Human Humoral Responses to Antigens of *Mycobacterium tuberculosis*: Immunodominance of High-Molecular-Mass Antigens

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The selection of antigens of *Mycobacterium tuberculosis* for most studies of humoral responses in tuberculosis patients has been restricted to molecules that were either immunodominant in immunized animals or amenable to biochemical purification rather than those that were reactive with the human immune system. Delineation of antigens that elicit humoral responses during the natural course of disease progression in humans has been hindered by the presence of cross-reactive antibodies to conserved regions on ubiquitous prokaryotic antigens in sera from healthy individuals and tuberculosis patients. The levels of cross-reactive antibodies in the sera were reduced by preadsorption with *Escherichia coli* lysates, prior to studying their reactivity against a large panel of *M. tuberculosis* antigens to which the human immune system may be exposed during natural infection and disease. Thus, reactivity against pools of secreted, cellular, and cell wall-associated antigens of *M. tuberculosis* was assessed by an enzyme-linked immunosorbent assay (ELISA). Initial results suggested that the secreted protein preparation contained antigens most frequently recognized by the humoral responses of pulmonary tuberculosis patients. The culture filtrate proteins were subsequently size fractionated by preparative polyacrylamide gel electrophoresis, characterized by reaction with murine monoclonal antibodies to known antigens of *M. tuberculosis* by an ELISA, and assessed for reactivity with tuberculous and nontuberculous sera. Results show that a secreted antigen of 88 kDa elicits a strong antibody response in a high percentage of patients with pulmonary tuberculosis. This and other antigens identified on the basis of their reactivity with patient sera may prove useful for developing serodiagnosis for tuberculosis.

Recent estimates by the World Health Organization suggest that approximately 90 million new cases and about 30 million deaths will occur due to tuberculosis during this decade (38). This resurgence of tuberculosis has led to renewed interest in developing improved vaccines, diagnostics, drugs, and drug delivery regimens for the disease. It is well established that cellular immunity is critical for protection against tuberculosis, and several laboratories are currently focused on defining the antigens of *Mycobacterium tuberculosis* that can elicit effective immunity (3) and on understanding the role of various cell populations in host-pathogen interactions (27, 36).

Humoral responses in tuberculosis have been studied for several decades, primarily for the purpose of developing serodiagnostic assays. Although some seroreactive antigens and epitopes have been identified, interest in humoral responses to *M. tuberculosis* has waned, since progress in the facile detection of corresponding antibodies has been limited. Studies based on crude antigen preparations revealed that healthy individuals possess antibodies, elicited by exposure to commensal bacteria, environmental bacteria, and vaccinations (4, 16, 18, 24, 27, 29, 48), that cross-react with several *M. tuberculosis* antigens. During the last decade, several antigens of *M. tuberculosis* have been isolated and characterized (51). However, a majority of the antigens obtained to date (the 71-kDa DnaK, 65-kDa GroEL, 47-kDa elongation factor Tu, 44-kDa PstA homolog,

40-kDa L-alanine dehydrogenase, 38-kDa PhoS, 23-kDa superoxide dismutase, 23-kDa outer membrane protein, 12-kDa thioredoxin, and 14-kDa GroES) have been found to possess significant homology with analogous proteins in other mycobacterial and nonmycobacterial prokaryotes (1, 2, 7, 9, 22, 28, 42, 43, 50–52), and almost all individuals (healthy or diseased) have antibodies to epitopes on conserved regions of these antigens, resulting in the cross-reactivity observed with the crude antigen preparations (17, 23, 35, 45).

To overcome the hindrance created by cross-reactive antibodies, some of the purified antigens such as the 38-kDa PhoS, 30/31-kDa antigen 85, 19-kDa lipoprotein, 14-kDa GroES, and lipoarabinomannan (LAM) (13, 19, 31, 34, 39, 40, 46) have been tested. However, the choice of these antigens was determined primarily by their availability, their immunodominance in animal immune systems, or the ease of biochemical purification rather than by their reactivity in the human immune response. The 38-kDa antigen has provided the highest sensitivity and specificity (14, 25, 29) to date, but the presence of anti-38-kDa antibodies is associated with treated, advanced, and recurrent tuberculosis (6, 15, 34). None of the antigens studied to date has emerged as a suitable candidate for development of a diagnostic assay for early tuberculosis. Since antigens and epitopes recognized during natural infection and disease progression in humans may differ substantially from those recognized by animals upon artificial immunization (5, 8, 26, 33, 35, 47), a selection of antigens based on their ability to stimulate the human immune system is required.

In this study, a systematic analysis of the reactivity of sera from tuberculosis patients was undertaken to delineate the

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major targets of human antibody responses. We observed that initial immunoadsorption of the sera with *Escherichia coli* antigens successfully reduced interference by cross-reactive antibodies, thus allowing a new approach to serological studies. The immunoadsorbed sera allowed identification of a high-molecular-weight secreted antigen of *M. tuberculosis* which is recognized by antibodies in a large proportion of patients and during earlier stages of disease progression and which will therefore be useful for diagnosis of tuberculosis.

