Association of viable *Mycobacterium leprae* with Type 1 reaction in leprosy

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Summary
The working hypothesis is that, viable *Mycobacterium leprae* (*M. leprae*) play a crucial role in the precipitation of Type 1 reaction (T1R) in leprosy.

**Material and Methods:** A total of 165 new multibacillary patients were studied. To demonstrate presence of viable *M. leprae* in reactional lesion (T1R+), three tests were used concurrently viz. growth in the mouse foot pad (MFP), immunohistochemical detection of *M. leprae* secretory protein Ag85, and 16s rRNA - using *in situ* RT-PCR. Mirror biopsies and non reactional lesions served as controls (T1R-).

**Findings:** A significantly higher proportion of lesion biopsy homogenates obtained at onset, from T1R(+) cases have shown unequivocal growth in MFP, proving the presence of viable bacteria, as compared to T1R(-) (P, 0.005). In contrast, few Mirror biopsies were positive in both T1R(+) and T1R(-).

With respect to Ag85, while the overall positivity was higher in T1R(+) (74%), however the intensity of staining (Grade $\geq$ 2+) was disproportionately higher in T1R(+) BT–BB lesions 11/20 (55%).

In the rebiopsies obtained during a repeat episode of T1R, Ag 85 as well as 16s rRNA, positivity (62% & 100%) was higher in T1R(+).

It is inferred therefore ‘viable’ bacteria are an essential component in T1R and difference in the quality of bacilli, not the quantity or the ratio of dead to viable play a role in the precipitation of T1R.

In conclusion, the findings show that ‘metabolically active’ *M. leprae* is a component/prerequisite and the secretory protein Ag 85, might be the trigger for precipitation of T1R.

**Keywords:** Leprosy, Viable *M. leprae*, Type 1 reaction, Mouse Foot Pad, Ag85, 16s r-RNA, *in situ* RT-PCR
Introduction

The two main types of reaction Type 1 (T1R) or reversal reaction and Type 2 (T2R) or Erythema Nodosum Leprosum (ENL), are important causes of morbidity and nerve function impairments (NFI) in leprosy.\(^1\) T1R is more frequent, afflicting \(\sim 30\%\) of borderline group [i.e. Borderline Tuberculoid (BT) to Borderline Lepromatous (BL)] of leprosy patients.\(^2\) Reactions and the associated NFI are therapeutically managed with immunosuppressive and anti-inflammatory drugs such as corticosteroids.\(^3–5\)

Notable features of T1R are:

(a) Symptoms and signs may be confined to the skin, the nerve(s), or may occur in both
(b) It is common in borderline (BT-BL) type of leprosy: 25 to 50\% patients experience T1R sometime during the course of disease, before, during and after treatment.\(^3\),\(^5\)
(c) A previous episode of reaction places a patient at higher risk of a repeat episode.\(^5\),\(^6\)
(d) That it has an immunological basis, but the trigger is largely unknown.\(^7\)

In the present study, we propose that metabolically active Mycobacterium leprae (\(M. leprae\)) in a skin or nerve lesion(s) are a trigger, that the threshold of cell mediated immune reactivity to whole \(M. leprae\) is decreased in the presence of \(M. leprae\) antigens. In brief, that incomplete killing or refractoriness to treatment or persistence of \(M. leprae\) increases the risk of reaction.

The proposal finds support from an earlier study conducted in this laboratory on skin biopsies in 25 smear negative BT patients manifesting late reversal reaction. The presence of viable \(M. leprae\) was demonstrated by growth in the foot pads of non immuno-suppressed Swiss white (S/W) mice in 12 patients (48\%). Secondly, the lesions showing sub-clinical T1R (histopathology only) scored higher in the Mouse Foot Pad (MFP) test (7/12 = 58\%) as against non-reaction cases (5/13 = 39\%) suggesting that viable \(M. leprae\) in a given site may be involved in the induction of T1R.\(^8\)

The present study elaborates on the earlier findings using additional tests for bacterial viability.

Materials and methods

STUDY POPULATION/ELIGIBILITY CRITERIA

Newly detected leprosy patients of either sex, aged between 15–60 years, with BT, BB or BL type of disease (Ridley-Jopling scale), and requiring a full course of World Health Organization Multibacillary – Multi Drug Therapy (WHO MB-MDT).

