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Selective Increases in Antibody Isotypes and Immunoglobulin G Subclass Responses to Secreted Antigens in Tuberculosis Patients and Healthy Household Contacts of the Patients

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***Mycobacterium tuberculosis*-specific antibodies (immunoglobulin M [IgM], IgE, IgG, and IgG subclasses) were determined in 164 tuberculosis patients (pulmonary involvement, $n = 135$; lymph node involvement, $n = 29$), 59 healthy household contacts (HC), and 51 healthy endemic donors (EC) by a quantitative enzyme-linked immunosorbent assay for reactivity with culture filtrate. Among the isotypes, significant differences between tuberculosis patient groups with either pulmonary or lymph node involvement and healthy control groups (HC and EC) were detected only for IgG ($P < 0.001$) and IgG1 ($P < 0.001$) antibodies. Pulmonary patients also showed a significant difference with IgM ($P < 0.01$) and IgE ($P < 0.05$) antibodies. HC showed elevation of only IgM antibodies compared with EC, indicating that IgM antibodies may be an indicator of recent infection with *M. tuberculosis*. These results suggest that the switching of IgM antibody response to IgG1 is a critical event in disease progression. Polyclonal IgG1, IgG3, and IgE antibodies also showed significant elevation ($P < 0.05$) in patients compared with EC. A strong correlation (rho = 0.254; $P < 0.003$) was observed between *M. tuberculosis*-specific IgG1 and polyclonal IgG1 in patients, suggesting that activations of antigen-specific and polyclonal antibodies are related events. No correlation was found between IgG1 antibodies and purified protein derivative skin test results. Since IgG1 antibody responses to culture filtrate are present only after disease establishment, IgG1 responses could provide a useful diagnostic marker of disease.**

Disease localization and protective immunity in mycobacterial disease are associated with T-cell responses, whereas humoral responses play little or no role in protection and are considered to be of marginal importance in the outcome of disease due to several intracellular pathogens, including tuberculosis and leprosy (reviewed in reference 20). The study of antibody responses in tuberculosis has therefore focused mainly on their usefulness as a diagnostic serological tool (3, 8, 15, 24), with little attention given to careful dissection of antibodies at the isotype and subclass level in relation to pathogenesis. Also unaddressed is the role of immunoglobulin G (IgG) subclass response in relation to the extent of pulmonary disease or to disease localization in different compartments. Switching of antibody responses from IgM to one of the other isotypes or IgG subclasses requires cytokines secreted by different subsets of T-helper cells (19, 27). The distribution of IgG subclasses may therefore provide valuable insights into differential T-cell activation and its relationship to disease progression in mycobacterial diseases. We have recently reported that in leprosy, selective increases in IgG1 and IgG3 antibodies to *Mycobacterium leprae* across the disease spectrum and both subclasses showed a highly significant correlation with bacterial load in the patients (12). The factors that regulate these IgG subclasses are still not well characterized and could well determine the disease progression in other mycobacterial infections. We have therefore extended our studies to isotype and IgG subclass responses in tuberculosis. The current study addresses the relationship between antibody isotypes (IgM, IgG, IgG subclass, and IgE antibodies) and the disease severity in pulmonary disease as well as the effect of localization of disease in different compartments (lymph node versus lung). The most significant antibody response in tuberculosis patients was observed for IgG1, which was raised irrespective of disease localization compared with that in healthy controls (household contacts [HC] and endemic controls [EC]). None of the other IgG subclasses showed a similar relationship with established disease.

