Isolation of *Mycobacterium leprae* from untreated borderline tuberculoid, mid-borderline and indeterminate cases using the mouse foot pad technique – a study of 209 cases

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**Summary** Using the mouse foot pad (MFP) system, isolation of *Mycobacterium leprae* was attempted in 209 skin biopsies obtained from 114 borderline tuberculoid (BT), 62 mid borderline (BB) and 33 indeterminate (I) untreated cases. Unequivocal growth in the foot pads of mice was seen in 100 (47.8%) cases. Of these 100 cases that showed growth in the mouse foot pad system, in 20 cases acid fast bacilli (AFB) were detected in small numbers (1+) in either smear or homogenate. The remaining 80 (42%) cases were negative for AFB in both smear and homogenate. The occurrence of viable bacilli and percentage take at 12 months was highest in BB (76 and 86%) followed by BT (38 and 75%) and I (30% and 52%) cases. In most of the BT (65%) and I (60%) cases, the first peak was seen only at 12 months. These results confirm that viable bacilli can be isolated and expanded from a good proportion of negative BT-BB cases using immunocompetent Swiss White mice.

**Introduction**

*Mycobacterium leprae* cannot be cultivated *in vitro*, but can be grown in the foot pads of mice (MFP), a system developed by Shepard in 1960.1,2 It is the method of choice for determination of viability and cultivation. There are several studies in which *M. leprae* were cultivated from untreated borderline lepromatous and lepromatous (BL-LL) patient tissues using MFP.2–5 However, no attempts have been made in the past to isolate *M. leprae* from smear negative borderline tuberculoid–mid borderline (BT–BB) cases due to low sensitivity of the system. Using the *in-vitro* method of reverse transcription-polymerase chain reaction (RT-PCR) and targeting the 16s ribosomal RNA, bacilli were detected in 67% (12/18) of paucibacillary (PB) cases.6 However, the bacilli detected by *in-vitro* methods cannot be used for further studies. Examination of infectivity in the MFP of serially diluted inocula derived from human skin biopsy specimens had revealed that the minimal infecting dose of *M. leprae* might be as small.
as one or two viable organisms. Acid fast staining requires at least $10^5$ organisms per gram of tissue for reliable detection. In one of our earlier studies on post-MDT recurrent lesions, viable *M. leprae* were isolated using MFP in 48% (12/25) of smear negative tuberculoid relapse cases. Encouraged by this finding, we studied a large group of untreated BB–BT cases, primarily to determine the sensitivity of the MFP system for detecting viable *M. leprae* in smear negative BT–BB lesions. We also hoped to expand the isolates for further drug sensitivity and genetic studies. The results obtained in 209 cases are presented and discussed in this paper.

Materials and methods

A total of 209 cases included in this study are a part of a larger cohort, studying the effect of corticosteroids on nerve damage and bacterial clearance. Between 2001 and 2005, 415 untreated patients were recruited into the study, of which 209 cases conforming to BT, BB or indeterminate type clinically and/or histopathology using the Ridley–Jopling classification are included here.

All the patients were clinically examined in detail and slit skin smears (SSS) were taken. Incisional skin biopsies obtained after informed consent were used for histopathology, bacteriological and MFP study as follows.

Each lesional biopsy obtained was divided into three parts. One part was frozen at $-70^\circ$C, and the second part was fixed in Formal Zenker and embedded in paraffin. The third part of the biopsy was homogenized and bacterial load per g weight was determined using standard protocol. The final volume of the suspension was maintained at 1 ml per 0.1 g of tissue sample. The detection limit of this method is $>1 \times 10^4$ *M. leprae* ml. The homogenate thus obtained, regardless of presence or absence of any AFB, was injected into both the hind foot pads (0.03 ml/foot pad and inocula size not exceeding $1 \times 10^4$ *M. leprae*) of a minimum of eight and a maximum of 10 immunocompetent Swiss White (S/W) mice. The Foundation is registered with the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA) (reg.no.424/01/a/CPCSEA, June 20, 2001) and the study had ethical clearance from them. The animals were housed in air-conditioned rooms and kept under a 12 h day/night cycle. Foot pad harvests were carried out at 6, 7 and/or 8 and 12 months post-inoculation using Shepard’s method, which is well standardized in this laboratory. A minimum of two counts per harvest was obtained at 6, 7 and 8 months. All the remaining mice were harvested at 12 months. At 12 months, a total of six to eight foot pad counts were obtained per sample. A known volume of foot pad homogenate was counted for acid fast bacilli (AFB) using a spot slide in a minimum of 200 microscopic fields per sample. The lower limit of detection by this counting method is $1 \times 10^4$ *M. leprae*/foot pad.

**DEFINITION OF SIGNIFICANT GROWTH IN THE FOOT PADS OF NORMAL S/W MICE**

One or more foot pads count showing $>1 \times 10^5$ *M. leprae* in the harvests carried out at 6 months or later were considered as a positive yield.

Results

**CLINICAL AND HISTOPATHOLOGICAL CLASSIFICATION**

All the 209 cases included in this study were untreated and had more than five skin/nerve lesions.
Using Trichrome modified Fite Ferracco (TRIFF) stained sections, the patients were further classified using the Ridley–Jopling classification. Thirty-three cases had indeterminate (I), 114 BT and 62 BB leprosy. Sixty-two BT and 34 BB cases had evidence of a type 1 reaction.

