Immunoglobulin G response against 10-kDa and 65-kDa heat-shock proteins in leprosy patients and their household contacts

R.E. Rojas, A. Segal-Eiras *

CINIBA (Centro de Investigaciones Inmunológicas Básicas y Aplicadas), Facultad de Ciencias Médicas, Universidad Nacional de La Plata, Calle 60 y 120, La Plata, 1900, Argentina

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Abstract

We measured antibody responses to recombinant Mycobacterium leprae 65-kDa (rML65) and 10-kDa (rML10) by indirect ELISA in sera from leprosy patients, household contacts and healthy controls in a leprosy-endemic area in the north east of Argentina. Serum antibody levels to those antigens were correlated with IgM anti-phenolic glycolipid I (PGL-I) levels, with bacterial index and the period of time under chemotherapy. Bacterial index positive (BI+) patients showed higher mean values when compared with BI negatives (BI−). Among lepromatous patients a positive correlation was observed between IgG antibody responses to both recombinant antigens and IgM antibody response to PGL-I. Anti-rML10 test detected a higher percentage of positives/total than anti-rML65 in all leprosy groups and healthy contacts. Bacterial load, leprosy clinical form and the time under chemotherapy were factors which could influence levels of the antibody response. The contribution of these antibody studies for a precise and early diagnosis in leprosy is discussed.

Keywords: Leprosy; Phenolic glycolipid I; hsp65; hsp10; Antibody response

1. Introduction

Although the prevalence of leprosy has declined as a result of the aggressive implementation of multiple drug therapy (MDT), the incidence has not fallen [1]; this emphasizes the need for new antigens to measure the disease transmission and the reservoirs of this infection. Furthermore, Mycobacterium leprae is one of the last remaining infectious pathogens which must be fully characterized antigenically.

Recent advances in molecular biology have allowed the identification and production of recombinant antigens. The greatest impact was the approach pioneered by Young et al. [2,3], resulting in the construction of a genomic library of M. leprae, the screening of recombinant clones with monoclonal and polyclonal antibodies and the identification of 12 different proteins or their genes [4]. Some of these products have been identified as members of the heat-shock proteins (hsp) family, among which the 70-kDa [5], the 65-kDa [6,7], and the 18-kDa hsp [8,9] (related respectively to DNA K protein, the GroEl protein, and a low molecular mass stress protein from soybean), have been demonstrated to be...
important antigens in eliciting B cell response [10]. Recently, the *M. leprae* 10-kDa hsp, which is homologous to the GroEs gene product of *Escherichia coli*, but for which a human homologue has not yet been identified, was also described as an immunodominant T cell antigen in leprosy [11-13]. Although this antigen has been identified as one of the major TH1 antigens, its role in antibody response has not been fully evaluated. Population studies involving defined antigens of *M. leprae* may allow the development of new specific serodiagnostic tests for early detection of leprosy. On the other hand, these studies may be useful in understanding the immunoregulatory mechanisms of antibody and immune complex formation in leprosy [14,15].

We have evaluated the IgG responses to recombinant antigens, i.e. *M. leprae* 65-kDa (rML65), and *M. leprae* 10-kDa (rML10) heat-shock proteins and the IgM response to PGL-I in sera from leprosy patients, as well as their contacts in an endemic area in north east Argentina. The aim was to establish their significance as useful tests for detecting leprosy as well as assessing disease status.

2. Materials and methods

2.1. Study populations

The population included patients with leprosy, household contacts and healthy controls from the same area. A total of 97 sera from adult patients (age range 20–87, mean 56 ± 13), classified according to the Ridley and Jopling criteria [16], were provided by the Dermatology Programme of the Iturraspe Hospital, Santa Fe, Argentina. The clinical classification of the patients were polar lepromatous (LL, n = 59), borderline lepromatous (BL, n = 5), borderline (BB, n = 10), borderline tuberculoid (BT, n = 7), polar tuberculoid (TT, n = 15). Both untreated and patients under multiple drug therapy (MDT) for periods ranging from a month to 58 months were included. Thirty-three patients had been under MDT for less than one year (7 of them were diagnosed and began the treatment at the moment of blood sample obtention). Thirty were under treatment between 1 and 4 years, whereas 13 patients received MDT for more than 4 years. A group of 21 patients under post-treatment surveillance was also included.

