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Immunoglobulin G1 (IgG1) and IgG3 Antibodies Are Markers of Progressive Disease in Leprosy

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***Mycobacterium leprae*-specific and polyclonal immunoglobulin G (IgG) subclass and IgE antibodies in leprosy patients across the histopathological spectrum were determined by using a quantitative enzyme-linked immunosorbent assay. Antibody responses to *M. leprae* sonicates were detected only in IgG1, -2, and -3 subclasses. Even at 100-times-lower dilutions, very little IgG4 and IgE antibody activity against *M. leprae* was detected in any group of leprosy patients. Quantitatively, antibody responses were highest at the lepromatous pole and decreased towards the tuberculoid pole. The greatest quantitative difference in antibodies between the lepromatous and tuberculoid poles was observed with IgG1 (140-fold), this was followed by the difference with IgG3 antibodies (32-fold). Polyclonal antibodies, on the other hand, were elevated for all four IgG subclasses as well as IgE in both lepromatous and tuberculoid leprosy patients compared with healthy controls from a leprosy-endemic area. Selective elevation of *M. leprae*-specific antibody responses in IgG1 and IgG3 subclasses, therefore, could not be attributed to selective polyclonal activation in these particular subclasses. Furthermore, polyclonal activation for IgE was observed in both lepromatous and tuberculoid leprosy patients, with higher levels in the tuberculoid group, which does not support selective TH2 activation in lepromatous leprosy patients. IgG1 and IgG3 antibodies also showed the highest Spearman rank correlation with the bacterial index in these patients (rho = 0.748 and $P < 0.001$ for IgG1; rho = 0.721 and $P < 0.001$ for IgG3). Thus, disease progression in leprosy showed a significant correlation with selective increases in IgG1 and IgG3 responses.**

Infection with *Mycobacterium leprae*, which resides and multiplies within the macrophage/monocyte system, can lead to the development of a spectrum of clinical manifestations depending on the type of immune response mounted by the host (22). T-cell responses are associated with the more localized or tuberculoid form of the disease (23), while antibody responses are associated with the more disseminated or lepromatous type of disease (17, 19). In leprosy patients, antibody concentrations increase in direct proportion to increase in the bacterial load (12). Antibody responses to *M. leprae* antigens in lepromatous leprosy patients, in the absence of detectable T-cell responses, have been shown to be elevated not only for immunoglobulin M (IgM) but also for IgA and IgG isotypes (17).

Switching of the antibody response from IgM to one of the other isotypes requires cytokines secreted by different subsets of T helper cells (18, 27). In the murine system, in vitro gamma interferon produced by TH1 cells induces IgG2a and IgG3 (7, 28); interleukin-4 (IL-4) produced by TH2 cells selectively stimulates IgG1 and IgE (8). Also, in the experimental mouse model TH1 cell activation has been shown to be associated with protection against infections with intracellular pathogens residing within the monocyte/macrophage system, while TH2 activation leads to progression of disease by suppressing activities of the TH1 subset and with augmentation of humoral responses, particularly of the IgG1 and IgE isotypes (26). The human counterparts of murine IgG subclasses are based on similarity in biological and functional activities. Murine IgG1 and human IgG4 are considered to be similar because of their property of binding to mast cells. Murine IgG3 and human IgG2 both recognize predominantly carbohydrate epitopes, while murine IgG2a and IgG2b and human IgG1 and IgG3 share the abilities to fix complement and bind to protein antigens. For humans, although TH1 and TH2 subsets have been identified on the basis of their cytokine profiles (24, 25), a detailed analysis of the regulation of different isotypes and IgG subclasses is still lacking. The most distinct requirement for isotype switching in humans has been experimentally shown for IL-4, which upregulates IgG4 and IgE (15). In another study, there was no relationship between IL-2, IL-4, IL-6, and gamma interferon secreted by T helper clones and their pattern of IgG subclass induction (5). Analysis of isotypes present in chronic diseases may therefore provide valuable insight not only into the differential activation of T-cell subsets but also into their role in disease progression. In this study, we have assessed both polyclonal and *M. leprae*-specific IgG subclasses and IgE responses across the leprosy disease spectrum and determined their relationships to both histopathology and the bacterial load in leprosy patients.

