Adjuvant Activity of *Mycobacterium leprae*

D. E. S. STEWART-TULL and M. DAVIES

*Department of Microbiology, Alexander Stone Building, University of Glasgow Garscube Estate, Bearsden, Glasgow, Scotland*

Received for publication 8 September 1972

*Mycobacterium leprae* organisms isolated from infected spleen and liver tissue by zonal centrifugation were shown to possess adjuvant activity. Histochemical examination of the footpad macrophage epithelioid granuloma showed that macrophages contained large amounts of hemosiderin after the injection of *M. leprae* and much smaller amounts after the injection of *M. tuberculosis*.

A marked adjuvant activity in both production of circulating antibody and development of a delayed-type hypersensitivity to ovalbumin was found by White et al. (8, 10) by using wax D (mixtures of peptidoglycolipid and glycolipid) from human strains of mycobacteria. Later, Stewart-Tull and White (6) showed that adjuvant-active peptidoglycolipids were present in the organisms of *Mycobacterium phlei* and *M. smegmatis* harvested from 7-day-old cultures. Wax D obtained from bovine strains of mycobacteria did not possess adjuvant activity.

In the present investigation, it was possible to obtain pure preparations of *M. leprae*, and it was decided to determine whether these organisms possessed a similar adjuvant activity.

**MATERIALS AND METHODS**

**Tissue containing *M. leprae***. The liver and spleen were obtained from a Chinese patient with lepromatous leprosy, post mortem. The tissue, which was kept frozen, was obtained from R. J. W. Rees, National Institute for Medical Research, London.

**Separation of leprosy bacilli from infected tissues**. The spleen and liver were disintegrated in a tissue emulsifier designed and manufactured by T. M. Connelly, Eaglesham, Glasgow. This disintegrator consisted of a stainless-steel tube containing a perforated, steel sieve plate. A polypropylene piston was used to turn cutting blades fixed at right angles to each other on either side of the sieve plate. Small pieces of tissue were placed in the stainless-steel tube, the cutting blades were located with the polypropylene piston, and manual force was applied.

The crude disintegrate was layered onto 55% sucrose and centrifuged at 4,000 rev/min for 20 min. The material (containing leprosy bacilli) which did not enter the sucrose solution was collected and centrifuged in an MSE model A zonal rotor in a Mistral 6L centrifuge. A linear gradient of 17 to 55% sucrose was used, and centrifugation was continued for 30 min at 4,000 rev/min. The gradient was pumped out of the rotor, and 5-ml fractions were collected. Smears of each fraction were prepared and stained by Ziehl-Neelsen method. The fractions containing acid-fast bacilli alone were pooled, and the organisms were sedimented by centrifugation.

**Preparation of the injection mixture**. Purified ovalbumin (crystallized and lyophilized, salt free, grade V, Sigma Chemical Co.) was used as the antigen in this investigation. The pellet of leprosy bacilli (ca 2.0 mg dry weight) was dissolved in 0.6 ml of Bayol 55 (kindly provided by Esso Petroleum Co. Ltd., Purfleet, Essex). Ovalbumin (10 mg) was dissolved in 0.2 ml of normal saline, and 0.2 ml of mannide mono-oleate (Arlacel Z, Atlas Powder Co. Wilmington, Del.) was added. The mineral oil containing the leprosy bacilli was added to this mixture which was emulsified by drawing up into a 1-ml syringe and expelling repeatedly into a glass tube.

Groups of guinea pigs were injected with either 0.1 or 0.2 ml of the water-in-oil emulsion into the left hind footpad. Control guinea pigs were injected with the same water-in-oil emulsion containing ovalbumin but without the leprosy bacilli, or with a saline solution containing ovalbumin.

**Delayed-type hypersensitivity to ovalbumin: corneal tests**. The guinea pigs were given an intracorneal injection of a solution containing 20 mg of ovalbumin per ml by the method of Stewart-Tull and White (6).

**Collection and treatment of sera**. After 3 weeks the animals were anaesthetized with ether, and blood was collected by cardiac puncture. Serum was separated after standing for 2 hr at 37 C and overnight at 4 C. All sera were stored at -20 C.

**Quantitative estimation of ovalbumin antibody levels in guinea pig sera**. Estimations were carried out by the method of Stewart-Tull and White (6), except that the protein content of the final precipitates was determined by the Lowry method (3) and spectrophotometric measurement at 280 nm. Standard curves were prepared for ovalbumin and guinea pig gamma globulins, and from these curves proportionality factors were determined. With the aid of these proportionality factors, the amount of antibody nitrogen per milliliter of guinea pig serum was calculated.
RESULTS

Isolation of M. leprae organisms from infected tissue by zonal centrifugation. Examination of the stained smears of each zonal centrifuge fraction showed that acid-fast bacilli were present in the less dense region of the gradient (Fig. 1), usually the initial 200 ml. Globi containing leprosy bacilli were found to penetrate the denser regions of the gradient. It was possible to obtain fractions which contained only M. leprae organisms, and the organisms from these fractions were used in the biological experiments.

Tests for biological activity of M. leprae. A positive biological effect of the water-in-oil emulsion containing M. leprae was based on the production of a local macrophage epithelioid granuloma in the injected footpad, an increase in the size of the homolateral iliac lymph nodes in the injected limb, and the induction of delayed-type hypersensitivity (corneal reaction). In each instance, the intensity of these reactions was recorded between the limits of 0 for no reaction and 3 for a strong reaction (Table 1). The injection of ovalbumin in normal saline did not produce any of these three effects. However, 400 μg of M. tuberculosis human strain C or 200 μg and 300 μg of M. leprae when incorporated into a water-in-oil emulsion containing ovalbumin produced positive biological effects.

