Evaluation of *Mycobacterium leprae* antigens in the monitoring of a dapsone-based chemotherapy of previously untreated lepromatous patients in Cebu, Philippines

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Summary Thirty-five previously untreated lepromatous patients receiving dapsone-based therapy were monitored throughout their 5-year period of treatment by serology and by pathology. Sequentially collected sera were used to evaluate the usefulness of four *Mycobacterium leprae* antigens as used in ELISA to monitor the progress of their therapy. ELISA results were compared with each other and with bacterial load over the treatment period and with duration of treatment. The ELISAs, based on the measurement of IgM antibody reactivity to the two neoglycoproteins (NDO and NTO) representing the phenolic glycolipid antigen of *M. leprae*, were found to be the most effective in monitoring treatment. A whole *M. leprae* based ELISA was less efficient in monitoring treatment because it failed to measure antibodies in 8 out of 35 patients and because it provided consistently lower values than either NTO or NDO. The ELISA-inhibition test based on the detection of antibodies to a species-specific epitope on the 36 K antigen of *M. leprae* was less suitable because of persistent reactivity during therapy, consequently resulting in no significant correlation with ELISA reactivities to NTO or NDO.

Introduction

Leprosy has been aptly described as a disease of 'a slow bacterium'. In terms of treatment, successful progress is often first noted after a year or more of drug therapy. Apparent progress

§ Deceased 8 January 1988
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during the first two years of treatment in incident multibacillary patients is frequently marred by reactional states. One of the difficulties in treatment is unsatisfactory quantitative measurement of a patient’s progress towards a successful outcome. Current measurement of a patient’s response to drug therapy is subjective: clinical observation and bacterial index (BI). Additional quantitative tests would be useful in assisting the clinician in evaluating the result of the medication. The ELISA offers two types of measurements that may be suitable for this purpose: antigen and class-specific antibody detection. Currently, positive antigen detection is limited to those patients with well established disease and very high BI values. Others have reported the inability to detect phenolic glycolipid-I antigen later than 4–6 weeks after initiation of treatment in these patients. Until better techniques become available for antigen detection, current methods will not serve to monitor therapy over the course of treatment. For this reason we have chosen to focus our efforts on evaluating antibody measurements over the course of therapy.

In this article we describe the results of 4 serological tests measuring antibody reactivity to 4 different Mycobacterium leprae antigens throughout the course of a dapsone-based treatment. The sera tested were collected during the course of a 5-year treatment protocol. Three tests measured IgM antibodies, two to neoglycoprotein antigens representing the phenolic glycolipid-I of M. leprae, one of which was a disaccharide antigen (NDO) and the other a trisaccharide antigen (NTO). A third antigen tested was whole irradiated M. leprae (MLEP). The fourth test (INH) was based on the inhibition of monoclonal antibody binding by human sera to a species-specific epitope on the 36 K protein antigen of M. leprae.

Test results were correlated with duration of treatment and bacterial index. Furthermore, the usefulness of the antigens represented by the 4 tests as analysed in relation to each other and in monitoring therapy of lepromatous leprosy patients.

Methods

CASE DATA

Sera for this study were collected from patients who participated in the Joint Chemotherapy Trial (JCT) conducted by the Leonard Wood Memorial Center for Leprosy Research in Cebu, Philippines in collaboration with the Sasakawa Memorial Health Foundation Tokyo, Japan and the Department of Health, Philippines. Sera of patients from two chemotherapeutic protocols (1A and 1C) of the JCT were used. Patients in group 1A received dapsone 100 mg/day, 6 times per week for 5 years and rifampicin 1·2 g once on admission. Patients in group 1C received dapsone 100 mg/day, 6 times per week for 5 years, clofazimine 100 mg 3 times per week for the first 24 weeks and rifampicin 1·2 g once on admission. The patients were previously untreated incident lepromatous (LL) cases free of any serious intercurrent disease and screened by chest X-ray to be free of tuberculosis and of erythema nodosum leprosum (ENL) on admission to the trial. All were clinically lepromatous and histologically LL with an average BI of 4·59 ± 0·4. A total of 38 patients were admitted over a 3-year period, sequentially and randomly allocated to either 1A or 1C. These patients were harbouring dapsone sensitive M. leprae, by mouse footpad inoculation. There were 17 males, ages 12 to 56 years old (average age 25·47 years), on admission to group 1A. Seventeen males, 12–55 years old (average age 22·41 years), and 3 females, 16–22 years old (average age 18·33 years), were admitted into group 1C. Eight patients in 1A and 8 patients in 1C dropped out during the course of the drug trial. Preliminary evaluation did not show any significant differences between the two therapy regimens (1A versus 1C) in terms of clinical improvement, bacteriologic reduction and occurrence of reactional states. Because of the low number of patients in each treatment group, the varying length of patient participation within the trial, no statistically significant differences in ELISA or BI values, and the lack of observable clinical difference in patient recovery between the therapy regimens, the data from the two groups were combined unless otherwise stated.
Sera from each patient were collected on admission and sequentially every 12 months during the 5-year period of therapy. As previously stated, however, some patients have missed collections or dropped out during the therapy period.

