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Determination of Circulating IgG Subclasses Against Lipoarabinomannan in the Leprosy Spectrum and Reactions[1]

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Lipoarabinomannan (LAM) is a crossreactive polysaccharide antigen present in association with the cell wall of *Mycobacterium leprae* and *M. tuberculosis* ([6]). It appears as a broad diffuse band with a molecular weight of 30–35 kDa on polyacrylamide gel electrophoresis (PAGE). It has many B-cell epitopes ([6, 7]) and induces both IgM and IgG types of antibodies ([12]). Further, *in vitro* studies have confirmed the suppressive effect of LAM on cell-mediated immunity against *M. leprae* ([11, 16, 20]).

In leprosy, humoral immune response to LAM has been studied by many authors ([12, 14, 15]). In addition, monitoring of IgG anti-LAM antibodies has been reported during chemotherapy ([8, 13, 19]). Roche, *et al.* ([19]) have reported the persistence of IgG anti-LAM antibodies following treatment, while a variable decline in the level of antibody with therapy was observed by Gelber, *et al.* ([8]). These differences in antibody levels may possibly be due to the variation in specific IgG subclass response. Further, although the antibody levels against LAM have been measured in erythema nodosum leprosum [(ENL), type 2 reaction] and reversal reaction (type 1), not much information is available on the level of IgG subclasses except the report of Dhandayuthapani, *et al.* ([4]). Since IgG subclasses have some pathogenic significance in many diseases, it needs further analysis in leprosy. In the present study, we have analyzed the IgG subclass responses to LAM in the sera of active leprosy patients, patients following effective chemotherapy (inactive cases) and in patients undergoing type 1 (reversal) and type 2 (ENL) reactions.

MATERIALS AND METHODS

Sera were collected from blood samples (5 ml each) drawn by antecubital venipuncture from 44 borderline lepromatous (BL)/lepromatous (LL) and 62 borderline tuberculoid (BT)/tuberculoid (TT) active leprosy patients, 18 lepromatous patients suffering from ENL reactions, and 15 patients with reversal reactions attending the outpatient department of the Central JALMA Institute for Leprosy, Agra, India. They were diagnosed clinically and bacteriologically, and were divided into a five-group spectrum approved by the Indian Association of Leprologists ([10]). The BL/LL patients were clinically active and bacteriologically positive. They were receiving multidrug therapy (MDT) varying from 1 month to 18 months. A few of the patients, about (10%), had not received any MDT. The BT/TT patients had raised, erythematous lesions with infiltration and well-defined margins. Some of these cases had thick cutaneous nerves near the lesions. They were mostly untreated with less than 3 months of treatment. Patients in reaction were given steroid therapy after collection of the blood samples.

Sera were also obtained from 39 inactive cases of BL/LL and 37 inactive cases of BT/TT leprosy. The "inactive" cases were previously active cases who had become inactive with treatment. A few of them had

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received dapsone monotherapy for 5 to 15 years; others had the recommended MDT for their disease status. These cases were free from clinical symptoms and were negative for acid-fast bacilli (AFB) in their skin smears. They had been released from treatment for from 3 to 10 years.

Serum samples from 17 healthy individuals who were contacts of leprosy patients were included as controls (HC).

Only those patients and healthy contacts who had given their consent were included in this study.

Antigens. Lipoarabinomannan (Ara-LAM) from rapid-growing mycobacteria species was obtained from Dr. P. J. Brennan, Colorado State University, Fort Collins, Colorado, U.S.A. (NIH contract no. 1-Al-05074).

Monoclonal antibodies. Mouse monoclonal antibodies to human IgG1 (Clone HP 6001), IgG2 (Clone HP 6014), IgG3 (Clone HP 6050) and IgG4 (Clone 6025) were obtained from Sigma Chemical Company, St. Louis, Missouri, U.S.A.