MATERIALS AND METHODS

Sera. The study population included 58 human immunodeficiency virus (HIV)-negative individuals with confirmed pulmonary tuberculosis. Of these, 16 were individuals attending the Infectious Disease Clinic at the Veterans Affairs Medical Center, New York, N.Y. All patients were *M. tuberculosis* culture positive, 9 of 16 patients were smear negative, 14 of 16 showed minimal to no radiological lesions, and all were bled either prior to or within 1 to 2 weeks of initiation of chemotherapy for tuberculosis. Eight serum samples were obtained from Leonid Heifitz and Lory Powell, Mycobacteriology Laboratory, National Jewish Center for Immunology and Respiratory Medicine, Denver, Colo. An additional 20 serum samples were provided by J. M. Phadtare, Pulmonary Disease Clinic, Grant Medical College, Bombay, India. Fourteen serum samples obtained from Lala Ram Sarup Tuberculosis Hospital, Mehrauli, New Delhi, India, were provided by S. Singh of the All India Medical Institute of Medical Sciences, New Delhi, India. A majority of these 42 patients were smear positive, had the radiological appearance of moderate to advanced pulmonary lesions, and were bled 4 to 24 weeks after initiation of chemotherapy.

The control populations consisted of the following groups. (i) The first group consisted of 16 HIV-negative, tuberculosis-negative, purified protein derivative (PPD) skin test-positive, healthy individuals. These individuals were either recent immigrants from countries of endemicity or involved in the care of tuberculosis patients in the Veterans Affairs Medical Center. (ii) Twenty-three HIV-negative, tuberculosis-negative healthy controls constituted the second group. Seven of these were PPD skin test negative, and the PPD reactivity of the remaining 16 individuals was unknown. (iii) The third group included 48 HIV-positive, asymptomatic healthy individuals with unknown PPD reactivity and with CD4 cells numbering >800. These individuals were included because tuberculosis has emerged as a major opportunistic disease in the HIV-infected population.

Antigens. The antigen preparations used in this study were total cellular sonicate (CS), total culture filtrate (CF), LAM-free CF proteins (LAM-free CFP), LAM, whole cell walls (CW), sodium dodecyl sulfate (SDS)-soluble cell wall proteins (SCWP), and cell wall core (CWC), all isolated from *M. tuberculosis* H37Rv.

CS was obtained from *M. tuberculosis* grown in Middlebrook 7H9 broth (Difco Laboratories, Detroit, Mich.) for 2 to 3 weeks. The bacilli were harvested by centrifugation at 1,000 rpm for 30 min, and the pellet was resuspended in phosphate-buffered saline (PBS) containing phenylmethylsulfonyl fluoride, EDTA, and dithiothreitol at a final concentration of 1 mM each. The suspension was frozen in liquid nitrogen and thawed (several times) to weaken the cell walls, after which the suspension was sonicated for 20 min at 4°C. The sonicate was centrifuged for 10 min at $150 \times g$, and the supernatant was collected.

To obtain the remaining antigens, *M. tuberculosis* was grown to the mid-log phase (14 days) in glycerol-alanine-salts medium. The cells were removed by filtration through a 0.22-μm-pore-size membrane, and the culture supernatant was concentrated by ultrafiltration with an Amicon (Beverly, Mass.) apparatus with a 10,000-molecular-weight cutoff membrane. The concentrated material (CF) was dialyzed against 100 mM ammonium bicarbonate and dried by lyophilization.

To obtain the LAM-free CFP, CF was suspended (7 mg/ml) in a buffer containing 50 mM Tris HCl (pH 7.4) and 150 mM NaCl, after which 20% Triton X-114 was added to obtain a final concentration of 4%. The suspension was allowed to rock overnight at 4°C. A biphasic partition was set up by warming the 4% Triton X-114 suspension to 37°C for 40 min, followed by centrifugation at $12,000 \times g$. The aqueous phase was reextracted twice with 4% Triton X-114 to ensure complete removal of the LAM, lipomannan (LM), and phosphotidylinositol-mannoside (PIM). The final aqueous phase was precipitated with 10 volumes of cold acetone, and the pellet was washed several times with cold acetone to remove residual Triton X-114. The LAM-free aqueous-phase CFP were suspended in 100 mM ammonium bicarbonate, aliquoted, and dried by lyophilization.

LAM, LM, and PIM were extracted from whole cells by mechanical lysis of the bacilli in PBS (pH 7.4) containing 4% Triton-X 114 in a Bead Beater (Biospec Products, Bartlesville, Okla.). Unbroken cells and cell wall material were removed by centrifugation at $12,000 \times g$, 4°C, for 15 min. The supernatant was collected, and a biphasic partition was set up. The detergent phase was obtained and back-extracted several times with cold PBS, and the macromolecules in the final detergent phase were precipitated with 10 volumes of cold acetone. The precipitate was collected by centrifugation and allowed to air dry. This material

(which contained the lipoglycans) was suspended in PBS, and residual proteins were removed by extraction with PBS-saturated phenol. The aqueous phase was collected, and after dialysis against distilled water, the lipoglycans were lyophilized. LAM was further separated from LM and PIM by size exclusion chromatography as described previously (11).

To isolate total CW, *M. tuberculosis* cells were inactivated by isothermal killing at 80°C for 1 h and suspended at a concentration of 0.5 g of cells/ml in a buffer containing PBS (pH 7.4), 4% Triton X-114, phenylmethylsulfonyl fluoride, pepstatin, EDTA, and DNase. The cells were disrupted in a Bead Beater with 0.1-mm-diameter zirconia beads. The lysed cells were first centrifuged at $3,000 \times g$ for 5 min to remove unbroken cells and then at $27,000 \times g$, 4°C, for 20 min. The resulting pellet was washed three times with cold PBS at room temperature. This final pellet was termed the CW.