**Indirect ELISA**

The ELISA used in this report was an indirect assay using 0.05 ml antigen suspension dried onto ‘U’ bottom microtitre plates (Dynatech, Alexandria, Virginia, USA). Antigen-coated wells were blocked to prevent nonspecific antibody binding by adding 0.075 ml of 5.0% goat serum or 5.0% BSA (bovine serum albumin) in phosphate buffered saline (PBS), pH 7.2 and incubating overnight at 4°C or for 2 hr at 37°C. Serum was diluted 1:500. An antihuman IgM conjugate was used to detect specific IgM activity. The remainder of the assay was performed as previously described.14-16

**Indirect ELISA antigens**

Three antigens were tested: whole armadillo-derived irradiated *M. leprae* and two neoglycoprotein antigens, one of which mimicks the two terminal sugars and the other mimicking the three terminal sugars of the phenolic glycolipid-I (PG-I) of *M. leprae*.7 These semisynthetic antigens are: ND-O-BSA (natural disaccharide) with octyl linkage to bovine serum albumin (BSA); and NT-O-BSA (natural trisaccharide) with an octyl linker arm attached to BSA.17 The octyl-linked neoglycoprotein antigens ND-O-BSA and NT-O-BSA, and whole *M. leprae* were provided under NIH contract (NO1 AI-52582) by Dr P Brennan, Colorado State University. The ELISA in which ND-O-BSA was used as antigen is termed NDO-ELISA; the ELISA using NT-O-BSA is NTO-ELISA; and the ELISA using *M. leprae* is MLEP-ELISA.

**Indirect ELISA antigen preparation**

Immunlon II plates (Dynatech Laboratories, Alexandria, Virginia 22314) were coated with antigen in a volatile coating buffer (0.01 M ammonium acetate/carbonate, pH 8.2). Whole *M. leprae* cells used as ELISA antigen were suspended in a volatile coating buffer at a concentration of 0.04 absorbance units at 420 nm. The ND-O-BSA and NT-O-BSA were diluted to 0.3 μg/ml from a stock solution of 100 μg/ml and mixed thoroughly. Each of the three antigens was coated on microtitre plates by adding 0.05 ml of antigen suspension to each well. The plates were incubated overnight at 37°C to dry the antigen onto the plate. Each well was examined for uniform coating and unevenly coated wells were eliminated from use. The antigen coated plates could then be stored for several months at room temperature.14

