Persister Studies in Leprosy Patients after Multi-Drug Treatment

U. D. Gupta, K. Katoch, H. B. Singh, M. Natrajan, and V. M. Katoch

ABSTRACT

Cutaneous biopsies were collected from leprosy patients who attended the out-patient department of the Institute for treatment at different intervals, i.e., 12 months, 18 months, 24 months, 36 months, and more after beginning the multi-drug treatment therapy (M.D.T.). The patients belonged to the two drug regimens; (i) standard multibacillary (MB) M.D.T. after 12, 24, and 36 months; or (ii) standard M.D.T. + Minocycline 100 mg once a month (supervised) + Ofloxacin 400 mg once a month supervised for 12 months. Biopsies were processed for mouse footpad inoculation and for estimating ATP levels by bioluminescence assay as per established methods. Viable bacilli were observed in 23.5% up to 1 year, 7.1% at 2 years, and in 3.84% at 3 years of M.D.T. by MFP and 29.4%, 10.7%, and 3.84% by ATP assay in the M.D.T. group at the same time period, respectively, but not in M.D.T. + Minocycline + Ofloxacin group after one year. The overall percentage of persisters was 5.55% by MFP and 7.14% by ATP assay up to 3 years of treatment.

RESUME

Des biopsies cutanées furent prélevées à intervalles successifs (12, 18, 24, 36 mois et plus) de patients hanséniens traités au service de consultation externe de l’Institut, après mise en œuvre de la polychimiothérapie (PCT). Les patients furent répartis en 2 types de PCT : (i) PCT multibacillaire standard après 12, 24 et 36 mois et (ii) PCT standard + Minocycline 100 mg une fois par mois (prise contrôlée) + Orofloxacine 400 mg une fois par mois en prise contrôlée pendant 12 mois. Les biopsies furent préparées pour le test d’inoculation à la patte de souris (IPS) et pour l’estimation des niveaux d’ATP par bioluminescence selon des méthodes bien établies. Des bacilles viables furent observés dans 23.5% des biopsies jusqu’à 1 an ; 7.1% après 2 ans et 3.84% après 3 ans de PCT par le test IPS et 29.4% ; 10.7% et 3.84% par test de l’ATP pendant les même temps après PCT, respectivement, mais pas chez le groupe PCT + Minocycline + Orofloxacine après 1 an. Le pourcentage global de patients avec bacilles persistants était de 5.55% d’après le test IPS et de 7.14% d’après le test à l’ATP après 3 années de traitement.

RESUMEN

Se trabajó con pacientes con lepra que acudieron al Instituto para su tratamiento. Los pacientes se asignaron a dos grupos, uno que recibió la poliquimioterapia (PQT) estándar para lepra multibacilar (MB) y otro que recibió la PQT estándar combinada con Minociclina (100 mg mensuales) y Ofloxacina (400 mg mensuales), ambas drogas administradas de manera supervisada por 12 meses. De cada paciente se tomaron biopsias de piel a los 12, 18, 24 y 36 meses o más, después de haber iniciado el tratamiento. Las biopsias fueron procesadas para su inoculación en la almohadilla plantar del ratón (APR) y para la medición de sus niveles de ATP por bioluminiscencia, de acuerdo a métodos ya establecidos. En el grupo tratado con PQT se observaron bacilos viables en el 23% de las biopsias a un año del seguimiento, en el 7.1% de las biopsias a los 2 años, y en el 3.8% a los 3 años usando la técnica de la APR, y en el 29.4% 10.7% y 3.84% de las biopsias usando el ensayo de ATP, a los mismos intervalos de tiempo. En las biopsias de piel del grupo tratado con PQT + Minociclina + Ofloxacina no se observaron bacilos después de un año de tratamiento. El porcentaje global de “persistentes” fue de 5.5% por el ensayo de la APR y de 7.14% por el ensayo de ATP a los 3 años del tratamiento.

