INFECTION AND IMMUNITY, July 1991, p. 2265–2273
0019-9567/91/072265-09$02.00/0

Vol. 59, No. 7

Identification of B- and T-Cell Epitopes within the MTP40 Protein of *Mycobacterium tuberculosis* and Their Correlation with the Disease Course†

JUAN C. FALLA, CARLOS A. PARRA,* MARCELA MENDOZA, LINA C. FRANCO, FANNY GUZMÁN, JAVIER FORERO, OSCAR OROZCO, AND MANUEL E. PATARROYO

Instituto de Inmunología, Hospital San Juan de Dios, Carrera 10 Calle 1, Bogotá, Colombia

Received 25 March 1991/Accepted 11 April 1991

Synthetic peptides derived from the amino acid sequence of MTP40, a recently characterized *Mycobacterium tuberculosis* protein, were tested by two different immunological assays in 91 individuals. For the purposes of this study, the population was distributed in four groups: active tuberculosis (TBC) patients with elevated bacillus loads (BK+), active TBC patients with low bacillus loads (BK−), healthy individuals living in the same household with tuberculous patients (HH), and normal individuals, who had presumably never been in contact with the bacilli (control). We found that T cells of individuals belonging to the HH group showed the highest and most frequent recognition of these peptides in a T-cell proliferation assay, while their antibodies showed the lowest recognition of these peptides when tested by enzyme-linked immunosorbent assay. In contrast, TBC patients revealed an inverse pattern of immune response. Interestingly, one of these peptides (P7) was recognized by T cells of 64% of the HH individuals and by 4.5% of normal donors. Another peptide (P4) was recognized by 55% of sera from BK+ patients and by 5.5% of normal donors. The results presented here indicate the existence of T- and B-cell epitopes within the MTP40 protein. Given the particular recognition pattern of this protein, added to the fact that it appears to be a species-specific antigen of *M. tuberculosis*, a detailed study of the immune response to it may be useful in the design of more accurate diagnostic tests and an improved vaccine against human TBC.

Tuberculosis is a chronic infectious disease caused, in the human host, by *Mycobacterium tuberculosis*, *M. bovis*, and *M. africanum*. The complex antigenic composition of mycobacteria and the presence of cross-reactive epitopes throughout the group (38) has made it difficult to develop efficient immunological methods for diagnosis. Although the tuberculin test has been helpful in roughly defining groups of tuberculous patients (42), more sensitive and specific assays that provide reliable diagnostic results are needed. On the other hand, the limited success of *M. bovis* BCG vaccination in developing countries has brought into doubt its effectiveness in controlling the disease (34, 43). For these reasons, new approaches are required to precisely identify epitopes within immunologically important antigens which might be useful for the diagnosis of and protection from the disease (3).

The development of serological methods for diagnosing tuberculosis has been the object of study of several groups (12). Tests based on the enzyme-linked immunosorbent assay (ELISA) and radioimmunoassays with purified protein derivative (PPD) have been developed, but the results have been disappointing (18, 39). Daniel and coworkers have performed ELISAs with antigen 5 (8, 33), which, although a well-characterized protein, is still not as specific as required for these purposes.

Although the use of antibodies in diagnosis has been attempted (8), little is known concerning their role in protection against tuberculosis. While many reports indicate that they are not important components mediating protection (7, 10), others have proposed that an antibody-mediated mechanism could play an important role in protection during the early stages of the bacillary infection (9). Several B-cell epitopes have been recently identified in mycobacterial antigens (1, 17, 40, 41, 44), but their roles in protective immunity remain to be demonstrated.

On the other hand, acquired resistance to mycobacterial infections has long been assumed to be mediated by T cells (13, 19, 20). Therefore, characterization and identification of *M. tuberculosis* antigens recognized by human T cells is required. As an important step towards this goal, mycobacterial antigens have been cloned by workers in several laboratories (2, 5, 11, 35), and recombinant proteins have been expressed and studied regarding their ability to induce T-cell activation, both in vivo and in vitro (21–24, 26–28, 32, 45). Although the available data suggest that most of these proteins are immunodominant targets in the response against mycobacteria (46), the fact that several of these antigens belong to the family of stress proteins (11, 35, 36) creates doubts about their possible usefulness.

Since BCG vaccination has failed to protect against the disease, our efforts have been directed towards the identification of specific epitopes expressed by *M. tuberculosis* (29) which could possess particular relevance in diagnosis and protection. Recently, one of these antigens was cloned in our laboratory from a genomic library by using a polyclonal antiserum. This 14-kDa protein was designated MTP40. Immunological and DNA hybridization studies suggest that this is a protein codified by a species-specific gene, exclusively present in *M. tuberculosis* and absent in *M. bovis* and *M. bovis* BCG, with no known homology to other reported mycobacterial antigens, either at the DNA or protein level (28a). In this study, we describe the cellular and humoral immune responses to synthetic peptides derived from the

* Corresponding author.

