ASBESTOS BODIES AND SIMILAR STRUCTURES

H.K. Koerten

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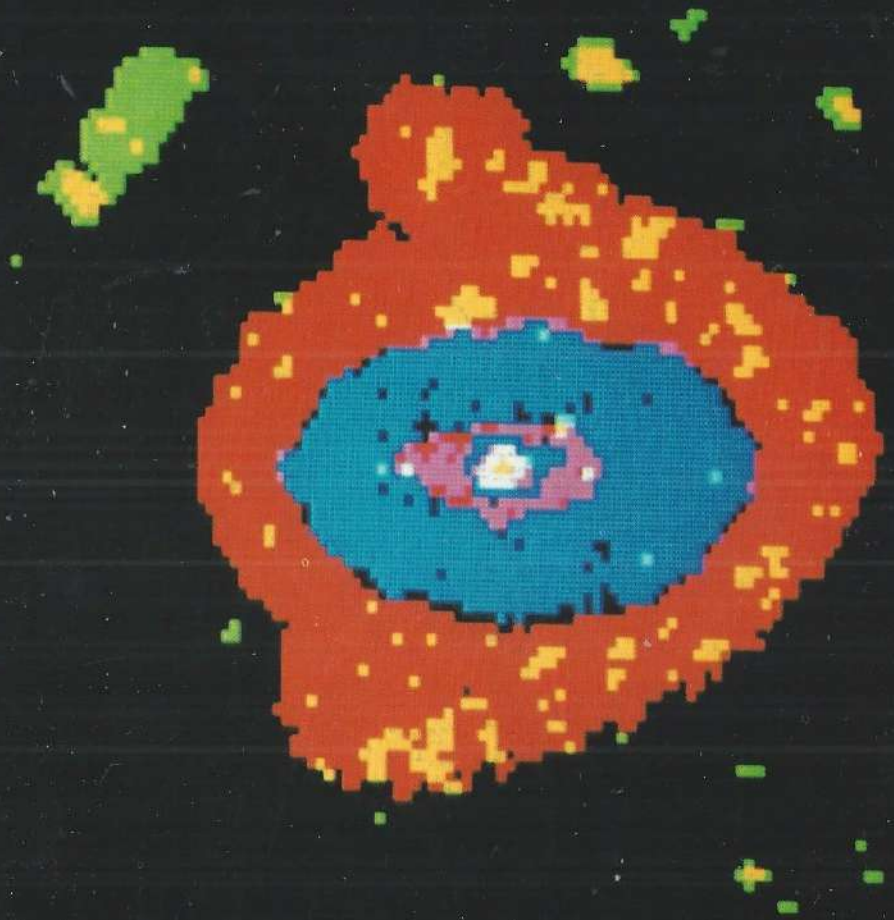

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ter nagedachtenis aan mijn vader
aan mijn moeder
aan Elly, Remco en Ernest

CONTENTS

CHAPTER I

GENERAL INTRODUCTION

1. *Asbestos*

Asbestos is a naturally occurring fibrous silicate with extremely thin fundamental fibrils that range from 20 to 200 nm in thickness. Because of these small diameters, fibrils with a length suitable for industrial use will have a very high length-to-diameter ratio. It is this ratio which determines whether a hydrous silicate is assumed to be asbestiform [1].

According to Hodgson [2], six types of asbestiform mineral can be distinguished, all of them deriving from two large groups of rock-forming minerals, i.e., the serpentines and the amphiboles.

Chrysotile $\{Mg_3[Si_2O_5](OH)_4\}$ is the only type of asbestos that belongs to the serpentine group and is by far the most frequently used for commercial purposes, its fine, silky white fibers having a high resistance to heat. The amphiboles include actinolite, amosite, anthophyllite, crocidolite, and tremolite. Crocidolite $\{Na_2Fe_2^{3+}(Fe^{2+}Mg)_3Si_8O_{22}(OH)_2\}$, also known as blue asbestos, has very strong, straight fibers and is especially resistant to acids. Among the amphiboles it is thought to be the most dangerous for human health.

Because of its special chemical and physical properties including resistance to acids and heat, great mechanical strength, and good insulating capacity, asbestos is applicable in a wide variety of manufacturing processes. Today, asbestos has gained economic value and is incorporated into more than 3000 products [3].

2. *Biological effects of asbestos*

2.1. *Asbestos related diseases*

Like other poorly digestible materials, asbestos introduced into an organism can induce a foreign-body reaction. This response is initially attended by an inflammatory reaction in the form of a local invasion by inflammatory cells, i.e., neutrophil granulocytes and monocytes [4]. Individuals who are regularly exposed to asbestos will inhale many asbestos fibers and show an increase in the number of asbestos fibers deposited in the lungs. Persistant exposure to asbestos can ultimately lead to asbestosis [5-8,14], an asbestos-related pneumoconiosis caused by excessive inhalation of asbestos fibers over a relatively long period.

Another health problem caused by asbestos exposure is lung cancer [5,8,9,10,13-15]. Lung cancer induced by asbestos seems to be associated with the inhalation of large amounts of asbestos fibers [17], and is known to occur preferentially in patients suffering from asbestosis. Furthermore, despite contradictory reports [18-20], the occurrence of asbestos-related lung cancer seems to be associated with smoking habits [18,19]. A second form of cancer related to asbestos exposure is mesothelioma, a rare carcinoma of mesothelial cells in the pleura [11,14-16,21,22] and peritoneum [21]. It is believed that the occurrence of a mesothelioma is related to exposure to any fibers of the amphibole type of asbestos [22].

The mechanism underlying the tumor-promoting potentiality of asbestos is, however, still uncertain, and various in vivo [23-25] and in vitro [26-28] studies have been performed to obtain more light on this topic. Although a causal relationship between asbestos dust and the occurrence of pneumoconiosis and cancer was established as early as 1927 [5], it was not until the 1970s that measures were taken to limit the use of and exposure to asbestos. This means that individuals working in asbestos mining, the production of asbestos-containing products, and in demolition, were still being severely exposed in the recent past.

Since asbestos-related cancers develop long after exposure starts (up to twenty-five years), a large number of patients who came into contact with asbestos before the precautionary measures were tightened up, must be expected.

2.2. *Asbestos cytotoxicity*

In addition to and as a part of the investigations on the tumor-promoting potential of asbestos fibers, studies were done on the cytotoxic effects of asbestos [29-33]. The findings show that both the fiber size and the type of asbestos have a strong influence on the cytotoxic effects of asbestos. The cytotoxicity of chrysotile fibers, as reflected by membrane damage and the release of lactate dehydrogenase [34,35], was found to be greater than that of the amphiboles, e.g. crocidolite [34].

2.3. *Asbestos bodies*

A phenomenon generally observed after asbestos exposure is the formation of asbestos bodies. A relationship between the numbers of asbestos bodies and the severity of asbestos exposure was first detected by Cooke [36]. Later, Steward and Haddow found that the presence of asbestos bodies was associated with asbestosis, and this led them to use the term asbestosis bodies [37]. Gloyne showed that the coat of an asbestos body is composed of iron-rich proteins [38]. It is this iron-rich coat that gives the asbestos bodies their characteristic golden-brown appearance in the light microscope. It is generally accepted that the coat of an asbestos body is produced by the surrounding cells, and macrophages are assumed to play a role in the process of asbestos body formation [39,40]. It is also known that the coat of asbestos bodies is rich not only in iron but also in acid mucopolysaccharides [39-41], and a relation between the presence of asbestos fibers and the formation of calcium deposits has also been described [42]. The exact mechanism underlying asbestos body formation is, however, still unknown, and several hypotheses have been put foreward [40,43-45].

The number of asbestos bodies detected after asbestos exposure and also the interval required for the development of an asbestos body, appear to be dependent on the type of asbestos [46] but also on the species in which the asbestos has loged [40]. Asbestos bodies have been frequently reported in man and the guinea pig, hamster, and mouse, but only twice in the rat [48,49]. In man, either the rate nor the duration of asbestos-body formation is known, but asbestos bodies were already present in the sputum of young children aged between 10 and 40 weeks [50].

In man, asbestos fibers and bodies are found most often in the lungs, but their presence has also been reported in other organs, e.g. the oesophagus, stomach, gut, liver, brain, and heart [51-55]. Migration of ingested asbestos fibers into various organs after subcutaneous injection of asbestos-containing suspensions [54] or ingestion [55] has been reported, but it is not known whether asbestos bodies are formed at these loci.

Since the characteristic iron-rich coat has also been seen after exposure to other fibrous materials [56-58], it has been suggested that the term ferruginous bodies be used until the nature of the core of the bodies is established [59].

3. *Value of asbestos bodies for diagnostic properties*

It has been assumed that asbestos-body formation combined with the presence of these bodies in lung or other tissue does not represent a disease, but the presence of such bodies has diagnostic value [60]. The number of

asbestos bodies in human sputum was found to be a logarithmic function of the duration of exposure counted in work days [61].

In tissue sections, the thickness (20-200 nm) and light-bending capacity of elementary asbestos fibrils make it impossible to detect these structures with the light microscope [62,63], and electron microscopy is required. If the type of asbestos has to be determined as well, transmission electron microscopy must be combined with X-ray microanalysis for positive identification of asbestos fibers [63]. Asbestos bodies are, however, easy visualized with the light microscope because of their characteristic golden-brown color and the range of the diameter (5-10 μm), and this provides information at the light-microscopical level about the seriousness of earlier asbestos exposures [64-66], which is valuable for diagnostic purposes and retrospective studies.

4. *Objectives of the study*

The present investigation concerned the morphological changes induced in mouse peritoneal macrophages by an intraperitoneally injected suspension of asbestos fibers, the formation of asbestos bodies in this body compartment, and the role of macrophages in the process. Since the cores of asbestos bodies are usually composed of asbestos of the amphibole type [47] and crocidolite is assumed to be the most carcinogenic amphibole [11], this type of asbestos was chosen for the present study. As a control for the specificity of the phenomena observed after exposure to crocidolite asbestos, the effects of non-fibrous, poorly digestible particulate material of a different nature were evaluated. For this purpose we selected two materials which unlike asbestos, do not disturb biological systems when introduced in solid form: the calcium phosphates hydroxyapatite and tri-calcium phosphate, both known for good biocompatibility [67].

The research started with the morphological investigation of the peritoneal cell population after intraperitoneal injection of crocidolite asbestos fibers. The results of this study are discussed in Chapter II.

Since asbestos bodies were not detected in the isolated peritoneal cell suspensions and because only a small proportion of the injected asbestos fibers were retrieved, a further investigation was performed (Chapter III). In this study special attention was paid to foreign-body granulomas seen on the surface of the peritoneal wall, the omentum, and the viscera. It describes the development and morphology of these granulomas and the occurrence of cytoplasmic ferritin and numerous iron-rich inclusion bodies in the cytoplasm of macrophages and multinucleated giant cells. In addition, the morphology and chemical composition of four different types of asbestos body found in these granulomas are described.

Although the data obtained in this study allowed us to formulate a hypothesis on the mechanism of asbestos-body formation, it was difficult to deduce the exact role of macrophages in this process and it could not be excluded that other cell types were involved in asbestos-body formation as well. Therefore, an investigation into macrophage/asbestos interaction in vitro was started (Chapter IV).

Chapter V reports the results of a study in which the data, obtained after the introduction of asbestos were compared with the findings after the introduction of the biomaterials. As already mentioned, use was made of granular hydroxyapatite, a calcium phosphate occurring naturally in crystalline form in bone [68], and granular tri-calcium phosphate, normally not present in crystalline form in biological systems. It is discussed in that chapter to what extent phenomena occurring after exposure to asbestos were also observed after the introduction of these materials and might thus be seen as general phenomena related to the presence of indigestible or poorly digestible materials in a biological system. A hypothesis concerning the observed phenomena is also put foreward in this Chapter.

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CHAPTER II

THE ENDOCYTOSIS OF ASBESTOS BY MOUSE PERITONEAL MACROPHAGES AND ITS LONG TERM EFFECT ON IRON ACCUMULATION AND LABYRINTH FORMATION [1]

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ABSTRACT

The effect of a single intra-peritoneal injection of crocidolite asbestos fibers on the peritoneal cell population was studied. Attention was paid to the changes in the proportions taken by the various types of cell in this population after peritoneal stimulation as well as the handling of asbestos fibers by the peritoneal cells and the formation of asbestos bodies.

Intraperitoneal administration of crocidolite led to an influx of inflammatory cells into the peritoneal cavity. The asbestos fibers were phagocytosed and gradually cleared from the peritoneal cavity. Long before this clearance was completed, the peritoneal cell population had returned to the steady state. The stimulated peritoneal macrophages showed increasing concentrations of iron in both lysosomes and the cytoplasm. At later time-points, residual bodies containing iron and asbestos fibers were seen frequently in macrophages, but asbestos bodies were not found. As a reaction to the administration of crocidolite asbestos, macrophages from the peritoneal cavity develop tubular systems (labyrinths) that increase in number and size.

INTRODUCTION

Asbestos is a fibrous mineral that is widely used in industry because of its physical and chemical properties. Exposure to asbestos fibers can lead to fibrotic and neoplastic diseases in the lungs and peritoneum [1,2,3]. In addition, asbestos is known to be cytotoxic [4,5] and to have an inhibitory effect on both colony

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formation in cell cultures [6] and cell proliferation [7,4].

Macrophages are assumed to play an important role in the reaction to asbestos exposure. A factor called the macrophage fibrogenic factor is released from macrophages after exposure to silica and asbestos [8,9]. Lysosomal enzymes are also released by mouse peritoneal macrophages after exposure to asbestos [10,11]. Further, the phospholipase activity of, and the prostaglandin (PG) release by macrophages are enhanced by asbestos [12,13], and these cells are also involved in the formation of what are called asbestos or ferruginous bodies, i.e., asbestos fibers surrounded by an iron-protein layer [14].

In the present study use was made of the amphibole crocidolite because the cores of asbestos bodies isolated from human lungs are generally composed of amphibole asbestos [15], and because this type of asbestos is known to be highly cytotoxic and carcinogenic [5]. To exclude the effect of other types of particle as could occur in the lung, the compartment chosen was the peritoneal cavity. The ultrastructure of the cell population of the mouse peritoneal cavity was investigated at various time intervals after the injection of asbestos. Recognition of cell types was based on morphological features and the localization of peroxidatic (PO) activity [16,17]. X-ray microanalysis was used for the detection of fibers and cytoplasmic iron as well as to measure the iron content of the lysosomes and residual bodies.

MATERIALS AND METHODS

Animals

Two differently bred strains of Swiss mice were used simultaneously: I, our own conventionally bred Swiss male mice, and II, SPF bred Swiss male mice obtained from the Central Animal Breeding Centre TNO (Zeist, The Netherlands). At the start of the experiments the mice were eight weeks old and weighed 20-25 grams. All mice were allowed to adapt for three days before the i.p. injection was given.

Asbestos administration

Crocidolite asbestos was suspended in Hanks' balanced salt solution, by the use of a Branson B12 sonifier for 3 min at 50 Watt, to a final concentration of 0.5 mg/ml. On day 0, 1 ml of this suspension was injected into the peritoneal cavity. Control animals were given 1 ml of the same solution containing no asbestos.

Isolation of the peritoneal cell suspension

After decapitation of the animals, 2 ml of a Ringer solution was injected intraperitoneally, followed by careful kneading of the abdomen for approximately 30 sec, after which the cell suspension was collected by suction. Peritoneal cells were isolated from unstimulated animals, and at intervals of 4, 16, 24, and 48 hours, 8 days, 2 weeks, and 1, 2, 6 and 8 months after asbestos administration. For each interval the cell suspensions from 3 mice were pooled.

Fixation

For the morphological studies, the cells were pelleted and the pellet was prefixed for 10 min at room temperature in 1.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4, 350 mOsmol). The cells were then washed at room temperature for 5 min in Ringer solution. Postfixation was performed for 30 min at 4°C in 1% OsO_4 in 0.1 M cacodylate buffer (pH 7.4).

Direct fixation was performed according to Brederoo and Daems [18]. In short, 2 ml phosphate-buffered saline was injected intraperitoneally, followed by careful kneading of the abdomen for approximately 30 sec. The cell suspension was collected by suction and without being washed brought into an excess of fixative, followed by the same procedure as described above.

Osmium(VI)-iron(II) complex fixation was performed according to de Bruijn et al. [19]. After glutaraldehyde prefixation, the cell pellet was resuspended in a freshly prepared solution of 1% OsO_4 and 1.5% of $K_3Fe(CN)_6$ in 0.1 M cacodylate buffer, for 16 h at 4°C, (pH 7.2).

Peroxidase cytochemistry

For the cytochemical demonstration of PO activity, the cells were prefixed in glutaraldehyde, washed in 0.1 M cacodylate buffer (pH 6.9) at room temperature for 5 min, and then preincubated for 30 min at room temperature in a medium containing 1 mg diaminobenzidine-4HCl per ml cacodylate buffer. Incubation was performed in the same medium supplemented with H_2O_2 to a

final concentration of 0.01%, after which the cells were washed and postfixed as for the morphological studies.

With this technique, three types of phagocyte could be distinguished: resident macrophages showing PO-positive reaction product in the nuclear envelope (NE) and rough endoplasmic reticulum (RER), exudate macrophages showing PO-positive granules, and PO-negative macrophages.

5'-nucleotidase cytochemistry

The demonstration of 5'-nucleotidase (5'-N) activity was performed according to Blok et al. [20]. In short, cells were fixed for 60 min at 4°C in 1% glutaraldehyde in buffer composed of 0.05 M Tris-maleate and 5% sucrose (TMB, pH 6.0). After three rinses (15 min each) in TMB (pH 7.2, 0-4°C) the cells were incubated for 15 min at 37°C in TMB (pH 7.2) containing 1.4 mM Adenosine 5' monophosphoric acid (Sigma Chem. Co.) 10 mM $Mg(NO_3)_2$, and 1mM $CeCl_3$.

Transmission electron microscopy

Fixed cells were washed and resuspended in a 2% agar solution at 60°C. After centrifugation, the agar was cooled down to 4°C to solidify, and then cut into small pieces measuring approximately 1 mm^3. These pieces were dehydrated in a graded alcohol series to alcohol 100% and embedded in Epon. Ultrathin sections were cut on an LKB microtome and stained with lead hydroxide. The sections were examined in a Philips EM 201 or 410. In each section, 300 to 400 cells were examined.

Scanning electron microscopy

Scanning electron microscopy (SEM) was performed according to the method described earlier [21]. In short, cells were fixed for 30 min at room temperature in 1.5% glutaraldehyde in 0.1 M cacodylate buffer, (pH 7.4, 350 mOsmol). After fixation, the cells were dehydrated in a graded alcohol series and dried under carbon dioxide in a Polaron critical point drier. The dried specimens were covered with a layer of gold and examined in a Cambridge Stereoscan S 180.

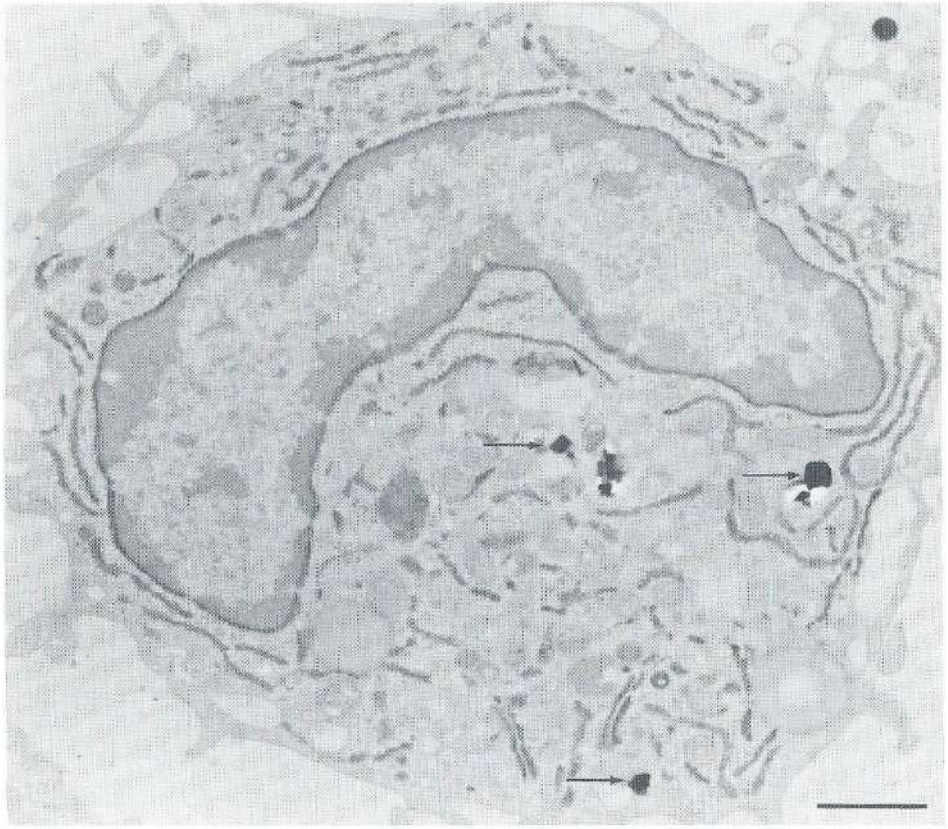


Fig. 1. Resident peritoneal macrophage of a conventionally bred mouse, isolated 48 h after asbestos injection. PO-positive reaction product is present in RER and NE. Fragments of ingested asbestos fibers are clearly visible (arrows). Bar: 1 μm.

X-ray microanalysis

For the identification of crocidolite asbestos fibers in the peritoneal cells and for determination of iron in the cytoplasm and inclusion bodies, X-ray microanalysis was performed with a Tracor Northern (TN) 2000 X-ray microanalyser connected to a Philips EM 400. Measurements were done in lead-stained sections on copper grids, prepared as for transmission electron microscopy (TEM).

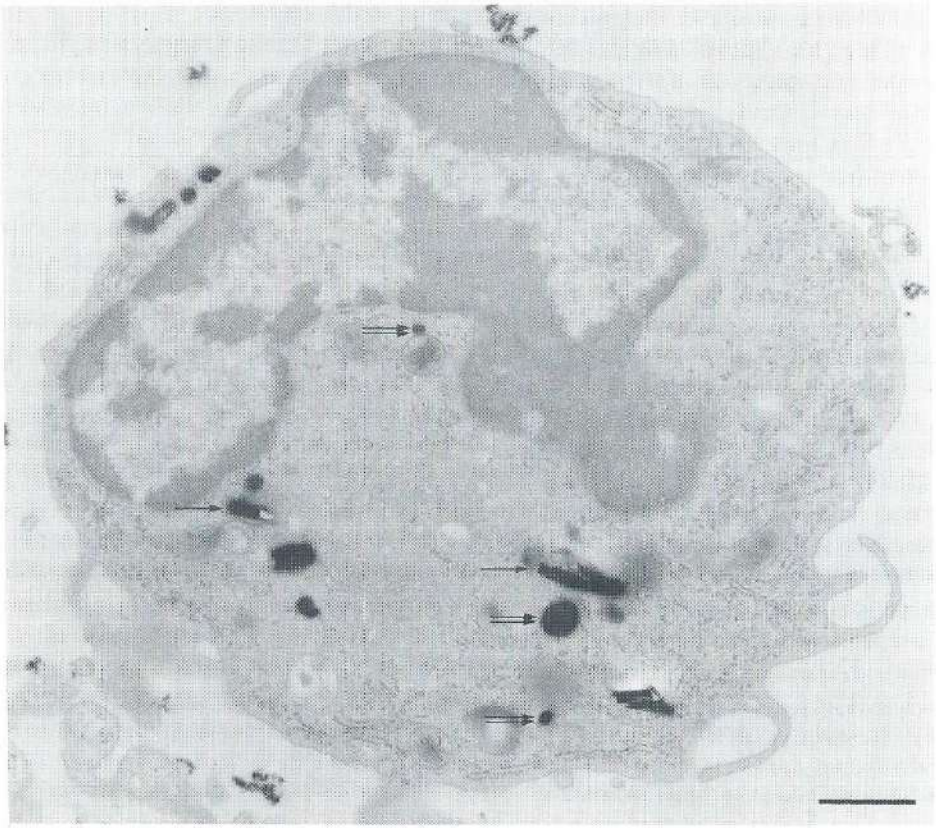


Fig. 2. Exudate macrophage of conventionally bred mouse, isolated 16 h after asbestos injection. Notice PO-positive granules (double arrows). Fragments of ingested asbestos fibers are clearly visible (arrows). Bar: 1 μm.

RESULTS

Peritoneal cell population

The unstimulated peritoneal cavity of the SPF mice contained resident macrophages (recognized by the PO-positive reaction product in the RER and NE; Fig. 1), PO-negative macrophages, lymphocytes, mast cells, and eosinophil granulocytes. The peritoneal cavity of the conventionally bred mice contained in addition, a small number of exudate macrophages (recognized by the presence of PO-positive granules; Fig. 2) and neutrophil granulocytes.

Fig. 3, a and b show the change in the relative proportion of phagocytes after asbestos administration. Fig. 3a gives the data on the conventionally bred Swiss mice. As this graph shows, an initial decrease of the proportion of resident

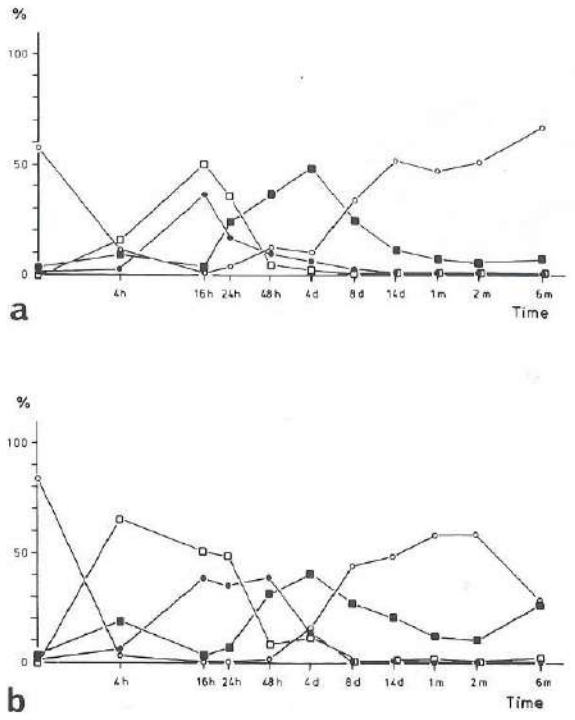


Fig. 3, a and b. Percentages of peritoneal cells, as a function of time after asbestos administration.
a: In the conventionally bred mice an initial efflux of resident macrophages (O) at the first time-points is followed by recovery of this group. At two weeks, the number of this type of cell is at the normal level. Exudate macrophages (●) and neutrophil granulocytes (□) enter the peritoneal cavity in response to the peritoneal stimulus. A maximum is reached at 16 h. At two weeks, their number has returned to normal as well. The PO-negative macrophages (■) show a peak at four days, after which their number slowly decreases during the course of the experiment.
b: The reaction of the SPF bred mice to the peritoneal stimulus is in principle the same as that in the conventionally bred group. One may speak of a slightly stronger reaction because the dip in the number of resident macrophages is more obvious. Also the peaks showing the highest percentages of exudate macrophages and neutrophil granulocytes is more pronounced. At six months, the values of the resident macrophages and the PO-negative macrophages deviated clearly from those of the conventionally bred mice.

macrophages was followed by an influx of neutrophil granulocytes and monocytes. At 16 h, the number of resident macrophages was lowest and the proportion of monocytes and granulocytes reached a maximum. During the course of the experiments, a return to the unstimulated situation, i.e., the original cell/cell ratio, was observed. Complete restoration occurred at 2 weeks.

The SPF mice showed a generally similar pattern. From Fig. 3b it is evident that in these mice, too, macrophages had disappeared at 4 h. There was also an influx of inflammatory cells, but the percentage of these cells, entering the peritoneal cavity appeared to be somewhat higher, and in the SPF mice the highest percentage of neutrophils in the peritoneal cavity was

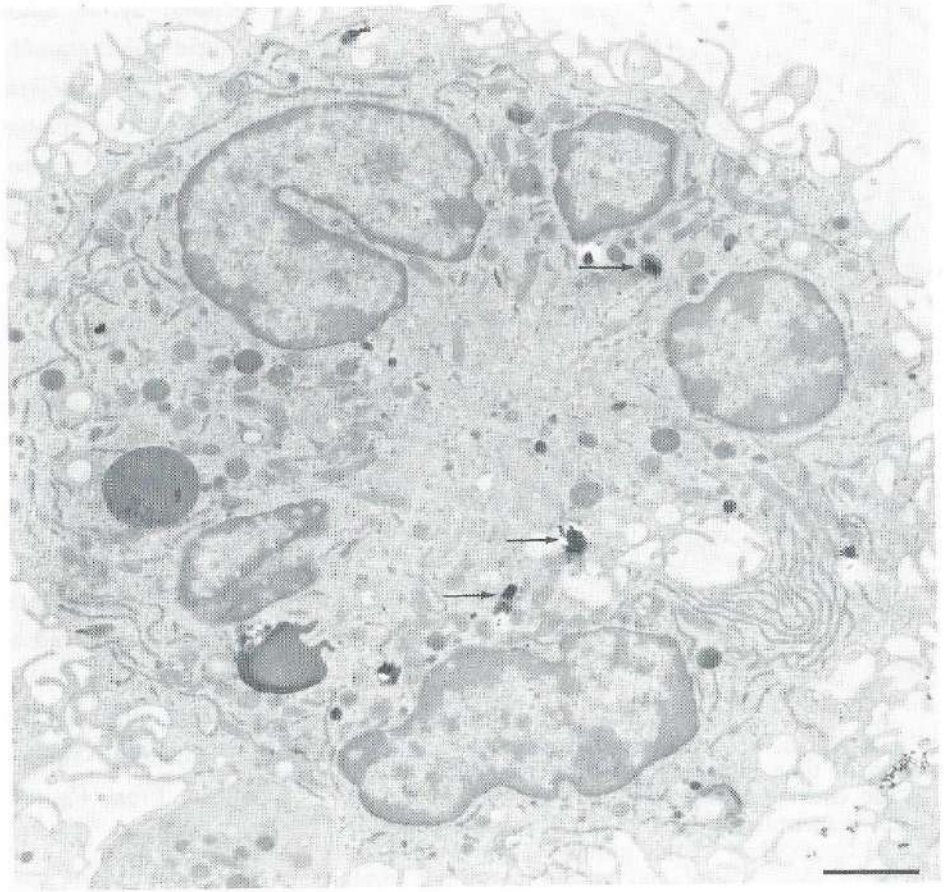


Fig. 4. Multinucleated giant cell of SPF bred mouse, isolated 48 h after asbestos injection. Fragments of ingested asbestos fibers are clearly visible (arrow). Bar: 2 μm.

reached at a different time, i.e., at 4 h. Furthermore, the number of neutrophils and exudate macrophages remained at a high level, longer than was the case for the conventionally bred mice. At 2 weeks, however, the cell population in the SPF mice too had recovered.