Demonstration of viable \(M. leprae\) in a skin or nerve lesion was done using three tests:

A. immunohistochemical detection of \(M. leprae\) secretory protein Ag 85;
B. detection of \(M. leprae\) specific 16s rRNA using \textit{In situ} reverse transcriptase polymerase chain reaction (\textit{in situ} RT- PCR)
C. Bacterial multiplication using Mouse Foot pad (MFP) technique.

Rationale for choosing a combination of tests for viability

(a) Ag 85, is a 30kDA protein, characterised as mycolyl transferase, secreted during growth and multiplication of \(M. leprae\).\(^9\),\(^10\) Hence presence of Ag 85 in the lesion suggests actively growing \(M. leprae\).
(b) RNA detection is a highly sensitive molecular biological tool. The presence of localization of *M. leprae* specific 16s rRNA will provide further confirmation.\(^\text{11,12}\)

(c) A positive MFP test, which is the gold standard for determining *M. leprae* viability,\(^\text{13}\) will clinch the findings in (a) and (b) above

**TREATMENT FOR LEPROSY**

All study patients were treated with 12 months MB-MDT regimen as per the National Leprosy Eradication Programme WHO/NLEP guidelines.\(^\text{2}\)

*Treatment for reaction/neuritis:* Patients presenting with, or developing (T1R) in the skin or nerve were treated with corticosteroids 40 mg (1mg/kg body weight) tapered to 5mg over 12 to 16 weeks.\(^\text{2}\)

*Determination of sample size:* The sample size was calculated on the basis of ‘sample size for comparison of proportions’.\(^\text{14}\) Based on the fact that around 45% of BT-BL patients present with or develop clinical T1R, at 95% confidence interval, a sample of \(\sim 150\) BT-BL cases was required to be recruited into the study.

*Recruitment and monitoring of patients:* A total of 165, newly diagnosed MB patients fulfilling the Ridley-Jopling classification as BT, BB and BL were recruited in the study.\(^\text{15}\) They were investigated at the baseline as described below and were followed up/monitored for a minimum period of 18 months thereafter.

*Statistical analysis:* Analysis was performed using SPSS version 19.0. The significance of association was tested using Chi-square test. The 95% confidence interval was calculated.

**A: Clinical investigations:**

Detailed history taking and systematic physical and neurological examination with a emphasis on signs and symptoms of reaction/neuritis and deformity (graded using WHO deformity grades) were recorded using a standard protocol created at the Foundation for Medical Research (FMR).

**ETHICAL CLEARANCE**

The study followed International Ethical Guidelines for Biomedical Research involving human subjects (CIOMS/WHO, 1993). This study received ethical clearance from the Ethics Committee of the Foundation for Medical Research. This included permission for skin and nerve biopsies. Written consent was obtained from individual study subjects before inclusion in the study using a standard consent form. No financial incentives were given to the patients. Travel expenses were reimbursed to the patients on the occasion. Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) cleared the use of animals in the study. The Foundation for Medical Research is registered with CPCSEA and has a valid registration number 424/RO/c/01/CPCSEA.
B: Laboratory investigations:

Slit skin smear examination (SSS): was done from at least three sites by trained paramedical staff. Slides were stained using Ziehl Neelsen Carbol Fushin (ZNCF), and scored for bacteriological index (BI).16

Collection of skin/nerve biopsies: Skin/nerve biopsies were obtained from patients after informed consent and collected as under:

At Registration (onset): Two incision skin biopsies were obtained from all the patients;

(a) Site showing maximum activity/reaction, referred to as Lesion (L) biopsy
(b) Non-lesion area (usually a mirror image site) to serve as control, referred to as Mirror (M).

In 10 Patients biopsy of an involved cutaneous nerve was obtained under local anesthesia. The skin overlying the nerve served as the inactive (non-lesion) M site.

During follow up: At the onset of a repeat episode of T1R, and at 3 months post-release from treatment from those who did not develop any T1R, only the lesion (L) site were biopsied.

Each biopsy was divided in to 3 parts, processed and studied as under

(1) One part was fixed in Formal-Zenker, processed and embedded in paraffin.

