IgG RESPONSE TO SOME MYCOBACTERIAL ANTIGENS IN SELECTED LEPROSY PATIENTS

Abdolnasser Rafi

Department of Pathobiology, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran

Abstract. Serum samples from selected leprosy patients with putative tuberculosis were tested by indirect ELISA to determine the level of IgG antibody against six mycobacterial antigen preparations. PCR-positive leprosy patients were confirmed with PGL-I ELISA. A ratio of antibodies to antigens of tuberculosis and leprosy was found to be a valuable serological marker for tuberculosis in long-treated leprosy patients.

INTRODUCTION

Over the past 80 years, several serological tests have been developed to detect *Mycobacterium leprae* infection (Melsom, 1983), however the results have not been satisfactory. Interest in the past few years in serodiagnosis of leprosy has been heightened by the development of serological tests based on supposedly *M. leprae*-specific antigenic determinants (Brett *et al,* 1983; Cho *et al,* 1983; Young and Buchanan, 1983; Klatser *et al,* 1985). The enzyme-linked immunosorbent assay (ELISA), sensitive serological technic based on phenolic glycolipid-I (PGL-I), one of the two most prominent capsular lipids of *M.leprae* (Hunter and Brennan, 1981), is now widely used in serological studies of leprosy. The ELISA technic (Engvall and Perlmann, 1971), is based on the assumption that either an antibody or antigen can be coupled to an enzyme and the resulting complex will retain both immunological and enzymatic activity. Indirect ELISA is suitable for the detection of IgG antibody.

The present study was carried out as part of the follow-up of a group of long-term-treated leprosy patients, a quarter of whom had been found to be PCR-positive for *M. leprae* (Rafi *et al,* 1995). This was suggestive of continued infection, re-infection or relapse in such patients and a few of these patients (not the same as those PCR-positive for *M. leprae*) were found to be PCR-positive for *M. tuberculosis* indicating infection with this organism (Rafi and Feval, 1995).

IgG antibody to six various mycobacterial antigen reagents was measured in serum samples from long-term-treated leprosy patients and also healthy volunteers, patients with active tuberculosis, and active lepromatous leprosy (LL) patients (as control groups) using and ELISA technic.

MATERIALS AND METHODS

Patients

In a preceding visit to Baba Baghi Leprosy Sanatorium, 279 leprosy patients with long histories (more than 10 years) of treatment for leprosy, agreed to skin-testing with 4 new tuberculins: Tuberculin (T), Leprosin A (LA), Scrofulin (S), and Vaccin (V) (Shield *et al,* 1977; Stanford *et al,* 1989; Ganapati *et al,* 1989), and to be randomized to receive an injection of saline as placebo or killed *M. vaccae* as immunotherapy (IT) (Stanford *et al,* 1990).

A year later, this study began on selected groups of these patients (n=43). They had received dapsone monotherapy over many years and agreed to provide sputum and skin tissue fluid specimens for PCR examination for tubercle and leprosy bacilli. Although these selected patients seemed to have been bacteriologically cured, they still had physical signs and manifestations of the earlier active disease. Recent clinical examinations, however, showed no obvious symptoms of any relapse in their leprosy. Their ages were between 30 and 80 years, and according to their clinical records, over the years, 22 had initial diagnoses of multibacillary (MB) leprosy, and 21 had initial diagnoses of paucibacillary (PB) leprosy. About half of them (21 patients) were selected as having Koch-type responses to tuberculin at skin-testing a year earlier suggestive of tuberculosis.

Serum samples

Form each of the above mentioned subjects, a 10 ml venous blood sample was obtained and then the

serum was separated carefully. Serum samples from 15 healthy volunteers, 11 patients with active tuberculosis, and 3 active lepromatous leprosy (LL) patients were also obtained as controls for the study.

