35	36.39 \pm 14.49	12.25 \pm 7.32	14.39 \pm 4.13	38.68 \pm 15.10	1.92 \pm 1.02 (3)
	Amosite (5)	1.41 \pm 0.26	39.50 \pm 5.32	16.01 \pm 2.42	14.25 \pm 3.36	30.24 \pm 4.93	3.92 \pm 1.18
62/63	Control (5)	1.26 \pm 0.12	45.04 \pm 11.72	13.33 \pm 5.96	10.81 \pm 2.00	30.82 \pm 9.20	1.42 \pm 1.31 (4)
	Amosite (5)	1.70 \pm 0.34*	37.84 \pm 9.55	20.39 \pm 4.74	15.08 \pm 3.78	26.69 \pm 6.47	2.96 \pm 1.67
128/129	Control (5)	1.28 \pm 0.24	40.32 \pm 10.42	21.89 \pm 7.42	12.42 \pm 3.58	25.37 \pm 7.08	1.32 \pm 0.46
	Amosite (5)	1.82 \pm 0.36*	32.23 \pm 7.65	21.85 \pm 8.12	12.19 \pm 2.96	33.73 \pm 6.97	2.09 \pm 0.82

* Significantly different from controls, $p < 0.05$ (Mann-Whitney Test).

^a Intrabronchial instillation of 1 mg amosite in 0.2 mL saline; controls received 0.2 mL saline only intrabronchially.

^b In parentheses: numbers of animals used.

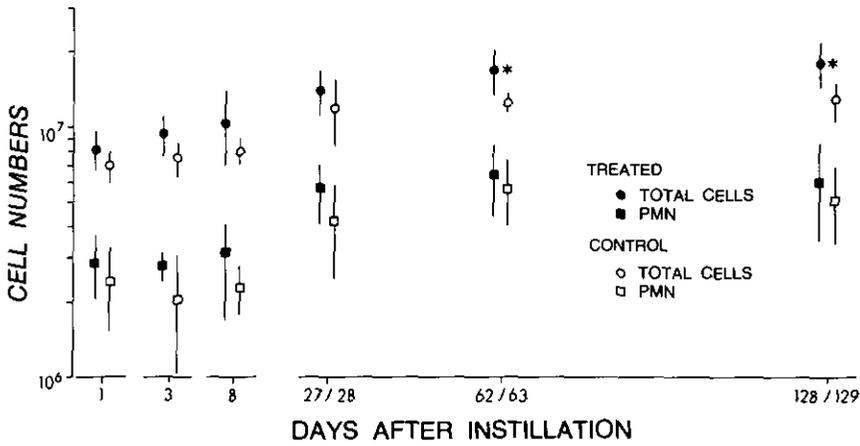


FIGURE 3. Cell kinetics of pleural lavage fluid after intrabronchial instillation of 1 mg amosite or 0.2 mL saline on day 0. Total cells and polymorphonuclear cells (PMN). The asterisk denotes significantly different, $p < 0.05$, two-tailed Mann-Whitney test.

