

CROSS-REACTIVITY OF ANTI-10kD HEAT SHOCK PROTEIN ANTIBODIES IN LEPROSY AND TUBERCULOSIS PATIENTS

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Summary The response to recombinant 10-kD heat shock protein (HSP) of *Mycobacterium leprae* (rML10) was evaluated by indirect ELISA in sera from leprosy patients, household contacts, tuberculosis patients and healthy controls in a leprosy-endemic area in the North East of Argentina. Some technical parameters were analyzed: within-assay and between-assay variability, dose-response curves and detectability indexes (specificity and sensitivity) of ELISA applied to measure anti-10kDa antibodies. High levels of these antibodies have already been reported in positive bacilloscopy patients; herein we have also demonstrated that tuberculosis patients sera cross-react with this *M. leprae* antigen. This test seems to have a low sensitivity and specificity for leprosy detection; it confirms that antibodies against highly conserved HSP antigens are important in the polyclonal response against mycobacterial epitopes in leprosy as well as in tuberculosis.

Key words: leprosy, tuberculosis, 10kD heat shock protein

Nowadays there are two main priorities in leprosy research regarding the impact of multidrug therapy (MDT) and the optimism generated by the elimination of leprosy as a public health problem¹. The first priority is the study of the immunopathogenic mechanism, which is related to the severity of disabilities caused by a long-term illness; the second one is the development of diagnostic tools which may facilitate the subclinical detection of new cases of leprosy. Up to now, serology has proved to be a limited value for leprosy diagnosis^{2, 3}; the most useful epidemiological test is the detection of anti-phenolic glycolipid (PGL-I) antibodies; its major utility is the confirmation of leprosy suspected cases but its sensitivity is low since a high proportion of paucibacillary patients lack elevated anti-PGL-I

antibodies. Other serodiagnostic markers such as heat shock proteins (HSP) have been less studied but their usefulness for leprosy diagnosis as far as sensitivity and specificity is concerned, still remains uncertain⁴⁻⁷.

The 10 kDa HSP is the major cytosolic protein of *M. leprae* (MCP-I), representing the most abundant protein (1% of the bacterial mass); MCP-I also occurs in a highly immunogenic peptidoglycan-bound form⁸; it is homologous to the GroES gene product from *Escherichia coli* (44% of homology)⁹, and it has previously been described by Mehra et al.¹⁰⁻¹². The homologous protein in *M. tuberculosis* displays 90% identity with the *M. leprae* 10-kDa protein and it has proved to be highly immunogenic in patients with tuberculosis (TB) infection¹³. The *M. tuberculosis* 10 kDa protein is also a secreted protein because it can be found in early culture filtrates¹⁴; specific T-cell clones to the *M. tuberculosis* 10 kDa protein were also shown to be reactive against *M. tuberculosis*^{15, 16}.

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The humoral immune response to the 10-kDa HSP in patients with leprosy and their convivents is still a subject of limited research; it should be relevant to investigate the antibody response against this single molecule, not only for its potential usefulness as a diagnostic marker, but also for its potential ability to induce autoimmune reactions.

The aim of the present work was to analyse the specificity and sensitivity of an ELISA applied to the measurement of anti-10kDa antibodies in leprosy diagnosis, as well as to determine the cross reactivity of TB sera.

Materials and methods

Study populations

A total of 97 sera of leprosy patients (age range 20 to 87, mean 56 ± 13), classified according to the Ridley and Jopling criteria¹⁷, were obtained from the Centro Dermatológico Dr. Manuel M. Giménez, Resistencia, Chaco, Argentina. The clinical classification of the patients were polar lepromatous (LL, $n = 59$), borderline lepromatous (BL, $n = 6$), borderline (BB, $n = 10$), borderline tuberculoid (BT, $n = 7$), polar tuberculoid (TT, $n = 15$). Patients were under MDT; 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 ($n = 22$). Healthy family contacts (HFC; $n = 39$), 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; $n = 43$) were obtained from blood donors at the Service of Hemotherapy; patients with active TB ($n = 15$) were also included. All of them bore pulmonary TB with positive bacilloscopy and were recently diagnosed, being under therapy for less than a week.

Peripheral blood was drawn aseptically and centrifuged; sera were aliquoted in Durham tubes and frozen at -70°C until use. Patients, household contacts and controls informed consent was obtained in all cases.

Antigen

The 10 kDa recombinant protein of *M. leprae* (rML10) was kindly gifted by Dr. M. Singh, GBF Braunschweig, Germany.