The SCWP were obtained by washing the CW twice with 2% SDS in PBS (pH 7.4) at room temperature. The tightly associated proteins were isolated by extracting the CW pellet three times with 2% SDS in PBS (pH 7.4) at 55°C. The 55°C 2% SDS extract was recovered, and the SDS was removed by use of an Extracti-Gel column (Pierce, Rockford, Ill.). The eluent from the column was dialyzed against twice-distilled H_2O, aliquoted, and dried by lyophilization.

The CWC (mycolyl-arabinogalactan-peptidoglycan complex) was generated as described by Daffe et al. (12) with minor modifications. Briefly, the SDS-insoluble material obtained after extraction of the SCWP was suspended in PBS–1% SDS–0.1 mg of proteinase K per ml and incubated for 20 h at 50°C. The insoluble material was pelleted by centrifugation, washed twice with 2% SDS at 95°C for 1 h, and collected by centrifugation. This was washed several times with water and 80% acetone to remove SDS.

Fractionation of LAM-free CFP. Fractionation by size was performed with a preparative SDS-polyacrylamide gel electrophoresis (PAGE) system (model 491 prep cell; Bio-Rad, Hercules, Calif.). CFP (20 to 25 mg) was loaded directly onto a 30-ml 10% preparative polyacrylamide tube gel containing a 6% stacking gel, which was poured in a casting tube with a 37-mm internal diameter. The running buffer used consisted of 25 mM Tris (pH 8.3), 192 mM glycine, and 0.1% SDS. The proteins were separated by electrophoresis using an increasing wattage gradient of 8 W for 3.13 h, 12 W for 2.5 h, and finally 20 W for 11.1 h. Proteins were eluted from the bottom of the tube gel with a constant flow of 5 mM sodium phosphate (pH 6.8). The initial 65 ml of eluant was collected as the void volume, after which 80 4.2-ml fractions were collected at a rate of 0.4 ml/min. Individual fractions were assayed by one-dimensional SDS-PAGE and pooled accordingly. SDS was removed from the pooled concentrated fractions by elution through an Extracti-Gel (Pierce) column. The pooled fractions were dried and stored frozen until tested.

Adsorption of sera with *E. coli* sonicate. Overnight cultures of *E. coli* (Y1090) grown in Luria-Bertani medium were centrifuged to obtain bacterial pellets that were treated as described for the *M. tuberculosis* sonicate, except that sonication was performed for 30 s. Each well of an Immulon 2 enzyme-linked immunosorbent assay (ELISA) plate (Dynatech, Alexandria, Va.) was coated with 200 μl of *E. coli* lysate suspended at 500 μg/ml in 20 mM carbonate buffer (pH 9.6) overnight. The plates were washed and blocked with 5% bovine serum albumin (BSA); Sigma Chemical Co., St. Louis, Mo.) in PBS for 90 min. HIV was inactivated by the addition of Triton X-100 (1% final concentration) to each serum sample, followed by heating at 55°C for 60 min. Samples from non-HIV-infected individuals were treated in the same manner to maintain consistency in sample preparation. Serum from each individual (20 μl) was diluted to 200 μl in PBS-Tween 20 (0.05%) in a 96-well tissue culture plate. The diluted serum samples were transferred to the *E. coli*-coated, blocked ELISA plate with a multichannel pipetter. The serum samples were exposed to the bound *E. coli* antigens for 90 min, after which they were transferred to another ELISA plate that had been coated with *E. coli* and blocked as described above. The serum samples were exposed to eight cycles of adsorption against *E. coli* antigens, after which they were transferred to a 96-well tissue culture plate, where sodium azide (1 mM final concentration) was added to each well. This protocol allows rapid and efficient processing of small volumes of multiple samples. Adsorbed serum samples were used within 1 week.

ELISA with *M. tuberculosis* antigens. Fifty microliters of antigen, suspended at 5 μg/ml (except CS and SCWP, which were used at 15 and 1 μg/ml, respectively) in coating buffer, was allowed to bind overnight to wells of ELISA plates. After three washes with PBS, the wells were blocked with 7.5% fetal bovine serum (Hyclone, Logan, Utah) and 2.5% BSA in PBS for 2.5 h at 37°C. Following this, sera were diluted to a 1:1,000 final dilution in PBS-Tween 20 (0.05%) containing 1% fetal bovine serum and 0.25% BSA, and 50 μl of each serum sample was added to each well. The antigen-antibody binding was allowed to proceed for 90 min at 37°C, after which the plates were washed six times with PBS-Tween 20 (0.05%), and 50 μl of alkaline phosphatase-conjugated goat anti-human immunoglobulin G (IgG; Zymed Laboratories, South San Francisco, Calif.) diluted 1:2,000 in the same diluent as that of the serum samples was added to each well. After 60 min, the plates were washed six times with Tris-buffered saline (50 mM Tris, 150 mM NaCl) and the Gibco BRL Amplification System (Life Technologies, Gaithersburg, Md.) was used for development of color. The plates were read at 490 nm after stopping the reaction with 50 μl of 0.3 M H_2SO_4.