LL and BL patients were grouped together (65 patients), and segregated into bacterial index positive (LBI+, n = 43) and bacterial index negative (LBI−, n = 22) lepromatous patients; TT and BT patients were also studied as one group (n = 22). Thirty patients bear B1 > 2, while 13 had B1 ≤ 2 considering all LBI+ patients; 23 out of 97 patients had been reported as suffering peripheral nerve enlargement, 18 of them were LL/BL patients and 5 TT/BB; 9/23 had active neuritis episodes. Finally, in 18 out of 97 patients a history of leprosy reactions was described. Other subsidiary diagnosis such as reversal reactions or ENL were not considered for purposes of analysis.

Healthy family contacts (HFC; n = 42), living in the same household as multibacillary (MB) and paucibacillary (PB) patients in the last 3 years, were included.

Negative control sera (healthy non-contacts, HNC; n = 40) were obtained from blood donors at the same hospital.

Serum samples were separated from 5 ml of venous blood and, after centrifugation, distributed in 250-ml in Durham tubes and frozen at −70°C until used.

2.2. Antigens

Natural disaccharide of PGL-I (3,6-di-O-methyl-β-D-glucopyranosyl-(1-4)2,3-di-O-methyl-α-L-rhamnopyranose) conjugated to bovine serum albumin (ND-O-BSA) and also the unconjugated BSA of the same lot were generously provided by Dr. R.J.W. Rees, National Institute for Medical Research, London, UK.

The 65-kDa and 10-kDa recombinant proteins of *M. leprae* (rML65, rML10) were kindly donated by Dr. M. Singh, GBF Braunschweig, FRG.

2.3. Monoclonal antibodies

The mAb F-4721-3 (mc 77401, reactive with the disaccharide epitope of the PGL-I, was provided by Dr. A.H. Kolk (Biomedical Research N.H. Swellingen Laboratory, the Netherlands).

MAB reactive with epitopes of 10-kDa and 65-kDa heat-shock proteins (hsp) were kindly supplied by Dr. Shinnick, Center for Disease Control, Atlanta,
GA; mAb ML30 (mc2009) and CS-43 (mc9249) recognize the 65-kDal hsp while CS-01 (mc 9245) reacts against 10-kDa hsp.

Both mAb and recombinant antigens were supplied through the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR).

2.4. PGL-I (ND-O-BSA) ELISA

IgM anti-PGL-I (ND-O-BSA) was measured by an enzyme linked immunosorbent assay (ELISA), according to the method described by Cho et al. [17]. Briefly, ELISA flat-bottom 96-well polystyrene microtiter plates (Nunc Immunoplate, Denmark) were coated with the ND-O-BSA or with the corresponding batch of BSA (100 ml per well) at a concentration of 3 mg/ml in 0.05 M carbonate buffer (pH 9.6); the plates were incubated for 1 h at 37°C and then overnight at 4°C. They were washed with phosphate-buffer saline (PBS) and blocked with 3% BSA in PBS (BSA-PBS, 200 ml/well) for 2 h at 37°C. After washing, serum samples at 1:100 dilution in 2% BSA-PBS containing 0.05% Tween-20 (PBST) were incubated in duplicate for 1 h at 37°C (100 ml/well). After washing four times with PBST, plates were incubated for 45 min at 37°C with horseradish peroxidase (HRP)-labeled rabbit anti-human IgM (Dako, Copenhagen, Denmark) diluted (1:1000) in BSA-PBST (100 ml/well). After washing thoroughly, freshly prepared 2,2’azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), (Sigma Chemical Co, MO), substrate solution was added and then incubated for 1 h in the dark.

