INTERNATIONAL JOURNAL OF LEPROSY Volume 39, Number 2
Printed in the U.S.A.

E. EXPERIMENTAL IMMUNOLOGY

Histologic Changes Evoked in Mice By Freund's Adjuvant, Complete With *M. leprae*

D. G. Jamison [1]

A previous investigation into the histologic changes evoked in mice by incomplete Freund's adjuvant established two main points, namely that the changes were demonstrable in the central as well as in the distal lymphnodes draining the injected footpad, and that the lymphatic vessels in the vicinity of the popliteal nodes showed considerable histologic changes. These reactions (particularly in the lymphatic vessels) may throw some light on the mode of action of Freund's adjuvant at least at the morphologic level. The local effects of this material are familiar, but it has long been recognized that many other effects of Freund's adjuvant are due to its spread to more distant tissues [Freund (3), White (6)]. Some years before Freund's adjuvant came into use, it was established that mineral oils facilitated the spread of killed mycobacteria. Several investigators, Rist (1938), Casals and Freund (1939) and Saenz and Canetti (1940), all implicated lymphatic pathways, but provided no supporting evidence. More recently, some direct evidence of lymphatic involvement by Freund's adjuvant has emerged [Freund and Lipton (4), Dale (2), Newbold (5)], but no histologic studies have been reported.

A comparative study of the histologic reactions evoked by Freund's adjuvants that have been "completed" with killed mycobacteria is now presented. As in the previous investigation, the emphasis has been on the development of distant as well as local changes, and particular attention has been paid to the lymphatic vessels. Since the mycobacterial components in Freund's adjuvants are interchangeable (White and Marshall, 1958; Shaw *et al.*, 1964), the effects of two killed mycobacteria have been examined, *Mycobacterium tuberculosis* and *Mycobacterium leprae*. *M. leprae* has apparently not been studied in this context before.

[1] D. G. Jamison, M.A.B.M., School of Anatomy, University of Cambridge, England.

MATERIALS AND METHODS

One hundred female Swiss albino mice were used, aged 8-12 weeks and weighing 15-20 gm. The animals were housed in metal cages and maintained on a pellet diet and water *ad libitum*.

Freund's incomplete adjuvant was obtained from the Difco Laboratories, Detroit, Michigan (Batch no. 468448). It contained Arlacel A (mannide monooleate) 1.5 ml. and Bayol F (paraffin oil) 8.5 ml.

Mycobacterium leprae was isolated from two skin biopsies from a patient with lepromatous leprosy who had received no treatment. The specimens were ground up in 5 ml. normal saline, a knife-point of trypsin (BDH 0.54 Anson units per gm.) was added, and the pH was adjusted to 7.6 with phosphate-saline buffer. The suspensions were incubated at 37°C overnight and the supernatant was then removed and centrifuged at 3000 r.p.m. for 20 minutes. The deposit was washed twice in normal saline, resuspended, and diluted to a standard opacity equivalent to that of a suspension of *M. tuberculosis* containing 1 mgm. of organisms per ml. [Calmette (1)]. The suspension was sterilized by autoclaving.

M. tuberculosis was isolated from a 3-week-old culture of *M. tuberculosis* H_{37} Rv, grown in Dubos medium. The culture was centrifuged at 3000 r.p.m. for 20 minutes and the supernatant was discarded. The deposit was washed in normal saline,

centrifuged twice, and, after the third centrifugation, resuspended in saline and diluted to a standard opacity in the same way as the preparations of *M. leprae*, described above. The suspension was sterilized by autoclaving.

The suspensions of mycobacteria were diluted with an equal quantity of saline or Freund's imcomplete adjuvant before injection. The mixture with Freund's incomplete adjuvant was emulsified by drawing it up with a syringe and expelling it again several times.

The mice were divided into six experimental groups. The symbols L+F indicate killed *M. leprae* and incomplete Freund's adjuvant.

L	indicates killed *M. leprae* in saline.
T+F	indicates killed *M. tuberculosis* and Freund's incomplete adjuvant.
T	indicates killed *M. tuberculosis* in saline.
F	Freund's incomplete adjuvant.
S	saline alone.

Two mice from each group were killed at six hours, 24 hours, 48 hours, five days, ten days, 15 days, 20 days, 30 days, 40 days and 50 days. All injections were made into the left hind footpad with an Agla micrometer syringe.