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MATERIALS AND METHODS

Study subjects. Patients with active tuberculosis presenting at Masoomeen Hospital between January 1991 and June 1994 ($n = 164$; pulmonary involvement, $n = 135$; lymphadenitis [LN], $n = 23$; LN with pulmonary involvement [LN/P], $n = 6$) were recruited for antibody studies. For the current study, patients with pulmonary tuberculosis who were positive for acid-fast bacilli in sputum and/or culture were included. LN patients showed characteristic caseous granuloma in histology, but none stained positive for acid-fast bacilli. LN/P patients showed histological evidence of caseous granuloma and radiological evidence of pulmonary involvement, and the sputum was also positive for acid-fast bacilli in five of these six patients. LN patients included in this study were those that responded to antituberculosis (ATT) chemotherapy. Pulmonary patients were further subdivided into moderate and advanced lung disease (PMD and PAD, respectively) groups on the basis of the extent of lung tissue involvement. The PMD group had disseminated lesions of moderate density that were confluent but not exceeding more than a total of one lung volume, without or with a single cavity measuring less than 4 mm and with miliary mottling; PAD lesions were more extensive than PMD lesions, exceeding one lung volume and with multiple cavities. The majority of patients (87%) had received <30 days of ATT chemotherapy. Fifty-nine healthy HC of patients with cavitory lung disease were recruited to represent the recently exposed population. Sera from 51 healthy donors who were employees of Aga Khan University representing a broad socioeconomic background and with no known direct exposure to active tuberculosis were included as EC. Among the controls groups, 87% of HC and 56% of EC were tuberculin skin test

positive (>10.0 mm) at the time of immunological assessment. There was no significant difference in the distribution of age and gender between patients and healthy controls.

Serum. Five milliliters of blood collected from both patients and controls was allowed to clot overnight at 4°C. 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. *Mycobacterium tuberculosis* culture filtrate (CF) was a gift from H. J. Kolk of The Royal Tropical Institute, Amsterdam, The Netherlands. Preparation of this CF has been described previously (33).

Reagents. Monoclonal antibodies specific for human IgG subclasses were HP 6069 (anti-IgG1) used in polyclonal IgG1 determination and kindly provided by R. G. Hamilton, Johns Hopkins University, Baltimore, Md. HP 6001 (anti-IgG1), HP 6002 (anti-IgG2), HP 6047 (anti-IgG3), HP 6023 (anti-IgG4), and HP 6029 (anti-IgE) prepared at the Centers for Disease Control and Prevention, Atlanta, Ga., were a gift from the late C. B. Reimer. The specificity, evaluation, and performance characteristics of these antibodies have been 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 purchased (Jackson Laboratories, Westgrove, Pa.) and diluted according to the manufacturer's recommendations. Preparation of purified rabbit anti-human IgE has been described in detail previously (9); the antibody was obtained by immunizing rabbits with the Fc fragments of human IgE myeloma, affinity purified over an IgE-Sepharose column and rendered Fcε specific by sequential passage over affinity columns of insolubilized IgG and $F(ab')_2$ fragments of human myeloma IgE.

Quantitation of IgE, IgM, IgG, and IgG subclasses to *M. tuberculosis* CF. IgG subclasses were quantitated by a slightly modified method as described previously for filarial antigens (13). Briefly, Immulon 4 plates (Dynatech, Alexandria, Va.) were coated with 100 μl of *M. tuberculosis* CF at 1 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 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 saturating 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. A serum pool containing high titers of antibody to *M. tuberculosis* CF was prepared and assigned arbitrary units of activity as described in Results and used as a reference calibrator. All test sera were assayed at a minimum of 3 dilutions, and the activity was expressed in units relative to the serum pool for IgG1 to IgG3 subclasses. For IgG4 and IgE antibodies, all sera were run at a dilution of 1:10 in a single assay and the results are expressed as optical density readings without converting into units of activity.

Quantitation of polyclonal IgG subclasses. The concentration of polyclonal IgG subclasses in serum was determined as detailed previously (14). Briefly, Immulon 4 microtiter plates were coated at saturating concentrations of a 1:1,000 dilution of HP 6069, HP 6002, and HP 6047 and 1:100 dilution 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 three 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 optical density was read at 410 nm in a microtiter plate reader (MR 600; Dynatech).

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 (myeloma PS; a kind gift from T. Ishizaka) with known amounts of IgE as previously reported (9). All test sera were run at a minimum of three serial dilutions. Values falling in the midrange of the dose-response curve were to used to calculate the concentration of IgE in test sera.