The optical density (OD) was read at 405 nm in a micro-ELISA reader (Titertek Multiskan Plus, Flow Lab., Finland); for each serum sample, the mean OD of BSA-coated wells was subtracted from the mean OD of ND-O-BSA-coated wells. A serum was considered ‘positive’ when the OD exceeded by 2 standard deviations (S.D.) the mean OD obtained from normal sera (> 0.080) at the same dilutions.

2.5. Indirect ELISA for measuring antibody response to recombinant hsp

Antibodies of IgG isotype to rML65 and rML10 were measured by indirect ELISA as described by Meeker et al. [18] with minor modifications. Optimal coating concentration for hsp was determined by titulation tests using mAbs. ELISA plates (Nunc Immuno plate, Denmark) were coated with 4 mg and 2 mg/ml of rML65 and rML10 respectively in 0.05 M carbonate buffer pH 9.6 and incubated at 37°C for 1 h and then at 4°C overnight in a humidified chamber. After washing and blocking with 3% BSA in PBS, appropriate dilution of serum samples in BSA-PBST (1:100) was added and incubated for 1 h at 37°C. The plates were then washed four times with PBST and incubated for 1 h with HRP-labeled rabbit anti-human IgG (Dako, Copenhagen, Denmark) diluted in BSA-PBST (1:2000). Subsequent steps were similar to those described for anti-ND-O-BSA Abs.

Reference sera (from control subjects, from lepromatous patients and the corresponding mAbs) were included every time the ELISA was performed, and the coefficient of variation per number of separate assays was less than 5%.

Samples with OD values above the mean value + 2 S.D. of the healthy non-contacts, > 0.235 for anti-rML65 Ab and > 0.090 for anti-rML10 Ab, were considered positive.

2.6. Statistical analysis

Correlation coefficient and regression analysis were performed using the PRIMER statistical package. Normality and homogeneity of variance (homoscedasticity) were done by Kolmogorov and Bartlett methods respectively [19]. Data were then analysed by Kruskal-Wallis with tied ranks tests and compared with χ² (0.05; n – 1); non-parametric tests for intergroup differences were done among LBI⁺, LBI⁻, TT/BT and HNC groups for each antibody [20].

3. Results

3.1. Anti-PGL-I (NDO-BSA) IgM response

Serum IgM antibody level to ND-O-BSA was significantly higher between LBI⁺ compared with LBI⁻ patients (x = 0.408 ± S.E.M. 0.069 vs. x = 0.086 ± S.E.M. 0.019; P vs. control < 0.001). Mean value in BB group was 0.142 ± S.E.M. 0.078, P <
0.05; BT/TT values were also statistically different when compared with HNC levels (P < 0.05); HFC values were not statistically different in relation to the HNC levels (Fig. 1A). The anti-PGL-I response showed a wide variation among the groups, including the BI+ patients; in this group, patients bearing high bacterial load (BI > 2) showed anti-PGL-I higher levels than in BI ≤ 2 patients (P < 0.05). The proportion of positive samples was increased along the leprosy spectrum, BT/TT 27%, BB 30%, LBI- 41% and LBI+ 67%; HFC and HNC showed low levels of antibodies, and the percentage of positive samples in the contact group was still lower than in paucibacillary patients, 19% (Fig. 2). All positive sera from the HFC group corresponded to LL household contacts.

3.2. IgG responses to rML65 and rML10

The antibody response to hsp was significantly important in LBI+ patients, similar to the findings obtained with the anti-ND-O-BSA antibody; BB and BT/TT patients had lower levels of both anti-recombinant hsp antibodies. In the BT/TT group there were three patients who showed strong response to the rML10 kDa, and it was not parallel to their responses to the other two antigens. Furthermore, these three patients were extremely different to the others in this group, concerning to their anti-rML10 kDa response (Fig. 1C). HFC were similar to HNC in their response to both recombinant antigens and also to the PGL-I. The highest levels of anti-rML65 and anti-rML10 were found in the LBI+ group (specially in those whose BI was > 2, P < 0.05), yielding a mean value of 0.243 ± S.E.M. 0.053 and 0.345 ± S.E.M. 0.083 respectively. On the other hand, LBI- patients showed a media of 0.202 ± S.E.M. 0.072 for the anti-rML65 and 0.112 ± 0.044 for the anti-rML10 (Fig. 1B,C). The number of positive cases was also dependent on the clinical form, with the highest percentages for the multibacillary group. When comparing the responses to both recombinant antigens, it could be seen that the number of positives for the anti-rML10 was higher than for the anti-rML65, and both were lower than anti-PGL-I, along all the leprosy spectrum (Fig. 2).