The optimal antigen and antibody concentrations for each antigen were determined by checkerboard titration with limited numbers of control and nontuberculosis sera prior to performing the ELISA with the total serum panel.
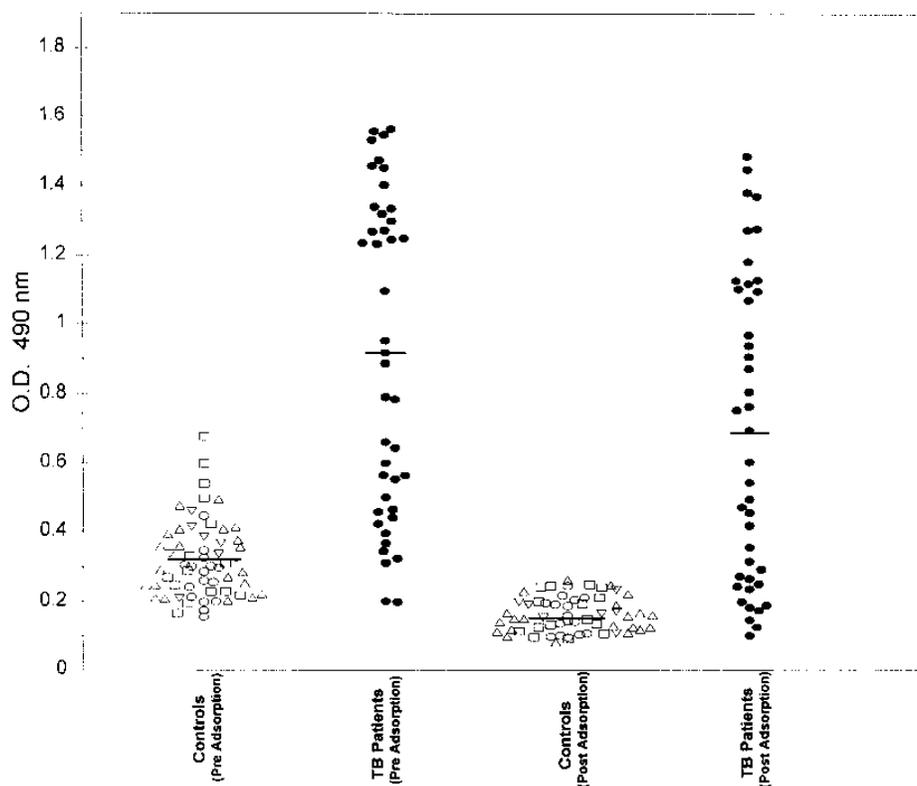
FIG. 1. Reactivity of sera from tuberculosis-negative, HIV-negative, and PPD-positive (○), tuberculosis-negative, HIV-negative, and PPD-negative (▽), and tuberculosis-negative, HIV-positive, asymptomatic (△) controls and from tuberculosis (TB) patients (●) with LAM-free CFP of *M. tuberculosis* H₃₇Rv before and after adsorption with *E. coli* lysate.

The ELISA with each of the sized fractions generated by preparative PAGE was performed as described as above, except that the coating antigen was used at a concentration of 2 μg/ml and the sera were tested at a final dilution of 1:200. Forty-two tuberculosis serum samples and 44 nontuberculosis controls (16 PPD-positive, 7 HIV-negative and PPD-negative, and 21 HIV-positive, asymptomatic individuals) were included in these ELISAs.

Characterization of known antigens of *M. tuberculosis* in the sized fractions of LAM-free CFP. The following monoclonal antibodies (MAbs) were obtained from the World Health Organization (courtesy of Thomas M. Shinnick, Centers for Disease Control, Atlanta, Ga.): IT-53, -45, -57, -42, -41, -56, -13, -64, -70, -43, -58, -46, -15, -23, -62, -47, -63, -49, -48, -59, -60, -44, -61, -52, -67, -68, -19, -51, -69, -4, -1, and -20, MLO4-A2, SAID2D, and CS-O1. The alternative names of the MAbs, the antigens they recognize, and the laboratories of origin were provided previously (20, 32, 51). Antiserum to the 50/55-kDa antigen, MPT32, was obtained from the National Institutes of Health (under contract 1-AI-25147) and provided by John T. Belisle.

The composition of the sized fractions was probed with the antibodies in an ELISA similar to what was used for assessment of reactivity with human sera, except that 50 μl of each of the antibodies defined above per well was used at a concentration recommended by the contributing laboratory. For these ELISAs, the second antibody was 50 μl of alkaline phosphatase-conjugated rabbit anti-mouse IgG or goat anti-rabbit IgG per well (1:2,000; Sigma).

SDS-PAGE and immunoblotting. All fractionations (LAM-free CFP and fractions thereof) were done on 10% SDS-polyacrylamide minigels, and the proteins were transferred onto nitrocellulose membranes before probing strips with the antibodies. To identify the antigens recognized by sera by ELISA in fraction 15, blots of total LAM-free CFP and fractions 10 and 15 were probed with (i) a pool of six tuberculosis serum samples that were positive for reactivity with LAM-free CFP by ELISA, (ii) a pool of six tuberculosis serum samples that were negative by ELISA, and (iii) a pool of six serum samples from PPD-positive healthy controls. All blots were screened for antibody binding by use of alkaline phosphatase-conjugated rabbit anti-human IgG and by subsequently developing the color reaction with BCIP-NBT (5-bromo-4-chloro-3-indolylphosphate toluidinium–nitroblue tetrazolium) substrate (Kirkegaard & Perry Laboratories, Gaithersburg, Md.).

Statistical analyses. The cutoff in all ELISAs was determined by use of the mean optical density (OD) plus 3 standard deviations (SD) of the control group. The Wilcoxon signed rank test for paired samples was used to compare preadsorbed and postadsorbed sera. The SD of these two groups were compared by use of the F test. The reactivity of tuberculosis sera with LAM-free CFP was compared with the reactivity with the other antigen preparations by the McNemar's paired test. The Graphpad Instat program was used for all statistical analyses.