Indirect ELISA procedure (Engvall and Perlmann, 1971; Nassau *et al,* 1976)

ELISA plates (Nunc-Immunoplates Maxisorp F-96) were coated individually with sonicated *M. tuberculosis* (son tb), secreted *M. tuberculosis* (sec tb), sonicated *M. leprae* (son lepr), PGL-I specific antigen of *M. leprae,* sonicated *M. vaccae* (son vacc), and sonicated *M. fortuitum* (son fortu) (supplied by Professor JL Stanford, UCLMS, London, UK), diluted to 5 µg/ml in coating buffer (pH 9.6), prepared in sterile nonpyrogenic water (Baxter). To coat the ELISA plates, 50 µl of antigen reagent was added to each well, and incubated overnight at 4°C in a humid environment.

The next day, ELISA plates were washed 3 times with PBS (pH 7.4)-Tween 20 (Sigma), and blocked at 37°C with 50 µl of PBS-Tween 20 containing 1% of bovine serum albumin (BSA) (Sigma) (in sterile nonpyrogenic water) in each well for 2 hours. They were washed again 3 times with PBS-Tween 20. Then serum samples, diluted 1:1,000 with PBS-Tween 20-1% BSA (the serum dilution had been optimized to 1:1,000 IgG estimations), were added in 50 µl volumes to duplicate wells. Next, ELISA plates were incubated at 37°C for 2 hours in a humid environment, and again washed 3 times with PBS-Tween 20-1% BSA.

Then peroxidase-conjugated rabbit antiserum to human IgG gamma chains (DaKo) was added at dilution of 1:2,000, 50 µl/well, and the ELISA plates were incubated overnight at 4°C in a humid environment.

On the last day, the ELISA plates were washed again 3 times and the reaction was developed by adding 50 µl of ABTS [2,2 -azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)] (Sigma) substrate dissolved in citrate-phosphate buffer (CPB) (pH 4.1) and hydrogen peroxide solution (20 volumes) (BDH) added just before use, to each well. After incubation of the plates in the dark for 30 minutes at 37°C in a humid environment, the enzymatic reaction was stopped by adding 50 µl of 0.2% sodium fluoride (Sigma) to each well. The optical density was read at 630 nm using an ELISA reader (Dynatech MR 5000).

Sensitivity and specificity results were calculated using the following formulae:

$$\text{Sensitivity} = \frac{\text{True positives}}{\text{True positives + False negatives}} \times 100$$

$$\text{Specificity} = \frac{\text{True negatives}}{\text{True negatives + False positives}} \times 100$$

The cut-off value for ELISA was calculated from the absorbance values from the healthy controls by adding 2 standard deviations to the mean value (Vikerfors *et al,* 1993).

RESULTS

All of the serum samples from individuals reacted to all antigens used in this study. The mean values of IgG antibody to various mycobacterial reagents in different groups are shown in Figs 1, 2. The greatest reaction of IgG antibody to mycobacterial antigens was found against sonicated and secreted *M. tuberculosis* antigen whereas the lowest reaction was obtained with PGL-I antigen. Statistical comparison of the results demonstrated the healthy control group to be significantly different from other groups with all antigen reagents used in this investigation, with the exception of PGL-I. With PGL-I ELISA no statistically significant difference was found between the healthy control group and the active TB patients or TB-PCR positive patients, however, significant differences were found between active LL and active TB groups ($p<0.001$). Differences were also found with PGL-I between Lepr-PCR positive and TB-PCR positive groups ($p<0.02$), and Lepr-PCR positive and Lepr-PCR negative groups ($p<0.001$). However, the difference between the two groups of TB-PCR positive and TB-PCR negative was not significant ($p<0.2$) (Fig 3).