**ELISA inhibition test**

Polystyrene ELISA microtitre plates (Dynatech) were coated with 100 μl per well of a soluble *M. leprae* preparation (0.5 μg/ml) in 0.05 M sodium bicarbonate buffer, pH 9.6, for 18 hr at 37°C. The plates were washed three times with PBS containing 0.05% Tween 20 (PBS/Tween). Simultaneously, to each well was added 20 μl of serum and 80 μl of peroxidase-labelled monoclonal antibody F47-9 diluted 1:1000 in PBS containing 0.1% Tween 20 and 0.6% BSA and the plates were incubated for 3 hr at 37°C.9,10 Sera were tested in duplicate. After washing with PBS/Tween, wells were incubated with 100 μl TMB-substrate solution (12 mg of 3,3',5,5'-tetramethylbenzidine (TMB) in 5 ml of ethanol added to 15 ml of 0.1 M citrate/phosphate buffer, pH 5.0; H₂O₂ added to a final concentration of 0.015%). Reactions were stopped after 15 min by adding 50 μl 2 M H₂SO₄. Resulting absorbance values were measured at 450 nm. Each test plate contained negative (OD = 1.5) and three positive (75; 50; 15% inhibition) control sera. The percentage inhibition of the sera was calculated as [(1-OD/ODₙₑₙ) × 100%].10
Statistical analyses were conducted on two types of data, those on actual ELISA and BI values over time and the other on percentages of these initial values over time. The initial value at the start of therapy for each patient was scored as 100% and subsequent test values over the course of therapy were adjusted as a percentage of that initial value. The purpose for converting ELISA values to percentages of initial values was to allow comparisons of ELISA value declines, since each patient’s pretreatment ELISA value would be different.

Wherever possible, indirect ELISA values for MLEP, NDO, and NTO were analysed using matched statistical analyses, i.e. repeated measures analysis of variance (ANOVA), paired t-test, or Wilcoxon sign rank sum test. This method was used to evaluate the difference in IgM reactivities to the different antigens for each given therapy duration period sample. When evaluating results for each antigen over time, the number of samples that could be analysed in this manner usually was small because of missing data, i.e. one missing serum sample for an individual would affect matched analysis over all time periods. Then analyses for independent samples were used (ANOVA or unpaired t-test), provided homogeneity of variance was fulfilled. If such was not fulfilled, then a nonparametric method was used, such as the Kruskal–Wallis ANOVA or Wilcoxon rank sum analysis. Both Pearson and Spearman correlation coefficients were calculated between ELISA values.

### Table 1. ELISA and BI values by treatment duration

<table>
<thead>
<tr>
<th>Test</th>
<th>Months of therapy</th>
<th>0</th>
<th>12</th>
<th>24</th>
<th>36</th>
<th>48</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
<tr>
<td>NDO-ELISA</td>
<td></td>
<td>1.81±0.78</td>
<td>1.25±0.71</td>
<td>0.79±0.63</td>
<td>0.63±0.51</td>
<td>0.42±0.36</td>
<td>0.35±0.29</td>
</tr>
<tr>
<td></td>
<td>(34)</td>
<td>(28)</td>
<td>(31)</td>
<td>(32)</td>
<td>(27)</td>
<td>(21)</td>
<td></td>
</tr>
<tr>
<td>NTO-ELISA</td>
<td></td>
<td>1.5±0.78</td>
<td>1.00±0.71</td>
<td>0.63±0.57</td>
<td>0.49±0.46</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>(34)</td>
<td>(27)</td>
<td>(30)</td>
<td>(24)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>MLEP-ELISA</td>
<td></td>
<td>0.76±0.7</td>
<td>0.33±0.7</td>
<td>0.18±0.13</td>
<td>0.13±0.13</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>(34)</td>
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<td>(30)</td>
<td>(24)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>INH-ELISA</td>
<td></td>
<td>70.5±21.97</td>
<td>46.41±30.0</td>
<td>43.95±29.15</td>
<td>38.58±28.7</td>
<td>32.7±29.1</td>
<td>35.18±26.4</td>
</tr>
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<td>(28)</td>
<td>(31)</td>
<td>(32)</td>
<td>(27)</td>
<td>(21)</td>
<td></td>
</tr>
<tr>
<td>BI</td>
<td></td>
<td>4.71±0.52</td>
<td>3.89±1.1</td>
<td>3.35±1.45</td>
<td>2.5±1.44</td>
<td>1.56±1.31</td>
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<td>(28)</td>
<td>(31)</td>
<td>(32)</td>
<td>(27)</td>
<td>(21)</td>
<td></td>
</tr>
</tbody>
</table>

NT, not tested.

1. At any given therapy duration period, MLEP-ELISA values are significantly lower than either NTO-ELISA or NDO-ELISA values (repeated measures ANOVA, p < 0.001).