Monoclonal antibody (Mab)

Mab CS-01 (mc 9245) reactive with an epitope of 10 kDa HSP were kindly supplied by Dr. Shinnick, Center

for Disease Control, Atlanta, Georgia, USA. This MAb and the recombinant antigen were supplied through the UNDP/World Bank/ WHO Special Programme for Research and Training in Tropical Diseases (TDR).

Antibody response to recombinant HSP evaluated by indirect ELISA

Antibodies of IgG isotype to rML10 were screened by indirect ELISA as described by Meeker et al.¹⁸ with minor modifications; optimal coating concentration for HSP was determined by titulation tests using Mab and pooled sera from LL patients as well as a pool obtained from HNC. ELISA plates (Nunc Immuno plate, Denmark) were coated with antigen concentrations ranging from 0.2 ng/ml to 20 $\mu\text{g/ml}$ in 0.05 M carbonate buffer pH 9.6 and incubated at 37°C for 1 hour and then at 4°C overnight in a humidified chamber. After washing and blocking with 3% bovine serum albumin (BSA) in phosphate- buffer saline (PBS), pooled serum samples in serial dilutions (1:25 to 1:51200) in BSA-PBS containing 0.05% Tween- 20 (BSA-PBST) were added and incubated for 1 hour at 37°C . The plates were then washed four times with PBST and incubated for 1 hour with horseradish peroxidase-labeled rabbit anti-human IgG (Dako, Copenhagen, Denmark) diluted in BSA-PBST (1:2000). After washing thoroughly, freshly prepared 2,2' azino- bis (3- ethylbenzthiazoline-6-sulfonic acid) (ABTS), (Sigma Chemical Co, Mo, USA), substrate solution was added and incubated at different times between 20 and 90 min in the dark. The optical density (OD) was read at 405 nm in a micro- ELISA reader (Titer-tek Multiskan Plus, Flow Lab., Finland); for each serum sample, the mean OD of BSA-coated wells was subtracted from the mean OD of rML10 kD coated wells. A serum was considered "positive" when the OD exceeded by 3 standard deviations (SD) the mean OD obtained from normal sera (cut off value = 0.090 at 405 nm) at the same dilution.

Dose response curves

The dose response curves were performed for six different antigen (rML 10kD) concentrations, ranging between 0.2 ng/ml and 20 $\mu\text{g/ml}$. The optical density values (OD) at 405 nm (determined at 20, 30 and 60 minutes of substrate incubation) were plot versus the logarithm of the inverse of each pooled serum dilution. The dose-response curve for LL pooled sera showed that the highest sensitivity, the maximum curve slope, was reached at a concentration between 2 and 20 $\mu\text{g/ml}$ of rML 10kD (Fig. 1); 1: 100 serum dilutions of pooled LL patients' sera rendered the best discrimination with control sample (HNC pooled sera) at 2-20 $\mu\text{g/ml}$ antigen concentration with a minimal immunoglobulin inespecific binding to the plate (Fig. 1). The OD values were also plotted versus the logarithm of the inverse of pooled HNC serum dilutions and as

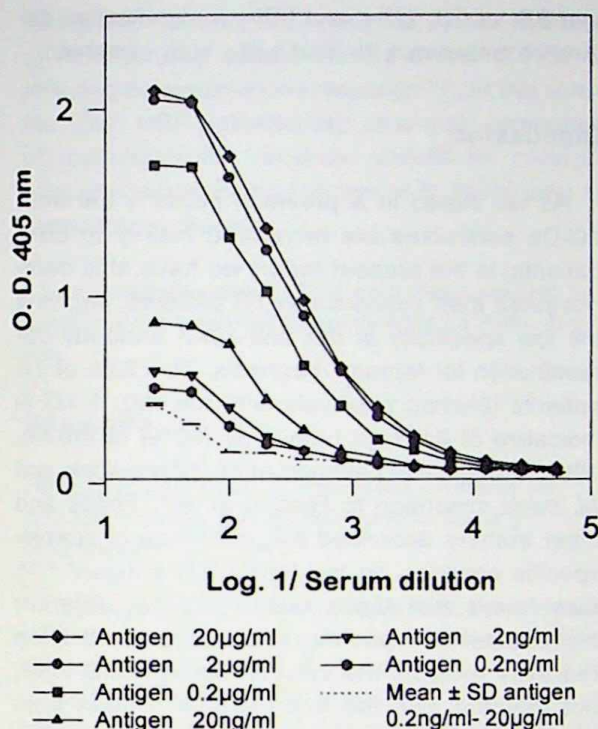


Fig. 1.— Optical density (OD) values at 405 nm determined after 60 min of substrate incubation were plotted versus the logarithm of the inverse of each pooled serum dilution. Full line curves represent the response of pooled LL sera at different antigen 10kD recombinant protein of *Mycobacterium leprae* concentrations; dashed line curve represents average response \pm SD of pooled healthy non contacts (HNC) sera at different antigen concentrations.

results were similar with different antigen concentrations, only one curve was drawn; each point of this curve represents the mean \pm SD of the OD determinations obtained from each pooled HNC sera dilution with the 6 different antigen concentrations (SD was $< 3\%$ of the mean) (Fig. 1).

