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EXPERIMENTAL LEPROMATOUS LEPROSY IN THE WHITE-HANDED GIBBON (*HYLOBATUS LAR*): SUCCESSFUL INOCULATION WITH LEPROSY BACILLI OF HUMAN ORIGIN

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Summary.—Leprosy bacilli of human origin were inoculated into a white-handed gibbon by the i.v. and i.p. routes, and also locally into ears, testis and around an ulnar nerve. The animal was observed closely during a period of nearly 15 years and did not exhibit any clinical evidence of cutaneous or neurological disease. At death, a wide range of tissues was taken for bacterial counts and histological examination, and a disseminated and progressive infection was demonstrated. Acid-fast bacilli were found in many sites; their morphological appearance, distribution in nerves, and pattern of multiplication in mouse foot-pads, and also the presence of anti-mycobacterial antibody in the serum and the absence of specific lymphocyte transformation were all in keeping with an infection by *Mycobacterium leprae*, at an early lepromatous stage. This is probably the first fully documented report of experimental lepromatous infection in a primate. The findings are discussed in relation to the long incubation period of lepromatous leprosy and the difficulties of diagnosing the disease at an early stage in man.

DESPITE NUMEROUS ATTEMPTS at experimental inoculation of *Mycobacterium leprae* into a wide range of animals by many different routes, none was proved successful until the discovery by Shepard (1960) of limited multiplication within the foot-pads of mice. The continuing search at that time for a more highly susceptible animal, together with the obvious advantages of establishing experimental leprosy in a primate and the availability of gibbons in Malaysia, prompted one of us (M.F.R.W.) to inoculate 3 of these animals with leprosy bacilli of human origin in 1961. Two died, but one survived, and was recently killed almost 15 years after inoculation. This communication describes the experimental procedure and the findings on bacteriological and histopathological examination.

MATERIAL AND METHODS

A. Inoculation.—In September 1961, a full-grown male white-handed gibbon, estimated to be 5 years old and weighing 5 kg, was inoculated with suspensions of *M. leprae* prepared from ear trimmings obtained that day from 2 previously untreated lepromatous patients in Malaysia. After removal of the epidermis, the tissue from each patient was cut up finely with scalpel and scissors. A crude suspension was prepared in sterile normal saline and centrifuged at a low speed to remove the larger lumps of tissue debris. The resulting supernatant was counted for acid-fast bacilli (AFB) by the method of Rees (1964). The suspension obtained from Patient No. 1 contained 2×10^9 AFB/ml with a morphological index (MI) (percentage of solid-staining, presumed viable AFB) of 9; that from Patient No. 2 contained $9{\cdot}6 \times 10^8$ AFB/ml with an MI of 10. Fifteen ml of the suspension from Patient No. 1 were inoculated i.v. (7 ml) and i.p. (8 ml). A total of 3·15 ml of the suspension from Patient No. 2 was inoculated locally, into the left testis and both ears and around the left ulnar nerve at the elbow. Therefore the inocula totalled $3{\cdot}3 \times 10^{10}$ AFB, of which approximately 3×10^9 were solid-staining. The AFB from both suspensions failed to grow on Loewenstein–Jensen slopes.

B. Follow-up.—The gibbon remained healthy throughout the 14 years 11 months of the experiment. At no time were there any clinical signs of leprosy. Skin biopsy samples were taken

in 1962, 1967 and 1970 from the left ear, the right ear and chin, and the left ear respectively. In addition, biopsy samples were taken of the right inguinal lymph node in 1962, submental muscle in 1967, and left triceps muscle in 1970.

In January 1974, the animal was skin-tested with lepromin (Wade–Mitsuda) and PPD (ITU of RT23).

C. Killed, August 1976.—The animal weighed 6 kg. It was anaesthetized with veterinary Nembutal (phenobarbitone sodium) and killed by bleeding from cardiac puncture, the blood being retained for immunological investigations. At postmortem examination a wide range of tissue samples was taken both for homogenization and bacterial counts, and for histopathology.

Histopathology.—Tissues taken included both ears, testes and ulnar nerves, skin of the external nose and lip, the right inferior turbinate bone, triceps, quadriceps and dartos muscles, right sciatic nerve, right inguinal lymph node, liver and spleen. Fixation was in formol-Zenker with transfer to 70% alcohol 2·5 h later, and in 10% formol saline. The tissues, embedded in wax and cut at 5 μm, were stained with the Fite–Faraco modification of Ziehl–Neelsen and a Masson's trichrome: "TRIFF" (Wheeler, Hamilton and Harman, 1965).

