

# IgM anti-phenolic glycolipid I and IgG anti-10-kDa heat shock protein antibodies in sera and immune complexes isolated from leprosy patients with or without erythema nodosum leprosum and contacts

R.E. Rojas <sup>a</sup>, S.O. Demichelis <sup>a</sup>, E.N. Sarno <sup>b</sup>, A. Segal-Eiras <sup>a,\*</sup>

<sup>a</sup> Centro de Investigaciones Inmunológicas Básicas y Aplicadas (CINIBA), UNLP, Calle 60 y 120, 1900 La Plata, Argentina

<sup>b</sup> Leprosy Laboratory, FIOCRUZ, Manginhos, Rio de Janeiro, Brazil

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## Abstract

The aim of the present work was to evaluate the levels of anti-PGL-I and anti-10-kDa heat shock protein antibodies in serum and immune complexes isolated from leprosy patients, convivents and controls. Leprosy patients with erythema nodosum leprosum or without it were included and a comparative study was done to investigate intergroup differences. Immune complexes were precipitated from serum by polyethylene glycol 3.5%; antibody levels were measured in sera and in dissociated immune complexes by ELISA. Serum antibody levels were then correlated with immune complex-associated antibody levels. The results showed that the erythema nodosum leprosum group differed from controls, contacts and non-erythema patients in their immune complex levels. IgM anti-PGL-I and IgG anti-10-kDa heat shock protein antibodies were constituents of the immune complexes in patients with erythema nodosum leprosum, who exhibited a significant difference in their immune complex composition compared with controls, contacts and non-erythema patients; while free antibody levels (anti-PGL-I and anti-10-kDa) did not differentiate between erythema and non-erythema patients, the measurement of immune complex-associated antibodies demonstrated a significant difference between the two clinical conditions. Furthermore, the measurement of immune complex-associated anti-PGL-I IgM made it possible to differentiate between contacts and controls. The significance of these results is discussed.

**Keywords:** Leprosy; Erythema nodosum leprosum; Immune complex; Phenolic glycolipid I; 10-kDa Heat shock protein

## 1. Introduction

The natural history of leprosy is characterized by a slow and progressive clinical course which may be interrupted by acute exacerbations called reactional

states. Two main forms of reactional states have been described: type 1 called reversal reactions and type 2, erythema nodosum leprosum (ENL) [1,2]. Type 1 reaction is one of the major causes of nerve damage in leprosy patients leading to disabilities of varying severity; it is characterized by episodes of increased inflammatory activity in skin and/or nerves in patients with borderline leprosy, whose immuno-

\* Corresponding author. Tel.: +54 (21) 214-756, ext. 346; fax: +54 (21) 259-115/259-116; e-mail: aeiras@satlink.com

logical status is unstable; these episodes are associated with a delayed cellular hypersensitivity to *Mycobacterium leprae* antigens. On the other hand, type 2 reaction occurs mainly in lepromatous leprosy, usually after 1 year of treatment, and involves systemic symptoms. Although ENL has been extensively studied in the past, there are major gaps in the immunological understanding of this reaction. The underlying mechanism is thought to be primarily humoral related to the Arthus phenomenon (type II hypersensitivity of the classification of Gell and Coombs) [3]. ENL is characterized by the deposition of extravascular immune complexes (IC), high antibody levels, a lower bacterial load and an enhanced T helper cell activity [4].

IC have been demonstrated along the clinical and immunopathological spectrum of leprosy and they are considered to be involved in the pathogenesis of reactions [5–7]. Furthermore, IC play an immunomodulatory role in leprosy according with Tyagi et al. [8,9] and Vaishnavi et al. [10].