MATERIALS AND METHODS

Study subjects. The study group consisted of 110 untreated leprosy patients newly registered at The Marie Adelaide Leprosy Center in Karachi, Pakistan. The criteria used for diagnosis of leprosy were standard clinical signs and histological confirmation, as previously described (11). The patients were classified as having lepromatous (LL), borderline lepromatous (BL), borderline tuberculoid (BT), or tuberculoid (TT) leprosy according to the histopathological features described by Ridley (22). Biopsies were obtained from active lesions, and the bacterial index (BI) was determined microscopically by staining tissue sections for acid-fast bacilli. BIs from 1 to 6 represent logarithmic increases in the number of bacilli (22). Patients presenting with reactional complications were not included in this study. Sera from 45 healthy donors who were employees of Aga Khan University, representing a broad socioeconomic background and with no known exposure to leprosy, were included as controls from a leprosy-endemic area. There was no significant difference between the distributions of age and sex in this group and those of the leprosy patients.

Antisera. Five-milliliter specimens of blood collected from both leprosy patients and controls were allowed to separate overnight at 4°C. For each specimen,

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serum was removed and centrifuged at 400 × *g* for 15 min; the clear supernatant was distributed in 100-μl aliquots and frozen at −70°C until use.

Antigens. Batch CD 114 of *M. leprae* soluble sonicates (MLSON) was kindly provided by R. J. W. Rees, National Institute of Medical Research, London, United Kingdom.

Reagents. Monoclonal antibodies specific for human IgG subclasses were HP 6069 (anti-IgG1), which was used in polyclonal IgG1 determination and kindly provided by R. G. Hamilton, Johns Hopkins University, Baltimore, Md., and HP 6001 (anti-IgG1), HP 6002 (anti-IgG2), HP 6047 (anti-IgG3), HP 6023 (anti-IgG4), and HP 6029 (anti-IgE), which were prepared at the Centers for Disease Control, Atlanta, Ga., and were a gift from C. B. Reimer. The specificity evaluation and performance characteristics of these antibodies are described in detail elsewhere (13, 14, 21). Goat anti-human IgG (Fc specific) and goat anti-mouse IgG (heavy-plus-light-chain specific) conjugated to alkaline phosphatase were commercially obtained (Jackson Laboratories, Avondale, N.J.) and diluted according to the manufacturer's recommendations. The preparation of purified rabbit anti-human IgE has been described in detail previously (9); this antibody was obtained by immunizing rabbits with the Fc fragments of human IgE myeloma, affinity purified over an IgE Sepharose column, and rendered Fc-ε specific by sequential passage over affinity columns of insolubilized IgG and $F(ab')_2$ fragments of human myeloma IgE. A secondary serum calibrator was standardized for IgE against patient (PS) myeloma, which was a kind gift of T. Ishizaka (Johns Hopkins University).

Quantitation of IgE, IgG, and IgG subclass antibodies to MLSON. Quantitation of IgG anti-MLSON has been described in detail previously (11). IgG subclasses were quantitated by using a slightly modified method as described for filarial antigens (13). Briefly, Immulon 4 plates were coated with 100 μl of MLSON at 4 μg/ml in carbonate buffer (pH 9.6) for 2 h at 37°C and then overnight at 4°C. Phosphate-buffered saline (PBS) containing 5% bovine serum albumin (BSA) was added and incubated for 2 h at 37°C to block free sites. One hundred microliters of sera diluted in PBS containing 0.05% Tween 20 and 1.0% BSA was added and incubated for 2 h at 37°C and then overnight at 4°C. Monoclonal antibodies specific for each of the IgG subclasses and IgE were added at saturation concentrations of 1:1,000 for HP 6001, HP 6002, HP 6029, and HP 6047 and 1:500 for HP 6023 and further incubated overnight at 4°C. Alkaline phosphatase-labeled goat anti-mouse IgG was incubated for 2 h at 37°C. The plates were finally developed with alkaline phosphatase substrate. Each incubation was followed by three washes with PBS containing 0.05% Tween 20 to remove unbound protein. In each assay a reference pool containing high titers of IgG subclass antibodies to MLSON was used to generate a dose-response curve; this was used as a calibrator which was assigned arbitrary units of activity, as described in Results. All test sera were run at a minimum of three dilutions, and the activity was expressed in units relative to the reference pool for IgG1, IgG2, and IgG3. Because of the absence of high-titer sera for IgG4 and IgE, sera were run at a single dilution of 1:10, and the results for these antibodies are expressed as optical density (OD) readings. To control for nonspecific binding, pooled sera from normal healthy donors provided the negative control for each assay.