IgM response to rML65 was also measured, and its level in patients sera were lower than IgG one with non-significant difference when comparing with HNC (data not shown).

3.3. Statistical analysis of intergroup differences for each antibody

The non-parametric Kruskal-Wallis test was performed in concordance to the non-normal data distri-
bution calculated by Kolmogorov test as well as the heterogeneity of variances studied by the Bartlett test. Results are shown in Table 1.

The anti-PGL-I antibody levels were different among all the groups in relation to HNC; non-significant difference was found between LBI$^+$ and TT/BT.

Concerning the anti-65-kDa antibody levels LBI$^+$ and LBI$^-$ groups were significantly different from TT/BT and HNC groups, whereas TT/BT vs. HNC and LBI$^+$ vs. LBI$^-$ did not show any difference among them.

All groups were statistically different ($P < 0.05$) when anti-rML10 kDa antibodies were analysed; three TT/BT patients sera included in this study were not considered for this analysis since their data were extremely dispersed compared with the other patients in the same group.

3.4. Correlation between antibody levels

Analysis of the data from BI$^+$ (multibacillar) patients and BI$^-$ (paucibacillar) patients demonstrated a good correlation between antibody titers in the former group and a poor correlation in the latter. The best correlation ($r = 0.698, P < 0.001$) was found between the antibody levels to ND-O-BSA and rML10 in LBI$^+$ patients; conversely, the correlation of antibody responses were not significant when BI$^-$ patients and HFC were studied (Table 2).

3.5. Correlation of lepromatous patients' antibody response and the period of multidrug chemotherapy

Analysis of data from LBI$^+$ patients under multidrug chemotherapy revealed that the anti-ND-O-BSA