The knowledge of the antigenic and antibody composition of the IC in leprosy may be useful for clarifying the immunopathogenesis of reactional states, especially of ENL; moreover, the presence of IC or their components could be considered as early markers of *M. leprae* infection. *M. leprae*-associated antigens as well as autoantigens have been mentioned as the main constituents of IC [11–14]. The presence of the 65-kDa antigen from *M. leprae* was clearly established with polyclonal antibodies. However, Sinha et al. [11] and we (manuscript submitted) failed to identify *M. leprae* antigens in IC through monoclonal antibody assays. Taking into account the difficulties in assessing IC antigenic moieties, it might be worthwhile to identify specific antibodies which should give indirect evidence of the antigen presence in circulating IC.

The aim of the present work was to investigate the presence of anti-phenolic glycolipid I (PGL-I) IgM and anti-10-kDa heat shock protein (hsp) IgG in IC isolated from sera of leprosy patients with and without ENL, as well as in household contacts and controls. The correlation between antibody levels in IC and in sera was calculated in order to determine the significance of these specific antibodies and their antigens in the immunopathogenesis of ENL and also in the subclinical infection.

## 2. Materials and methods

### 2.1. Patients and controls

The population in the study included patients with leprosy, household contacts and healthy controls from the same area. Sera of 29 patients (age range 15–61, mean  $40 \pm 13$ ), classified according to the Ridley and Jopling criteria [15], were provided by the Leprosy Laboratory IOC, FIOCRUZ, Mangunhos, Rio de Janeiro, Brazil. Patients were divided into two groups: (a) patients with ENL (ENL,  $n = 19$ ); (b) patients without ENL (NENL,  $n = 10$ ). Patients with ENL belonged to the BL or LL clinical forms. The group without ENL included five patients with the BL/LL form while the other five were BT; both untreated patients and patients on multi-drug therapy (MDT) for periods ranging from 1 to 72 months were included. Twelve patients had been on MDT for less than 1 year (two of them were diagnosed and began the treatment at the moment the blood sample was obtained); 12 had been on treatment for 1–4 years, while eight patients had received MDT for more than 4 years.

Healthy family contacts (HFC,  $n = 13$ ), sharing the same household as the multibacillary patients in the last 3 years, were included.

Negative control sera (healthy non-contacts, HNC;  $n = 15$ ) were obtained from blood donors of the same area.

Serum samples were separated from 10 ml of venous blood and after centrifugation, they were aliquoted in 250  $\mu$ l and frozen at  $-70^\circ\text{C}$  until use.

### 2.2. Antigens

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

The 10-kDa recombinant protein of *M. leprae* (rML10) was a kind gift of Dr. M. Singh (GBF, Braunschweig, Germany).

### 2.3. Monoclonal antibodies (MAB)

The MAb F-4721-3 (mc 7740), reactive with the disaccharide epitope of PGL-I, was provided by Dr. A.H. Kolk (Biomedical Research, N.H. Swellengrebel Laboratory, The Netherlands).

MAB CS-01 (mc 9245) reactive with the epitope of the 10 kDa hsp was kindly supplied by Dr. Shinnick (Center for Disease Control, Atlanta, GA, USA). Both MAB and recombinant antigen were supplied through the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR).

### 2.4. Polyethylene glycol (PEG) precipitation test

Circulating IC were detected by the 3.5% PEG-6000 precipitation test [16]. Briefly, 100 µl of freshly collected sera was diluted 1:10 with borate buffer pH 8.4; an equal volume of 7% PEG (Sigma, St. Louis, MO, USA) in borate buffer was added and the mixture was kept overnight at 4°C. The precipitate was collected by cold centrifugation at 8000 rpm and washed once with chilled 3.5% PEG. Finally the pellet was reconstituted in 2 ml NaOH 0.1 M and the optical density (OD) of the mixtures was read at 280 nm; each serum was assayed in duplicate and in three different experiments. Samples with OD values above the mean+3 S.D. of the HNC were considered positive.

