J. Anat. (1994) 185, pp. 471–479, with 7 figures Printed in Great Britain

Experimental modulation of the reactivity of pleural milky spots (Kampmeier's foci) by Freund's adjuvants, betamethasone and mycobacterial infection

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(*Accepted 7 April 1994*)

ABSTRACT

We studied the response of milky spots in the parietal pleura of the rat and mouse to intrapleural instillation of immunomodulatory agents such as complete or incomplete Freund's adjuvants and betamethasone, and also to infection by mycobacteria (*M. avium*). Both incomplete (mineral oil) and complete (mineral oil plus dead mycobacteria) adjuvants, as well as *M. avium* infection, induced a striking increase in the size and cellularity of the pleural milky spots whereas betamethasone caused a slight atrophy. The extensive inflammatory infiltrates observed after adjuvant injection differed between milky spots reactive to complete and incomplete Freund's adjuvants. Fifteen days after adjuvant administration, the pleural milky spots of rats were still enlarged and hypercellular but differences were noted in the size of milky spots of the pleura between the 2 adjuvant treatments: animals submitted to injection of complete Freund's adjuvant showed an increase in the size of milky spots from d 3 to d 15, while the size of milky spots of the incomplete Freund's adjuvant treated group showed a decrease in size from d 3 to d 15. The milky spots at d 15 were well organised: reticulin fibres permeated the whole area of the milky spot and the different cell types were evenly distributed. Histiocytes, which were previously confined to the inner layer, were now the main cell type in all areas of milky spots. A moderate number of mast cells and a few eosinophils were also seen. Complete Freund's adjuvant caused the formation of granulomas in the milky spots, a change that was not detected in animals treated with incomplete adjuvant. The enlarged pleural milky spots that were found in *M. avium*-infected mice showed mononuclear cells that contained numerous mycobacteria revealed by staining with the Ziehl–Neelsen method. Our data indicate that milky spots are areas of the parietal pleura with an elective role in the mounting of local inflammatory and immunological reactions of the pleural space. Milky spots may therefore be considered as important defence structures of the pleural membranes, reacting in accordance with the nature of the stimuli reaching the pleural space.

Key words: Rat; mouse; immunomodulation; macrophages; fibrosis; reticulin fibres.

INTRODUCTION

The milky spots (MS) of the pleural space, also known as Kampmeier's foci, were first described by von Recklinghausen in 1863 in the rabbit, and their presence was documented later in the pleural leaflets of several animals (Maximow, 1927; Mixter, 1941). Identical structures have been found in other locations, for instance in the human greater omentum (Seifert, 1921). In 1901, Marchand first suggested that these MS might have a phagocytic function; these phagocytic capacities were confirmed subsequently in other studies using different particles introduced into the pleural space (Webb, 1931; Kanazawa et al. 1979; Shimotsuma et al. 1991; Pereira & Grande, 1992).

In the pleura the MS are confined to the parietal leaflet. In laboratory animals the MS are particularly numerous in the retrocardiac pleural folds, an area of the parietal pleura that extends between the pericardium and the diaphragm. These pleural areas are

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the same site as where we have recently found accumulations of particles injected in the pleural space of dogs (Pereira & Grande, 1992). In man the retrocardiac pleural folds do not exist and MS are found in the pleura that is located over the oesophagus and aorta (Kampmeier, 1928). This different positioning of MS in man is probably related to the rotation to the left of the heart during fetal life. The human newborn also presents MS in the pleural adipose organs located in the parietal pleura (Aharinejad et al. 1992).

Based on findings in the omentum, it was proposed that MS may have the function of an intestinal thymus (Koten & Otter, 1992), although the MS were also considered to be no more than rudimentary regional lymph nodes (Beelen, 1992; Shimotsuma et al. 1992). So far, little attention has been paid to the study of functional features of the MS of the pleura despite their postulated role in the protection of the mediastinum from noxious agents reaching the pleural space (Cooray, 1949). Their presence was considered to be associated with the very low frequency of mediastinitis detected in large series of autopsies when compared with the frequency of pleural diseases (Cooray, 1949).

The purpose of the current experimental study was to investigate the dynamics of MS of the pleural space, namely the response of these structures to immunomodulatory stimuli and infectious agents reaching the pleural space. Accordingly, in this work we have investigated the structural changes of MS of the rat and mouse pleura in response to the instillation of well known immunomodulators and to local infection with mycobacteria. For that we have studied by light and electron microscopy the structural changes of MS of the rat pleura in response to the intrapleural instillation of an immunosuppressive drug (betamethasone) and of adjuvants that cause strong B cell (incomplete Freund's adjuvant) or T cell (complete Freund's adjuvant) responses, and also to intrapleural injection of *M. avium* bacilli, an infectious agent that is often isolated from pleural exudates of AIDS patients (Hawkins et al. 1986; Contreras et al. 1988; Collins, 1989; Modilevsky et al. 1989; Prince et al. 1989; Tsang et al. 1992).