At necropsy, the following tissues were removed and fixed in Bouin's solution: skin and subcutaneous tissues of the injected footpad, ipsilateral and contralateral popliteal and lumbar lymphnodes, and fibromuscular tissues from the ipsilateral popliteal fosa. Five-μm parraffin sections were stained with hematoxylin and eosin and, where necessary, with hematoxylin and Van Gieson, Giemsa's stain, and Ziehl-Neelsen's stain, and by Masson's trichrome method and Sweets and Davenport's modified silver impregnation method.

The histologic and bacteriologic results were assessed independently and were not collated until the end of the experiment.

RESULTS

Effects of one subcutaneous injection of killed *M. leprae* and Freund's incomplete adjuvant (group L+F)

Extensive changes are seen in the footpad and these spread to involve proximal and distal draining lymphnodes and also the lymphatic vessels on the injected side. No abnormalities are seen in the contralateral tissues.

In the footpad a scanty inflammatory infiltrate, in which polymorphs predominate, appears six hours after injection. By 24 hours, this infiltrate is increased in amount and is more pleomorphic in composition. Polymorphs are still numerous, but some of them are already degenerate; lymphocytes and macrophages are present in several areas. The increase in lymphocytes and other mononuclear cells is more marked at 48 hours (Fig. 1) and, by five days, (Fig. 2), the foot presents a characteristic histologic appearance. There are a

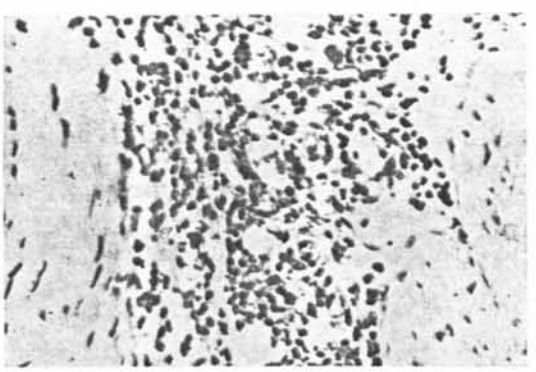

FIG. 1. Group L + F. Footpad. 48 hours. Dense inflammatory infiltrate in which polymorphs predominate. Hematoxylin and eosin × 300.

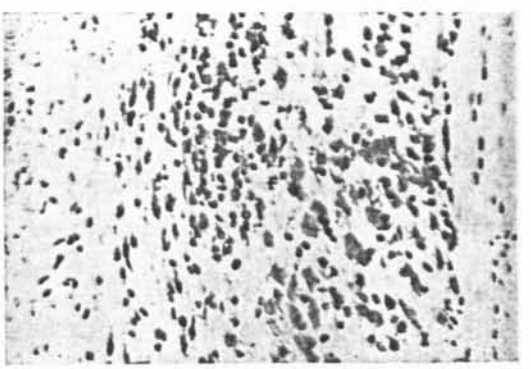

FIG. 2. Group L + F. Footpad. 5 days. An outlying part of the infiltrate in which large pleomorphic mononuclear cells are beginning to appear. HE + 400.

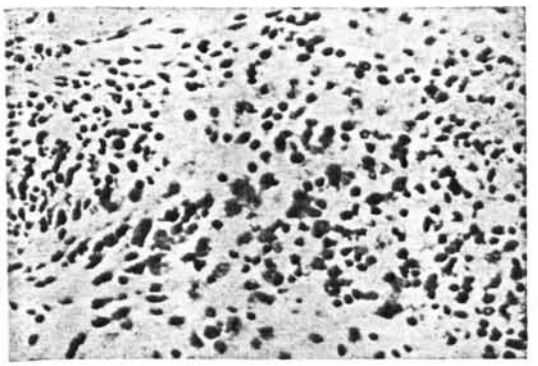

Fig. 3. Group L + F. Footpad. 30 days. Dense sheets of pleomorphic mononuclear cells: some are in mitosis and may contain fat vacuoles. HE × 400.

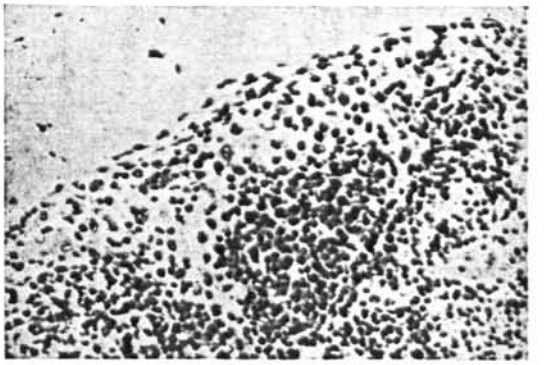

FIG. 4. Group L + F. Popliteal lymph node. 30 days. Large pleomorphic mononuclear cells, some in mitosis in the subcapsular sinus. HE × 400.