TABLE.—*Yields of* M. leprae *obtained from samples of 11 Tissues Taken at Necropsy*

Tissue	Yield of AFB
Dartos	$1{\cdot}7\times10^6$*
Ear, left	$<1{\cdot}5\times10^5$†
Ear, right	$<1{\cdot}5\times10^5$
Muscle, right quadriceps	$<1{\cdot}5\times10^5$
Muscle, left triceps	$<1{\cdot}5\times10^5$
Nerve, left ulnar	$7{\cdot}7\times10^5$
Nerve, right ulnar	$3{\cdot}1\times10^5$
Nose and upper lip, left	$1{\cdot}5\times10^5$
Nose and upper lip, right	$1{\cdot}2\times10^6$
Testis, left	$<1{\cdot}5\times10^5$
Testis, right	$4{\cdot}6\times10^5$

* Positive suspensions were inoculated on to Loewenstein–Jensen slopes; no mycobacterial growth was obtained.

† No bacilli counted; minimum level of sensitivity $1{\cdot}5\times10^5$ bacilli (8×10^4 AFB/ml).

Bacterial counts.—Samples of 11 tissues (Table) were homogenized in glass hand homogenizers in 2 ml of 1% BSA in water, and counted for AFB.

Five of the 6 positive suspensions were each used to inoculate both hind foot-pads of a group of 6 CBA mice, the inoculum varying between $4{\cdot}6\times10^3$ and $1{\cdot}26\times10^4$ per foot-pad. All positive suspensions were inoculated on to Loewenstein–Jensen slopes.

The mice were killed between 6 and 9·5 months after inoculation. Nine foot-pads in each group were counted for evidence of multiplication of AFB, and 3 were fixed for histological examination.

Two foot-pad suspensions, one obtained from a mouse inoculated with AFB obtained from dartos, and the other from the right ulnar nerve, were passaged to further groups of mice (the inoculum being 1×10^4 AFB/foot-pad) and tested for dapsone sensitivity (Rees, 1967), using 0·01, 0·001 and 0·0001% dapsone in the mouse diet.

Immunology.—Cultures of lymphocytes obtained from 20 ml cardiac whole blood were set up, and stimulated with whole *M. leprae* antigen (Myrvang *et al.*, 1972), BCG, and PHA according to the lymphocyte transformation test (LTT), using a micro-method.

Serum obtained from the gibbon blood was sent in dry ice to the National Institute for Medical Research, London, where it was examined by immunodiffusion for the presence of antimycobacterial antibodies using *M. leprae* and BCG as antigens.

RESULTS

A. Follow-up

The tissues taken in 1962, 1967 and 1970 were uniformly negative for AFB (Dr Croft, personal communication). The lepromin test measured 3 mm at 3 days and 0 mm at 3 weeks; that is, both Fernandez and Mitsuda reactions were negative. The tuberculin test was also negative.

B. Autopsy

The animal appeared healthy. Macroscopically no significant abnormalities were detected, although the left testis appeared a little smaller and softer than the right.

Histopathology

All tissues showed bacteria morphologically resembling *M. leprae*, with the exception of the right testis (in which tissue they were, however, present in countable numbers on homogenization; Table). They were found singly, in small groups and in globi. Approximately 30% of all free-standing bacilli were solid-staining along their entire length. Most bacilli were located in macrophages, but they were also observed in Schwann cells (Figs 2 and 3), perineurial cells, fibroblasts, endothelial lining cells of vessels, pericytes