† This article is dedicated to the memory of Juan C. Falla.

MTP40 protein, by using peripheral blood lymphocytes (PBL) and sera from active tuberculosis patients (BK+ and BK−), healthy individuals living in the same household with tuberculosis patients (HH), and normal donors (control). The results of these studies clearly indicate the presence of T- and B-cell epitopes within the MTP40 protein. The implications of these findings for the development of a novel generation of diagnostic and immunoprophylactic methods against tuberculosis are discussed.

MATERIALS AND METHODS

Subjects. A group of 27 patients (age range, 35 to 60 years) who had been diagnosed as suffering from active tuberculosis and undergoing a 6-month chemotherapy schedule were selected. Of these individuals, 25 were in the first week of antibiotic therapy (rifampin, streptomycin, isoniazide, and pirazinamide) and were still bacteriologically positive upon sputum examination (BK+). Pulmonary tuberculosis had been clinically and bacteriologically diagnosed in 23 of them, while ganglionar and renal tuberculosis had been diagnosed by biopsy in the other 2. The last two patients of this group had not responded to treatment, and were still BK+ despite being in the third and fourth months of chemotherapy. This group will be referred to as BK+. Another group of 17 active tuberculosis patients was included in the study. All of these individuals had been BK+ at the time of diagnosis but had since become nonbacilliferous and negative on sputum examination (BK−) after drug treatment. All of these patients were in their third to sixth month of chemotherapy at the time of this study. This group will be referred to as BK−. All the samples from tuberculous patients (either BK+ or BK−) were obtained from ambulatory patients at the Neumology Service of the Hospital Santa Clara and Hospital San Juan de Dios in Bogotá, Colombia, with previous consent from the patients. The third group was composed of 25 healthy individuals who had been living in the same household with active tuberculous patients for at least 1 year after the patients developed the clinical disease (HH). The last group was composed of 22 healthy donors, volunteer first-year medical students from the Universidad Nacional de Colombia who presumably had never been in contact with the disease. Of these individuals, 11 had been vaccinated with BCG.

Peptide synthesis. Ten peptides were synthesized at the Instituto de Inmunología by using the solid-phase peptide synthesis method described by Merrifield (25) and the simultaneous multiple-solid-phase peptide synthesis method described by Houghten (15). The amino acid sequences of the synthesized peptides are shown in Table 1 in single-letter codes.

TABLE 1. Amino acid sequences of synthetic peptides derived from the MTP40 protein sequence

Peptide	Residue[a]	Sequence[b]
P1	1–16	MLGNAPSVVPNTTLGM
P2	13–28	TTLGMHCGSFGSAPSNG
P3	29–49	WLKLGLVEFGGVAKLNAEVMS
P4	59–74	MLGTGTPNRARINFNC
P5	70–90	INFNCEVWSNVSETISGPRLY
P6	86–106	GPRLYGEMTMQGTRKPRPSGP
P7	115–134	ASMLGTVTNSPGVPAVPWGA
P8[c]	17–34	HCGSFGSAPSNGWLKLGL
P9[c]	48–66	MSPTTPSRQAVMLGTGTPN
P10[c]	102–122	RPSGPRMPPDPGTASMLGTVT

[a] Position of each peptide within the MTP40 protein.
[b] Single-letter code.
[c] Not used in the present study.

$H_{37}R_v$ sonic extract. *M. tuberculosis* $H_{37}R_v$ (TMC-102; Trudeau Mycobacterial Collection) was grown on Sauton medium and harvested after complete growth. The bacilli were sonicated at 0°C for 15 min, followed by a 5-min interval, and the procedure was repeated four times. The sonic extract was centrifugated at 150,000 × *g* for 1 h at 4°C. The supernatant was removed, and the protein concentration was determined by the Lowry assay. This material was stored in aliquots at −70°C until needed.