MATERIALS AND METHODS

Animals

Forty-five Wistar rats and 18 CD1 mice were used in this study. Wistar rats and CD1 mice were obtained from a local breeder (Gulbenkian Institute of Science, Oeiras). The animals were kept under standard housing conditions and had unrestricted access to food (commercial chow) and water.

Experimental protocols

Adjuvant and betamethasone treatment. The rats were divided in 4 groups of 10 animals each and an additional control group of 5 animals was also employed. Each of the 4 groups of 10 rats were injected in the right pleural space through the 4th intercostal space, at the level of the anterior axillary line, either with 100 µl of complete Freund's adjuvant (85% of paraffin oil and 15% of mannide mono-oleate containing 1 mg/ml of *Mycobacterium tuberculosis*, H37Ra, ATCC 25177, Sigma, F-4258), or incomplete Freund's adjuvant (85% of paraffin oil and 15% of mannide mono-oleate, Sigma, F-5506), or betamethasone (100 µg) or physiological saline (control). The additional control group of 5 animals was left untreated. The rats were killed 3 (72 h) and 15 d after the injection in the pleural space (5 animals in each timing). The retrocardiac fold of the pleura was excised, fixed and processed for light microscopy and transmission electron microscopy.

Infection with mycobacteria. CD1 mice were used to study the alterations in the structure of pleural milky spots induced by mycobacterial infection. The 18 mice were divided into 6 groups of 3 animals. Animals from 5 of these groups were infected by intrapleural injection of 0.1 ml of saline containing 10^7 viable *Mycobacterium avium* bacilli (ATCC 25291 strain; serotype 2). The 6th group of mice was used as control. The different groups of mice were killed after 3 h, and 1, 3, 7 and 21 d of infection.

Light microscopy

Whole retrocardiac pleural folds were fixed in toto in 10% formaldehyde and embedded in paraffin wax. Serial 5 µm sections were obtained and stained by Giemsa, periodic acid-Schiff (PAS), MSB (Martius-Scarlet-Blue) and Manuel's method for reticulin (Prophet et al. 1992). The sections were observed in a Zeiss Axioplan microscope. The sections of pleural folds from mice infected with *M. avium* were stained by the Ziehl–Neelsen method to visualise the bacilli.

Fixation and processing for electron microscopy

The pleural folds were fixed in 0.1 M cacodylate buffer containing 4% formaldehyde–1.25% glutaraldehyde–10 mM calcium chloride (Silva, 1984) for 24 h at room temperature. In order to help identify and