RESULTS

Effect of adsorption of sera with *E. coli* lysate. The reactivities of sera from 38 HIV-negative nontuberculous individuals (16 positive, 7 negative, and 15 unknown for PPD reactivity), 21 HIV-infected asymptomatic individuals, and 42 tuberculosis patients with the LAM-free CFP were evaluated before and after depletion of cross-reactive antibodies by adsorption with *E. coli* lysate (Fig. 1). There was no difference in the reactivities of the different subgroups of the control sera. The OD (mean ± SD) of the unadsorbed control sera was 0.316 ± 0.111, and that of the same sera postadsorption was 0.165 ± 0.05 (Table 1). This reduction in reactivity was statistically significant ($P < 0.0001$). In addition, the SD of the control serum group postadsorption was significantly lower ($P < 0.0001$) when compared with the SD of the same serum preadsorption (Fig. 1 and Table 1). The OD (mean ± SD) for the preadsorbed tuberculosis sera was 0.911 ± 0.454, and the same sera postadsorption had an OD of 0.694 ± 0.440 (Fig. 1). Although the reactivity of the adsorbed tuberculosis sera was also reduced significantly as compared with that of preadsorbed sera ($P < 0.0001$), the SD of the preadsorbed and postadsorbed tuberculosis serum groups were similar (Table 1). Thus, significant levels of cross-reactive antibodies that could be removed by adsorption against *E. coli* lysate were present in both the control and test sera. For the control group, removal of these antibodies reduced the baseline serum reac-

TABLE 1. Comparison of preadsorbed sera with *E. coli* adsorbed sera

Source of sera	OD (mean ± SD)		Wilcoxon test *P* value[a]	F test *P* value[b]
	Preadsorption	Postadsorption		
Controls	0.316 ± 0.111	0.165 ± 0.050	<0.0001	<0.001
Tuberculosis patients	0.911 ± 0.454	0.694 ± 0.440	<0.0001	NS[c]

[a] Wilcoxon signed rank test comparing the preadsorbed and postadsorbed sera.

[b] F test comparing the standard deviations of the preadsorbed and postadsorbed sera.

[c] NS, not significant.

tivity. However, as expected, despite the decreased antibody levels, the variability between individual tuberculosis sera was unaffected. By using the mean OD ± 3 SD of the respective control sera as cutoff values, antibodies reactive to LAM-free CFP were detectable in 25 of 42 (60%) of the unadsorbed tuberculosis serum samples (Fig. 1). When tested postadsorption, antimycobacterial antibodies became detectable in 4 of 17 (24%) additional, previously negative serum samples, raising the sensitivity to 69% (Fig. 1).

These experiments were also analyzed by using the highest OD in the control serum group as the cutoff (29), as was done by other investigators. Prior to adsorption, ODs obtained with 59 control serum samples ranged from 0.16 to 0.68 (Fig. 1). Twenty-four of the 42 (57%) tuberculosis serum samples had ODs greater than the highest control value. After adsorption, the range of ODs with the same control serum samples was 0.08 to 0.25, and 31 of 42 (74%) tuberculosis serum samples were found to be antibody positive. Thus, antibodies to *M. tuberculosis* antigens were now detectable in 7 of 18 (39%) additional, previously negative serum samples. In view of the increased sensitivity obtained with adsorbed sera, all sera were hereafter preadsorbed prior to use in any assay.

Reactivity of the adsorbed sera with different antigenic preparations of *M. tuberculosis*. The reactivity of sera from 87 nontuberculosis controls and 58 tuberculosis patients with different antigen preparations of *M. tuberculosis* were analyzed (Table 2). With the total CF preparation, which contains all the secreted antigens (proteins and nonproteins), 39 of 58 (67%) of the serum samples from tuberculosis patients had detectable antibodies, while 2 of 87 control serum samples were positive. With the LAM-free CFP, 41 of 58 (71%) of the tuberculosis serum samples were antibody positive and none of the 87 control serum samples were reactive. Thirty-six tuberculosis patients (62%) had antibodies to the CS, as had 2 of 87 of the control subjects. CW of *M. tuberculosis* were tested with sera from 48 tuberculosis patients and 54 nontuberculosis con-

TABLE 2. Reactivity of sera with antigens of *M. tuberculosis*

Antigen	% Sensitivity (*n*)	% Specificity (*n*)	*P* value[a]
CF	67 (58)	98 (87)	NS
LAM-free CFP	71 (58)	100 (87)	
CS	62 (58)	98 (87)	NS
CW	58 (48)	99 (54)	NS
LAM	55 (58)	98 (87)	0.039
SDS-cell wall proteins	52 (58)	99 (87)	0.015
CWC	8.6 (58)	100 (87)	<0.0001