Serological tests. Sera were assayed by ELISA according to the following procedure: four 96 high-binding-capacity microwell modules (no. 4-69914; NUNC, Roskilde, Denmark) were coated with each peptide. A total of 150 μl per well of a solution of 10 mg of each peptide per ml in coating buffer ($NaHCO_3$– 0.1 M Na_2CO_3, pH 9.2) was left for 1 h at 37°C, then for 48 h at 4°C, and finally for 1 h at 37°C. Control wells were coated with buffer devoid of peptide under the same conditions. The plates were washed twice with phosphate-buffered saline–0.05% Tween-20 (PBST), and 100 μl of each serum diluted 1:20 in PBST with 1% goat serum as a blocking agent (PBST-GS) was added per well. Sera were incubated for 1 h at 37°C and washed five times with PBST. After adding 100 μl of anti-human immunoglobulin G peroxidase conjugate (Sigma no. A-8785) per well, diluted 1:1,000

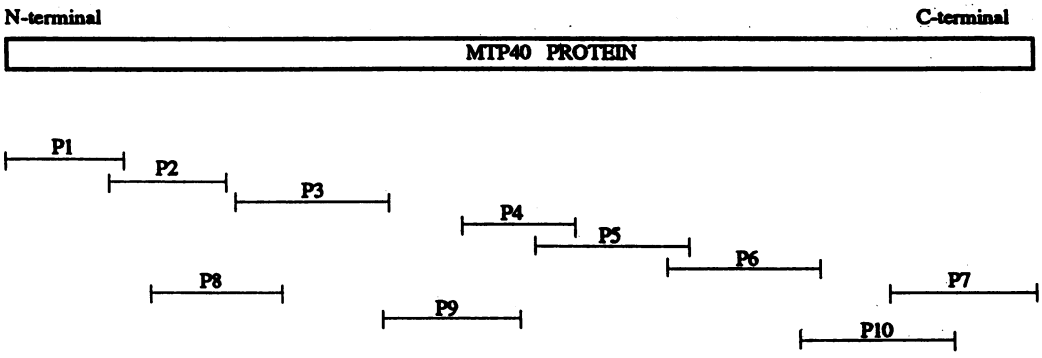

FIG. 1. Schematic representation of the MTP40-derived synthetic peptides. The sequences of the peptides used in this study were taken from the deduced amino acid sequence of the *mtp40* gene. Peptides P1 to P7 were used in both the ELISAs and the lymphoproliferative assay, while peptides P8, P9, and P10 were not used.