### 2.5. Isolation of IC by PEG precipitation

IC isolation was performed by precipitation of serum in 3.5% PEG-6000, according with Zubler et al. [17,18]. Briefly, 200 µl of serum was diluted 1:5 in phosphate buffered saline (0.15 M, pH 7.4) (PBS) containing 0.02 M disodium EDTA (PBS-EDTA); an equal volume of 7% PEG (in PBS-EDTA) was added, and the mixture was kept overnight at 4°C. The precipitate was collected by cold centrifugation and washed once with chilled 3.5% PEG. Finally the pellet (enriched IC) was reconstituted in PBS up to a volume of 100 µl.

### 2.6. Dissociation of IC

Dissociation was performed according to the

methodology of Yamashita et al. with some modifications [19]. Briefly, to 100 µl of the precipitates obtained from serum, 100 µl of cold 0.2 M HCl-glycine, pH 2.8, was added and incubated at 4°C for 15 min; then, the solution was neutralized with 50 µl of 1 M K<sub>2</sub>HPO<sub>4</sub> and diluted by adding 2% BSA with Tween 20. Finally, the dissociated IC were tested by an enzyme-linked immunosorbent assay (ELISA) within 30 min.

### 2.7. PGL-I (ND-O-BSA) ELISA

Anti-PGL-I (ND-O-BSA) IgM in serum and in dissociated IC was measured by ELISA, according to the method described by Cho et al. [20]. Briefly, ELISA flat-bottom 96-well polystyrene microtiter plates (Nunc Immunoplate, Roskilde, Denmark) were coated with ND-O-BSA or with the corresponding batch of BSA (100 µl per well) at a concentration of 3 µg ml<sup>-1</sup> 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 PBS and blocked with 3% BSA in PBS (BSA-PBS, 200 µl per well) for 2 h at 37°C. After washing twice, serum samples at 1:100 dilution in 2% BSA-PBS containing 0.05% Tween-20 (PBST) or IC at 1:20 dilution were incubated in duplicate for 1 h at 37°C (100 µl per well). Then plates were washed four times with PBST and 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 µl per well). After washing thoroughly, freshly prepared 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (Sigma), the substrate solution was added and incubated for 1 h in the dark. The main difference between the ELISA for serum and for IC was the dilution factor, 1:20 for IC and 1:100 for serum.

The OD was read at 405 nm in a micro-ELISA reader (Titertek Multiskan Plus, Flow Lab., Finland); for each serum or IC 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' if the OD exceeded by 3 S.D. the mean OD obtained from normal sera at the same dilutions. To correlate data, three titrated pools of serum showing high, medium and low antibody lev-

els were included in each plate, while PBS with 0.5% BSA was tested as the control (zero point activity). After reading, a curve was drawn for each plate, using the zero and standard values, the slope and correlation coefficient were calculated. If these data were not satisfactory (slope too low, fit below 98%), plates were discarded and the assays were repeated.

### 2.8. Recombinant 10-kDa hsp ELISA

Antibodies of the IgG isotype to rML10 were measured by indirect ELISA as described by Meeker et al. with minor modifications [21]. The optimal coating concentration for hsp was determined by titration tests using MAb. ELISA plates (Nunc) were coated with  $2 \mu\text{g ml}^{-1}$  rML10 in 0.05 M carbonate buffer pH 9.6 and incubated at  $37^\circ\text{C}$  for 1 h and then at  $4^\circ\text{C}$  overnight in a humidified chamber. After washing and blocking with 3% BSA in PBS, appropriate dilutions of serum samples or dissociated IC samples in BSA-PBST were added and incubated for 1 h at  $37^\circ\text{C}$ . The plates were then washed four times with PBST and incubated for 1 h with HRP-labeled rabbit anti-human IgG (Dako) diluted in BSA-PBST (1:2000). Subsequent steps were similar to those described for anti-ND-O-BSA Abs.

Reference sera from control subjects and lepromatous patients were included every time the ELISA was performed and the coefficient of variation in a number of separate assays was less than 5%.