[a] *P* value obtained by McNemar's paired test to compare the reactivity of tuberculosis sera with LAM-free CFP with the reactivity with other antigens. NS, not significant.
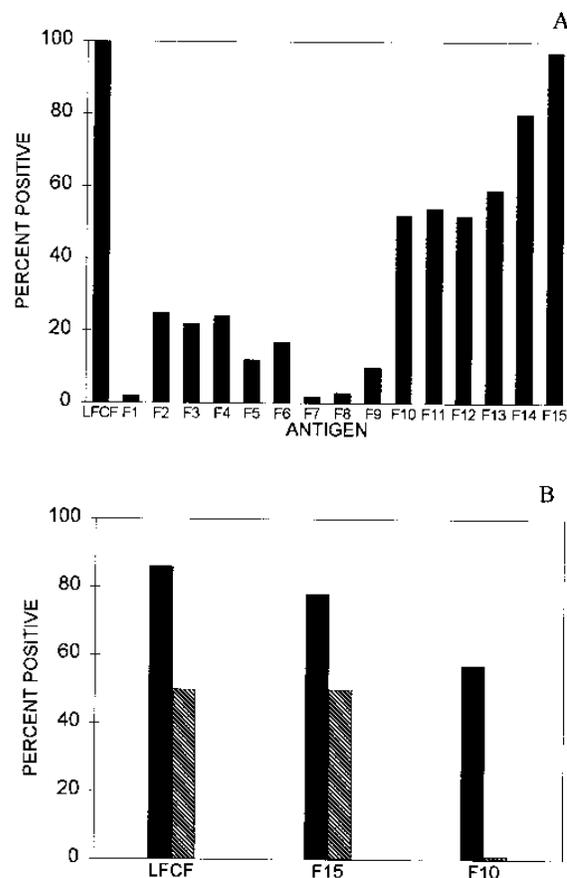

FIG. 2. (A) Reactivity with fractions of LAM-free CFP of sera of tuberculosis patients which were reactive with total LAM-free CFP. (B) Comparison of reactivity of advanced, partially treated (■) and early, minimally treated (□) tuberculosis patients with LAM-free CFP (LFCF) and fractions 10 and 15. F, fraction.

trols, and 28 of 48 (58%) tuberculosis patients were antibody positive, when only 1 of 54 controls had antibodies to this antigen. The reactivities of the tuberculosis sera with the CF, CS, and CW preparations was not significantly different from the reactivity with LAM-free CFP (Table 2). With SCWP, only 52% (30 of 58) tuberculosis patients were antibody positive, although 99% of the control subjects lacked antibodies. Fifty-five percent (32 of 58) of tuberculosis patients had antibodies to LAM, while only two of the controls were positive. Antibodies to the CWC were detectable in only 8.6% of the patients. Reactivity of tuberculosis sera with the SCWP, LAM, and CWC antigen preparations was significantly lower than the reactivity with the LAM-free CFP preparation (Table 2). Since the highest sensitivity and specificity were obtained with the LAM-free CFP, it was used for all further analysis.

Seroreactivity to fractions of LAM-free CFP. To narrow the search for the serologically dominant antigens in LAM-free CFP, reactivity of sera from 42 tuberculosis patients and 44 healthy control subjects was tested with the sized fractions. Seventy-two percent (30 of 42) of the tuberculosis serum samples had antibodies to the unfractionated LAM-free CFP, while none of the controls was positive (Table 3). The serum samples that showed positive reactivity with the total LAM-free CFP were compared with those showing reactivity with the 15 fractions (Fig. 2A). Less than 25% of the patients who were reactive with the total LAM-free CFP showed reactivity with

TABLE 3. Reactivity of fractions of LAM-free CFP

Fraction no.	Reactive antibodies[a]	Reactive patients (%)
10	IT-62, IT-23	33
11	IT-62, IT-23	37
12	Anti-MPT32	36
13	Anti-MPT32	42
14	IT-41	55
15	IT-41, IT-57	72

[a] Murine MAbs include IT-62 and IT-23 (anti-38 kDa), IT-41 (anti-71 kDa), and IT-57 (anti-82 kDa). Anti-MPT32 antiserum was raised in rabbits.
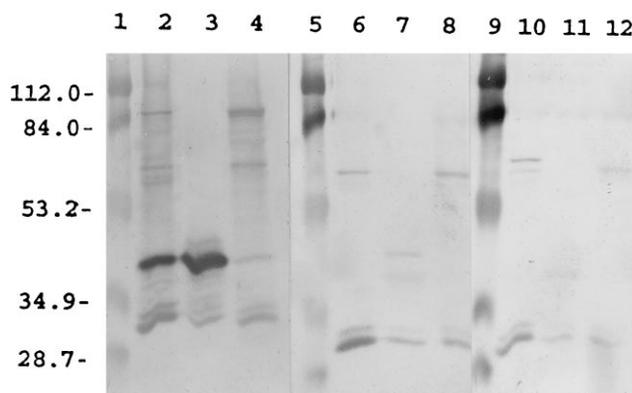

FIG. 3. Immunoblot analysis of total LAM-free CFP and fractions 10 and 15 (see Materials and Methods). Lanes: 1, 5, and 9, molecular mass markers; 2, 6, and 10, LAM-free CFP; 3, 7, and 11, fraction 10; 4, 8, and 12, fraction 15. Blots with lanes 1 to 4, 5 to 8, and 9 to 12 were probed with pools of sera (1:200) from ELISA-positive tuberculosis patients, ELISA-negative tuberculosis patients, and PPD-positive healthy controls, respectively. Numbers on the left are molecular masses in kilodaltons.

antigens in fractions 1 to 9. In contrast, 50 to 60% of the serum samples were reactive with antigens in fractions 10 to 13, 80% were reactive with antigens in fraction 14, and 96% were reactive with antigens in fraction 15 (Fig. 2A). Combinations of fractions 14 and 15, 12 and 13, 10 and 14, and 10, 14, and 15 failed to show any improvement over the use of fraction 15 alone (data not shown). None of the serum samples that failed to react with the total LAM-free CFP were reactive with any of the fractions.