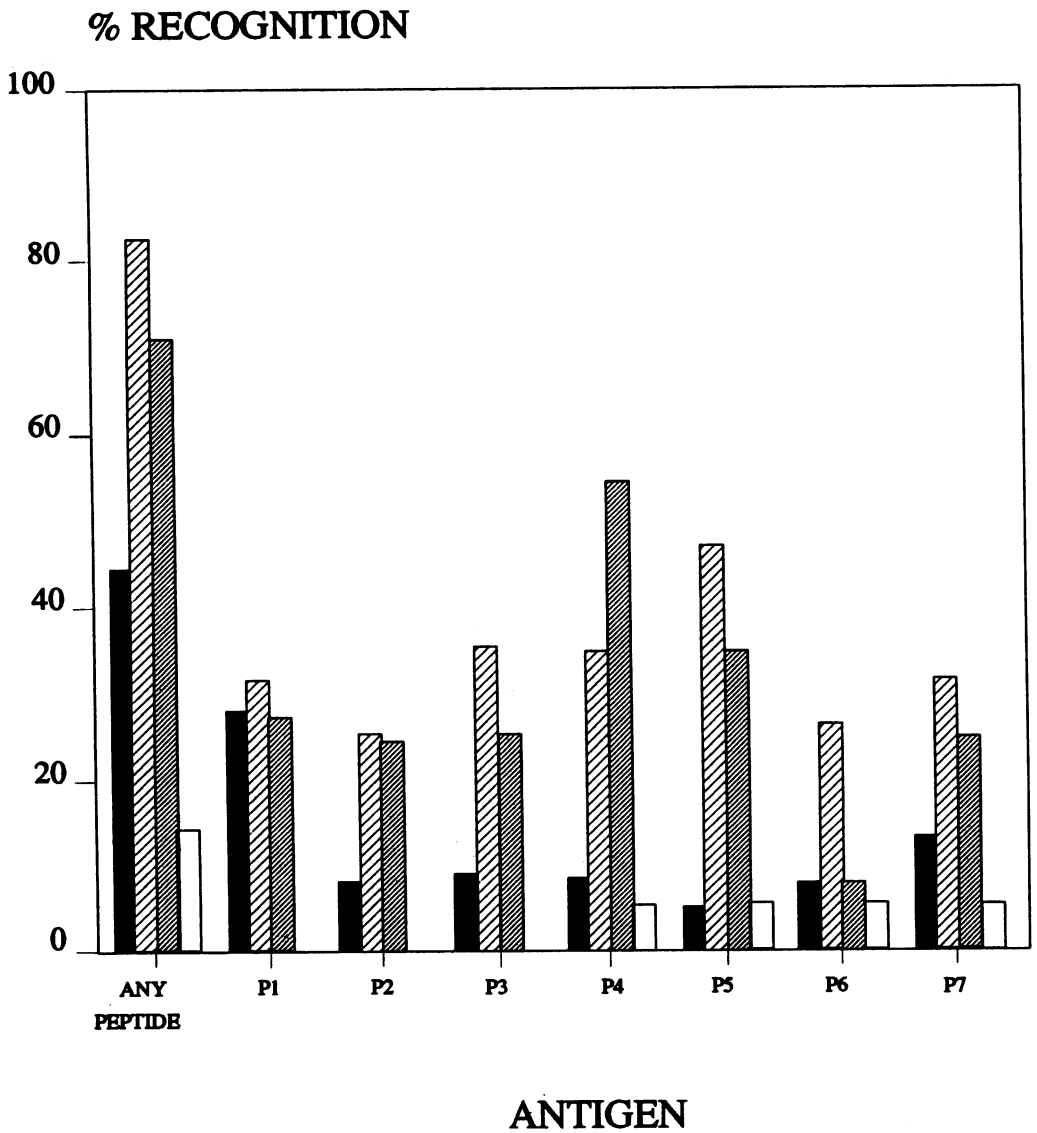

FIG. 2. Recognition of MTP40-derived peptides by sera of the four groups of studied individuals, as assessed by ELISA. Bars represent the degree of recognition in terms of the percentage of responders within each particular group. Patient symbols: ■, HH; ▨, BK+; ▨, BK−; □, normal. The percentage of individuals within each group who recognized at least one of the seven peptides is also shown (any peptide).

(vol/vol) in PBST-GS, the plates were incubated for 1 h at 37°C. The plates were then washed five times with PBST, and 100 μl of substrate solution (25 mg of OPD [*o*-phenyldiamine] and 30 μl of H_2O_2 per 10 ml of citrate phosphate buffer, pH 5.0) per well was added. The reaction was performed at room temperature in the dark for 5 min and stopped by the addition of 50 μl of 2 N sulfuric acid per well. Finally, the plates were read in an ELISA plate reader at a wavelength of 492 nm. Sera giving an optical density 2 standard deviations above the mean of normal donor sera were considered positive.

Lymphocyte proliferation assay. PBL were isolated from heparinized whole blood by Ficoll-Hypaque centrifugation (Sigma, Poole, United Kingdom) and resuspended in RPMI 1640 culture medium (Flow Laboratories) containing 10% of fetal calf serum, 2 mM L-glutamine, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 100 IU of penicillin per ml, and 40 μg of gentamicin per ml. Cells (1.5×10^5) per well were then seeded in 96-well flat-bottom microtiter plates with different antigen concentrations for 5 days at 37°C in humidified air with 5% CO_2. The cultures were then pulsed with 0.8 μCi of [*methyl*-^{3}H]thymidine (Amersham International, Amersham, United Kingdom) per well. Approximately 16 h later, the cells were harvested onto glass fiber filter strips, and [^{3}H]thymidine incorporation was measured in a liquid scintillation apparatus (Beckman L-9000). Cultures were performed in triplicate, and the numbers of counts per minute were converted into stimulation indices (SI). Lymphocyte proliferation to antigen was considered positive when the values were 2 standard deviations above the mean values obtained for the 22 normal donors.

Statistical analysis. The Kolmogorov-Smirnov procedure was used to test the normal distribution of humoral and cellular responses. An analysis of variance was performed to examine significant differences for each of the peptides

TABLE 2. Analysis of the humoral immune response among the different study groups

Group	Mean OD_{492} with antigen[a]						
	P1	P2	P3	P4	P5	P6	P7
Control	0.202	0.145	0.225	0.320	0.205	0.277	0.134
BK+	0.274	0.162	0.309	0.457	0.289	0.292	0.176
BK−	0.302	0.199	0.380	0.404	0.416	0.341	0.228
HH	0.259	0.143	0.190	0.384	0.175	0.269	0.147

[a] OD_{492}, optical density at 492 nm. The statistical significance values of the analysis of variance comparing the four groups were 0.3378 (P1), 0.1916 (P2), 0.0002 (P3), 0.0002 (P4), 0.0001 (P5), 0.2557 (P6), and 0.0352 (P7). For peptides P3, P4, and P5, the following pairs of groups were significantly different ($P = 0.05$): BK+/HH, BK−/HH, and BK−/Control (P3); BK+/HH and BK+/Control (P4); and BK−/HH and BK−/Control (P5).

among the studied groups. The proportions of positive humoral and cellular responses (2 standard deviations above the mean for the control group) for three of the four groups studied (BK+, BK−, and HH) were compared through the proportions difference test.