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In pre-multi-drug therapy (M.D.T.) era, persistence of drug sensitive Mycobacterium leprae and emergence of drug resistant mutants despite prolonged therapy with DDS was reported to be the cause of treatment failures in lepromatous patients \(^{(14,21)}\). With the introduction of rifampicin, it was expected that in addition to a rapid decrease in the infectivity of multibacillary (MB) cases, the above problems would also be taken care of if drugs were used alone \(^{(22)}\) or in combination \(^{(13)}\). With the M.D.T. of leprosy, the results have been satisfactory as it has been generally effective in reducing the viable load as well as duration of treatment in MB cases. However, the persistence of drug sensitive viable organisms has been demonstrated after varying durations of treatment at different sites by several workers \(^{(6,8,9,11,16,20)}\). These persisting bacilli have special significance as they have the potential of causing relapse in MB cases after M.D.T. \(^{(8,12)}\). This study has been initiated to gain an overview of this problem and follow the recent trends in multibacillary cases treated with M.D.T.

**MATERIALS AND METHODS**

One hundred twenty six biopsies from ninety six borderline lepromatous (BL)/polar lepromatous (LL) patients attending the outpatient department of Central JALMA Institute for Leprosy and Other Mycobacterial Diseases were included in this study. The age of the patients ranged from 16 to 60 years. All these patients did not suffer from any chronic disease like diabetes mellitus, tuberculosis, hypertension, etc., and showed no clinical evidence of resistance. The patients belonged to the two drug regimens: (i) standard MB M.D.T. after 12, 24, and 36 months; (ii) standard M.D.T. + Minocycline 100mg once a month (supervised) + Ofloxacin 400mg once a month (supervised) for 12 months \(^{(7)}\). Before starting the treatment, these patients were examined in detail, clinical findings were charted and recorded, and smears were taken from different sites for calculation of bacterial index (B.I.). At the start of therapy, the average B.I. ranged from 2 to 5+ for regimen 1 (mean 3.6), and from 1 to 4+ for regimen 2 (mean 2.21) on the Ridley scale \(^{(15)}\). The biopsies were processed for mouse footpad inoculation and bacillary ATP assay \(^{(5,11)}\) as used earlier by us.

**Mouse footpad inoculation.** The footpads were homogenized and the bacterial enumeration was done as described by D’Arcy and Rees \(^{(2)}\). A batch of five random bred BALB/C mice was taken and each hind mouse footpad was inoculated with 0.03 ml suspension containing 5000 to 10,000 bacilli. The bacilli were harvested at six months and eight months (50% at each stage) after inoculation and acid–fast bacilli (AFB) were counted \(^{(3)}\). The footpad pools were used for enumeration of the bacilli. The percentage of viable persisters being low, even a 10-fold increase in the harvest count was taken as evidence for bacillary growth \(^{(10)}\).

**ATP assay.** The biopsies were processed, bacillary ATP was extracted and assayed as per the technique standardized in our laboratory \(^{(11)}\). ATP levels were estimated and expressed as pg/million of AFB. Cultures were set up in the final preparation to rule out contamination with any cultivable mycobacteria or any other organism.

**RESULTS**

The details of specimens showing viability after different durations of M.D.T. by mouse footpad as well as ATP are presented in Table 1. Out of 126 biopsies, 71 biopsies belonged to patients treated with standard M.D.T. regimen while 55 biopsies belonged to patients treated with standard M.D.T. + 100 mg of Minocycline + 400 mg of Ofloxacin once a month (supervised). After one year of treatment, out of 17 biopsies (MDT), 4 were found to be positive for viable M. leprae by mouse footpad and 5 by ATP method while out of 55 biopsies belonging to M.D.T. + Minocycline + Ofloxacin group, none was found to positive. The range of B.I. of these patients were 2 to 5+ (average 3.60) and 1 to 4+ (average 2.2), respectively. By the mouse footpad method, the Fisher exact test of viability of M. leprae at one year between regimen 1 (4/17) and regimen 2 (0/55) is highly significant (p = 0.002). Similarly, by the ATP method, the Fisher exact test of viability of M. leprae at one year between regimen 1 (5/17) and regimen 2 (0/55) is highly significant. The results from the percentage of patients with viable bacilli at all time periods from regimen 1 (7/126) with viable bacilli from regimen 2 at one year is statistically significant (p = 0.02).
TABLE 1. Percentage of biopsies showing positivity for viable M. leprae after different durations of multi-drug therapy.