### Table 1

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Difference ($R_A - R_B$)</th>
<th>S.E.</th>
<th>Statistical critical value</th>
<th>Significance level ($P$)</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-PGLI</td>
<td></td>
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<tr>
<td>LBI$^+$ vs. HNC</td>
<td>52.2–21.5 = 30.7</td>
<td>0.84</td>
<td>36.50</td>
<td>&lt; 0.001</td>
<td>s.d.</td>
</tr>
<tr>
<td>LBI$^-$ vs. HNC</td>
<td>36.3–21.8 = 14.5</td>
<td>1.31</td>
<td>11.04</td>
<td>&lt; 0.001</td>
<td>s.d.</td>
</tr>
<tr>
<td>TT/BT vs. HNC</td>
<td>34.9–23.7 = 11.2</td>
<td>1.70</td>
<td>6.55</td>
<td>&lt; 0.05</td>
<td>s.d.</td>
</tr>
<tr>
<td>LBI$^+$ vs. LBI$^-$</td>
<td>35.5–20.9 = 14.6</td>
<td>1.64</td>
<td>8.87</td>
<td>&lt; 0.005</td>
<td>s.d.</td>
</tr>
<tr>
<td>LBI$^+$ vs. TT/BT</td>
<td>38.1–19.8 = 18.3</td>
<td>1.31</td>
<td>13.91</td>
<td>&lt; 0.001</td>
<td>s.d.</td>
</tr>
<tr>
<td>LBI$^-$ vs. TT/BT</td>
<td>22.3–18.9 = 3.4</td>
<td>4.00</td>
<td>0.85</td>
<td>N.S.</td>
<td>n.d.</td>
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<tr>
<td>Anti-rML65</td>
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<tr>
<td>LBI$^+$ vs. HNC</td>
<td>47.9–34.7 = 13.2</td>
<td>2.04</td>
<td>6.47</td>
<td>&lt; 0.05</td>
<td>s.d.</td>
</tr>
<tr>
<td>LBI$^-$ vs. HNC</td>
<td>37.7–26.3 = 11.4</td>
<td>1.98</td>
<td>5.73</td>
<td>&lt; 0.05</td>
<td>s.d.</td>
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<tr>
<td>TT/BT vs. HNC</td>
<td>27.4–32.9 = 5.5</td>
<td>3.92</td>
<td>1.41</td>
<td>N.S.</td>
<td>n.d.</td>
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<tr>
<td>LBI$^+$ vs. LBI$^-$</td>
<td>30.5–32.2 = 1.7</td>
<td>14.16</td>
<td>0.12</td>
<td>N.S.</td>
<td>n.d.</td>
</tr>
<tr>
<td>LBI$^+$ vs. TT/BT</td>
<td>37.3–21.3 = 16.0</td>
<td>1.46</td>
<td>10.89</td>
<td>&lt; 0.001</td>
<td>s.d.</td>
</tr>
<tr>
<td>LBI$^-$ vs. TT/BT</td>
<td>27.1–14.6 = 12.5</td>
<td>1.07</td>
<td>11.63</td>
<td>&lt; 0.001</td>
<td>s.d.</td>
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<tr>
<td>Anti-rML10</td>
<td></td>
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<tr>
<td>LBI$^+$ vs. HNC</td>
<td>52.5–32.9 = 19.6</td>
<td>1.21</td>
<td>16.14</td>
<td>&lt; 0.001</td>
<td>s.d.</td>
</tr>
<tr>
<td>LBI$^-$ vs. HNC</td>
<td>37.4–28.9 = 8.5</td>
<td>2.06</td>
<td>4.11</td>
<td>&lt; 0.05</td>
<td>s.d.</td>
</tr>
<tr>
<td>TT/BT vs. HNC</td>
<td>41.7–26.9 = 14.8</td>
<td>1.42</td>
<td>10.42</td>
<td>&lt; 0.005</td>
<td>s.d.</td>
</tr>
<tr>
<td>LBI$^+$ vs. LBI$^-$</td>
<td>34.5–24.8 = 9.7</td>
<td>2.32</td>
<td>4.17</td>
<td>&lt; 0.05</td>
<td>s.d.</td>
</tr>
<tr>
<td>LBI$^+$ vs. TT/BT</td>
<td>35.6–23.5 = 12.1</td>
<td>1.68</td>
<td>7.17</td>
<td>&lt; 0.01</td>
<td>s.d.</td>
</tr>
<tr>
<td>LBI$^-$ vs. TT/BT</td>
<td>22.7–16.2 = 6.5</td>
<td>1.19</td>
<td>5.46</td>
<td>&lt; 0.05</td>
<td>s.d.</td>
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</table>

$R_A - R_B$: difference between mean ranks.
S.E.: standard error.
PGLI: phenolic glycolipid I.
rML65: recombinant 65-kDa protein.
rML10: recombinant 10-kDa protein.
LBI$^+$: bacterial index positive lepromatous leprosy patients.
LBI$^-$: bacterial index negative lepromatous leprosy patients.
TT/BT: borderline tuberculoid and tuberculoid leprosy patients.
HNC: healthy non-contacts.
N.S.: not significant.
s.d.: significantly different.
n.d.: not significantly different.
Table 2
Correlation between IgG response to recombinant antigens and anti-PGL-I IgM response in leprosy patients and contacts