RESULTS

Peptide synthesis. Ten different peptides (P1 to P10), derived from the amino acid sequence of the MTP40 protein, were initially synthesized to perform epitope mapping experiments on this protein (Fig. 1). In order to use the minimum number of different peptides that together could span most of the length of the entire protein, three of these (P8 to P10), which significantly overlap with other peptides, were not included in the present study. It is important that, in choosing the peptide sequences (Table 1), no algorithm prediction was taken into account.

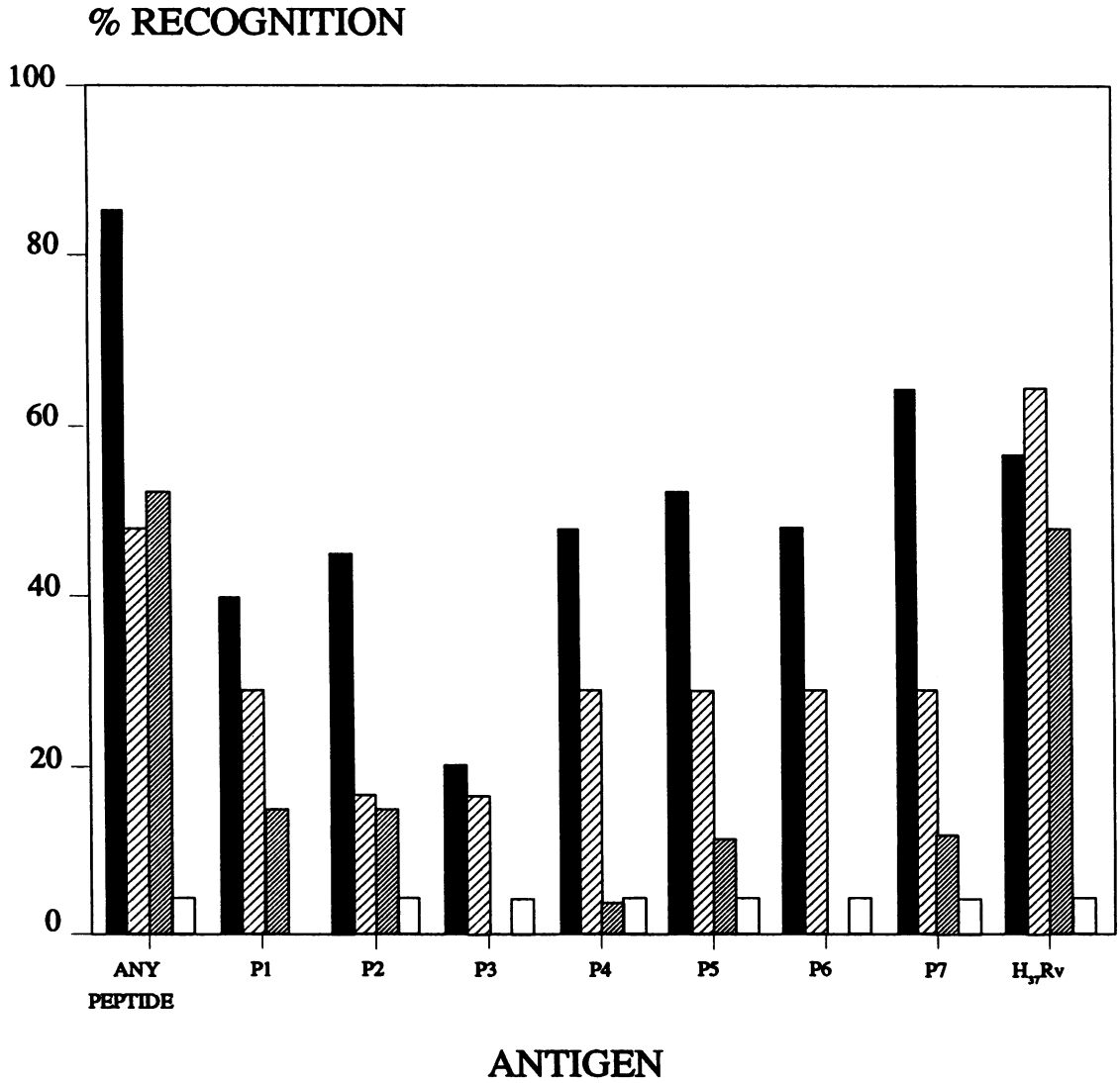

FIG. 3. Lymphocyte proliferation in each of the four groups of individuals studied in response to stimulation with MTP40-derived peptides. Bars represent the degree of recognition in terms of the percentage of responders within each particular group. Patient symbols: ■, HH; ▨, BK+; ▨, BK−; □, normal. The percentage of individuals whose lymphocytes were stimulated by at least one of the seven peptides is also shown (Any peptide). $H_{37}R_v$ represents the response to the *M. tuberculosis* sonic extract.

