INFECTION AND IMMUNITY, Dec. 1994, p. 5673–5678
0019-9567/94/$04.00+0
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Vol. 62, No. 12

Immune Responsiveness and Lymphokine Production in Patients with Tuberculosis and Healthy Controls

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Received 21 July 1994/Returned for modification 30 August 1994/Accepted 26 September 1994

The aim of the present study was to determine the profile of immune responsiveness that differentiates patients with tuberculosis (TB) from healthy tuberculin-positive controls. Forty-five patients with pulmonary TB and 16 healthy tuberculin-positive controls, all human immunodeficiency virus negative, were studied. Patients had decreased reactivity to tuberculin, diminished proliferative response to purified protein derivative (PPD), lower concentrations of interleukin-2 (IL-2) and gamma interferon in PPD-stimulated cultures, no increase in the percentage of γ/δ cells in PPD-stimulated cultures, and higher immunoglobulin G antimycobacterial antibodies compared with control subjects. Furthermore, controls exhibited decreased production of IL-4 by PPD-stimulated cells. Multivariate discriminant and factor analyses demonstrated divergent patterns of immune reactivity against mycobacterial antigens. The association of IL-4 and immunoglobulin G antibody levels in patients, in contrast to the high reactivity to tuberculin, increased proliferation to PPD, and higher levels of IL-2 and gamma interferon observed in healthy controls suggested that most TB patients exhibit a TH2 pattern of immune responsiveness while tuberculin-positive healthy individuals have a TH1 pattern.

Patients with tuberculosis (TB) frequently have depressed cellular and increased humoral immune responses against mycobacterial antigens (3, 21, 24, 30, 46). Previous reports have shown negative tuberculin skin test reactions (20, 24, 28, 32, 46), changes in circulating lymphocyte populations (3, 30, 34, 37, 40), diminished proliferative responses to specific antigens (16, 19, 24, 38), alterations in the production of lymphokines (13, 15, 38, 41), defects in macrophage antimycobacterial activity (22), decreased activity of natural killer cells (31), and increased antibody levels (7, 13, 16, 21, 42, 45, 46). These observations indicate that with TB there is a disregulation of the immune response which could be related to the function of the TH1 and TH2 $CD4^+$ T-lymphocyte subsets (25). These cells represent functionally distinct cellular populations. TH1 cells produce mainly interleukin-2 (IL-2) and gamma interferon (IFN-γ) and, therefore, are involved in the activation of macrophages and cytotoxic cells. TH2 lymphocytes produce IL-4, IL-5, and IL-6, which are responsible for B-cell differentiation and activation. Although there is evidence that exposed healthy subjects have TH1-like responses to mycobacterial antigens (9, 11, 12, 15, 33, 39), no comprehensive evaluation has been made regarding the type of responses that TB patients could manifest compared with tuberculin-positive healthy controls. In this study we tried to identify the components of the cellular and humoral immune responses which could discriminate adult tuberculous patients from tuberculin-positive healthy controls.

Subjects studied. Forty-five adult patients with newly diagnosed, bacteriologically confirmed, pulmonary TB, untreated or treated for a maximum of 2 weeks, were referred from the Tuberculosis Control Programs of Hospital La María, Instituto Metropolitano de Salud, Instituto de los Seguros Sociales, Dirección Seccional de Salud de Antioquia, and Hospital Universitario San Vicente de Paul, Medellín, Colombia. Sixteen healthy, tuberculin-positive, adult individuals were included as controls. All subjects studied were informed about the objectives of the study and voluntarily agreed to participate in it. Twenty-four (53%) patients were males, and 21 (47%) were females. Nine (56%) controls were males, and 7 (44%) were females. The ages of patients ranged from 16 to 71 years, with a mean (± standard error of the mean) of 38.3 ± 2.1 years. For controls, ages varied from 25 to 72 years, with a mean of 43.6 ± 2.9 years. Thirty-nine (87%) patients were receiving ambulatory treatment and six (13%) were hospitalized at the time of the study. There were three (7%) patients who had pleural complications. One patient with ganglionary TB and one with endometrial TB had radiological evidence of simultaneous lung compromise; therefore, they were included as patients with pulmonary TB.

