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Experiences with mouse foot pad inoculation of leprosy bacilli originating from the Congo

BY

S. R. PATTYN and P. G. JANSSENS

Summary — Four strains of *M. leprae* originating from Central Africa have been isolated on mouse foot pads in our laboratory.

The results are in complete agreement with those formerly described by Shepard.

No strains were obtained from patients treated with DDS or Ciba 1906 during 2 to 6 months.

Conservation of lepromatous tissue on ice for 7 days or at — 13 °C for 44 days keeps at least some of the bacilli viable.

The generation time of the isolated bacilli was 25 to 31 days.

Comparative experiments with other mycobacteria showed *M. leprae* to differ sufficiently in its behaviour in the mouse foot pad to be able to be diagnosed as such.

During the past three years we have inoculated a total of eleven suspensions of acid-fast-bacilli (AFB) obtained from cutaneous biopsies of leprosy patients in Leopoldville, République du Congo, into the foot-pads of mice, a technique first described by C. C. Shepard (1960).

Data on the results obtained are the subject of this paper.

Material and Methods

The biopsy specimens were obtained by Dr J. P. Bastin from negro patients seen at the leprosarium of Leopoldville. They were send to Antwerp immediately or after various time lapses, at low temperatures as will be indicated under results. Transportation was on wet ice in ampoules containing Hanks balanced salt solution with penicillin 100 mcgr per ml. At arrival in our laboratory the specimens were kept in the refrigerator until processing and inoculation.

Harvests and countings were made following the techniques described by C. C. Shepard (1960) an agla-micrometer syringe being used to make drops of 2 mcl. Inoculations in white mice were done in one or both hind foot-pads 0.03 ml per foot.

Suspensions for inoculation and harvests were inoculated on Loewenstein Jensen medium and incubated for periods up to one year at 37 °C and 33 °C.

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Results

Three batches of material were received, respectively in march 1961, april 1962 and december 1962.

Table 1 gives the data on the specimens received and the results obtained. Four strains were be established, all originating from untreated lepromatous cases. Inoculation of 5 treated lepromatous cases and 2 untreated borderline ones resulted in failure.

TABLE 1

Results of mouse foot pad inoculation of *M. leprae*

Strain number	Clinical form dis.	Treatment	Date of biopsy	Conservation	Date of recept Antwerp	Conservation	Date of inoc.	% solid	Result
22991	L	No	23-03-61	4 °C	24-03	4 °C	27-03	No	+
58	L	No	19-04-62	4 °C	20-04	4 °C	21-04	4	+
3/63	L	No	03-12-62	— 13 °C	15-12	4 °C	19-12	40	+
144	L	No	02-11-62	— 13 °C	15-12	4 °C	3-1-63	25	+
37	L	Ciba 1906 2 months	17-04-62	4 °C	20-04	4 °C	25-04	50	—
55	BL	No	17-04-62	4 °C	20-04	4 °C	25-04	5	—
22434	BL	No	17-03-61	4 °C	24-03	4 °C	27-03	No	—
36	L	Ciba 1906 2 months	17-04-62	4 °C	20-04	4 °C	26-04	0	—
36 B	L	Ciba 1906 10 months	10-12-62	— 13 °C	15-12	4 °C	18-12	30	—
58 B	L	DDS 6 months	10-12-62	— 13 °C	15-12	4 °C	26-12	30	—
85	L	Ciba 1906 6 months	10-12-62	— 13 °C	15-12	4 °C	3-1-63	5	—

L = lepromatous leprosy.
BL = borderline leprosy.

Conservation in Leopoldville between biopsy taking and mailing was kept to a minimum for the first specimens but gradually extended for the two other batches. Moreover, whereas the first

two batches were kept in the refrigerator, the third was put into an electric deep-freeze whose temperature was — 13 °C.

As can be seen in table 1 positive results were obtained not only with very fresh specimens which had not been kept at 4 °C, but also with specimens kept at — 13 °C for 12 and 44 days, followed by conservation periods at 4 °C of 4 and 16 days respectively.