An unusual phenomenon was noticed at the six-month time-point in the SPF mice. Here, the proportion of resident macrophages was lower than after two months and the number of PO-negative macrophages appeared to be increased. However, when the proportions of resident and PO-negative macrophages are summed, the normal pattern emerges and the graph resembles that of the

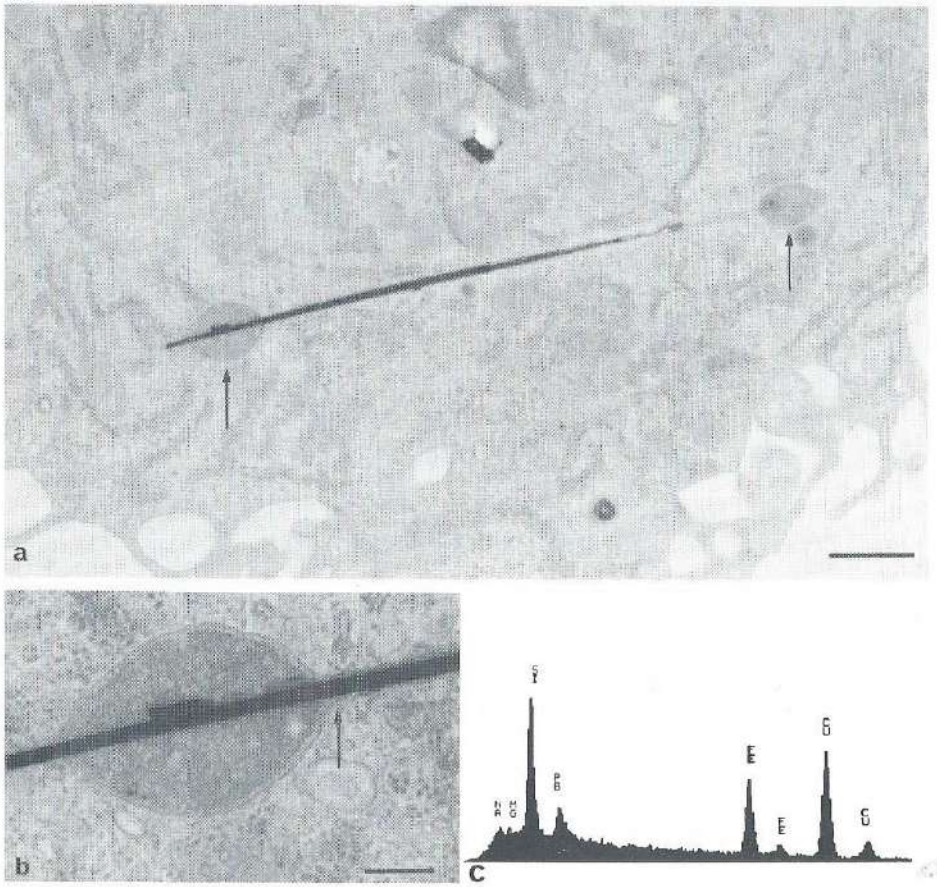
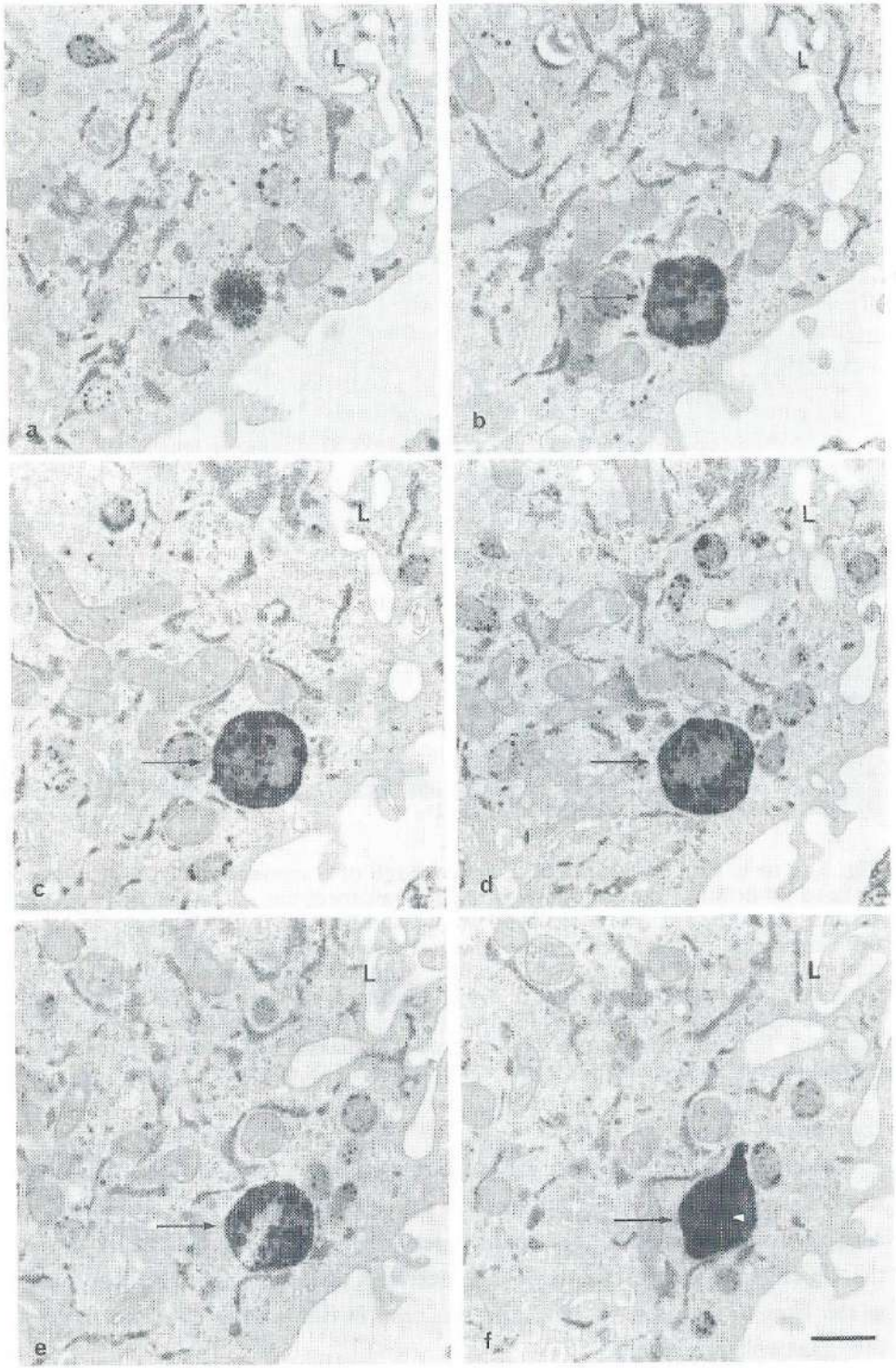


Fig. 5, a to c. Part of a peritoneal macrophage of a conventionally bred mouse, isolated 24 h after asbestos injection with X-ray spectrum. a: In the plane of the section an asbestos fiber is visible. Two lysosomes fused with the phagosome at the extremities of the fiber can be seen (arrows). Bar: 1μm. b: High magnification of part of the fiber shown in a. The lysosomal membrane continues along the fiber (arrows). Bar: 0.2 μm. c: X-ray spectrum of the fiber shown in a and b. The relative proportions of Na, Mg, Si, and Fe are typical for crocidolite asbestos. Besides these elements Pb, and Cu introduced by the preparative procedure are also present. (Vertical full scale 2,048 counts.)

conventional strain.

After asbestos stimulation, some giant cells with three to five nuclei per section were seen at 48 h (Fig. 4). The number of giant cells then increased, reached a maximum number of about 2 per cent of the total cell population on the 4th day, and then showed a gradual decrease. Giant cells were seldom found at the later time-points. No alteration was seen in the number of nuclei present per giant cell per section.



	4h-4d	8d	2w	1m	2m	6m
Res macrophages	<0.1	0.5±0.6	6.5±4.2	6.7±6.4	18.5±7.7	31.3±19
PO⁻ macrophages	<0.1	1.2±0.9	1.3±2.6	4.6±5.3	8.2±8.4	6.2±10

Tab. I. Relative proportion of resident - and PO-negative macrophages, showing one or more labyrinths in the plane of the section at various time intervals after a single i.p. asbestos administration. Numbers shown are percentages of cells ± standard deviation.

Asbestos uptake

The results of SEM showed that the length of the crocidolite fibers ranged from 0.5 μm to 60 μm, and that fibers of all sizes were attacked by the peritoneal phagocytes. TEM showed that the majority of the asbestos fibers was ingested by the various types of macrophages.

The ingested fibers were present in phagosomes and secondary lysosomes, which were strongly elongated when they contained long fibers. In such cases the limiting membrane of these organelles lay close to the ingested fibers; this sometimes obscured membranes, creating the impression that fibers lie entirely or partly free in the cytoplasm (Fig. 5a). However, at high magnification, a limiting membrane could be seen in most cases (Fig. 5b).

On the basis of morphological criteria, no cytotoxic effect of fiber-macrophage interaction was observed. X-ray microanalysis showed that all fibers were of the asbestos-type crocidolite (Fig. 5c). Comparison of X-ray spectra of non-injected and ingested fibers showed no difference in the relative peak heights of the elements in question.

As early as 4 h after administration of crocidolite asbestos, about 60% of the total macrophage population showed ingested fibers in the plane of the section. At 6 months, asbestos fibers were still found in about 5% of the macrophages. There was a decrease in not only the number of fibers per

Fig. 6. Serial sections of a residual body in a macrophage of a SPF bred mouse, isolated 6 months after asbestos injection. The odd sections of this series (a: section 1, b:section 3, etc. up to f: section 11) illustrate the finding that small asbestos fibers (arrowhead) in residual bodies (arrow) are only visible in some of the sections. In the right top corner of the sections part of a labyrinth is visible (L). Bar: 0.5 μm.

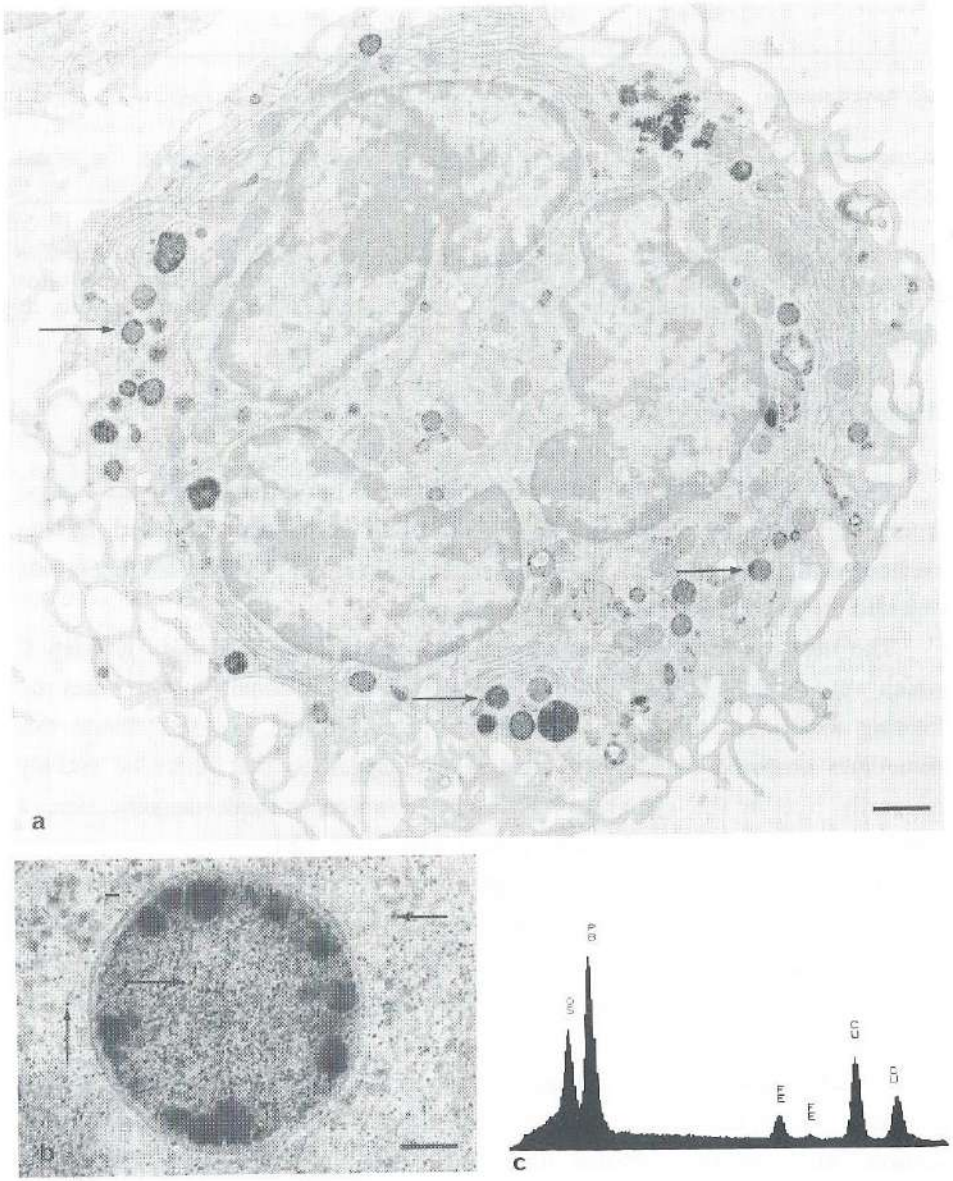


Fig. 7, a to c. Macrophage from SPF bred mouse, isolated six months after asbestos injection. a: Numerous inclusion bodies are present in the cytoplasm (arrows). Bar: 1 μm. b: High magnification of an inclusion body. Iron particles are clearly visible in the inclusion body as well as in the cytoplasm (arrows). Bar: 0.1 μm. c: X-ray spectrum of the inclusion body in b, showing the presence of iron. (Vertical full scale 2,048 counts.)

macrophage but also in the average size of the fibers (data not shown). Fibers seen after shorter intervals were frequently long and composed of several adjacent elementary fibers. However, especially at 2 months and later, most of the macrophages showed only elementary fibers, which were situated in residual bodies. At later time points an increasing number of cells lacking asbestos fibers was seen in the plane of the section. However, serial sections still showed the presence of small crocidolite fibers in residual bodies (Fig. 6a to f).

The presence of iron

After i.p. administration of asbestos, a number of inclusion bodies was found in the macrophages (Fig. 7a). At high magnification a morphology suggesting the presence of iron was observed (Fig. 7b). Both the number of macrophages with inclusion bodies and the number of these bodies per macrophage increased during the course of the experiments.

On day 8, neither control nor asbestos-stimulated mouse macrophages with inclusion bodies containing iron were found. After two months, 90% of the macrophages from asbestos-stimulated mice had inclusion bodies containing iron, whereas 28% of the macrophages from the control animals had such inclusion bodies. At six months 90% of the macrophages from asbestos-stimulated mice still showed inclusion bodies and 42% of the macrophages from control animals had one or more of these inclusion bodies in the cytoplasm. Initially, X-ray microanalysis of the inclusion bodies showed no iron, but after two and six months the same technique showed the presence of iron (Fig. 7c). Therefore, it may be concluded that not only the number of inclusion bodies increased but also the amount of iron per inclusion body. Also at the later time-points cytoplasmic iron micelles were recognized (Fig. 7b). The concentration of this iron was, however, too low to be detected by X-ray microanalysis.

Despite our finding of iron and the presence of asbestos fibers in iron-containing phagosomes and residual bodies, no mature asbestos bodies [14] were found.

Occurrence of labyrinths

Labyrinths, which are normally rare in mouse peritoneal macrophages, are composed of globular complexes of interconnected channels with a diameter of about 250 nm. In some cases coated vesicles are continuous with the membranes

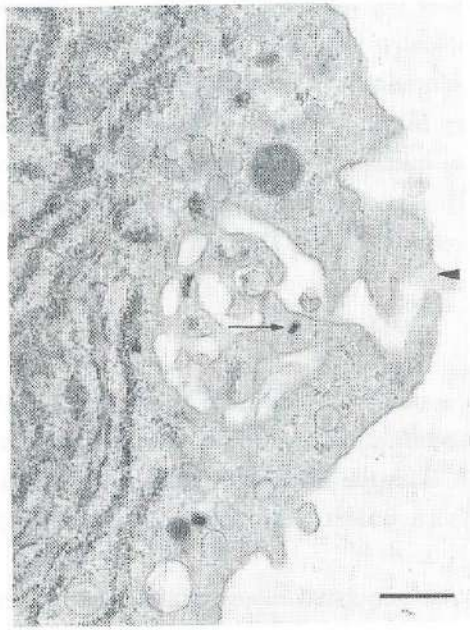


Fig. 8. Labyrinths are seldom found in macrophages of unstimulated mice. In this section the interconnected channels of such a labyrinth are in contact with the extracellular space (arrowhead). Characteristic fragments of PO-positive material (arrow) can be seen in the cytoplasm penetrating the labyrinth. Bar: 0.5μm.

of the channels. Between the channels small fragments of PO-positive material are seen (Fig. 8). On day 8 after asbestos administration, the number of labyrinths was higher than in the steady state and also compared with the control animals (Tab. I). As can be seen in the same table, there was a gradual increase in the percentage of cells showing a labyrinth in the plane of the section. At 6 months, about 36% of the total macrophage population displayed a labyrinth. The average size of the labyrinths increased from rather small between 4 and 48 hours, to very large from 2 to 6 months (Fig. 9 and 10). At 8 months, the number and size of the labyrinths had returned to the range of the unstimulated situation. To make certain that these alterations in size and frequency were not due to variations in the fixation procedure, we fixed cells from control and asbestos-stimulated mice according to the direct fixation method as described by Brederoo and Daems [18] as well. Unlike those authors' findings in the guinea pig, there was no significant difference in the number of labyrinths in material fixed according to our standard method and the material treated by direct fixation.

In some cases labyrinths appeared to be connected to a system consisting of plate-like structures with a rather constant diameter of about 35 nm (Fig 11a and b). This part of the labyrinth too showed contact with coated vesicles, suggesting communication between the labyrinth tubules and the extracellular space. A three-dimensional computer reconstruction of serial sections confirmed the impression that the whole labyrinth, including the lamelliform part, was in contact with the extracellular space and could therefore be considered to be a complex invagination of the cell membrane. Evidence supporting this contact

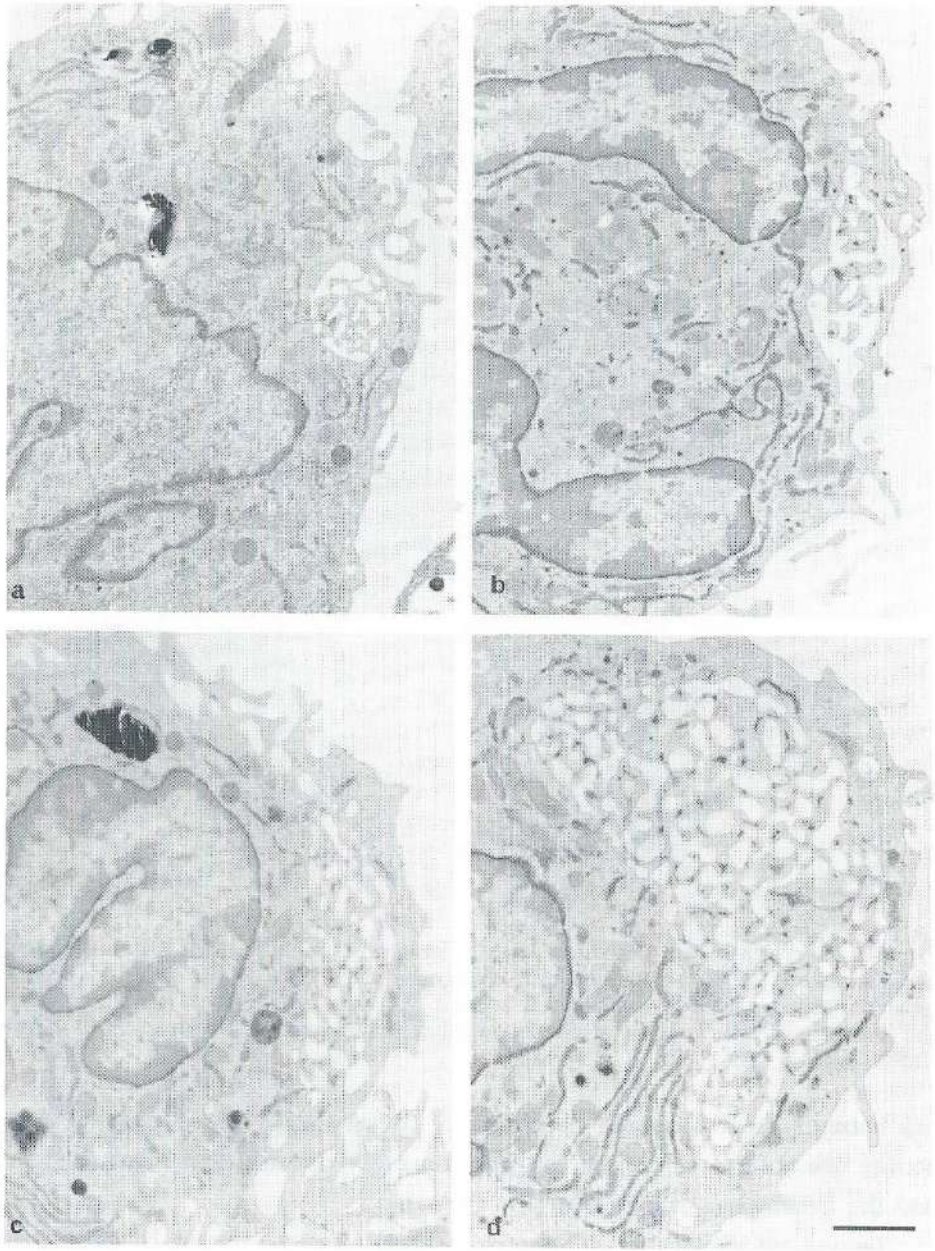


Fig. 9. Micrographs showing the continued increase in the size of the labyrinths after a single asbestos injection. a: at 24 h, b: at one month, c: at 2 months, and d: at 6 months. Bar: 1 μm.

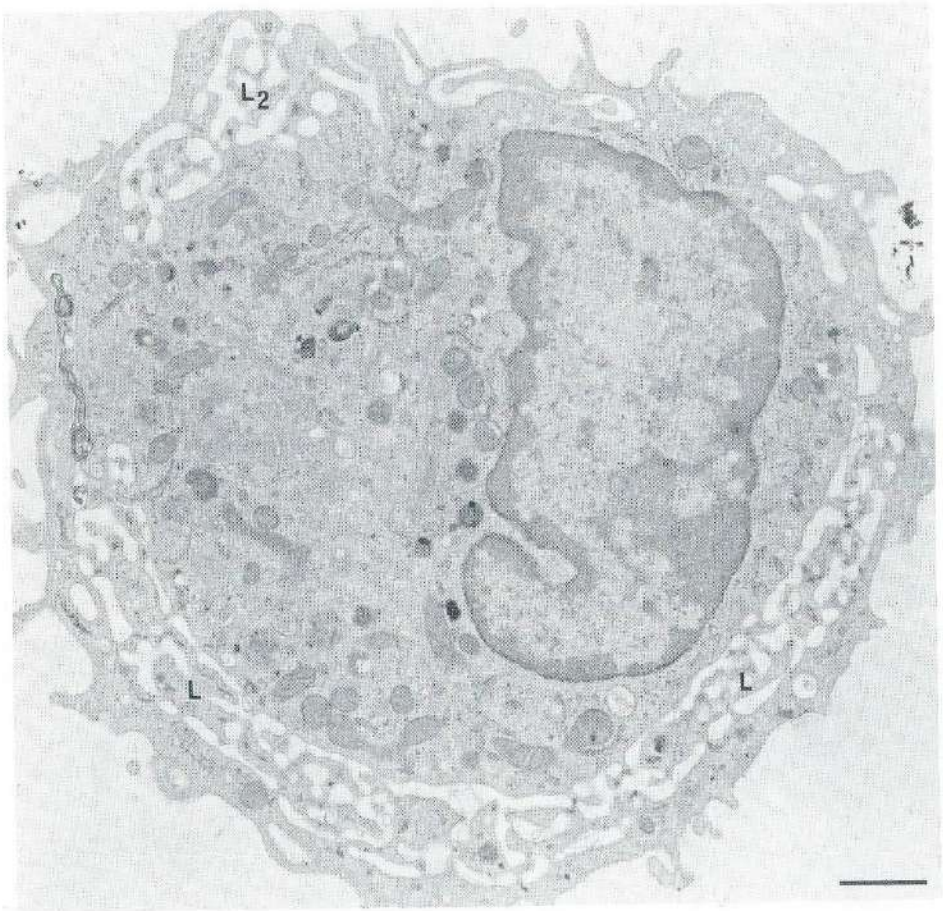


Fig. 10. Macrophage of SPF bred mouse, isolated 6 months after administration of asbestos. A labyrinth (L) extends along the perimeter of the cell. A second labyrinth can be seen in this section as well (L_2). One of a set of serial sections, the seventh of which showed the establishment of contact between both labyrinths. Bar: 1 μm.

has been obtained in mouse bone-marrow cultures [22]. In this material 5'-N activity was not only present at the cell surface but also in the wide tubular part and the lamelliform part of labyrinths (Fig. 11b).

To find out whether the PO-positive fragments enclosed by the labyrinths are parts of the endoplasmic reticulum, we applied the fixation technique developed by de Bruijn et al. [19]. The results show clearly that in view of their size and

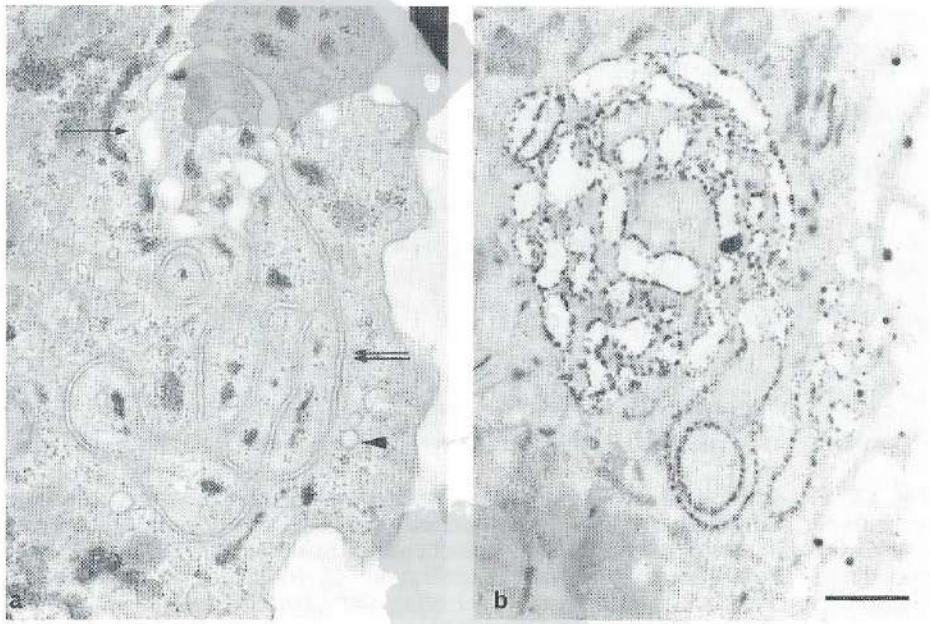


Fig. 11, a and b.
a: Labyrinth consisting of two tubular systems, viz, the commonly observed wide tubular part (arrow), which is connected to a lamelliform part (double arrow). The latter is composed of plates separated by a rather constant spacing (approximately 35 nm). Occasional bristle-coated vesicles are seen fusing with the membrane of the lamelliform part (arrowhead).
b: Labyrinth in a cell from bone-marrow culture after cytochemical treatment for the demonstration of the ectoenzyme 5'-nucleotidase. Reaction product is present in both the wide tubular and the lamelliform part, confirming the contact of both parts with the extracellular space. Bar: 0.5 μm.

location the PO-positive fragments should indeed be considered to represent endoplasmic reticulum (Fig. 12).

DISCUSSION

In the present study, asbestos was endocytosed by macrophages, and six months after the injection of this material, asbestos-containing macrophages were still present in the peritoneal cavity of mice. Crocidolite administration led to increasing numbers of labyrinths in the peritoneal macrophages, as well as to an accumulation of iron in the cytoplasm and the lysosomes. These effects will be discussed in detail here.

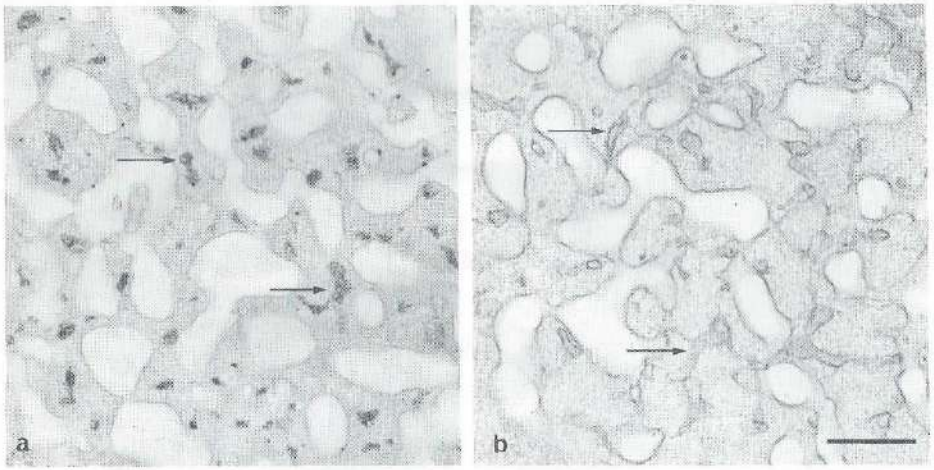


Fig. 12, a and b. Detail of two labyrinths isolated at six months, fixed according to different procedures. a: Labyrinth fixed with 1.5% glutaraldehyde followed by 1% OsO_4, (standard method). The cytochemical reaction for PO-activity was performed. Note the characteristic distribution of fragments of PO-positive material (arrows) in the cytoplasm penetrating the labyrinth. b: Labyrinth fixed according to the osmium(VI)-iron(II) complex method, without PO reaction. The distribution and dimensions of the fragments of endoplasmic reticulum are similar to those of the PO-positive fragments in a (arrows). Bar: 0.25 μm.

Endocytosis of asbestos

The effect of a single intraperitoneal asbestos injection on the composition of the peritoneal cell population was in general, similar to that described earlier [23] and was identical in the two types of mice used. There was only a slight difference in the time at which changes in the composition of the peritoneal cell populations occurred as a reaction to the inflammatory stimulus. In both types granulocytes and monocytes entered the peritoneal cavity shortly after the injection of asbestos, and the proportion of macrophages normally residing in the peritoneal cavity diminished initially. This phenomenon has been reported by other authors as well [17,24]. Within 14 days a steady state in terms of cell/cell ratios was restored. Compared with the results of earlier studies on the effect of various stimuli on the composition of the peritoneal cell population [23], crocidolite asbestos is a strong stimulus under which large numbers of inflammatory cells migrate to the peritoneal cavity.

The asbestos fibers were gradually cleared from the peritoneal cavity. Shortly after the injection, peritoneal macrophages were found to contain large numbers

of phagocytosed fibers, ranging from long thick fiber bundles to small fragments of elementary fibers. Despite the presence of these fibers in the cytoplasm, the cells showed a normal morphology and excessive cell death was not seen.

The numbers of fiber-containing cells and of fibers present per cell gradually decreased. Because, as is generally accepted, asbestos can remain in tissue for many years [2,3] and thus gives rise to asbestos-related diseases, it is not probable that the number of fibers is reduced by intracellular digestive activity.

In spite of the gradual decrease in the number of asbestos containing cells, intracellular fibers were found even as late as six months after asbestos administration. Since phagocytosis of macrophages by macrophages was never seen either in the present experiments or in our previous studies, it is improbable that these fragments represent the residue of phagocytosed macrophages. Furthermore, the asbestos fibers were present in residual bodies, which makes recent uptake of these fibers very unlikely. Hence, the fiber-containing macrophages must have been present in the peritoneal cavity throughout the experimental period.

These findings lead to the conclusion that macrophages are able to remain in the peritoneal cavity for a very long time. This assumption is supported by the findings of de Bakker et al. [25] who described the presence of iron in resident peritoneal macrophages isolated 8 months after one intraperitoneal injection of iron dextran. The gradual increase in the number and size of the labyrinths in the peritoneal macrophages after asbestos administration points in the same direction, as does the gradual increase in the iron content of the cytoplasm and lysosomes. Other data too support the considerable longevity of peritoneal resident macrophages (for a review, see Daems [17]).

Iron accumulation

In a study on the involvement of pulmonary macrophages in the formation of asbestos bodies, Suzuki and Churg [14] described iron-containing inclusion bodies in chrysotile-stimulated macrophages. As early as two weeks after exposure to chrysotile asbestos, alveolar macrophages contained iron-positive granules. Fusion of these granules with the chrysotile-containing residual bodies led to increased iron concentrations in the latter, resulting in immature asbestos bodies. In the present study increasing numbers of macrophages from animals injected with asbestos showed increasing numbers of iron-containing inclusion bodies and at the later time-points also cytoplasmic ferritin. X-ray microanalysis

showed that, besides the increase in the number of iron-containing inclusion bodies, there was also an increase in the iron concentration per inclusion body. The control animals given only buffer, also had iron in inclusion bodies at the later time-points. Iron accumulation has indeed been described in aging macrophages [26]. Because the iron level was higher in asbestos-stimulated mice than in the control animals, we conclude that this kind of stimulus was responsible for the large amount of iron accumulated by the macrophages. The accumulation of iron is not associated only with the presence of asbestos; other particles too can give rise to the formation of cytoplasmic ferritin, e.g. contact with the biomaterial ß-Whitlokite [27] or to the formation of pseudo asbestos bodies [28].