ELISA for HIV antibodies. Serum antibodies against human immunodeficiency virus type 1 (HIV-1) antigens were studied at the reference laboratory of the Dirección Seccional de Salud de Antioquia with a commercial enzyme-linked immunosorbent assay (ELISA) kit (Vironostika anti-HIV Uniform micro-ELISA system; Organon, Teknika, Boxtel, The Netherlands). Confirmation was done by Western blot (immunoblot) (HIV-1 Western blot kit; Epitope, Beaverton, Oreg.). None of the subjects studied had antibodies against HIV-1.

ELISA for antimycobacterial antibodies. Determination of serum immunoglobulin G (IgG) antibodies against mycobacteria was made with an ELISA test developed in our laboratory (24a). Briefly, plates with 96 U-bottom wells (Nunc, Roskilde, Denmark) were coated with 100 μl of a *Mycobacterium bovis* BCG sonicate (31) at a concentration of 6 μg/ml. Duplicates of sera (100 μl per well), diluted 1:320 in BLOTTO, were tested by incubating them for 1 h at 37°C. After the plates were washed, 100 μl of peroxidase-labeled goat anti-human IgG (Sigma Chemical Co., St. Louis, Mo.), diluted 1:1,000 in BLOTTO, was added to each well, and the plates were incubated for 1 h at 37°C. The reaction was developed with 50 μl of a solution of 16.5 mM *ortho*-phenylenediamine (Sigma Chemical Co.) in 0.27 M Tris-citrate buffer (pH 6) (Sigma Chemical Co.) and 0.024% hydrogen peroxide (Sigma Chem-

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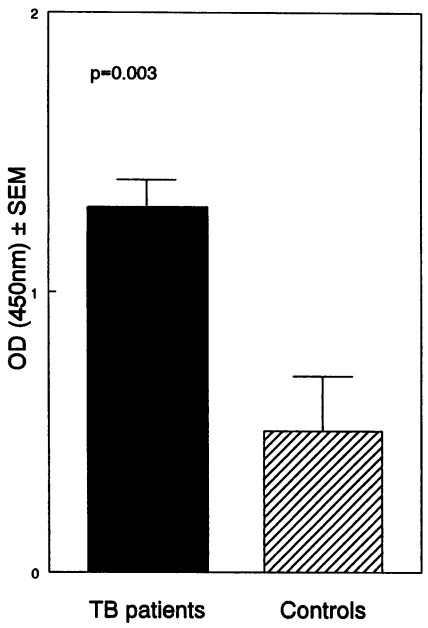

FIG. 1. IgG anti-*M. bovis* BCG sonicate. OD_{450nm}, optical density at 450 nm.

ical Co.). The reaction was stopped with 0.23 M phosphoric acid (Merck, Darmstadt, Germany) and 10 mM HCl (Merck, Darmstadt, Germany). The A_{450} was read in an ELISA reader (Dynatech, Alexandria, Va.). The level of IgG antimycobacterial antibodies in TB patients was 1.3 ± 0.1 (standard error of the mean). This value was significantly higher ($P = 0.003$) than that for controls, whose mean A_{450} value was 0.5 ± 0.2 (Fig. 1).

Tuberculin skin test. Patients and controls received 0.1 ml (5 tuberculin units [TU]) of purified protein derivative (PPD, batch CT6; Connaught, Willowdale, Ontario, Canada) intradermally, immediately after blood sample collection. The induration was measured 72 h later and recorded as the average of two perpendicular diameters in millimeters. The mean induration of the tuberculin skin test reaction in patients was 8.8 ± 0.9 mm, which was significantly lower ($P = 0.009$) than that of 14.3 ± 1.7 mm found in the control group (Table 1).

Cell isolation and culture. Peripheral blood mononuclear cells (PBMC) were isolated from 50 ml of defibrinated blood on Ficoll-Hypaque (Sigma Chemical Co.), washed twice, and resuspended in RPMI-1640 (ICN-Flow, Costa Mesa, Calif.) supplemented with 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 24 mM sodium bicarbonate, 100 IU of penicillin per ml, 50 μg of gentamicin per ml, and 2.5 μg of amphotericin B (E. R. Squibb I. A. Corp. & Son, Cali, Colombia) per ml. For proliferation assays, the medium was supplemented with 10% pooled human heat-inactivated serum; in the cultures used for cytokine production, 10% heat-inactivated fetal calf serum (CELLect Gold; ICN-Flow) was used.