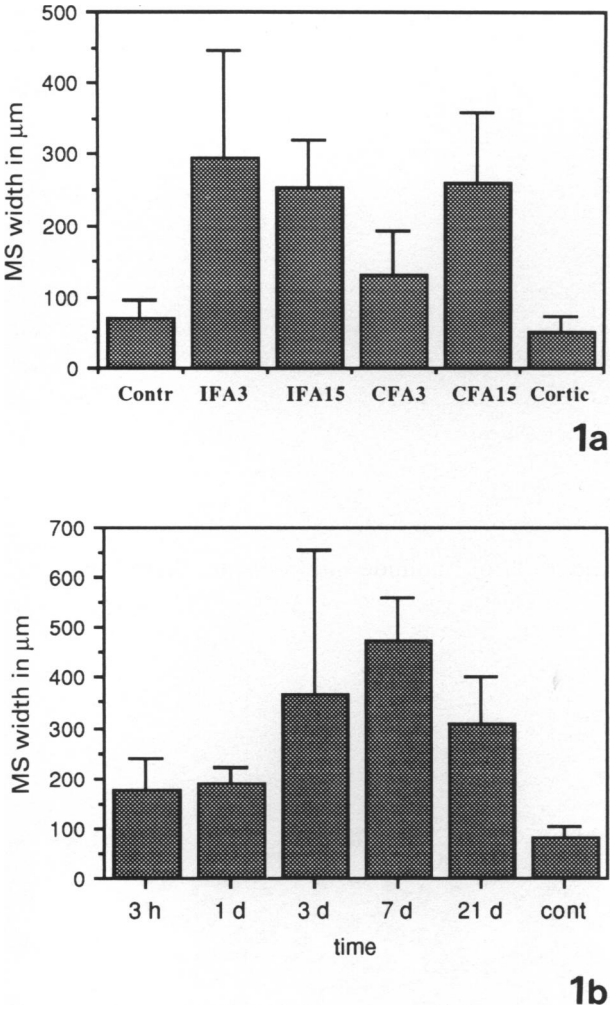

Fig. 1. (*a*) Effect of intrapleural instillation of adjuvants (complete Freund's adjuvant, CFA, and incomplete Freund's adjuvant, IFA) and corticosteroid (betamethasone) on the width of milky spots of the rat retrocardiac pleural folds. The values for milky spot width are given as means ± S.D. The treated rats were killed 3 (72 h) and 15 d after IFA and CFA injection and 3 d (72 h) after corticosteroid instillation. The values measured after 3 d of IFA treatment and after 3 and 15 d of CFA injection were significantly higher ($P < 0.01$) than control values. (*b*) Changes in the width of milky spots (MS) of the mouse rectrocardiac pleural folds in response to infection of the pleural space with 10^7 viable bacilli of *Mycobacterium avium*. The values for MS width are represented as means ± S.D. The different groups of animals were killed up to 21 d after the mycobacterial inoculation. All groups of infected mice showed a statistically significant increase ($P < 0.01$) in the width of MS when compared with control mice (cont). In the infected groups, the values for MS width observed in rats after 7 and 21 d of infection were significantly higher ($P < 0.01$) than the equivalent values measured after 3 h and 1 d of infection.

excise the small patches of MS out of the retrocardiac pleural folds, we added 0.5% methylene blue stain to the fixative solution. The samples were washed in cacodylate buffer and distilled water, and postfixed in a ferrocyanide-reduced osmium solution made up of 0.1% potassium ferrocyanide and 1% osmium tetroxide in distilled water (Águas, 1982; Neiss, 1984). The specimens were washed in water, stained in toto in 1% uranyl acetate (Silva, 1973), dehydrated in graded ethanols, and embedded in Epon. Some excised aldehyde-fixed MS were postfixed in a routine (unreduced) osmium tetroxide solution (1% osmium tetroxide–10 mM calcium chloride) and embedded in the hydrophilic resin LR White (London Resin Co. Ltd) or in Epon. All resin-embedded specimens were sectioned in an LKB ultramicrotome and thin sections stained with uranyl acetate (Silva, 1973) and lead citrate (Reynolds, 1963). The preparations were viewed in a JEOL electron microscope.

Quantification of milky spot size

We have determined the average width of MS in paraffin sections of pleural folds from the different experimental groups of rats and mice. For that we chose sections taken perpendicular to the MS surface and we measured at random the width of 3 MS per animal in at least 3 animals of each group. Statistical comparison of the quantitative values obtained was made using the Student's t test. The numerical data obtained in the different groups of animals are given in Figure 1.

RESULTS

Pleural MS of untreated rats and mice

In the control groups of rats and mice (untreated and saline instilled animals) the MS were composed of small aggregates of cells surrounding a vascular core and lined by mesothelial cells of the parietal pleural leaflet. The cell population of the MS of the control groups was composed mainly of histiocytes, plasma cells, mast cells and lymphocytes. They were located between the mesothelial layer of the parietal pleura and the adipose tissue (Fig. 2).

Pleural MS of rats treated with incomplete Freund's adjuvant

The incomplete Freund's adjuvant (IFA) treatment caused hyperplasia of the mesothelial layer that covered the MS. This reactive hyperplasia changed the morphology of the epithelium from a simple into a stratified organisation. The phenomenon was more evident at d 3 (72 h) than at d 15 after the treatment. Interestingly, at d 15, we also observed detachment of the mesothelial layer. The detachment was peculiarly confined to the MS areas.

At both time points after the IFA injection there was a striking enhancement in the size of the MS—which measured 296 ± 150 µm at d 3 and 252 ± 69 µm