Statistical analysis. All statistical analyses were carried out using Microsoft Excel, Cricket graph, and Statview software packages on a Macintosh microcomputer. The Student *t* test was carried out to assess the significance of differences in antibody among patients and control groups. The Spearman rank correlation test was used to determine the relationship between different parameters.

RESULTS

Quantitation of IgG subclasses and IgE antibodies to *M. tuberculosis* CF. (i) Development of a serum calibrator and performance characteristics. Antigen-specific antibody responses in individual patients usually show log distribution, and therefore, optical density readouts at a single dilution do not allow quantitative assessment of differences between groups. To overcome this limitation, we developed a reference serum calibrator for each of the isotypes and IgG subclasses except IgG4 and IgE, since antibodies in these two isotypes did not show log distribution when screened at a 1:10 dilution. Independent serum pools were generated by pooling sera with high titers for either IgM, IgG, or IgG subclass antibodies against CF. These pools were assigned arbitrary units of activity, with 1 U being equivalent to an optical density of ~0.1. Dose-response curves for IgG and IgG1, IgG2, and IgG3 subclass antibodies against culture filtrate (after transformation of the dose-response curve into units per milliliter) are shown in Fig. 1. A four-degree polynomial equation was used to calculate the units in individual sera by using the midrange values. Although this system allows us to determine quantitative log differences among groups for an individual IgG subclass or isotype, it still does not allow comparison of concentrations between different IgG subclasses or isotypes which are related to the sensitivity of detection of the different antibody probes.

(ii) Determination of antibody activity in sera of tuberculosis patients. Figure 2 shows the IgM, IgG, and IgE antibody responses to *M. tuberculosis* CF in tuberculosis patients with involvement of different compartments. Pulmonary patients showed significantly elevated antibody responses in all three isotypes (IgG, $P < 0.001$; IgM, $P < 0.01$; and IgE, $P < 0.05$) compared with those in the healthy control group. Patients with lymph node involvement showed significant differences with IgM ($P < 0.05$) and IgG ($P < 0.01$) antibodies. Patients with both lymph node and pulmonary involvement showed raised IgG ($P < 0.001$) only. Interestingly, the only antibody that was significantly elevated in the HC group was IgM ($P < 0.001$). This could be due to current exposure to *M. tuberculosis*, as these were contacts of patients with cavitory lung disease at the time of testing.

When IgG antibody was dissected into its subclasses (Fig. 3), only IgG1 was significantly raised in all patient groups (LN, $P < 0.004$; LN/P, $P < 0.0004$; PMD, $P < 0.02$; and PAD, $P < 0.004$) compared with that in HC and EC. The only other antibody which approached significance in the pulmonary involvement group was IgG3 (LN/P, $P = 0.06$; PMD, $P = 0.05$; PAD, $P = 0.08$). IgG2 and IgG4 did not show any consistent pattern of elevation.

Quantitation of polyclonal antibodies. To see if the *M. tuberculosis*-specific IgG subclass response were related to differential polyclonal activation of these particular isotypes, we also determined the concentrations of polyclonal antibodies for each IgG subclass and IgE. Table 1 shows the concentration of polyclonal IgE and IgG subclass antibodies. Polyclonal activation was selectively observed for IgG1, IgG3, and IgE in patient groups compared with EC. The only exception was IgE in the LN group, which was raised but did not achieve statistical significance. Interestingly, HC also showed selective polyclonal activation of the same antibodies (IgG1, IgG3, and IgE), although antigen-specific IgG antibodies in these subclasses could not be detected.

Correlation of antigen-specific IgG1 with polyclonal IgG1 and purified protein derivative (PPD) skin test. To see if induction of antigen-specific responses were related to polyclonal activation or delayed-type hypersensitivity, which is an