Samples with OD values above the mean+3 S.D. of the HNC were considered positive.

### 2.9. Statistical analysis

The normality of the data was evaluated by Kolmogorov-Smirnov and the homogeneity of variances by Bartlett's method; non-parametric data testing for unequal sample sizes (Kruskal-Wallis with tied ranks) was performed in order to investigate similarities among groups and compared by  $\chi^2$  ( $P < 0.05$ ;  $n-1$ ). Non-parametric multiple contrasts (by tied ranks) were done in order to test intergroup differences among ENL, NENL, HFC and HNC, it was calculated by the same method for each antibody in sera and in IC [22].

In order to investigate the correlation between the presence of free antibodies (anti-PGL-I and anti-10-

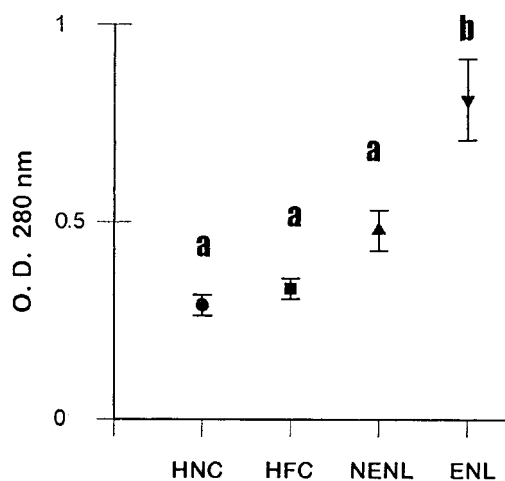
kDa) in sera and as components of IC, a simple Spearman ( $P < 0.05$ ) rank correlation coefficient was performed; the same test was applied to investigate the correlation between antibody levels in IC vs. IC levels. The non-parametric Kendall correlation analysis was also performed.

## 3. Results

### 3.1. IC levels with the 3.5% PEG precipitation test

Leprosy patients with ENL yielded the highest levels of IC when compared with all the other groups (Fig. 1A). This observation was also outstanding when comparing ENL with NENL group ( $x = 0.813 \pm 0.103$  (S.E.M.) vs.  $x = 0.479 \pm 0.051$ ,  $P < 0.05$ ) (Table 1).

The percentage of positives samples (number of positives/total) showed marked differences between groups, with 74% in the ENL group, 30% in NENL and no positive samples in HFC (Table 2).



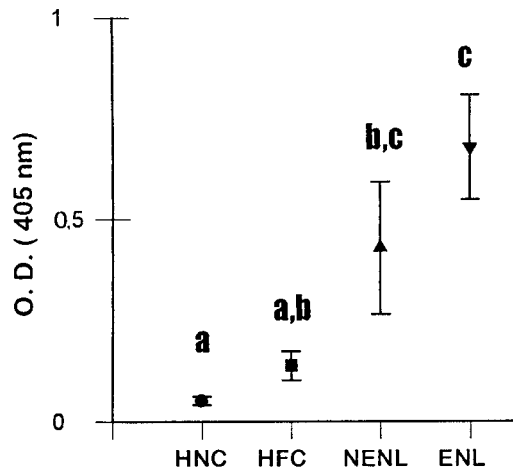
### A) SERUM IMMUNE COMPLEXES

Fig. 1. Serum IC levels (A) measured by OD at 280 nm. Levels of anti-PGL IgM in sera (B), anti-10-kDa IgG in sera (C), IC-associated anti-PGL IgM (D) and IC-associated anti-10-kDa IgG (E) measured by OD at 405 nm in different groups of patients and controls: HNC ( $n = 15$ ), HFC ( $n = 13$ ), NENL ( $n = 10$ ) and ENL ( $n = 19$ ). a, b, c indicate significant differences ( $P < 0.05$ ) among groups by Kruskal-Wallis multiple comparisons (with tied ranks).