ELISA. ELISA plates (Nunc, Roskilde, Denmark) were coated with 100 µl of LAM (5 µg/ml) in 0.05 M bicarbonate buffer, pH 9.6, at 37°C overnight. The plates were washed three times in phosphate buffered saline (PBS) and then blocked with 1% bovine serum albumin (BSA) in PBS for 1 hr. After washing three times with PBS-Tween (0.1%) the sera (100 µl, 100-fold dilution) were added in duplicate to the plates. The plates were incubated at 37°C for 2 hr and then washed three times with PBS-Tween; 100 µl of mouse anti-human IgG1, IgG2, IgG3 and IgG4 monoclonal antibodies were added to the plates, and they incubated at 37°C for 90 min. The monoclonal antibody to IgG1 was used at a 1:3000-fold dilution whereas other monoclonals were used at a 1:5000-fold dilution. The plates were then washed three times and 100 µl of peroxidase conjugated goat anti-mouse IgG (1:10,000) (Sigma) was added to each well. The plates were then incubated at 37°C for 90 min. After washing, OPD substrate (ortho-phenylene diamine dihydrochloride containing H_2O_2) was added to develop color. The reaction was stopped after 30 min with 5 N H_2SO_4 and the optical density (OD) was measured at 492 nm in an ELISA reader (Titertek Multiskan; Flow laboratories, Rockville, Maryland, U.S.A.).

The dilution of serum was determined from the standard curve drawn by taking the OD values of the pooled sera of BL/LL, BT/TT patients and healthy contacts tested in a serial dilution. The OD values of the dilution used in this study (1:100) were read in the linear part of the standard curve. Sera from healthy contacts were included along with test samples in every ELISA. The mean OD + 3 S.D. of healthy contacts was used as the cut-off point to determine the positivity for each subclass.

Statistical analysis. Data were analyzed using Student's *t* test and the chi-squared test.

RESULTS

The levels of the IgG subclass antibodies to LAM have been analyzed in active BL/LL and TT/BT leprosy patients and in patients with reactions (Table 1). The mean antibody level of IgG2 obtained in active TT/BT patients was significantly higher compared to the healthy contacts ($p < 0.01$). In all healthy contacts, the values for the IgG subclasses were within the cut-off point. IgG2 antibodies were found to be at a higher level in all groups of patients. The mean antibody levels of IgG1, IgG2 and IgG3 were significantly higher ($p < 0.01$ for each subclass) in active BL/LL patients than those obtained in active TT/BT patients. The IgG3 response was found to be at a lower level than the IgG1 and IgG2 antibody responses. In all groups of patients the mean antibody level and seropositivity for IgG4 antibodies were found to be low. The positivity values for IgG1 (48%), IgG2 (66%) and IgG3 (39%) in BL/LL patients were significantly higher than the IgG1 (16%), IgG2 (39%) and IgG3 (11%) positivity values for the TT/BT patients (Table 2).

Further, the IgG subclass responses in inactive cases (BL/LL, and BT/TT, after successful chemotherapy) were determined (Table 1). Even with disease inactivity and bacterial negativity, 10 out of 39 (26%) of the BL/LL cases were positive for IgG1 antibodies; whereas 21 out of 44 active BL/LL patients (48%) were positive for the same antibody (Table 2). Similarly, 17 out of 39 inactive BL/LL patients (44%) were

TABLE 1. *IgG subclass levels (mean ± S.D.) to LAM in the sera of active and inactive cases of leprosy and leprosy cases with reactions (ENL and reversal reactions).*

IgG sub-classes	HC[a] (N = 17)	TT/BT[b]		BL/LL[c]		ENL[d] (N = 18)	RR[e] (N = 15)
		Active (N = 62)	Inactive (N = 37)	Active (N = 44)	Inactive (N = 39)		
IgG1	0.02 ± 0.01	0.025 ± 0.027	0.017 ± 0.013	0.074 ± 0.074[g]	0.035 ± 0.034[h]	0.04 ± 0.04	0.15 ± 0.48[j]
IgG2	0.07 ± 0.02	0.15 ± 0.13[f]	0.148 ± 0.131	0.25 ± 0.17[g]	0.143 ± 0.12[h]	0.23 ± 0.20	0.34 ± 0.34[k]
IgG3	0.018 ± 0.01	0.023 ± 0.022	0.014 ± 0.01	0.044 ± 0.043[g]	0.013 ± 0.011[h]	0.023 ± 0.02[i]	0.04 ± 0.08
IgG4	0.027 ± 0.01	0.031 ± 0.023	0.02 ± 0.02	0.033 ± 0.026	0.018 ± 0.016	0.034 ± 0.028	0.02 ± 0.03