2. NDO-ELISA values were significantly higher than NTO-ELISA values (repeated measures ANOVA, p < 0.001), only for the initial samples, i.e. 0 months; at later treatment durations samples, ELISA values were not significantly different between NDO or NTO antigens.

3. For all test values, results from 12 months to the rest of therapy period are significantly lower than at 0 months.

* Results are expressed as the mean value±standard deviation. The number of samples tested is given in parentheses.
results and in combination with BI or INH results. Coefficients of determination (i.e. \( r^2 \)) were calculated as the ratio of regression sum of squares over the total sum of squares. Data that were converted to percentages of prior values were evaluated using similar methods. All probabilities presented are two-tailed.

Results

**ELISA and BI values during therapy**

When ELISA and BI values were compared between therapy groups IA and IC, no statistically significant differences were found between the values, nor were there observable clinical differences in bacteriologic reduction and occurrence of reactional states. Consequently, individuals from the two therapy groups were combined and analysed as one group.

The mean ELISA and BI values ± standard deviations during the course of treatment are presented in Table 1. For all treatment duration samples, ELISA values were significantly higher using NDO or NTO as antigens than using *M. leprae* as antigen \((p<0.001\) by repeated measures ANOVA). At the initial pretreatment sample, NDO values were significantly higher than NTO values (average difference of 0.316), \( p < 0.001\) by paired \( t\) test; however, for other treatment duration samples the NDO-ELISA and NTO-ELISA values were not significantly different.

After one year of therapy the mean values of all four ELISAs and the BIs were significantly lower \((p < 0.01)\) than the initial values at the beginning of therapy and stayed significantly lower throughout the therapy period. As the values begin to taper off between 24 and 60 months of therapy (Table 1), differences year-to-year may not be significantly different, but the values are nevertheless significantly lower than initial values. Eight patients who showed no reactivity in the MLEP-ELISA throughout the therapy period had high values in the other three tests.

No significant increases or decreases in any of the ELISA values were noticed during episodes of ENL, which occurred in half of the patients during the course of chemotherapy.

![Graph of ELISA and BI declines over the course of dapsone treatment of lepromatous patients](image_url)

**Figure 1.** ELISA and BI declines over the course of dapsone treatment of lepromatous patients. Graphic representation of the mean values presented in Table 2.
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Table 2. ELISA and BI percentages by treatment duration

<table>
<thead>
<tr>
<th>Test</th>
<th>0</th>
<th>12</th>
<th>24</th>
<th>36</th>
<th>48</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDO-ELISA</td>
<td>100 ±27.4</td>
<td>43.6 ±26.0</td>
<td>31.9 ±17.1</td>
<td>23.7 ±14.7</td>
<td>22.9 ±16.3</td>
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</tr>
<tr>
<td>(34)</td>
<td>(28)</td>
<td>(31)</td>
<td>(32)</td>
<td>(27)</td>
<td>(21)</td>
<td></td>
</tr>
<tr>
<td>NTO-ELISA</td>
<td>100 ±26.8</td>
<td>41.1 ±22.2</td>
<td>28.9 ±15.4</td>
<td>NT</td>
<td>NT</td>
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<td>(27)</td>
<td>(30)</td>
<td>(24)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MLEP-ELISA</td>
<td>100 ±32.0</td>
<td>23.9 ±17.0</td>
<td>19.3 ±11.8</td>
<td>NT</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td>(26)</td>
<td>(21)</td>
<td>(23)</td>
<td>(17)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>INH-ELISA</td>
<td>100 ±44.3</td>
<td>59.8 ±35.8</td>
<td>56.0 ±43.7</td>
<td>43.5 ±36.0</td>
<td>49.5 ±31.9</td>
<td></td>
</tr>
<tr>
<td>(34)</td>
<td>(28)</td>
<td>(31)</td>
<td>(32)</td>
<td>(27)</td>
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<tr>
<td>BI</td>
<td>100 ±23.6</td>
<td>71.5 ±29.6</td>
<td>52.6 ±29.1</td>
<td>33.1 ±27.3</td>
<td>28.8 ±20.2</td>
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<td>(34)</td>
<td>(28)</td>
<td>(31)</td>
<td>(32)</td>
<td>(27)</td>
<td>(21)</td>
<td></td>
</tr>
</tbody>
</table>

1 The data from each assay were adjusted by assigning the initial value as 100% at the start of therapy for each patient. Subsequent values for each patient over the course of treatment were calculated as percentages of initial values.
2 Results are expressed as the mean value ± standard deviation. The number of samples tested is given in parentheses.
NT, not tested

For all test values, results from 12 months to the rest of therapy period are significantly lower than at 0 months (p < 0.01 by ANOVA or Kruskal-Wallis test).