Quantitation of polyclonal IgG subclasses. The concentrations of polyclonal IgG subclasses in serum were determined as detailed previously (14). Briefly, Immulon 4 microtiter plates (Dynatech, Alexandria, Va.) were coated with optimal dilutions of monoclonal antibodies (1:1,000 for HP 6069, HP 6002, and HP 6047 and 1:100 for HP 6023) and incubated at 4°C overnight. After the free sites were blocked with 5% BSA in PBS for 2 h at 37°C, the plates were incubated with sera. At least four serial dilutions of test sera were run in each assay. A World Health Organization reference serum (67/97) with known amounts of the four subclasses was run as a standard. The sera were incubated for 2 h at 37°C and subsequently overnight at 4°C. The plates were probed with goat anti-human IgG conjugated to alkaline phosphatase and subsequently developed with the appropriate substrate. Between each incubation the plates were washed three times to remove unbound proteins. The reaction was stopped with 3 N NaOH, and the OD was read at 410 nm in a microtiter plate reader (Dynatech MR 600).

Quantitation of polyclonal IgE. Plates were coated with purified rabbit anti-human IgE at 2 μg/ml. Sera were incubated for 2 h at 37°C and subsequently overnight at 4°C. Mouse monoclonal antibody HP 6029 was added at a 1:1,000 dilution and incubated overnight at 4°C. Goat anti-mouse IgG conjugated to alkaline phosphatase was used as the revealing probe (described above) and was followed by the appropriate substrate. Between each step the plates were washed three times with PBS containing 0.05% Tween 20. An eight-point calibration curve was developed with a standard reference serum (PS myeloma) with known amounts of IgE, as previously reported (9). All test sera were run at a minimum of three dilutions.

Statistical analysis. All statistical analyses were carried out with Microsoft Excel Cricket graph and Statview software packages on a Macintosh microcomputer. The interdilution coefficient of variation was calculated for the mean reduction in the dose-response curve for five dilutions. Nonparametric analysis (Mann-Whitney U test) was carried out to assess the significance of differences in antibody to *M. leprae* among patient groups. Spearman rank correlation was used to analyze the relationship of IgG subclasses and IgE to the BI in leprosy patients.

RESULTS

Quantitation of IgG subclass and IgE antibodies to MLSON. (i) Development of a serum calibrator and performance characteristics. In the absence of a serum calibrator with known weight/volume concentrations of *M. leprae*-specific IgG subclasses, we developed an alternative reference serum calibrator for IgG subclasses which was defined in terms of its antibody-binding activity. Sera from 48 patients with lepromatous disease were screened for antibody activity in all IgG subclasses against MLSON. A reference pool was generated by using equal volumes of five sera giving ODs of >2.0 at a 1:1,000 dilution with IgG1-, IgG2-, and IgG3-specific monoclonal