[a] HC = Healthy contacts.
[b] TT/BT = Tuberculoid and borderline tuberculoid leprosy.
[c] BL/LL = Borderline lepromatous and lepromatous leprosy.
[d] ENL = Erythema nodosum leprosum.
[e] RR = Reversal reactions.
[f] Significantly higher compared to corresponding healthy contacts.
[g] Significantly higher compared to corresponding active BT/TT group.
[h] Significantly lower compared to corresponding active BL/LL group.
[i] Significantly lower compared to corresponding active BL/LL group.
[j] Significantly higher compared to active TT/BT group.
[k] Significantly higher compared to active TT/BT group.

positive for IgG2 antibodies. In the corresponding active group, 29 out of 44 patients (66%) were found positive. Only 5% (2 out of 39) of the inactive group were IgG3-seropositive in comparison to 39% (17 out of 44) in the active group of BL/LL patients.

However, following chemotherapy, a significant fall ($p < 0.01$) in all subclass levels, except for IgG4, was observed in BL/LL patients. On the other hand, no significant changes in the mean antibody levels or in the values of seropositivity were observed in BT/TT patients after chemotherapy.

There was a significant reduction in the IgG3 level in ENL cases when compared with active BL/LL patients ($p < 0.05$). In patients undergoing type 1 reaction, the levels of IgG1 ($p < 0.002$) and IgG2 ($p < 0.05$) subclasses were found to be higher than in the active TT/BT group. The seropositivity for the IgG1 subclass was significantly higher in patients with type 2 (ENL) reaction ($p < 0.04$) as compared to type 1 (RR) reaction.

DISCUSSION

In the present study, we have used a crossreactive carbohydrate antigen, LAM, to demonstrate IgG subclass response in active TT/BT and BL/LL patients, in inactive cases after successful chemotherapy, and in patients undergoing reactional episodes. The mean level and seropositivity value of IgG1 were observed to be significantly higher in active BL/LL patients than in active TT/BT patients. A similar enhancement of the IgG1 level to LAM in lepromatous patients was reported by Dhandayuthapani, *et al.* ([4]).

The finding that the IgG2 subclass against LAM was predominant in all groups and remained at a higher level during leprosy infection is in agreement with the report of Dhandayuthapani, *et al.* ([4]). An augmented IgG2 response is a generalized feature of bacterial infections ([9]), and it has been established that the IgG2 response is mostly induced against carbohydrate epitopes ([2]). LAM has been reported to be presented by the CD1b molecule to αβ T-cell receptor-bearing lymphocytes. T cells activated by LAM produced gamma interferon (IFN-γ) and were cytolytic ([21]). In the present study, LAM would have induced an elevated IgG2 response in both the BT/TT and BL/LL groups of patients.

On the whole, IgG3 antibody levels were found to be low compared to the levels of IgG1 and IgG2. The mean levels and seropositivity values for IgG1, IgG2 and IgG3 were significantly higher in BL/LL patients compared to TT/BT patients. In our previous study, we have demonstrated the IgG1 and IgG3 enhancement in BL/LL patients compared to BT/TT patients to whole *M. leprae* sonicated antigens ([3]). Since LAM is one of the major immunogens of whole sonicated antigens of *M. leprae,* the

TABLE 2. *Percentage of seropositivity for IgG subclasses to LAM in active and inactive cases of leprosy and leprosy cases with reaction.*

IgG subclasses[a]	HC (17)	BT/TT		BL/LL		BL/LL ENL[d] (N = 18)	RR[e] (N = 15)
		Active (N = 62)	Inactive (N = 37)	Active (N = 44)	Inactive (N = 39)		
IgG1	(0)	16(10)[b]	8(3)	48(21)[c]	26(10)[d]	39(7)	6(1)
IgG2	(0)	39(24)	38(14)	66(29)[c]	44(17)[d]	61(11)	46(7)
IgG3	(0)	11(7)	3(1)	39(17)[c]	5(2)[d]	11(2)[e]	13(2)
IgG4	(0)	7(4)	5(2)	11(5)	8(3)	11(2)	6(1)

[a] Cut off for IgG1 ≥ 0.05; IgG2 ≥ 0.15; IgG3 ≥ 0.05; IgG4 ≥ 0.06.
[b] Numbers in parentheses are positive individuals.
[c] Significantly higher compared to corresponding active TT/BT group.
[d] Significantly lower compared to active BL/LL group.
[e] Significantly lower compared to corresponding active BL/LL group.

results obtained with both preparations were similar. The level of IgG4 antibodies was found to be low and similar in both groups of patients.