Lymphocyte proliferation. PBMC were adjusted to 10^6 cells per ml in complete medium. Triplicates of 100 μl of the cell suspension were plated in 96 U-bottom-well plates (Nunc). Cells were stimulated either with 5 μg of PPD (Connaught) per ml at the time of initiation of the culture or with 1 μg of phytohemagglutinin (Sigma Chemical Co.) per ml, added after 72 h of culture. Nonstimulated cells were included as a control. Cultures were incubated for 5 days at 37°C in 5% CO_2. Six hours before harvesting, 0.5 μCi of 5'-[^{3}H]thymidine (14.5 Ci/mmol [specific activity]; Amersham, Buckinghamshire, United Kingdom) was added. Cells were collected in a cell harvester (PHD, Cambridge, Mass.), and the radioactivity was measured in a liquid scintillation counter (model 1211; LKB, Turku, Finland).

TABLE 1. Tuberculin skin test results and lymphocyte proliferation in patients with TB and healthy controls

Group	Tuberculin skin test reaction (avg diam [mm])[a]	Net PBMC proliferation response (cpm) to[b]:	
		PPD	PHA
Patient	8.8 ± 0.9	6,405 ± 1,537	22,887 ± 4,845
Controls	14.3 ± 1.7	10,577 ± 2,234	22,566 ± 12,828
P[c]	0.004	0.03	NS[d]

[a] Results, with 5 TU of tuberculin, are expressed as the average diameter of induration (in millimeters) ± standard errors of the means.
[b] Net results, results for stimulated cells minus results for unstimulated cells. PPD and phytohemagglutinin (PHA) were added to concentrations of 5 and 1 μg/ml, respectively.
[c] *P* value by the Mann-Whitney U test.
[d] NS, not significant.

The net proliferation of PBMC in patients in response to PPD was (mean ± standard error of the mean) 6,405 ± 1,537 cpm; these responses were lower than the proliferation response exhibited by healthy controls (10,577 ± 2,234 cpm; *P* of 0.03) (Table 1). The proliferative responses to phytohemagglutinin were similar for patients and controls (22,887 ± 4,845 cpm versus 22,566 ± 12,828 cpm, respectively).

Cytokine measurement. In another set of cultures, 2×10^6 PBMC per well in 1.5 ml volumes were plated in 24-well plates (Nunc) with or without 5 μg of PPD per ml. After 96 h, the supernatants were collected and stored at −70°C. Cells were frozen in culture medium containing 20% fetal calf serum and 10% dimethyl sulfoxide (Merck) and stored in liquid nitrogen until used for lymphocyte subpopulation determinations.

IL-2, IL-4, and IFN-γ concentrations were determined by using commercial ELISA kits (Intertest-2, Intertest-4, and Intertest-γ, respectively; Genzyme Co., Boston, Mass.) following the methods recommended by the manufacturer. PBMC from TB patients produced negligible amounts of IL-2 in response to PPD (Fig. 2), in contrast to those of controls, whose cells produced significantly higher quantities of IL-2 after stimulation with PPD ($P = 0.001$). Although cells from both controls and patients produced IFN-γ in response to PPD stimulation, the production of IFN-γ in TB patients was significantly lower ($P = 0.04$) than that in control subjects. The cells from patients did not produce significant quantities of IL-4 in response to PPD. However, in controls, the production of IL-4 decreased in PPD-stimulated cultures compared with that in unstimulated cultures ($P = 0.0001$).

Determination of lymphocyte populations by immunofluorescence. Cell surface markers were studied after in vitro culture with or without PPD. CD3/CD19 and CD4/CD8 markers were detected by double-direct immunofluorescence with fluorescein- and phycoerythrin-labeled murine monoclonal antibodies (Simultest T/B, Simultest Helper/Suppressor; Becton-Dickinson, San Jose, Calif.). Detection of α/β and γ/δ T cells and CD25 (IL-2 receptor-positive) cells was done by indirect immunofluorescence with murine monoclonal antibodies against the specific cell surface marker as the first antibodies (anti-human T-cell receptor-α/β-1, anti-human T-cell receptor-γ/δ-1, and anti-human IL-2 receptor, respectively;