TABLE 3. Analysis of the cellular immune response among the different study groups

Group	Mean SI (mean 1/SI) with antigen[a]							
	P1	P2	P3	P4	P5	P6	P7	$H_{37}R_v$
Control	0.94 (1.11)	1.13 (0.96)	1.26 (0.90)	0.99 (1.06)	1.01 (1.04)	1.01 (1.04)	1.01 (1.02)	2.42 (0.79)
BK+	1.11 (1.00)	1.32 (0.83)	1.10 (0.99)	1.01 (1.04)	1.12 (0.95)	1.02 (1.01)	1.11 (0.96)	3.06 (0.53)
BK−	1.14 (1.08)	2.12 (0.87)	1.99 (0.87)	1.55 (0.86)	1.58 (0.84)	2.91 (0.85)	1.63 (0.85)	5.58 (0.31)
HH	2.12 (0.84)	4.34 (0.65)	3.16 (0.70)	3.00 (0.74)	3.04 (0.68)	4.02 (0.65)	3.28 (0.63)	5.33 (0.35)

[a] Values for each antigen correspond to the mean of the SI; those in parentheses correspond to the mean of the inverse value of SI (1/SI). The statistical significance of the analysis of variance comparing the 1/SI value for each of the four groups was 0.1035 (P1), 0.0094 (P2), 0.0205 (P3), 0.0008 (P4), 0.0003 (P5), 0.0003 (P6), 0.0001 (P7), and 0.0030 ($H_{37}R_v$). For P2 through P7 and $H_{37}R_v$, the following pairs of groups were significantly different ($P = 0.05$): Control/HH (P2), BK+/HH (P3), Control/HH and BK+/HH (P4 through P7), Control/HH and Control/BK− ($H_{37}R_v$).

Antibody reactivity. Sera from tuberculous patients, as tested by ELISA, showed a high frequency of antibody reactivity toward synthetic peptides derived from the MTP40 protein. It is interesting that recognition of the peptides by antibodies was more frequent in tuberculous patients than in HH individuals. Figure 2 shows these results in terms of percentages. While peptide P4 was the most widely recognized by BK+ patients (55%), the other peptides had, in general, higher recognition values by BK− patients. In contrast, low serological reactivities to most MTP40 peptides were observed for HH individuals and normal donors. A total of 84% of sera from BK− and 70% of sera from BK+ patients recognized at least one of these peptides, while only 44% of HH individuals and 14% of normal donors did.

The optical density values obtained by ELISA were tested by the Kolmogorov-Smirnov procedure. The null hypothesis for normality was not rejected ($P > 0.10$) for the original values in each of the groups of patients, except for peptides P2 and P3 in the BK+ group and for peptides P2 and P6 in the HH group. As shown in Table 2, a statistically significant difference, as evidenced by an analysis of variance, was found between the different groups of individuals studied. Pairs of groups significantly different are also shown for peptides P3, P4, and P5.

Lymphocyte proliferation assay. The functional viability of lymphocytes was evaluated by studying their responses to the mitogen concanavalin A on parallel microcultures. Background proliferation varied from 300 to 5,500 cpm. The results of this assay showed that all lymphocyte preparations were equally viable. A statistical analysis of the response to concanavalin A in terms of its SI did not reveal any significant difference between the responses to concanavalin A by cells of the different study groups (data not shown).

The peptides tested in the lymphoproliferative assays were used at two different concentrations, 5 and 25 μg/ml; the $H_{37}R_v$ sonicate was used at a concentration of 5 μg/ml. A positive response to at least one of the MTP40-derived peptides was observed in 84% of the HH individuals, 48% of the BK− patients, 52% of BK+ patients, and 4.5% of the normal donors (Fig. 3). By taking into account the responses to any peptide and to concanavalin A, in addition to the fact that 48% of the BK+ patients and 65% of the BK− patients responded to the soluble fraction of $H_{37}R_v$ in this assay, the possibility of the existence of an immunosuppressive mechanism in tuberculous patients was discarded.

The percentages of positive PBL responses of the populations under study are also shown in Fig. 3, where it can be seen that, for all peptides, proliferative responses were always the highest for HH individuals, followed by the responses in BK− patients, BK+ patients, and normal donors. Of all the peptides, P7 showed the highest recognition value (64%) by T cells of HH individuals. This same peptide was recognized by 29% of BK− patients, 11% of BK+ patients, and 4.5% of normal donors.