In this study, we have also determined the levels of the IgG subclasses in inactive cases of leprosy following adequate chemotherapy. Although a significant fall in all IgG subclasses, except IgG4, was observed in the group of BL/LL patients after the disease became inactive with therapy, persisting levels of IgG1 (26%) and IgG2 (44%) subclasses could be detected in the sera of inactive cases (Table 2). The mean antibody level and seropositivity rate of IgG3 were found to be low in inactive cases. An earlier study reported the decline in IgG antibody level to LAM after 4 years of therapy ([8]). In another study, however, an increase in IgG anti-LAM antibody levels following treatment in a group of Nepali patients was reported ([19]). Although no changes in the mean antibody level or in the seropositivity rate were observed in the BT/TT patients after chemotherapy, a high level of IgG2 along with a higher seropositivity rate were observed in inactives cases.

The persistence of IgG1 and IgG2 antibodies in the sera of inactive (cured) patients may be due to the presence of LAM somewhere in the tissue(s). It is known that drug-sensitive *M. leprae* can persist for many years after effective chemotherapy ([18]) and, therefore, can stimulate antibody production ([24]). Although the inactive cases in the present study were free from live or dead bacilli as revealed from the skin smears (from five sites), the possibility of any "persisters" remaining in internal structures cannot be ruled out ([18]). It is also possible that due to the slow degradable nature of *M. leprae* LAM might remain in tissues for a long time, even after bacterial clearance. Further, LAM being a common antigen among bacterial species, other bacterial infections may lead to an anamnestic antibody response in the host. However, this possibility probably can be ruled out because none of the healthy contacts in the present study was seropositive for these two subclasses.

In our earlier observations, we have noted the persistence of IgG1 and IgG2 antibodies to whole *M. leprae* sonicated antigens in inactive cases of BL/LL leprosy ([3]). In the present study we have also observed the persistence of these two IgG subclasses to LAM in inactive cases of leprosy. Contrary to the above, in a recent study by Fujieda, *et al.* ([5]) *in vitro* culture could not demonstrate the production of IgG2 from IgD + B cells (i.e., B cells having IgD receptor) in the presence of LAM and CD1b-restricted T cells from leprosy lesions. This discrepancy may be due to the difference in the experimental setup because in the present study the response has been measured in an *in vivo* situation in patient sera.

Regarding antibody level in reactions, Miller, *et al.* ([15]) have reported that the occurrence of ENL reaction had no significant effect on the total level of IgG antibody. However, they could observe a significant triphasic pattern with an initial fall of antibody preceding the clinical development of the reversal reaction, followed by a sharp rise coincident with the appearance of symptoms of a type 1 reaction and, finally, a second decline occurring several weeks to months after the onset of reaction. In con-

trast, Roche, *et al.* have observed no effect on IgG anti-LAM levels during reactional episodes ([19]). A decreased level of IgM antibody to pure glycolipid and no change to a crude soluble antigen of *M. leprae* in patients with ENL reaction have been reported ([1]). On the other hand, lower levels of IgG subclasses were observed in our study in lepromatous patients with ENL reactions compared to active BL/LL patients. A significant fall in the IgG3 level and a lower seropositivity value for the IgG3 subclass could be seen in patients with ENL reactions compared to the active BL/LL group. This supports our earlier observation ([3]). IgG3 antibody perhaps may be primarily deposited as immune complexes in tissues, which might account for a fall in the IgG3 level in serum. But Dhandayuthapani, *et al.* have different observations; they have found a relatively higher level of IgG4 antibody against sonicated extract and a decreased IgG1 antibody level to LAM in patients with ENL reactions ([4]).

Possibly our report is the first to analyze the IgG antibody response in a subclass level to LAM in patients with reversal reactions. Since most of the TT/BT patients suffer from reversal reactions, we have compared the IgG subclasses in TT/BT patients with and without reactions.