few degenerate polymorphs and eosinophils, but mononuclear cells predominate and include small lymphocytes, medium-sized mononuclear cells (several of which contain cytoplasmic fat spaces), and distinctive large mononuclear cells. These cells have a round or oval nucleus, prominent chromatin, a well-developed nuclear membrane and variable amounts of basophilic cytoplasm. Some of the cells are in mitosis and others contain large cytoplasmic vacuoles. The number of large mononuclear cells increases at 10 and 15 days and, at 20 and 30 days, these cells dominate the histologic picture (Fig. 3). In some areas they form dense sheets and their cell membranes are so poorly defined as to suggest a syncytial arrangement. Mitotic figures are still present 30 days after injection. Later the inflammatory reaction declines. Certain negative features of the response may be noted. The infiltrate remains diffuse and there is no localization around specific structures such as nerves. Discrete granulomata are not formed. There is no local necrosis or accumulation of multinucleate giant cells. Plasma cells are rarely seen.

Parallel observations were made on the distribution of *M. leprae* in these lesions. At six hours, the organisms lie free in extracellular spaces. By 24 hours, the organisms are confined to macrophages, which later accumulate around the dilated dermal lymphatics. This localization is apparent five days after the injections and is constant. Mycobacteria are not distributed diffusely throughout the inflammatory infiltrate and the large pleomorphic mononuclear cells do not appear to participate in their phagocytosis.

The popliteal lymph-nodes are histologically normal at six and 24 hours. A mild reaction is apparent at 48 hours: the superficial sinuses become dilated and contain increased numbers of histiocytes, but the pulp remains quiescent. More marked changes involving both sinus and pulp elements are seen at five days. The mononuclear cells in the sinuses are increased in number and are somewhat pleomorphic; several large, immature-looking cells resembling those described in the footpad are present and a few of them are in mitosis; others contain cytoplasmic vacuoles. In addition, there is follicular hyperplasia. These changes persist until 30 days (Fig. 4). Mycobacteria lie free in the peripheral sinuses at six and 24 hours, but at 48 hours they are first seen in the cytoplasm of macrophages; thereafter all mycobacteria are intracellular. They remain localized to the sinus histiocytes; isolated mycobacteria were seen in macrophages in the substance of the pulp in one instance only, at 40 days.

In the popliteal connective tissues, perilymphangitis is apparent as early as 24 hours after injection. The perimysial vessels

are first involved and the infiltrate resembles that present in the footpad. Perineural, as well as perimysial, vessels are affected at 48 hours and the degree of involvement subsequently increases. Some lymph channels appear to be totally obstructed; others are partially occluded and also widely dilated. The distribution of the infiltrate is characteristically eccentric in relation to the lumen of the vessel and there appears to be swelling and proliferation of lymphatic endothelium in addition to infiltration by inflammatory cells. After the first few days, the infiltrate is composed mainly of mononuclear cells, but the distinctive large pleomorphic mononuclear cells (seen in the feet) are not identified around the dilated lymphatic vessels (Fig. 5). These changes are less obvious after 30 days and, as they decline, alterations in the lymphatic endothelium become more apparent; in some sections, the endothelial cells are swollen and contain large cytoplasmic vacuoles (Fig. 6). Small numbers of mycobacteria are present at six hours and at five, 15 and 30 days. At six hours, they are seen free in a presumptive lymphatic vessel running close to a nerve, but no cellular response is apparent. In the later phases, mycobacteria are encountered mainly within mononuclear cells infiltrating the walls of lymphatic vessels, but their presence in the popliteal lymphatics is less constant than in more distal structures.

Lumbar lymphnodes are normal at six, 24 and 48 hours, and at five and ten days. At 15 days, there are reactive changes in the sinuses with foamy macrophages similar to those described in the popliteal nodes; there is also some follicular activity. These changes persist and slight follicular hyperplasia is still apparent at 50 days, although the sinus reactions are reduced by this time. The presence of mycobacteria is more variable than in the popliteal nodes although their distribution is similar. Small numbers of mycobacteria are seen at five, 15 and 30 days in sinus histiocytes.