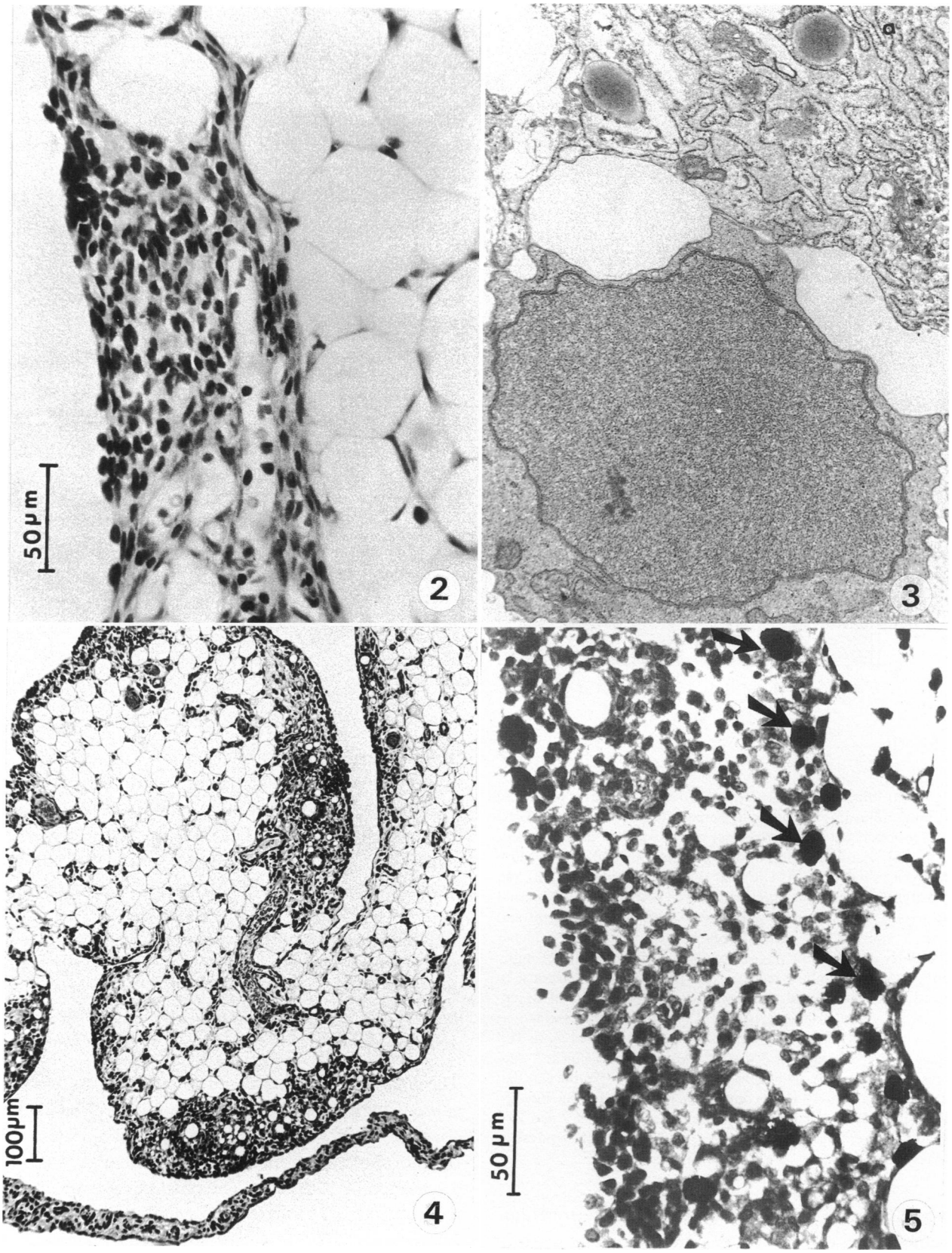

Fig. 2. Light micrograph of a pleural milky spot from an untreated rat. This structure is made up of nests of mononuclear cells located in the submesothelial layer of the parietal pleura. Martius Scarlet Blue stain, ×400.

Fig. 3. Thin-section electron micrograph illustrating exuberant cell membrane interactions between milky spot cells from a rat treated for 3 d with incomplete Freund's adjuvant. The cell at the upper half of the figure is a plasma cell showing the characteristic enlarged cisternae of rough endoplasmic reticulum. Bar, 1 µm.

at d 15, whereas the width of control MS was 72 ± 25 μm—and of its cellularity. This was the result of the attraction of inflammatory cells and also of the local response of the structural elements of MS. At d 3, we observed inflammatory infiltrates made up mostly of histiocytes and lymphocytes. These infiltrates also contained neutrophils, eosinophils, mast and plasma cells. PAS staining revealed that the plasma cells showed no zonation in the MS. An increase in reticulin fibres was observed in the deeper areas of the MS.

On thin section electron microscopy, the plasma cells presented a characteristic hypertrophy of the rough endoplasmic reticulum and of the Golgi apparatus. Interestingly, there were extensive cell membrane interactions between the plasma cells and the connective tissue cells (Fig. 3). Some of the stromal cells appeared to be binucleated, thus suggesting cell division.

At d 15 after the IFA treatment, the MS contained a large number of macrophages but no granulocytes. Reticulin fibres were seen scattered throughout the whole width of MS. No granulomas were found in these samples.

