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Comparison of IgM, IgG and IgA responses to *M.leprae* specific antigens in leprosy

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SUMMARY **Antibodies of IgM, IgG and IgA classes against *M.leprae* specific antigens (PGL-I, ND-O-BSA, and NT-O-BSA) were determined in the sera of 80 leprosy patients (28 untreated, 34 treated lepromatous and 18 tuberculoid), 25 tuberculosis patients and 33 normal individuals of Northern Thailand. No strong distinction in reactivity could be found between the three antigens. The IgM antibody assay yielded more positive results than assays for IgG and IgA. It was found that the positivity rates of IgM antibodies to all three antigens were highest in untreated lepromatous leprosy (82%). In tuberculoid leprosy, the positivity rates of IgM, IgG and IgA to the antigens were more variable, ranging from 22 to 50 percent. Patients with tuberculosis and normal individuals did not produce IgM antibodies against the antigens. The results suggested that the determination of IgM against the three antigens is a more sensitive and specific test for active leprosy than those of IgG and IgA. The relationship between the duration of treatment and IgM antibody levels in lepromatous leprosy (LL) was studied. Untreated LL patients had significantly higher IgM and IgA antibody levels than treated patients. There was no difference in IgG antibody levels between the two groups, and the levels of both groups were higher than normal controls. Serial determination of IgM antibodies in 7 LL patients revealed that treatment was strongly associated with progressive decrease in IgM antibody levels against all three antigens.**

Although several effective chemotherapeutic agents are now available for the treatment of leprosy, delayed diagnosis and drug resistant organisms render drug treatment of diagnosed cases an ineffective means for eradication of this disease. Immunoassays and reagents, both specific and non-specific to *Mycobacterium leprae (M.leprae),* have been developed in several laboratories to facilitate early diagnosis of leprosy and to monitor the effectiveness of chemotherapy in leprosy control programs. One such reagent which has been increasingly used and available in sufficient amounts for immunoassays is phenolic glycolipid-I (PGL-I).[1] PGL-I has been chemically and structurally elucidated,[2] and subsequently demonstrated to be *M.leprae*-specific.[3] It was found that in the trisaccharide portion of PGL-I, the terminal sugar, 3, 6-di-O-methyl-B-D-glucopyranosyl substitute, is the primary antigenic determinant and is essential for the recognition by specific antibodies of *M.leprae*-infected patients, especially the lepromatous type.[2,4] Two synthetic antigens have been derived from the original PGL-I antigen, using the trisaccharide determinant of PGL-I as the basic structure of the new antigens. Subsequently, disaccharide and trisaccharide compounds are conjugated to bovine serum albumin and are thus called natural-disaccharide (ND-O-BSA) and natural-trisaccharide (NT-O-BSA) conjugates respectively. These carbohydrate-containing antigens of *M.leprae* have been reviewed recently.[5] Although work has been done on the IgM antibody response to PGL-I in leprosy patients,[3,6,7] studies comparing reactivity of all three major antibody classes (i.e. IgM, IgG and IgA) to the *M.leprae* specific antigens both in the natural form (PGL-I) and its synthetic forms (ND-

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O-BSA and NT-O-BSA) have been limited. [8,9]

In this report we studied *M. leprae*-specific responses of all three major antibody classes (IgM, IgA and IgG) in sera of leprosy, tuberculosis patients and normal individuals in Northern Thailand. The responses to three *M.leprae* antigens were compared. The effect of treatment on the antibody responses in lepromatous patients was also studied.

MATERIALS AND METHODS

Sera

Sera of 80 leprosy patients attending the McKean Rehabilitation Center, the major leprosy treatment center in Northern Thailand, were collected from 62 lepromatous (LL/BL) and 18 tuberculoid (TT/BT) patients. The patients were examined and the type of disease classified by clinical and histopathological criteria as described by Ridley & Jopling. [10] Most of the patients were under treatment with diamino diphenylsulfone (DDS), clofazimine (B663) and rifampin. The lepromatous patients (LL/BL) were arbitrarily divided into 2 groups: a) 28 new patients untreated or treated for less than one year, and b) 34 old patients treated for more than one year. All sera were collected between September 1986 and February 1988 and stored as 0.5 ml aliquots at -20° C without addition of glycerine. Sera from 25 bacteriologically proved tuberculosis (TB) patients were kindly provided by Dr.S.Makonkawkeyoon, Department of Clinical Immunology, Faculty of Associated Medical Sciences, Chiang Mai University. Also included in the study were sera of 33 normal healthy individuals, of which 20 were blood bank donors and 13 RIHES staff.