The source of the iron is uncertain, but it seems improbable that in the present study the iron in the cytoplasm and inclusion bodies was derived from the asbestos fibers. X-ray spectra of the native fibers found at the early time-points showed the same Si/Fe ratio as the small fibers found in the residual bodies 6 months after asbestos administration. The iron could not have originated from totally disintegrated asbestos fibers, because free silicon was not detected in the cells. Furthermore, it is unlikely that the iron occurring in inclusion bodies had been released by endocytosed erythrocytes [14], because erythrophagocytosis was rarely seen in our material.

On these grounds, more likely explanations of the elevated iron level would seem to be either a modification of the cellular iron metabolism resulting in blockage of the outflow of iron from the macrophages [29] or an enhanced uptake of iron by the stimulated macrophages [30].

Despite the accumulation of iron in the peritoneal macrophages, mature asbestos bodies were not found in our material. This might be due to a clearance of long asbestos fibers, the basis of asbestos body formation [31], from the peritoneal cavity. If this were the case, blocking of clearance by traumatization of the diaphragm, see Leak [32], might result in the formation of asbestos bodies in the peritoneal cavity.

Whether or not long asbestos fibers are cleared from the peritoneal cavity indeed, is yet uncertain. It is also possible that isolation of these fibers from the peritoneal cavity is difficult because long asbestos fibers have partly or totally penetrated into the peritoneal wall, a phenomenon also described for lung [33], and are therefore absent in the isolated cell suspensions. Whether one of the above mentioned explanations for the absence of long asbestos fibers in the isolated peritoneal cell suspensions is the case, will be subject of future

estigations.

currence of labyrinths

An unexpected phenomenon was the high number of labyrinths in the itoneal macrophages after intraperitoneal administration of crocidolite asbestos ers. These structures, originally described in peritoneal resident macrophages the guinea pig [18], are seldomly present in normal mouse peritoneal crophages [34]. The labyrinths are globular complexes of interconnected mbrane-limited channels that are continuous with the plasma membrane. The sence and number of labyrinths in the guinea pig has been reported to be pendent on the method used for fixation, and the occurrence of these uctures to be restricted to the resident-macrophage population [18]. In the sent study the visibility of labyrinths was not related to the fixation condi-ns nor was the occurrence of the labyrinths restricted to the resident crophage population. In addition, human monocytes are known to be able to velop labyrinths in vitro [35]. The fact that in either this or a previous study] labyrinths were never observed in monocytes or monocyte-derived peritoneal crophages, might have been due to the disappearance of this type of cell from peritoneal cavity before labyrinths could develop.

nctional significance of labyrinths

The present results have led us to believe that labyrinths are functionally portant structures whose size and number are the result of cellular alterations ermined by a long-term reaction to the presence of asbestos. It has been gested that the labyrinths could serve as a pathway for transport between oplasmic elements (e.g. the endoplasmic reticulum) and the extracellular space]. This idea was also put forward by Gerrard [37] and White [38], who cribed in platelets an open canalicular system morphologically resembling yrinths, and pointed to a relation between the excretion of cellular products l this system. Furthermore, there is a striking resemblance between the lings in earlier investigations [18] and in the present study i.e., the intimate ntact between elements of the PO-positive endoplasmic reticulum and the ules of the labyrinths on the one hand, and the relation between the PO-itive dense tubular system and the open canalicular system in platelets on the er.

Biochemical investigations [39,40] showed PG synthetase to be present in the RER, and the presence of PO activity itself is related to PG synthesis [41,42]. In addition the release of PG is also suppressed by PO inhibitors, such as aminotriazole and sodium azide, which inhibit the PO activity in the RER and NE [41].

In the light of the report describing PG release by platelets [38] and taking into account that the release of PGE_2 by macrophages increases after exposure to asbestos [13], it can be speculated that the formation of labyrinths is related to PG synthesis. However, in unpublished immunological experiments in which we attempted to demonstrate the localization of PGE_2 [42], there was no label found above the labyrinths. This could be due to the preparative procedure used, because the tubules of the labyrinth communicate with the extracellular space and therefore PG originally present could easily be dissolved and washed away during preparation of the material. On the other hand, label was found above cytoplasmic vacuoles and lysosomes, which is in agreement with the findings of Darte and Beaufay [40], who describe the presence of PGE_2 in lysosomes and related vacuoles.

In sum, in the present study the i.p. administration of crocidolite asbestos fibers led to higher iron levels in inclusion bodies and residual bodies in mouse peritoneal macrophages, which might be the basis for asbestos body formation. Furthermore, the present findings point to a prolonged presence of the resident macrophages in the peritoneal cavity of the mouse.

Lastly, a clear relationship was found between the administration of crocidolite asbestos and the presence in macrophages of labyrinths, which increased in number and size. Although it can be speculated that these labyrinths have an important function in the synthesis and/or release of prostaglandins by mouse peritoneal resident macrophages, the localization of PGE_2 in these structures was not demonstrated with the methods used.

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CHAPTER III

ASBESTOS BODY FORMATION AND IRON ACCUMULATION IN MOUSE PERITONEAL GRANULOMAS AFTER THE INTRODUCTION OF CROCIDOLITE ASBESTOS FIBERS [2]

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ABSTRACT

This report describes the cell biology of the development of asbestos bodies after a single intra-peritoneal injection of a suspension of crocidolite asbestos fibers into the mouse peritoneal cavity. The majority of the injected fibers were found in aggregates of peritoneal macrophages, exudate cells, and fibrous tissue. These aggregates developed into granulomas containing not only numerous asbestos fibers but also cells of various types, including macrophages, multinucleated giant cells, fibroblasts, plasma cells, granulocytes, and mast cells. Cytoplasmic ferritin was abundantly present in macrophages and giant cells. In addition, iron-rich inclusion bodies were detected.

The results of this study show that asbestos body formation can occur outside the pleural cavity. Asbestos body formation occurred in the granulomas after one month and longer periods. On the basis of morphological criteria, various types of asbestos body were distinguished. X-ray microanalysis showed that variations in the density of the coat could to a certain extent be attributed to the presence of chemical elements in various concentrations. Evidence is presented that asbestos body formation is an extracellular phenomenon.

INTRODUCTION

Asbestos or ferruginous bodies, to be referred to in the following as asbestos bodies, come into existence as a reaction to the presence of poorly digestible fibrous material in the lungs or pleural cavity and consist of an iron-rich layer of protein deposited around the fiber in question [1]. The mechanism of asbestos

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body formation is not known, but several possibilities have been postulated [2-6].

The occurrence of asbestos bodies in biopsy specimens of human lung tissue is used to determine asbestos exposure in occupational medicine [7,8], and an association has been established between the presence of asbestos bodies and the development of fibrosis [9]. The number of asbestos bodies present in broncho-alveolar lavage fluid or lung tissue can be used as a measure of asbestos exposure [10]. It is assumed that an asbestos-body count of more than one per 10^9 cells in lung lavage fluid is an indication of considerable occupational exposure to asbestos [11]. The formation of asbestos bodies has been induced under experimental conditions in various mammalian species besides man, i.e., in the mouse, rat, guinea pig [5], and hamster [6]. Few reports are available on the presence of asbestos bodies outside the lungs or pleural cavity, and it is still uncertain whether asbestos body formation occurs at those sites [12,13].

The present report, which concerns a continuation of our research on the effect of crocidolite asbestos fibers introduced into the mouse peritoneal cavity [14], presents the results of an electron-microscopical study on the formation of asbestos bodies in granulomas in that compartment. In addition, the chemical composition of the asbestos bodies as determined by X-ray microanalysis is discussed.

MATERIALS AND METHODS

Animals

Use was made of SPF bred male Swiss mice which were obtained from the Central Institute for the Breeding of Laboratory Animals TNO (Zeist, The Netherlands) and were 8 weeks old and weighed 20 grams at the start of the experiments. All mice were allowed three days for adaptation before the i.p. injection was given.

Asbestos

With a Branson B12 sonifier (3 min at 50 Watt), crocidolite asbestos was suspended in Hanks' balanced salt solution to a final concentration of 0.5 mg/ml. On day 0, 1 ml of this suspension was injected into the peritoneal cavity. Control animals received 1 ml of the same solution without asbestos.

Isolation of free peritoneal cells and granulomas

At regular intervals a number of the animals were decapitated and injected intra-peritoneally with 2 ml Ringer solution. The abdomen was then carefully kneaded for approximately 30 sec, after which the peritoneal cell suspension was collected by suction. Cells were isolated in this way at 4, 16, 24, and 48 hours, 1 and 2 weeks, and 1, 2, 4, 6, and 8 months after asbestos administration. The peritoneal wall and viscera of the same animals were examined and granulomas were dissected.

A second group of animals were fixed by perfusion before the granulomas were dissected, but the peritoneal cell suspension of this group was not collected.

Fixation and peroxidase cytochemistry

The free peritoneal cells were pelleted and the pellet was prefixed for 10 min at room temperature in 1.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4, 350 mOsmol). The cells were then washed at room temperature for 5 min in 0.1 M cacodylate buffer (pH 6.9) and pre-incubated for 30 min at room temperature in a medium containing 1 mg diaminobenzidine-4HCl per ml cacodylate buffer. Incubation was performed in the same medium supplemented with H_2O_2 to a final concentration of 0.01%.

Postfixation was performed in 1% OsO_4 in 0.1 M cacodylate buffer for 30 min at 4°C (pH 7.4). The granulomas found in animals from which the peritoneal cell suspension had been collected were fixed by immersion in the same glutaraldehyde solution for 1 hour at room temperature.

Perfusion fixation was performed at room temperature by perfusion of the total body via the left ventricle of the heart. The peripheral circulation was rinsed with a Ringer solution for 30 sec, followed immediately by 1.5% glutaraldehyde for 10 min. Fixation was then continued by immersion of the tissue blocks in the same fixative for 1 hour.

Next, some of the granulomas were processed for electron microscopy without any further fixation. Others were postfixed in the 1% OsO_4 solution as described above for 1 hour. A third group was postfixed in the modified OsO_4 fixative according to de Bruijn et al. (1973) [15] for 24 hours at 4°C.

Extraction of the asbestos bodies

Extraction of asbestos bodies was done according to Smith et al. (1972) [16]. In short, dissected granulomas were placed in a glass vessel containing domestic laundry bleach (5.25% sodium hypochlorite) to digest the tissue. When all of the tissue had been dissolved, the suspension was centrifuged and the supernatant decanted. The residue was then rinsed and filtered on a membrane filter.

Transmission electron microscopy (TEM)

The tissue blocks were dehydrated in a graded series of alcohol up to 100% and embedded in Epon. Ultrathin sections were cut on an LKB microtome, stained with lead hydroxide, and examined in a Philips EM 410.

Scanning electron microscopy (SEM)

After glutaraldehyde fixation, the blocks were dehydrated in a graded alcohol series up to 100% and critical point dried under carbon dioxide in a Balzers model CPD 020 critical point drier. The dried specimens were covered with a layer of gold about 6 nm thick in a Polaron sputter coater, and examined in a - Cambridge Stereoscan S 180 at an acceleration voltage of 15 to 20 kV and a tilting angle of about 30° relative to the electron beam.

X-ray microanalysis (XRMA)

X-ray microanalysis can be used to determine chemical elements present in accurately defined structures in electron-microscopical sections [14]. For this purpose, the electron beam is focused on the structures of interest and the X-ray signal resulting from the specimen-beam interaction is used to obtain a spectrum reflecting the presence of chemical elements in the indicated area. XRMA was applied to identify the crocidolite asbestos fibers and to determine the chemical composition of the matrix of inclusion bodies as well as the coats of asbestos bodies.

X-ray microanalytical spot analyses were performed with a Tracor (TN) 2000 X-ray microanalysor attached to a Philips EM 400 scanning transmission electron microscope. The sections were collected on copper grids which were placed in a low-background holder at an angle of 108° relative to the electron beam. Measurements were done for 100 sec lifetime, with a spot diameter of 100 nm and an accelerating voltage of 80 kV.

X-ray maps were made with the same instrumental configuration, with a spot size of 50 nm and an accelerating voltage of 80 kV. For this purpose, the electron beam was controlled by the Tracor computer to move over the specimen in a raster pattern. The computer program allows the detection of only four chemical elements simultaneously, and windows to detect phosphorus, chlorine, calcium and iron were set. A fifth window, ranging from 4600 to 5000 eV, was set for background subtraction. Per pixel point a dwell-time of 0.5 s was used.

RESULTS

Peritoneal cell population

As already reported [14,17], the composition of the peritoneal cell population changed in response to the introduction of foreign material into the peritoneal cavity.

At 4 hours, an influx of neutrophil granulocytes had already taken place. SEM showed that, in contrast to the situation in the unstimulated peritoneal cavity, many cells were attached to the peritoneal wall and numerous asbestos fibers were scattered over the peritoneal wall, omentum, and viscera.

At 16 and 24 hours, more exudate cells had invaded the peritoneal cavity. TEM showed that the majority of these cells were granulocytes and monocytes, many of which contained partially or completely ingested asbestos fibers.

At 48 hours the number of exudate cells had decreased, and after the eighth day granulocytes and monocytes were seldom seen, but the total number of cells collected from the peritoneal cavity remained relatively high during the further course of the experiment. Even at eight months the average cell count was $16.4 \times 10^6 \pm 2.1 \times 10^6$ for the group given crocidolite. The corresponding values for the control animals were $5.3 \times 10^6 \pm 0.2 \times 10^6$ cells, i.e., within the range of the average number of cells present in the unstimulated peritoneal cavity.

Although the number of cells found in the peritoneal cavity at 4, 6, and 8 months was still rather high compared with the control animals, the proportional composition of these cell suspensions was the same as in the control group. Starting on day 14, 80% of the cell suspensions collected from the asbestos-sti-

mulated animals had a red coloration suggesting the presence of blood. Erythrocytes were indeed found among other cell types in TEM sections, whereas the corresponding suspensions of the control group were devoid of such cells.

Aggregate and granuloma formation

Fiber/cell aggregates could be recognized macroscopically already after four days by the bluish colour of the crocidolite asbestos fibers. Aggregates smaller than 2 mm were frequently found against lobes of the liver and on the diaphragm, stomach, gut, and omentum, but aggregates of 2 mm and larger were only seen in contact with the omentum.

Scanning electron microscopy showed that aggregation of asbestos-containing phagocytes had already occurred at 4 hours. These early aggregates were still very small (< 0.1 mm), the location was variable, and the structure was still very loose (Fig. 1a). At 24 hours, the aggregates became more distinct and a network of fibrous material containing cells and asbestos fibers was observed (Fig. 1b). The peritoneal wall and viscera were still strewn with free cells at this time-point, but free asbestos fibers were seldom seen. The size of the aggregates sometimes increased to 1 mm and larger during the first four days, and SEM showed that their structure had become more compact. In this period the free cells on the peritoneal wall and viscera disappeared (Fig. 1c). By day four, all free cells had disappeared and mesothelial cells had started to cover the aggregates.

Encapsulation of the aggregates by mesothelial cells was completed at two weeks. SEM of loci after this and longer intervals showed only heightened areas with here and there a single asbestos fiber penetrating through the covering mesothelial layer (Fig. 1d).

Transmission electron microscopy showed that during the first twenty-four hours exudate cells, i.e., monocytes and neutrophil granulocytes, entered the aggregates, which became larger during this period. Besides the above-mentioned exudate cells, there were also macrophages, mast cells, and fibroblasts in the aggregates. The greater part of the extracellular space was occupied by unorganized collagen fibers forming a network around the cells and the asbestos fibers.

On day four, the majority of the aggregated cells were macrophages with some multinucleated giant cells, both containing asbestos fibers. Small asbestos fibers were present in inclusion bodies, either phagosomes or secondary lysosomes, and incidental asbestos fibers apparently lying free in the cytoplasm were seen. Large asbestos fibers were in general only partially surrounded by one or

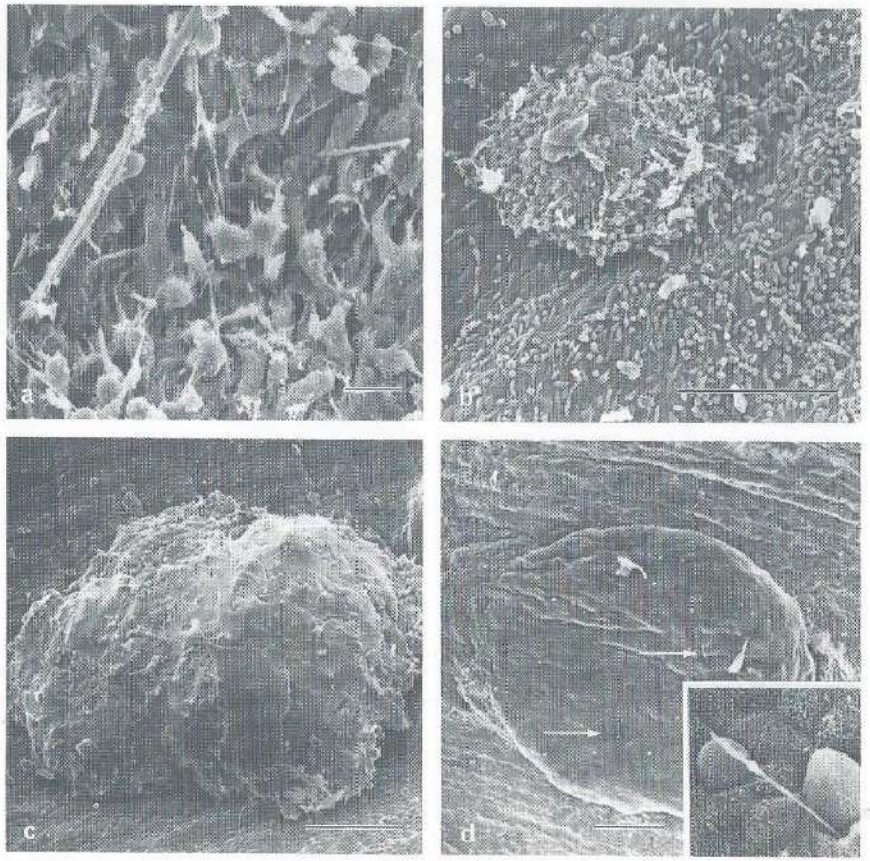


Fig. 1, a-d. Scanning electron micrographs showing the development of asbestos granulomas. a: Aggregation of asbestos fibers and free peritoneal phagocytes seen against the omentum four hours after the injection of suspended asbestos fibres. Note the difference in size between the large asbestos fibers and the phagocytes. Bar: 10 μm. b: Aggregation of asbestos fibers and free peritoneal phagocytes at twenty-four hours. The aggregate is now large relative to the one in a, and the structure is distinctly more compact. Numerous free peritoneal cells are attached to the mesothelial cells of the peritoneal wall. Bar: 100 μm. c: Aggregate of asbestos fibers and free peritoneal cells, seen at forty-eight hours. The structure of the aggregate is very compact. There are no longer any free peritoneal cells or asbestos fibers against the peritoneal wall. Bar: 100 μm. d: Asbestos-containing granuloma along the peritoneal wall seen at one month. The granuloma is completely covered by the mesothelial cells of the peritoneal wall. Incidental asbestos fibers penetrating through the layer of mesothelial cells are visible (arrows and inset). Bar: 100 μm.

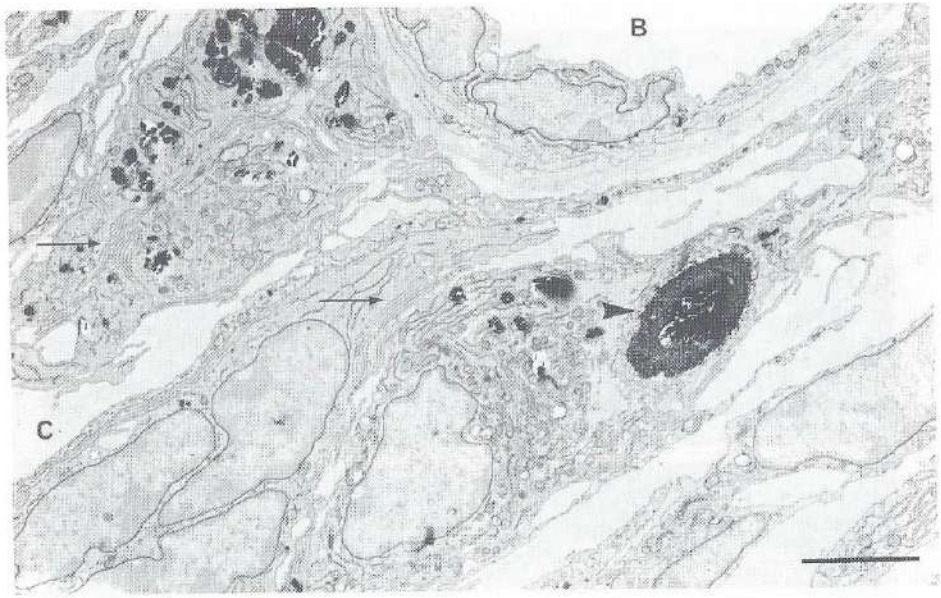


Fig. 2. Transmission electron micrograph showing a granuloma against the diaphragm at six months. The interdigitating folds of the plasma membranes of various macrophages and giant cells can be seen (arrows). A number of asbestos fibers is present, one of which has induced formation of an asbestos body (arrowhead). Sectioning artifacts due to the hardness of the asbestos fibers are visible. B: blood vessel, C: collagen. Fixation: glutaraldehyde, osmium(VI)-iron(II) complex. Bar: 10 μm.

more macrophages or giant cells. At this time-point each section of giant cells contained three to five nuclei which were randomly distributed through the cytoplasm.

TEM of aggregates after fourteen days to one month showed that encapsulation by mesothelial cells had been completed, and newly formed blood vessels were seen regularly. Starting at one month, the aggregates became transformed into vascularized granulomas containing lymphocytes, fibroblasts, macrophages, giant cells, plasma cells, and, in smaller numbers, mast cells and eosinophil granulocytes. The organization of the collagen in the extracellular space was better than that seen at the shorter intervals. Between day fourteen and one month there were more multinucleated giant cells than after the shorter intervals. Giant cells containing fifteen to twenty nuclei, sometimes in the peripheral cytoplasm, were regularly seen. Pairs of centrioles were present in the centre of these cells. At these time-points the macrophages and giant cells showed thin cytoplasmic flaps which at sites of contact with neighbouring cells

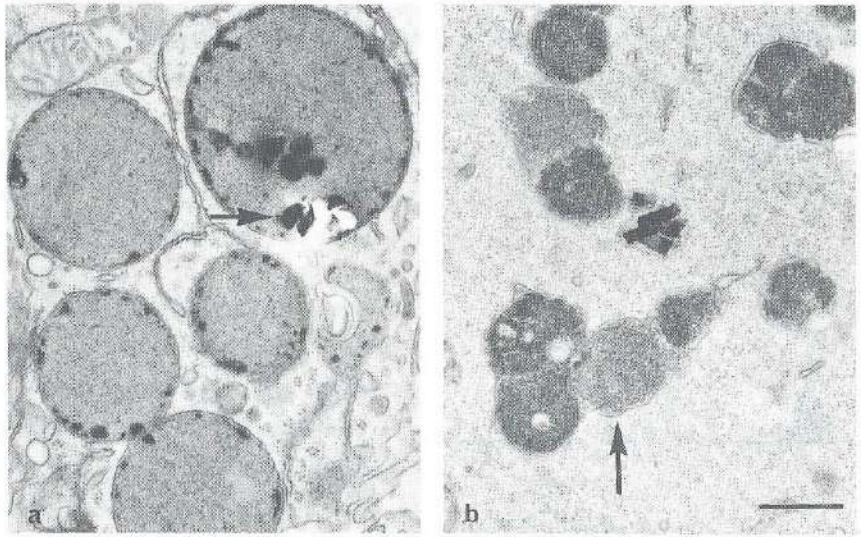


Fig. 3, a and b. Electron micrographs showing type I and II inclusion bodies. a: Type I inclusion bodies with the homogeneous matrix consisting of evenly distributed iron micelles. Small asbestos fragments are sometimes enclosed by the inclusion bodies (arrow). Peripherally there is a zone containing dark osmiophil droplets. Between this zone and the enclosing membrane a thin halo is visible. Cytoplasmic ferritin is present in the cytoplasm surrounding the inclusion bodies. b: Type II inclusion bodies. The matrix of this type is variably dense due to different concentrations of iron micelles, suggesting fusion of single small inclusion bodies. Here too cytoplasmic ferritin is abundantly present. Fixation: glutaraldehyde, osmium(VI)-iron(II) complex. Bar: 0.5 μm.

were folded, thus forming a number of layers (Figs. 2 and 6).

Although fibroblasts and collagen fibers were seen throughout the granulomas, the bulk of the connective tissue was present in the periphery, forming a capsule directly under the layer of mesothelial cells. The cells in the granulomas showed a normal morphology, and cell death caused by the presence of asbestos fibers was not observed. Morphological site-related differences between granulomas were not found.

Iron-containing inclusion bodies

Macrophages and giant cells found in aggregates and granulomas two weeks or longer after the introduction of asbestos frequently contained large numbers of dark inclusion bodies. On the basis of morphological criteria, two types of inclusion body were distinguished (Fig. 3). The first type comprised smooth

round inclusion bodies with a fine homogeneously distributed granular content consisting of micelles measuring about 6 nm. Droplets of osmiophil material were as a rule present in the periphery of these inclusion bodies. The limiting membrane was separated from the electron-dense content by an electron-lucid halo about 20 nm wide (Fig. 3a).

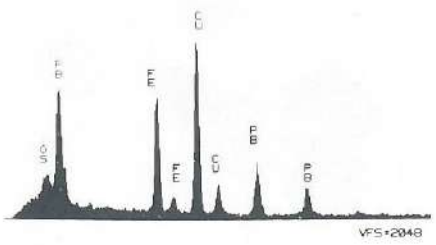


Fig. 4. X-ray microanalysis spectrum of one of the inclusion bodies in 3b (arrow), showing the presence of iron. (Vertical full scale 2,048 counts.)

The second type of inclusion body was in general smaller and more heterogeneous as to shape and content (Fig. 3b). Like the first type, the smaller bodies of this type contained small micelles of about 6 nm which were homogeneously distributed, but the osmiophil droplets and the halo characteristic for the first type were not encountered. The micelles in the larger inclusion bodies of the second type were heterogeneously distributed, suggesting the occurrence of fusion between inclusion bodies of the second type. XRMA demonstrated the presence of iron in both types of inclusion body (Fig. 4). Small asbestos fibers were seen regularly in both types, although less frequently in the second. The two types of inclusion body were never found together in the cytoplasm of one cell.

Cytoplasmic ferritin was frequently encountered in macrophages and giant cells of the granulomas (Fig. 3, a and b), but the presence of cytoplasmic ferritin was not related to the presence or absence of either type of inclusion body.

Asbestos body formation

TEM performed on granulomas dissected after one month or more showed asbestos bodies (Fig. 2). The dimensions of these bodies were variable, and in cross-sections the diameter ranged from 0.5 μm to 15 μm. The majority, however, lay in the range of 5 μm to 10 μm. This variation of the diameter started at one month and continued through the longest interval of 8 months.

The macrophages and giant cells in the immediate vicinity of the asbestos bodies showed an organelle-free zone. These zones were often permeated by

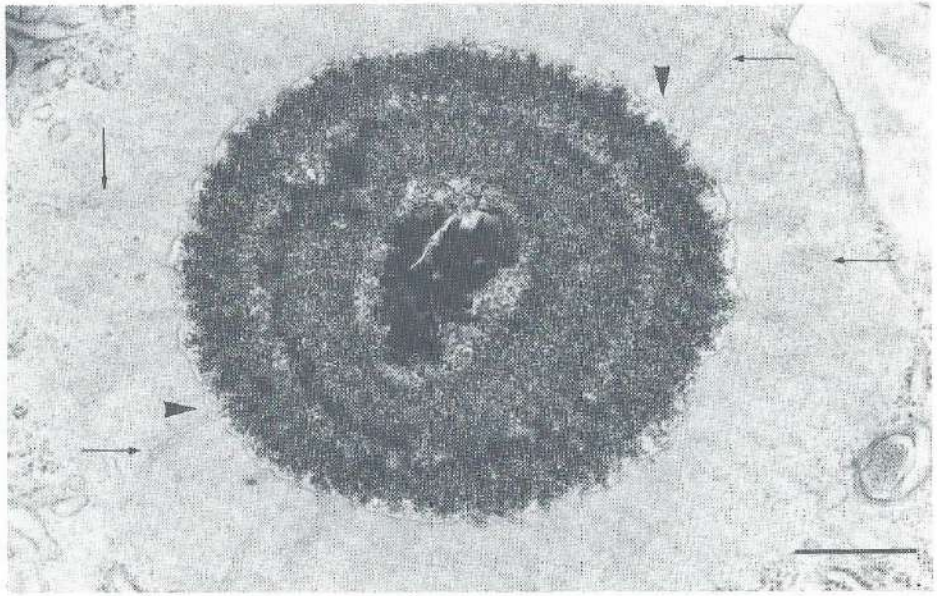


Fig. 5. Asbestos body in a granuloma against the diaphragm at six months. The asbestos body is surrounded by an organelle-free zone which is traversed by small tubular structures (arrows). Sometimes it can be seen that these structures are in direct contact with the plasma membrane enclosing the asbestos body (arrowheads). Fixation: glutaraldehyde, osmium(VI)-iron(II) complex. Bar: 0.5 μm.

small tubular structures (Figs. 5, 8, and 11) whose membrane was continuous with the plasma membrane (Fig. 5). The amount of cytoplasmic ferritin detected in the organelle-free zones was distinctly smaller than that in the surrounding cytoplasm.

Longitudinal sections through the asbestos bodies frequently showed spots where two macrophages made contact. At these places there was a connection between the extracellular space and the space surrounding the asbestos body (Fig. 6). In transverse sections, where spirally wound plasma membranes were often seen, there was again a direct connection between the space occupied by asbestos fibers and bodies and the extracellular space (Figs. 7a and 12).

We sometimes saw two or more asbestos bodies enclosed by the same cell or group of cells. Free asbestos bodies were never found in our material. Ultrathin sections in which the asbestos bodies were oriented such that the central asbestos fiber lay more or less in the plane of the section gave the impression that especially the longest asbestos fibers provided the basis for

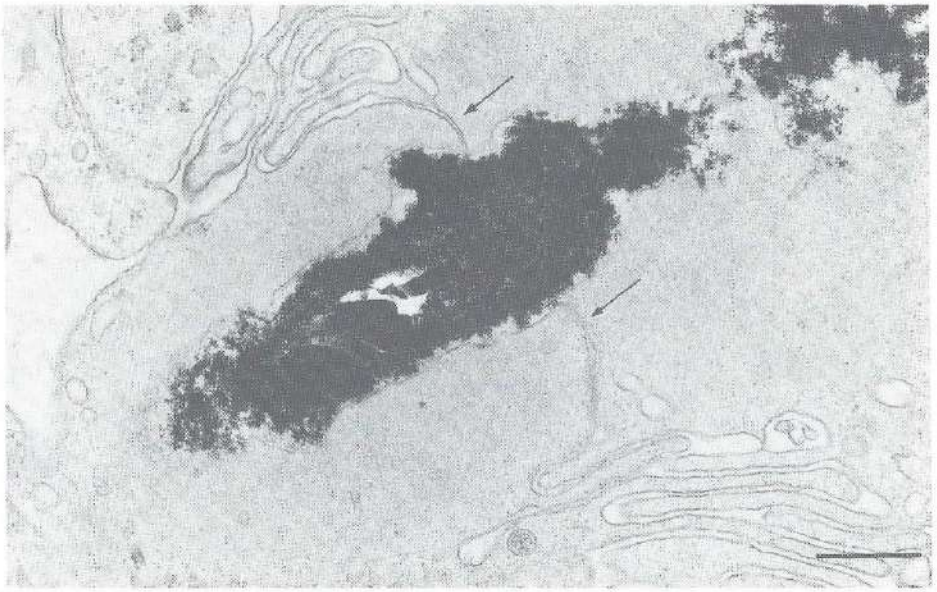


Fig. 6. Part of a longitudinally sectioned asbestos body. A connection between the space holding the asbestos body and the extracellular space is visible (arrow). The plasma membranes of the two cells enclosing the asbestos body are folded, thus forming interdigitating contacts. Fixation: glutaraldehyde, osmium(VI)-iron(II) complex. Bar: 0.5 μm.

asbestos body formation.