Higher levels of IgG1 and IgG2 were observed in patients with type 1 reactions compared to those of active TT/BT patients. Miller, *et al.* also had observed a sharp rise in the total level of IgG during a type 1 reactional episode ([15]). A reversal reaction seems to be associated with a sudden increase in cell-mediated immunity against *M leprae* antigens ([17]). Enhancement of IgG1 in patients with reversal reaction could be explained by a heightened cell-mediated immunity which would have induced production of more IFN-γ in response to LAM by activated $\alpha\beta$ T cells. Following the increases in IFN-γ levels, raised levels of IgG1 and IgG3 are common features of viral infections ([22]). However, in the present study the rise in the IgG2 subclass level was a prominent feature and further contradicted the *in vitro* finding of Fujieda, *et al.* ([5]).

It will be of interest to further characterize the antigen responsible for evoking the IgG1 and IgG2 responses in inactive cases of leprosy by localizing LAM in the tissues. Further, the level of IgG3 in lepromatous patients and the IgG1 and IgG2 subclasses in TT/BT patients would be worth studying in trying to predict the occurrence of type 2 and type 1 reactions, respectively.

SUMMARY

IgG subclasses against lipoarabinomannan of mycobacteria were analyzed in the sera of leprosy patients. Patients with active leprosy [tuberculoid and lepromatous, patients undergoing erythema nodosum leprosum (ENL) and reversal reactions] and inactive cases (tuberculoid and lepromatous who were cured after chemotherapy) were included in this study. Active lepromatous patients had higher levels of IgG subclasses, except IgG4, compared to active tuberculoid patients. Some of the inactive cases (lepromatous patients cured after chemotherapy) were positive for the IgG1, IgG2 and IgG3 subclasses. However, their levels are lower than active lepromatous cases. On the other hand, no difference in the subclass levels between the active and inactive tuberculoid groups could be observed. While a significant fall in the level of IgG3 in ENL was observed as compared to lepromatous leprosy without ENL, higher levels of IgG1 and IgG2 were found in patients with reversal reactions compared to their active counterparts without reactions.

RESUMEN

Se analizaron las subclases de IgG contra lipoarabinomanana de micobacterias en los sueros de pacientes con lepra. En el estudio se incluyeron pacientes con lepra activa (tuberculoides y lepromatosos, pacientes con eritema nodoso leproso, ENL, y pacientes con reacción reversa) asi como casos inactivos (tuberculoides, los pacientes lepromatosos activos tuvieron los niveles más altos de todas las subclases, excepto IgG4. Algunos de los casos inactivos (lepromatosos curados con PQT) fueron positivos para las subclases IgG1, IgG2 e IgG3. Sin embargo, sus niveles fueron menores que los de los casos lepromatosos activos. Por otro lado, no se observaron diferencias en los niveles de las subclases entre los grupos tuberculoides activo e inactivo. Aunque se observó una caida significativa en el nivel de IgG3 en los cases con ENL (comparados con los casos sin ENL), los niveles de IgG1 e IgG2 fueron mayores en los casos activos con reacciones reversas que en los casos sin reacción.

RÉSUMÉ

Les sous-classes d'IgG dirigés contre le lipo-arabino-mannane de mycobactérie furent analysés à partir de sérums de patients hanséniens. Les patients présentant une lèpre active [tuberculoïde et lépromateuse, patients souffrant d'érythème noueux lépreux (ENL) et de réactions réverses] et des individus avec une lèpre inactive (tuberculoïdes et lépromateux qui ont été guéris par la chimiothérapie) ont été inclu dans cette étude. Les patients qui présentaient une lèpre lépromateuse active avaient des niveaux plus élevés de sous-classes d'IgG, à l'exception des IgG4, que les patients tuberculoïdes. Certains cas inactifs (patients lépromateux guéris après chimiothérapie) étaient positifs pour les sous-classes IgG1, IgG2 et IgG3. Cependant, leur niveaux étaient plus bas que chez les cas de lèpre lépromateuse active. En revanche, il n'a pas été observé de différences de niveaux de sous-classes entre les groupes de lèpre tuberculoïde active et inactive. Tandis qu'une baisse significative du niveau d'IgG3 fut observée chez les patients atteints d' ENL, comparé aux cas de lèpre lépromateuse sans ENL, des niveaux plus élevés de IgG1 et IgG2 étaient présents chez les patients souffrant de réactions réverses, comparés à leurs homologues actifs sans réactions.

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