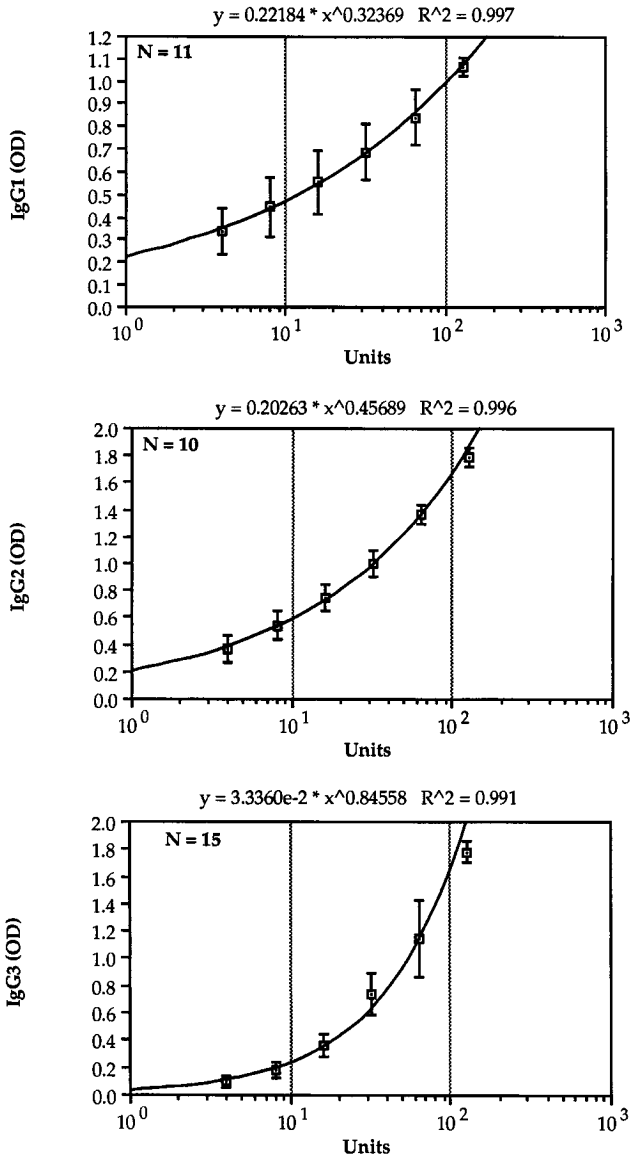

FIG. 1. Reproducibility of calibration curves used in assignment of antibody activity to individual sera. The reference serum was assigned 25,600 arbitrary units of antibody activity against *M. leprae* for all three IgG subclasses. Each point represents the mean (± standard deviation) obtained in several assays for that concentration. N, number of assays analyzed. The ordinate shows readings of OD at 410 nm; the abscissa shows units of antibody activity. The logarithrimic fit and the coefficient of determination (R^2) are shown for the calibration curves of all three IgG subclasses.