The basic structure of an asbestos body was found to consist of a central asbestos fiber surrounded by an iron-containing coat. The thickness of the coat varied. In some cases only a thin coat of tenuously divided micelles was visible (Fig. 7a and 7b), but in others a thick, densely packed coat was seen (Fig. 2 and 8). Several different types of asbestos body could be distinguished on the basis of the ultrastructure and chemical composition of the coat. One type (type I) concerned asbestos bodies with a coat containing homogeneously distributed dark micelles measuring about 6 nm (Fig. 8a). XRMA of this type of asbestos body showed without exception high peaks for iron (Fig. 8b). Small asbestos fragments scattered through the coat of these asbestos bodies were frequently seen (Fig. 8a inset).

Another type (type II) had a lamellated coat in which concentric rings containing iron micelles with alternating densities were seen. This type too had small asbestos fragments but these were mainly restricted to the darker lamellae (Fig. 9a, and 10a). Furthermore, small needle-like crystals resembling apatite crystals

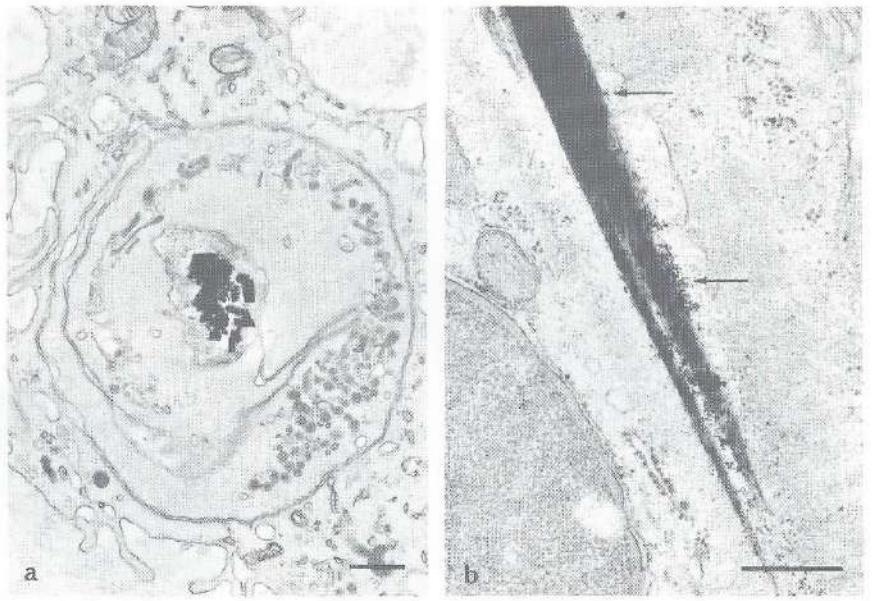


Fig. 7 a and b.
a: Transverse section through a young asbestos body. A spirally wound plasma membrane provides a connection between the extracellular space and the space containing the asbestos body. Note the sparsely distributed iron micelles in the coat of this asbestos body. Fixation: glutaraldehyde, osmium(VI)-iron(II) complex. Bar: 1 μm.b: Longitudinal section through a young asbestos body. The asbestos body coat contains a small number of iron micelles (arrows). Fixation: glutaraldehyde, osmium(VI)-iron(II) complex. Bar: 0.5 μm.

were sometimes seen, mainly on the edges of the lamellae and in the peripheral zones (Fig. 9b). XRMA of these needles gave peaks for calcium and phosphorus (Fig. 9c). Low concentrations of calcium were sometimes also detected by XRMA in the darker rings, but needle-like crystals like those just described were never found in these loci. XRMA of the rings showed that the darker rings had spectra with higher iron peaks than the lighter rings did. To obtain exact data on the distribution of the various chemical elements over the total coat of an asbestos body, X-ray maps of a number of coats were made (Fig. 10b). These maps confirmed the finding that the darker rings were usually characterized by a high concentration of iron. Calcium was more prominent in the lighter rings. These X-ray maps also indicated that phosphorus was always co-localized with the calcium, and that at places with high concentrations of iron there was also an elevated chlorine concentration.

The third type, which was only seen occasionally, comprised asbestos bodies

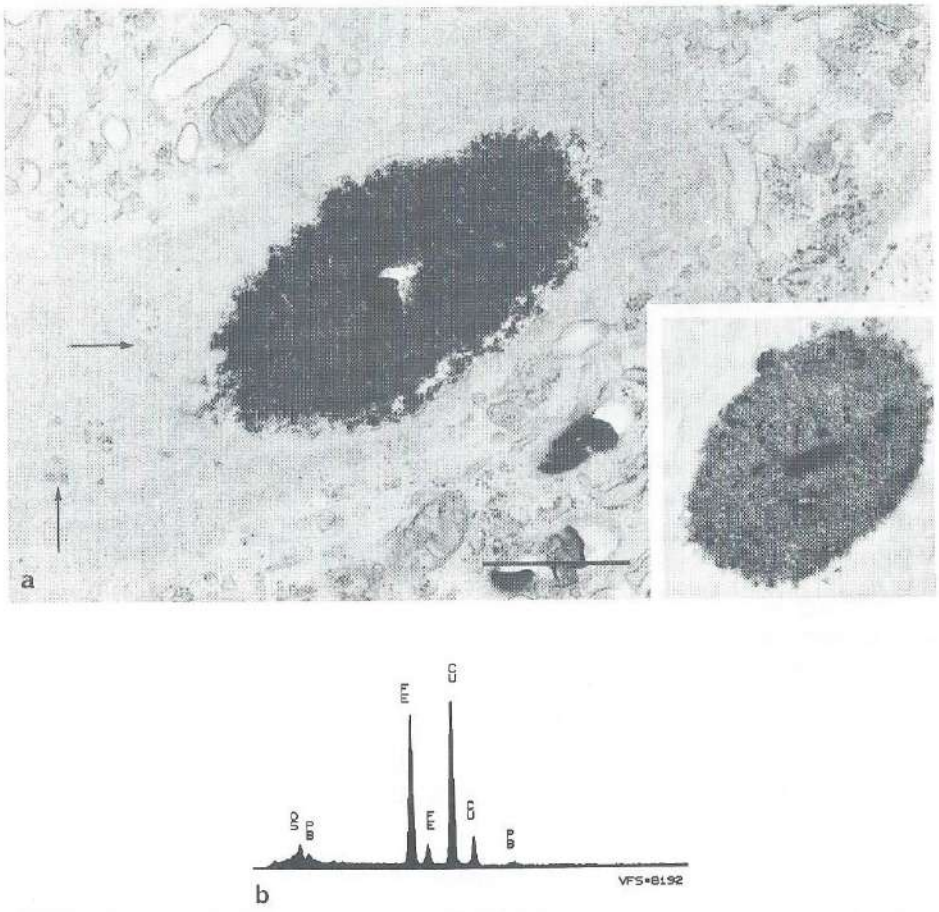


Fig. 8 a and b.
a: Type I asbestos body. The central asbestos fiber is covered by a densely packed homogeneous iron-rich coat. The space containing the asbestos body is limited by the plasma membrane of the surrounding phagocyte. The cytoplasm bordering the asbestos body is free of organelles and traversed by small tubules (arrows). Fixation: glutaraldehyde, osmium(VI)-iron(II) complex, Bar: 1 μm. Inset: Type I asbestos body fixed with glutaraldehyde alone to visualize the numerous asbestos fragments (arrows). b: X-ray microanalysis spectrum of the coat of the asbestos body in a. The spectrum shows that the coat has a high iron content. (Vertical full scale 8,192 counts.)

with an electron-lucid coat. Here the absence of electron density was due to the relatively low numbers of iron micelles in the various lamellae (Fig. 11). Small asbestos fragments, as detected in asbestos bodies of types I and II, were never seen in the coat of type III asbestos bodies. XRMA of this type of asbestos

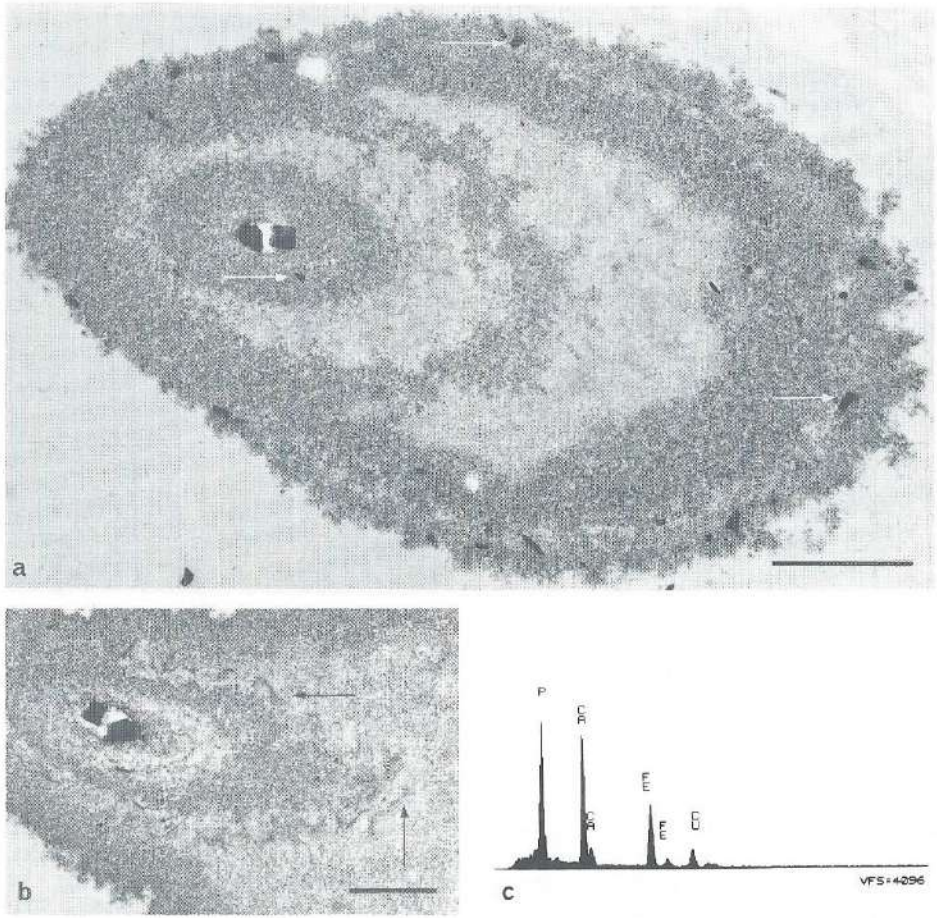


Fig. 9 a-c.
a: Type II asbestos body. The central asbestos fiber is covered by a heterogeneous coat with an annular structure. In the darker rings of this coat small asbestos fragments can be seen (arrows). Bar: 1 μm.b: Part of a type II asbestos body. Small needle-like crystals are present in this asbestos body (arrows). Fixation: glutaraldehyde only. Bar: 0.5 μm.c: X-ray microanalysis spectrum of the area containing the needle-like structures of the asbestos body shown in b. The spectrum shows distinct peaks for iron as well as for calcium. (Vertical full scale 4,069 counts.)

body showed that only low concentrations of iron were present, and calcium was never found.

The fourth type of asbestos body contained dark non-granular material. Remarkably, the coat of such asbestos bodies was always damaged by ultrathin sectioning, leaving only fragments of the coats in question (Fig. 12a). XRMA of

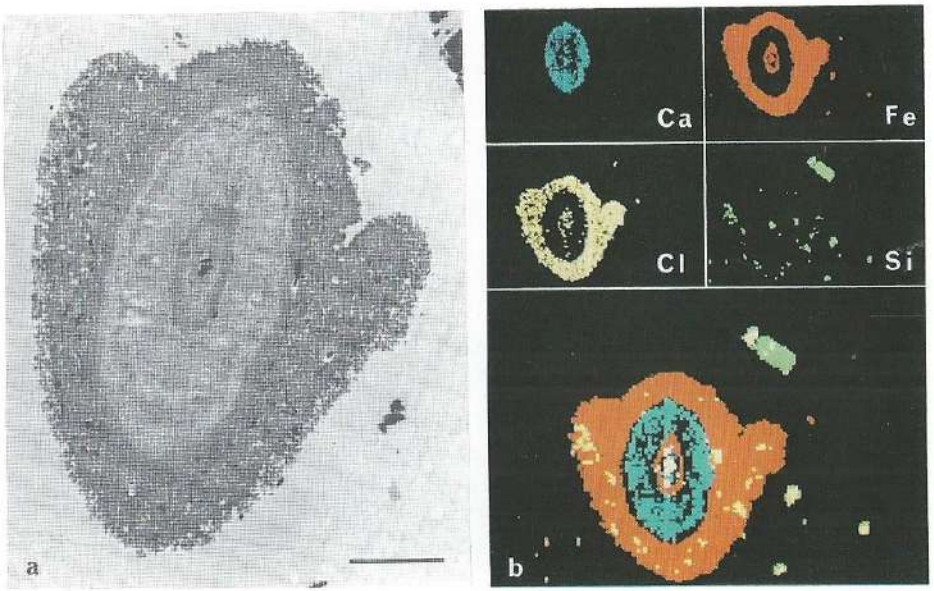


Fig. 10 a and b. Type II asbestos body.
a: Transmission electron micrograph showing the characteristic annular morphology and asbestos fragments in the dark peripheral zone. Apatite crystals are not visible but the presence of calcium was demonstrated by X-ray microanalysis (XRMA).
b: XRMA map of the same asbestos body. The four colors represent chemical elements for which windows were set. Each dot indicates a positive signal (= counts minus background at selected window). Top: Localization of the individual elements in the coat of the asbestos body and in the asbestos fibers, the latter recognized from the silicon signal. Bottom: composition showing the localization of the elements iron, calcium, and silicon. Note the distinctive distribution of iron and calcium. Fixation: glutaraldehyde alone. Bar: 2 μm.

the non-granular coats gave relatively high peaks for calcium and phosphorus, but iron was also present in these areas (Fig. 12b). Calcium phosphate crystals were never seen in the type IV asbestos body.

Finally, there were asbestos bodies showing both the granular structure seen in the first and second types and the non-granular material occurring in the fourth type. However, when these two kinds of deposition were encountered in one asbestos body, they always occurred in different areas, the centre being occupied by the non-granular, calcium-rich deposits and the peripheral zone showing a high concentration of iron micelles (Fig. 13).

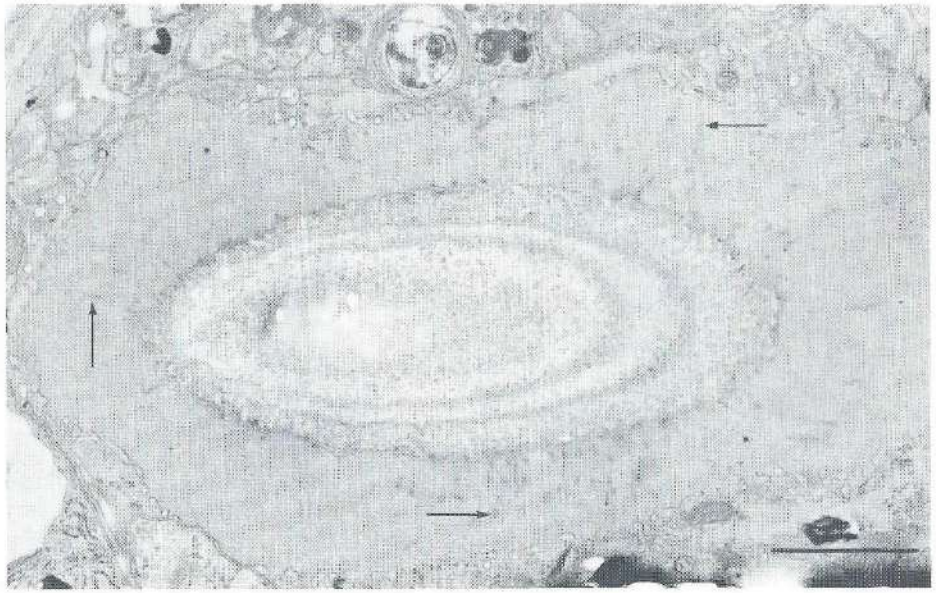
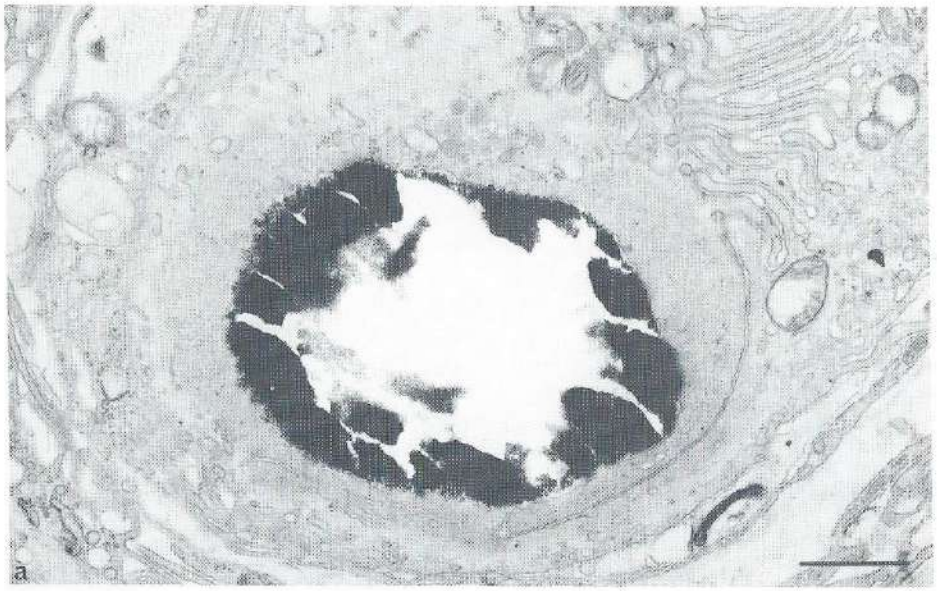


Fig. 11. Type III asbestos body showing resemblance to the type II asbestos body. However, the electron density is low due to a very low concentration of iron micelles. The impression of an annular morphology is caused by non-iron electron-dense material. Note the numerous tubules traversing the organelle-free cytoplasmic zone (arrows). Fixation: glutaraldehyde, osmium(VI)-iron(II) complex. Bar: 2 μm.

Extraction of asbestos bodies

TEM sections of ingested fibers, especially the long asbestos fibers, are seldom oriented such that the total fiber lies in the plane of the section. For determination of the size distribution of the asbestos fibers in the granulomas and to obtain information about the relation between the dimensions of the fibers and the presence of a coat, we used the method described by Smith and Naylor [16] to extract asbestos fibers and asbestos bodies from the granulomas. This method gives total dissolution of cellular material without affecting the asbestos fibers, including the coats. Corn et al. [18] showed that the fiber loss associated with this method is negligible. Therefore, material obtained with this procedure was used to collect information about possible differences between the asbestos fibers in the injected and the recovered populations. SEM of the same material showed that the size distribution of the recovered fibers differed from that of the



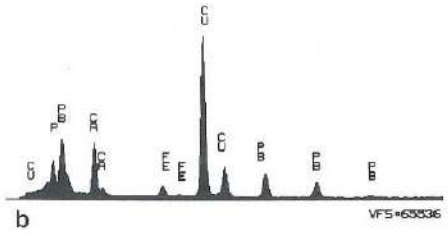


Fig. 12 a and b.
a: Type IV asbestos body. The coat of this type of asbestos body, and with it the central asbestos fibers, were generally removed by ultrathin sectioning. Although this type of asbestos body does not resemble those of types I, II or III, the morphology of the cells surrounding the asbestos bodies is similar. Note the presence of the typical organelle-free zone and the interdigitating plasma membranes. b: X-ray microanalysis spectrum of the asbestos body in a. There are distinct peaks for calcium and iron. Fixation: glutaraldehyde, osmium(VI)-iron(II) complex. Bar: 1 μm. (Note that the vertical full scale is 65,536 counts.)

injected fibers (Fig. 14). As Fig. 14 shows, there were relatively few recovered fibers with a length between 0.5 and 10 μm compared with the originally injected population. Fibers of this class were, however, frequently found in residual bodies of the free peritoneal macrophages (see chapter II) [14] and in Kupffer cells.

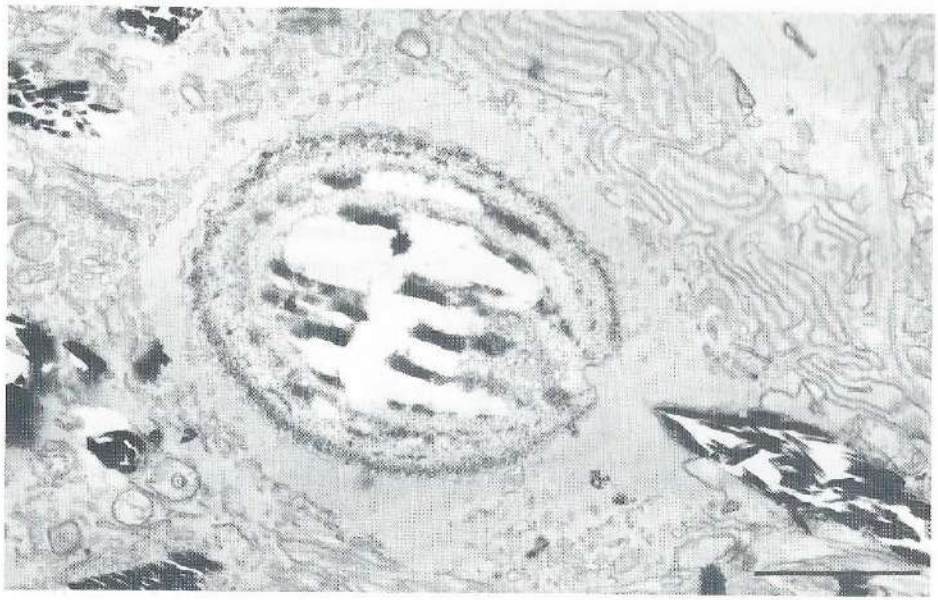


Fig. 13. Asbestos body showing the characteristics of types II and IV, but each in separate areas, i.e. the centre occupied by the structure as described for type IV and the periphery occupied by a structure resembling type II. Fixation: glutaraldehyde, osmium(VI)-iron(II) complex. Bar: 1 μm.

Of the recovered asbestos fibers, 0.1 to 1% were sometimes covered totally but a larger percentage partially by a round to oval coat (Fig. 15). The formation of an asbestos body proved to be independent of the thickness of the asbestos fiber. However, when the coat had been deposited on a thin fiber, that fiber always lay in approximately the centre of the deposited material (Fig. 15, a and b) whereas the coat formed on thick fibers was generally limited to only one side of the fiber (Fig. 15c). The length of the fibers was also found to be important in asbestos body formation. Asbestos bodies were never seen on fibers shorter than 10 μm.

In agreement with the TEM observations, the coats of asbestos bodies had a maximum diameter of about 15 μm, but the diameter of this coat generally did not exceed 5 to 10 μm. With very long fibers, more than one and sometimes numerous coats were present along one fiber and large areas remained uncoated (Fig. 15b).

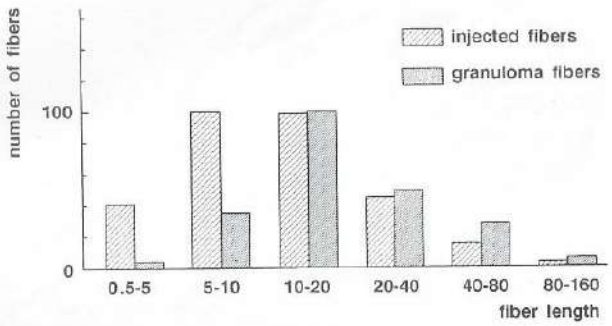


Fig. 14. Histogram showing the size distribution of the originally injected fiber population and of the population recovered by extraction. The histogram shows that the relative distributions of the original and recovered populations are significantly different for the groups representing the smaller fibers.

DISCUSSION

In 1929, Steward and Haddow [19] were the first to point to a relation between the presence of asbestos fibers and the "bizarre and distinctive golden brown bodies" described by Marchand [20]. This led Steward and Haddow to introduce the term asbestosis bodies in their paper. Later, this term was changed to asbestos body [1], because it was assumed that the phenomenon in question was restricted to the interaction between tissue and asbestos fibers. Gross et al. [21] showed that asbestos bodies were present after the instillation of other indigestible particles as well, and this led them to introduce the term ferruginous bodies. For the present report, however, preference was given to the term asbestos body because a suspension of pure crocidolite asbestos fibers had been chosen for the study.

Although the introduction of asbestos fibers in various loci outside the lungs and pleural cavity has been subject of several investigations [22,14] and although asbestos bodies have sometimes been found outside the lungs [12,13], asbestos body formation at these loci has not been demonstrated and it remained

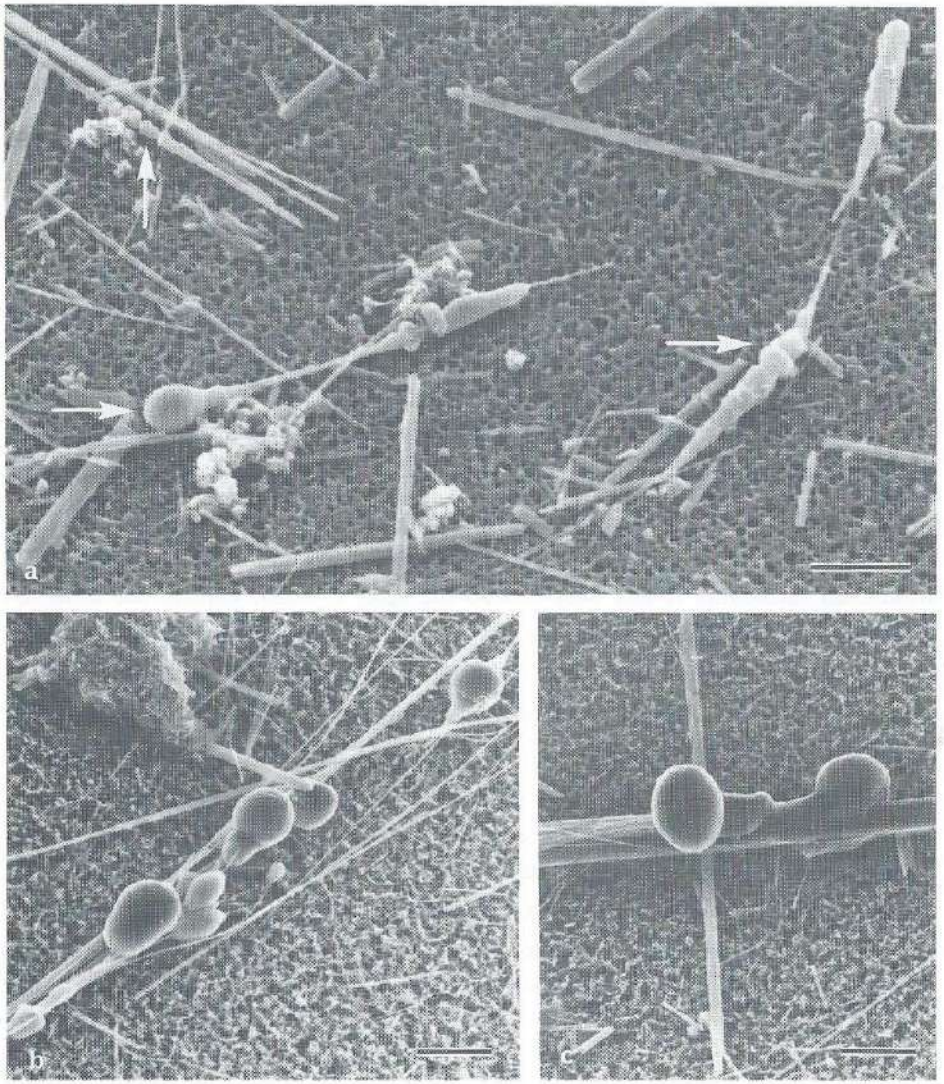


Fig. 15 a-c. Scanning electron micrographs of isolated asbestos bodies.
a: Asbestos fibers recovered with the tissue-dissolving method. Some fibers are coated and form typical beaded asbestos bodies (arrows). Bar: 20 μm. b: Part of a very long asbestos fiber (total length about 1000 μm), isolated with the same method. Numerous asbestos bodies are seen on this fiber. Bar: 10 μm. c: Asbestos fiber from the same specimen as in b. Asbestos bodies have formed against a thick part of the fiber. Note that the coat is restricted to one side of the asbestos fiber. Bar: 10 μm.

uncertain whether it could occur here. In the present study, however, the formation of asbestos bodies was observed in peritoneal granulomas after the introduction of crocidolite asbestos into the mouse peritoneal cavity. It was therefore of interest to find out whether the asbestos bodies formed at these sites resemble those produced in the lungs and pleural cavity [5,6] and, if so, whether the mouse peritoneal cavity would serve as a useful model for study of the process of asbestos body formation in vivo.

Aggregates and foreign body granulomas

SEM showed that asbestos fibers aggregate rapidly after being injected as a sonificated suspension of unclustered fibers. It seems likely that these aggregates offer the basis for granuloma formation. The development of granulomas as a reaction to the presence of foreign material such as asbestos fibers is a well-known phenomenon [5]. The mechanism underlying asbestos fiber accumulation is still unknown, but various possibilities may be suggested. For example, anchoring of long asbestos fibers penetrating between mesothelial cells and the attachment of macrophages containing other asbestos fibers as well, might induce granuloma formation. If this were indeed the case, it would explain the observation made by de Bakker et al. [23], i.e., that peripheral macrophages of the milky spots are exposed to the lumen of the peritoneal cavity. If the mechanism suggested above is correct, it would also explain why the largest aggregates are found in contact with the omentum. At these sites an active process rather than random penetration would lead to the presence of larger amounts of asbestos fibers serving as anchors for the aggregation of cells and fibers. During the longer intervals other cell types such as fibroblasts, mast cells, and plasma cells would arrive in the aggregates, vascularization would occur, and ultimately foreign body granulomas would be formed. Whether such a process actually occurs is an interesting subject for future investigations.

Iron pools

Iron can be found in at least three forms in the macrophages and giant cells of the granulomas: as cytoplasmic ferritin, in inclusion bodies showing a homogeneous distribution pattern of iron micelles probably also representing ferritin, and in inclusion bodies with some resemblance to the hemosiderin-containing siderosomes described by Ghadially [24].

No indications as to the source of the iron in the macrophages and asbestos bodies were obtained in the present study. It seems probable to assume that this iron was derived from contacts between asbestos fibers and the erythrocytes present in suspensions of cells from asbestos-stimulated animals, as described by Hahon [25] and Brody [26]. However, since remnants left by erythrophagocytosis were seldom seen in either macrophages from granulomas or in the free peritoneal macrophages studied in this investigation, we assume that the erythrocytes found in the asbestos-stimulated animals derived from bleeding occurring at the moment of isolation and we therefore consider it justifiable to conclude that the source of the iron is not the phagocytosis and digestion of erythrocytes. Another possibility is that the greater amount of iron in the macrophages and giant cells is related to an effect of the presence of indigestible material in the peritoneal cavity on the iron metabolism of these types of cell. Such a phenomenon is known to occur when macrophages are exposed to other indigestible particles. For example, in rat macrophages van Blitterswijk et al. [27] found an enhanced iron content induced by the contact with the poorly digestible biomaterial ß-Whitlockite implanted in the rat middle ear. Alterations in the iron metabolism of macrophages exposed to contact with other types of poorly digestible or completely indigestible material will be investigated in future studies.