Pleural MS of rats treated with complete Freund's adjuvant

The animals treated with complete Freund's adjuvant (CFA) showed hyperplasia of the mesothelial cell layer and a striking increase in the size and cellularity of the MS that caused fusion of multiple individual MS, making an almost continuous covering of the surface of the pleural folds. This change was similar to what was observed in the IFA injected rats (Fig. 4). The marked inflammatory infiltrates that were observed at d 3 contained numerous histiocytes and mast cells. Mast cells were confined to the basal layer of the MS (Fig. 5). Reticulin fibres were identified only in the deeper areas of the MS. In contrast to the IFA group, plasma cells were rarely seen in the MS of the CFA-treated rats. Eosinophils were present in the CFA treated rats although in fewer numbers than in the IFA group. Electron microscopy observations of the MS at d 3 showed that mast cells and eosinophils were embedded in an unorganised connective tissue network that corresponded to the reticulin fibrosis seen by light microscopy. The connective tissue showed very irregular profiles that involved marked cell processes and indentations. Some of these cells appeared to be binucleated. Dead cells were detected either free in the stroma or inside macrophages.

At d 15 after the CFA administration, the MS remained hypercellular and showed the formation of granulomas (Fig. 6*a*, *b*). These were recognised because of the concentric organisation of cells of epithelioid nature and also because of the presence of a particular arrangement of the reticulin skeleton (Fig. 6*b*). Numerous multinucleated giant cells were also seen scattered in the MS. Blood capillaries, that were scarce at d 3, were now conspicuously seen in all of the MS, as well as the reticulin skeleton that at d 3 was confined to the basal layer. At d 15 we also found a rich vasculature associated with the blood supply of the hypercellular MS.

Our quantitative analysis of the MS width (Fig. 1*a*) showed that CFA induced an enhancement in their size that was particularly evident 15 d after the adjuvant injection. At this time the values for MS width was 259 ± 100 μm which are significantly higher ($P < 0.01$) than those of controls (72 ± 25 μm). Interestingly, at d 3 the width of MS from CFA treated rats (130 ± 63 μm) was significantly lower ($P < 0.01$) than those of IFA injected animals (296 ± 150 μm).

Pleural MS of betamethasone treated rats

Corticosteroid treatment led to a general hypocellularity of the pleural MS. The MS were already difficult to identify with the stereomicroscope that was used to collect the samples. This is because they contained only small agregates of cells located underneath the mesothelial layer. The anti-inflammatory treatment also led to a marked change in the stroma of the retrocardiac pleural folds expressed by a differentiation of the adipose tissue from a multiloculated cell population into an uniloculated one. On average the values of width of MS of this group of rats were lower (50 ± 24 μm) than those of controls (72 ± 25 μm); the 2 groups were not, however, statistically different.

Pleural MS of mice submitted to intrapleural infection with M. avium

We found that the injection of mycobacteria into the pleural space of mice led to a significant increase in the

Fig. 4. Low magnification of an area of the retrocardiac pleural fold of a rat treated with incomplete Freund's adjuvant (IFA). The IFA injection caused fusion of adjacent milky spots, resulting in a continuous layer of lymphoid tissue. × 100.

Fig. 5. Light micrograph of pleural milky spot (MS) from a rat treated with complete Freund's adjuvant killed 3 d after the intrapleural instillation of adjuvant. The hypercellular MS shown in this micrograph (Giemsa staining) illustrates the peculiar accumulation of mast cells (arrows) in the basal area of the MS. × 400.