<table>
<thead>
<tr>
<th>Duration of treatment</th>
<th>Range and average BI at the time of biopsy</th>
<th>MFP+(%)</th>
<th>ATP +%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(1)</td>
<td>(2)</td>
</tr>
<tr>
<td>6 months to 1 yr of treatment</td>
<td>3.6 (2–5+) 2.21 (1–5+)</td>
<td>44/17 (23.5%)</td>
<td>0/55 (0%)</td>
</tr>
<tr>
<td>&gt;1 yr to 2 yrs of treatment</td>
<td>2.55 (1–5+)</td>
<td>2/28 (7.1%)</td>
<td>3/28 (10.7%)</td>
</tr>
<tr>
<td>&gt;2 yrs to 3 yrs of treatment</td>
<td>1.81 (1–3+)</td>
<td>1/26 (3.84%)</td>
<td>1/26 (3.84%)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>7/126 (5.55%)</td>
<td>9/126 (7.14%)</td>
</tr>
</tbody>
</table>

BI: Bacteriological index; MFP: Mouse Footpad; ATP: Bioluminescence assay; (1) conventional MDT; (2) Conventional MDT + Minocycline 100 mg once a month supervised + Ofloxacin 400 mg once a month supervised; (3) Overall.

Similarly, out of 28 biopsies from patients who had M.D.T. up to 2 years, 2 biopsies showed AFB counts by mouse footpad and 3 were positive for bacillary ATP. The mean B.I. of these biopsies ranged from 1 to 5+ (average 2.6). Further, out of 26 biopsies from patients who had taken up to 3 years of M.D.T. or more, 1 biopsy showed positivity by mouse footpad as well as by ATP assay. Statistically, the differences between the two methods were non–significant. The B.I. of the patients ranged from 1 to 3+ (average 1.81). Overall, out of 126 biopsies included in this study, 7 (5.55%) showed evidence of viability by mouse footpad, whereas 9 (7.14%) showed positivity by ATP bioluminescence. The results of quantitative relationship between bacillary ATP and mouse footpad showed that when ATP levels were in the range of 0.36 to 3.59 pg/million, both techniques were equally good (positives were 7/126). However, two cases whose bacillary contents were in the range of 0.039 to 0.04 pg/million bacilli did not show growth by mouse footpad (Table 2).

The correlation of initial B.I. and M. leprae viability after chemotherapy was analyzed and is presented in Table 3. At one year of treatment with standard M.D.T., it was observed that in biopsies in initial B.I. up to 2+, no viability was observed by either of the method. Further, in biopsies with initial B.I. of 2 to 3.9+, viability was observed in 4/53 (7.55%) and 5/53 (9.4%) biopsies by mouse footpad and ATP, respectively. However, in biopsies with initial B.I. of 4+ and more, higher viability was observed [3/13 (23.1%) by MFP, and 4/13 (30.8%) by ATP]. The differences in viability in biopsies between group 2 to 3.9+ and 4+ and more, the differences were significant.