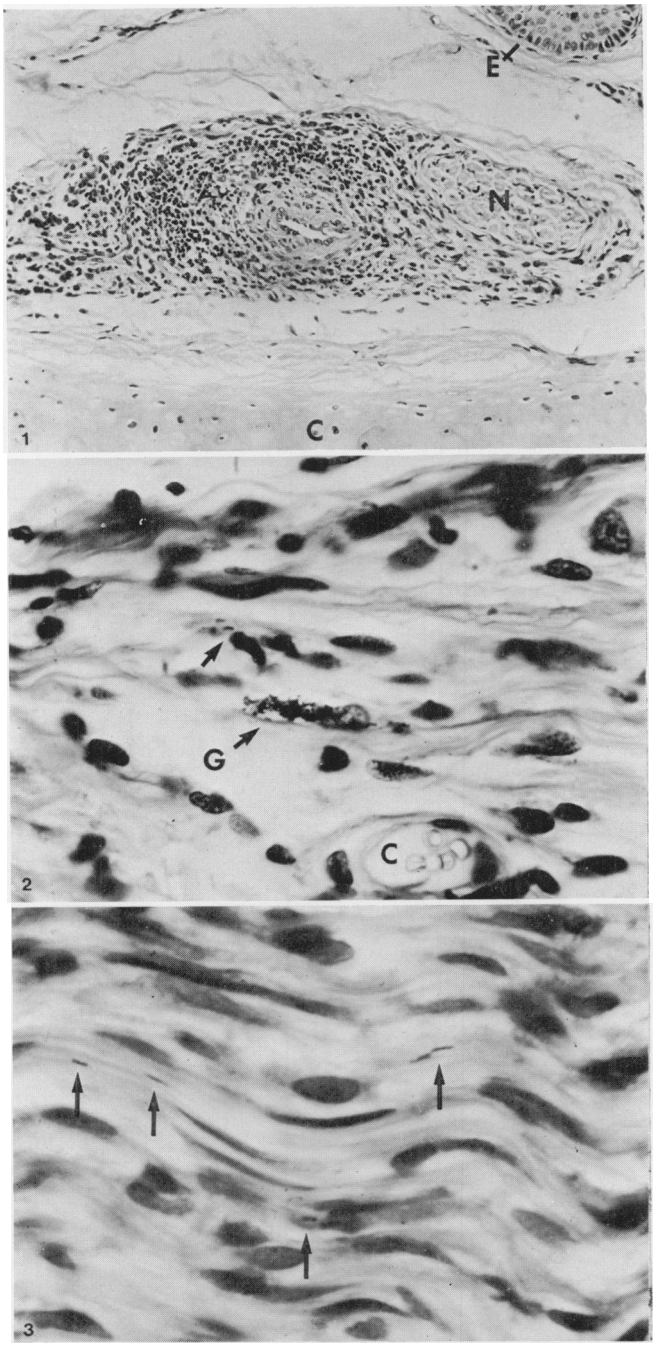

FIG. 1.—Left ear. Between epidermis (E) and cartilage (C), a neurovascular bundle in the dermis shows considerable infiltration, mainly perivascular, with lymphocytes and a few histiocytes. N—nerve. The ear was a locally inoculated site; similar pathology, with occasional epithelioid change in histiocytes, was present in most parts of the section. TRIFF. ×165.

FIG. 2.—Nerve, right ulnar. Bacilli in globus form (G) lie in the cytoplasm of a Schwann cell and there are a few single bacilli adjacent to this (arrowed). C—capillary. TRIFF. ×660.

FIG. 3.—Nerve, left ulnar. Single bacilli (arrowed), several of them solid-staining, lie in the endoneurial area of nerve. Fite–Faraco. ×660.