<table>
<thead>
<tr>
<th>IgG response to recombinant antigens</th>
<th>Correlation to anti-PGL-I IgM response in patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>BI+ patients</td>
<td>BI- patients</td>
</tr>
<tr>
<td>rML65kDa</td>
<td>( r = 0.515 )</td>
</tr>
<tr>
<td>( P &lt; 0.001 )</td>
<td>( P = 0.56 )</td>
</tr>
<tr>
<td>rML10kDa</td>
<td>( r = 0.698 )</td>
</tr>
<tr>
<td>( P &lt; 0.001 )</td>
<td>( P = 0.082 )</td>
</tr>
</tbody>
</table>

- **r**: correlation coefficient.
- **P**: statistical significance.
- **BI+**: bacterial index positive patients.
- **BI-**: bacterial index negative patients.
- **HFC**: healthy family contacts.
- **PGL-I**: phenolic glycolipid I.
- **rML65kDa**: recombinant M. leprae 65-kDa protein.
- **rML10kDa**: recombinant M. leprae 10-kDa protein.

**BSA** response showed a marginal correlation with the time (in months), \( r = -0.319 \), \( P = 0.05 \); 90% of patients with the highest antibody levels to anti-ND-0-BSA (\( > 0.800 \)) was under treatment for less than 15 months. No correlation was observed between the period of chemotherapy in relation to anti-rML65 as well as to anti-rML10 IgG levels. Furthermore, patients with iters above 0.800 for anti-rML10 received MDT for less than 14 months. Patients recently diagnosed (without MDT at the moment of blood sample extraction, \( n = 7 \)) showed the highest mean levels for the three antibodies (anti-PGL-I, \( x = 1.054 \) S.E.M. = 0.2; anti-65-kDa hsp, \( x = 0.697 \) S.E.M. = 0.16; anti-10-kDa hsp, \( x = 1.08 \) S.E.M. = 0.23), behaving as a different group in relation to patients receiving MDT (\( P < 0.05 \)).

The group of patients under post-treatment surveillance and patients still under treatment (> one year) showed the lowest levels for the three antibodies. Both groups were different from those who received chemotherapy for less than one year (\( P < 0.001 \)).

**4. Discussion**

*M. leprae* causes a spectral disease; the resistance correlates with a TH1-strong immune response against the mycobacteria, whereas susceptibility is preferentially associated with a TH-2 function [21,22]. Among leprosy patients, those with LL forms show predominantly TH-2 responses with high antibody levels, while TT patients are mainly TH-1 responders with adequate cellular reactions. It is now becoming clear that the divergence into TH1 and TH2 cells is regulated by the innate immune response at the outset of infection. The characterization of *M. leprae* antigens may allow for the clarification of the cause of this divergence [23,24].

Identification of species-specific epitopes on certain *M. leprae* antigens, for instance PGL-I, has led to the development of species-specific serological tests for leprosy [25–28]; extensive work has been done on PGL-I antibody levels, although its usefulness when tested on large population studies is doubtful and often controversial [29–32]. However, this test may contribute a better understanding of the immune response to the *M. leprae* [33,34].

It is considered more appropriate to use purified antigens instead of complex derivatives from mycobacteria to improve the study of *M. leprae* relevant epitopes, the immune profile of leprosy patients as well as healthy endemic controls. Some of the most important antigens are the heat-shock proteins; these may play a role in eliciting protective immunity against mycobacterial infections since some of them induce a predominant TH-1 response in leprosy contacts [13] and, as an immunodominant antigen they are able to elicit a strong delayed-type hypersensitivity reaction in guinea pigs sensitized with killed *M. leprae* [11]. However, the precise role of hsp in protective immunity is open to discussion. Indeed, since mycobacterial hsp are closely related to human hsp, these stress proteins might be involved in autoimmune response [35–39]; according to this concept, we have studied the antibody response to some of these antigens.