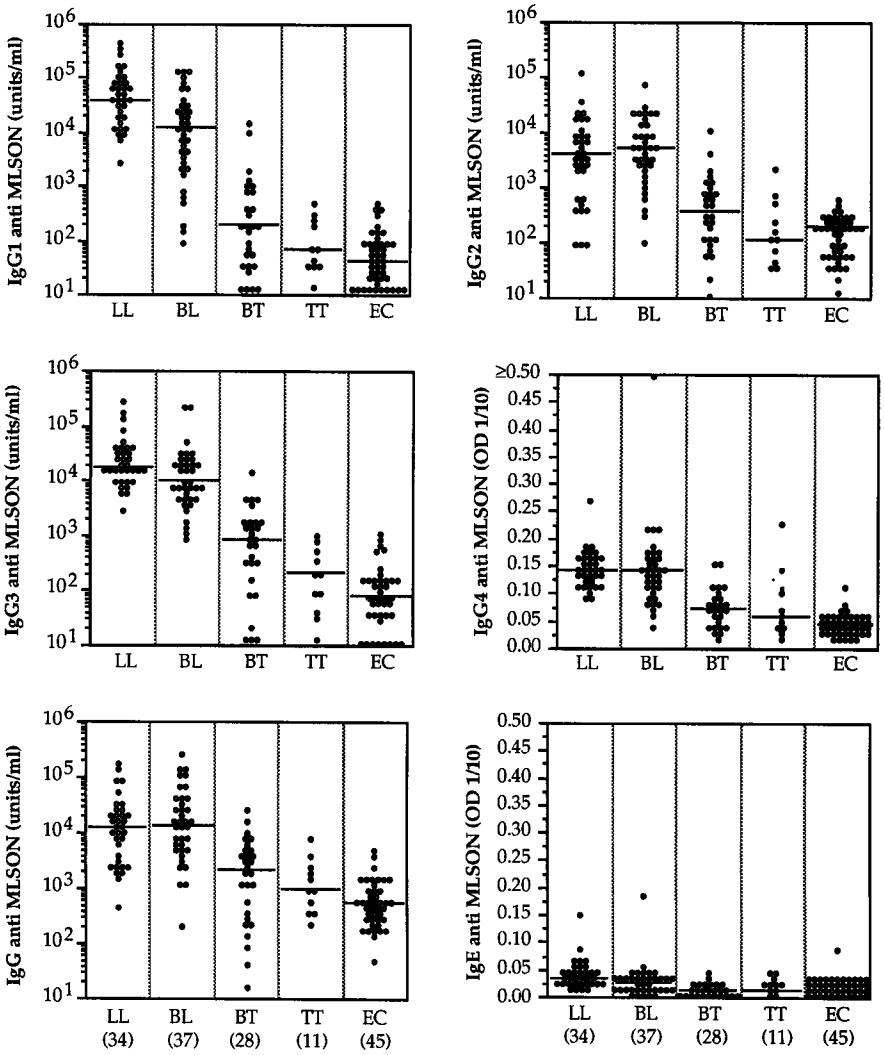

FIG. 2. Distribution of *M. leprae*-specific IgE and IgG subclasses across the leprosy disease spectrum. Results are expressed as units of antibody activity assigned from the calibration curve for IgG1, IgG2, and IgG3 anti-*M. leprae* activities; IgG4 and IgE anti-*M. leprae* activities are expressed as OD at a single dilution of 1:10. Patient groups were LL, BL, BT, and TT types of leprosy. EC, healthy controls from a leprosy-endemic area. The number of individuals tested in each group is indicated in parentheses. Horizontal bars show geometric mean levels for each group.

antibodies. This pool was assigned a value of 25,600 arbitrary units of activity for all three IgG subclasses (IgG1, IgG2, and IgG3), since this was the end point titer for all three subclasses determined in 10 to 15 different assays. The mean OD values obtained in these assays for an eight-point reference curve, after transformation of the dilution curve into units per milliliter, are shown in Fig. 1. The dose-response curves for all three IgG subclass antibodies to MLSON were highly reproducible as seen by the standard deviations around the mean values for 10 to 15 assays. The coefficient of determination for a logarithimic fit was >99% for all three IgG subclasses. The interdilution coefficient of variation as determined by mean reduction in activity for five dilutions was 2.5% for IgG1, 4.6% for IgG2, and 3.5% for IgG3 between 16 and 128 U. This calibrator was therefore found to be acceptable for determination of values for sera distributed over several log units. Such a calibrator was not necessary for IgG4 and IgE antibodies, since these antibodies did not show a log distribution of MLSON-specific antibody activity.

(ii) Determination of antibody activity in sera of leprosy patients. Figure 2 shows the IgG subclass and IgE antibody responses to MLSON (across the disease spectrum). Very high concentrations of antibody were detected in IgG1, IgG2, and IgG3 compared with those for controls from a leprosy-endemic area. IgG4 showed some activity in the LL and BL groups of patients, but the binding was low (OD, <0.2) even at a low dilution of 1:10. IgE antibodies showed even lower binding. The low antibody activity was not due to an inability of these reagents to bind to the isotypes, since these same reagents detected both antigen-specific IgG4 and IgE in sera of patients with filariasis (data not shown), which are known to contain high titers of both these isotypes (9, 13). Although the significant differences observed among the lepromatous and tuberculoid groups were expected, it was of interest that IgG1 (P = 0.0002) and IgG3 (P = 0.008) showed significant differences even among the subgroups of lepromatous leprosy (LL and BL) patients. For tuberculoid patients (BT and TT groups), only IgG3 was significantly different (P = 0.02).

Table 1 summarizes mean antibody levels and 95% confi-

TABLE 1. *M. leprae*-specific antibody isotypes and IgG subclasses in leprosy

Isotype or subclass	Antibody concn[a] for group(s):		
	LL and BL (n = 71)	BT and TT (n = 39)	EC[b] (n = 45)
IgG1	52,038 ± 17,849 (100–409,600)	916 ± 867 (10–14,400)	83 ± 33 (10–544)
IgG2	10,026 ± 4,171 (100–121,600)	882 ± 632 (10–12,000)	177 ± 39 (10–600)
IgG3	33,324 ± 13,488 (1,000–332,800)	1,353 ± 769 (10–14,000)	194 ± 81 (10–1,280)
IgG4	0.167 ± 0.050 (0.044–1.924)	0.079 ± 0.015 (0.021–0.235)	0.049 ± 0.005 (0.021–0.117)
IgE	0.039 ± 0.007 (0.009–0.186)	0.017 ± 0.004 (0.000–0.046)	0.013 ± 0.004 (0.000–0.090)