1-a Histopathology: Five micron thick paraffin embedded sections stained with Trichrome modified Fite-Faraco (TRIFF) were examined for acid-fast bacilli (AFB), the characteristics of the granuloma and used for leprosy classification using the Ridley-Jopling scale.15

1-b Immunohistochemical detection and localization of Ag85: The paraffin embedded tissue sections were also used as described below. Paraffin sections of lesional and mirror image biopsies were subjected to indirect immunoperoxidase staining using rabbit raised polyclonal antibody (kindly supplied by Prof. Harald Wiker, University of Bergen, Norway) for detection of the antigen. Peroxidase-conjugated swine anti-rabbit (Dakocytomation) served as a secondary antibody. Endogenous peroxidase activity was blocked using 3% H2O2 and 1% foetal calf serum was used for non-specific blocking. The reaction was developed with 3-3’–diaminobenzedine (DAB) as chromogen substrate and counter-stained with haematoxylin.17

(2) Detection of M. leprae specific 16s rRNA using in situ RT PCR
The Second part of the biopsy collected in 10% buffered formalin was used for the detection of 16s rRNA, by direct in situ RT-PCR, this work was conducted at National JALMA Institute for Leprosy and other Mycobacterial Diseases (NJIL & OMD).

In situ reverse transcriptase polymerase chain reaction (in situ RT-PCR) In situ localisation of M. leprae 16s rRNA was carried out using Fast start Taq DNA polymerase, Digoxigenin (DIG) labeled nucleotides, anti DIG antibody labeled with alkaline phosphatase and 5-Bromo-4-Chloro-3'-indolylphosphate - Nitro Blue Tetrazolium (BCIP-NBT) as substrate chromogen assisted detection. A dark blue signal in the cells indicated presence of rRNA, hence of viable M. leprae. Later DAB was preferred; presence of brown precipitate of DAB in inflammatory macrophages, In Schwann cells in the nerve biopsies (number of nerve biopsies = 3) and in dermal nerves in skin biopsies was recorded.11,12
Assessment of *M. leprae* viability using mouse foot pad method (MFP):
The third part of the biopsy was used for MFP study using the standard method. In brief, the
tissue was homogenized with a glass homogenizer within 24 hours of collection. A fixed
volume (i.e. 0·001ml) of the homogenate was uniformly smeared on spot slides, dried and
stained with ZNCF. The number of AFBs was determined using standard protocol outlined by
World Health Organization. The bacillary load per gram weight of tissue was determined,
adjusted to $1 \times 10^4$ AFB/30 μl of homogenate and injected into both hind foot pads of normal
Swiss white (S/W) mice. Skin homogenates that scored negative for AFB were injected
without further dilution. The bacillary count was counted at 6th, 7th, 8th and 12th months post-
inoculation. A foot pad yield of $\geq 1 \times 10^5$ was considered positive.

Results

As depicted in Table 1, of the 165 subjects recruited for this study male to female ratio was
2·3:1 and the average age recorded was 32 ± 14.

CLINICAL

An account of clinical samples examined using different methods is shown in Table 2a. Time Scale for the collection of biopsy specimen at onset as well as re biopsies is depicted in Table 2b.

Frequency of T1R and its recurrence in the subjects: Of the 165 patients recruited for
the study 63 were from T1R (+) group. Of these 63, 58 (35%) were seen with T1R at
registration of which majority had skin only reaction (39 = 67%), 16 (27%) had skin +
nerve reaction and three had only nerve reaction. In the remaining, five (3%) cases, a 1st
episode of T1R was recorded within the first 3 months of starting MDT. Among the 58
cases presenting with T1R, 40 (69%) had single episode and 18 (31%) had multiple (2 or
more) episodes of T1R. In the five cases that developed an incidence of T1R; two had
multiple episodes. Thus in the T1R (+), 20 (32%) had more than one episode of T1R during
the course of this study (Table 3). Whereas, in T1R (−) (n = 102), 11 (10%) cases
developed episode of reaction at any time point after 3 months, of which one had two
episodes.

LABORATORY INVESTIGATIONS

Biopsy figures and results are depicted in Tables 4, 5a, 5b and 6.

<table>
<thead>
<tr>
<th>Variables</th>
<th>No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total no cases</td>
<td>165</td>
</tr>
<tr>
<td>Male patients</td>
<td>115</td>
</tr>
<tr>
<td>Female patients</td>
<td>50</td>
</tr>
<tr>
<td>Male to Female ratio</td>
<td>2·3:1</td>
</tr>
<tr>
<td>Average Age &amp; SD</td>
<td>32 yrs ± 14</td>
</tr>
</tbody>
</table>
I. Histopathological Classification (Ridley-Jopling) and frequency of T1R in the different leprosy types

Of the 165 lesion biopsies studied from 165 patients, histopathological features of BT leprosy were seen in 112 (68%) mid-borderline (BB) in 13 (8%) and BL type of leprosy in 40 (24%) of cases. Frequency of T1R was highest among BB (69%) followed by BT (36%) and BL (35%) (Table 4).