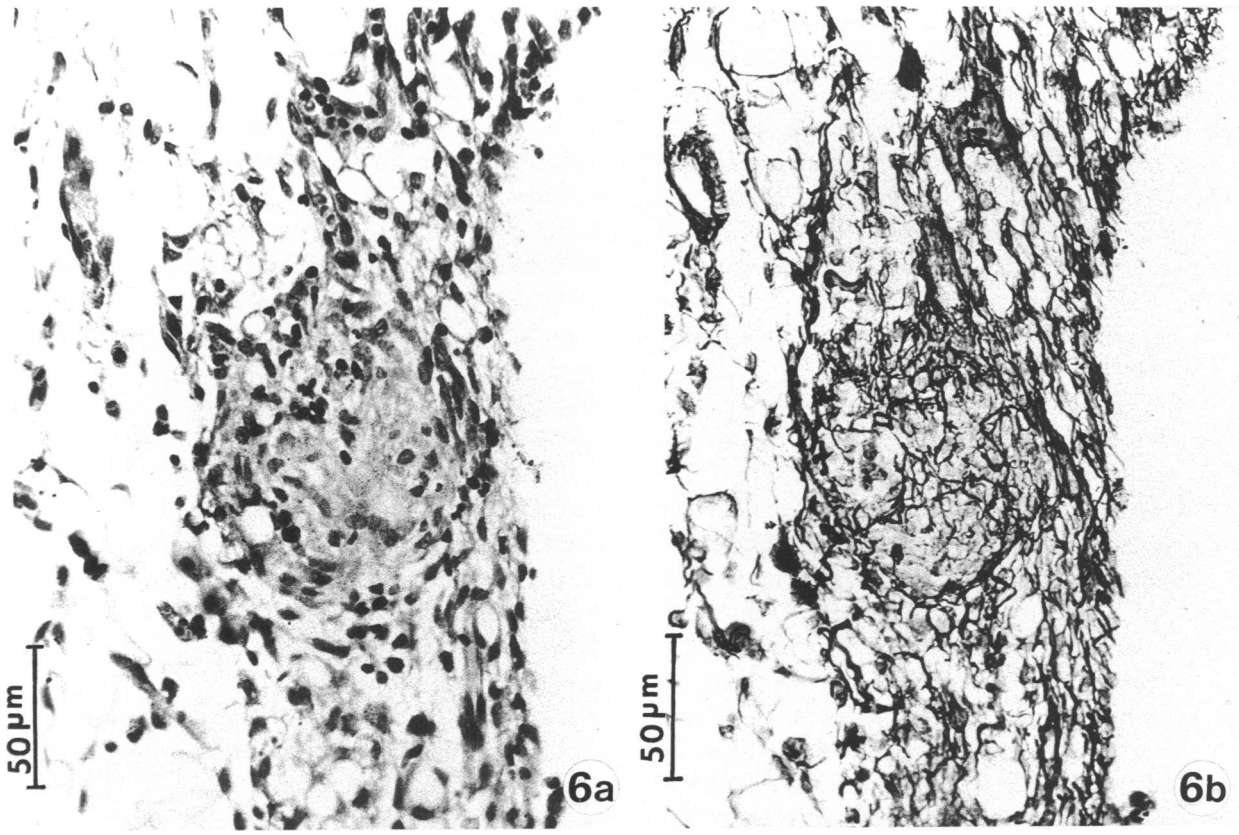

Fig. 6. Light micrographs of pleural milky spots from rats treated with complete Freund's adjuvant (CFA) 15 d before killing. (*a*) A granuloma is present in the centre of the figure. MSB stain, ×400. (*b*) Reticulin fibres are seen permeating the whole width of the granuloma. Manuel's stain, ×400.

cellularity of MS as illustrated in the graph of Figure 1*b*, giving our quantative data on the width of the MS. The figure shows that the MS increase up to d 7 of *M. avium* infection. At this time the values for MS width are 474 ± 86 µm which are significantly higher ($P < 0.001$) than those of controls (82 ± 23 µm). The marked enhancement in the size of MS caused by the infection is documented in Figure 7*a*, corresponding to 7 d of infection, where mononuclear cells become the predominant cell type of the MS. The MS had an important participation in the removal of the mycobacteria present in the pleural space since we observed numerous Ziehl–Neelsen positive bodies inside macrophages of the MS, shown in Figure 7*b*.

DISCUSSION

We have investigated here the response of the milky spots (MS) of the retrocardiac folds of the pleura (also known as Kampmeier's foci) to different kinds of immunomodulatory substances and also to an experimental infection of the pleural space that was produced by the injection of *M. avium*. The pleura is a serosal membrane that contains lymphoepithelial formations, known as MS, which are scattered in its parietal leaflet. In man, these bodies were demonstrated in the human mediastinal pleura by Kampmeier (1928) and in the chest wall of newborns (Aharinejad et al. 1990). Their role in the overall physiopathology of the pleura and of pleural space is often neglected or misunderstood (Antony et al. 1992).

A number of recent studies were devoted to the cellular composition of the omental and mediastinal MS (Beelen et al. 1980; Shimotsuma, 1991; Inoue & Otsuki, 1992). Little attention, however, has been focused on the dynamics of the pleural MS under different experimental conditions. We have therefore investigated the response of these MS to the presence in the pleural cavity of well characterised immunomodulators. Using the rat as our animal model, we performed intrapleural instillations of incomplete Freund's adjuvant (IFA), of complete Freund's adjuvant (CFA) and of a corticosteroid (betamethasone). These substances were chosen because their action on the immune system has been well established in a number of previous studies (Herbert, 1968; Warren et al. 1986; Woodard, 1990). IFA is known to cause a strong inflammatory reaction that is associated with a nonspecific stimulation of humoral