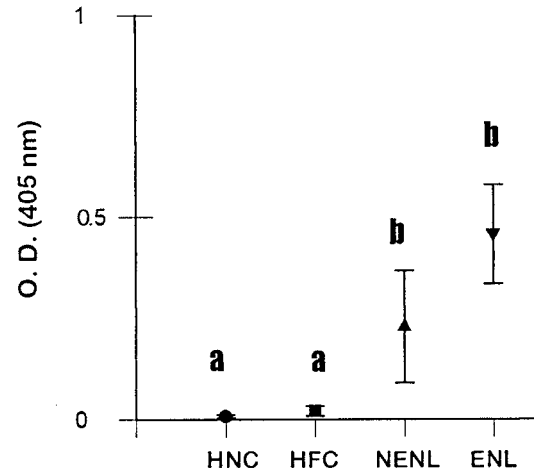
### 3.2. Anti-PGL-I (ND-O-BSA) IgM response in sera

Serum IgM antibody levels to ND-O-BSA were higher in ENL patients than in NENL patients ( $x = 0.679 \pm 0.130$  vs.  $x = 0.429 \pm 0.164$ ), however,

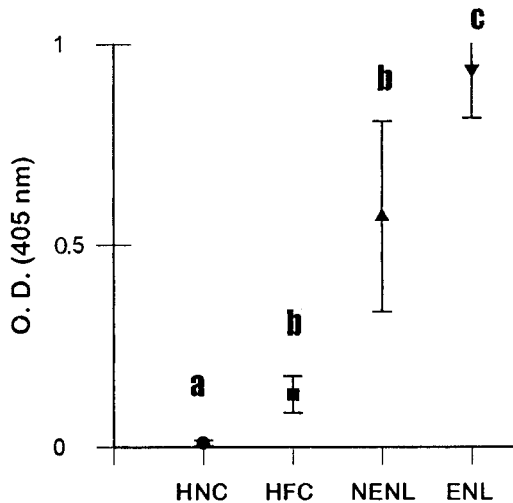
this difference was not statistically significant (Fig. 1B). The ENL group showed significant differences when it was compared with the HNC ( $P < 0.05$ ) and HFC groups ( $P < 0.05$ ). HFC values were not statistically different compared to the healthy control



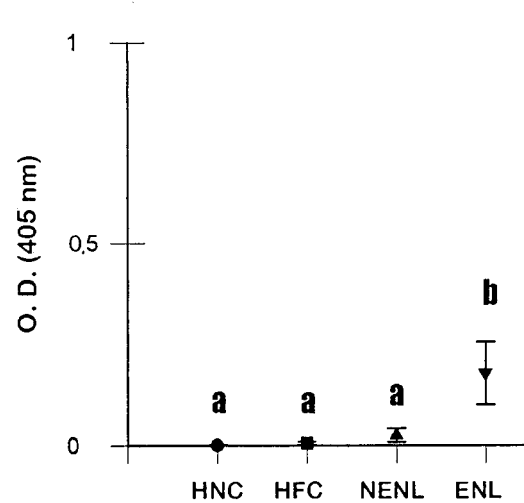
B) ANTI-PGL-I IgM IN SERA



C) ANTI-10kD IgG IN SERA



D) IC ASSOCIATED ANTI-PGL-I IgM



E) IC ASSOCIATED ANTI-10kD IgG

Fig. 1. (Continued).

Table 1

Anti-PGL-I and anti-10-kDa antibody values in sera and in precipitated IC from sera in ENL and NENL patients, contacts and controls

Clinical form	<i>n</i>	Anti-PGL-I in serum	IC-associated anti-PGL-I	Anti-10-kDa in serum	IC-associated anti-10-kDa	IC levels
HNC	15	0.052 ± 0.011	0.010 ± 0.007	0.008 ± 0.004	0.001 ± 0.001	0.289 ± 0.026
HFC	13	0.138 ± 0.036	0.130 ± 0.045*	0.021 ± 0.012	0.006 ± 0.004	0.331 ± 0.026
NENL	10	0.429 ± 0.164*	0.570 ± 0.237*	0.229 ± 0.139*	0.025 ± 0.017	0.479 ± 0.051
ENL	19	0.679 ± 0.130*	0.938 ± 0.122*	0.458 ± 0.123*	0.178 ± 0.078*	0.813 ± 0.103*

Results are means ± S.E.M.