Further, all the cases with clinical evidence of T1R also had histopathological evidence of T1R \( (n = 65) \). In addition, 18 cases (11%) had only histopathological evidence of T1R (sub-clinical).

Among the re-biopsies, 19 (68%) were BT leprosy, three were mid-borderline (BB) and six were BL type of leprosy (Table 8).

II. Bacteriological findings in relation to T1R (Table 5a)

All the 165 cases were scored for the presence of AFB in tissue homogenates and/or in tissue sections (histopathology), 31 (19%) scored positive and others \( (n = 134) \) were negative.

Of the 31 AFB positive in ‘L’, 16 (52%) were T1R (+) and 15 (48%) were T1R (−).

Of the 134 AFB negative patients, 47 (35%) were T1R (+) and 87 (65%) were T1R (−).

Among the 28 re-biopsies, three scored positive for AFB; all three had repeat episodes of T1R and were from T1R (+) group.

III. Immunohistochemical detection and localization of Ag 85: (Figures 1 & 2)

(a) As compared to AFB scores, percentages of both ‘L’ and ‘M’ scoring positive with Antigen 85 are significantly higher in all three types of leprosy i.e. BT, BB and BL, proving its higher sensitivity. (b) Positive scores with Ag 85 are significantly higher in ‘L’ as compared to ‘M’ particularly in BT lesions. c) Overall positive scores (%) is higher in T1R(+) (74%) group as compared to T1R (−) (66%) but the difference is not statistically significant \( (P = 0.6) \). (Table 5a)

Table 2a. Account of number of clinical samples examined at onset and re-biopsies using different methods

<table>
<thead>
<tr>
<th>Test</th>
<th>Lesion (onset)</th>
<th>Mirror (onset)</th>
<th>Lesion (rebiopsies)</th>
<th>Mirror (rebiopsies)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histopathology</td>
<td>162</td>
<td>162</td>
<td>28</td>
<td>22</td>
</tr>
<tr>
<td>MFP</td>
<td>158</td>
<td>141</td>
<td>28</td>
<td>21</td>
</tr>
<tr>
<td>Ag 85 detection</td>
<td>76</td>
<td>73</td>
<td>23</td>
<td>18</td>
</tr>
<tr>
<td>16s RNA- in situ RT PCR</td>
<td>28</td>
<td>28</td>
<td>9</td>
<td>8</td>
</tr>
</tbody>
</table>

Table 2b. Time Scale of biopsies collected at onset and re-biopsies in T1R (+) and T1R (−) cases

<table>
<thead>
<tr>
<th>Type</th>
<th>Onset</th>
<th>3–6 m</th>
<th>7–9 m</th>
<th>10–12 m</th>
<th>&gt;12 m</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1R (+)</td>
<td>63</td>
<td>7</td>
<td>2</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>T1R (−)</td>
<td>102</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>13</td>
</tr>
</tbody>
</table>
A semi-quantitative assessment of Ag 85 positivity is depicted in Table 6. Notably, 20/38 (52%) L biopsies from T1R (+) cases scored ≥ 2+ and 11/20 (55%) were BT-BB cases, indicating that intensity of staining is disproportionately higher among the BT lesions showing T1R.

Localization of Ag85:

Localization of Ag 85 staining was restricted to areas where M. leprae could be found viz. strong antigen 85 positivity was seen in dermal nerves mainly in the Schwann cells and in the inflammatory cells such as macrophages (Figure 3). Occasionally muscle spindles in the skin also scored positive for Ag 85.

An account of antigen 85 positivity recorded in the skin/dermal nerves:

Skin lesions from 26 patients scored positive for Ag 85 in the dermal nerves. Notably 19 among them (73%) were with T1R (+), showing a strong association between presence of Ag 85 within the dermal nerve and T1R.

A similar positive association was also seen in the re–biopsies. Time scale of the re-biopsy is given in Table 2b. Of the 23 re-biopsies (all lesions), including 13 with T1R (+) and 10 without T1R (−), the proportion of cases with positivity in T1R (+) was 8/13 (62%), four among them were BT cases and T1R (−) was 5/10 (50%), was not statistically significant.