Fig 4 shows that in PGL-I ELISA amongst long-term-treated leprosy patients, 12/43 (27.9%) were found to be seropositive. Of the 12 seropositive patients in this group, nine were Lepr-PCR positive (75%) and the sensitivity and specificity of PGL-I ELISA for Lepr-PCR positive patients reached 81.8% and 90.6% respectively. Sensitivity and specificity of the other ELISAs for Lepr-PCR positive patients in this study were as follows:

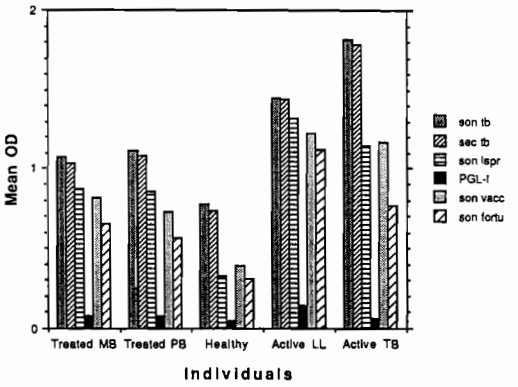

Fig 1–IgG response to mycobacterial antigens in MB and PB patients and control groups.

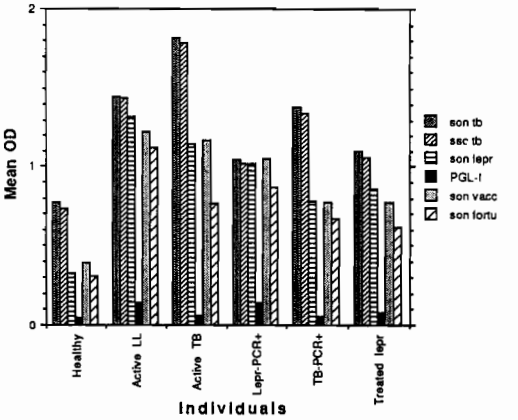

Fig 3–IgG to PGL-I in six different groups.

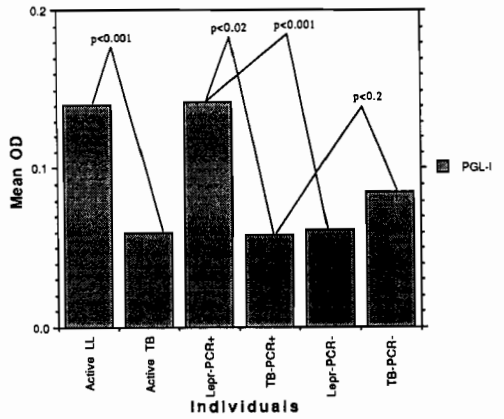

Fig 2–IgG response to mycobacterial antigens in control groups, PCR-positive patients and treated leprosy patients.

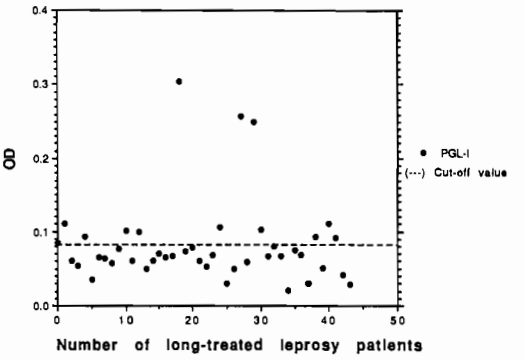

Fig 4–OD results with PGL-I for long-treated leprosy serum group.

1) Sonicated *M. leprae* (son lepr) ELISA had a sensitivity of 100% and specificity of 15.6%.
2) Sonicated *M. tuberculosis* (son tb) ELISA had a sensitivity of 45.5% and specificity of 44.7%.
3) Secreted *M. tuberculosis* (sec tb) ELISA had a sensitivity of 54.5% and specificity of 46.9%.
4) Sonicated *M. vaccae* (son vacc) ELISA had a sensitivity of 100% and specificity of 46.9%.
5) Sonicated *M. fortuitum* (son fortu) ELISA had a sensitivity of 100% and specificity of 50%

Considering the high specificity of PGL-I ELISA in comparison with those for other antigen reagents, 9/11 (81.8%) of the Lepr-PCR positive patients found in the previous *M. leprae* DNA study could be confirmed with PGL-I ELISA.

Amongst active LL patients, 3/3 (100%) were found to be seropositive for PGL-I whilst amongst active TB patients this ratio was 2/11 (18.2%), and in the healthy group was 1/15 (6.7%). In contrast, none of the TB-PCR positive patients of the previous *M. tuberculosis* DNA study were found to be seropositive for PGL-I (0/6, 0%).