\**P* < 0.05.

population (Table 1). The anti-PGL-I antibody response showed a variable intensity among the groups, including the ENL patients; anti-PGL-I antibody values yielded 74% positives in the ENL group, 50% in NENL and 23% in HFC (Table 2).

### 3.3. IgG responses to rML10 in sera

The antibody response to the 10-kDa hsp was elevated in ENL patients, similar to that obtained against PGL-I; ENL and NENL patients were significantly different from HFC and controls, both groups being similar to each other (Fig. 1C). NENL patients had lower anti-10-kDa antibody levels than ENL, but this difference lacked statistical significance. The highest levels of anti-rML10 were found in the ENL group followed by NENL, with averages of  $0.458 \pm 0.123$  (*P* vs. control < 0.05) and  $0.229 \pm 0.139$ , respectively (*P* vs. control < 0.05). The number of anti-10-kDa positive samples was also dependent on clinical status, showing the highest percentage in the ENL group (68%) (Table 1).

The IgM response to rML10 was also measured; low levels in patient sera were found compared to the IgG ones, without any statistical difference when compared with HNC (data not shown).

Table 2

Percentage of positive sera in different groups of leprosy patients and contacts

Clinical form	<i>n</i>	Anti-PGL-I in serum	IC-associated anti-PGL-I	Anti-10-kDa in serum	IC-associated anti-10-kDa	Precipitated IC
HFC	13	23	38	15	15	0
NENL	10	50	70	40	20	30
ENL	19	74	89	68	68	74

The cut-off for each assay was calculated as the average of the HNC group +3 S.D. Those values exceeding the cut-off level were considered positive.

### 3.4. Determination of anti-PGL IgM in IC precipitated from sera (IC associated anti-PGL-I IgM)

After dissociation, IC precipitated from serum were tested for the presence of anti-PGL-I IgM; all patients and contacts differed significantly from the control group (Fig. 1D). The highest levels belonged to the ENL patients which differed significantly from all the other groups, while the NENL group behaved similar to HFC. The average values obtained from the ENL group were  $0.938 \pm 0.122$ , followed by the NENL group ( $x = 0.570 \pm 0.237$ ) and decreasing in HFC ( $x = 0.130 \pm 0.045$ ) (*P* vs. control < 0.05) (Table 1). The anti-PGL IgM in IC showed the highest percentage of seropositive samples: 89% in ENL, 70% in NENL and 38% in HFC groups when compared with serum anti-PGL-I, serum anti-10kD, IC associated anti-10kD and precipitated IC determination (Table 2).

### 3.5. Determination of anti-10-kDa hsp IgG in IC precipitated from sera (IC-associated anti-10-kDa IgG)

The profile of anti-10-kDa antibodies in the different groups was similar to the IC precipitation profile