[a] Antibody concentrations are given as units for IgG1, IgG2, and IgG3 and as OD at a 1:10 dilution for IgG4 and IgE. Results are expressed as group means ± 95% confidence interval. The range is shown in parentheses. For LL and BL versus EC: IgG1, $P < 0.001$; IgG2, $P < 0.001$; IgG3, $P < 0.001$; IgG4, $P < 0.026$; IgE, $P < 0.001$. For BT and TT versus EC: IgG1, $P = 0.002$; IgG2, $P = 0.013$; IgG3, $P < 0.0001$; IgG4, $P = 0.0004$; IgE, $P = 0.041$. For LL and BL versus BT and TT, $P < 0.001$ for all four subclasses of IgG and for IgE. Significance was determined by the Mann-Whitney U test.
[b] EC, healthy controls from a leprosy-endemic area.

dence intervals among the major subgroups. Since units of antibody activity are dependent on the affinity of the probing reagent, antibody units among different IgG subclasses may not be equivalent in terms of concentrations. To overcome this limitation, the ratios of antibody activities in patient groups to those in healthy controls from a leprosy-endemic area were compared for each IgG subclass. The greatest difference in mean antibody activity between patient groups and healthy controls was observed for IgG1 (630-fold for LL-BL and 11-fold for BT-TT) antibodies, followed by IgG3 (171-fold for LL-BL and 7-fold for BT-TT) antibodies. IgG2 showed a 56-fold elevation for LL-BL and a 5-fold elevation for BT-TT, while only a 2- to 4-fold difference was observed in cases of IgG4 and IgE.

To see if these quantitative differences in IgG1 and IgG3 antibodies could be accounted for by differential polyclonal activation among IgG subclasses in lepromatous and tuberculoid leprosy patients, we also determined the concentrations of polyclonal antibodies for each IgG subclass and IgE.

Quantitation of nonspecific polyclonal antibodies. Table 2 shows the concentration of each IgG subclass and IgE isotype. In the lepromatous leprosy patient group, although all four IgG subclasses as well as IgE were equally elevated compared with those in healthy controls from a leprosy-endemic area, the ratio of MLSON-specific antibody was highest for IgG1 and IgG3, as shown above. Surprisingly, in the BT-TT group, IgG1, with the highest ratio of MLSON antibody activity compared with controls, was the only subclass which did not show significant polyclonal activation. Similarly, IgG4 and IgE showed significant polyclonal activation in both the LL-BL and BT-TT groups compared with healthy controls, but no MLSON-specific antibody activity was detected in these isotypes. Thus, the presence or absence of an antibody response to MLSON is unlikely to be accounted for by polyclonal activation alone.

Correlation of IgG subclass antibodies and bacterial load. Disease progression in leprosy is accompanied by an increase in the bacterial load within lesions, which is expressed as a BI of 1 to 6, representing logarithmic increases in bacterial numbers. Figure 3 shows the regression analysis between *M. leprae*-specific IgG subclass antibodies and the BI within biopsied lesions as tested by the Spearman rank test. Among the four IgG subclasses, IgG1 showed the highest correlation (rho = 0.748, $P < 0.001$), followed by IgG3 antibodies (rho = 0.721, $P < 0.001$). These results suggest that IgG1 and IgG3 may play an important role in disease progression.

DISCUSSION

Disease localization and protective immunity in leprosy have been shown to be associated with T-cell responses. The study of antibody responses has therefore focused mainly on their usefulness as a diagnostic serological tool, with little attention given to careful dissection of antibodies at the isotype and subclass levels in relation to pathogenesis.

The most significant finding in the current study was the selective increase in the levels of IgG1, IgG2, and IgG3 *M. leprae*-specific antibodies across the disease spectrum. In addition, levels of both IgG1 and IgG3 anti-MLSON showed an extremely high correlation with the bacterial load in patients, indicating that these antibodies may be markers of disease progression. The elevation in these IgG subclasses was not due to selective polyclonal activation, since all IgG subclasses and IgE showed concentrations significantly higher than those for controls from a leprosy-endemic area. The elevation in polyclonal IgE antibodies was high across the leprosy spectrum, although no MLSON-specific IgE antibody was detected. Similar elevations of polyclonal IgG subclasses and IgE in leprosy patients have been reported for the Vietnamese population