In addition, the production of increased serum antiovalbumin levels was a manifestation of positive adjuvant activity. The quantitative precipitin test indicated that the mean values of antibody N (μg/ml of serum) were 78 for ovalbumin alone, 376 for ovalbumin in water-in-oil emulsion containing M. tuberculosis C, and 192 and 148 for ovalbumin in water-in-oil emulsion containing 200 μg and 300 μg of M. leprae, respectively (Table 1). A gamma-globulin arc was produced by these sera against ovalbumin in immunoelectrophoretic analyses.

Pigmentation of injected footpads. A number of animals were injected with the water-in-oil emulsion containing M. leprae and were subsequently given another injection 3 months later into the same footpad. It was noticed that the injected footpads of two animals, pink in color before injection, were markedly pigmented and bluish-black in color. Sections of the footpad skin and granuloma were stained by Fontana silver method for melanin and Perl Prussian blue method for ferric iron (hemosiderin). The section of footpad skin and granuloma from an animal which received one injection of water-in-oil emulsion containing M. tuberculosis strain C showed the presence of relatively little hemosiderin within the macrophages of the granuloma (Fig. 2). However, guinea pigs which received booster doses of a water-in-oil emulsion containing M. leprae possessed approximately 20 times as much hemosiderin in the macrophages of the local granuloma (Fig. 3).

Sections stained by Fontana silver method did not show any increase in the quantity of melanin either in the melanocytes or in the macrophages in the local granuloma.

DISCUSSION

Previous investigations have shown that when ovalbumin is injected in a water-in-oil emulsion together with killed M. tuberculosis or the peptidoglycolipid (wax D) derived from these organisms an increased level of antiovalbumin antibody was produced (1, 5, 6, 8–10; Stewart-Tull, Ph.D. thesis, Glasgow University, Glasgow, Scotland, 1966). The adjuvant activity of the wax D of M. phlei and M. smegmatis was controlled by the age of the culture (6). The injection of M. leprae in a water-in-oil emulsion containing ovalbumin stimulated the formation of the local granuloma in the footpad and swollen lymph nodes and delayed hypersensitivity to the protein antigen. Elevated serum antibody N levels were also found which were comparable to those produced by other acid-fast organisms. The results indicate that M. leprae obtained from infected tissues is capable of producing an adjuvant effect. It is interesting that a surface network of adjuvant-active peptidoglycolipid filaments was found on mycobacterial organisms (7) and recently has been shown to be present in M. leprae (2). The surface network was less dense on M. leprae obtained from infected tissue than it was on M. tuberculosis grown in artificial culture media. From the results

FIG. 1. Smear of M. leprae organisms stained by Ziehl-Neelsen's method.
Table 1. The effect of M. tuberculosis and M. leprae on the production of a local granuloma, increase in size of homolateral lymph nodes, delayed-type hypersensitivity, and production of antiovalbumin antibody in the guinea pig

<table>
<thead>
<tr>
<th>Fraction and dose</th>
<th>No. of animals</th>
<th>Intensity of reactiona</th>
<th>Production of antiovalbumin</th>
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<tbody>
<tr>
<td>2.0 mg of ovalbumin in 0.2 ml of normal saline</td>
<td>5</td>
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<td>0</td>
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<tr>
<td>2.0 mg of ovalbumin plus 400 μg of M. tuberculosis C in water-in-oil emulsion</td>
<td>3</td>
<td>3 ulc</td>
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<td>3.5</td>
<td>3c</td>
</tr>
<tr>
<td>1.0 mg of ovalbumin plus 200 μg of M. leprae in water-in-oil emulsion</td>
<td>3</td>
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<td>3</td>
<td>3</td>
</tr>
<tr>
<td>2.0 mg of ovalbumin plus 300 μg of M. leprae in water-in-oil emulsion</td>
<td>3</td>
<td>2.5</td>
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<td>2</td>
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</tbody>
</table>

a Recorded between the limits of 0 for no reaction and 3 for a strong reaction.

b Arithmetic mean of sample.

c Ulceration of foot.

d Chemosis.

Increased levels of hemosiderin in macrophages of the footpad granuloma. It is possible that the increased levels of hemosiderin might have been caused by local hemorrhage after the injection of the water-in-oil emulsion into the guinea pig footpad. However, this phenomenon of increased pigmentation in the footpad has never been previously observed by the authors, in animals injected with Freund complete adjuvant. On the other hand, the hemosiderin might have been separated...
from the patient’s spleen during the preparation of the pure suspension of *M. leprae*. This would seem unlikely since only 33% of the animals injected with the water-in-oil emulsion containing ovalbumin and *M. leprae* showed the increased pigmentation of the footpad. Consequently, it would seem that the increased amount of hemosiderin may be related to an intense inflammatory reaction which could result from delayed hypersensitivity to *M. leprae*.

This finding that the pigment is mainly due to increased amounts of hemosiderin would suggest that the reports in the literature attributing a phenoloxidase system to *M. leprae* oxidizing 3,4-dihydroxyphenylalanine to pigmented products (4) should be accepted with caution when dealing with an in vivo model. The precise mechanism of this increased pigmentation is still under investigation.

ACKNOWLEDGMENTS

One of us (D.E.S.-T.) is indebted to J. A. Milne, Dermatology Department, Glasgow University, for his guidance in the histological examination of footpad sections and to R. J. W. Rees for providing tissue infected with *M. leprae*.

LITERATURE CITED