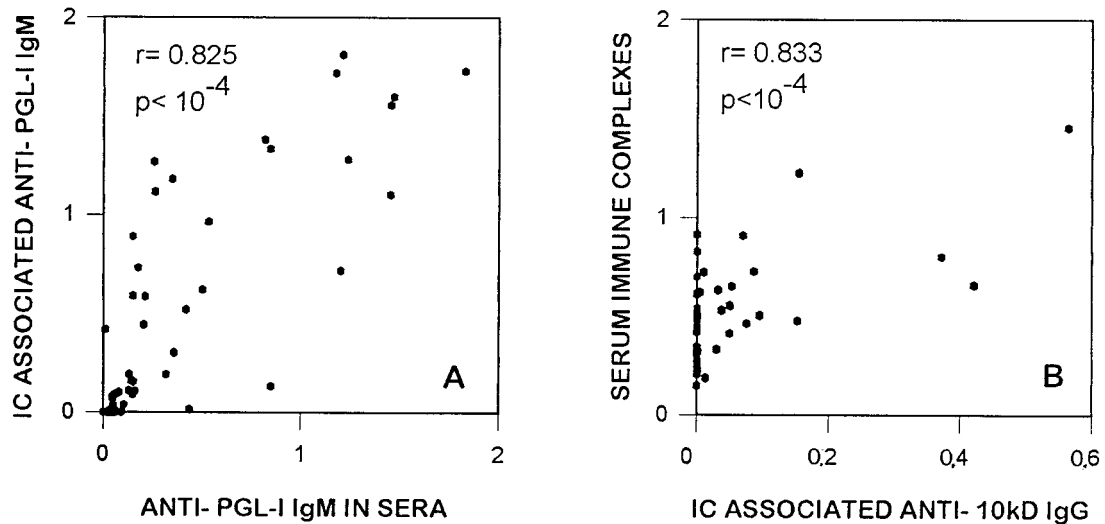


Fig. 2. A: OD correlation between anti-PGL-I IgM levels in sera and IC-associated anti-PGL IgM levels. B: Correlation between IC-associated anti-10-kDa IgG levels and serum IC levels. The correlation coefficient and significance level for each Spearman test ( $n=57$ ) are shown.

(Fig. 1A,E). Only ENL patients showed significant differences with all the other groups, with an average of  $0.178 \pm 0.078$  ( $P$  vs. HNC  $< 0.05$ ), while the NENL IC-associated anti-10-kDa yielded a low average of  $0.025 \pm 0.017$  ( $P$  vs. control non-significant). Neither the NENL group nor the HFC were different from the HNC. The seropositive percentage for each group was the lowest considering all the assays performed, except in the ENL group where 68% positive samples was found (Table 2).

### 3.6. Correlation analysis

In order to study the significance of the results, the correlation between free antibodies and IC-associated antibodies was investigated; the Spearman correlation coefficient obtained between anti-PGL-I antibodies and IC-associated anti-PGL-I was  $r = 0.825$  ( $P < 0.01$ ). A significant correlation was also obtained with the non-parametric Kendall analysis with a  $\tau = 0.6442$  ( $P < 0.0001$ ). In contrast, a poor correlation between free anti-10-kDa antibodies and IC-associated anti-10-kDa antibodies was found ( $r = 0.483$ ;  $P < 0.01$ ).

Because of the similarities between the statistical profiles obtained with IC-associated anti-10-kDa antibodies and IC determination itself, the Spearman

correlation test was applied to the results obtained in both assays; surprisingly, a high correlation coefficient of 0.833 ( $P < 0.01$ ) was achieved (Fig. 2). This high correlation was confirmed by the non-parametric Kendall analysis with the following result:  $\tau = 0.4197$  ( $P < 0.0005$ ).

## 4. Discussion

IC may be involved in the immunopathogenesis of ENL and their possible role as early antigenic reservoirs in leprosy subclinical infection can be suspected; in this sense, the knowledge of their antigenic and antibody composition should be of value. *M. leprae* natural antigens have been studied by biochemical techniques and by recombinant technology as well; two of the most important *M. leprae* antigens identified are PGL-I and the 10-kDa hsp. PGL-I is the most immunoreactive antigen of the *M. leprae* cell wall; it elicits an important IgM response in leprosy patients which reacts in parallel with the bacterial load [23–26]. Conversely, the 10-kDa hsp is the major cytosolic protein of *M. leprae* (MCP-I) representing its most abundant protein (1% of the bacterial mass). MCP-I also occurs in a highly immunogenic peptidoglycan-bound form [27,28]. Mehra et

al. demonstrated its role as a potent T cell antigen in leprosy [29] and, on the other hand, we have demonstrated that the 10-kDa hsp is able to elicit an IgG response, especially in lepromatous leprosy patients [30].