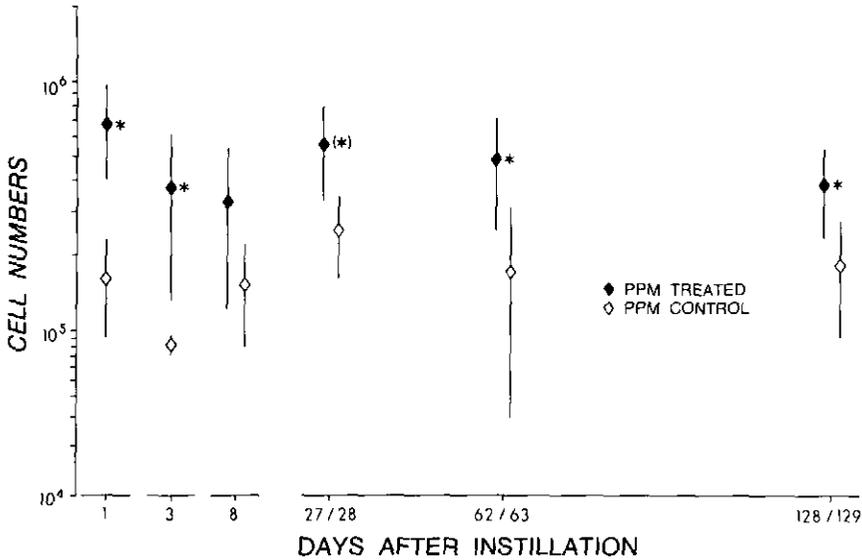


FIGURE 4. Cell kinetics of pleural lavage fluid after intrabronchial instillation of 1 mg amosite or 0.2 mL saline on day 0. Peroxidase positive macrophages (PPM). The asterisk denotes significantly different, $p < 0.05$, two-tailed Mann-Whitney test; (*) denotes $p < 0.05$, one-tailed Mann-Whitney test.

a subgroup of the macrophages, could be identified by the blue-stained granules in their cytoplasm (some of these slides were too weakly stained and had to be excluded, as indicated in the tables). A significant increase in the PPM fraction was found on days 1 and 3 and a trend towards higher percentages was observed on the following sacrifice days (Table 2).

The difference between the amosite and control groups became more obvious when absolute PPM

numbers of the pleural lavage were compared (Fig. 4). The average level of the pleural PPMs from day 1 through day 128/129 was $0.474 (\pm 0.240) \times 10^6$ cells for the amosite group, and $0.160 (\pm 0.092) \times 10^6$ cells for the control group. This threefold increase in the amosite group is significant at $p < 0.001$.

Amosite fibers were found in the lung lavage fluid of the treated animals on all sacrifice days, decreasing in numbers with time. In pleural lavage samples, no fibers could be detected.

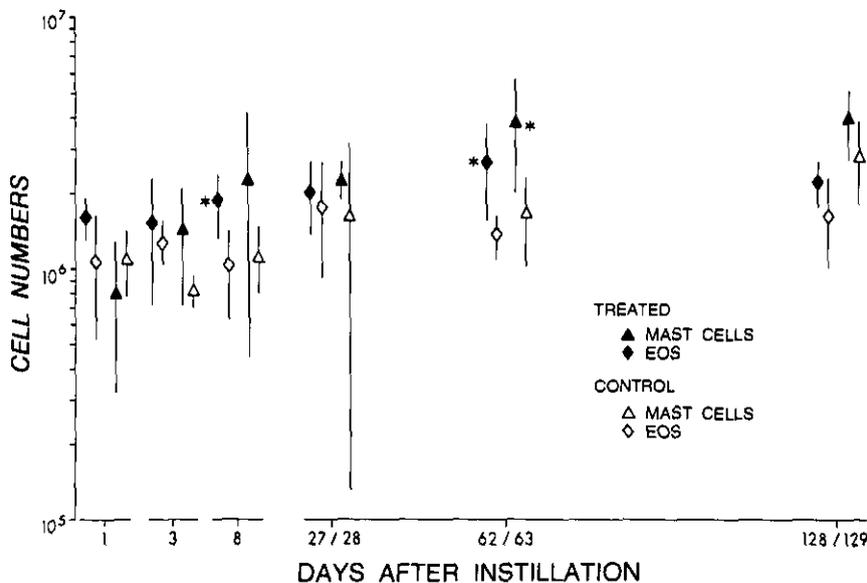


FIGURE 5. Cell kinetics of pleural lavage fluid after intrabronchial instillation of 1 mg amosite or 0.2 mL saline on day 0. Mast cells and eosinophils (EOS). The asterisk denotes significantly different, $p < 0.05$, two-tailed Mann-Whitney test.

Discussion

In vitro and *in vivo* studies evaluating the effect of amosite fibers on AMs gave different results regarding the degree of toxicity (13); however, cytotoxic effects as judged by decreased viability of AMs have been demonstrated (14). In the present study, the number of AMs 24 hr after intrabronchial administration of amosite decreased, and their cell volume and diameter was significantly increased compared to those of saline-instilled controls. This could indicate an acute cytotoxic effect of the instilled amosite.