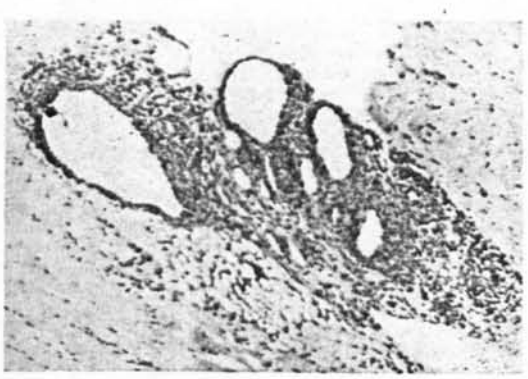

FIG. 5. Group L + F. Connective tissues from popliteal fossa. 15 days. Dilated lymphatic vessels, surrounded by dense inflammatory infiltrate. HE × 130.

FIG. 6. Group L + F. Connective tissues from popliteal fossa. 50 days. Perineural lymphatic vessel showing perilymphangitis infiltrate and degenerative changes in the lining lymphatic endothelium. HE × 255.

Effect of one subcutaneous injection of killed *M. leprae* (group L)

In contrast to the reactions encountered in mice injected with *M. leprae* and Freund's incomplete adjuvant (group L+F), no histologic changes are found in animals that received *M. leprae* alone. No inflammatory infiltrates are seen in the feet in many blocks cut at several levels, or in and around lymphatic vessels in the popliteal connective tissues; the popliteal and lumbar lymphnodes remain normal throughout the experiment.

Effects of one subcutaneous injection of killed *M. tuberculosis* and Freund's incomplete adjuvant (group T+F)

The anatomical distribution, time-sequence and morphologic features of the

reactions found in this group are similar to those described in mice given an injection of *M. leprae* and incomplete Freund's adjuvant (group L +F), although the intensity and duration of the reaction is much greater.

Effects of one subcutaneous injection of killed *M. tuberculosis* (group T)

In contrast with previous observations with *M. leprae* (group L), it was found that one injection of killed *M. tuberculosis* induces a histologic reaction. The changes are, however, inconspicuous and confined to the footpad and the popliteal lymphnode.

In the injected footpad a mixed inflammatory response composed mainly of polymorphs is seen at six hours. The proportion of small lymphocytes and mononuclear cells subsequently rises and a few of the large pleomorphic mononuclear cells appear at 48 hours. The reaction is most intense at five days, but declines thereafter. There is minimal cellular infiltration at ten and 15 days, but, from 20 days onward, the footpad is histologically normal.

An equally transient morphologic response is seen in the draining popliteal lymphnodes. These remain normal until five days, when there is a slight increase in the number of sinus histiocytes and some follicular hyperplasia. This is still apparent at ten days, but the histologic appearance is normal by 20 days. The other tissues examined are histologically normal.

Effects of one subcutaneous injection of Freund's incomplete adjuvant (group F)

The histologic changes produced by one injection of Freund's incomplete adjuvant are now described.

In the footpad, the inflammatory exudate resembles that observed in groups L+F at six, 24 and 48 hours. By five days, mononuclear cells are numerous, but large pleomorphic forms are rarely seen. The infiltrate remains predominantly mononuclear and there is no increase in the number of large pleomorphic forms: by day 20, the reaction is declining. The popliteal lymphnodes show an increase in sinus histiocytes at 24 hours. Many of the cells subsequently develop foamy cytoplasm by five days, but these changes have largely receded by day 20. The pulp is inactive throughout the period of observation. Perilymphangitis is apparent in the popliteal connective tissues at 24 hours, but the inflammatory response is more transient and less extensive than in groups L+F and T+F; endothelial changes are not found. Reactions in the lumbar lymphnodes are similar to those observed in the popliteal nodes, but their appearance is delayed and they are usually less intense; by 20 days, there is considerable intracellular and extracellular accumulation of fat in the sinuses, but reactive changes are not seen in the pulp.

Effects of one subcutaneous injection of saline (group S)

The histologic changes induced by 0.02 ml. saline are transient and confined to the footpad. A scanty acute inflammatory infiltrate appears at six hours and is more intense at 24 hours; it begins to decline at 48 hours and by five days the dermis appears normal.

DISCUSSION

Three main conclusions emerge from these experiments. First, Freund's incomplete adjuvant (group F) induced a mononuclear cell reaction in the footpad and in proximal and more central draining lymphnodes, together with a striking lymphangitis. Second, when Freund's incomplete adjuvant was "completed" with killed *M. leprae* or killed *M. tuberculosis* (groups L+F and T+F), either mixture induced similar histologic changes. The morphologic reactions in the footpad, lymphnodes and lymphatic vessels were more intense than those in group F and were sustained for longer periods of time; they were also modified qualitatively since the local histologic response was dominated by large pleomorphic mononuclear cells. There was no apparent difference between the lesions seen in groups T+F and L+F. Third, when killed *M. leprae* or *M. tuberculosis*

were injected alone, histologic reactions either failed to develop or were greatly reduced.