Characterization of antigens in sized fractions of LAM-free CFP with murine MAbs. To determine which of the previously defined proteins were present in the seroreactive fractions, reactivity of sized fractions with 36 murine MAbs and with anti-MPT32 antiserum was assessed by ELISA. The results with fractions 10 to 15 are shown (Table 3). Murine MAbs IT-62 and IT-23, both of which recognize epitopes on the 38-kDa protein, reacted exclusively with fractions 10 and 11. Fractions 12 and 13 were reactive only with the rabbit antiserum to MPT32. Fraction 14 reacted with MAb IT-41, which recognizes an epitope on the 71-kDa DnaK protein. Fraction 15 showed reactivity with MAbs IT-41 and IT-57; the latter MAb reacts with an 82-kDa antigen (Table 3).

Comparison of reactivities of advanced and early tuberculosis patients. In view of the reported association of the anti-38-kDa antibodies with advanced and treated tuberculosis, and because no two cohorts of patients can be identical, the reactivity of sera from treated, relatively advanced tuberculosis patients (sera from Bombay, India, and Denver, Colo. [see Materials and Methods]) and sera from untreated (or minimally treated) early tuberculosis patients (from Veterans Affairs Medical Center, New York, N.Y. [see Materials and Methods]) in our cohort were compared. Reactivities of the two groups of patients with LAM-free CFP, fraction 10 (which contains the 38 kDa antigen), and fraction 15 are shown in Fig. 2B. Eighty-two percent (23 of 28) of the advanced and 50% (7 of 14) of the early tuberculosis patients had antibodies to the total LAM-free CFP. Serum samples from all but one of the advanced tuberculosis patients (22 of 28) and all 50% of the early tuberculosis patients that were reactive with the LAM-free CFP were also reactive with antigens in fraction 15. In contrast, although 57% (16 of 28) of the advanced tuberculosis patients were reactive with fraction 10, none of the serum samples from untreated patients with relatively early disease was reactive.

Immunoblot analyses of fractions. Since each of the fractions can be expected to contain proteins besides those that were identified by the murine MAbs, LAM-free CFP, fraction 10, and fraction 15 were fractionated by SDS-PAGE, immunoblotted, and probed with a serum pool from six tuberculosis patients who were ELISA positive with the LAM-free CFP (Fig. 3, lanes 2 to 4). Serum pools from six ELISA-negative tuberculosis patients (lanes 5 to 8) and six healthy controls

(lanes 10 to 12) were tested as negative controls. Proteins of 65- and 30-31 kDa in the LAM-free CFP and in the two fractions were reactive with all three serum pools. The antibody-positive tuberculosis serum pool recognized at least 10 additional distinct bands in the fractionated total LAM-free CFP (Fig. 3, lane 2). The molecular masses of the antigens ranged from 33 to 112 kDa. The 38-kDa antigen was the thickest band to be seen, showing that it is present in large amounts in the LAM-free CFP. Since it is a strongly seroreactive antigen in several patients (Fig. 2B), the 38-kDa antigen appeared as a prominent band. Another dark band was observed at 88 kDa, but this antigen is present in smaller amounts in the LAM-free CFP, and the band was much thinner than that of the 38-kDa antigen. Weaker reactivity with antigens of 33, 36, 58, 60, 62, 70, and 84 kDa was also observed. Fraction 10 contained large amounts of the 38-kDa antigen, which was the strongest band, and relatively smaller amounts of other seroreactive proteins ranging from 30 to 43 kDa. In contrast, fraction 15 contained several high-molecular-mass antigens ranging from 72 to 88 kDa and a small amount of the 38-kDa antigen (which was not detected by anti-38-kDa MAbs) (Table 3). Strong seroreactivity with a doublet at 88/84 kDa and weaker reactivity with 78- and 72-kDa antigens were found. Sera from tuberculosis patients who were ELISA negative with fraction 15 (Fig. 3, lanes 6 to 8) or from healthy controls (Fig. 3, lanes 10 to 12) showed no reactivity with the 88/84-kDa antigens. Since the 72- to 88-kDa antigens are absent in fraction 10, the reactivity of ELISA-positive tuberculosis sera with antigens in fraction 15 must be directed towards these antigens. That the 88-kDa antigen is strongly reactive in fraction 15 and in the total LAM-free CFP suggests that this antigen may be responsible for the reactivity with individual serum samples observed in the ELISA.

DISCUSSION

The reactivity of sera from normal healthy individuals (14, 24) to antigens of *M. tuberculosis* has been a major hindrance in the direct analysis of the humoral immune responses in tuberculosis patients. Several studies (16, 21, 48) have reported that sera from control individuals recognize several antigens of *M. tuberculosis*. Since most proteins of *M. tuberculosis* isolated to date possess significant homology with analogous proteins

(1, 2, 7, 9, 22, 28, 42, 43, 51, 52) in other prokaryotes, we reasoned that the reduction of cross-reactive antibodies to these proteins may enrich for antibodies to mycobacterium-specific antigens and mycobacterium-specific epitopes of conserved proteins (17, 35, 45) and thus allow recognition of antigens with strongly seroreactive determinants. The choice of *E. coli* lysates for this purpose was based on its being a commensal organism, known to possess many conserved bacterial proteins. The current work demonstrates that when specificities of 98 to 100% were maintained with all the different antigen preparations of *M. tuberculosis* tested, the LAM-depleted CFP provided the highest sensitivity, although the difference in reactivity with CS, CF, and CW was not statistically significant. Since LAM migrates as a broad band in the 30- to 40-kDa region on gels, antibodies reactive to LAM would obscure the other antigens in this region on immunoblots. The major components of cell walls, i.e., the core, SCWP, and LAM, showed reduced reactivity when tested as individual preparations, and they did not react with any tuberculosis serum that was not reactive with the LAM-free CFP. For these reasons, we proceeded with this preparation for our studies.