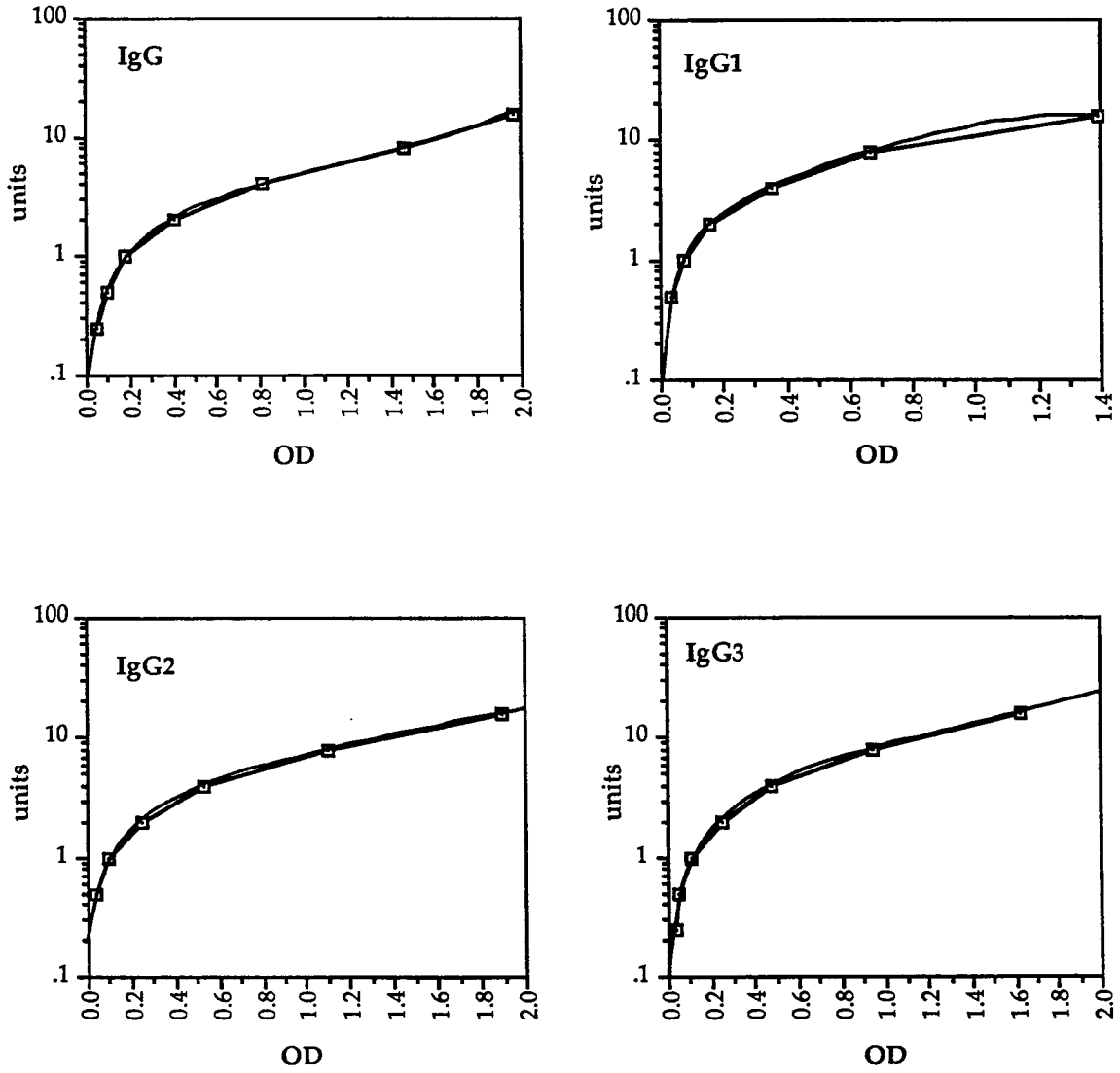

FIG. 1. Dose-response curves for IgG, IgG1, IgG2, and IgG3 to *M. tuberculosis* CF. The reciprocal of serum dilution giving an optical density of ~0.1 was assigned as units of activity per milliliter of neat serum: IgG, 16,000 U; IgG1, 3,200 U; IgG2, 6,400 U; and IgG3, 3,200 U. Calibration curves are shown as optical density (OD) relative to units. A four-order polynomial fit used to assign units of antibody activity in individual test sera is shown.