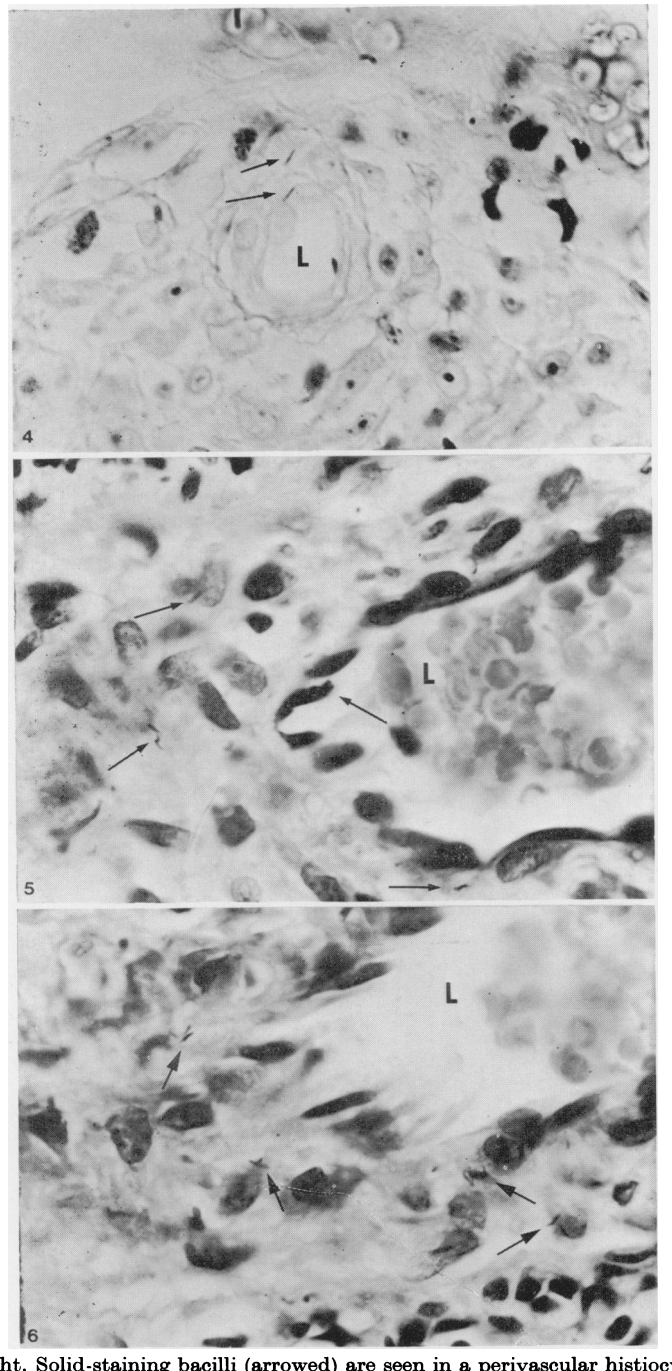

FIG. 4.—Ear, right. Solid-staining bacilli (arrowed) are seen in a perivascular histiocyte (upper) and in the cytoplasm of an endothelial lining cell (lower). L—lumen. TRIFF. × 660.

FIG. 5.—Testis, left. A group of bacilli (arrowed) lies in the cytoplasm of an endothelial lining cell of a venule (L—lumen), and there are several other bacilli in perivascular histiocytes (arrowed). Fite–Faraco. × 660.

FIG. 6.—Testis, left; same vessel as Fig. 5. Serial sections reveal further bacilli (arrowed) in endothelial lining cells and perivascular histiocytes. L—lumen. Fite–Faraco. × 660.

(Figs 4–6) and Kupffer cells in the liver. At the locally inoculated sites (left testis, left ear, right ear and around the left ulnar nerve) there was a local infiltration of lymphocytes and histocytes (Fig. 1), and in the ears some of the latter showed a tendency towards epithelioid change. Giant cells were not found and there was no evidence of necrosis or caseation in any tissue. Although the changes seen microscopically were more intense in the left (inoculated) than the right (uninoculated) ulnar nerve, the only locally inoculated tissue to show structural changes was that of the left testis, where quite large areas of seminiferous tubules, interstitial tissue and cells were fibrosed or atrophic. In most of the uninoculated sites where bacilli were found—often in considerable numbers—there was no evidence of a cell-mediated immune response, but in some parts of the ulnar and sciatic nerves there was a localized and dense lymphocytic infiltration, mainly around endoneurial blood vessels. Even in these nerves, however, the main picture was of widely scattered bacilli, singly, in small groups and in globi and with absolutely no cell response. The right inferior nasal turbinate showed thinned but intact squamous epithelium with a submucosa in which there were numerous bacilli, mainly in macrophages, but also in endothelial lining cells of various vessels, and in nerves. Striated muscle did not show bacilli within fibres, but they were present in considerable numbers between them, and were also seen in a muscle spindle. Liver was normal in structure and showed only a very occasional bacillus, non-solid staining, in a Kupffer cell. Spleen showed ageing changes with sparse follicles and only an occasional bacillus in blood vessel wall. Liver and spleen had profuse haemosiderin deposits, but no pigmentary or other evidence of malaria. Amyloid was not found in any tissue.

Histopathological changes in mouse foot-pad tissues

Foot pads from 9 mice were examined at $9\frac{1}{2}$ or $11\frac{1}{2}$ months after local inoculation and a combined lymphocytic–histiocytic infiltrate was found in all except one and bacilli were present in all except 2. In the remaining 6 animals, the appearances were entirely in keeping with those known to be consistently produced by experimental infection with *M. leprae* in the mouse model (Shephard, 1960; Rees, 1964), including the finding of bacilli within nerves.

Bacteriology

Six of the 11 gibbon tissues which were homogenized were found to contain AFB (Table), the yields (in 2 ml) varying between $1{\cdot}55 \times 10^5$ and $1{\cdot}7 \times 10^6$ AFB. The tissue samples were of the order of 10–100 mg. The yields per gram of tissue were therefore compatible with early lepromatous leprosy.