Table 1

Correlation between seropositivity for PGL-1 and skin test positivity with 4 new tuberculins.

Skin-testing	No. of skin test positive* patients	No. (%) of PGL-1 seropositive patients amongst the skin test positive group (tested a year later)
Tuberculin	39	8 (20.5%)
Leprosin A	13	7 (53.8%)
Scrofulin	15	4 (26.7%)
Vaccin	14	6 (42.9%)

*Reactions of 2 mm or more are taken as positive responses with these reagents[1].

Correlation between seropositivity for PGL-I and skin test positivity to 4 new tuberculins tested a year earlier are shown in Table 1. Most correlation was obtained between seropositivity for PGL-I and skin test positivity to leprosin A.

When the long-term-treated leprosy patients were separated into MB and PB groups, no considerable difference was found between them.

Individually comparison of those long-treated leprosy patients who had received immunotherapy a year earlier with those who had received placebo, showed no statistically significant difference with any of the reagents.

DISCUSSION

In leprosy, it has been shown that the decrease in antibody activity was most significant in the IgG assay (Brett *et al,* 1983). IgG antibody levels therefore appear to be the most sensitive indicator especially during the long-term treatment of leprosy.

In the present study, PGL-I ELISA proved to be a satisfactory technic for the detection of IgG antibody to *M. leprae*. In comparison with antibody levels detected to the crude antigens used in this study, very low levels of IgG antibody to the PGL-I antigen of *M. leprae* were found. A similar finding has been reported before, using glycolipid antigen of *M. leprae* compared with crude sonicates (Brett *et al,* 1983). However, in this investigation of PGL-I ELISA no statistically significant differences were found between groups of healthy control persons, active TB patients, TB-PCR positive leprosy patients, and TB-PCR negative leprosy patients. On the other hand, statistically significant differences were found between groups of active LL and active TB patients, Lepr-PCR positive and TB-PCR positive leprosy patients, Lepr-PCR positive and Lepr-PCR negative leprosy patients. This suggests that PGL-I ELISA can be specifically used as a diagnostic method in serological studies of leprosy patients.

The high specificity of PGL-I ELISA obtained in this study confirms the effectiveness of this technic for the serodiagnosis of leprosy patients.

The finding of the greatest seropositivity for PGL-I in active LL and Lepr-PCR positive leprosy patients also supports the value of PGL-I ELISA in the serodiagnosis of leprosy patients.

The finding of more correlation between PGL-I seropositivity and leprosin A-positivity but not with the three other skin test reagents (tuberculin, scrofulin, and vaccin) (Table 1), shows the usefulness of leprosin A skin-testing for leprosy.

The difference in PGL-I IgG between long-treated MB and PB patients was not significant. An explanation for this may be that the PB patients studied included more Lepr-PCR positive cases than were found among MB patients. In support of this, in another study (Lefford *et al,* 1991) it was found that the PGL-I IgM ELISA may have its greatest diagnostic value in PB disease. In the same study, it was inferred that casual exposure to *M. leprae* or subclinical infection, is insufficient to induce a detectable humoral immune response to PGL-I. Further studies of antibodies to PGL-I on treated MB and PB patients are needed to establish

the difference between these two groups.

From the data on mean values of different groups (Figs, 1, 2), taking the following steps might lead to a means of serodiagnosis for tuberculosis in leprosy patients:

1) In patients with mycobacterial disease, an increase of the mean value for son tb above the mean value for son lepr, enables calculation of the son tb/son lepr ratio.
2) Exclusively in active TB and TB-PCR positive groups the son tb/son lepr ratio exceeds 1.5 and reaches to high ratios of 1.6 and 1.8 respectively.

Considering the latter step, it appears that son tb/son lepr ratios of 1.5 and above indicate infection with *M. tuberculosis*. Further investigations are needed to confirm this and determine the usefulness of crude antigens of *M. tuberculosis* in the serodiagnosis of tuberculosis.