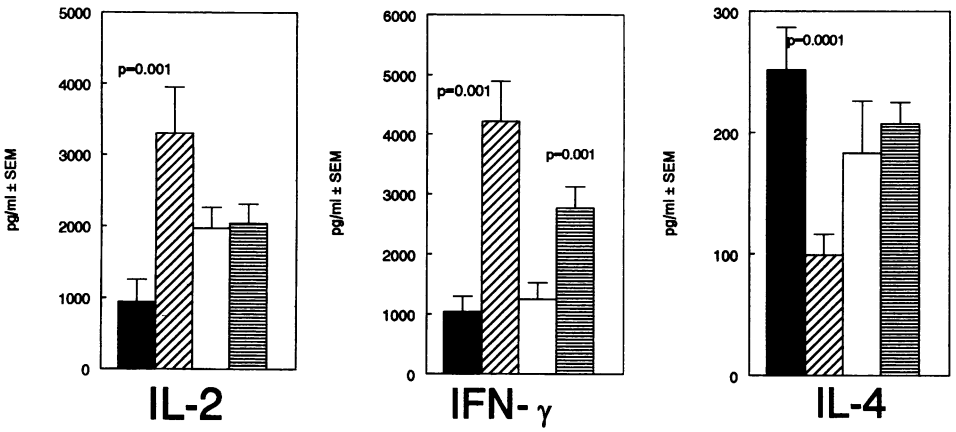

FIG. 2. Production of IL-2, IFN-γ, and IL-4 by TB patients and tuberculin-positive healthy controls. Lymphokine concentrations were detected by ELISA in supernatants from 96-h PBMC cultures. Controls: ■, unstimulated; ▨, PPD stimulated. Patients: □, unstimulated; ▤, PPD stimulated.

Becton-Dickinson) and fluorescein isothiocyanate-labeled goat anti-mouse antiserum (Becton-Dickinson) as the second antibody. Staining was done according to the manufacturer's instructions. Only cells with peripheral fluorescence were counted in an epifluorescence microscope (Zeiss, Oberkochen, Germany), and the percentage of positive cells was determined in at least 200 cells per sample.

The net change in the percentage of positive cells found after stimulation with antigen, compared with that in unstimulated cultures (Fig. 3), was not significant for CD19 (B cells), CD3 (total T cells), CD4, CD8, CD25 (IL-2 receptor-positive) and α/β cells. However, the percentage of γ/δ cells in PPD-stimulated cultures from patients with pulmonary TB diminished 1.6% ± 2.1% with respect to that in unstimulated cultures, while in controls the percentage increased 8.8% ± 4.0% ($P = 0.02$).

Multivariate statistical analysis. Discriminant analysis using the variables that showed a significant difference in the specific response to mycobacterial antigens (tuberculin skin test; IgG antimycobacterial antibodies; PPD-induced proliferation; IL-2, IFN-γ, and IL-4 production; and changes in γ/δ cells) clearly discriminated patients from controls ($P = 0.001$). None of the controls' responses were similar to those of the patients, while only 12.5% of patients showed responses similar to those of the controls. Factor analysis was used to determine the relative weight of each variable among the variables used in the discriminant analysis. Factor analysis arrays the variables into different linear functions that have a certain probability of modeling the interactions between them, while distinguishing the variables from each other as much as possible. According to the weights assigned to each variable, a vectorial space in which each subject can be located can be constructed (Fig. 4). Factor 1 separated IL-4 and IgG antimycobacterial antibodies from the other variables (skin test, lymphocyte proliferation, IL-2 and IFN-γ production, and changes in γ/δ T cells), which are more relevant to cell-mediated immunity. Patients with pulmonary TB were widely distributed with respect to both factors, although most of them contributed negatively to factor 1. In contrast, all healthy controls were located on the right side of the plot, where the vectors corresponding to the variables related to cell-mediated responses were found.