Antigen

Phenolic glycolipid I, natural disaccharide octyl and natural trisaccharide-octyl-bovine serum albumin (PGL-I, ND-O-BSA and NT-O-BSA) were kindly supplied by Dr. P.J. Brennan (Colorado State University, Colorado, U.S.A.). [11,12]

ELISA assay

This assay was carried out following the methodology described by Cho *et al.* [3] Briefly, the stock solution of PGL-I stored in chloroform: methanol (2:1 by volume) at 4° C, was suspended in absolute ethanol to a concentration of 2 μg/ml. One hundred μl was added to each well of flat-bottomed polystyrene microtiter plates (Dynatech Laboratories Alexandria, VA.) The plates were incubated at 60° C for 2 hr, washed four times with phosphate buffer saline 0.15M, pH 7.4 (PBS), and nonspecific sites were blocked with PBS containing 5 percent bovine serum albumin (fraction V, Sigma) (PBS/BSA). The plates were incubated at 37° C for 1 hr in a moist chamber and after the PBS/BSA was aspirated, 100 μl of serum diluted 1:300 with PBS containing 20 percent normal goat serum (PBS/NGS) were added and incubated at 37° C for 1 hr. The plates were washed with PBS, and goat anti-human IgM, IgG and IgA peroxidase conjugates (Cappel Laboratories), diluted 1:2000 in PBS/NGS, were added at 100 μl/well. After a 1 hr incubation the plates were washed, and 100 μl of O-phenylenediamine-H_2O_2 substrate in citrate phosphate buffer, pH 5.0, were added followed by a 30-min incubation at 37° C. The reaction was stopped with 2.5N H_2SO_4 and the optical density (O.D.) was read at 492 nm using a Titertek Multiskan Reader (Flow Lab., Bonn, Federal Republic of Germany).

ND-O-BSA and NT-O-BSA antigens were dissolved in coating buffer (carbonate-bicarbonate buffer, pH 9.6) at a dilution of 1:2000 and 1:5000, respectively and 100 μl of diluted antigen were added to wells of microtiter plates which were subsequently incubated overnight at 37° C. Procedures were identical to those described for PGL-I, except that PBS containing 0.05 percent tween (PBST) was used for washing, PBST containing 1 percent BSA (1% BSA/PBST) for blocking, and PBST containing 10 percent NGS (10% NGS/PBST) was used for diluting sera and antibody peroxidase conjugates. Each serum was tested in duplicate on antigen coated wells and on control wells coated with PBS/BSA.

For reference, positive and negative sera were pooled from active lepromatous leprosy patients and normal blood bank donors, respectively. They were included in each plate to correct for the variation in reading between plates. Results were expressed as the difference in O.D. between mean O.D. of wells with antigen and mean O.D. of wells without antigen.

Statistical analysis

Student's *t* test was used. [13]

RESULTS

Optimal conditions for the ELISA using *M.leprae* specific antigens

A checkerboard titration showed that optimum conditions were established using PGL-I of 2 μg/ml for coating, serum dilution of 1:300 and HRP-conjugated antihuman IgM dilution of 1:2000 (Fig. 1). Similar titrations were carried out for optimum concentrations of NT-O-BSA and ND-O-BSA antigen, and dilutions of HRP-conjugated anti-human IgG and IgA. The dilution of test serum was fixed at 1:300 as for IgM antibody detection. Using a positive serum which gave a high O.D. for IgM antibodies (O.D. >1.400), the highest O.D.'s for IgG and IgA antibodies could be acheived with concentrations of NT-O-BSA and ND-O-BSA at 0.04 μg CHO/ml and 0.06 μg/CHO/ml respectively, whereas the dilutions of HRP-conjugated anti-human IgG and IgA were both at 1/2000.