Overall, 13/23 (57%) cases were Ag 85 +ve, indicating presence of viable bacilli among cases receiving 6–12 months of MB-MDT (Table 7).

IV. In situ reverse transcriptase polymerase chain reaction (in situ RT-PCR) (Figures 4 & 5)

(a) Overall, the proportion of cases scoring positive in the 16s rRNA detection method was higher than Ag85 in both ‘M’ and ‘L’ biopsies indicates higher sensitivity of the former.

(b) In the lesions the scores were higher in T1R (+) group as compared to T1R (−) (15/16 (94%) and 10/12(83%) respectively) however the difference was statistically not significant (Table 5a).

Table 3. Account of T1R at onset, developed within 3 months and its recurrence

<table>
<thead>
<tr>
<th>Site of Reaction</th>
<th>At registration (n = 58) (%)</th>
<th>Developed within 3 m (n = 5)</th>
<th>Total (n = 63) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Only Skin</td>
<td>39 (67)</td>
<td>5</td>
<td>44 (70)</td>
</tr>
<tr>
<td>Skin+Nerve</td>
<td>16 (27)</td>
<td>0</td>
<td>16 (25)</td>
</tr>
<tr>
<td>Only Nerve</td>
<td>3 (5)</td>
<td>0</td>
<td>3 (5)</td>
</tr>
<tr>
<td>No of reaction episodes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single</td>
<td>40 (69)</td>
<td>3</td>
<td>43 (68)</td>
</tr>
<tr>
<td>≥2 episodes</td>
<td>18 (31)</td>
<td>2</td>
<td>20 (32)</td>
</tr>
</tbody>
</table>

Table 4. Overall frequency of T1R and leprosy class at onset (RidleyJopling, histopathological scale)

<table>
<thead>
<tr>
<th>Variables</th>
<th>BT</th>
<th>BB</th>
<th>BL</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1R (+)</td>
<td>40 (36%)</td>
<td>9 (69%)</td>
<td>14 (35%)</td>
<td>63 (38%)</td>
</tr>
<tr>
<td>T1R (−)</td>
<td>72</td>
<td>4</td>
<td>26</td>
<td>102</td>
</tr>
<tr>
<td>Total</td>
<td>112</td>
<td>13</td>
<td>40</td>
<td>165</td>
</tr>
</tbody>
</table>
**Type 1 reaction and viable Mycobacterium leprae**

**Table 5a.** Results of lab tests i.e. AFB, Ag85, 16s rRNA and MFP in relation to T1R(+) and T1R(−) in different leprosy class expressed as No. positive/No. tested in the Mirror (M) and lesion (L) sites

<table>
<thead>
<tr>
<th>Leprosy Class</th>
<th>Groups</th>
<th>Biopsy site</th>
<th>AFB detection (in Homogenate or histopathology)</th>
<th>Ag85 detection</th>
<th>16s rRNA- detection (In situ RT-PCR)</th>
<th>Growth in the Mouse Foot Pad</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>BT (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>BB (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>BL (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1R (+)</td>
<td>M</td>
<td>1/37</td>
<td>0/8</td>
<td>2/13</td>
<td>3/58 (5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>3/40 (7)</td>
<td>2/9 (22)</td>
<td>11/14 (79)</td>
<td>16/63 (25)</td>
<td></td>
</tr>
<tr>
<td>T1R (−)</td>
<td>M</td>
<td>0/62</td>
<td>0/4</td>
<td>1/20</td>
<td>1/86 (1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>2/72 (3)</td>
<td>0/4</td>
<td>13/26 (50)</td>
<td>15/102 (15)</td>
<td></td>
</tr>
<tr>
<td>T1R (+)</td>
<td>M</td>
<td>4/20 (20)</td>
<td>1/7 (14)</td>
<td>5/10 (50)</td>
<td>10/37 (27)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>13/21 (62)</td>
<td>5/7 (71)</td>
<td>10/10</td>
<td>28/38 (74)</td>
<td></td>
</tr>
<tr>
<td>T1R (−)</td>
<td>M</td>
<td>5/23 (22)</td>
<td>1/3</td>
<td>6/10 (60)</td>
<td>12/36 (34)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>14/25 (56)</td>
<td>2/3</td>
<td>9/10 (90)</td>
<td>25/38 (66)</td>
<td></td>
</tr>
</tbody>
</table>