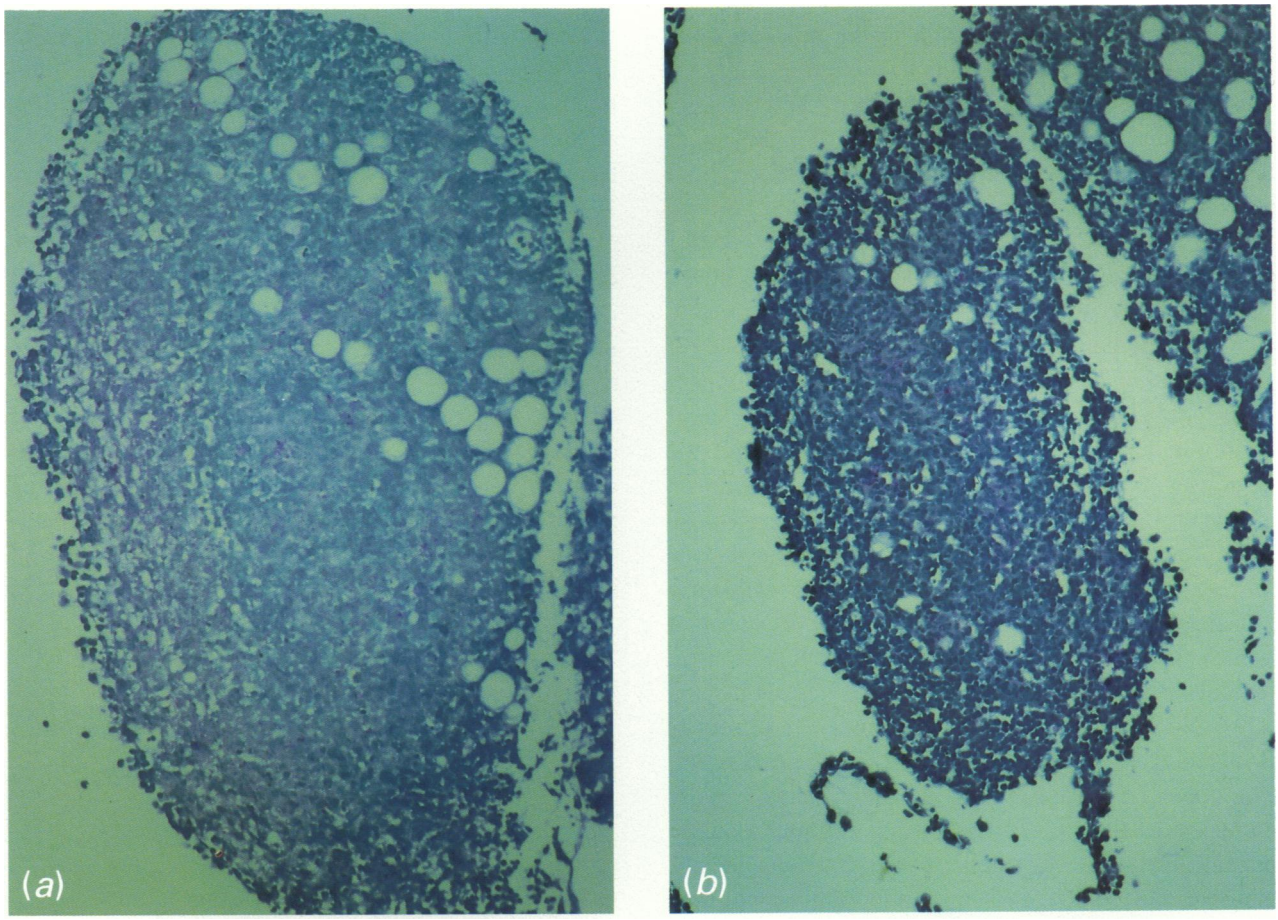

Fig. 7. Light micrographs of paraffin sections of pleural milky spots (MS) of mice submitted to mycobacterial (*M. avium*) infection of the pleural space. To show the distribution of the mycobacteria in the MS, the sections were stained by the Ziehl–Neelsen method followed by toluidine blue. (*a*) High magnification of an enlarged MS from a mouse after 7 d of mycobacterial infection, showing that mycobacteria have been ingested by macrophages of the MS. (*b*) General view of the same section. (*a*) × 200; (*b*) × 100.

immunity, whereas the inflammation produced by CFA induces a marked cellular immune reaction due to the mycobacterial antigens contained in the adjuvant (Bomford, 1980). In contrast, the corticosteroid betamethasone causes suppression of local immune reactions (Salman & Rose, 1990).