Dose-response curves showed that an antigen concentration of 2 µg/ml was adequate to measure anti-10kDa levels in sera diluted 1/100, since the highest slope of the curve was between 2 and 20 µg/ml of antigen. The peak of absorbance was observed at 60 min of incubation (Fig 1); however evaluation at 20 and 30 min also rendered enough discriminative absorbance compared with HNC pooled sera.

Reproducibility assessment

The assay reproducibility was tested by two methods: the coefficient of variation (CV%) and a *t*-test for the difference between 2 assays.

The CV% expressed the SD as a percentage of the mean (CV% = SD/mean \times 100); it was referred to the

within-assay variation or to the between-assay variation and it was calculated for a single sample and its duplicate and also for mean values of two groups of duplicates. An ELISA is acceptable when the within-assay variation is less than 2-3% and the between-assay factor is less than 10%²¹.

The *t*-test for detecting the difference between two assays was calculated as follows²¹:

$$t_{df = n - 1} = \frac{\text{difference in means of two distributions}}{SD / \sqrt{n}}$$

Whether the estimated *t* value was not significant (95% confidence), both distributions were comparable and the reproducibility of the assay was considered acceptable.

The within-assay CV% for a single sample was 1.38% and for means of duplicates was 0.94%; on the other hand between-assay CV% were 7.94% and 7.88% for a single sample and for means of duplicates respectively. Therefore the reproducibility was acceptable since the within-assay and between-assay variation were less than 3% and 10% respectively. In addition, when the average difference in means of two distributions was estimated and the *t*-test applied, the factor obtained was 0.43, without statistical significance (95% confidence interval), showing that both assays were comparable.

Sensitivity and specificity assessment

The sensitivity for leprosy diagnosis was estimated as the percentage of positive results in leprosy group (it was calculated for each clinical form and for the total leprosy population).

The specificity for leprosy detection was calculated as the percentage of negative results in HNC, TB and also in these two populations considered together (HNC + TB).

Statistical analysis

Normality and homogeneity of variance (homoscedasticity) were calculated by Kolmogorov and Bartlett methods respectively¹⁹. Non parametric multiple comparisons were performed by Kruskal-Wallis with tied ranks tests and compared with $\chi^2_{(0.05; n-1)}$, in concordance to the non normal data distribution calculated by Kolmogorov test as well as the heterogeneity of variances studied by the Bartlett test. Non parametric comparisons were calculated among HNC, TB and leprosy groups for anti-10kD antibody by the method suggested by M. Hollander and D. A. Wolfe²⁰; no data were excluded.

Results

Sensitivity and specificity for leprosy detection. According to the dose-response curve, 2 µg/ml

antigen concentration and 1:100 serum dilution were selected to perform the antibody determination in all serum samples.

The sensitivity for leprosy detection was 44% for LBI+ patients (19/43), 36% for LBI- (8/22), 14% for BT/TT (3/22), 10% for HFC (4/39) and 33% for TB patients (5/15) (Table 1).

The specificity for leprosy detection varied according to the population considered; the specificity calculated in relation to the HNC was 100%, to the TB group was 66% while when this two populations were grouped together, the specificity was 91%.

Antibody levels in leprosy and TB population. Optical density (OD) values determined at 405 nm representing antibody levels were compared. LBI+ patients showed the highest levels of anti-10kDa antibodies ($x \text{ OD} = 0.345 \pm 0.083$) and these levels decreased along the leprosy spectrum, according with our previous report²². TB patients showed a mean value of 0.129 ± 0.052 , being different from the control group ($p < 0.05$) (Table 1).

The Kruskal-Wallis analysis showed that HNC did not differ from HFC, TT/BT and BB, although it was different from LBI+ and LBI- and TB. While the non parametric multiple comparison did not show any difference either among HFC, TT/BT

and BB, or TB, LBI+ and LBI-, a significative difference between LBI- and LBI+ was observed.