indicator of TH1 responses, Spearman rank correlation was used to analyze the relationship. Polyclonal IgG subclass responses showed a significant association with the respective antigen-specific antibody responses (IgG1, rho = 0.254, $P < 0.003$; IgG2, rho = 0.317, $P < 0.0002$; IgG3, rho = 0.278, $P < 0.0013$; IgG4, rho = 0.211, $P < 0.014$) in patients with pulmonary tuberculosis. However, no significant association was observed between tuberculin skin test reaction size and either the antigen-specific IgG1 (rho = 0.089; $P = 0.338$) or polyclonal IgG1 (rho = 0.083; $P = 0.369$) antibodies, indicating that the IgG1 response is independent of TH1 responses.

DISCUSSION

It has been suggested that similar to leprosy, tuberculosis is a spectral disease with an inverse relationship between severity of disease and cellular immune responses, as assessed by tuberculin skin tests and in vitro lymphocyte blastogenic responses, and a direct relationship with humoral responses (11, 17). These studies were based on small groups, and the patients had been on variable lengths of ATT treatment. The major strengths of our study are that we have used large group sizes, particularly with the pulmonary patients, and the majority of patients had received <30 days of ATT treatment. Duration of chemotherapy is an important consideration, since we were interested in identifying immune profiles related to disease establishment whereas ATT treatment can result in an alteration of the immune profile, particularly with respect to the antibody responses. The most significant finding in the current study was the presence of *M. tuberculosis*-specific IgG1 antibodies in patients with established disease without any correlation with the presence or absence of cellular responses. Specific IgG1 antibody was raised in all tuberculosis patients irrespective of disease localization (lymph node versus pulmonary compartment) compared with that in healthy controls (HC and EC). IgG1 antibody was not detected in HC despite evidence of recent infection, as suggested by the significantly raised IgM antibodies compared with those in healthy EC.