For each one of the groups of individuals studied, the distribution of SI values was tested by the Kolmogorov-Smirnov procedure. For the original values, the null hypothesis for normality was rejected ($P < 0.001$). However, when inverse transformations of the original SI values (1/SI) were subjected to the same test, a normal distribution for all of the peptides, except for P1 in all groups of individuals and for P7 in the BK+ group, was found. Therefore, these 1/SI values were used to analyze differences among the groups studied. As is shown in Table 3, statistically significant differences, as evidenced by the analysis of variance, were apparent for each one of the peptides, except for P1.

Relationship between cellular and humoral reactivities. Figure 4 schematizes the intensities of the humoral (lines 1 to 7) and cellular (lines 8 to 14) responses to each of the MTP40-derived synthetic peptides for three of the four groups of individuals studied (HH, BK+, and BK−). Line 15 shows the intensity of the cellular response to the $H_{37}R_v$ sonic extract. Cutoff points 1, 2, and 3 were taken as the means of the values for the normal donor group plus 2, 3, and 5 standard deviations, respectively (Fig. 4). To schematize the responses, the values were obtained from the statistical analyses shown in Tables 4 (humoral response) and 5 (cellular response).

Also shown in Fig. 4 are the results obtained for the PPD tuberculin test, prior to bleeding, in HH individuals. No association was found between the tuberculin status and the response to the individual peptides or the sonic extract. Prominent examples of this situation can be observed for patients 73 and 80.

It is also interesting that the intensity and frequency of cellular recognition of the antigens is maximal in HH individuals, compared with tuberculous patients, while the recognition of peptides by antibodies is more frequent with those from tuberculous patients than from HH individuals (Fig. 4). Figure 4 shows that there exists a greater proportion of positive responses (2 standard deviations above the normal donor group mean) to all peptides, from B cells (46 of 175) than from T cells (16 of 175) in BK+ patients ($P < 0.0001$, obtained through the proportions difference test), while in HH individuals, the proportion of positive B-cell responses (19 of 175) is lower than the proportion of positive T-cell responses (79 of 175) ($P < 0.0001$). In BK− patients, the proportions of positive B-cell and T-cell responses (38 of 119 and 31 of 119, respectively) are not significantly different ($P = 0.13$).