**BACTERIOLOGICAL FINDINGS**

Five cases (1 BT, 4 BB) had few acid fast bacilli in the slit skin smear graded as (1 +), but the skin biopsy homogenate was negative. Fifteen cases (2 BT, 12 BB and 1 indeterminate) scored positive in homogenate (between $1 \times 10^6$ and $8 \times 10^7$ AFB/g wt) but were smear negative; none were both smear and homogenate positive. Thus smear and homogenate combined, 20/209 (9.5%) cases scored positive for acid fast bacilli (AFB) and others were negative in both.

**MOUSE FOOT PAD TEST RESULTS**

All the 20 smear or homogenate positive (1 +) cases mentioned above had growth in the MFP system. In addition, 80 cases [80/189 (42.3%)] that were both smear and homogenate negative also showed growth in the MFP system. Thus, positivity using the normal S/W mice was seen in 100/209 (47.8%) cases. Mouse footpad growth was highest in the BB cases (47/62 = 75.8%), followed by BT (43/114 = 37.7%) and 1 cases (10/33 = 30.3%) (Table 1). Among the cases showing viable bacilli, 26/43 (60.4%) BT cases and 27/47 (57.4%) of BB cases had a type 1 reaction.

The proportion of foot pads showing unequivocal growth (% take) between months 6 and 8 and at 12 months were 20 and 86% in BB, 17 and 75% in BT and 3.3 and 52% in I groups, respectively (Table 2). It was also noted that 65% of BT, 60% of I and 38% of BB cases the first peak with a significant fold increase of $>10^5$ AFB/FP was obtained only at 12 months.

**Discussion**

Using immunocompetent Swiss white mice, we have been able to isolate *M. leprae* in 100/209 cases (47%) that were mostly smear negative and had less than $1 \times 10^4$ *M. leprae* per ml of tissue (0.1 g) homogenate. Twenty were either smear or homogenate positive (1 +), and the remaining 80 cases [80/189 (42.3%)] were both smear and homogenate negative.

**Table 1.** MFP positivity in BT, BB and indeterminate cases in relation to AFB status

<table>
<thead>
<tr>
<th>Leprosy class</th>
<th>MFP + ve in smear or homogenate + ve cases (%)</th>
<th>MFP + ve in both smear and homogenate – ve cases (%)</th>
<th>Overall MFP + ve (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BT</td>
<td>3/3</td>
<td>40/111 (36)</td>
<td>43/114 (37-7)</td>
</tr>
<tr>
<td>BB</td>
<td>16/16</td>
<td>31/46 (68)</td>
<td>47/62 (75-8)</td>
</tr>
<tr>
<td>Indeterminate</td>
<td>1/1</td>
<td>9/32 (28)</td>
<td>10/33 (30-3)</td>
</tr>
<tr>
<td>Total</td>
<td>20/20 (100)</td>
<td>80/189 (42.3)</td>
<td>100/209 (47.8)</td>
</tr>
</tbody>
</table>
Levy and Murray have shown that serially diluted inocula containing 5 AFB/FP, a fold growth of 10^6 AFB/FP was obtained at 280 days (i.e. 9·3 months) with a doubling time of 12·8 days. In keeping with this, a significant fold increase in the foot pad was obtained only at 12 months in the majority of I (60%) and BT (65%) cases and 38% of the BB cases. It is important to extend the foot pad harvests up to 12 months. The total yield as well as the proportion of foot pads showing unequivocal growth was highest in BB, followed by BT and I cases. These results indicate a graded difference in the inocula size in I, BT and BB cases.

Eight of the isolates were repassaged into normal S/W mice. On repassage, a normal pattern of growth, i.e. first peak between 6 and 8 months and second peak at 12 months, was seen in all cases (results not shown). One could use this specific and time tested system for expansion of isolates for further studies.

Another interesting finding is the higher occurrence of viable bacilli in cases with a type 1 reaction (T1R) in both BT and BB groups, although this was not statistically significant. Speculatively, therefore, viable bacterial foci may have a role to play in reaction precipitation, and if confirmed, this would be in contrast to the generally held view that killed M. leprae products may be acting as a trigger for T1R.13

In leprosy control, it was hoped that providing MDT to all newly detected cases would not only lead to healing of the patients, but also prevent further spread of the infection. However, the new case detection rate in general has not decreased significantly.14 In a review on risk factors among contacts, it was stated that contact with MB patients had an 8-fold increased risk, whereas contact with tuberculoid or paucibacillary leprosy, the risk was 4 times higher.15 In addition, drug resistant strains continue to be reported in new cases, as well as relapses even in areas of the world with successful implementation of MDT.16–19 Shin et al. have studied polymorphism, using molecular typing method. However, they were not able to study the cases where the bacterial load was low.20 Thus mouse foot pad method remains an indispensable tool, and the bacilli expanded using this method could well be used for strain typing, for screening of drug resistance, understanding the mode of transmission of disease and for epidemiological investigations.

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References