The viability of the bacilli as judged from Ziehl-Neelsen stains varied between 0-5 and 42 p. cent. There was a relatively good correlation between the percentage of solid staining bacilli observed and the end result. That this is not always the case is demonstrated by the two patients who have been biopsied twice : viz. 58 (+ 58 B) and 36 (36 B). It is rather surprising that 36 B after 10 months of treatment shows 30 p. cent solid staining bacilli where as the first biopsy showed no solid staining. This may however be due to the biopsies having been taken from different sites of the body.

Inoculations were done with about 10^4 bacilli, the evolution of the infection being followed at monthly or bimonthly intervals.

Passages were made when the number of bacilli showed an increase to 5.10^5 - 10^6.

The oldest strain (22491) is now in its 5th mouse passage, since its primary inoculation almost 3 years ago. Subinoculations were done respectively after 6, 7, 10 and 9 months and increases in the number of bacilli have been of the order of 100 × in a time lapse of 7-9 months.

The maximum number of bacilli hervested has been 3.5×10^6 per foot-pad. Calculations of generation time give results varying between 25 and 31 days.

The mice did not develop any clinical symptoms.

Numerous inoculations or original suspensions and passage material on Loewenstein-Jensen media incubated at 37 °C and 33 °C and observed for periods up to one year did not give growth of AFB.

On several occasions we searched for AFB in the fore feet of mice inoculated in the hind feet. Special care was taken by thorough cleaning of all instruments to avoid any possible contamination of such harvests by AFB through instruments. In a few instances we were able to find bacilli in the front-feet, but always faintly stained and few in number. In one case we tried subinoculation into new mice, which are now under observation.

We tried also passages of the oldest strain into rats and hamsters. These animals were inoculated intratesticularly and in the foot-pads. Newborn rats were also inoculated in the foot pads.

In hamsters the bacilli were still present after 7 months in the foot pads, but without evidence of multiplication. The bacilli disappeared from the testicles during the one year observation period.

In rats the AFB disappeared in the feet and the testicles. Experiments with newborn rats were also negative.

In one experiment a group of mice was administered suramin 1 mg twice a week durin 3 ½ months without more rapid multiplication in these animals as compared to the control group. This experiment was done because Hilson and Elek (1957) have shown that the multiplication of *M. lepraemurium* in mice is enhanced by the administration of suramin.

We inoculated several other species of mycobacteria into mouse foot pads in order to compare their behaviour with that observed with AFB isolated from leprosy patients. These species are : *M. balnei, ulcerans, fortuitum, lepraemurium, marianum, kansasii, avium,* a scotochromogen and a Battey strain. *M. balnei* multiplies at a very much higher rate, the generation time being less than one day. Inoculation is followed by clinical disease within a week, characterised by swelling and ulceration. Histologically there is a granulomatous inflammatory reaction with necrosis and large numbers of long AFB scattered over the whole area.

M. ulcerans on the other hand has a generation time of 10-12 days clinical symptoms appear after 6-8 weeks, there are again swelling and ulceration. Histologically there is as with balnei a granulomatous inflammatory reaction but with much more oedema.

After inoculation of *M. fortuitum, marianum, kansasii, avium,* a scotochromogen, and the Battey organism, the bacilli disappear gradually from the foot pad during the following three weeks, they may however be found in the feet for a period of up to 3 months but in ever diminishing numbers.

Discussion

We have thus been able to confirm on a small scale with African strains of *M. leprae* the findings of Shepard, which he made with North American and Philippine strains of the same bacillus. During the same period, Rees (personal communication) in London has been able to establish experimental infection of mouse foot pads with *M. leprae* from S. E. Asia (Malaya) and W. Africa.

The great question which arises is that of the identity of the observed mycobacteria. Shepard made a masterly comment on this in his first paper on the subject (1960). In a later he has

given immunological proof of the identity of one of his isolates by preparing a lepromin with it (1963).

Now we know a certain number of properties of *M. leprae* in mouse foot pads, which enables us to differentiate it from all other known mycobacteria, while it can readily be differentiated from most other mycobacteria by its failure to grow on suitable bacteriological media. And at this moment of our knowledge the most important differentiation in the experimental animals is with *M. lepraemurium*.

M. leprae strains do not give rise to clinical symptoms in the mouse foot pads nor do they give rise to generalized infection in this animal after I. P. inoculation (which *M. lepraemurium* does) *M. leprae* probably does not multiply in rat testicles (which *M. lepraemurium* does). Furthermore the number of bacilli recovered from mouse foot pads is much lower with *M. leprae* than with *M. lepraemurium*.