Following phagocytosis of fibers, AMs release a leukocyte-mobilizing factor (15,16). Accordingly, we have found a significant increase in PMN numbers in lung lavages of treated animals which remained increased until day 62. The PMN increase was paralleled by a simultaneous elevation in PPM numbers. We had observed a similar parallel increase in both PMN and PPM numbers in a different study, in which low amounts of toxic and excessive amounts of inert substances had been instilled into lungs of rats (17). Those and the present results led us to suggest, that the stimulus for PMN and PPM recruitment is the same either in time or in substance. However, the present study invalidates the latter suggestion since an increase in the numbers of PPMs in the pleural cavity occurred without a simultaneous change in PMN numbers (see below). A very transient increase in PPMs and PMNs of the lung lavage was also found in the saline-instilled ani-

mals, meaning that the irritation of the intrabronchial instillation provokes a slight response.

It appears that there are two phases in the pulmonary PPM response to instilled amosite, an initial high influx of short duration, lasting for about 3 days, and a more prolonged increase until day 62. The first phase could reflect an acute recruitment of AM from the circulating blood, as suggested by Bowden et al. (18). In the presence of cytotoxic fibers, the increased PPM level is maintained until most of the fibers are cleared or phagocytosed and the stimulus for recruitment of new cells has ceased.

Whether airway peroxidase, which is found in the mucous glands of the upper airways and their secretions (19), is picked up by the AM is not known. This alternative explanation for peroxidase positivity in AMs, however, is not very likely, since PPMs were also found in the pleural space. We believe that there is sufficient evidence for the notion that PPMs represent newly arrived macrophages (10,11).

In contrast to the lung lavage results, no increase in PMN numbers was seen in pleural lavage samples. However, PPMs of the pleural cavity showed an immediate (after 24 hr) increase, and their numbers stayed high throughout the study.

What is the stimulus for PPM recruitment to the pleural space? Bryks and Bertalanffy (8), in a study of pleural cell kinetics, found an increased proliferative response of mesothelial cells after intratracheal instillation of chrysotile. This was already significant on day 5, the earliest time point studied by them. To explain this increase, they considered that

an early arrival of fibers at the pleura may be responsible. Whether a proliferative mesothelial cell response can explain the influx of PPMs through the release of a chemotactic factor is speculative at best for now. However, the early cell response seen in our study would suggest a considerably shorter time in which fibers reach the pleura: from less than 5 days (5) to less than 1 day. Alternatively, fibers reaching the pleura may be a stimulus for PPM recruitment, either directly or after phagocytosis by resident macrophages. A third possibility for PPM appearance in the pleural space could be a general recruitment of new macrophages from the circulating blood into the lung after intrabronchial administration of amosite and after its penetration into lung parenchyma. These macrophages would then enter both the airways and the pleural space. Further investigations to clarify this phenomenon are needed.

The fact that no increase in PMNs in the pleural space was found suggests that the mobilizing factors for PPMs and PMNs are different. Also, the lack of a transient PPM response in the pleural space of the saline-instilled group—in contrast to the observation of the lung lavage—would favor the idea that airway irritation alone is not the cause of the PPM response in the pleural cavity.

Only relatively few of the instilled fibers are expected to reach the pleura, and if they enter the pleural space they may be cleared through the stomata of the parietal pleura (7). Hence, the negative results of the search for fibers in the pleural lavage fluid are not necessarily surprising. Careful examinations of both visceral and parietal pleura of the animals of this study for amosite fibers are now in progress. A significant increase in total cell numbers of the pleural lavage beginning at day 62/63 is partly due to an increase in mast cells, eosinophils and mesothelial cells. Its significance remains to be studied further.

In summary, the intrabronchial instillation of 1 mg of amosite leads to an immediate and lasting increase of PMN and PPM numbers in the airways, which is judged as an indicator of amosite toxicity to the lung. In addition, as early as 24 hr after administration, pleural lavage samples also showed an increase in PPM numbers, which may be indicative for the arrival of fibers at pleural sites. This PPM response was maintained throughout the duration of the study, up to day 128. Future studies have to show if this PPM response is involved in the pathogenesis of pleural plaques or calcification processes.

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