TABLE 2. Polyclonal antibody isotypes and IgG subclasses in leprosy

Isotype or subclass	Antibody level[a] for group(s):		
	LL and BL (n = 71)	BT and TT (n = 39)	EC[b] (n = 45)
IgG1	11.93 ± 1.09 (3.2–29.4)	8.93 ± 0.82 (4.8–14.7)	8.41 ± 1.06 (3.2–19.2)
IgG2	5.16 ± 0.63 (1.6–13.5)	3.98 ± 0.45 (1.6–6.7)	3.33 ± 0.49 (1.24–10.24)
IgG3	2.01 ± 0.28 (0.44–8.0)	1.51 ± 0.25 (0.3–3.2)	1.11 ± 0.18 (0.34–2.8)
IgG4	1.24 ± 0.24 (0.03–4.8)	1.09 ± 0.20 (0.2–2.56)	0.85 ± 0.21 (0.030–2.94)
IgE	2,791 ± 804 (36–18,000)	3,245 ± 2,612 (16–50,000)	1,191 ± 661.2 (8–10,400)

[a] Antibody levels are given as milligrams per milliliter for IgG subclasses and as nanograms per milliliter for IgE. Results are expressed as group means ± 95% confidence interval. The range is shown in parentheses. For LL and BL versus EC, $P < 0.001$ for all four subclasses of IgG and for IgE. For BT and TT versus EC: IgG1, $P > 0.10$; IgG2, $P = 0.016$; IgG3, $P = 0.023$; IgG4, $P = 0.026$; IgE, $P = 0.025$. For LL and BL versus BT and TT: IgG1, $P = 0.0004$; IgG2, $P = 0.024$; IgG3, $P = 0.024$; IgG4, $P > 0.10$; IgE, $P > 0.10$. Significance was determined by the Mann-Whitney U test.
[b] EC, healthy controls from a leprosy-endemic area.