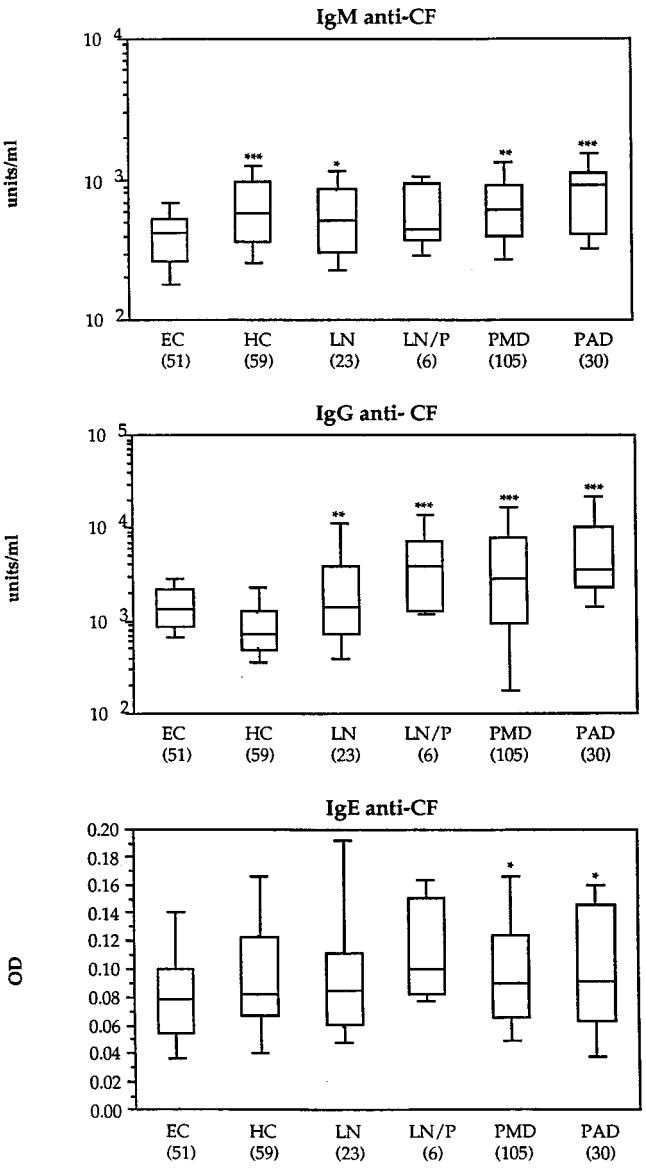

FIG. 2. Comparison of antibody isotype (IgM, IgG, and IgE) responses to *M. tuberculosis* CF in different groups. The number in each group is given in parentheses; horizontal bars indicate the 10th, 25th, 50th, 75th, and 90th percentiles for each group. The Student *t* test was carried out to assess the significance of differences between patient groups and EC (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). OD, optical density.

None of the other IgG subclasses or IgE antibodies showed such consistent elevation in tuberculosis patients. One earlier report using PPD antigens did not show similar elevation of IgG1 in tuberculosis patients (15). One explanation may be that PPD contains denatured antigens while CF is produced without subjecting the proteins to denaturation (32). While T cells are known to react equally well with native and denatured proteins, antibody binding to denatured antigens can be decreased. This is substantiated by our previous observation that IgM and IgG antibodies showed higher reactivity to CF compared with PPD, while in a lymphocyte proliferation assay, PPD was consistently more active than CF in the same patient groups (unpublished data). In leprosy, we have reported a similar elevation of antigen-specific IgG1 and IgG3, and the strongest association of IgG1 antibodies was observed with bacterial load rather than cellular responses (12).

In the murine system, switching of antibody responses from IgM to one of the other isotypes is dependent on different cytokines. Gamma interferon produced by TH1 cells induces IgG2a and IgG3 in vitro (6, 28); interleukin-4 (IL-4) produced by TH2 selectively stimulates IgG1 and IgE (7). The human counterparts of murine IgG subclasses are based on similarities in biological and functional activities. Murine IgG2a and IgG2b and human IgG1 and IgG3 share the ability to fix complement and bind to protein antigens (25). 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. In humans, although TH1 and TH2 subsets have been identified on the basis of their cytokine profiles (22, 23), 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 (16). In another study, there was no relationship between IL-2, IL-4, IL-6, and gamma interferon secreted by T-helper clones and their IgG subclass induction pattern (4). Our studies suggest that IgG1 responses are independent of T-cell activation, since IgG1 antibodies do not show any association with either skin test or lymphocyte blastogenic responses which are suppressed in pulmonary tuberculosis but not in tuberculous LN. Depressed PPD-stimulated blastogenesis in patients with pulmonary tuberculosis appears due, at least in part, to a suppressive influence of monocytes (5, 26, 30). The mechanism of suppression involves direct stimulation by PPD (34) of monocytes primed during the course of tuberculosis to produce suppressive predictors such as IL-2R (31) and transforming growth factor β (29). It is of interest to speculate that some of the mediators (cross-modulatory cytokines) overexpressed by activated monocytes in tuberculosis could themselves contribute to the increased antibody levels by promoting isotype switching and postswitch differentiation of B cells and are not due to the presence or absence of activated T cells. In the murine model recent data strongly suggest that the monocyte-derived transforming growth factor β plays an important role in switching IgM responses to IgG2b (18).

No significant responses were observed with the IgG2 subclass antibodies in the patients compared with those in EC, although IgG2 has been reported to be elevated in response to the carbohydrate antigen lipoarabinomannan of *M. tuberculosis* (2) as well as to the PPD antigens in tuberculosis patients (24). IgG2 preferentially recognizes T-independent carbohydrate antigens (1, 35). CF obtained in the log phase contains relatively low levels of carbohydrates (32) such as lipoarabinomannan, and therefore, IgG2 antibodies may not be as reactive with CF as with purified lipoarabinomannan. Furthermore, carbohydrates are subclass antibody stable and may survive the denaturation steps in the preparation of PPD, resulting in denaturation of protein antigens but enrichment of carbohydrates, leading to lower reactivity with IgG1 but higher reactivity with IgG2. These observations raise an important issue of bias that can be introduced by the use of different crude antigenic preparations. For T-cell-dependent antibody responses to protein antigens, CF may be a more suitable antigen preparation than PPD.