Since the LAM-free CFP contains over a hundred different secreted proteins (44, 49), most of which are as yet undefined, and since we wanted to determine which of these proteins were the most frequent targets of the humoral immune response in tuberculosis patients, reactivity of sera with size-fractionated antigens was assessed. The reactivity of tuberculosis sera with antigens in fraction 15 suggested that a high-molecular-mass secreted antigen(s) of *M. tuberculosis* elicits antibodies in a majority of tuberculosis patients. Since fraction 15 contained antigens reactive with murine MAbs IT-41 and IT-57, the antibodies in the sera of tuberculosis patients could be directed against either these antigens or other as-yet-undefined, high-molecular-mass antigens. Screening of a lambda gt11 expression library with these two MAbs yielded clones that produced recombinant 71- and 82-kDa proteins; however, neither antigen showed significant reactivity with the tuberculosis patient sera (data not shown). Immunoblot analyses revealed that the main seroreactive antigens in fraction 15, which were absent in fraction 10, had molecular masses of 88 and 84 kDa. The dominant reactivity of the 88-kDa protein with the antibody-positive tuberculosis serum pool, in both the LAM-free CFP preparation and fraction 15, suggests that this antigen may be responsible for the strong antigenicity observed with the latter. No reactivity of IT-62 and IT-23 was seen with fraction 15, and the faint 38-kDa band observed in the blot is probably a degradation product of the higher-molecular-mass antigens.

Although the 38-kDa PhoS protein has provided the best sensitivities and specificities for serodiagnosis of tuberculosis to date, the presence of anti-38-kDa antigen antibodies has been shown to correlate with the extent of pulmonary disease and antituberculosis therapy (30, 34). Several studies (6, 10, 13, 15, 21, 34, 47) in different populations (from China, Bolivia, Argentina, and South and North America) showed that the sensitivity with the 38-kDa antigen ranged from 45 to 90%, being higher in populations where more patients present with advanced disease.

Our results with the 38-kDa antigen are similar to those of other investigators (21, 47) in that about 60% of the patients with advanced tuberculosis had anti-38-kDa antibodies. None of the patients with minimal disease was reactive with the 38-kDa antigen. However, reactivity with antigens in fraction 15 was obtained in 82% of the advanced and 50% of the early tuberculosis patients, even though these antigens were present in much smaller amounts in the LAM-free CFP. Thus, antibodies to the antigens in this fraction are detectable both

earlier and more frequently during the course of active tuberculosis than antibodies to the 38-kDa antigen are (Fig. 2B), suggesting that it is more commonly immunogenic in the patients.

Using a similar approach, but with unadsorbed sera, Verbon et al. (47, 48) reported that 29, 50, and 50% of tuberculosis serum samples react with antigens of 12, 16, and 24 kDa, respectively. Based on reactivity with MAbs (data not shown), these antigens would be present in fractions 2 through 6 in the present study. However, less than 20% of tuberculosis serum samples showed reactivity with these fractions. Since the 12- and 16-kDa proteins are heat shock proteins, antibodies to conserved regions of these antigens would be depleted in our sera. Besides, antibodies in human sera are directed towards conformational epitopes on these proteins (47), which may have been destroyed during the fractionation procedure. Either or both of these factors would contribute to the decreased reactivity observed with the lower-molecular-mass antigens in this study.

Comparative studies with recombinant 38- and 12-kDa antigens and the corresponding native proteins obtained from cultures of *M. tuberculosis* show that human sera have poor reactivity with the former (47). In addition, reactivity of human sera with overlapping peptides of 12 and 16 kDa was 20 to 50% lower than the reactivity with the native antigens (47). These and other studies on reactivity of human and murine sera with antigens of *M. tuberculosis* suggest that in contrast to the murine antibodies, human antibodies elicited during natural disease progression recognize glycosylated, conformational epitopes (41) on the native proteins. Currently, experiments aimed at isolating and obtaining purified 88-kDa antigen are under way so that disease-associated seroreactive epitopes on this antigen may be defined to allow achievement of higher sensitivity. Also, the use of purified antigen and epitopes will obviate the requirement of adsorbing the sera for obtaining high sensitivities. Whether immune complexes containing the 88-kDa antigen exist and can be detected in the sera of patients who lack these antibodies remains to be tested. Interestingly, recent studies by Raja et al. (37) showed that immune complexes in the sera of smear-negative tuberculosis patients, and not healthy controls, contained antigens of >70 kDa.

This study illustrates that a direct analysis of the humoral immune responses will help identify new antigens that were not found to be dominant when murine MAbs were raised (20, 32) and that have not been described to date. It also shows that depletion of antibodies to cross-reactive regions on common bacterial proteins enables recognition of antigens with strong seroreactive determinants. The use of these antigens, selected on the basis of their reactivity with the human immune system during active disease progression, may prove useful for development of serodiagnostic assays for tuberculosis.

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