To test the reproducibility of the ELISA assay, aliquots of pooled positive lepromatous leprosy serum

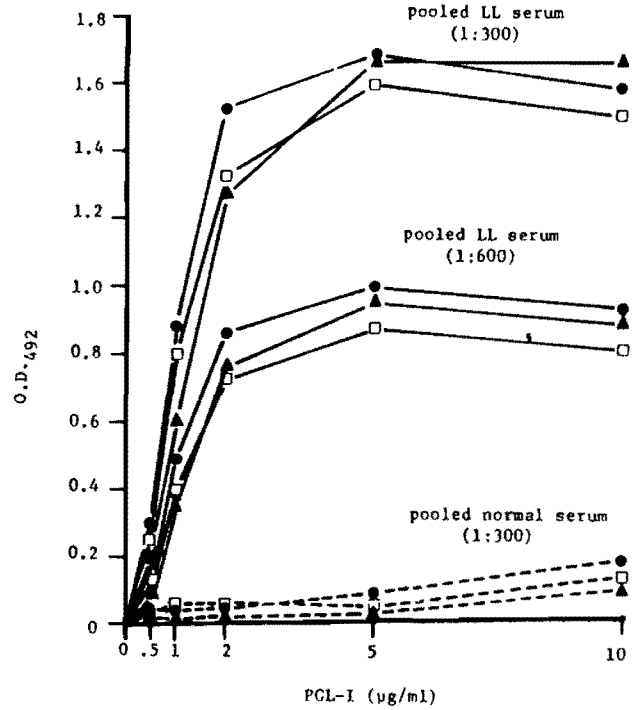

Fig. 1 Block titration of IgM antibodies against PGL–I in pooled sera from lepromatous leprosy patients (———) and normal individuals (– – – –). The sera were diluted at 1 : 300 and 1 : 600, and anti–human IgM conjugate at 1 : 500 (●), 1 : 1,000 (□) and 1 : 2,000 (▲).

were tested for within, among plates and also day-to-day variations. Results of assays done during April 1987 to June 1987 (n = 12) showed a mean (± SD) O.D. of 1.391 ± 0.082 for IgM antibodies to NT-O-BSA antigen, giving the coefficient of variability (C.V.) of the assay at 5.92 percent.

M. leprae-specific antibodies in sera of leprosy patients

Almost all lepromatous leprosy (LL/BL) sera were highly reactive to all three *M. leprae* antigens, with mean O.D. (± S.E.) for IgM antibody of 0.766 ± 0.124, 0.881 ± 0.127 and 0.815 ± 0.121 against PGL-I, ND-O-BSA and NT-O-BSA respectively (Table 1). IgM antibodies in sera of tuberculoid (TT/BT) leprosy were less reactive to the antigens, resulting in O.D.'s at 0.205 ± 0.088, 0.181 ± 0.078 and 0.201 ± 0.086 against NT-O-BSA, PGL-I and ND-O-BSA respectively. Sera of tuberculosis patients and normal controls showed very low or no IgM antibodies against all three antigens. By taking the mean O.D. + 3 S.D. of normal controls