ELISA and BI declines by percentages of prior values

The data from each assay were standardized by assigning the initial value at the start of therapy for each patient to be 100%. Over the course of treatment subsequent values for each patient were calculated as a percentage of the initial value. The average and standard deviations of these values are presented in Figure 1 and Table 2. There was no significant difference between NDO- and NTO-ELISA percentages, both with average declines to approximately 30% of the initial values after 3 years of treatment. Values for NDO-ELISA declined to an average 70% of initial ELISA values after 1 year of treatment, to 43.6% after 2 years, and to 22.9% after 5 years. Comparatively, MLEP-ELISA reflected a faster decline after the first year of treatment (to 39.8%, Table 2) and by 2 years of treatment average ELISA values were 23.9% of original values. ELISA-INH values reflected a slower decline, being 40-50% of initial values after 4-5 years of therapy. The BI values declined to 28.8% of initial values after 5 years of treatment.

Correlations between ELISAs and BI

Overall correlations (Pearson) between ELISA results and BIs for each treatment duration period and between the different tests are presented in Table 3. Spearman correlations also were calculated but conclusions and probabilities were similar to Pearson coefficients; the latter are presented in the results.
Table 3. Correlation coefficients between ELISAs and BI

<table>
<thead>
<tr>
<th></th>
<th>NDO-ELISA</th>
<th>NTO-ELISA</th>
<th>MLEP-ELISA</th>
<th>INH-ELISA</th>
<th>BI</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDO-ELISA</td>
<td>—</td>
<td>0.817*</td>
<td>0.604†</td>
<td>0.329†</td>
<td>0.444†</td>
</tr>
<tr>
<td>NTO-ELISA</td>
<td>0.866†</td>
<td>—</td>
<td>0.682†</td>
<td>0.376†</td>
<td>0.570†</td>
</tr>
<tr>
<td>MLEP-ELISA</td>
<td>0.354†</td>
<td>0.492†</td>
<td>—</td>
<td>0.233*</td>
<td>0.297†</td>
</tr>
<tr>
<td>INH-ELISA</td>
<td>0.369†</td>
<td>0.369†</td>
<td>0.366†</td>
<td>—</td>
<td>0.095‡</td>
</tr>
<tr>
<td>BI</td>
<td>0.399†</td>
<td>0.258†</td>
<td>0.285†</td>
<td>0.071†</td>
<td>—</td>
</tr>
</tbody>
</table>

The bold printed figures represent correlation coefficients of percentages of initial values (see Table 2). The normal printed figures represent correlation coefficients of test values (see Table 1).

* p < 0.05  
† p < 0.01  
‡ not significant

Percentages of ELISA and BI values were significantly correlated ($r = 0.444$ for NDO-ELISA, $p < 0.01$ at 172 df; $r = 0.570$ for NTO-ELISA, $p < 0.01$ at 123 df; and $r = 0.297$ for MLEP-ELISA, $p < 0.05$ at 123 df). Similarly, the OD values of the ELISAs showed significant correlations with BI values ($r = 0.399$ for NDO-ELISA, $p < 0.001$ at 172 df; $r = 0.258$ for NTO-ELISA, $p < 0.01$ at 123 df; and $r = 0.285$ for MLEP-ELISA, $p < 0.05$ at 123 df). It should be noted, however, that with coefficients of determination (i.e. $r^2$) of approximately 0.16, 0.07, and 0.08, respectively, the degree to which an ELISA value can be predicted by a BI value, or vice versa, is unreliable. INH-ELISA did not correlate with BI, either by test value or by percentages.