We have quantified the effect of intrapleural administration of these substances in pleural MS. This quantification was done by measuring the width of MS instead of counting individual MS. The latter type of quantification was not possible in the adjuvant stimulated animals since the increase in size of MS led to fusion of individual MS into a single layer on the retrocardiac pleural folds. We thus performed quantitative evaluation of the MS width which revealed striking differences in the effect of the 3 immunomodulatory agents on the size of MS. As expected from an immunocompetent structure, the reaction of MS to CFA and IFA was not identical. CFA induced a sustained reaction without reduction in the size of MS at d 15, whereas IFA caused an acute reaction that was expressed by a greater width of the MS at d 3 than at d 15. Also as expected, the injection of the immunosuppressive betamethasone resulted in a reduction of the average MS size.

In our experiments, we found on the one hand that the IFA treatment led to the presence of a significant number of plasma cells, which were not observed in CFA samples, and on the other that numerous granulomas were present in MS after the injection of CFA in the pleural space. The high reactivity of the MS to either of the 2 inflammatory adjuvants did not involve the formation of cell clusters, in contrast with what has been reported for omental MS (Shimotsuma et al. 1992). Mast cells had, however, a particular topography in the CFA-induced hypercellular MS since they were located in the basal portion of the MS, an area that displayed a rich reticulin skeleton. These different locations of mast cells in the IFA and CFA treated rats may be related to different pathways of mast cell activation: an antigenic pathway that involves the preliminary binding of immunoglobulin E, and a nonantigenic pathway described in the rat peritoneal mast cells (Landry et al. 1992). The methods

used in our work did not allow the separation of different mast cell populations in our samples.

Interestingly, connective tissue fibres were initially absent from the areas of MS growth but they were later seen penetrating the whole width of the lymphoid patch. These observations suggest that, in the long term, a localized area of reticulin fibrosis may persist as an indication of a previous local immune reaction.

Because the intrapleural injection with CFA and IFA may be considered too strong a stimulation of this serosal space that would be difficult to compare with pathological processes affecting the pleural membrane, we have decided also to investigate the response of MS to infection, a relatively common insult of the pleural space. To study infection we chose to inject mycobacteria, which are one of the more common infectious agents isolated from pleural effusions. The mice were inoculated with *M. avium*, a species that may cause severe pulmonary infections in patients with AIDS (Hawkins et al. 1986; Contreras et al. 1988; Collins, 1989; Modilevsky et al. 1989; Prince et al. 1989; Tsang et al. 1992). In these infection experiments we found that the pleural MS rapidly increased their width due to an enhancement in mononuclear cells. The data, therefore, confirmed the result obtained with the immunomodulators and thus showed that even in an experimental model of a clinical situation, the pleural MS react as immunocompetent structures. In addition, the injection of the microbial particles allowed us to verify that pleural MS play an important role in the clearance and phagocytosis of infectious particles that reach the pleural space.

The investigation of the immunophysiology of pleural MS is a particularly timely endeavour because of the recent publication of contradictory views on the nature of MS. Koten & Otter, (1992) proposed that MS may function as an intestinal thymus, whereas Shimotsuma et al. (1992) and Beelen (1992) saw no reason to consider MS as more than rudimentary regional lymph nodes. All these previous interpretations were based on results using MS in the omentum.

A recent study by us on the mechanisms of clearance of the pleural cavity of the dog has revealed a crucial role for the retrocardiac pleural fold MS in the removal of particles present in this serosal space. We found large quantities of tungsten particles inside MS macrophages after the instillation of a suspension of tungsten powder in the pleural space (Pereira & Grande, 1992). This was confirmed in the present study since we found numerous *M. avium* bacilli ingested by mononuclear cells located in MS. Taken together with our current findings, namely the absence of a clear cell cluster zonation of the MS, our data make us favour the previous proposal of Shimotsuma & Simpson-Morgan (1991) and Beelen (1992) who have sided against a thymus-like function for MS.

In conclusion, the structural features that we have observed in the reactive MS indicate they are capable of mounting both a strong humoral (as in IFA-treated rats) or cellular (as in CFA-treated rats) immune response, as well as removing by phagocytosis microbial particles that enter the pleural space. These data offer good evidence that MS work as a fully capable lymphoepithelioid organ reacting in accordance with the nature of the stimuli present in the pleural space and may therefore protect the mediastinum from noxious agents coming from the serosal space.

ACKNOWLEDGEMENTS

The technical assistance of António Moreno, A. Costa e Silva, Emanuel Monteiro, José Aurélio Mexedo and Duarte Monteiro is gratefully acknowledged. We also thank the Service of Experimental Surgery (ICBAS) for technical help. This investigation was supported by grants from the Portuguese Research Council (JNICT).

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