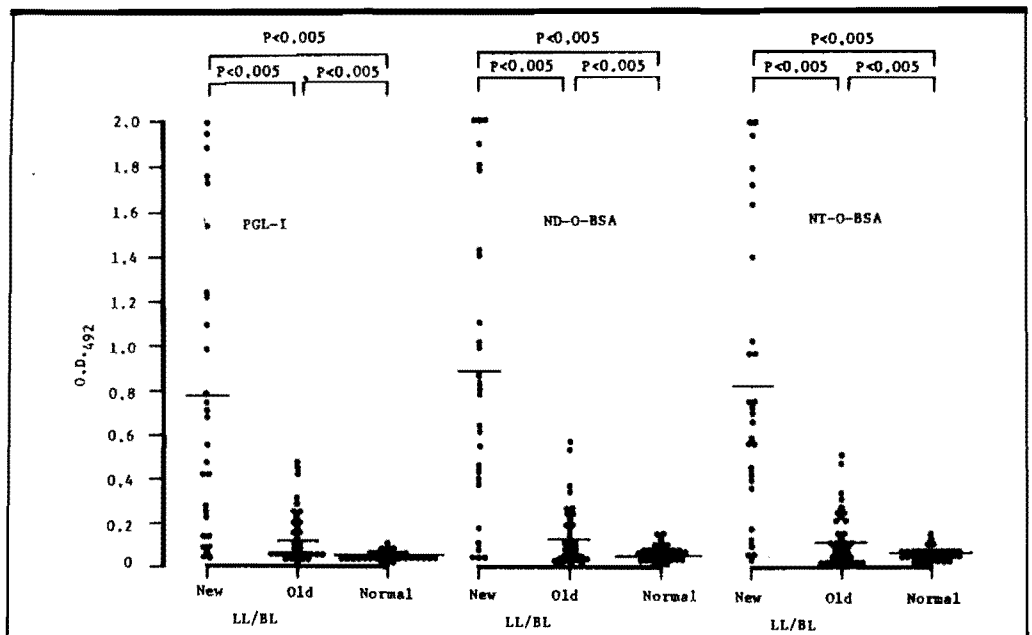

Fig. 2 IgM antibodies to *M. leprae* specific antigens (PGL–I, ND–O–BSA and NT–O–BSA) in sera of lepromatous leprosy patients with duration of treatment less than 1 year (New), more than 1 year (Old), and normal controls.

able 1. Antibodies against *M. lepree* specific antigens in sera of leprosy patients (LL/BL, TT/BT)[a], tuberculosis patients (TB) and normal controls (NC).

	No. sera tested	IgM		IgG		IgA	
		O.D. 492	No. (%)+ve[b]	O.D. 492	No. (%)+ve	O.D. 492	No. (%)+ve
PGL–1							
LL/BL	28	0.766 ± 0.124[c]	23 (82)	0.180 ± 0.032	14 (50)	0.127 ± 0.014	14 (50)
TT/BT	18	0.181 ± 0.078	5 (28)	0.211 ± 0.059	9 (50)	0.083 ± 0.006	3 (17)
TB	25	0.044 ± 0.003	1 (4)	0.100 ± 0.008	8 (32)	0.078 ± 0.006	5 (20)
NC	33	0.047 ± 0.003	0 (0)	0.050 ± 0.004	0 (0)	0.065 ± 0.002	0 (0)
ND–O–BSA							
LL/BL	28	0.881 ± 0.127	23 (82)	0.213 ± 0.054	15 (54)	0.139 ± 0.032	18 (64)
TT/BT	18	0.201 ± 0.086	4 (22)	0.289 ± 0.102	6 (33)	0.032 ± 0.009	5 (28)
TB	25	0.031 ± 0.006	0 (0)	0.075 ± 0.016	4 (16)	0.027 ± 0.002	2 (8)
NC	33	0.051 ± 0.006	0 (0)	0.041 ± 0.002	0 (0)	0.022 ± 0.001	0 (0)
NT–O–BSA							
LL/BL	28	0.815 ± 0.121	23 (82)	0.257 ± 0.078	20 (71)	0.150 ± 0.032	19 (68)
TT/BT	18	0.205 ± 0.088	5 (28)	0.073 ± 0.073	8 (44)	0.025 ± 0.010	2 (11)
TB	25	0.029 ± 0.004	0 (0)	0.049 ± 0.008	5 (20)	0.020 ± 0.002	1 (4)
NC	33	0.050 ± 0.005	0 (0)	0.029 ± 0.002	0 (0)	0.012 ± 0.002	1 (3)

a) Treated for less than 1 year.
b) Criteria for positivity: Mean + 3SD of normal controls
c) $\bar{X} \pm SE$