Endocytosis of asbestos fibers and the formation of asbestos bodies

Asbestos fibers were found in macrophages, monocytes, and granulocytes shortly after they were injected into the peritoneal cavity, and with increasing time numerous asbestos-containing macrophages and giant cells were found in peritoneal granulomas. Long asbestos fibers were found to be preferred as the basis for asbestos body formation. TEM of the granulomas and SEM of the extracted asbestos fibers and asbestos bodies showed that a proportion of the asbestos fibers was too long to be ingested by a single macrophage or even a multinucleated giant cell. It was this part of the fiber population in particular that acquired a coat and became asbestos bodies. In addition, it was found that the membrane surrounding the asbestos body was continuous with the plasma membrane of the surrounding macrophages or giant cells. This indicates that asbestos body formation takes place extracellularly. In this investigation the coat of the asbestos bodies was always surrounded by and in direct contact with macrophages or giant cells. The finding that especially these cell types showed an elevated iron level in inclusion bodies and their cytoplasm led to the

conclusion that these cells are responsible for the formation of asbestos bodies. The fact that we observed a number of different types of asbestos body raises the question whether changes in the condition of the macrophages involved in the process of asbestos body formation are responsible for the differences found between the asbestos bodies we observed.

The various configurations found in one and the same asbestos body might reflect repeated contact of the same asbestos body with different macrophages. Repeated phagocytosis of asbestos bodies by cultured alveolar macrophages has been reported [28], but our findings do not show whether this process takes place in vivo as well. Since, however, free asbestos bodies were never seen in our material, we assume that some other factor, for example different conditions of the macrophages involved in the process of asbestos body formation, is responsible for this phenomenon.

Although the present results do not provide unequivocal proof, we assume that, like iron, calcium is deposited in the coats of the asbestos bodies by macrophages surrounding the asbestos fibers. This assumption is supported by earlier reports describing the deposition of calcium by macrophages [5,29].

The pathway along which various elements such as calcium and iron enter the asbestos body coat is still unknown. Perhaps the small tubules traversing the organelle-free zones play a role in the transport of these elements. In other situations similar structures seem to be involved in the transport of calcium ions, e.g. odontoblasts during enamel formation [30]. The impression is also gained that the lysosomal system is involved in deposition of material, e.g. the iron in the coat of the asbestos bodies. The presence of asbestos fragments in both the iron-containing lysosomes and the darker parts of the coats points to such deposition. If continued deposition of the lysosomal content were indeed responsible for the formation of the asbestos body coat, the variable thickness and density of the asbestos body coat would reflect a maturational sequence. In our opinion, young asbestos bodies have only a thin coat with few iron micelles, whereas the coats of mature asbestos bodies are thicker (5-10 μm) and often show an annular morphology (compare Fig. 7 a and b with Figs. 2 and 6).

Limitations on the ultimate size of asbestos bodies, e.g. as described by other authors for pulmonary asbestos bodies [5], were also observed in this study. The increase in the size of the asbestos bodies stopped when a range of 5-10 μm was reached. A relation between the limitation of the size of asbestos bodies and the ingestion of a complete asbestos fiber by fusion of macrophages or giant cells as suggested by Davis [5] is improbable, because asbestos bodies

of 5 to 10 μm were already seen after one month. Because the dimensions found at the longest intervals were in the same range and the extraction experiments showed that asbestos bodies formed on fibers too long to be ingested were also present in the granulomas, it must be concluded that the size of asbestos bodies is limited by other factors besides total engulfment of such fibers.

The suggestion made by Mace et al. [31] that asbestos body formation is induced by a kind of defense mechanism triggered by toxic effects of the asbestos fibers seems to us doubtful. In our opinion the formation of asbestos bodies is more a matter of chance as to whether a cell of the right type (macrophage or giant cell) will encounter the right fiber, i.e., a fiber with a length such that it cannot be completely ingested and will therefore remain in the extracellular space. When such a situation arises, the macrophage or giant cell may seal off the fiber and exocytose the content of its lysosomes into the space thus obtained in an attempt to digest the fiber in question extracellularly. That this process actually takes place has been shown by another study done by our group, in which asbestos fibers were added to a macrophage culture (see chapter IV). That study showed that the process involved is similar to that of bone resorption by osteoclasts, the material to be digested being sealed off from the environment and a space created in which digestive activity is performed by the exocytosis of agents synthesized by the osteoclast. However, the difference between the two processes is that the asbestos fibers cannot be degraded by the lysosomal enzymes and the digestive activity may therefore continue for a very long time, leading to accumulation of the indigestible debris in the lysosomes. The presence of small asbestos fragments in the coat of the asbestos bodies supports this hypothesis.

If the formation of asbestos bodies is indeed a matter of chance, that would also explain why certain parts of an asbestos fiber remain uncoated. In the process of granuloma formation these parts are embedded in the fibrous network and therefore cannot be reached by the phagocytes.

In sum, it may be concluded from the present results that asbestos body formation is an extracellular phenomenon that can occur outside the lungs and pleural cavity, e.g. in the mouse peritoneal cavity. Because the asbestos bodies found in this study showed a strong resemblance to those found in the pleural cavity, it seems justified to conclude that the mouse peritoneal cavity can serve as a model for the study of processes related to asbestos body formation. Since these processes reflect the functioning of macrophages in contact with indigestible particles, it seems possible that the phenomena observed in this study

hold as well to more general phenomena occurring as a result of macrophage-material interactions. Studies of this kind are of importance for an understanding of the biocompatibility of materials and will be subject of future investigations.

Acknowledgements

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CHAPTER IV

THE FORMATION OF ASBESTOS BODIES BY MOUSE PERITONEAL MACROPHAGES: AN IN VITRO STUDY [3]

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ABSTRACT

To study the mechanism of asbestos body formation, UICC crocidolite asbestos fibers were added to a culture of mouse peritoneal macrophages. Small asbestos fibers were totally ingested by the macrophages, but fibers too long to be completely ingested remained as a consequence extra-cellular. These long asbestos fibers became the basis for asbestos body formation. The basic mechanism underlying asbestos body formation was found to be the exocytotic activity of macrophages.

The number of iron-rich inclusion bodies was dependent on the availability of iron in the culture media, and the same holds for the amount of iron in the asbestos body coat. This means that asbestos body formation is a phenomenon that occurs accidentally when macrophages come into contact with long fibers in an iron-rich environment. A time-dependent increase in the number, average size, and rate of segmentation of the asbestos bodies was observed. The present report is the first to describe asbestos body formation in vitro.

INTRODUCTION

The presence of asbestos- or ferruginous bodies has been frequently reported by authors who used material obtained from patients or laboratory animals exposed to asbestos fibers or some other fibrous material with biologically inert characteristics [1-7]. In addition, the mechanism of asbestos body formation has been the subject of a number of investigations [8-10]. In an earlier study we showed that the process of asbestos body formation was not restricted to the pleural cavity and the lungs, but could also take place in foreign body gra-

[3] This chapter has been submitted for publication

nulomas in the mouse peritoneal cavity (see chapter III) [7]. It was concluded from that study that asbestos body formation is a phenomenon, that occurs when macrophages and/or multinucleated giant cells meet indigestible fibers too large to be totally ingested by a single macrophage or giant cell. The same study provided indications that asbestos body formation is the result of exocytotic activity of the macrophages. The complex structure of the granulomas made it difficult to determine not only the exact mechanism underlying the deposition of material around an asbestos fiber but also whether asbestos body formation is restricted to macrophages.

To obtain more information about the process of asbestos body formation, we performed an in vitro study in which mouse peritoneal macrophages were cultured in the presence of UICC crocidolite asbestos fibers.

MATERIALS AND METHODS

Animals

The Spf bred Swiss male mice obtained from HARLAN CPB (Zeist, The Netherlands) were 6 weeks old and weighed 20 grams at the start of the experiments. All mice were allowed three days to adapt before the peritoneal cell suspension was collected.

Cell collection and culture

The animals were decapitated, 2 ml HBSS was injected intra-peritoneally, the abdomen was then carefully kneaded for approximately 30 sec, after which the peritoneal cell suspension was collected under sterile conditions. The cells were counted in a Coulter counter (Coulter Electronics Model DN) and pelleted in a cryocentrifuge for 5 min at 90xG and 4°C. The cells were re-suspended in DMEM to a concentration of 0.5 x 10^6 cells per ml, and 2-ml samples were brought into Greiner petri dishes (Qualitat FC, 35/10) containing sterile cover glasses. After incubation for 2 hours at 37°C in an atmosphere composed of 95% air and 5% CO_2, the petri dishes were washed with DMEM to remove the non-adherent cells and the adherent population was cultured for 16 hours in DMEM containing 20% FCS and 5 μg/ml UICC crocidolite asbestos fibers. The petri dishes were then washed again to remove excessive asbestos and culture was resumed. Control cultures were treated in the same way but without the addition of asbestos. The following culture media were used:

Control medium: DMEM with 20% FCS

Iron-rich medium: DMEM with 20% FCS and 1.8 mM iron, in the form of iron dextran.

Manganese rich medium: DMEM with 20% FCS and 1.8 mM manganese in the form of $MnCl_2$.

Colloidal Gold

At the interval of 1 week some cultures were refreshed with a solution containing 1% BSA-gold. These cultures were used to study the deposition of particles into the space containing the asbestos bodies, and to study the pathway along which these particles are transported to these loci.

Fixation

For both LM and TEM the specimens were fixed at 4, 6, 8, 10, and 12 weeks after the start of the culture in 1.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4, 330 mOsmol) for 10 min at room temperature and then washed in a Ringer solution. For TEM the cells were postfixed in 1% OsO_4 in 0.1 M cacodylate buffer. A part of the specimens was postfixed with the modified OsO_4 fixative according to de Bruijn et al. (1973) [11] for 24 hours at 4°C. An other part was fixed with glutaraldehyde only to allow undisturbed XRMA.

Enzyme cytochemistry

Peroxidase

To determine the initial composition of the peritoneal cell population, we performed cytochemical staining to detect peroxidatic activity in the various cell types. For this purpose, the cells were fixed in glutaraldehyde as described above, washed at room temperature for 5 min in 0.1 M cacodylate buffer (pH 6.9), and pre-incubated for 30 min at room temperature in a medium containing 1 mg diaminobenzidine-4HCl per ml cacodylate buffer. Incubation was performed in the same medium supplemented with H_2O_2 to a final concentration of 0.01%.

Acid phosphatase

The method described by Hulstaert (1983) [12] was used to detect acid phosphatase activity in the macrophages. The cells were prefixed in glutaraldehyde, washed for 5 min in 0.1 M cacodylate buffer (pH 7.4) followed by 5 min in 0.08 M TMB (pH 5.0), frozen at -24°C in 0.08 M TMB, pre-incubated for 30 min at 37°C in 0.08 M TMB containing 1 mMol $CeCl_3$. Incubation was performed in the same solution to which 1.5 mg/ml ß-glycerophosphate was added. The specimens were then washed in a Ringer solution before being further processed for TEM. $CeCl_3$ was added to the incubation solution as a tracer to demonstrate the localisation of the ß-glycerophosphate.

For the controls, the same procedure was performed but with the omission of ß-glycerophosphate.

Light microscopy

After fixation with 1.5% glutaraldehyde, the specimens were rinsed with a Ringer solution and stained with Prussian blue according to Bunting for the demonstration of iron [13]. The preparations were stained with Kernechtrot according to Domagk [14] to visualize the nuclei. The stained specimens were dehydrated in a graded alcohol series up to alcohol 100% and embedded in Entellan embedding medium.

The preparations were examined by conventional brightfield light microscopy as well as with a Bio-Rad Lasersharp MRC-500 laser scanning microscope.

Transmission electron microscopy

The preparations were dehydrated in a graded alcohol series up to 100% and embedded in Epon, ultrathin sections were cut on an LKB microtome and stained with lead hydroxide. The sections were examined in a Philips EM 410 transmission electron microscope.

X-ray microanalysis

X-ray microanalysis was performed with a Tracor (TN) 2000 X-ray microanalyser attached to a Philips EM 400 scanning transmission electron microscope. The system geometry of this instrument is described before [15]. The

sections were collected on copper grids, which were placed in a low-background holder. The holder was tilted at an angle of 108 degrees relative to the electron beam. Measurements were performed during 100 s livetime; the spot diameter was 100 nm and the accelerating voltage 80 kV.

RESULTS

Cell suspensions

The TEM studies showed that, according to the localization of reaction product after incubation for peroxidatic activity, the majority of the cells in the peritoneal cell suspension collected from unstimulated SPF mice are peritoneal resident macrophages (Fig. 1) and lymphocytes.

Control cultures

The macrophages were still vital at the end of the longest interval studied (12 weeks), and the total number of macrophages determined at various intervals after the start of the experiment did not alter significantly. Some of the culture dishes contained very flat cells with a large oval to round nucleus. On basis of their morphology, these cells were identified as fibroblasts. The fibroblasts gradually increased in number during the course of the experiments, but their presence or absence had no noticeable effect on the vitality of the macrophages or on the investigated phenomena.

On the eighth day of culture, a number of multinucleated giant cells were seen. Initially, there were only two to three nuclei in the cytoplasm of these giant cells. The number of multinucleated cells increased slowly during the further course of the experiment, but the total number of nuclei per giant cell remained relatively low. Giant cells containing more than five nuclei were not seen.

TEM showed that the morphology of the macrophages underwent characteristic alterations during culture. At 24 and 48 hours the nuclei became circular and the plasma membrane had a rather smooth contour. Further an increasing number of cytoplasmic vacuoles were present (Fig. 2). After the longer culture periods there were many cytoplasmic vacuoles (Fig. 3) and the formation of blebs (cytoplasmic bulges) was sometimes observed. Occasionally, lipid droplets were seen. The cells in cultures incubated to demonstrate peroxidatic activity

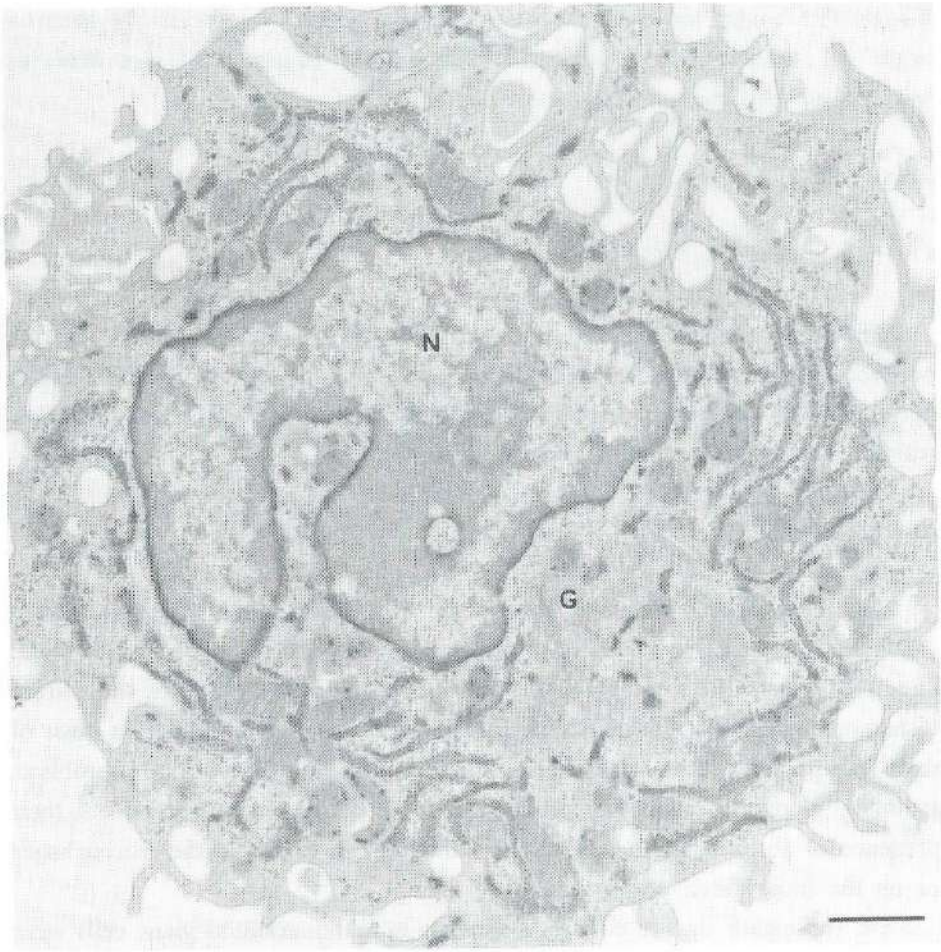


Fig. 1. Transmission electron micrograph showing a peritoneal resident macrophage from an unstimulated mouse peritoneal cavity. The preparation was processed for the demonstration of peroxidase activity. Note peroxidase reaction product in the rough endoplasmic reticulum and the nuclear envelope. Fixation: glutaraldehyde, OsO_4. N: nucleus, G: Golgi area. Bar:1 μm.

were peroxidase negative at 24 hours or longer after the start of the culture.

Endocytosis of asbestos fibers

At 16 hours, the macrophages had endocytosed the majority of the asbestos fibers. A number of the asbestos fibers were too long to be completely ingested by one macrophage or multinucleated cell. In general, a number of macrophages

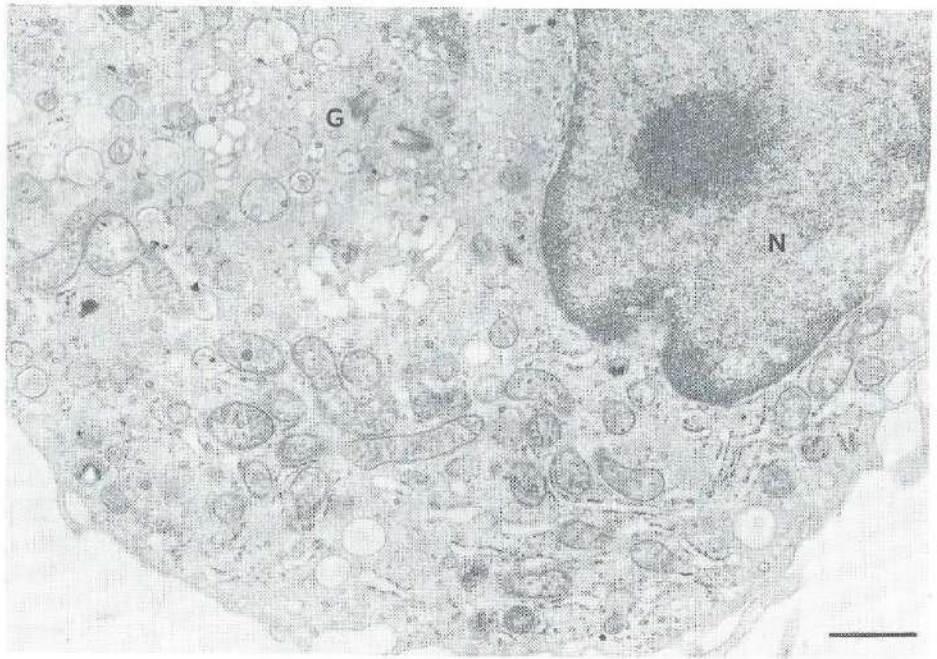


Fig. 2. Transmission electron micrograph of a mouse peritoneal resident macrophage after 48 hours of culture. The plasma membrane has become distinctly smoother. PO-positive reaction product was no longer present in cells of this type. Fixation: glutaraldehyde, osmium(VI)-iron(II) complex. N: nucleus, G: Golgi area. Bar: 1 μm.

were attached to these long fibers at the same time (see e.g. Fig. 6a). Total engulfment of a relatively small fiber (about 10 μm long) by one macrophage or multinucleated cell was sometimes impossible because two macrophages had started to endocytose it simultaneously.

A small proportion of the asbestos fibers were endocytosed by fibroblasts if such cells were present in the culture dishes. However, these were always fibers small enough to be completely ingested by a single cell.

TEM of the macrophages attached to asbestos fibers showed the typical organelle-free zones seen in the in vivo experiments [7] (Fig. 4). At places where two macrophages met each other, the contact between them was intimate.

Deposition of iron

Despite the low concentration of iron in the standard DMEM culture

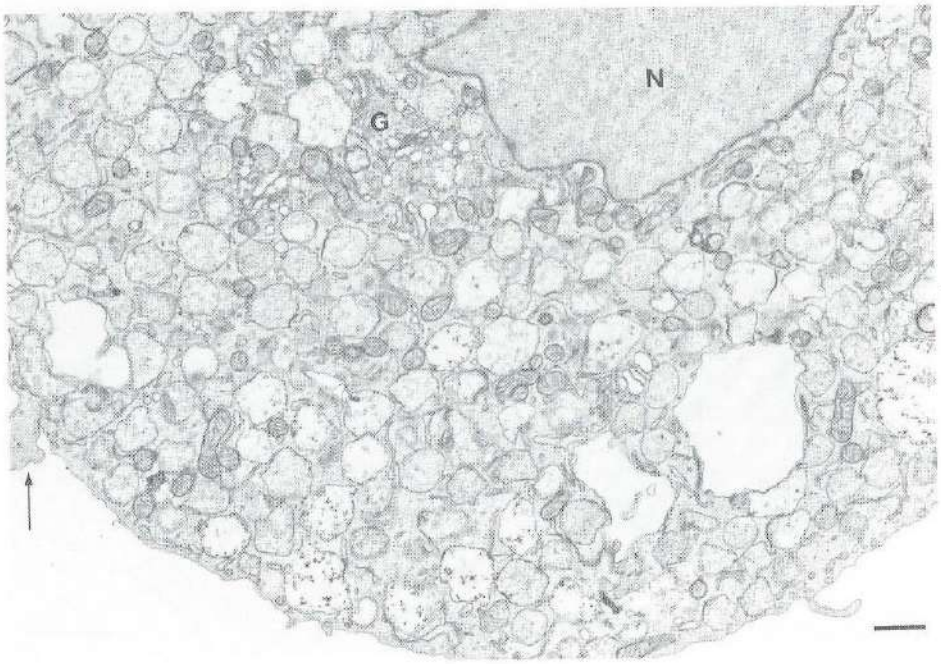


Fig. 3. Transmission electron micrograph of a mouse peritoneal resident macrophage after 4 weeks of culture. Blebs were sometimes observed on the plasma membrane of such cells (arrow). Many cytoplasmic vacuoles are visible. Fixation: glutaraldehyde, osmium(VI)-iron(II) complex. N: nucleus, G: Golgi area. Bar: 1 μm.

medium, light microscopy performed on material fixed 6 weeks or longer after the addition of asbestos fibers occasionally showed extremely thin layers of Prussian blue stainable material along asbestos fibers (not shown).

The addition of iron dextran to the culture media led to the formation of numerous electron-dense inclusion bodies in the macrophages. TEM showed that the number of inclusion bodies per macrophage was variable and that within one cell-type the electron density of the inclusion bodies was variable as well (Fig. 5a). These electron-dense bodies contained iron, as was demonstrated by XRMA (Fig. 5b). The values obtained for the net count rate with XRMA showed that variation in electron density referred to variation in the iron concentration. Although additional iron was present in the culture media throughout the total culture period, a persisting increase in the number of inclusion bodies per macrophage was not seen.

As in the control cultures, the cells incubated in the media containing

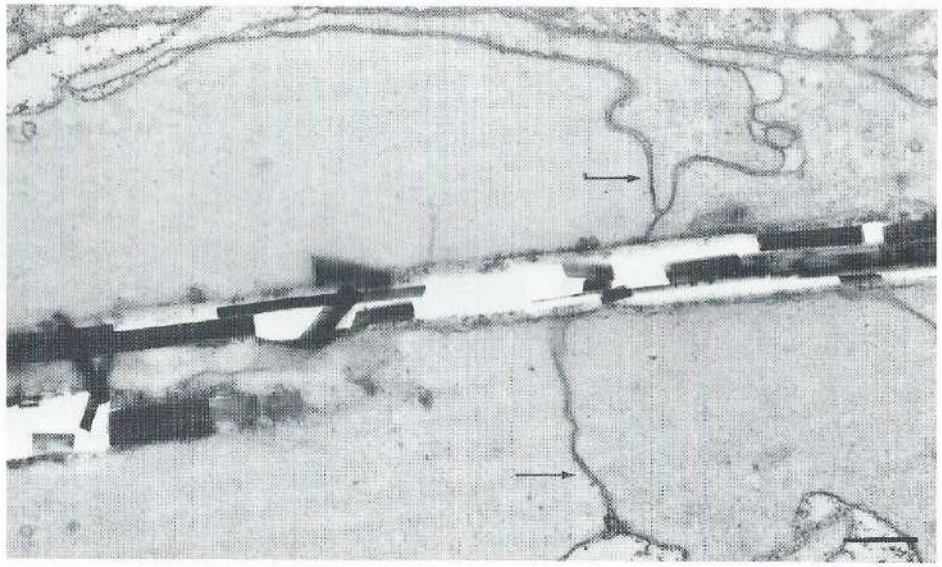


Fig. 4. Transmission electron micrograph at a relatively high magnification of a part of an asbestos fiber surrounded by a number of cells. The micrograph shows the place where two macrophages make contact. Communication between the space containing the asbestos fiber and the extracellular space can be seen (arrows). Note the organelle-free cytoplasm, surrounding the asbestos fiber. Fixation: glutaraldehyde, osmium(VI)-iron(II) complex. Bar: 0.25 μm.

asbestos and higher concentrations of iron were still vital at the end of the longest culture period.

Formation of asbestos bodies

As already mentioned, macrophages cultured in the control and the iron-rich media contained small asbestos fragments, and macrophages exposed to asbestos fibers too long to be completely ingested attached themselves to such fibers. LM of material cultured with asbestos fibers for four weeks and longer before being studied with Prussian blue showed that asbestos body formation had occurred (Fig. 6a). At four weeks the coats were still thin and segmentation was only seen occasionally, but the thickness of the asbestos bodies increased with increasing duration of the culture period and segmentation became more prominent (Fig. 6b). Confocal laser microscopy was found to be valuable for studies concerning the rate of segmentation, since it revealed the typical beaded appearance of asbestos bodies (Fig. 7). As the graph in Fig. 8 shows, the proportion of

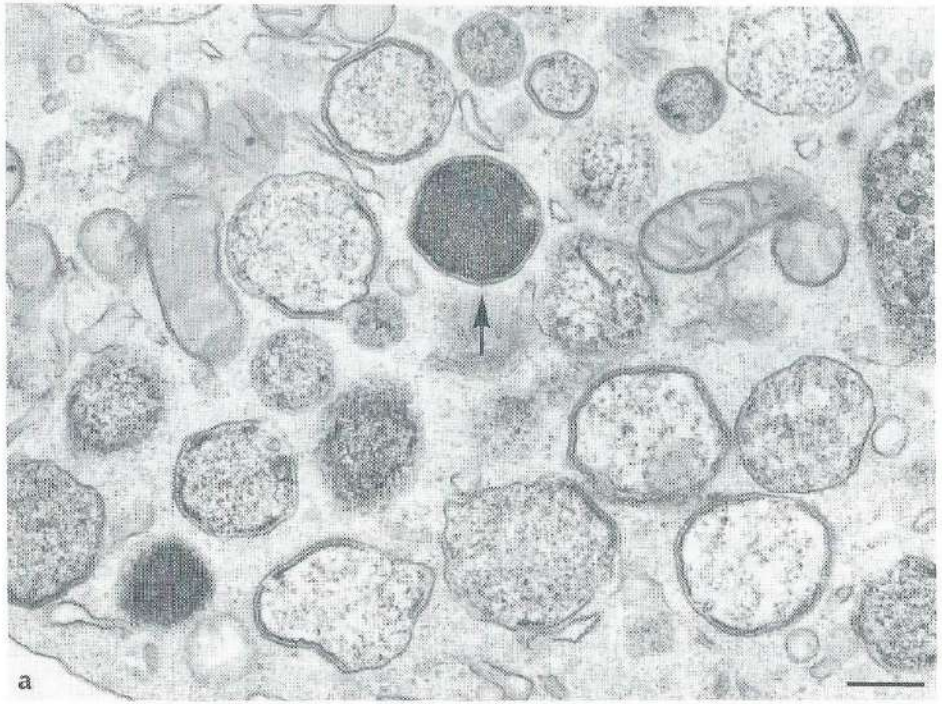


Fig. 5, a and b.
a: Transmission electron micrograph of a part of a macrophage cultured in iron-rich medium. Inclusion bodies containing variably dense material are visible. Fixation: glutaraldehyde, osmium(VI)-iron(II) complex. Bar: 0.5 μm.
b: X-ray microanalysis spectrum of the dark inclusion body in a (arrow), showing the presence of iron. (Vertical full scale: 4,096 counts).

asbestos bodies of the beaded type increased with time. This graph is based on the data obtained from randomly chosen fields measuring 10,000 X 3,000 μm.

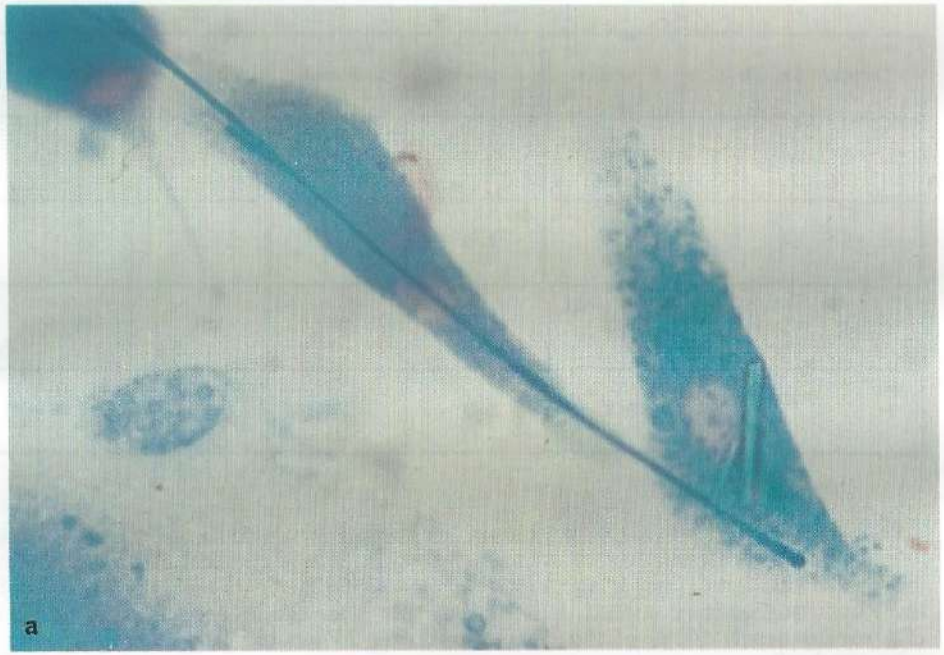
	Percentage of coated asbestos fibers				
Length in μm.	4 weeks	6 weeks	8 weeks	10 weeks	12 weeks
0 - 25	0.0	0.0	0.4	0.0	0.0
25 - 50	9.0	2.0	25.7	24.5	69.9
50 - 75	33.3	36.4	100	100	100
> 75	47.8	100	100	100	100

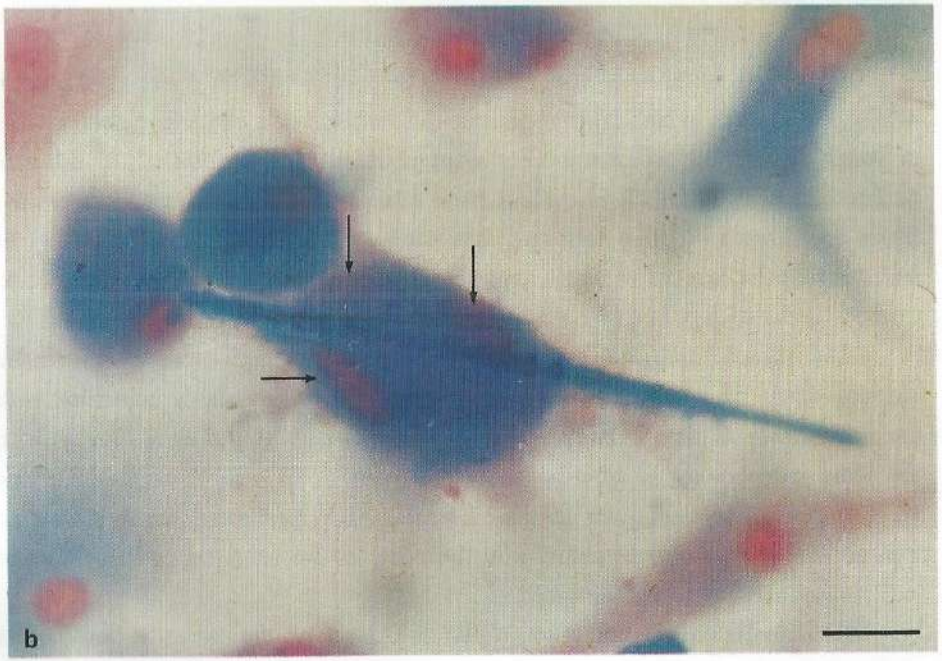
Tab. 1. Results of an evaluation yielding the relative proportion of asbestos fibers giving rise to the formation of an asbestos body. It is clear that this proportion increases with time. This is accompanied by a decrease in the average length of the fibers that became coated. It is evident that after the longest interval studied (12 weeks) nearly 100% of the fibers measuring 25 μm and longer have become asbestos bodies.