Key: T1R (+) - reaction group, T1R (−) - no reaction group, M – Mirror biopsy, L – Lesion biopsy, BT- Borderline tuberculoid, BB- Borderline, BL- Borderline lepromatous, AFB- Acid fast bacilli, 16s rRNA- In situ RT-PCR (in situ reverse transcriptase polymerase chain reaction)

\( a \) – p value: 0.00008 [OR = 5.7, RR: 2.7 (95%CI)] (between T1R (+) and T1R (−) group [BT class])

\( b \) – p value: 0.00018 [OR = 0.27, RR: 0.52 (95%CI)] (between T1R (+) and T1R (−) group [total values])

(c) In the re-biopsies, all 4 from T1R (+) and 3/5 from T1R (−) scored positive for *M. leprae* specific 16s rRNA by in situ RT-PCR, further ascertaining the presence of viable bacteria in patients on treatment (MB-MDT) or having completed treatment (Table 7).

V. Viability of *M. leprae* assessed through Mouse foot pad test

Mouse foot pad test results of 158 Lesion (L) & 141 Mirror (M) biopsies under different leprosy type are depicted in Table 5. The former includes 11 nerve biopsies. Overall, 74 (46%)

**Table 5b.** Comparative analysis of number of cases positive for 2 (i.e. Ag85 and MFP) and 3 (i.e. Ag85, 16s rRNA and MFP) tests in smear + ve and smear − ve T1R (+) and T1R(−) groups at onset in select cases

<table>
<thead>
<tr>
<th></th>
<th>Smear + ve</th>
<th>Smear − ve</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 Tests + ve</td>
<td>3 Tests + ve</td>
</tr>
<tr>
<td>T1R (+)</td>
<td>8</td>
<td>11</td>
</tr>
<tr>
<td>T1R (−)</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>
of L [40 in T1R (+) and 34 in T1R (−)] & six (4%) of M scored positive in the MFP test. All six M biopsies that scored positive were from BL cases. A significantly higher proportion of L biopsies from T1R (+) patients tested positive in the MFP test, confirming the presence of viable *M. leprae* 40/60 (66%) as compared to T1R (−) 34/98 (35%) and this difference is statistically highly significant (*P* = 0.00018) (Table 5a).

**MFP FINDINGS IN AFB–VE CASES**

Among the 74 MFP +ve cases in ‘L’ biopsies, 24/40 (60%) in T1R (+) and 24/34 (70%) in T1R (−) were AFB–ve in the tissue homogenates. Majority were BT-BB type of lesion. Four cases in T1R (−) which were AFB–ve and MFP +ve developed an episode of reaction at a time point after 3m and were BT-BB. Overall, MFP positivity among the AFB–ve biopsies was 24/42 (51%) in T1R (+) and 24/87 (27%) in T1R (−) which was also significant (*P* = 0.01).

A small number of mirror (M) biopsies from both the groups i.e. T1R (+) two (3%) and T1R (−) four (5%) respectively scored positive in the MFP and difference was not significant.

None of the 28 re-biopsies, including 15 T1R (+) and 13 T1R (−) cases subjected to MFP test showed any fold increase/growth in MFP (Table 7).

Comparative analysis of all three tests viz growth in MFP, detection of Ag85 and 16srRNA, wherever done, in smear +ve and smear –ve patients, reveal that proportion scoring all tests negative is higher in the T1R (−) [8/20 = 40%] as compared to T1R (+) [4/26 = 15%]. While a positive result is an indicator of, or in case MFP test confirms the presence of viable bacteria, a negative finding does not rule out the presence of viable bacteria, considering that a small part of biopsy specimen was tested. (Table 5b)

To summarise, at onset while the MFP test showed a highly significant difference in the positive score between the two groups, whereas in the other two tests i.e. Ag 85 and 16s rRNA, the overall proportion of positive scores was higher in T1R (+) group but the difference was statistically not significant. Thus antigen based tests displayed lower discriminatory power. In case of repeat biopsies, however the antigen based tests showed a better discriminatory power while mouse foot pad test was of no value.
In leprosy around 25–50% of borderline group of patients (BT to BL) experience Type 1 Lepra reaction during the course of the disease before the initiation of treatment, during and even after the completion of MDT and is the commonest cause of morbidity.\textsuperscript{3,5} Reaction and ensuing nerve damage is managed by the use of immunosuppressive/anti-inflammatory drugs such as corticosteroids.\textsuperscript{2} However, the important question of what precipitates such a reaction, which is key to its prevention/prediction and management remain unclear.