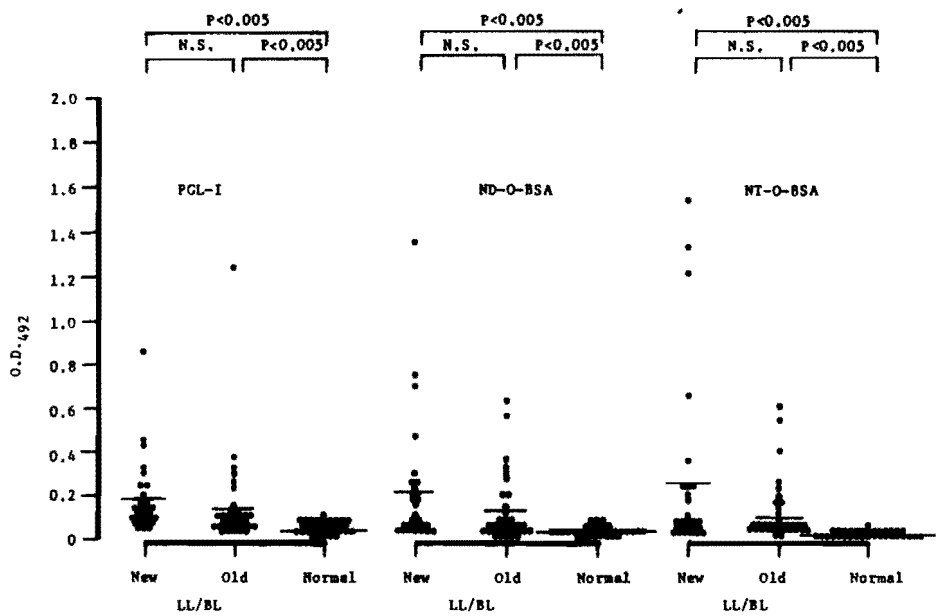

Fig. 3 Same as Fig. 2., but for IgG antibodies.

as a cut-off value, i.e. 0.095, twenty three out of 28 (82%) lepromatous sera were positive for IgM antibodies against all three antigens (Table 1). Tuberculoid patients had IgM antibodies at a much lower positive rate (28%), since only five out of 18 sera were positive. The levels of IgM antibodies to PGL-I in tuberculosis patients were not significantly different from the levels in normal control subjects ($p > 0.05$). Only 1 out of 25 (4%) TB serum gave an O.D. at 0.098, which was only marginally higher than the cut-off value. There was highly significant correlation between the IgM antibody level obtained with any pair of antigens : PGL-I *VS* ND-O-BSA ($r = 0.9602$, $p < 0.001$), PGL-I *VS* NT-O-BSA ($r = 0.9539$, $p < 0.001$) and ND-O-BSA *VS* NT-O-BSA ($r = 0.9879$, $p < 0.001$).

Antibody response of the IgG and IgA classes against the three antigens were found to have different patterns. Unlike IgM response, only 50-71 percent of LL/BL were IgG positive for the antigens. Moreover, 16-32 percent of TB sera were also

Table 2. IgM antibody levels against *M. leprae* specific antigens in sera of individual lepromatous leprosy patients during treatment[a].

LL patients	Month of blood drawing	Ig IgM antibody levels (mean O.D. 492)		
		PGL–1	ND–O–BSA	NT–O–BSA
R–002	0	0.456 (100)[b]	0.343 (100)	0.312 (100)
	6	0.324 (71)	0.244 (71)	0.233 (75)
	10	0.223 (48)	0.186 (54)	0.172 (55)
R–008	0	0.674 (100)	0.869 (100)	0.744 (100)
	2	0.292 (43)	0.490 (56)	0.462 (62)
	4	0.269 (40)	0.384 (44)	0.391 (52)
	5	0.223 (33)	0.294 (34)	0.308 (41)
R–009	0	0.559 (100)	0.800 (100)	0.659 (100)
	1	0.630 (113)	0.706 (88)	0.582 (88)
	9	0.349 (62)	0.402 (50)	0.327 (50)
R–013	0	0.779 (100)	1.096 (100)	0.958 (100)
	1	0.548 (70)	0.886 (81)	0.765 (80)
	2	0.423 (54)	0.693 (63)	0.608 (63)
R–017	0	0.236 (100)	0.355 (100)	0.327 (100)
	1	0.209 (88)	0.328 (92)	0.306 (93)
	6	0.102 (43)	0.167 (47)	0.136 (41)
R–023	0	1.874 (100)	1.903 (100)	1.720 (100)
	1	2.000 (106)	1.972 (103)	1.763 (102)
	5	1.624 (87)	1.770 (93)	1.547 (90)
R–028	0	0.736 (100)	1.015 (100)	1.017 (100)
	2	0.449 (61)	0.653 (64)	0.683 (67)
	4	0.371 (50)	0.504 (50)	0.476 (47)

a) All, except R–009, were treated for less than 1 year.
b) Percent of level of first bleeding (in parentheses).