The relative number of coated fibers also increased with time, as shown in Table 1. The data in this table represent the findings in a randomly chosen field in which fibers and asbestos bodies were counted until a total of 10 asbestos bodies had been found. It is evident from table I that the proportion of coated fibers increased with time too, and that almost all of the fibers 25 μm long or more had given rise to asbestos body formation by the end of the 12th week. Coated fibers with a length below 25 μm were rarely observed.

TEM showed that the coats were composed of the same characteristic electron-dense material as in the in vivo study [7] and invariably coincided with the appearance of type I asbestos bodies (Fig. 9a and b). Asbestos bodies with the same morphology as asbestos bodies of types II, III, or IV, as found in the in vivo study, were not seen in this material. XRMA showed the presence of iron and frequently also of chlorine in the coats of the asbestos bodies. Calcium and phosphorus were not detected.

The time-dependent increase in the thickness of the asbestos-body coats observed in the light microscopical preparations was confirmed by the TEM





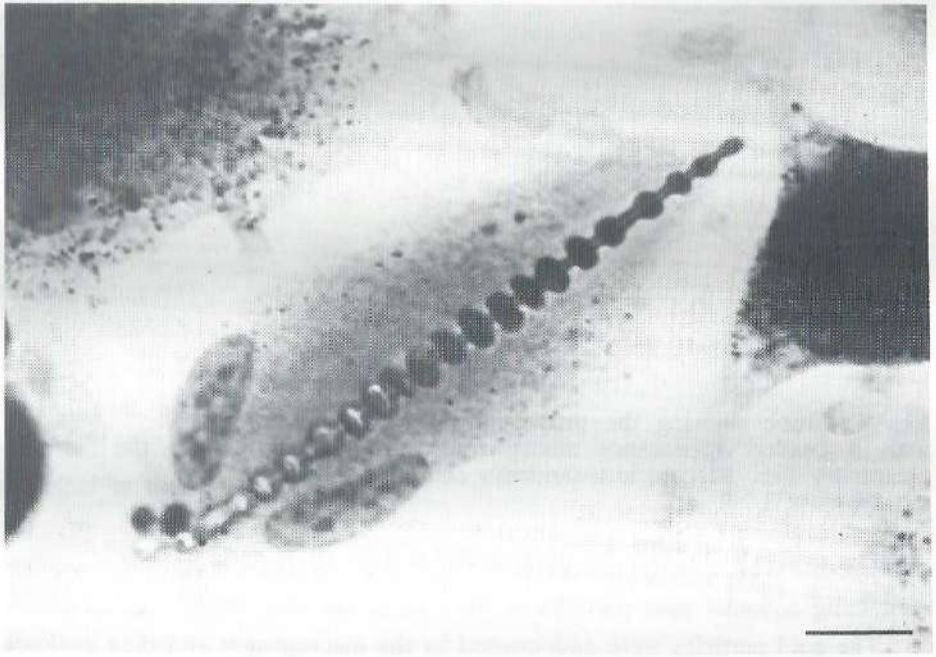


Fig. 7. Light micrograph showing a segmented asbestos body fixed after 12 weeks of culture. The micrograph was made with a Polaron laser scanning microscope, which revealed the beaded appearance of the asbestos body clearly. Bar: 5 μm.

observations, which also showed an increase in the number of iron micelles (compare Fig. 9a with Fig. 10b).

Culture in manganese-rich medium

Coating of the fibers in manganese-rich media remained at the level of the control cultures. Here too, very thin Prussian blue positive coatings were

Fig. 6 a and b.
a: Light micrograph of macrophages stained with Prussian blue and Kernechtrot and attached to a long asbestos fiber. The cells were fixed after 4 weeks of culture. A blue stain, indicating iron deposition, is visible along the asbestos fiber.
b: Light micrograph showing a segmented asbestos body fixed after 8 weeks of culture. The preparation was stained with Prussian blue and Kernechtrot. Note the presence of at least 3 nuclei in the cytoplasm of the large cell containing the largest part of the asbestos body (arrows), and also the intense staining of the asbestos body coat, pointing to a relatively high iron concentration. Bar:5μm.

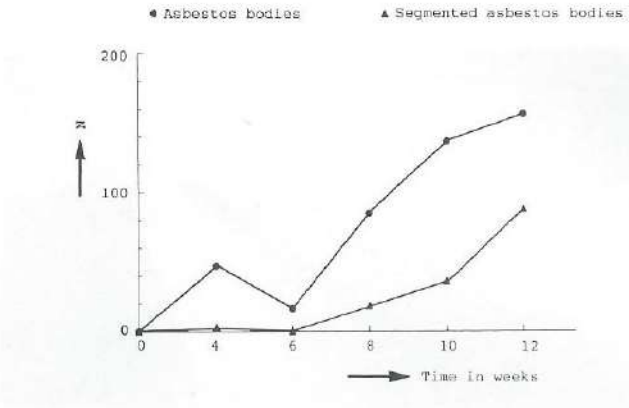


Fig. 8. Graph showing the proportion of asbestos bodies with a beaded appearance among the total number of asbestos bodies detected in a randomly chosen area measuring 10,000 x 3,000 μm.

sometimes detected. Manganese was not detectable by XRMA in inclusion bodies in the macrophages or in deposits on long asbestos fibers.

The incorporation of colloidal gold

For investigation of the involvement of inclusion bodies in the process of asbestos body formation, a number of cultures were given a medium containing colloidal gold particles at the end of the first week of culture.

The gold particles were endocytosed by the macrophages and then enclosed in the inclusion bodies (Fig. 10a). These gold particles were also incorporated into the coats of the asbestos bodies (Fig. 10b).

Acid phosphatase activity

To determine the nature of the inclusion bodies, we processed a number of cultures for the demonstration of acid phosphatase activity. The tracer used for the localization of the Na-ß-glycerophosphate was $CeCl_3$. A cerium signal was demonstrated with XRMA in the inclusion bodies and in the coat of the asbestos bodies (Fig. 10c). Point analyses performed in the nucleus and cytoplasm were negative for cerium which was also found to be absent in

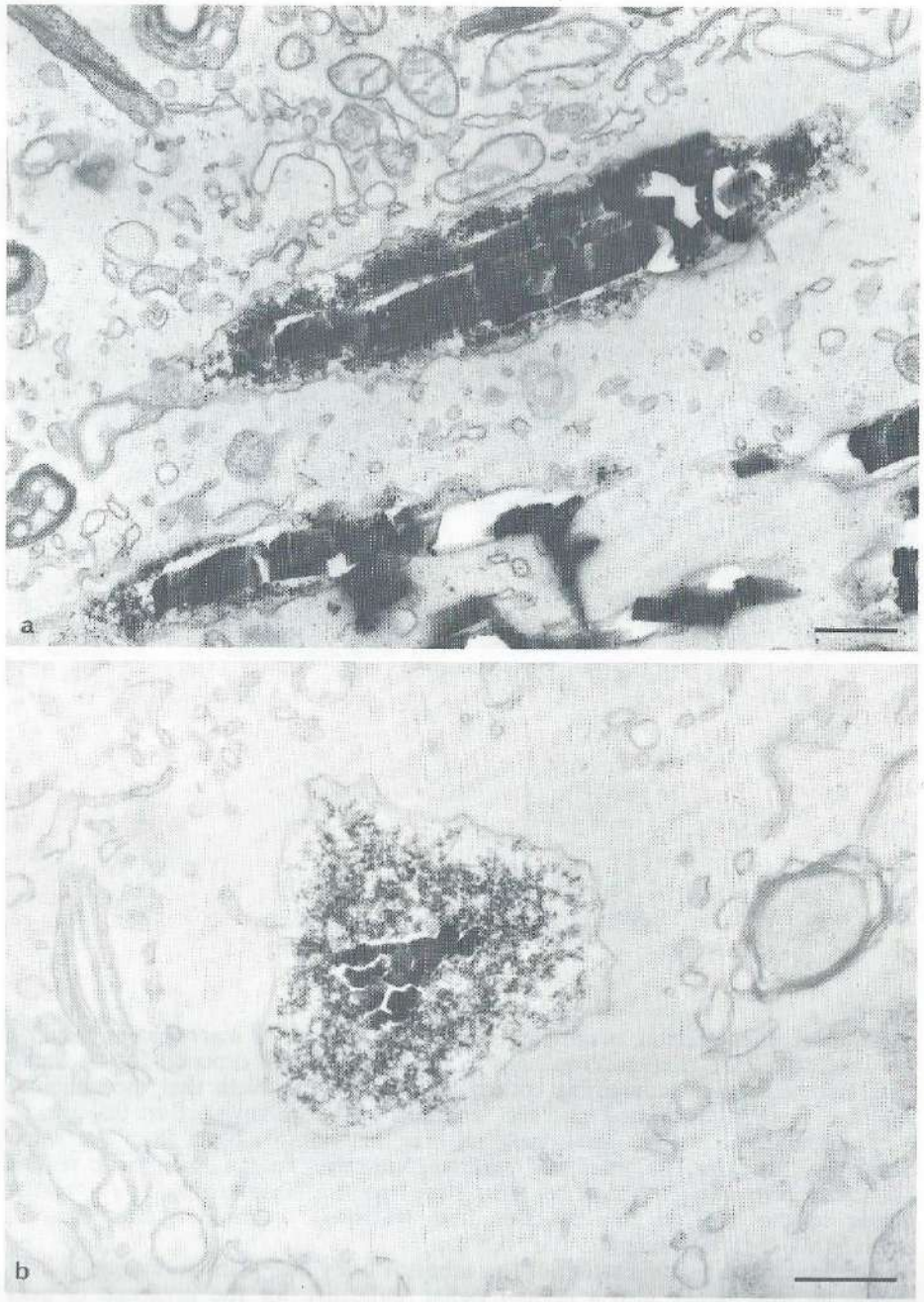


Fig. 9, a and b.
a: Transmission electron micrograph showing a longitudinal section through an asbestos body at 6 weeks of culture. The deposits of iron along the fiber are clearly visible. Note the characteristic organelle-free zone surrounding the asbestos fibers.Fixation: glutaraldehyde, osmium(VI)-iron(II) complex. Bar: 0.5 μm.
b: Transmission electron micrograph showing a transverse section through an asbestos body at 6 weeks of culture. Similar dark deposits and an organelle-free zone are clearly visible. Fixation: glutaraldehyde, osmium(VI)-iron(II) complex. Bar: 0.5 μm.

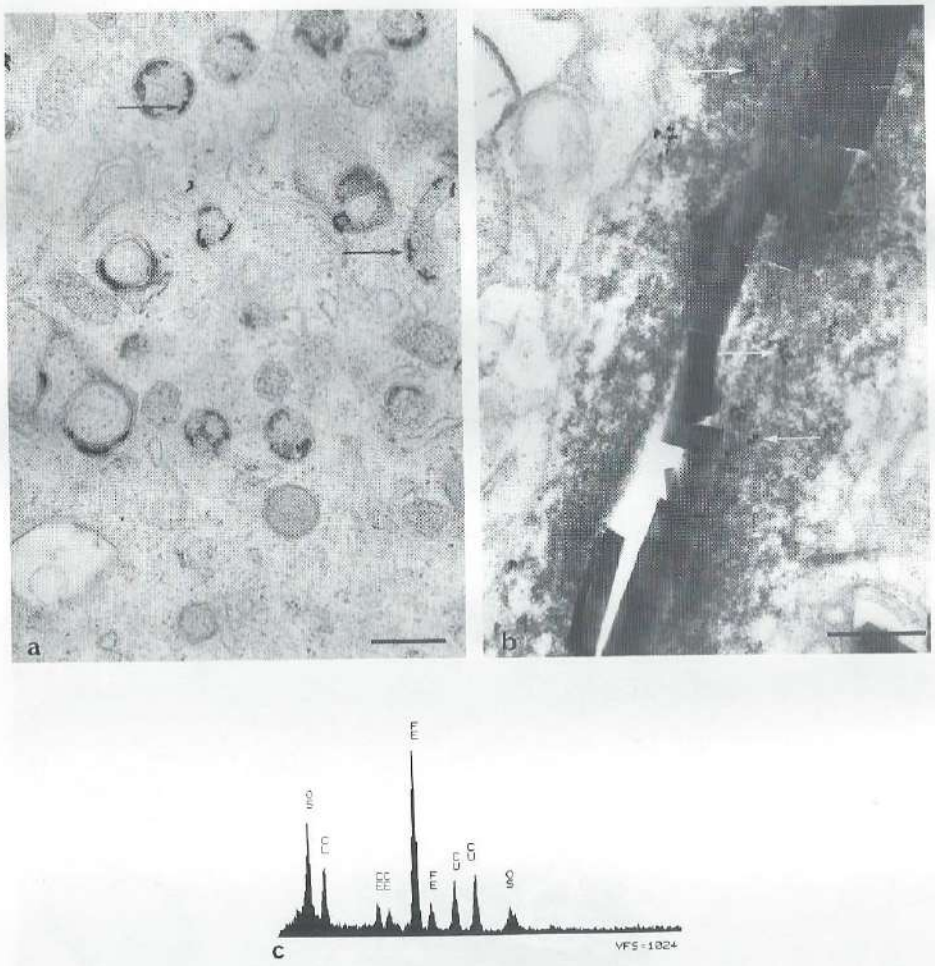


Fig. 10, a - c.
a: Transmission electron micrograph showing a part of a macrophage fixed at 4 weeks of culture. The macrophages in this culture were exposed for 24 hours to a culture medium containing colloidal gold particles. Note that the colloidal gold has been incorporated into the inclusion bodies (arrows). Bar: 0.5 μm.
b: Transmission electron micrograph showing an asbestos body from the same culture at 12 weeks. Colloidal gold particles, indicating fusion of inclusion bodies like those in a, with the space containing the asbestos body are visible at various places in the coat of this asbestos body (arrows). Fixation: glutaraldehyde, osmium(VI)-iron(II) complex. Bar: 0.5 μm.
c: X-ray microanalysis spectrum of the asbestos body in b. This technique shows, beside an iron signal, also a cerium signal, indicative for the presence of acid phosphatase in the asbestos body coat. (Vertical full scale 1,024 counts.)

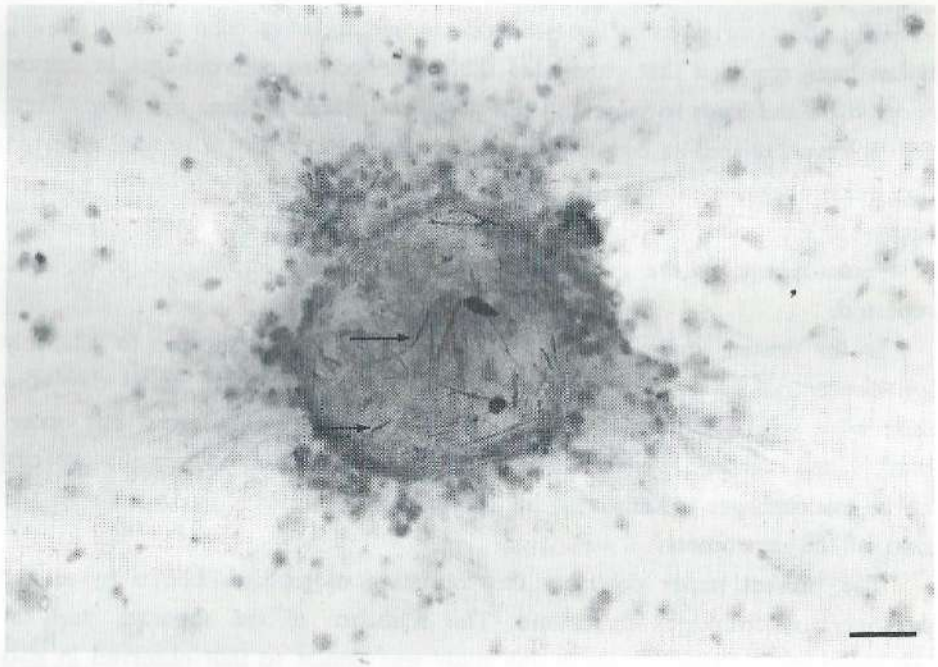


Fig. 11. Light micrograph showing a cell aggregate containing many asbestos fibers (arrows). Aggregates of this type were frequently seen in culture dishes in which not only macrophages but also fibroblasts were present. The relatively large number of asbestos fibers in such aggregates suggests an active transport of asbestos fibers to these locations. Bar: 50 μm.

inclusion bodies and the asbestos bodies in the control experiments where ß-glycerophosphate was omitted.

Formation of cell aggregates

Aggregation of cells was seen in culture dishes in which both fibroblasts and macrophages were present (Fig. 11). LM and especially TEM showed that these relatively large concentrations of cells contained both fibroblasts and macrophages. Numerous asbestos fibers as well as asbestos bodies were present in the aggregates. Collagen was, as a rule, present in the extracellular space of the aggregates.

DISCUSSION

The effects of asbestos fibers on cells in culture have been studied [16-20]. It has been reported that crocidolite asbestos added to macrophages in culture is cytotoxic and leads to increased cell death [21], [22,23]. These cytotoxic effects are, however, related to high concentrations of asbestos. Bey et al. [24] reported that macrophages in culture exposed to low concentrations of crocidolite asbestos fibers remained vital for a period of ten days. The formation of asbestos bodies in a macrophage culture assay has, to the best of our knowledge, never been reported.

In the present study, macrophages survived after the exposure to relatively low concentrations of crocidolite. Even after a culture period of 84 days, the cells were still vital and a marked decrease of cell numbers did not occur. Further, the ingestion of asbestos fibers did not reduce the endocytotic capacity of the macrophages as shown by the uptake of gold particles one week after the start of the experiment.

The present paper describes the formation of asbestos bodies by mouse peritoneal macrophages in culture. The sequence of the separate steps in asbestos body formation seen in this study was similar to that observed in vivo by Botham and Holt [8], who distinguished a number of steps in the process of asbestos body formation around anthophyllite asbestos fibers in the lungs of guinea pigs. The next steps described by Botham and Holt and also by us in an earlier report [7] are successively the development of giant cells, the formation of an iron-containing coat along the fibers, the development of an irregular outline of the coat, the formation of young asbestos bodies, and finally the formation of mature asbestos bodies. These steps and roughly also the duration of each step as observed in the present study, are very similar to those reported by Botham and Holt as well as the data obtained in our in vivo study. According to Botham and Holt the source of the iron essential for the formation of the iron-rich coat is hemolysis. Although in our opinion the digestion of erythrocytes by macrophages was not the source for the iron [7], the present investigation has shown that an increase of the iron concentration in the culture media is indeed related to increase in asbestos body formation. In addition, the present study showed the occurrence of a number of phenomena:

1. Macrophages cultured in iron-rich media contain many iron-rich inclusion bodies.
2. Iron-rich inclusion bodies are lysosomal in origin, as can be demonstrated by the use of an acid phosphatase reaction.
3. Small gold particles endocytosed by the macrophages are found in the inclusion bodies and also in the asbestos bodies.

On the basis of these observations and the results of our previous work, the following mechanism of asbestos body formation is postulated. Macrophages in the vicinity of asbestos fibers attach to these fibers in an attempt to ingest them. If, however, a fiber is too long to be completely ingested, the macrophage can only surround part of the fiber, and this gives rise to a micro-environment between the macrophage and the asbestos fiber. This type of contact between the macrophages and the long fibers leads to exocytosis of the lysosomal contents, which are deposited in the space between the fiber and the macrophage. Thus, when an iron compound is present in the lysosomes, an asbestos body comes into existence. Enhancement of the iron concentration in the culture media led to an increase in the number and average size of the asbestos bodies. This observation supports the hypothesis that asbestos body formation is dependent on the contents of the lysosomes and is therefore a coincidental phenomenon. This hypothesis is also supported by the findings in cells cultured in media containing colloidal gold particles. These particles initially present in lysosomes were later found in the asbestos body coats. In material cultured in a medium rich in manganese, an element that does not accumulate in macrophage lysosomes did not reappear in the inclusion bodies or the asbestos body coats. No indication pointing to increased asbestos body formation was obtained.

Because coating of asbestos fibers was only found at places where contact occurred between macrophages and asbestos fibers, and because the lysosomal contents accumulated at that site, we concluded that the macrophages seal off this part of the extracellular space, thus forming a separate compartment from which the exocytosed lysosomal contents cannot escape.

The formation of such a compartment and the exocytosis of lysosomal contents into it suggests analogy with the process of bone resorption by osteoclasts, where the osteoclasts form an extracellular micro-environment into which the lysosomal enzymes are released [25].

Asbestos bodies were sometimes formed on asbestos fibers that were actually short enough to be ingested by a single macrophage or giant cell. These were fibers which had been approached by two macrophages simultaneously, making engulfment of the fiber by only one of them impossible. This means that the fiber will not be ingested and can serve as the basis for asbestos body formation.

As Fig. 8 shows, an increase in the number of asbestos bodies showing the characteristic beaded appearance was observed in the material studied in this investigation. Since the rate of segmentation increased as a function of time, we conclude that this phenomenon is related to maturation and does not depend on mechanical forces as suggested by Mace et al. [26].

Asbestos bodies with the characteristic morphology or chemical composition of types II, III, and IV [7] were not seen in this study. This means that the formation of different types of asbestos body is related to systemic variations that are not initiated in stable and strictly controlled macrophage cultures.

The occurrence of cell aggregates resembles the aggregation and granuloma formation found in our in vivo experiments [7]. A chemotactic response induced by the interaction between asbestos fibers and macrophages or fibroblasts is probably responsible for this aggregation. Such a factor is indeed known to be released in the supernatant of cultures of rat alveolar macrophages exposed to crocidolite asbestos fibers [27]. The presence of relatively large numbers of asbestos fibers in these aggregates suggests that the fibers were transported to these loci by macrophages.

In sum, the present findings show that the formation of asbestos bodies by mouse peritoneal macrophages can be initiated in vitro. Further, the results support our earlier assumption that asbestos body formation is at least partially the result of exocytotic activity of macrophages. Finally, this study has yielded a model making it possible to obtain more information on the mechanism of asbestos body formation.

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CHAPTER V

THE EFFECTS OF CROCIDOLITE ASBESTOS AND CALCIUM PHOSPHATE CERAMICS ON MOUSE PERITONEAL MACROPHAGES

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ABSTRACT

The effects of intraperitoneally injected suspensions of crocidolite asbestos fibres and suspensions of granulated hydroxyapatite or tri-calcium phosphate were compared. Initially, all types of material induced an inflammatory reaction characterized by the influx of neutrophil granulocytes and monocytes into the peritoneal cavity, but at later time-points the presence of the particules in the mouse peritoneal cavity led to morphological changes in the free peritoneal macrophages, i.e., the formation of iron-rich inclusion bodies and the development of tubular systems (labyrinths). The number of inclusion bodies and especially the number and size of the labyrinths increased with time and also according to the type of material injected. Labyrinths were most numerous in crocidolite asbestos-stimulated macrophages, relatively scarce in the tri-calcium phosphate group, and absent after hydroxyapatite stimulation.

All materials gave rise to the formation of foreign-body granulomas whose structure was dependent on the nature of the injected material. Like the free peritoneal macrophages, the macrophages in all granulomas contained iron-rich inclusion bodies. Labyrinths were not found in the macrophages from granulomas. As reported (Chapter III and ref. 1), asbestos bodies were plentiful in the crocidolite asbestos-stimulated mice. Structures similar to asbestos bodies were also detected in the tri-calcium phosphate stimulated animals and in smaller numbers in the hydroxyapatite-stimulated animals. Like the results of our studies on the mechanism of asbestos body formation (Chapters III and IV, ref. 1 and 2), the present findings show that macrophages encountering relatively large, poorly digestible particles are triggered to exocytose their

lysosomal contents into a micro-environment by a process resembling that of bone resorption by osteoclasts.

In addition, X-ray microanalysis showed that Fe, Si, Al, Cr, and Ni were present in inclusion bodies of macrophages and multi-nucleated giant cells involved in hydroxyapatite and tri-calcium phosphate breakdown but not in macrophages found in animals stimulated with crocidolite asbestos.

INTRODUCTION

In reports on the biological performance of biomaterials, the macrophage/material interaction at the implantation site is generally considered to be of great importance [3-6]. However, analysis of the macrophage/material interaction is complicated by the wound reaction caused by surgical implantation of a medical device or an artificial organ made of such biomaterials. This wound reaction will initially exceed the tissue reaction [6] and may thus obscure the macrophage/material interaction. Such complications can be avoided experimentally by injecting the foreign substance into the mouse peritoneal cavity, a technique commonly used in studies on the function and origin of macrophages [7] and on macrophage/material interactions [3,8-10].

In the present study, the effects of three intra-peritoneally injected materials were compared. For this purpose use was made of a suspension of crocidolite asbestos, known to be a strong inflammatory stimulus [8], and suspensions of granular hydroxyapatite and tri-calcium phosphate, both known as bio-active resorbable calcium phosphates [11-14] that give very low inflammatory reactions (for a review, see van Blitterswijk [15]).

MATERIALS AND METHODS

Animals

SPF bred female Swiss mice, obtained from Harlan Central Animal Breeding Center (Zeist, The Netherlands), were 6 weeks old and weighed about 20 grams at the start of the experiments. All mice were allowed to adapt for at least three days before the experiment was started.

Materials

The calcium phosphates used were hydroxyapatite and tricalcium phosphate, both in a granulated form with a particle size smaller than 40 μm (Fig. 1 a and b). The asbestos was UICC crocidolite asbestos with fiber lengths ranging from <0.1 μm to 1000 μm. (Fig. 1c).

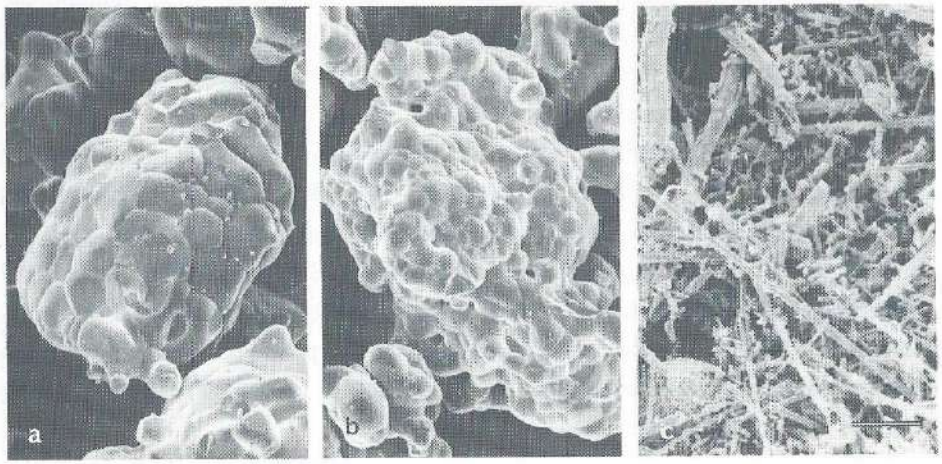


Fig. 1 a - c. Scanning electron micrographs of the three materials under study. a: hydroxyapatite, b: tri-calcium phosphate, c: crocidolite asbestos. Bar: 5 μm.

Administration of the materials

All three materials were suspended in Hanks' balanced salt solution (HBSS). Per animal, 1 ml of the suspension under study was injected into the peritoneal cavity on day 0. To obtain comparable numbers of particles, we used a suspension of crocidolite asbestos with a final concentration of 0.5 mg/ml, and hydroxyapatite and tri-calcium phosphate were suspended to a final concentration of 5 mg/ml. Control animals were given 1 ml HBSS alone. A second control group was untreated, and the isolated peritoneal cell suspension of this group was examined to establish the composition of the initial population.

Isolation, fixation, and peroxidase cytochemistry

The free peritoneal cells were isolated and fixed at intervals of 8, 16, 24, and 48 hours, 4, 8 and 16 days, and 1, 2, and 6 months. The procedures used for isolation, fixation, and peroxidase cytochemistry have been described in detail elswhere [8]. In short, the cells were collected by suction, fixed in 1.5% glutaraldehyde, and incubated in a medium containing 0.1% diaminobenzidine-4HCl and 0.01% H_2O_2 for the demonstration of peroxidatic activity. Postfixation was performed with 1% OsO_4. For each interval studied the cell suspensions of three mice were pooled.

The procedures used for the isolation and fixation of granulomas have also been described in detail [1]. In short, after decapitation of the animals the granulomas were dissected and fixed by immersion in 1.5% glutaraldehyde. For a second group fixation was performed by perfusion of the total body with 1.5% glutaraldehyde before removal of the granulomas. For transmission electron microscopy blocks of tissue were postfixed in OsO_4 or in a modified OsO_4 fixative according to de Bruijn et al. (1973) [16]. Scanning electron microscopical specimens were not postfixed.

Transmission electron microscopy (TEM)

The fixed tissue blocks were dehydrated in a graded series of alcohol up to 100% and embedded in Epon. Ultrathin sections were cut on an LKB microtome, stained with lead hydroxide, and examined in a Philips EM 410.

Scanning electron microscopy (SEM)

After glutaraldehyde fixation, the blocks were dehydrated in a graded alcohol series up to 100%. For freeze fracture some of the dehydrated specimens were brought into supercooled liquid nitrogen, fractured with a cold razor blade, and critical point dried under carbon dioxide in a Balzers model CPD critical point drier. The dried specimens were then covered with a layer of gold about 6 nm thick in a Balzers sputter coater model MED 010 and examined in a Cambridge Stereoscan S 180 at an acceleration voltage of 15 to 20 kV and a tilting angle of about 30°.

X-ray microanalysis (XRMA)

X-ray microanalytical spot analyses were performed with a Tracor (TN) 2000 X-ray microanalysor attached to a Philips EM 400 scanning transmission electron microscope. The system geometry of this instrument has been described elsewhere [17].

Sections collected on copper grids were placed in a beryllium low-background holder which was tilted at an angle of 108 degrees to enhance the X-ray yield. X-ray point analyses were done with a spot diameter of 100 nm during 100 sec lifetime.

RESULTS

Free peritoneal cell population

The first response to the introduction of the foreign materials into the peritoneal cavity was an influx of neutrophil granulocytes and monocytes. The number of neutrophils detected after introduction of the various materials was dependent on the type of stimulus used. The introduction of crocidolite asbestos into the peritoneal cavity induced a relatively strong influx of neutrophil granulocytes compared with the effect of hydroxyapatite and tri-calcium phosphate (Fig. 2), which gave numbers in the range of the control group given only HBSS. On day 4 the total number of neutrophils had returned to normal values in all animals.

Besides variations in cell numbers, the introduction of particulate suspensions also led to morphological changes in the peritoneal macrophages. Various iron-containing inclusion bodies, some containing small fragments of the injected material, were seen. The average number of inclusion bodies per cell section (0.5 ± 1.2 at the start of the experiments) increased with time, and differences related to the nature of the stimulus were observed. At six months the numbers of inclusion bodies were 2.6 ± 1.1 for the control, 3.7 ± 1.2 for hydroxyapatite, 3.7 ± 1.8 for tri-calcium phosphate, and 5.7 ± 2.0 for crocidolite asbestos. Although the inclusion bodies were generally more electron dense after the longer intervals, no significant variation in the average iron concentration per inclusion body or relation to the type of stimulus was shown by XRMA.