Forty-three of the 44 mouse foot-pads counted between 6 and $11\frac{1}{2}$ months after inoculation showed typical limited multiplication of *M. leprae*, the yields varying between $7{\cdot}8 \times 10^4$ and $3{\cdot}5 \times 10^6$ AFB.

On passage, the control mice also showed typical limited multiplication, but not the mice fed dapsone in their diet; the organism was therefore fully sensitive to dapsone.

Immunology

In the LTT, the gibbon lymphocytes responded to PHA, but there was no response to either *M. leprae* or BCG. Antimycobacterial antibodies were detected in the gibbon serum.

DISCUSSION

To date, the only non-immunosuppressed animal found to develop lepromatous leprosy is the armadillo (Kirchheimer and Storrs, 1971), and another animal model more closely resembling the human disease in its pathogenesis and immunology could prove to be very valuable. However, attempts to transmit leprosy to primates have been few, despite their long life-span, and biological similarity to man. Marchoux and Bourret

(1908) and Nicolle and Blaizot (1911) inoculated chimpanzees, but failed to obtain convincing evidence of progressive disease. In 1912, gibbons were inoculated at the Institute for Medical Research in Kuala Lumpur, but the experiment was subsequently abandoned. Gunders (1958) described the development of nodules in a chimpanzee inoculated i.v. with *M. leprae* 11 months previously, but the lesions regressed at 14 months, and the infection appeared to resemble self-healing borderline tuberculoid leprosy. Nevertheless, this report gave direct encouragement both to ourselves, and also to Binford (1965), who studied one infant and 23 adult chimpanzees for 5–7 years after inoculation with *M. leprae* and who obtained, in the infant chimpanzee only, evidence of self-limiting lesions resembling those described by Gunders (Binford, personal communication).

The findings in the present study are strongly indicative of a disseminated infection with *M. leprae*. The histopathological findings, especially the presence of AFB in Schwann and perineurial cells, were typical of leprosy in man. The limited multiplication of the AFB in the mouse foot-pad, and their exquisite sensitivity to dapsone in the mouse diet (Shepard, McRae and Habas, 1966; Rees, 1967) were typical for *M. leprae*.

We classified the disease as early lepromatous (LL) leprosy on the character of the host cells, principally undifferentiated macrophages or histiocytes, the presence of globi, the negative lepromin test, the negative lymphocyte transformation test (LTT) on specific antigenic (*M. leprae*) stimulation, and the finding of circulating antimycobacterial antibodies. Although a total body count for AFB was not carried out, the numbers of bacilli found in the tissue samples, the fact that some 30% of individually identified AFB were solid-staining and the widespread involvement of the endothelial lining cells of various blood vessels suggested that the infection had entered a phase of rapid bacterial multiplication with haematogenous spread. There were already numerous bacilli in the nasal submucosa, lending support to the concept that human lepromatous patients may begin to excrete leprosy bacilli in their nasal secretions well before their disease can be easily diagnosed.

In our experimental design, it was decided to give a single large inoculum, rather than repeated small serial inocula such as could well occur in human contacts of leprosy patients. In this way, the date of infection was known. Superinfection was most improbable, as the gibbon was kept 1 km from the wards (where newly admitted infectious patients were treated) and few even of the long-stay inmates entered the fenced-off area surrounding its cage. Although the animal attendants were ex-patients, they were all well and regularly treated, and none showed any evidence of relapse or of sulphone resistance. Furthermore, as the gibbon's strain of *M. leprae* was fully dapsone-sensitive, superinfection from a dapsone-resistant patient is excluded.

An incubation period of nearly 15 years from inoculation to the development of widespread, but still clinically inapparent infection in the gibbon is in keeping with experience in man. Although indeterminate and tuberculoid leprosy are common in children, lepromatous leprosy is rare before puberty, and may reach a peak incidence around the fourth decade (Bechelli, Martinez Dominguez and Patwary, 1966). It is interesting that Donham and Leininger (1977) have recently described spontaneous disease resembling borderline-lepromatous (BL) leprosy in a chimpanzee imported into the United States from Sierra Leone, citing *M. leprae* as the causative organism (although results of lepromin and of mouse foot-pad inoculation tests are still awaited), and suggesting that the animal might have been infected naturally from human sources before importation. The animal was estimated to be 5–7 years old at the time when signs of the disease were first detected.