The results of the present study confirm previous reports

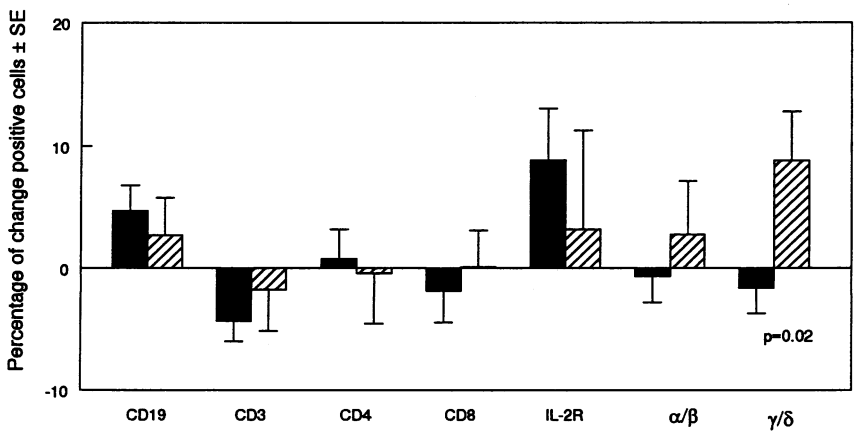

FIG. 3. Changes in percentage of lymphocyte subpopulations in 96-h PBMC cultures stimulated with PPD (5 μg/ml) or unstimulated. The percentage of change equals the percentage of positive cells in cultures with PPD minus the percentage of positive cells in cultures without PPD. CD3/CD19 and CD4/CD8 were detected by double direct immunofluorescence. CD25 (IL-2 receptor), α/β, and γ/δ cells were detected by indirect immunofluorescence. ■, patients; ▨, controls.

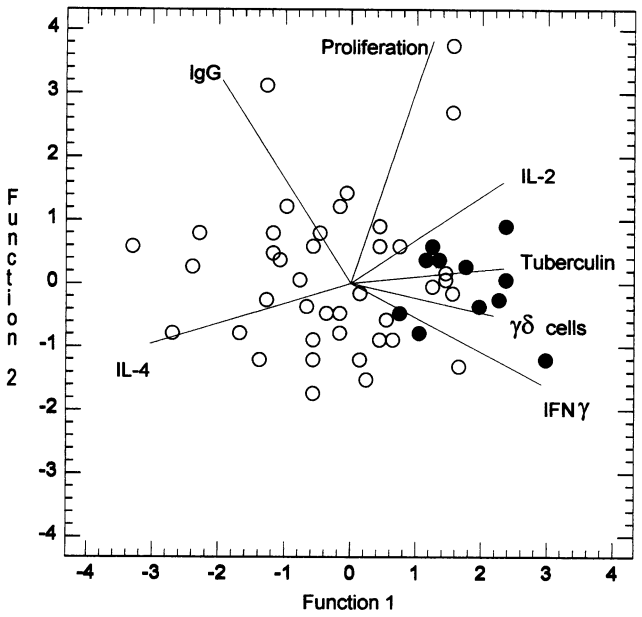

FIG. 4. Distribution of variables that showed significant differences between TB patients (○) and controls (●) in vectorial space generated by multivariate factor analysis and locations of subjects studied according to the two main functions generated.

showing that patients with TB, compared with tuberculin-positive healthy subjects, have altered immune responsiveness to mycobacterial antigens (1, 4, 13, 21, 24, 30, 46). Our patients had diminished reactivities to the tuberculin skin test, lower PPD-induced lymphocyte proliferation, decreased in vitro production of IL-2 and IFN-γ, no increase in the percentage of γ/δ cells in stimulated cultures, and higher levels of serum IgG antimycobacterial antibodies. Diminished tuberculin skin test reactions have been found associated with low in vitro proliferative responses to PPD (4, 20, 24, 28, 30, 46). Decreased IL-2 and IFN-γ concentrations have also been reported in supernatants of peripheral blood leukocytes cultures from patients with pulmonary TB which have been stimulated in vitro with mycobacterial antigens (13, 38, 41). The levels of IgG antimycobacterial antibodies are often increased in tuberculous patients (7, 13, 16, 42, 45, 46). However, to our knowledge, this is the first report showing differences in in vitro production of IL-4 by PBMC from tuberculous patients and healthy sensitized controls.