The elevated response of anti-65-kDa antibodies in LBI+ are coincident with those reported by Ilangumaran et al. [10]; antibody levels against 65-kDa hsp yielded high values in groups with positive bacilloscopy where a bacterial overload was present, whereas the BT/TT group had low levels of anti-65-kDa antibodies, similar to HFC and HNC individuals. The presence of anti-hsp 65-kDa antibody in patients with a positive bacterial index suggests the expected immune reaction against this antigen associated with *M. leprae*; also, the anti-65-kDa hsp may be induced by other mycobacteria and by shared
epitopes with autologous molecules [40–42]. In tuberculosis leprosy, nerve lesions appear to follow cell-mediated immune response to *M. leprae*, while in LBI [4], Schwann cells are infected with many *M. leprae* resulting in demyelination of peripheral nerves. Launois et al. found that some nerve epitopes recognized by patients’ antibodies are shared by the 65-kDa hsp of *M. leprae* [43]. Autoantibodies recognizing the same molecules could participate in nerve damage, helped by T-cells in the pathogenesis of the tuberculoid form. However, in our study we could not demonstrate such an association since patients with nerve damage (such as nerve enlargement or active neuritis) did not differ significantly in their anti-65-kDa hsp levels from patients with mild nerve injuries (data not shown in results).

It is interesting to point out that the cut-off value for anti-rML65 kDa was higher than that corresponding for the other antigens (anti-ND-O-BSA and anti-rML10 kDa). This difference could be due to the high degree of homology between mammalian molecules and mycobacterial 65-kDa [44–46] and the lack of shared autologous epitopes with the other two antigens (PGL-I and rML10 kDa).

The 10-kDa antigen of *M. leprae* is considered a major T-cell antigen; it has been suggested as being potentially useful in the immunoprophylaxis of leprosy [13]. It is quantitatively prominent, about 1% of the *M. leprae* bacterial mass, and is actually considered to be the most promising protein as regards a vaccine for leprosy [12]. Lepromatous patients with positive bacilloscopy yielded a high 10-kDa hsp antibody mean value which was more discriminative than anti-rML65 kDa for BI+ and BI– patients. Furthermore, those patients with BI > 2 showed anti-10-kDa hsp levels significantly higher than those obtained in BI ≤ 2 (P < 0.05). The response to rML10 kDa was rather more adequate to detect patients and reservoirs than anti-rML65 kDa but less than anti-ND-O-BSA; the latter rendered the highest percentages of positives in all the clinical forms and in contacts. The current study of the antibody response against 10-kDa hsp is one of the first reported; the group of patients with positive bacilloscopy and high 10-kDa antibody levels showed a significant correlation with anti-ND-O-BSA values, \( r = 0.698 \) and \( r = 0.636 \) with anti-65-kDa hsp; thus, results suggest the possible usefulness of testing these antibody levels, specially against PGL-I and hsp 10-kDa in positive bacilloscopy patients, to establish the persistence of *M. leprae* overload as well as its associated proteins.

The development of vaccines taking into account the inclusion of hsp may offer some difficulties, since they are highly conserved across different species in nature and mycobacterial hsp can be closely related to human proteins [39,40]. These antigens included in vaccines may be able to induce an autoimmune disease, specially related to dominant determinants of mycobacterial hsp causing an immune response against normal cryptic epitopes of their human homologues. The 65-kDa hsp has a similar sequence with the human 63-kDa mitochondrial protein P1; both proteins 63- and 65-kDa have epitopes recognized by cytolytic T cells; this is an explanation sustaining that 65-kDa protein could be involved in the mechanism of injuries in rheumatic arthritis [38]. The possibility that 10-kDa hsp of *M. leprae* has a human homologue and whether this hsp contains cross-reactive T and B-cell epitopes needs to be fully elucidated. It is difficult to assess how these antibody assays might be useful in supplementing the clinical diagnosis itself. This is an important subject since, in areas where leprosy is endemic, the clinical diagnosis is mostly the sole criterion; it is complemented by the slit-skin smear study and the bacterial index determination. It must be noted that not one of the HNC showed positivity for the anti-PGL-I or the anti-10-kDa hsp antibodies, while 19 and 13% of the HFC were positive respectively. The real impact of these tests in assessing new cases of leprosy must be evaluated in a follow-up study; the possibility of investigating different antibodies against *M. leprae* epitopes may contribute for the precise and early diagnosis of the disease.

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