**Correlations between ELISAs**

The correlation between NDO-ELISA and NTO-ELISA results is very high ($r = 0.866$, $p < 0.001$ at 123 df for ELISA values; $r = 0.817$, $p < 0.001$ at 123 df for percentages), indicating that both the ELISA values are similar and the rates of decline in those values are comparable. Please refer to Table 3. Correlations between either NDO-ELISA or NTO-ELISA with MLEP-ELISA are considerably lower, primarily since the values for MLEP-ELISA are significantly lower than either NDO-ELISA or NTO-ELISA. Correlations between any of the indirect ELISAs and INH-ELISA are similarly low, primarily due to the consistent reactivity over time with INH-ELISA. The coefficients of determination results (i.e. $r^2$) for MLEP-ELISA or INH-ELISA either with each other or compared to NDO-ELISA or NTO-ELISA are quite low (ranging from about 0.13 to about 0.24); whereas, the $r^2$ for NDO-ELISA compared with NTO-ELISA is quite high (about 0.75), indicating good agreement between both results.

**Discussion**

Our findings indicate a decline in ELISA reactivity and a decline in BI over the course of dapsone-based therapy. All patients were previously untreated lepromatous leprosy cases. During the course of treatment, these patients received two different dapsone-based chemotherapeutic protocols, differing only in the administration of clofazimine (for 24 weeks, group JCTI-C) at the beginning of the therapy period. Both groups received a single dose of rifampicin at the start of the therapy period and thereafter dapsone monotherapy for 5 years. Clinical and serological evaluation did not
indicate any significant difference between the two regimens (JCTI-A and JCTI-C), consequently, the data from the two treatment groups were combined for statistical analysis.

ELISAs measuring IgM antibodies to the NDO and NTO antigens showed a significant decline to approximately 30% of initial values after 3 years of therapy ($p < 0.01$ by Kruskal–Wallis test) (Tables 1 and 2). Similar declines of antibody levels during therapy, using these and other antigens, have been previously described. The overall NDO- and NTO-ELISAs showed comparable and statistically significant correlations with BI (Table 3), although variation was observed with individual patients. The coefficients of determination (i.e. $r^2$), however, are quite low, indicating that the degree to which an ELISA value can be predicted from a BI value likewise is low. As with BI, an ELISA measurement at a single point in time cannot be used to indicate effective treatment. Thus sequential sera taken during the course of treatment should be monitored and compared with the sample collected at initiation of treatment.

The overall NDO- and NTO-ELISAs were highly correlated with each other ($r = 0.866$ for ELISA values and $r = 0.817$ for ELISA percentages, $p < 0.001$ for both, Table 3).

Although the MLEP-ELISA correlated significantly (Table 3) with the NDO- and NTO-ELISAs, several findings suggest that the MLEP-ELISA does not detect antibody reactivity to the sugar epitope of the phenolic glycolipid-I of M. leprae. At any given sampling time period, MLEP-ELISA values are significantly lower than either NTO-ELISA or NDO-ELISA values ($p < 0.001$ by repeated measures ANOVA). The MLEP-ELISA showed a much faster decline during the first year of treatment than the other ELISAs. In addition, the sequential sera of 8 patients were negative from the beginning of therapy with the MLEP-ELISA, while being positive in the other ELISAs. This makes the MLEP-ELISA less efficient in monitoring patients on treatment.

The INH-ELISA showed a decline to approximately 50% after 5 years of treatment (Table 2). No significant correlation was found with the BI over the course of treatment (Table 3), which could be attributed to the persistence of activity during therapy. In 9 out of 35 patients no decline in their INH-ELISA activity was found throughout the treatment period. Similar findings have been reported with an ELISA based on the detection of antibodies to the lipoarabinomannan of M. leprae.7,28

In contrast to others27 we found no relationship between the occurrence of ENL and changes in ELISA values.

Sequential monitoring with the ELISAs measuring IgM antibodies to the sugar epitope of the phenolic glycolipid-I of M. leprae were found to be the most suitable for monitoring lepromatous patients under chemotherapy. The NDO- and NTO-ELISA may be better quantitative measurements of progress during chemotherapy than the BI. The MLEP-ELISA is less suitable because it can fail to detect antibodies in some patients. The INH-ELISA is less suitable because of persistent reactivity during the course of therapy in some patients.

Acknowledgments

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