M. lepraemurium multiplies in the mouse foot pad but with a generation time of 15 days so that an inoculum of 10^4 attains 10^6 or more after only 3 months. This is a much more rapid multiplication than that observed after inoculation of AFB from leprosy.

Histologically there is also a difference between *M. leprae* and *M. lepraemurium* in that in the latter infection the granulomatous reaction is much more important and extensive, the bacilli which are also longer, extend and proliferate up to the superficial layer of the dermis.

M. leprae can be isolated in mice from lepromatous cases with relative ease, but isolation seems to be much more difficult from cases treated with DDS or Ciba 1906 during 2-6 months.

Our results also show that, at least, some lepromas may be frozen at temperature of — 13 °C for 44 days and still contain viable bacilli.

With the technique proposed by Shepard we are certainly for the first time able to learn much more about *M. leprae* and the physiopathology of the disease it causes.

Samenvatting — Vier stammen *M. leprae* afkomstig uit Centraal Afrika werden in muisvoetzolen geisoleerd.

De resultaten stemmen helemaal overeen met die van Shepard.

Geen isolaten werden bekomen uit patienten die sedert 2 en 6 maand met DDS en Ciba 1906 waren behandeld.

In lepromateus weefsel bewaard op ijstemperatuur gedurende 7 dagen of aan — 13 °C gedurende 44 dagen blijft tenminste een gedeelte der bacillen leven.

De generatietijd der bacillen bedroeg 26 tot 31 dagen.

Vergelijkend onderzoek met het gedrag van andere mycobacterien in de muisvoetzool toonde aan dat dit van *M. leprae* voldoende ervan verschilt om als zodanig te kunnen herkend worden.

Résumé — Quatre souches de *M. leprae* d'Afrique centrale furent isolées dans la plante de pied de souris.

Les résultats observés sont en accord avec ceux décrits par Shepard.

Aucune souche ne pût être obtenue à partir de malades traités pendant 2 et 6 mois avec DDS et Ciba 1906.

Au moins une partie des germes dans un tissu lépromateux reste vivante après conservation pendant 7 jours sur glace, ou pendant 44 jours à — 13 °C.

Les temps de génération des bacilles varient entre 25 et 31 jours.

Des études comparatives sur le comportement d'autres espèces de mycobactéries dans la patte de souris ont montré que l'évolution de l'infection à *M. leprae* est suffisamment caractéristique pour être reconnue comme telle.

Zusammenfassung — 4 Stämme von *M. leprae* aus Zentralafrika wurden auf Fusssohlen der Maus isoliert. Die beobachteten Resultate stimmen mit den von Shepard beschriebenen überein.

Kein Stamm konnte von Patienten gewonnen werden, die während 2-6 Monaten mit DDS und Ciba 1906 behandelt worden waren.

Zum mindesten ein Teil der Keime bleibt im lepromatösen Gewebe bei einer Konservierung auf Eis 7 Tage lang oder 44 Tage bei — 13 °C am Leben.

Die Zeit des Wachstums der Bazillen schwankt zwischen 25 und 31 Tagen. Vergleichende Untersuchungen über das Verhalten anderer Mykobakterienarten in der Fusssohle der Maus haben gezeigt, dass die Entwicklung der Infektion mit *M. leprae* hinlänglich charakteristisch ist, um als solche erkannt zu werden.

Resumen — Cuatro cepas de *M. leprae* de Africa central fueron aisladas en la planta de ratones. Los resultados observados estan de acuerdo con los descritos por Shepard.

Ninguna cepa pudo ser obtenida a partir de enfermos tratados durante dos y seis meses con DDS y Ciba 1906.

Al menos una parte de los gérmenes, en el tejido lepromatoso, permanece viva despues de conservaciòn durante siete dias sobre hielo, ó durante cuarenta y cuatro a — 13 °C.

El tiempo de generación de los bacilos varia entre venticinco y treinta y un dias. Estudios comparativos sobre el comportamiento de otras especies de micobacterias en la pata de ratones mostraron que, la evolución de la infección por *M. leprae,* es suficientemente caracteristica para ser reconocida como tal.

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