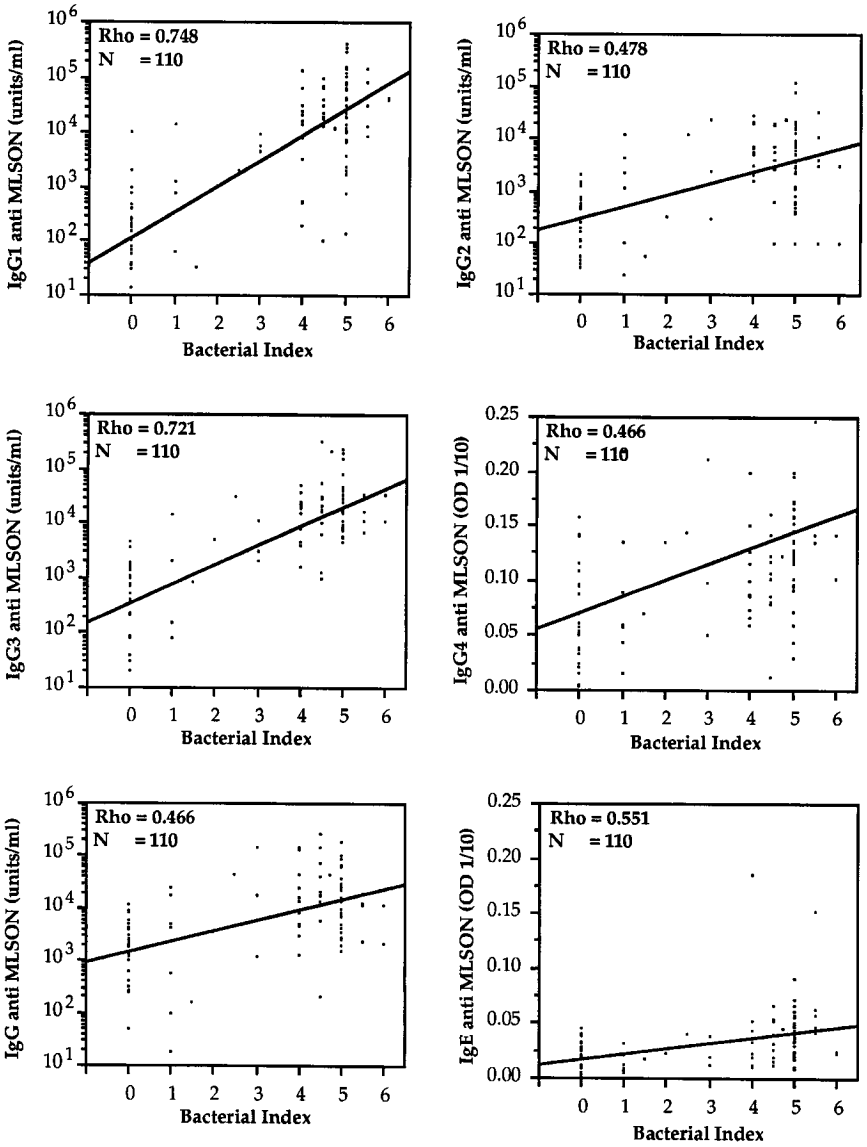

FIG. 3. Regression analysis for *M. leprae*-specific IgE and IgG subclasses. Antibody activity is expressed as units per milliliter for IgG1, IgG2, and IgG3 and as OD at a single dilution of 1:10 for IgG4 and IgE. The BI in skin biopsies was determined by histology. Linear regression lines are shown. Correlation as determined by the Spearman rank test is shown (Rho) for each of the antibody subclasses and IgE.

(29). As expected for a parasite-endemic area, the baseline concentrations of polyclonal IgE were also higher for controls.

Previously, Dhandayuthapani et al. (6) reported that IgG2 was the predominant subclass binding to MLSON in leprosy patients. In their study a different panel of monoclonal antibodies was used, and quantitative comparison among different subclasses was based on OD readings at a single dilution. These comparisons are not valid in the absence of a reference serum with known weight/volume concentrations of MLSON-specific antibodies, and the use of single dilutions would be unable to measure a difference of greater than 1 log unit in antibody concentrations. We have overcome these two limitations by assigning arbitrary units based on antibody activity to our reference serum pool, and therefore we were able to determine differences in antibody concentrations spread over several logs. Second, we have evaluated the ratio of antibody activity to the baseline antibody activity in the control group, which would normalize the differences in sensitivities for different subclasses. The greatest difference in antibodies to MLSON between the patient and healthy control groups was detected for IgG1 > IgG3 > IgG2. Very low antibody activity was detected in IgG4 and IgE isotypes. This could have been due to an inability to bind the relevant subclass, which is not likely since these reagents effectively bound these two isotypes in polyclonal assays. A second limitation may have been introduced by competition with other predominant antibodies. We have used conditions of antigen excess to overcome this limitation. Further analysis of these isotypes after fractionation of sera may provide additional insights. However, it is unlikely that these antibodies are present in functional concentrations, since allergic activity in leprosy patients has not been reported. Our results, therefore, strongly suggest a prominent elevation of IgG1 and IgG3 antibodies followed by IgG2 antibodies in

patients compared with the relative activity in controls from a leprosy-endemic area.

Detection of IgG2 in both lepromatous and tuberculoid types of leprosy is not surprising, since IgG2 antibodies are directed mainly to type 2 carbohydrate antigens (1, 30) and have been shown to be recognized in a T cell-independent manner in the murine system (28). IgG1 and IgG3 antibodies, on the other hand, are directed to protein antigens and are upregulated by gamma interferon secreted by the activated TH1 subset (7). It was therefore surprising to detect the highest levels of IgG1 and IgG3 in patients with lepromatous leprosy, in whom TH1 responses are low to undetectable as assessed by lepromin skin tests (16) and gamma interferon secretion in response to *M. leprae* antigens (20). These results, therefore, suggest that IgG1 and IgG3 antibody responses in leprosy may be relatively independent of cytokines secreted by the TH1 subset or that gamma interferon is provided by alternate cell sources such as the gamma-delta T cells, which have been shown to be activated by mycobacterial antigens (10) and to secrete gamma interferon (4). The presence of IgG1 and IgG3 in the early stages of infection may enhance bacterial uptake and the clearance of pathogens via the Fc receptor on macrophages. In lepromatous leprosy patients, genetic defects in macrophages, such as a low capacity for respiratory burst activity (2), would allow *M. leprae* to survive and multiply intracellularly instead of being cleared. This process may be further enhanced by virulence factors such as phenolic glycolipids, which scavenge reactive oxygen intermediates and inhibit bactericidal action (3), leading to further progression of disease.

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