Labyrinths, i.e., globular structures composed of intercon-

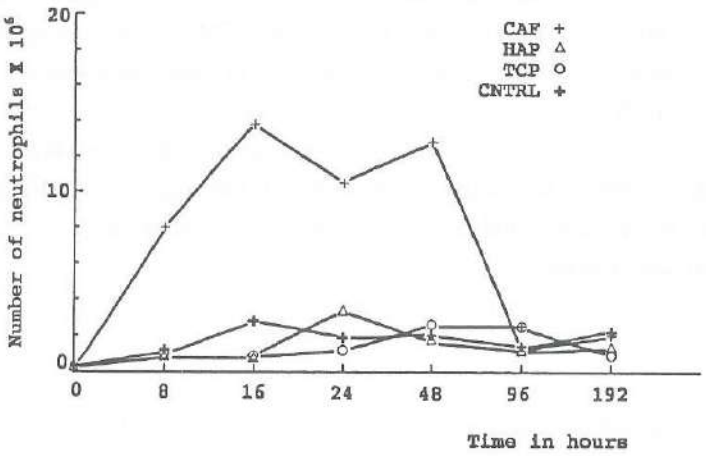


Fig. 2. Numbers of neutrophil granulocytes found after intraperitoneal injection of the three materials. As this graph shows, there was a strong influx of neutrophil granulocytes in response to the introduction of crocidolite asbestos. The numbers of neutrophils remained at the control level after exposure to hydroxyapatite and tri-calcium phosphate.

nected channels with a diameter of about 250 nm (Fig. 3), were detected in peritoneal resident macrophages from animals given crocidolite asbestos or tri-calcium phosphate but not in those of hydroxyapatite-stimulated animals. As in an earlier study [8], not only the proportion of the peritoneal resident macrophages containing these typical invaginations of the plasma membrane but also the average size of the labyrinths increased as a function of time after crocidolite asbestos stimulation. Labyrinths were also seen one month and longer after tri-calcium phosphate stimulation, but these labyrinths remained relatively small compared with those induced by crocidolite asbestos (compare Fig. 3a and 3b). The total number of labyrinths seen in the tri-calcium phosphate-stimulated macrophages was too low to indicate a time-dependent frequency.

Peritoneal granulomas

Aggregates up to 3 mm in diameter were seen in close proximity with the omentum and peritoneal viscera after the intra-peritoneal injection of all types of particulate material. Aggregates present after two or more weeks were

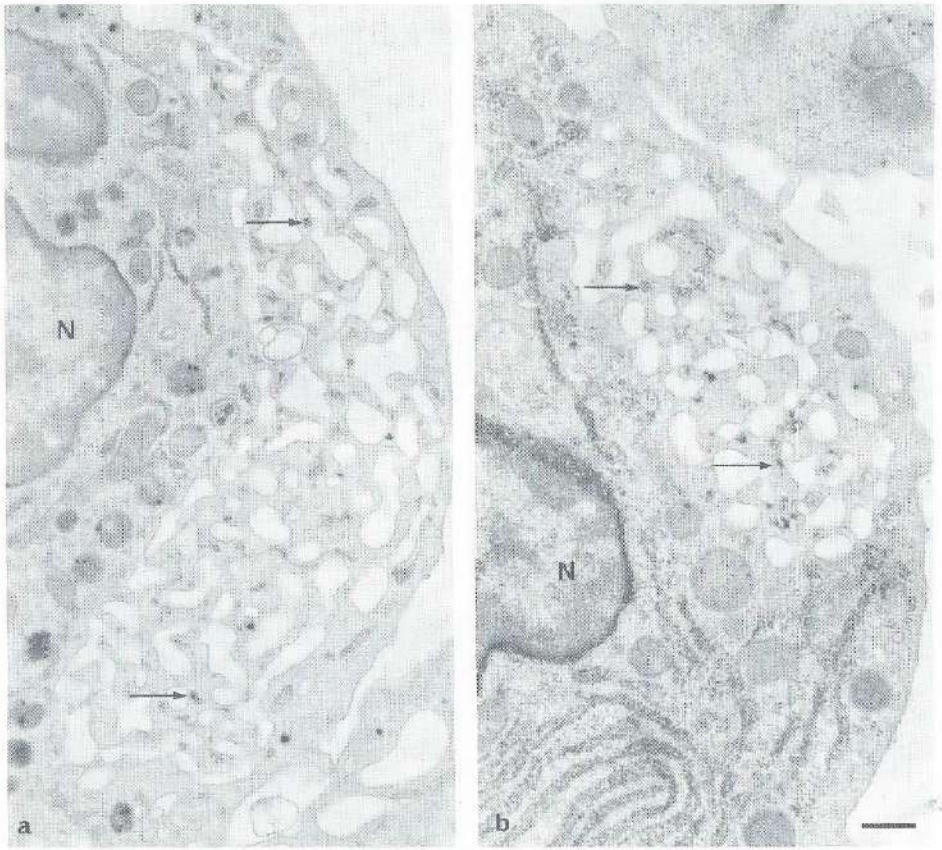


Fig. 3 a and b. Transmission electron micrographs of labyrinths found at six months. a: Labyrinth after crocidolite asbestos stimulation. This labyrinth has the average size of the labyrinths found after asbestos stimulation. Note the small fragments of peroxidase-positive rough endoplasmic reticulum in the cytoplasm (arrows). b: One of the largest labyrinths found after tri-calcium phosphate stimulation. Note that here too, fragments of endoplasmic reticulum are present in the cytoplasm of the labyrinth (arrows). Fixation: glutaraldehyde, OsO_4. N = nucleus. Bar: 0.5 μm

studied by light microscopy (LM), which showed that they were encased in capsules formed by connective tissue (Fig. 4). In addition to fibroblasts and collagen, the crocidolite asbestos granulomas contained lymphocytes, mast cells, macrophages, and multinucleated giant cells with 20 to 30 nuclei per sectioned syncytium. Long and short asbestos fibers, ingested or surrounded by the macrophages and multinucleated giant cells, were seen throughout these granulomas.

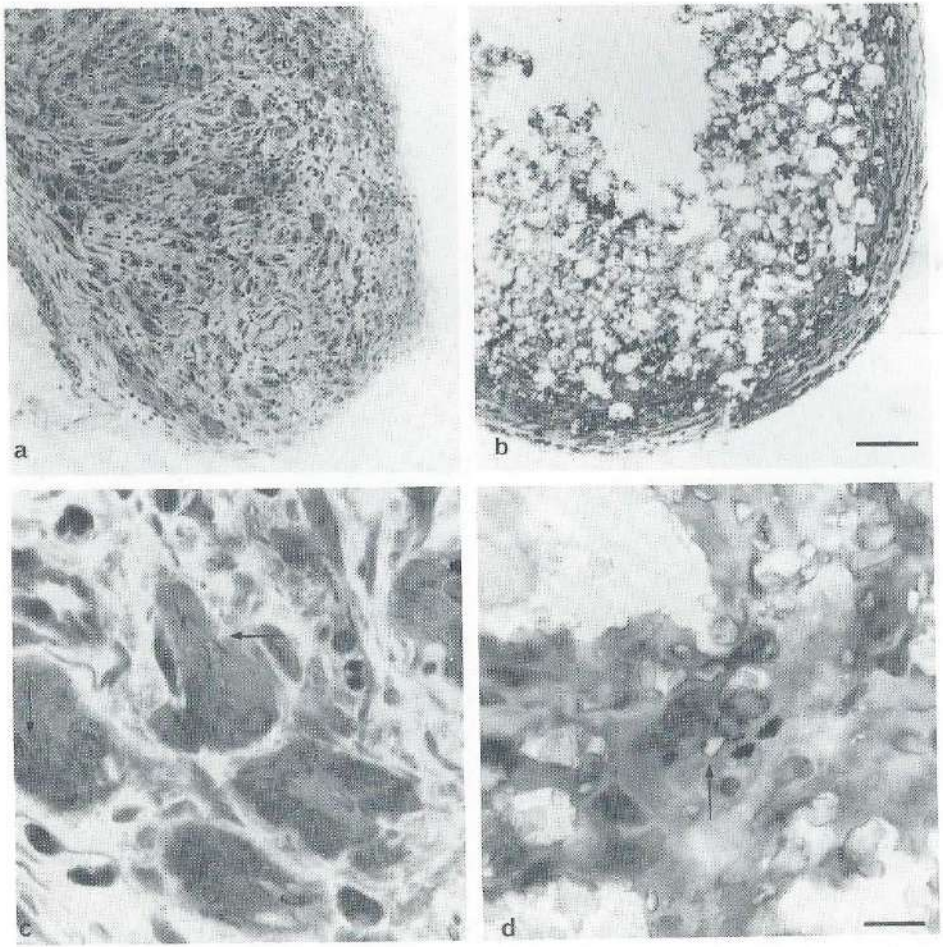


Fig. 4, a-d. Light micrographs of granulomas at one month. a: crocidolite asbestos-induced granuloma. b: hydroxyapatite granuloma after the same interval. The center of this granuloma is composed almost exclusively of the biomaterial. Bar: 100 μm.
At a higher magnification individual giant cells and macrophages containing the injected materials are visible. c: crocidolite asbestos containing giant cells found in the granuloma of a. Many asbestos fibers can be seen (arrows). d: part of the granuloma of b. In this micrograph a giant cell containing hydroxyapatite crystals can be observed (arrow). Bar: 10 μm.

The hydroxyapatite and tri-calcium phosphate granulomas were basically the same as the crocidolite asbestos granulomas and contained similar cell types. Here too, macrophages and multinucleated giant cells containing small particles were seen, but the numbers of macrophages and giant cells were

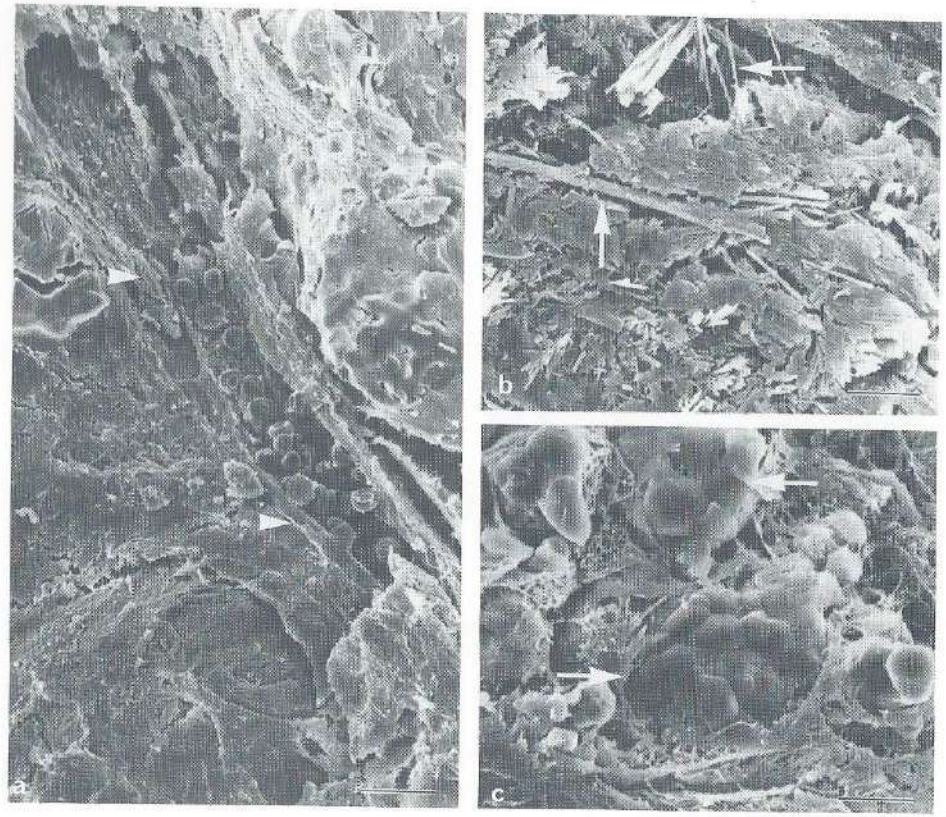


Fig. 5, a-c. Scanning electron micrographs of freeze-fractured granulomas. a: Peripheral part of a hydroxyapatite-induced granuloma after two months. Note the blood vessel lying diagonally (arrowheads) and some cells crammed with material. Bar: 10 μm. b: Center of a crocidolite asbestos-induced granuloma at six months. Note the relatively small intercellular spaces and the numerous asbestos fibers (arrows). c: Central area of a tri-calcium phosphate granuloma, also at six months. The large intercellular spaces are occupied by collagen fibers. Various cells containing material can be seen (arrows). Bar: 5 μm.

smaller and the number of nuclei per giant cell was lower (5 to 15). Furthermore, most of the biomaterial was concentrated in the center of the granulomas (Fig. 4b). Particles too large to be phagocytosed were still in the extracellular space and were surrounded by macrophages, multinucleated giant cells, and collagen.

The SEM findings supported the LM observations. The injected particles accumulated together with exudate cells in aggregates, where they were held together by a loose fibrous network. At two weeks the aggregates were

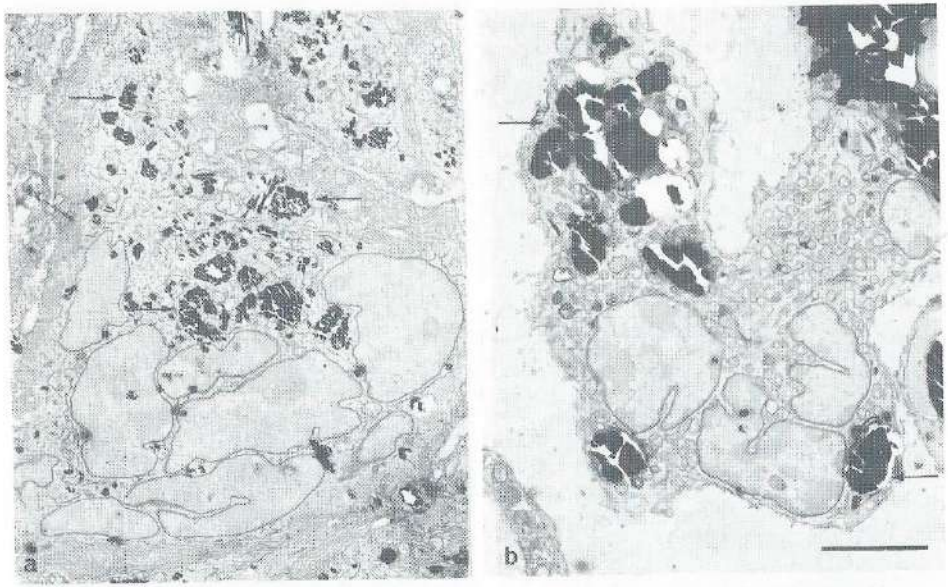


Fig. 6, a and b. Transmission electron micrographs of giant cells in the central area of granulomas at 2 months. a: Crocidolite asbestos granuloma. Several asbestos fibers are visible (arrows). Note the relatively small intercellular spaces and interdigitating folds of the plasma membranes. b: Tri-calcium phosphate-induced granuloma. The giant cell contains large particles of biomaterial (arrows). The large extracellular spaces are occupied by collagen. Fixation: glutaraldehyde, osmium(VI)-iron(II) complex. Bar: 5 μm.

covered with a layer of mesothelial cells. In freeze-fractured granulomas at two weeks and longer SEM showed that fibrous tissue was still present inside the granulomas. Vascularization had occurred in all granulomas at one month and longer (Fig. 5a). For all three materials, comparison of the SEM images showed that at the longer intervals the internal structure visualized after freeze fracturing differed according to the stimulus used. The crocidolite-asbestos granulomas had a high cell density and relatively small intercellular spaces (Fig. 5b). Asbestos fibers were distributed throughout these granulomas. The intercellular spaces in the granulomas containing hydroxyapatite and tri-calcium phosphate were larger. The cells, many of which contained crystals of the biomaterial, were separated by areas of fibrous tissue (Fig. 5c). Morphological differences between granulomas found after stimulation with hydroxyapatite or tri-calcium phosphate were not observed with SEM.

TEM showed the same general morphological features of the granulomas.

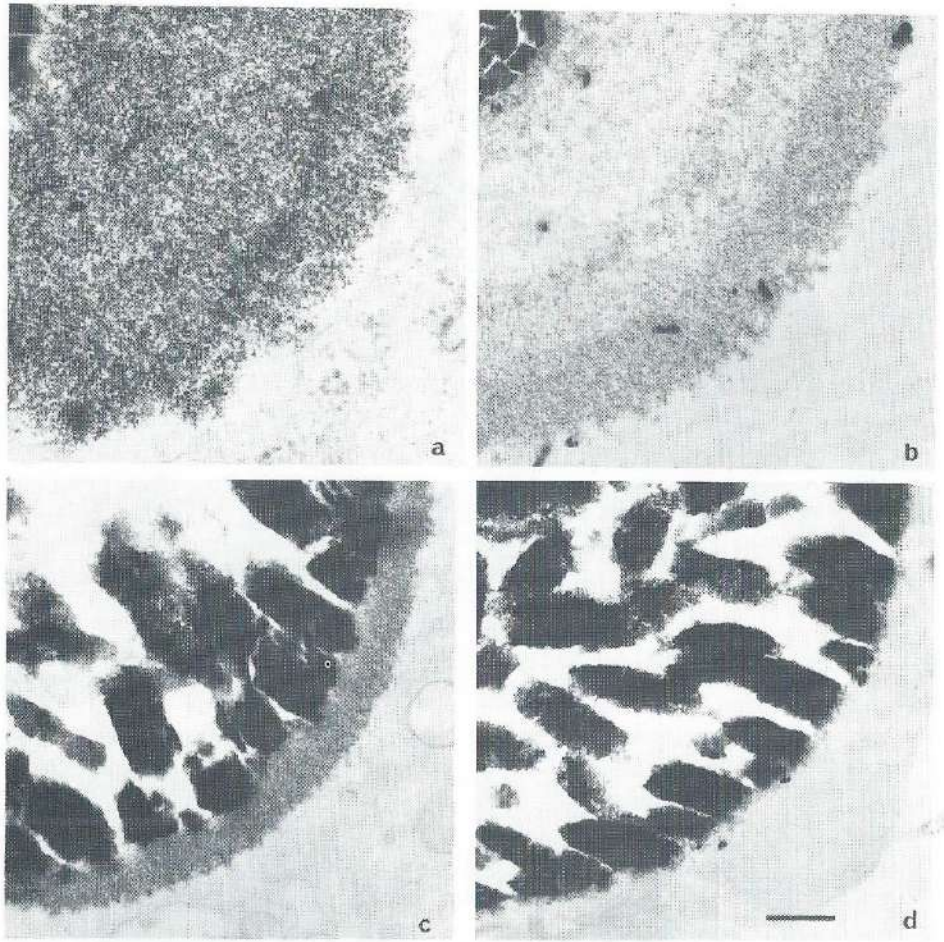
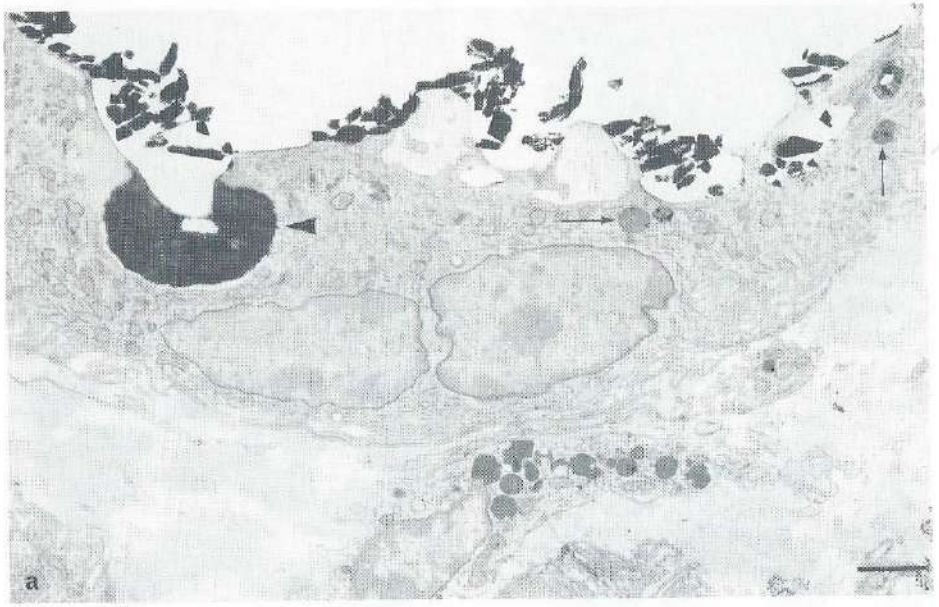
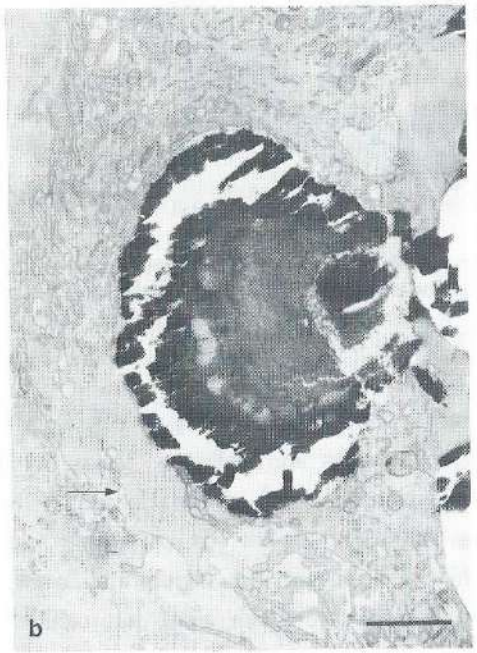
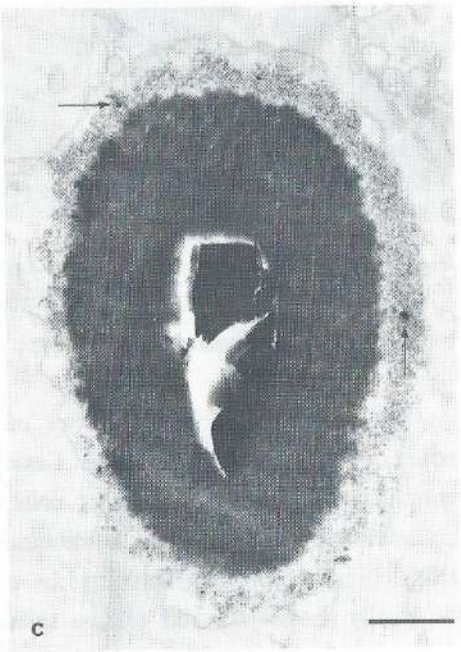


Fig. 7, a-c. Transmission electron micrograph of the types of asbestos body found most frequently in the crocidolite-induced granulomas. a: asbestos body of type I, b: asbestos body of type II, c: asbestos body of combined types I and IV, and d: asbestos body of type IV. Bar: 1 μm.

At two weeks and longer, a layer of mesothelial cells covering a capsule of connective tissue was observed. Besides fibroblasts and collagen, there were lymphocytes, plasma cells, mast cells, macrophages, and multinucleated giant cells. The crocidolite-asbestos granulomas differed from the hydroxyapatite and tri-calcium phosphate granulomas by a higher density of the various cell types, especially phagocytes and plasma cells, and by the relatively small intercellular spaces in the former (Fig. 6a and b).







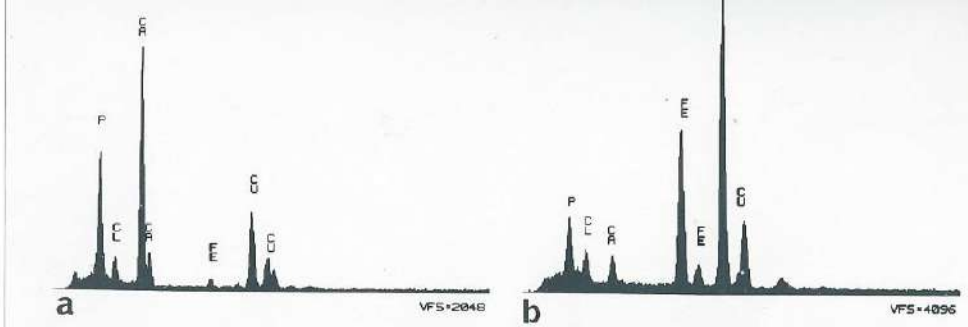


Fig. 9, a and b. X-ray microanalytical spectra of the deposit in Fig. 8c.
a: Spectrum of the central deposit. Note the high peaks for calcium and phosphorus. (Vertical full scale, 2,048 counts.)
b: Spectrum of the granular ring. The locally high iron concentration is clearly shown by this spectrum. (Vertical full scale, 4,096 counts.)

As in SEM and LM, the central area of the granulomas showed striking differences according to the type of stimulus used. Crocidolite asbestos was distributed throughout the granulomas, and the structure of the granulomas was relatively homogeneous, whereas in those induced by hydroxyapatite and tri-calcium phosphate most of the biomaterial was encountered in the center. No morphological differences between hydroxyapatite and tri-calcium phosphate granulomas were seen with TEM. After the longer intervals all granulomas were vascularized but crocidolite asbestos granulomas were distinctly more vascularized than the others.

Extracellular depositions

At one month and longer, asbestos bodies were frequently seen in the granulomas containing crocidolite asbestos. As in an earlier study [1], four different types of asbestos body were found. The most frequently detected types were: type I with a homogeneous iron-rich coat with small asbestos

Fig. 8, a-c. Transmission electron micrographs of a tri-calcium phosphate granuloma. Sectioning artifacts caused by the hardness of the material can be seen. a: Giant cell attached to a particle of biomaterial. Iron-containing inclusion bodies, some of which containing calcium phosphate particles, are present (arrows). A typical deposit resembling an asbestos body is visible at the tissue/material interface (arrowhead). Bar: 2 μm. b: Higher magnification of a deposit resembling type IV. Note the organelle-free zone adjacent to the deposit (arrow). Bar: 1 μm. c: Deposit of combined type I/IV. Note the biomaterial in the central area and the granular ring encircling the dark material. Small biomaterial fragments are visible in the granular ring (arrows). Fixation: glutaraldehyde, osmium(VI)-iron(II) complex. Bar: 1 μm.

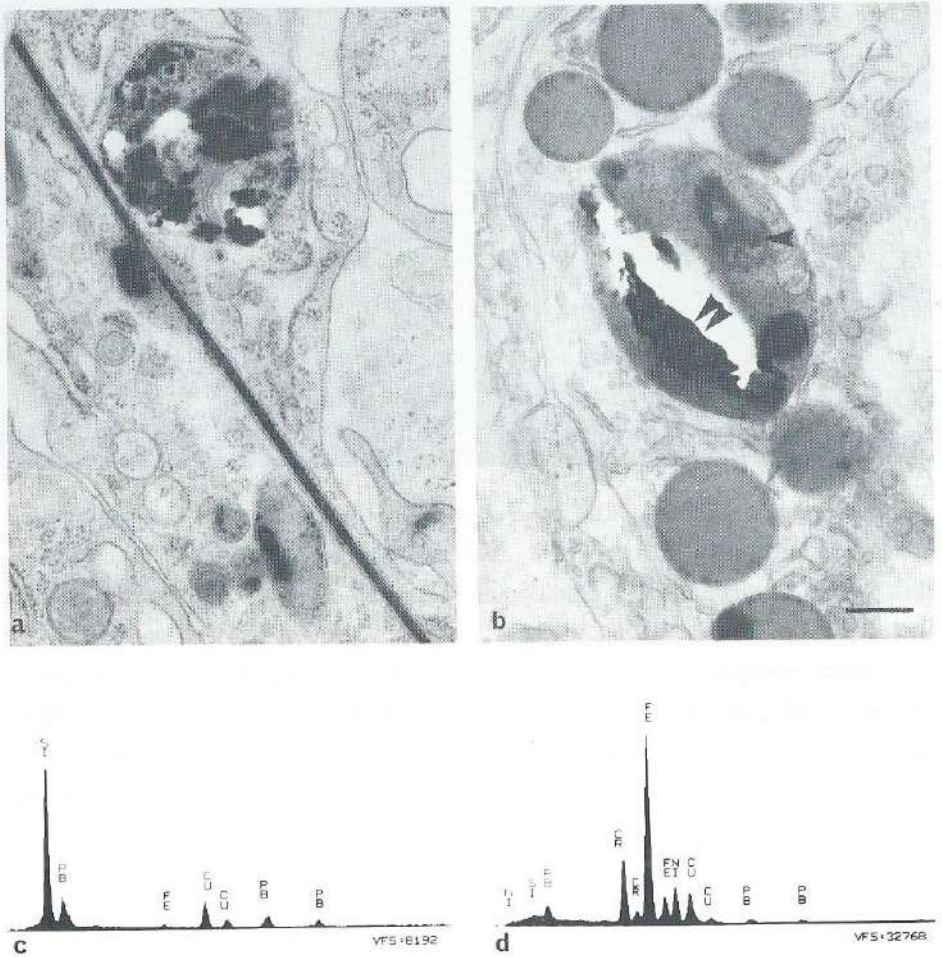


Fig. 10, a-d. a: Section showing inclusion bodies in a macrophage in a crocidolite asbestos-induced granuloma at six months. X-ray microanalysis invariably gave the typical crocidolite-asbestos spectrum when spot analyses of the dark inclusions were made. Note the long asbestos fiber contained by one inclusion body. b: Inclusion bodies in a macrophage of a tri-calcium phosphate granuloma at six months. Inclusions with variable electron density can be seen. Fixation: glutaraldehyde, osmium(VI)-iron(II) complex. Bar: 0.5 μm. c: X-ray microanalytical spectrum showing the presence of silicon. The spectrum represents the structure marked with an arrowhead. (Vertical full scale, 8,192 counts.) d: X-ray microanalytical spectrum showing the presence of iron, chromium, and nickle. This spectrum represents the structure indicated by double arrowheads (Vertical full scale, 32,768 counts.)

particles dispersed through the matrix of the coat (Fig. 7a), type II an annular morphology (Fig. 7b), and type IV with a homogeneous calcium-rich coat (Fig. 7d). Combinations of type I and type IV were seen regularly

(Fig. 7c).

Structures similar to asbestos bodies were also present in the granulomas containing one of the calcium phosphates. However, differences related to the type of calcium phosphate were observed. Granulomas of hydroxyapatite-stimulated animals seldom showed deposits resembling asbestos bodies, but tri-calcium phosphate gave rise to the formation of numerous deposits with a morphological resemblance to asbestos bodies (Fig. 8a). The types of deposit showed a similarity to the type IV asbestos body (Fig. 8b) and to the combination of asbestos bodies of types I and IV (Fig. 8c). As in true asbestos-body formation, small fragments of tri-calcium phosphate were present in granular rings. Deposits resembling type I asbestos body were not seen in the tri-calcium phosphate-induced granulomas.

XRMA of the matrices of the extracellular deposits showed that, like the type-IV asbestos body, they were composed of calcium phosphate-rich material containing iron in a low concentration (Fig. 9a). The deposits representing type I/IV gave similar XRMA patterns in a spot analysis of the center, whereas the granular rings showed high concentrations of iron (Fig. 9b).

Trace element accumulation

As already mentioned, macrophages and giant cells in all types of granuloma had round to oval inclusion bodies in their cytoplasm, and XRMA showed that iron was present in the matrix of these inclusion bodies. A number of the inclusion bodies contained particles of the originally injected material (Fig. 10a).

Furthermore, phagocytes present in granulomas induced by tri-calcium phosphate and less so in those induced by hydroxyapatite contained some inclusion bodies in which another kind of material was present (Fig. 10b). In such structures XRMA identified the elements silicon, aluminum, chromium, and nickle (Fig. 10 c and d). Both the local concentration and the ratio of these elements were variable. Inclusion bodies containing this type of material were never found in the granulomas induced by crocidolite asbestos.

DISCUSSION

The mouse peritoneal cavity as compartment has frequently been used to study the origin and function of macrophages [7,18,19] as well as the reaction

of macrophages to the introduction of solutions differing in nature [1,7-10].

The increasing need for new biomaterials in reconstructive surgery has created a need for reliable tests to assess tissue tolerance [20,21]. Since macrophages generally come into direct contact with implanted biomaterials, this type of cell is assumed to be the most important of the cells functioning at the implant interface and reacting with the implant [3,4,22]. This means that extensive study of the macrophage/biomaterial interaction is required. The mouse peritoneal cavity has proven to be a suitable compartment for such studies [9,10].