Pulmonary patients also showed a trend towards higher concentrations of IgG3 and IgG4 antibodies, but these responses did not achieve statistical significance. Despite the low levels of *M. tuberculosis* IgE antibody detected, a significant difference was observed in the pulmonary group compared with EC. Similarly, patients with both pulmonary and lymph node in-

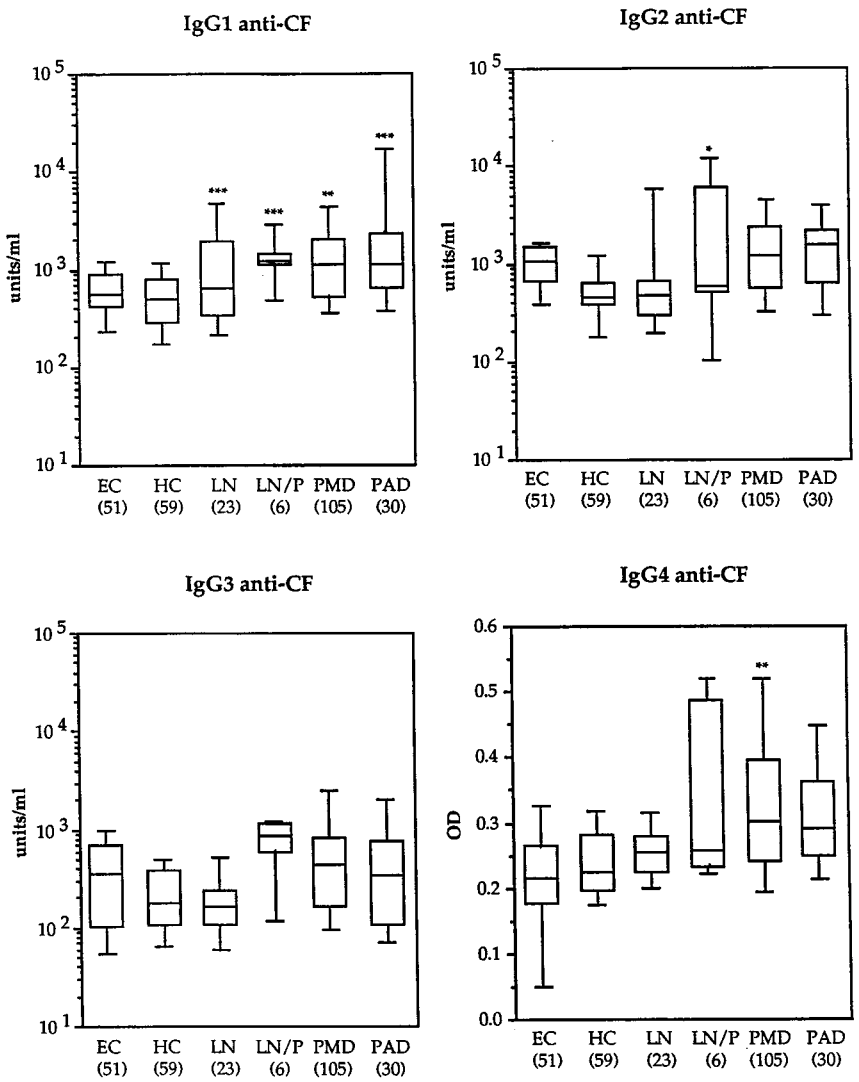

FIG. 3. Comparison of IgG subclass antibodies to *M. tuberculosis* CF in each of the groups. All other parameters are same as described in the legend to Fig. 2.

volvement also showed a trend towards higher responses in IgG3, IgG4, and IgE subclasses, while patients with tuberculous LN alone did not show detectable responses in IgG3 and IgG4.

M. tuberculosis-specific IgE antibodies also showed a significant difference between EC and pulmonary patients. Because of the extremely low optical density readouts with IgE antibodies to *M. tuberculosis*, the significance of the difference between EC and pulmonary patients remains questionable. One reason for such low levels may be the blocking effect by *M. tuberculosis*-specific antibodies of other IgG subclasses; further studies need to be carried out to address this issue.