The general pattern of response observed in the two groups studied showed that tuberculin-positive healthy controls exhibited a vigorous cell-mediated immune response while the majority of the tuberculous patients showed low T-cell reactivity and augmented humoral responses. It is interesting that factor analysis showed that IL-4 production and IgG antimycobacterial antibodies were distinct from proliferation responses to PPD, tuberculin skin test, IL-2 and IFN-γ production, and changes in γ/δ cells in PPD-stimulated cultures. Furthermore, the distribution of patients and controls correlated with the distribution of these variables. It is likely that this distribution could represent the influence of functionally divergent TH1 and TH2 cellular types of immune response (26, 31, 35, 36). TH1 responses appear to mediate functions related to cytotoxicity and macrophage activation and therefore play an important role in combatting intracellular microorganisms (26). On the other hand, TH2 responses are effective in helping B cells to produce antibodies and are important in combatting extracellular bacteria and in the induction of humoral immunity. Thus, we suggest that the TH1 phenotype dominated the response of normal PPD-sensitized controls, as has been suggested by other investigators (9, 11, 12, 25, 33, 39), while a TH2 type of immune response appears to predominate in most of the patients with pulmonary TB. The demonstration of TH1 and TH2 phenotypes in mice infected with *M. bovis* BCG (14) or vaccinated with killed *Mycobacterium leprae* (27, 43) has been reported. In humans, T-cell clones, obtained from tuberculin-positive subjects stimulated with mycobacterial antigens present a TH1 pattern of lymphokine production (11, 12, 33). However, it should be noted that under normal in vivo conditions, TH1 and TH2 patterns should coexist in a balance so that a protective immune response develops, while under pathological conditions this balance can be shifted in either direction. Interestingly, it has been shown that patients with leprosy exhibited TH1 and TH2 patterns of cytokine production that correlate with the clinical spectrum of the disease (44). Patients with tuberculoid leprosy and a reversal reaction had increased expression of IL-2, IFN-γ, and lymphotoxin whereas patients with lepromatous leprosy or erythema nodosum leprosum had increased expression of IL-4, IL-5, and IL-10. More recently, it has been reported that in tuberculin-positive healthy subjects, in vitro stimulation with PPD preferentially induced IFN-γ and very few IL-4-producing cells (9). In addition, it has been shown that after in vitro stimulation with live virulent *M. tuberculosis*, cells from TB patients expressed levels of IL-2 and IFN-γ mRNA lower than those of sensitized controls (15). Our results are in agreement with these reports and further show that there are significant differences in the production of IL-4, a TH2-derived cytokine, between TB patients and controls.

The finding that cells from control subjects exhibited negative net production of IL-4 after in vitro stimulation with PPD may suggest that under normal conditions the immune system possesses a mechanism for suppression of IL-4 production after stimulation with a TH1 antigen. It is tempting to speculate that the high concentration of IFN-γ and IL-2 attained in controls as a consequence of PPD stimulation could be actively inhibiting IL-4 production in normal individuals (23), whereas in TB patients this suppressive mechanism does not work properly or, alternatively, there could be an active suppression of IL-2 production, as had been suggested by Toosi et al. (38). Another possibility is that γ/δ cells, which react in vitro to mycobacterial antigens, could secrete IFN-γ and IL-2 (5, 8, 29, 35) and hence participate in the regulation of the cytokine secretion pattern. In our study, PBMC of control subjects, stimulated with PPD, showed a higher increase in γ/δ cells than did those of TB patients. However, the increase in γ/δ cells could be secondary to the production of IL-2 by cells from control individuals, rather than a direct response of γ/δ cells to PPD, since most of these cells seem to recognize non-protein antigens (6).

The antagonistic cytokine secretion patterns found in patients and controls could correlate with the capability of activating macrophages and control mycobacterial intracellular replication (17, 18). On the other hand, there is the possibility that the differences in immune responsiveness observed between controls and patients are influenced by macrophages. It has been reported that TH1 and TH2 responses are conditioned by the antigen-presenting cell. Macrophages are better presenting cells for TH1 cells, whereas B cells preferentially induce TH2 activation (10).

The analysis of the influence of each variable in the context of the other variables indicative of the immune reactivity, and

in the two groups of subjects studied, discriminated patients and controls. The demonstration of different patterns of immune responsiveness in patients with TB is an important contribution to the comprehension of the pathogenesis of the disease. Future studies must try to unravel the basis of selection of these cytokine patterns and further explore the nature of alterations of the immune response in TB patients in order to find new possibilities for therapeutic intervention.

We thank all the administrative personnel and clinical staff of the health institutions in which patients were contacted, especially to Rodrigo Ramírez, Marta L. Osorio, Fernando Bedoya, and José R. Maya for their collaboration and Ruth Ramírez of the Laboratory of the Dirección Seccional de Salud de Antioquia for the serological studies of HIV infection. We also acknowledge Mary Fujiwara, Ken Morgan, and Ellen Buschman for their valuable help in the preparation of the manuscript.

This work was funded by COLCIENCIAS.

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