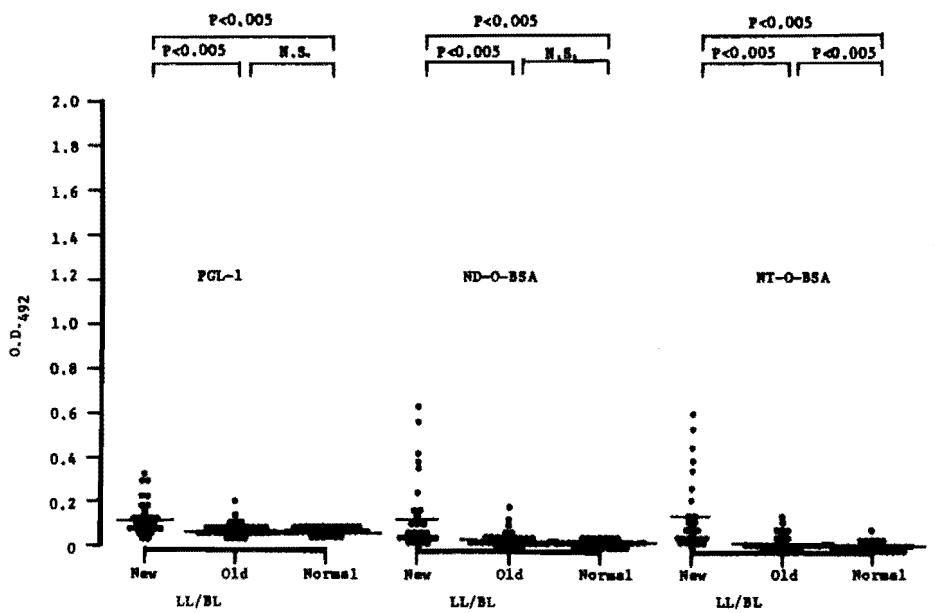

Fig. 4 Same as Fig. 2., but for IgA antibodies.

positive. For the IgA response, only 50-68 percent of LL/BL sera, 11-28 percent of tuberculoid sera, 4-20 percent of tuberculosis sera and more importantly, 3 percent of normal controls were also positive (Table 1).

Effect of treatment on antibody responses to *M.leprae* specific antigens

The relationship between chemotherapy and levels of antibodies against *M.leprae* specific antigens was studied.

Fig. 2, 3 and 4 are scattergrams of antibodies against *M.leprae*-specific antigens in the sera of 34 untreated and 28 treated lepromatous patients compared to sera of 33 normal controls. It was found that untreated lepromatous patients had significantly higher IgM antibodies than treated patients ($p < 0.005$). The latter group, however, still had higher IgM levels than normal controls ($p < 0.005$) (Fig. 2). This pattern of IgM antibody response was demonstrable against all three antigens tested. In contrast to IgM antibody response, there was no significant difference in *M.lepare*-specific IgG antibody levels between untreated and treated lepromatous patients (Fig. 3). Nevertheless, both groups of patients had significantly higher IgG antibody levels than normal controls ($p < 0.005$) (Fig. 3). The pattern of IgA antibody response to *M.leprae* specific antigens was slightly different from those of IgM and IgG antibodies. Untreated lepromatous patients had significantly higher IgA antibody levels than treated patients ($p < 0.005$) and normal controls ($p < 0.005$) (Fig. 4). Treated lepromatous patients had comparable levels of IgA against PGI-I and ND-O-BSA to normal controls. Interestingly, IgA antibody levels against NT-O-BSA in treated lepromatous patients were statistically higher than the levels in normal controls ($p < 0.005$) (Fig. 4).

Follow-up of IgM antibodies in leprosy patients during treatment

Levels of IgM antibodies were studied in lepromatous patients after they had been treated for some time. As shown in Table 2, sera drawn from 7 lepromatous patients showed declining IgM antibody activity against all three antigens after they had been treated. The rate of decrease of IgM antibody activity to half of the initial activity was variable among patients. The IgM antibody activity in sera of some patients (R-008, R-013) dropped rapidly within 2 months after treatment, whereas others showed 50 percent drop in activity when they had been treated for 10 months (R-002) or more than 2 years (R-009). These observations were true for all three *M.leprae* specific antigens tested.