Polyclonal activation was also observed for the same IgG isotypes (IgG1, IgG3, and IgE) for which antigen-specific antibodies were detected, suggesting that antigen-specific responses are related to polyclonal activation in these patients. However, specific antibodies cannot be a reflection of polyclonal activation alone, since HC did not show specific antibodies despite polyclonal activation in all the relevant isotypes, viz., IgG1, IgG3, and IgE. Whereas elevated IgE responses in HC could be attributed to more frequent parasitic infections due to lower socioeconomic background, elevation in IgG1 and IgG3 is more difficult to explain. One explanation may be that polyclonal responses precede antigen-specific responses. In the case of the HC group, the intense exposure to mycobacteria may initially lead to the activation of the innate arm of the immune system, including IgM antibodies, NK cells, and $\gamma\delta$ T cells, which are known to be activated by mycobacterial antigens (10). The lipopolysaccharide-like mycobacterial antigens such as lipoarabinomannan and the cytokines secreted by the activated cells may provide the permissive signals required for polyclonal activation in HC (25). However, switching of antigen-specific IgM to IgG antibody requires additional signals which are elaborated when the infection becomes patent. The strong association observed between polyclonal and antigen-specific IgG subclass antibody responses suggests that initial polyclonal expansion of B cells is a natural strategy for increasing the repertoire of antigen-specific B cells by the host.

Since IgG1 antibodies are associated only with disease establishment and not with infection alone, factors which result in switching of IgM responses to IgG1 antibodies during infection may be critical to disease progression and need to be further characterized. Obviously these observations need to be extended to other extrapulmonary tuberculosis infections in

TABLE 1. Distribution of polyclonal IgG subclasses and IgE

Isotype	Mean mg/ml[a] ± 95% confidence interval (range) in:					
	EC (n = 51)	HC (n = 59)	LN (n = 23)	LN/P (n = 6)	PMD (n = 105)	PAD (n = 30)
IgG1	5.96 ± 0.61 (1.8–13.86)	7.03 ± 0.73 (2.0–18.0)	7.48 ± 1.31 (2.44–16.8)	8.93 ± 2.08 (6.0–12.8)	8.5 ± 0.57 (3.76–24.0)	10.2 ± 1.5 (3.6–20.8)
IgG2	2.91 ± 0.33 (0.55–5.52)	2.36 ± 0.34 (0.07–7.2)	2.37 ± 0.50 (0.4–5.6)	3.17 ± 1.31 (1.6–6.0)	3.59 ± 0.27 (1.1–9.6)	4.4 ± 0.94 (0.89–12.8)
IgG3	0.84 ± 0.12 (0.28–2.17)	1.31 ± 0.18 (0.32–3.2)	1.35 ± 0.28 (0.43–3.04)	1.4 ± 0.49 (0.8–2.4)	1.52 ± 0.16 (0.5–6.1)	1.57 ± 0.33 (0.32–4.2)
IgG4	0.72 ± 0.15 (0.05–2.60)	0.74 ± 0.18 (0.02–4.8)	0.76 ± 0.36 (0.11–4.16)	0.77 ± 0.32 (0.32–1.28)	1.12 ± 0.16 (0.06–5.7)	0.98 ± 0.25 (0.13–3.36)
IgE	506 ± 192 (8.0–3,900)	1,863 ± 687 (40–14,000)	954 ± 625 (80–5,096)	2,040 ± 2,759 (35–9,000)	2,809 ± 932 (5.6–37,500)	2,002 ± 844 (35–10,000)

[a] IgE results are expressed as nanograms per milliliter. The Student t test was used to determine the significance of differences in antibodies among different groups. Significantly ($P < 0.05$) raised values compared with EC values are underlined.

which skin test anergy has been observed in a high percentage of patients and samples for microscopy and culture are difficult to obtain. For diagnostic purposes the presence of specific IgG1 could be a useful adjunct marker of disease.

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