**DISCUSSION**

M.D.T. campaigns have led to a major decline in the prevalence of leprosy. However, it continues to be an important public health problem in many parts of the world. Despite the regular administration of M.D.T., live bacilli persist in a section of leprosy cases. A number of workers have demonstrated these live persisters by growth in mouse footpads inoculated by M. leprae in pre-M.D.T. (13, 14), as well as post-M.D.T. era (5, 9, 16, 17, 18). W.H.O. and the some national agencies such as in India have recently recommended that treatment in MB cases be stopped after 1 year of treatment. In this study, 23.5% of the specimens showed growth by mouse footpad while 29.4% of the specimens showed growth by ATP assay in patients treated with conventional M.D.T. after 1 year of treatment (Table 1). On the other hand, none of the specimens showed growth by mouse footpad as well as ATP assay in patients treated with M.D.T. + Minocycline + Ofloxacin, clearly indicating that addition of Minocycline and Ofloxacin in the treatment regimen was quite effective as no viable persisters were detectable after 1 year of treatment (7). However, in the present
study as well as in other studies live bacilli have been demonstrated after one year of treatment with conventional M.D.T. Out of the 17 specimens in the up to one year M.D.T. group, 6 had received M.D.T. for 6 months out of which one showed growth (having initial B.I. of 5+), and 11 received M.D.T. for 1 year and growth was seen in 3 patients (having initial B.I. of 4+ and more, and 3 to 4+ after one year of treatment). These observations clearly indicate that there is a potential risk associated with stopping the therapy at one year mainly in such patients who are having high initial B.I. However, the adequacy of one year treatment in such cases can only be known after experience of follow-up studies become available. Up to 2 years of M.D.T. (13 to 24 months), 2 out of 28 biopsies (7.1%) showed growth in mouse footpad and significant ATP was detected in 3 out of 28 (10.7%) of the biopsies. In patients who had taken M.D.T. from 25 to 36 months, 1 out of 26 (3.84%) biopsies was positive by both, i.e. mouse footpad as well as ATP assay. Overall, 7 out of 126 (5.55%) and 9 out of 126 (7.14%) biopsies by mouse footpad and ATP assay were observed to be positive which are in agreement with earlier reports where persister rates of 9 to 16% varying periods of MDT have been reported (5, 6, 9, 19, 20). On the other hand, much higher persister rates has also been reported by Shetty, et al. (16, 17, 18) in nerves and skin of leprosy patients. There has been good concordance between viability determination by mouse footpad and ATP when ATP levels were in the range of 0.36 to 3.59 pg/million but when ATP levels were lower mouse footpad failed to detect any positivity as reported earlier by Gupta, et al. (5).

In the present investigation, the patients of standard M.D.T. were on continuous M.D.T. until smear negativity (at least 2 years). It is difficult to foresee how these patients would have behaved if they had been on one year fixed duration M.D.T. Persisters have been reported to be the cause of relapses after 4 to 9 years in well conducted drug trials with adequate follow-up (12). There are reports which suggest that patients with high pre-treatment M. leprae loads are at higher risks of relapse if the treatment is stopped after 2 years W.H.O.-M.D.T./Fixed Duration Therapy compared to patients treated till point of smear negativity (14). Further, it is apparent that in biopsies with initial B.I. of 1 to 1.9, the M.D.T. alone or in combination with minocycline and ofloxacin, no viable organisms were observed. But when the initial B.I.s were 2 to 3.9+ or = 4, the percentage of specimens showing viable organisms increased (7.55% and 22.1% by mouse footpad and 9.4% and 30.8% by ATP). All the specimens in which viable organisms could be demonstrated beyond one year had the initial B.I. of ≥ 4+ (Tables 1 and 3). Other studies at our institute have also shown that highly bacillated cases dropping out of treatment up to 12 to 18 months had higher relapse rates (8). These cases are very small proportion of all leprosy cases as <2% of current cases have B.I. of 3+ or more (unpublished data by Katoch, et al.). It will be interesting to observe the progress

<table>
<thead>
<tr>
<th>ATP content (pg/million)</th>
<th>Positive by ATP</th>
<th>Positive by MFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.36–3.59</td>
<td>7/126</td>
<td>7/126</td>
</tr>
<tr>
<td>0.04–0.359</td>
<td>1/126</td>
<td>0/126</td>
</tr>
<tr>
<td>0.02–0.039</td>
<td>1/126</td>
<td>0/126</td>
</tr>
<tr>
<td>Total</td>
<td>9/126</td>
<td>7/126</td>
</tr>
</tbody>
</table>

**Table 3.** Correlation of initial B.I. and M. leprae viability after chemotherapy.

<table>
<thead>
<tr>
<th>Initial B.I.</th>
<th>No. of biopsies</th>
<th>MFP+</th>
<th>ATP+</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–1.9+</td>
<td>60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2–3.9+</td>
<td>53</td>
<td>4(7.55%)</td>
<td>5(9.4%)</td>
</tr>
<tr>
<td>4–5+</td>
<td>13</td>
<td>3(23.1%)</td>
<td>4(30.8%)</td>
</tr>
<tr>
<td>126</td>
<td></td>
<td>7</td>
<td>9</td>
</tr>
</tbody>
</table>
of such patients after stopping M.D.T. at other centers where M.D.T. is being given. Further, there is a need to carry out surveillance studies in larger number BL/LL patients to know the trends of persisters as well as the resultant relapses.

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