DISCUSSION

Before any laboratory technique can be applied to clinical studies, the technique has to be optimised and standardised. The ELISA technique in this study, using *M.leprae*-specific antigens (PGL-I, ND-O-BSA and NT-O-BSA), was initially set up as reported by Cho *et al.* [3] In preliminary experiments, coating of polystyrene plates with PGL-I was accomplished by diluting PGL-I in absolute ethanol and incubating the plates at 60° C for 2 hr. This was slightly different from the procedure that was described by Cho *et al* [3] in which the PGL-I was suspended, sonicated and finally diluted in carbonate-bicarbonate coating buffer. We found that the solubility of PGL-I in this coating buffer was poor and resulted in low O.D. readings at 492 nm with known positive sera. When absolute ethanol was used for the PGL-I coating process, sonication of the PGL-I solution was not required (unpublished observations).

Studies on antibody responses to *M.leprae* using purified PGL-I and synthetic neoglycoproteins have been previously carried out by several investigators. [3,6,7,11] However, most of them studied antibodies of either the IgM class alone or IgM and IgG antibodies, and only with one or two of the antigens used in our study. [8,9] Douglas and co-workers [8] employed PGL-I and a synthetic antigen representing the terminal sugar of PGL-I, namely M-BGG, to study their effectiveness in monitoring antibody levels during chemotherapy. By the end of the 1st year of treatment, antibody levels to M-BGG had declined by 42 percent, by the end of the 2nd year by 61 percent and at the end of 3 years by 68 percent. The same group also found the measurement of IgM antibodies to NT-O-BSA and ND-O-BSA to be the most effective in monitoring treatment. [9] The effectiveness of IgM measurement has been confirmed by the present study which demonstrated that the IgM positivity rate was highest in lepromatous leprosy patients and lower in tuberculoid patients as compared with normal controls. Only 4 percent of tuberculosis sera gave false positive reactions for IgM antibodies to PGL-I antigen. The false positivity among healthy controls and patients with tuberculosis against *M.leprae* specific antigen may be due either to non-specific binding of sera to the plastic plate or due to a specific, albeit weak, antigen-antibody reaction, especially in individuals living in endemic areas of leprosy. [11,14] The false positivity was not observed when ND-O-BSA and NT-O-BSA were used (Table 1). Since there was good correlation between the ELISA values to any pair of antigens used in our study, it is recommended that assay of IgM antibodies to only one of these antigens would be adequate.

In assays for IgG and IgA antibodies to *M.leprae* specific antigens, more cross-reactivity against the three antigens tested was found with sera from patients with tuberculosis and normal controls (Table 1). This confirms the view that these assays are unlikely to be specific for leprosy infection. It is suggested that the ELISA for IgM antibodies to *M.leprae* specific antigens, at least to the three

antigens used in this study, should be the test of choice for the study of *M.leprae* infection, since it gives the highest positive results in leprosy patients with the fewest false positives in tuberculosis patients and normal controls. Nevertheless, the assay for IgM antibodies in tuberculoid patients was positive for only a small proportion of the population (28%). This sensitivity might be improved by using other as yet undefined glycoconjugates.

The ELISA using PGL-I was thought to be a highly specific technique for the detection of antibodies in leprosy infection since there was low cross reactivity from BCG vaccination or previous infection with *M.tuberculosis.* [15] There have been only a few limited studies exploring the possibility of using the ELISA technique for detection of antibodies against PGL-I, ND-O-BSA and NT-O-BSA as a test for the diagnosis of preclinical or subclinical leprosy infection. [14,16] Although the results of this study indicate that the ELISA for IgM antibodies to *M.leprae* specific antigens is highly sensitive and specific, it is crucial to determine its predictive value in asymptomatic contacts of leprosy patients. [17] A seroepidemiologic study to determine the predictive value of ELISA positivity in household contacts of patients with leprosy is being conducted in Northern Thailand, and it is hoped that the results of this ongoing study will contribute to a better leprosy control program.

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