Our results showed that the ENL group differs considerably from controls, contacts and NENL patients in IC level. They displayed increased levels of IC and also a high percentage of seropositivity in the IC precipitation assay. Thus, our results are in agreement with the suspected role of IC in the immunopathogenesis of ENL.

This work allowed us to demonstrate that IgM anti-PGL-I and IgG anti-10-kDa hsp antibodies are constituents of the IC in patients with ENL. This group exhibited a significant difference in IC composition compared with controls, contacts and NENL.

The free antibody (anti-PGL-I and anti-10-kDa) determinations showed a clear distinction between ENL patients and controls or contacts; however, these assays did not differentiate ENL from NENL. Interestingly, although free antibody levels (anti-PGL-I and anti-10-kDa) did not differentiate between ENL and NENL patients, the measurement of IC-associated antibodies made evident the difference between the two clinical conditions. Furthermore, the measurement of the IC-associated anti-PGL-I antibody was the sole assay which differentiated between contacts and controls.

As regards the correlation analysis, it is worth while to emphasize that the levels of free anti-PGL-I were highly correlated with IC-associated anti-PGL-I antibody levels. This fact may indicate that anti-PGL-I incorporation into the IC is dependent on the serum level.

Concerning the anti-10-kDa response it is important to emphasize the outstanding correlation between IC-associated anti-10-kDa antibodies and IC levels. The anti-10-kDa antibodies in IC seemed to be preferentially associated with IC formation rather than specific antibody production; this high correlation was mainly observed in the ENL group. In individuals without ENL the presence of anti-10-kDa antibodies in IC is dependent on their serum levels; this could be attributable in ENL patients to the fast delivery into the circulation of the 10-kDa hsp, an intracellular mycobacterial antigen, its binding with specific antibodies, rapidly forming IC and finally

determining the consequent decrease of serum free antibodies. This behavior is completely different from that observed in the IC-associated anti-PGL antibodies; this divergence may be related to the distinct way these two antibodies are produced and also to the nature of the corresponding antigens. The production of IgM is fast and matches the appearance of the antigen in blood; thus the increment of PGL-I in the circulation leads to a rapid production of antibody; then the continuous production of this antibody facilitates its incorporation in the IC. In contrast, the induction of anti-10-kDa IgG may be slower; thus, the sudden delivery of the 10-kDa antigen in ENL serum leads to IC formation and the decrease of the antibody in sera. Conversely, in leprosy patients without ENL the scarce delivery of the 10-kDa protein induces slow and progressive antibody production. It must be underlined that the 10-kDa hsp is mainly an intracellular protein, thus its presence in blood is important when acute release in serum takes place such as happens in ENL.

Although HFC and HNC did not show significant differences between each other either in IC levels or in free antibody values, they exhibited an evident divergence in IC-associated anti-PGL-I antibodies; when IC-associated anti-PGL antibodies were measured the HFC group behaved as the NENL patients, and this fact may be attributable to a subclinical infection; therefore, the measurement of these IC-associated antibodies could be of value for early leprosy detection.

These findings may be of value for predicting the occurrence of ENL and may improve the knowledge of the immunopathogenic role of mycobacterial antigens. The study of their specific antibodies in IC, especially anti-10-kDa, could be valuable for monitoring the development of ENL since this IC-associated antibody was greatly increased in this pathological condition; in this sense, a follow-up study may be recommended in order to clarify this relationship. Furthermore, the knowledge of the role of the 10-kDa antigen in the formation of IC must be emphasized since this antigen is currently regarded as a candidate for a subunit anti-leprosy vaccine [31]; therefore, its role in inducing IC formation should be elucidated and this work may contribute to this elucidation.



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