There were marked differences between the experimental groups in the distribution of large pleomorphic mononuclear cells at the site of injection. Freund's incomplete adjuvant, alone, evoked small numbers of these cells, but the response was greatly augmented if killed *M. tuberculosis* or *M. leprae* were added. A few pleomorphic mononuclear cells were seen when killed *M. tuberculosis* were injected alone, but none appeared in response to killed *M. leprae*. This difference between killed *M. tuberculosis* and killed *M. leprae* (groups T and L) is difficult to explain, particularly as *M. leprae* appeared to be as effective as *M. tuberculosis* in producing an enhanced response when combined with Freund's incomplete adjuvant. The function of these large mononuclear cells was not apparent; some of them contained intensely vacuolated cytoplasm, suggesting phagocytosis of lipid material, but no intracellular mycobacteria were demonstrated. It is most improbable that they are concerned with antibody formation. Their nature and origin were also uncertain and the non-committal term "pleomorphic mononuclear cells" seems more appropriate than "epithelioid cells," since we believe that they differ from the latter in several respects.

Histologic changes in the lymphnodes were most marked in mice injected with one of Freund's complete adjuvants. In groups T+F and L+F, prominent reactions were seen in both the popliteal and the more central lumbar nodes. Changes in the sinuses occurred in both groups, but there was sustained moderate follicular hyperplasia, unaccompanied by medullary plasmacytosis, in group T+F.

The changes in the popliteal lymphatic vessels provide one explanation for the dissemination of mycobacteria observed in groups L+F and T+F. Such changes are clearly associated with the presence of Freund's incomplete adjuvant in the inoculum. The relation of the inflammatory infiltrates to the lymphatic vessels was often difficult to determine. Although such lesions have been described as perilymphangitis, some of them show clear evidence of inward extension toward the lumen of the vessel, and proliferation of the lining endothelial cells was often seen. The large pleomorphic mononuclear cells did not appear in these infiltrates, but there were many smaller mononuclear cells that contained acid-fast bacilli. Mycobacteria were also demonstrable in endothelial cells lining the lymphatic vessels. It is curious that similar changes were not found in the dermal lymphatics of the foot, a situation much closer to the original inoculum. It is possible that some segments of the lymphatic pathway are more susceptible to damage than others. A difference of this kind may reflect the established variations in fine structure between lymphatics from different regions. Lastly, it is clear that lymphangitis is not a prerequisite for dissemination, since isolated mycobacteria were occasionally seen in distal lymphnodes in groups L and T.

SUMMARY

The morphologic response to Freund's adjuvant completed with killed *M. leprae* has been studied in mice by comparing the histologic reactions to a single 0.02 ml injection of this completed adjuvant, with similar injections of Freund's incomplete adjuvant, saline suspensions of killed *M. leprae* and of saline alone into the left hind footpad. The mice were killed at intervals from six hours to 50 days after injection, and the injected footpad, popliteal and lumbar lymph nodes, and popliteal connective tissues were examined histologically. The local and distant morphologic reactions evoked by Freund's incomplete adjuvant alone were found to be enhanced, sustained, and partially modified by the addition of killed *M. leprae*. In the absence of Freund's incomplete adjuvant killed *M. leprae* produced no local response and there was little evidence of dissemination.

It is suggested that the enhanced spread of *M. leprae* in the presence of Freund's incomplete adjuvant is related to the intense reactive changes that are induced by Freund's incomplete adjuvant in the draining lymphatic vessels.

REFERENCES

1. Calmette, A. L'infection bacillaire et la tuberculose. Masson et Cie, Paris, 1922, p. 248.
2. Dale, M. M. The effect of Freund's adjuvant on lymphatics in the mouse's ear. Brit. J. Exper. Path. **41**(1960) 46.
3. Freund, J. Some aspects of active immunization. Ann. Rev. Microbiol. **1**(1947) 291.
4. Freund, J. and Lipton, M. M. Experimental allergic encephalomyelitis after excision of injection site of antigen-adjuvant emulsion. J. Immunol. **75** (1955) 454.
5. Newbould, B. B. Chemotherapy of arthritis induced in rats by mycobacterial adjuvant. Brit. J. Exper. Path. **45**(1964) 375.
6. White, R. G. Role of adjuvants in the production of delayed hypersensitivity. Brit. Med. Bull. **23**(1967) 39.