The present investigation concerned the effects of three different materials on the mouse peritoneal cell population and in particular the macrophages.

Peritoneal cell population

The composition of the peritoneal cell population altered in response to the introduction of all three particulate materials. In the present study, crocidolite asbestos led to by far the greatest increase of the numbers of neutrophil granulocytes, and we therefore concluded that this material caused a stronger inflammatory reaction than hydroxyapatite and tri-calcium phosphate did. The neutrophil counts after the introduction of hydroxyapatite and tri-calcium phosphate were in the range of the control group given only HBSS, which led us to conclude that hydroxyapatite and tri-calcium phosphate are weak inflammatory stimuli. This could be expected, because both hydroxyapatite and tri-calcium phosphate were developed to serve as biotolerant materials (for a review, see van Blitterswijk [15]).

Iron accumulation

It is known that the iron concentration in phagocytes increases with age [23]. In an earlier study we found that the introduction of asbestos fibres into the mouse peritoneal cavity can lead to accumulation of iron in the peritoneal macrophages [8] in the form of cytoplasmic ferritin as well as to an increased number of iron-containing inclusion bodies. Although the source of the iron in the inclusion bodies was not established in that study, the iron definitely did not derive from the crocidolite asbestos and it was therefore assumed that macrophages in contact with indigestible material are induced to accumulate iron from another source. A long-term in vitro investigation [2] showed that

an increase of the iron concentration in the media of macrophage cultures leads to an increase in the amount of iron in macrophages, as shown by the formation of many iron-rich inclusion bodies. We therefore conclude that to a certain extent the iron pool in macrophages reflects the availability of iron. This assumption is supported by observations of other authors, who have shown that both a hemorrhage and injection of blood give rise to the formation of iron-rich inclusion bodies [24-27].

An increased availability of iron in the present investigations could have been due to leakage of new blood vessels formed during the process of granuloma formation. This is supported by the accumulation of iron in macrophages present in the granulomas induced by tri-calcium phosphate and hydroxyapatite and also seen after implantation of hydroxyapatite in the rat middle ear [14]. Furthermore, the present study showed that the amount of iron accumulated was dependent on the type of stimulus used. Since crocidolite asbestos led to the formation of more iron-rich inclusion bodies than the two biomaterials did, the amount of iron present in macrophages in the direct vicinity of an implant must to a certain extent be related to chemical or physical properties of the implant. Such correlation between the type of implant and the rate of iron accumulation could be valuable in studies on the biocompatibility of materials. The importance of the phenomenon of iron accumulation will be the subject of future investigations.

Labyrinth formation

As reported earlier [8], mouse peritoneal macrophages could be induced to form labyrinths by exposure to crocidolite asbestos, and the number and the size of labyrinths increased with time. Further, the findings suggested a relation with an enhanced synthesis of prostaglandin.

Labyrinth formation was seen in the present study too, and the presence of labyrinths was not only found to be a time-dependent phenomenon occurring after exposure to poorly digestible materials, but also appeared to be related to the type of stimulus. Numerous labyrinths appeared after crocidolite asbestos stimulation. After tri-calcium phosphate stimulation labyrinths were seen much less frequently, and although they increased in size they remained considerably smaller than those observed after crocidolite asbestos stimulation (compare Fig. 3a and 3b). In our hands hydroxyapatite did not induce the formation of labyrinths. This difference in response indi-

cates that labyrinth formation is a material-dependent phenomenon. The functional significance of the labyrinths has been discussed elswhere [8]. The possibility of a relationship between the occurrence of labyrinths and prostaglandin synthesis and between the formation of labyrinths and biocompatibility will be investigated in due course.

Extracelluar deposits

Extracellular deposition of materials normally present in the lysosomes of peritoneal macrophages has already been described in relation to the formation of asbestos bodies [1,2]. Those studies led us to conclude that where macrophages and/or multinucleated giant cells encounter particles too large to be completely ingested they seal off a part of that material to form a micro-environment between cell and material. Next, these cells exocytose their lysosomal content into this micro-environment in an attempt to digest the asbestos fibers extracellularly by a process resembling bone resorption by osteoclasts. Materials present in the lysosomes by chance are also deposited in this micro-environment, and this leads to extracellular deposits. On the basis of morphological and chemical criteria, different types of extracellular deposits formed by this process can be distinguished. In one of our previous reports [1] we described four types of asbestos body, and in that study we saw combinations of types I and IV as well. In the present study, too asbestos bodies were formed after crocidolite asbestos stimulation and the four types were again recognized. Deposits resembling type-IV asbestos bodies and the combination of types I and IV were abundant in the tri-calcium phosphate-induced granulomas, but were seldom found in granulomas formed in reaction to hydroxyapatite.

The formation of deposits on non-asbestos fibrous materials has been reported [28,29], but such formation on non-fibrous materials has, to the best of our knowledge, never been described. Only one report, i.e., by van Blitterswijk et al. [14], mentions the formation of electron-dense material at the tri-calcium phosphate interface. The morphology of the material described by these authors resembled the type IV asbestos body, but no phagocytes were seen in the vicinity. This led the authors to assume that the electron-dense structures represented the lamina limitans seen when bone formation ceases [30].

Since the electron-dense deposits we saw were in close proximity with

macrophages or giant cells and an iron-rich layer containing small fragments of biomaterial was found on a number of occasions, we concluded that the structures seen in the present study were not related to a lamina limitans but rather to the process of extracellular deposition [1,2].

Thus the present study has provided evidence supporting the hypothesis that the process leading to asbestos-body formation is not a specialized process occurring only when macrophages encounter asbestos fibers or a select group of other fibrous materials, but is a general form of behavior shown by macrophages and giant cells when they make contact with large particles of poorly digestible materials.

That the formation of extracellular deposits is known to be related to the non-degradable character of the material that induces their formation [1], suggests that the difference between the numbers of extracellular deposits after injection of crocidolite asbestos on the one hand and tri-calcium phosphate or hydroxyapatite on the other, is related to biodegradability. In other words, hydroxyapatite would be more easily resorbed than tri-calcium phosphate. However, hydroxyapatite and tri-calcium phosphate are known to be at least equally resorbable, and many authors have described tri-calcium phosphate as the most resorbable material of the two (for a review, see van Blitterswijk [15]). It therefore seems more likely that the observed differences in the formation of extracellular depositions found in the present study are related to the surface characteristics of the materials in question.

If such a relation existed, it would mean that the formation of extracellular deposits could provide direct information on the bioactivity of materials. The extent to which extracellular deposits are indeed related to and a measure of bioactivity will be the subject of future investigations.

Trace element accumulation

The release of chemical elements at the interface between soft tissue and metallic or ceramic implants has been described (for a review, see Mitchel [31]), but reports on the release of elements from ceramics are rare. The presence of various elements in macrophages and multinucleated cells in contact with implanted ceramics, i.e., tri-calcium phosphate and hydroxyapatite, was first described by van Blitterswijk et al. [14]. In their study based on XRMA, these authors demonstrated the presence of iron, silicium, titanium, aluminum, chromium, calcium, phosphorus, and magnesium in the lysosomes

of macrophages at the tissue/biomaterial interface. In another paper the accumulation of this type of element after the intraperitoneal injection of biomaterials was reported [10]. The present study showed trace element accumulation too, and we assumed that these elements were released during the process of digestion. The assumption that these elements did indeed derive from the implanted ceramics, is supported by the absence of such elements in the granulomas induced by the poorly degradable crocidolite asbestos fibers.

Although we observed no toxic effects ascribable to the accumulated elements in this or previous studies [10,14,32], it is known that the release of trace elements, especially aluminum, can cause bone disease [33-35]. In our opinion, prolonged accumulation of trace elements from degradable ceramics can lead to toxic concentrations at the material/tissue interface, which in turn can influence the biocompatibility of materials left in situ for long periods.

Since it is not known to what extent these elements are transported by the macrophages to other organs where their liberation could cause even more damage [33], it seems important to include studies on the release and toxicity of these elements in the evaluation of the biocompatibility of biomaterials.

In sum, it is concluded from the results of the present study that the peritoneal cavity of the mouse is a suitable compartment for the investigation of macrophage/biomaterial interactions. Furthermore, our work on this problem has shown that iron accumulation and labyrinth formation occur in response to the presence of poorly digestible particles. The value of these observations for biocompatibility assessment is uncertain and will be studied.

The present study has shown that accumulation of trace elements is associated with degradation of biomaterials; that this accumulation can lead to relatively high concentrations of these elements after prolonged implantation; and that toxic levels may be reached locally.

The results have also shown that extracellular deposition of the lysosomal content, leading ultimately to the formation of asbestos bodies, is not restricted to macrophages exposed to asbestos, but is a phenomenon associated with encounters between macrophages or multinucleated giant cells and particles that are too large to be completely ingested.

If, as seems likely, the number of extracellular deposits is a measure of the surface activity of biomaterials, the occurrence of such deposits would be important for an understanding of the behavior of biomaterials at implantation

sites. The formation of extracellular deposits in this context will therefore be the subject of future investigations as well.

Acknowledgements

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CHAPTER VI

Asbestos bodies, i.e., asbestos fibers coated with an iron-rich protein layer, are frequently found in individuals who have been exposed to asbestos. In light microscopy asbestos bodies are, in contrast to uncoated asbestos fibers, easily observed. For this reason, asbestos bodies can serve in light microscopical diagnosis as an indication of asbestos exposure.

As early as 1906, Marchand [1] described "bizarre and distinctive golden-brown bodies", sometimes present in sputum and biopsy specimens of human lung tisue. In 1929, Steward and Haddow [2] pointed to a relation between the presence of asbestos fibers and these golden-brown bodies, and they introduced the term asbestosis body. Later, Gloyne used the term asbestos bodies [3], because he assumed that the phenomenon in question was restricted to the interaction between tissue and asbestos fibers. He also showed that the golden-brown color of an asbestos-body is due to iron-rich proteins in the asbestos body coat. Gross et al. [4] found that asbestos bodies were present after the instillation of other indigestible fibrous particles as well, and this led them to introduce the term ferruginous bodies.

Studies on the process of asbestos-body formation have since then been published [5-9], but consensus had not been reached as to the exact underlying mechanism and the role of macrophages in this process. Furthermore, it was not known whether the process of asbestos-body formation is limited to materials with a fibrous morphology.

The studies reported in this thesis were performed to investigate the effects of intraperitoneally injected particulate materials on the composition of the peritoneal cell population and on the morphology of the macrophages in that population. We also wanted to investigate the process of asbestos-body formation and the role of macrophages in this process. Finally, we wanted to find out whether asbestos-body formation occurs only with asbestos and some other fibrous materials i.e. glass [10,11], respectively zeolite fibers [12], or is a general phenomenon occurring when large particles of poorly digestible materials are encountered by macrophages.

The free peritoneal cell population

The free peritoneal cell population in the unstimulated peritoneal cavity of experimental animals has been described in earlier reports [13-15]. These studies showed that two types of cell predominate in the peritoneal cavity:

lymphocytes (30%) and peritoneal resident macrophages (60%), the latter characterized by peroxidatic activity in the rough endoplasmic reticulum and the nuclear envelope. The origin and function of the peritoneal resident macrophages have been amply discussed in the past, and two theories concerning the origin are held [14,16-18].

The injection of sterile agents into the mouse peritoneal cavity leads to an influx of neutrophil granulocytes and monocytes. The latter cell type differentiates into the exudate macrophage, recognized by the presence of peroxidase positivity in lysosomes [13-15].

It has been shown [15] that the number of cells that migrate to the mouse peritoneal cavity in response to stimulation depends on the type of stimulus used. According to the results of that study, strong inflammatory stimuli such as paraffin oil cause large numbers of neutrophil granulocytes to migrate to the peritoneal cavity, whereas weak inflammatory stimuli such as an isotonic solution of sodium chloride cause only a slight increase in the number of neutrophils.

As described in this thesis, the injection of various particulate materials also induced an inflammatory reaction. The number of neutrophil granulocytes returned to normal values within four days, and the original composition of the total peritoneal cell population was restored within eight days. Further, as described in Chapter II, the introduction of a poorly digestible particulate material, i.e., crocidolite asbestos fibers, led to macrophage fusion and the formation of multinucleated giant cells. In view of these findings it is evident that the intraperitoneal introduction of crocidolite asbestos fibers, which gives rise to a strong influx of neutrophil granulocytes into this body compartment, is a strong inflammatory stimulus. The results described in Chapter V support this conclusion. On the same grounds it may be concluded that hydroxyapatite and tri-calcium phosphate, are weak inflammatory stimuli (Chapter V), which is in agreement with studies on these materials at other implantation sites (for a review see van Blitterswijk) [19].

Iron accumulation

With increasing age increased amounts of iron, as indicated by increased numbers of ferritin particles in lysosomes, can be observed in Kupffer cells [20]. An age-related increase in the iron content of lysosomes was also observed in the present study, in this case in peritoneal macrophages of

animals given only a physiological saline solution.

The intraperitoneal introduction of asbestos fibers amplified this effect. Under these conditions macrophages with numerous iron-rich inclusion bodies in their cytoplasm were found (see e.g. Chapter II, Fig. 7). Furthermore, other particulate materials, i.e., hydroxyapatite and tri-calcium phosphate, also gave rise to an increase in the number of this type of inclusion body. This is in agreement with the results of another study [21] in which an increased concentration of cytoplasmic ferritin was found in macrophages and multi-nucleated giant cells wich had been exposed to tri-calcium phosphate. We also had the impression that the electron density, and thus the iron concentration in the inclusion bodies, were dependent on the type of stimulus used, the highest levels being seen after crocidolite asbestos stimulation. XRMA, however, did not seem to be sufficiently sensitive to demonstrate such a relation.

The origin of the iron could not be established conclusively in the present investigations, but asbestos could be excluded as a source because the XRMA spectra obtained from crocidolite asbestos before injection and after the longest intervals showed constant Fe/Si ratios. The observation of iron accumulation after exposure to the iron-free calcium phosphates also indicates that the iron must have come from another source. It seems likely that the source is extracellular iron. This assumption is supported by reports of other authors, who observed an increased number of iron-rich inclusion bodies after hemorrhage [22], injection of blood [23], or injection of colloidal iron [24,25]. Strong support for this assumption is provided by the results indicating that cultured macrophages accumulate iron in response to an increased iron concentration in the culture medium (see Chapter IV).

The increased iron concentration in the mouse peritoneal macrophages suggested that this iron could form the basis for asbestos-body formation. Because asbestos bodies formed in the lungs can be isolated by bronchoalveolar lavage [26-28] and are regularly found in the sputum of individuals exposed to asbestos dust [27,28], it seemed probable that free alveolar macrophages were the cells involved in the process of asbestos-body formation. Since free macrophages are also present in the peritoneal cavity, we started by looking for asbestos bodies in the isolated peritoneal suspensions. However, no asbestos bodies were found in these suspensions, and only a small number of predominantly short asbestos fibers was present in the free peritoneal macrophages.

Continuation of the investigations showed that the long asbestos fibers were trapped in foreign-body granulomas and had induced the formation of asbestos bodies at those sites. The occurrence of asbestos bodies in these granulomas is discussed below.

The formation of labyrinths

The investigations reported in Chapters II and V showed that crocidolite asbestos fibers induce the formation and development of labyrinths, i.e., globular complexes of interconnected membrane-limited channels whose membranes are continuous with the plasma membrane. These structures, originally described in peritoneal resident macrophages of the guinea pig [29], are seldom seen in unstimulated mouse peritoneal macrophages [30] and have a relatively small diameter (approximately 1.5 μm). However, under the experimental conditions applied, the number of labyrinths increased with time, until at 6 months more than 30% of the peritoneal macrophages contained these structures. Not only the number but also the size of the labyrinths increased, and labyrinths with a diameter of 7 μm and larger were frequently present at the two-month and longer intervals (Chapter II). The biological significance of the labyrinths could not be determined, but it seems likely that they serve to enlarge the cell surface and act as a pathway for transport between cell organelles and the extracellular space [31].

The intimate contact between elements of the peroxidase-positive endoplasmic reticulum and the tubules of the labyrinths shows a striking resemblance to the PO-positive dense tubular system and the open canalicular system in blood platelets [32,33].

In the light of reports describing PG release by platelets [32] and taking into account the finding that the release of PGE_2 by macrophages increases after exposure to asbestos [34,35] and other poorly digestible materials [36], a relationship between the occurrence of labyrinths and PG synthesis seems likely. This is supported by biochemical investigations [37,38] showing that PG synthetase is present in the RER, and by studies reporting a relation between the presence of PO activity in the RER and PG synthesis [39,40]. It has also been shown that the release of PG is suppressed by PO inhibitors such as aminotriazole and sodium azide, which inhibit PO activity in the RER and NE of peritoneal resident macrophages too [39].

Labyrinth formation was also induced in peritoneal macrophages by intraperitoneal injection of tri-calcium phosphate but did not occur after the introduction of hydroxyapatite (Chapter V). This suggests that labyrinth formation is related to the presence of particulate materials of a certain nature and might provide information on biocompatibility. The extent to which the occurrence of labyrinths in mouse peritoneal macrophages is related to PG synthesis and expresses biocompatibility remains to be investigated.

The formation of foreign-body granulomas

The SEM studies showed that the injected asbestos fibers and peritoneal cells aggregate rapidly and offer a basis for granuloma formation. Although the formation of foreign-body granulomas is a well-known phenomenon [9,41], nothing is known about the mechanism by which the originally separate asbestos fibers accumulate in the peritoneal cavity. It is, however, probable that macrophages attached to asbestos fibers are involved in the transport of these fibers to certain loci, guided by chemotactic factors. The results of the in vitro experiments support this assumption. In these experiments too the formation of aggregates of cells and numerous asbestos fibers was observed. Since the asbestos fibers were added to the cultures as a homogeneous suspension and because the macrophages containing asbestos fibers were initially equally divided over the culture dishes, it may be concluded that during incubation the macrophages migrated to form the aggregates observed at the longer culture times. Because they were not seen in the control cultures, it may be concluded that the aggregates were the result of an active transport of fibers to these loci by macrophages.

Asbestos-body formation

The foreign-body granulomas formed after an intraperitoneal injection of asbestos contained many asbestos fibers which had been ingested by macrophages and multinucleated giant cells. Some of these fibers, however, were too long to be ingested and remained in the extracellular space. At various places such fibers were surrounded by macrophages and/or multinucleated giant cells, and it was here, at the interface between these cells and the long asbestos fibers, that the process of asbestos-body formation was found to occur.

The smallest fiber length that can give rise to the formation of an

asbestos body was established after extraction of the asbestos fibers from the granulomas. These studies showed that asbestos bodies were never formed on fibers shorter than 10 μm and generally appeared on much longer fibers. From the LM observations in the in vitro study which showed that asbestos-body formation was restricted to incompletely ingested fibers, it may be concluded that asbestos bodies are formed extracellularly. This conclusion is supported by TEM observations at the site of contact between two cells attempting to ingest the same asbestos fiber. At these places the membranes embracing the asbestos body were often continuous with the plasma membranes, thus forming a connection between the space containing the asbestos body and the extracellular space (see, e.g., Chapter III, Fig. 6).

On the basis of the morphology of the asbestos-body coats and especially their chemical composition determined by X-ray microanalysis, four different types of asbestos body were distinguished: type I, with a homogeneous iron-rich coat in which small asbestos fragments are dispersed; type II with an annular morphology in which iron and calcium predominate in alternating layers; type III with an annular morphology and very low levels of chemical elements; and type IV, an asbestos-body type with a homogeneous coat composed predominantly of calcium and phosphorus.

The dominance of iron in the asbestos-body coats and the high iron content of the inclusion bodies, particularly those of macrophages in contact with asbestos bodies, led us to assume that especially these inclusion bodies are involved in the process of asbestos-body formation.

The finding of small asbestos fragments in the inclusion bodies and in the iron-rich parts of the asbestos bodies in both the in vivo and the in vitro studies is, in our opinion, an indication of such involvement. We assume that after being endocytosed by a macrophage, such fragments are enclosed in the lysosomal system. When such a macrophage makes contact with an asbestos fiber that is too long to be ingested, fusion of intracellular lysosomal vesicles with the plasma membrane will occur and the contents of these vesicles are secreted into a micro-environment between the macrophage and the asbestos fiber. This phenomenon, called exocytosis [42] is a common process that occurs in almost all types of cell that secrete macromolecules.

That small particles are indeed endocytosed and transported to the space containing the asbestos body was demonstrated by the experiments in which small particulates, i.e., 15 nm colloidal gold particles, were added to the culture media. These gold particles were, like the asbestos fragments, found in

the iron rich inclusion bodies and in the asbestos-body coats.

The accumulation of gold particles in the inclusion bodies, together with the morphology of the latter, suggested a lysosomal nature of these organelles. Cytochemically, the inclusion bodies were found to contain acid phosphatase, which confirmed their lysosomal nature. The observation that acid phosphatase was also present in the coats of the asbestos bodies provided further evidence of the occurrence of exocytosis of the lysosomal contents. Two types of inclusion body were recognized, one resembling those found in the free peritoneal macrophages (Chapter II) and the other showing morphological resemblance to siderosomes, for instance those described by Ghadially et al. [22]. Both types contained the small asbestos fragments. In a recently started study it was demonstrated, by the use of electron energy loss spectrometry, that chlorine is colocalised with iron in the inclusion bodies and in the iron-rich parts of the asbestos bodies. XRMA showed that the iron to chlorine ratio of the second type is of the same order of magnitude as that of asbestos body type I and of the iron-rich rings of asbestos body type II (unpublished observations, Koerten and de Bruijn). We therefore think that the second type of inclusion body was preferentially involved in the process of asbestos-body formation.

The study described in Chapter V showed, to the best of our knowledge for the first time, that the process of extracellular deposition also occurs when macrophages meet poorly digestible materials of a non-fibrous nature, i.e., granulated hydroxyapatite and tri-calcium phosphate. We therefore concluded that the process of extracellular deposition, on which the formation of asbestos bodies is based, is a general process that occurs when macrophages make contact with relatively large and poorly digestible particles.

Only one other report mentions the formation of structures with a somewhat comparable morphology at the tri-calcium phosphate interface in the rat middle ear [43]. However, in that study phagocytes were not present at such sites, and this led the authors to assume that their observations concerned a lamina limitans, as normally observed when the process of bone formation ceases temporarily [44].

Taking the foregoing observations into account, we arrived at the hypothesis that: asbestos bodies are formed extracellularly by exocytotic activity of macrophages or giant cells. The formation of asbestos bodies is a matter of chance depending on whether a cell of the right type (macrophage or giant

cell) encounters the right particle, i.e., one with dimensions such that it cannot be completely ingested and will therefore remain in the extracellular space. When such a situation arises, the macrophage or giant cell seals off the particle and exocytoses the contents of its lysosomes into the space thus obtained, in an attempt to digest the particle extracellularly. The process in question is similar to that of bone resorption by osteoclasts, where the material to be digested is sealed off from the surroundings and a space created in which digestive activity is performed by the exocytosis of agents synthesized by the osteoclast. However, the two processes differ in that the lysosomal enzymes do not degrade the materials we used effectively and exocytosis might therefore continue for a very long time, leading to the accumulation of material derived from the lysosomes, which in turn would lead to extracellular deposition (Fig. 1).

The fact that asbestos bodies are found more frequently after the introduction of an amphibole than after the introduction of chrysotile [45,46] can be explained by this hypothesis. Chrysotile is more easily fragmented into short fibers which can be completely ingested [47] and extracellular deposition will thus not occur.

The origin of the calcium phosphate crystals in asbestos bodies of type II and in the homogeneous deposits of calcium and phosphorus in the type IV asbestos body is not explained by the results of these studies. As mentioned in Chapter III, the small tubules traversing the organelle-free zones in the cytoplasm of macrophages and giant cells may play a role in the deposition of calcium. It is, however, also conceivable that the hydroxyapatite, continuously present as a saturated solution in extracellular spaces and body fluids, is deposited due to changes in the pH [48], the asbestos or iron particles functioning as nucleation points.

Segmentation of asbestos bodies

It is known that asbestos bodies are sometimes segmented, and segmentation was seen in the present studies too. The in vitro experiments showed that the number of asbestos fibers that became coated and the rate of segmentation were both dependent on the duration of the culture period. Almost all fibers with a length of 25 μm or longer were coated at 12 weeks, and about 50% of these asbestos bodies were segmented. The mechanism underlying this segmentation is not known, but its occurrence in vitro disproves the claim of Mace et al. [49] that segmentation of asbestos bodies is the result of expiratory movements, because movements of this kind could not occur during culture. The beaded appearance of asbestos bodies might be due to continued expansion of the surrounding membrane due to fusion of the lysosomes with the plasma membrane.

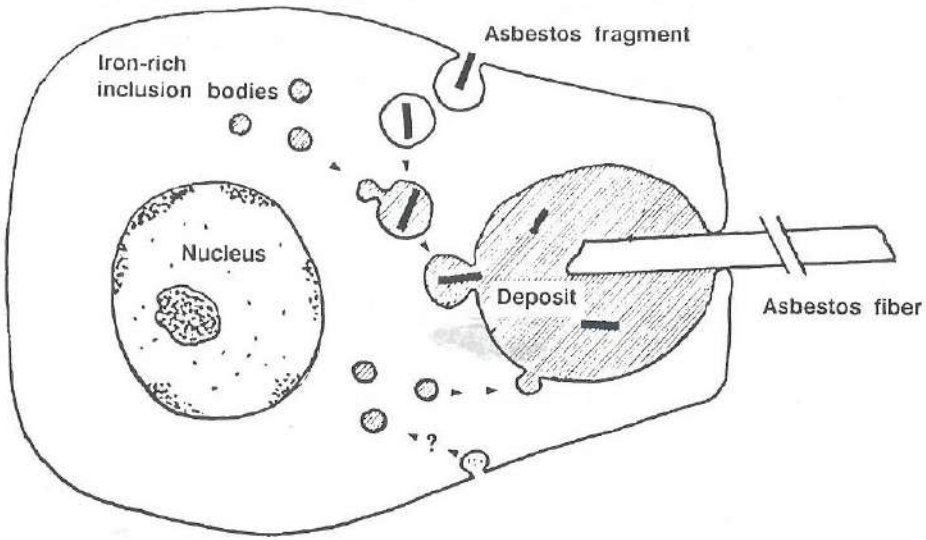

Fig. 1. Schematic representation of the assumed pathway of iron and small particles in macrophages during the process of asbestos-body formation. Such particles and iron are endocytosed by macrophages and enclosed in lysosomal structures. The lysosomes then fuse with the extracellular micro-environment in an attempt to achieve extracellular digestion. The content of the lysosomes is thus deposited by chance in this micro-environment, where it accumulates to form an asbestos body or some other type of extracellular deposit.

Occurrence of asbestos bodies outside the lungs

The presence of asbestos bodies outside the pleural cavity has been reported [50,51] but although several investigators have introduced asbestos fibers at several sites outside the lungs and pleural cavity [52-54], it is not

known whether asbestos-body formation can occur there [52].

The above-described observations (Chapter III) and even more so the results of the in vitro study (Chapter IV), which showed for the first time that asbestos-body formation also takes place when macrophages in culture are exposed to asbestos fibers prove that asbestos-body formation is not restricted to the lungs and that this process can occur at any place where macrophages and long asbestos fibers make contact.

Nomenclature

As already mentioned, a number of other poorly digestible fibrous materials, e.g. zeolite and glass [10-12], are reported to induce the formation of the characteristic iron-rich coat. This led Gross et al. [4] to suggest the use of the term ferruginous body instead of asbestos body. However, since the present study has shown that the chemical composition of the coat of asbestos bodies is variable as well, and can consist partly or mainly of non-ferrous elements, i.e., calcium and phosphorus, it is evident that the term ferruginous body does not completely cover the nature of the extracellular depositions. It therefore seems preferable to reserve the term asbestos body for those cases where there is certainty that the core of the structure is asbestos in nature, and to refer to extracellular deposits as a more general term for such structures.

Trace element accumulation

Experiments on the biocompatibility of calcium phosphate ceramics have shown that hydroxyapatite and tri-calcium phosphate are subject to biodegradation (for a review see van Blitterswijk and Grote) [19]. It has also been shown that, as a result of the digestive activity of macrophages functioning at the material interface, trace elements are released from these materials and accumulate in residual bodies [55,56]. The study described in Chapter V too showed the release and accumulation of these elements (see Chapter V, Fig. 10).

Processes occurring at the tissue/material interface are assumed to be of decisive importance for the functioning of biomaterials [57], and it is known that the release of both particulates and metal ions from implants can have toxic effects to the host tissue (for a review see Michel) [58]. The above-cited

studies did not show any toxic reactions due to components released from hydroxyapatite and tri-calcium phosphate implants. Although toxic reactions were also not seen in the study described in Chapter V it remains possible that very long implantation times give rise to certain toxic effects identical to those reported after implantation of other materials [59,60]. Since the extent to which the released elements are transported to other organs where their liberation might cause severe damage [61], is not known either, we are of the opinion that biomaterials intended for clinical application should be extremely pure. Trace element accumulation studies to assess this purity should therefore be part of the evaluation of newly developed biomaterials.

Future work

The results of the studies reported in this thesis show that a number of phenomena occur when macrophages are exposed to poorly digestible materials. Especially effects occurring after contact with asbestos fibers and biomaterials, for example labyrinth formation and the formation of extracellular deposits, are assumed to be general phenomena related to macrophage/material interactions. The finding that such deposits were seen more frequently in the granulomas formed after exposure to crocidolite asbestos or tri-calcium phosphate than in those occurring after exposure to hydroxyapatite, is considered to be of great importance. Because the formation of these structures cannot be related to biodegradability, it seems probable that the described formation of extracellular deposits is also related to interface characteristics of the materials studied. Because processes occurring at the biomaterial/tissue interface are considered to be of crucial importance for the functioning of the biomaterial, the meaning of the extracellular deposits in relation to material characteristics will be studied in more detail in the near future.

It would seem useful, too, to include studies on labyrinth formation, iron accumulation, and trace-element accumulation in biocompatibility studies. The inclusion of such parameters in general biocompatibility tests might help to avoid the occurrence of unwanted and unexpected phenomena at the tissue/biomaterial interface.

Application of the two models used in the investigations described in this thesis, i.e., the animal model with intraperitoneal injection of materials and the in vitro model, facilitates the investigation of the systemic and pure

wound reaction.

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SUMMARY

Asbestos is a fibrous mineral with extremely fine filaments which are almost impervious to the effects of chemicals and temperature. These characteristics make asbestos suitable for many industrial applications, and it is widely used. During the mining and processing of asbestos and the production of goods containing asbestos, the fibers can occur in the air as dust and if they are inhaled will remain in the lungs. The specific characteristics of asbestos mean that it is not susceptible to biodegradation, and the presence of asbestos fibers in the lungs can lead to a chronic tissue reaction.

The exposure of lung tissue to asbestos dust can give rise to serious diseases such as asbestos-induced pneumoconiosis as well as malignant tumors among which mesothelioma is the most serious. Furthermore, phenomena whith less serious effects. The latter are exemplified by the development of asbestos bodies, which are composed of an asbestos fiber around which the host forms an organic coat with a high concentration of iron and sometimes of calcium and phosphorus as well. Although many investigators have attempted to explain the meaning of asbestos bodies and although it is suggested by these authors that macrophages play a role in their genesis, the exact mechanism underlying their formation is not yet understood.

The studies reported in this thesis made use of an animal model to collect more information about the processes responsible for the occurrence of asbestos bodies and in particular about the role of macrophages in this process. It was also hoped that the results would indicate whether the observed phenomena only occur in the presence of asbestos fibers or represent a general reaction to exposure to relatively indigestible substances.

The mechanism underlying the formation of asbestos bodies can be investigated by studying the process in the lungs of animals which have inhaled the fibers, but this procedure, used by some authors, has the drawback that the animals inhale other kinds of dust particle at the same time. However, it is known that asbestos bodies are sometimes present in sputum and that they can be isolated from the lungs by lavage with physiological solution of saline. In view of these findings, we assumed that free pulmonary macrophages are capable of forming asbestos bodies.

The occurrence of free macrophages in the peritoneal cavity and the knowledge that dust particles cannot reach this cavity led us to take the peritoneal cavity as compartment for our investigations. Because, as already