Discussion

As we stated in a previous paper²², the anti-10kDa antibodies are increased mainly in LBI+ patients; in the present report we have also demonstrated their existence in TB patients, showing the low specificity of the anti-10kD antibody determination for leprosy diagnosis. The 33% of TB patients reacting positively with the rML 10kD is indicative of the high homology (90%) of the ML 10kD with the 10kD antigen of *M. tuberculosis* and *M. bovis* according to Rivoire et al.⁹. These and other authors described the existence of species-specific epitopes on the HSP10kD antigen^{8, 9, 23}; they found that Mabs raised against different mycobacterial species showed a restricted reactivity towards the corresponding 10kD HSP, not reacting with the homologous protein from other mycobacterial species; for example Mab CS01⁹ reacts with 10kD from *M. leprae* and Mab SA12²³ reacts with BCG-a 10kD HSP. Despite the expression of these species-specific epitopes on the 10kD HSP, the polyclonal response against this antigen in patients with different mycobacterial diseases, may involve species common epitopes as found in the present work; therefore the detection of these antibodies lack enough specificity for leprosy detection. Moreover, when the intergroup differences were analysed by means of the Kruskal-Wallis test it could be observed that patients with active TB were similar to lepromatous patients (LBI+ and LBI-) as far as the anti-10 kD antibody level was concerned. This fact may be attributable to the heterogeneous response in these groups, which showed values highly dispersed with respect to the mean, and also to the higher bacterial load of these groups compared with the others (BB, BT/TT, HFC and HNC).

Although this assay has not shown enough specificity or sensitivity for leprosy detection and epidemiologic screening, it allowed to demonstrate that a significant proportion of leprosy and TB patients harbor high levels of anti-ML10kD antibodies. This finding must be considered of value

TABLE 1.— Average levels of anti-10kDa antibodies estimated by their OD values in different groups of leprosy patients, conviviants and tuberculosis patients

Clinical Form (n)	Mean OD value ± S.E.	p	DI+
LBI+ (43)	0.345 ± 0.083	< 0.001	44%
LBI- (22)	0.112 ± 0.044	< 0.05	36%
BB (10)	0.013 ± 0.002	N.S.	0%
BT/TT (22)	0.156 ± 0.086	N.S.	14%
HFC (39)	0.038 ± 0.01	N.S.	10%
TB (15)	0.129 ± 0.052	< 0.05	33%
HNC (43)	0.026 ± 0.003		0%

LBI+: lepromatous leprosy patients with positive bacilloscopy; LBI-: lepromatous leprosy with negative bacilloscopy; BB: borderline leprosy; BT/TT: borderline tuberculoid and tuberculoid leprosy patients; HFC: healthy family contacts; TB: tuberculosis patients. HNC: healthy non contacts; S.E.: standard error. DI+: detectability index of positives (OD: optical density value > 3 SD of HNC mean). N.S.: non significant.

taking into account the highly conserved structure of HSP among species and the role of HSP in inducing an autoimmune response^{24,29}. In this sense the 10kD HSP potential use as a main component of mycobacterial vaccines should be carefully evaluated considering the risk of to developing an autoimmune disease^{9, 11}.

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Resumen

Reacción cruzada de anti-10kD proteína de choque térmico en pacientes con lepra y tuberculosis

Se evaluó la inducción de anticuerpos contra la proteína recombinante de 10kD de *Mycobacterium leprae* (rML10) mediante un ELISA indirecto en el suero de pacientes con lepra, en convivientes de primer grado, en pacientes con tuberculosis así como en controles sanos de un área endémica de lepra en el Noreste de Argentina. Se analizaron los siguientes parámetros de control de calidad del método: la variabilidad intraensayo e interensayo, las curvas dosis-respuesta y los índices de detectabilidad (especificidad y sensibilidad); como habíamos demostrado en un trabajo previo, en los pacientes con lepra con baciloscopia positiva se obtuvieron los niveles más elevados de anticuerpos anti-10kD; en el presente trabajo fue posible demostrar la reacción cruzada entre los sueros de pacientes con tuberculosis y con lepra mediante este antígeno de *M. leprae*. Este ELISA ofrece una especificidad y sensibilidad limitadas para la detección de pacientes con lepra; se confirma que los anticuerpos contra antígenos altamente conservados en la naturaleza como son las HSP, son importantes en la respuesta policlonal contra las micobacterias tanto en lepra como en tuberculosis.

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Still one may remember that in medical sciences as well as in politics, minorities have a surprising way of becoming majorities in a very short time. What we need most is more facts rather than opinions.

De todos modos debería recordarse que en las ciencias médicas lo mismo que en la política, las minorías tienen una sorpresiva forma de convertirse en mayorías. Lo que más necesitamos son hechos y no opiniones.

William B. Coley (1862-1936)

Some Thoughts on the Problem of Cancer Control. *American Journal of Surgery*, 1931