The present study is built around a working hypothesis viz; viable \textit{M. leprae} play an important role in the precipitation of Type 1 reaction in leprosy. To demonstrate the presence of viable \textit{M. leprae} in a reacting lesion, three tests were applied concurrently viz. growth in

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figures1.png}
\caption{Skin lesion biopsies from a BB case stained with Antigen 85 antibody. Note positive but weak Ag 85 staining at onset (Figure 1) with out T1R vs the a-re-biopsy obtained during an episode of T1R (Figure 2) showing higher intensity of staining (arrow).}
\end{figure}
the mouse foot pad, immunohistochemical detection of \textit{M. leprae} secretory protein Ag 85, and detection of \textit{M. leprae} specific 16s rRNA – using \textit{in situ} RT-PCR.

It is important to state the known limitations in the use of these methods viz:

(1) viable bacteria are present in the normal course of the untreated disease
(2) none of the test/s quantifies the number of viable bacteria in a given sample
(3) none of these tests by itself or in combination fulfill the desired sensitivity and specificity

Taking these limitations into account, our first assumption is, since T1R is an immune mediated Delayed-Type Hypersensitivity (DTH) type of reaction mounted by the host against bacteria there should be higher /better killing of bacteria at the site of reaction which in turn should result in decrease in number of viable bacteria. It is anticipated therefore, overall positive score (%) should be lower in T1R (+) lesions/cases, in a given test denoting viability.

Our second assumption is, if there is a higher positive score during or before a clinical reaction and its persistence during a relapse, this could be a good indication of their involvement in the process.

\textbf{Table 7.} Results of various test in re biopsies i.e. detection of Ag85, 16s rRNA- \textit{In situ} RT-PCR and MFP methods in Lesion (L) and Mirror (M) expressed as No. positive/No. tested among T1R (+) and T1R (−) groups

<table>
<thead>
<tr>
<th></th>
<th>Lesional re-biopsy</th>
<th>Mirror re-biopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TIR (+)</td>
<td>TIR (−)</td>
</tr>
<tr>
<td>Ag85 positive</td>
<td>8/13 (62)</td>
<td>5/10 (50)</td>
</tr>
<tr>
<td>16s RNA–\textit{in situ} PCR</td>
<td>4/4</td>
<td>3/5</td>
</tr>
<tr>
<td>Viability using MFP test</td>
<td>0/15</td>
<td>0/13</td>
</tr>
</tbody>
</table>

\textit{Key:} T1R (+) - reaction group, T1R (−) - no reaction group, 16s rRNA- \textit{In situ} RT-PCR - \textit{In situ} reverse transcriptase polymerase chain reaction.
In this study, comparison is between a reaction lesion (skin or nerve) T1R (+) with no reaction T1R (−) lesions. Only patients presenting with or developing clinical signs of T1R within the first 3 months of initiation of MDT in the skin with or without neuritis are considered as T1R (+) cases.

As anticipated, presence of viable bacteria, were demonstrated in both T1R (+) and T1R (−) group of patients. However, there was a marked difference in the quality or metabolic state of *M. leprae* detected in the two groups was evident from the following three findings;

1. A significantly higher proportion of lesion biopsy homogenates obtained at onset, from T1R (+) cases scored positive (have shown unequivocal growth in MFP test, proving the presence of viable bacteria), as compared to T1R (−) (*P* < 0.005). Also, MFP positivity among AFB –ve L biopsies was significantly higher in T1R (+) (*P* < 0.05). In contrast, a small proportion of non lesion biopsies (mirror site) scored positive in both T1R (+) and T1R (−) group and the difference was not statistically significant.

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**Figure 4.** Demonstration of *M. leprae* specific 16 s rRNA using in situ RT PCR, BT skin lesion collected during 2nd episode of T1R at 6 months. Dermal nerve showing positive signal (arrow).

**Figure 5.** Skin lesion from a BT case, collected during 2nd episode of T1R at 13 months. *M. leprae* specific 16s rRNA positive signal (arrow) is seen in a small dermal vessel.
(2) With respect to Ag85, while the overall positive scores were higher in the T1R (+) group but the difference was not statistically significant, the intensity of staining (grade ≥ 2+) was disproportionately higher in BT – BB lesions with T1R (+) (11/20 = 55%).

(3) In the re biopsies obtained during a repeat episode of T1R, Ag 85 as well as 16s rRNA, positive scores (%) were higher in the T1R (+) lesions.