In conclusion, the comparative results of PGL-I ELISA work and previous *M. leprae* DNA study demonstrated that PGL-I ELISA can differentiate between leprosy patients positive or negative by PCR. Thus it could be applied in relapse or re-infection studies of leprosy. A ratio of antibodies to antigens of tuberculosis and leprosy was found to be potentially useful serological marker for tuberculosis in leprosy patients.

ACKNOWLEDGEMENTS

I would like to thank Dr MH Modayen, Director, and Dr F Feval, leprologist of Baba Baghi Leprosy Hospital of Iran for their assistance and support. Professor JL Stanford's supply of the mycobacterial antigens from UCLMS, London is gratefully acknowledged.

REFERENCES

Brett SJ, Draper P, Payne SN, Rees RJW. Serological activity of a characteristic phenolic glycolipid from *Mycobacterium leprae* in sera from patients with leprosy and tuberculosis. *Clin Exp Immunol* 1983; 52: 271-9.

Cho SN, Yanagihara DL, Hunter SW, Gelber RH, Brennan PJ. Serological specificity of phenolic glycolipid I from *Mycobacterium leprae* and use in serodiagnosis of leprosy. *Infect Immunol* 1983; 41: 1077-83.

Engvall E, Perlmann P. Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. *Immunochem* 1971; 8: 871-4.

Ganapati R, Revankar CR, Lockwood DNJ, *et al.* A pilot study of three potential vaccines for leprosy in Bombay. *Int J Lepr* 1989; 57: 33-7.

Hunter SW, Brennan PJ. A novel phenolic glycolipid from *Mycobacterium leprae* possibly involved in immunogenicity and pathogenicity. *J Bacteriol* 1981; 147: 728-35.

Klatser PR, De wit MYL, Kolk AH. An ELISA-inhibition test using monoclonal antibody for the serology of leprosy. *Clin Exp Immunol* 1985; 62: 468-73.

Lefford MJ, Hunegnaw M, Siwik E. The value of IgM antibodies to PGL-I in the diagnosis of leprosy. *Int J Lepr* 1991; 59: 439-40.

Melsom R. Serodiagnosis of leprosy: the past, the present, and some prospects for the future. *Int J Lepr* 1983; 51: 235-52.

Nassau E, Parsons ER, Johnson GD. The detection of antibodies to *Mycobacterium tuberculosis* by microplate enzyme-linked immunosorbent assay (ELISA). *Tubercle* 1976; 57: 67-70.

Rafi A, Donoghue HD, Stanford JL. Application of polymerase chain reaction for the detection of *Mycobacterium leprae* DNA in specimens from treated leprosy patients. *Int J Lepr* 1995; 63: 42-7.

Rafi A, Feval F. PCR to detect *Mycobacterium tuberculosis* DNA in sputum samples from treated leprosy patients with putative tuberculosis. *Southeast Asian J Trop Med Public Health* 1995; 26: 253-7.

Shield MJ, Stanford JL, Paul RC, Carswell JW. Multiple skin testing of tuberculosis patients with a range of new tuberculins, and a comparison with leprosy and *M. ulcerans* infection. *J Hyg* 1977; 78: 331-48.

Stanford JL, Bahr GM, Rood GAW, *et al.* Immunotherapy with *Mycobacterium vaccae* as an adjunct to chemotherapy in the treatment of pulmonary tuberculosis. *Tubercle* 1990; 71: 87-93.

Stanford JL, Stanford CA, Ghazi-Saidi K, *et al.* Vaccination and skin test studies on the children of leprosy patients. *Int J Lepr* 1989; 57: 38-44.

Vikerfors T, Olcen P, Wiker H, Watson JD. Serological response in leprosy and tuberculosis patients to the 18-kDa antigen of *Mycobacterium leprae* and antigen 85 B of *Mycobacterium bovis* BCG. *Int J Lepr* 1993; 61: 571-80.

Young DB, Buchanan TM. A serological test for leprosy with a glycolipid specific for *Mycobacterium leprae*. *Science* 1983; 221: 1057-9.