Why the leprosy bacillus should remain apparently dormant for so many years remains a mystery, although the finding of lymphocytes at the sites of local inoculation, and of an epithelioid tendency of some of the histiocytes in the infiltrate in the ears, suggestive of borderline (BB) leprosy, indicate that initially the animal was able to mount a cell-mediated immune response, albeit a weak one. Because repeated lepromin testing might have affected the animal's specific cell-mediated immunity (CMI), no such test was performed in the first decade. It was found to be negative at 12·3 years, at a time when the gibbon's total bacterial (antigenic) load was probably still small. Should this experiment be repeated in another group of gibbons, it would be of great interest to perform serial specific LTTs at regular intervals, as well as other immunological investigations, in an endeavour to discover the natural history and nature of the CMI deficit in lepromatous leprosy.

We believe that this is the first reported case of a successful and disseminated infection with *M. leprae* in the gibbon, and probably the first fully documented account of experimental lepromatous infection in a primate. This animal was mature at the time of inoculation, and was considered to be aged 5 years. It was about 20 years old at the time of killing, the normal life-span of the gibbon being of the order of 30 years (Segal, unpublished). Further well-conceived experiments involving the inoculation of gibbons with living leprosy bacilli appear indicated, although the prospect of 15–20 years' follow-up would seem somewhat daunting.

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REFERENCES

BECHELLI, L. M., MARTINEZ DOMINGUEZ, V. & PATWARY, K. M. (1966) WHO Epidemiologic Random Sample Surveys of Leprosy in Northern Nigeria (Katsina), Cameroon and Thailand (Khon Kaen). *Int. J. Lepr.*, **34**, 223.

BINFORD, C. H. (1965) The Inoculation of Human Leprosy in the Chimpanzee: Initiation of a Long-term Project. *Int. J. Lepr.*, **33**, 666.

DONHAM, K. J. & LEININGER, J. R. (1977) Spontaneous Leprosy-like Disease in a Chimpanzee. *J. Infect. Dis.*, **136**, 132.

GUNDERS, A. E. (1958) Progressive Experimental Infection with *Mycobacterium leprae* in a Chimpanzee. A Preliminary Report. *J. Trop. Med. Hyg.*, **61**, 228.

INSTITUTE FOR MEDICAL RESEARCH, Kuala Lumpur (1913) *Twelfth Annual Report, 1912*. Kuala Lumpur: FMS Government Printing Office, p. 26.

KIRCHHEIMER, W. R. & STORRS, E. H. (1971) Attempts to Establish the Armadillo (*Dasypus novemcinctus* Linn.) as a Model for the Study of Leprosy. 1. Report of Lepromatoid Leprosy in an Experimentally Infected Armadillo. *Int. J. Lepr.*, **39**, 693.

MARCHOUX, E. & BOURRET, G. (1908) Essai d'inoculation de la Lèpre en Chimpanze. *Bulletin de la Societé de Pathologie Exotique*, **1**, 416.

MYRVANG, B., GODAL, T., RIDLEY, D. S., FROLAND, S. S. & SONG, Y. K. (1972) Immune Responsiveness to *Mycobacterium leprae* and Other Mycobacterial Antigens throughout the Clinical and Histopathological Spectrum of Leprosy. *Clin. exp. Immunol.*, **14**, 541.

NICOLLE, C. & BLAIZOT, L. (1911) Essai de Reproduction de la Lèpre chez le Chimpanze et les Singes Inférieurs. *Archives de l'Institut Pasteur de Tunis*, 275.

REES, R. J. W. (1964) Limited Multiplication of Acid-fast Bacilli in the Foot-pads of Mice Inoculated with *Mycobacterium leprae*. *Br. J. exp. Path.*, **45**, 207.

REES, R. J. W. (1967) Drug Resistance of *Mycobacterium leprae* Particularly to DDS. *Int. J. Lepr.*, **35**, 625.

SHEPARD, C. C. (1960) The Experimental Disease that Follows the Injection of Human Leprosy Bacilli into Foot-pads of Mice. *J. exp. Med.*, **112**, 445.

SHEPARD, C. C., MCRAE, D. H. & HABAS, J. A. (1966) Sensitivity of *Mycobacterium leprae* to Low Levels of 4,4′-Diaminodiphenyl Sulfone. *Proc. Soc. exp. Biol. Med.*, **122**, 893.

WHEELER, E. A., HAMILTON, E. G. & HARMAN, D. J. (1965) An Improved Technique for the Histopathological Diagnosis and Classification of Leprosy. *Lep. Rev.*, **36**, 37.