It can be inferred therefore ‘viable’ bacteria are an essential component and probably play a crucial role in Type 1 reaction.

Conversely, difference in the quality of bacilli and not the quantity or the ratio of dead to viable; play a role in the precipitation of Type 1 reaction in a given site.

Supportive evidence for the above statement also comes from a preliminary study finding, using real time PCR (results not shown). A higher cDNA copy numbers of ilyA gene (indicator of metabolically active M. leprae) in lesion (L) biopsies from T1R (+) cases as compared to T1R (−) cases were detected.

Another study using real time analysis demonstrates presence of significant amount of mRNA for the hsp18 gene, in reaction lesions indicating the existence of live bacilli in reversal reaction cases. Other documented evidence viz (a) higher incidence of viable M. leprae in cases with sub-clinical (histopathological) evidence of T1R as compared to T1R (−) cases. (b) higher occurrence of viable M. leprae among patients with late onset reaction are also in support. Further, bacteriologically positive patients are 3-2 times at a higher risk of developing Type 1 reaction is in consonance with the earlier documented findings. One of the widely accepted views is that, M. leprae products (antigens) are involved in the precipitation of T1R. It is believed, M. leprae antigens such as PGL-1 and 18 kDa antigen (stress protein) may be involved in the precipitation of T1R. Autoimmune response to bacillary antigens as a cause for T1R precipitation was suggested by Naafs. As opposed to these our study demonstrated the presence/involvement of metabolically active M. leprae in the precipitation of T1R.

Notably in the re-biopsies collected during or on completion of treatment, all four from T1R (+) and 3/5 from T1R (−) scored positive for M. leprae specific 16s rRNA by in situ RT-PCR, ascertaining the presence of viable bacteria. Proportion of cases scoring positive with antigen 85 were higher in T1R (+) lesions. None scored positive in the MFP test is attributable to its limitation as a test.

Besides the strong association seen between viable bacteria and Type 1 reaction, detection of Ag 85 positivity in 13/23 (57%) and M. leprae specific 16s r RNA in 7/9 (77%) of re-biopsies, fact that they were patients receiving > 6 months or having completed 12 months of MB-MDT, imply that M. leprae remain refractory to antileprosy treatment is worrisome.
This finding has an important bearing on the management of T1R cases as well as raises question regarding the efficacy of MDT.

Reactions that occur post release from treatment in around 5–10% of cases, termed as ‘late reversal’ reaction, are generally being treated with corticosteroids alone under the assumption that these reactions are precipitated by bacterial products (antigens).\textsuperscript{25,26} Findings from this study allow us to emphatically state that any long term treatment with steroids must be covered with anti-leprosy treatment.

Not surprisingly, a strong association was seen between T1R and the localisation, increased frequency as well as intensity of antigen 85 staining in the dermal nerves. The Schwann cells in particular are a known reservoir of \textit{M. leprae}.\textsuperscript{27} It is conceivable therefore, survival of \textit{M. leprae} within the Schwann cells/nerves, increases the vulnerability of patients towards reaction and neuritis.

This study using three viability assessment methods viz. detection of \textit{M. leprae} secretory protein i.e. Ag 85, detection of \textit{M. leprae} specific 16s r RNA using \textit{in situ} RT PCR and growth in MFP, firstly demonstrates the presence of viable \textit{M. leprae} in the reaction lesions in a significantly higher proportion of cases in the mouse foot pad test that is the gold standard, confirming the presence of viable \textit{M leprae}, are an essential component of T1R, thus strengthening our hypothesis. Importantly, the bacilli are ‘metabolically active’ and results of the three viability tests complement each other.

We are aware that the three tests used in this study have no practical value in predicting or diagnosing T1R.

Preliminary data (results not shown) show over-expression of \textit{M. leprae} gene associated with growth and metabolic activity viz \textit{tlyA} gene in real time PCR. This is in line with the positive association seen between metabolically active \textit{M. leprae} and T1R. Study using Expression analysis of genes related to metabolism and virulence of \textit{M. leprae} from human host by micro-array has shown over expression of \textit{accA3} gene in patients with reaction.\textsuperscript{28} These certainly have the potential to be developed as a marker for predicting T1R.

In conclusion, our study findings show that ‘metabolically active’ \textit{M. leprae} is a component/prerequisite and the secretory protein Ag 85, might be the trigger for precipitation of T1R.

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