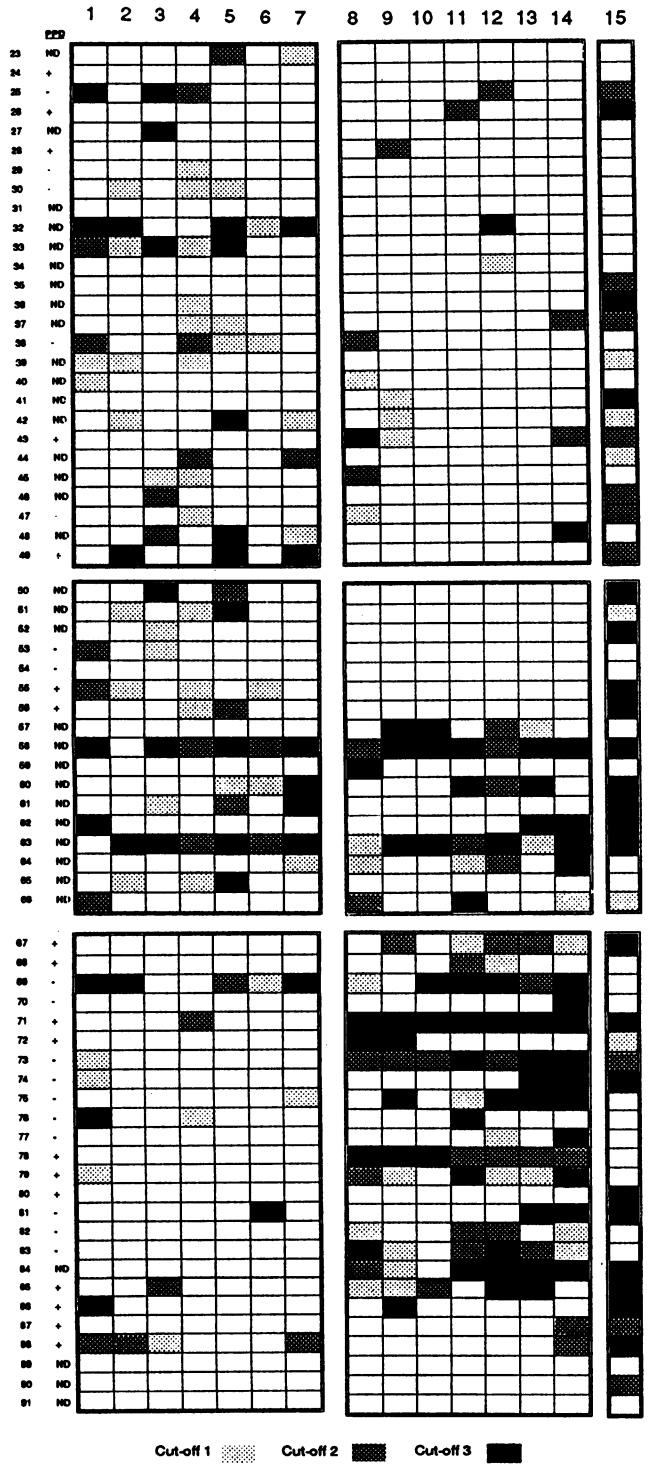

FIG. 4. Schematic representation of serological responses and lymphocyte proliferation to seven MTP40-derived synthetic peptides and the $H_{37}R_v$ sonic extract from 69 subjects belonging to three of the four groups of individuals studied: active BK+ tuberculosis patients (no. 23 to 49), active BK− tuberculosis patients (no. 50 to 66), and HH individuals (no. 67 to 91). Lines 1 through 7 at the top represent the degree of antibody response, as assessed by ELISA, when peptides P1 to P7 were used as antigens. Lines 8 through 14 represent the degree of lymphocyte proliferation when peptides P1 through P7 were used as antigens. Line 15 represents the degree of lymphocyte proliferation when *M. tuberculosis* $H_{37}R_v$ was used as an antigen. PPD tuberculin skin test results were graded as follows (diameter of patch): +, >10 mm; −, <10 mm; ND, no data available. Cutoffs 1 through 3 were taken as the means of the values for the normal donor group plus 2, 3, and 5 standard deviations, respectively. The data from which these values were taken are described in Tables 4 and 5 for the humoral and cellular immune responses, respectively.

DISCUSSION

We have recently cloned a 14-kDa protein from *M. tuberculosis*, which we have designated MTP40. This appears to be a highly species-specific protein not present in other slow- or fast-growing mycobacteria. To assess the immunological relevance of this protein in humans infected and/or exposed to *M. tuberculosis*, a panel of synthetic peptides was used to test the humoral and cellular responses of selected groups of individuals with different degrees of bacillary burden.

The use of synthetic peptides derived from immunogenic proteins in the development of new tools to combat infectious diseases is gaining wide acceptance. These provide means by which single epitopes of these proteins can be easily constructed, thus simplifying the elucidation of the role of immunogenic proteins in protection (30, 31).

Peptide mapping of the MTP40 antigen, described in this paper, demonstrated that it contains several human B- and T-cell epitopes. With respect to the B-cell epitopes, we have found that peptide P4 was recognized by more than half (55%) of the sera taken from active BK+ patients, in whom the bacillus load is presumably the greatest among the four groups of persons studied. In contrast, only 5.5% of the normal donor serum samples, in which the bacillus content is believed to be nonexistent, reacted to the peptide. This wide difference in reactivity suggests that P4 may represent an immunodominant B-cell site within MTP40. These results were confirmed in a subsequent screening of a larger panel of sera, in which 57% of 196 BK+ patients and 2.5% of 120 normal donors reacted with this peptide (data not shown).

Several other peptides (P1, P3, P5, and P7) were recognized by a high proportion of sera from tuberculous patients, while sera from HH individuals reacted to the same peptides in a significantly lower proportion. These results suggest that the presence of anti-MTP40 peptide antibodies may be indicative of infection with *M. tuberculosis*. Furthermore, there may even exist a direct relationship between the bacillus load and antipeptide antibody levels. Careful quantification of both parameters, antipeptide antibody levels and the bacillus load, in longitudinal studies with tuberculous patients, including those with minimal tuberculosis (6), would be required to verify this hypothesis. The use of a mixture of peptides, with the aim of widening the range of recognition, could also be attempted in these studies.

Serological diagnosis in tuberculosis is of great concern (16). Most nonspecific reactions in these assays probably arise because mycobacteria contain antigens that are widely shared. If reliable serological assays are to be developed, they will depend on highly specific antigen preparations that do not allow the recognition of epitopes shared with environmental microorganisms. Furthermore, serodiagnosis will depend on developing tests that are simpler or cheaper or on ones that possess characteristics that render them more favorable than the sputum smear examination. Synthetic peptides derived from proteins exclusive of *M. tuberculosis* may fulfill these requirements; we propose peptides, such as P4, as candidate antigens. However, a detailed study of the

TABLE 4. Statistical analysis of the humoral immune response

Antigen	Mean OD_{492}[a]						
	Control (SD)	BK+	BK−	HH	Cutoff[b]		
					1	2	3
P1	0.202 (0.070)	0.274	0.303	0.270	0.342	0.411	0.551
P2	0.145 (0.028)	0.162	0.199	0.143	0.020	0.228	0.283
P3	0.225 (0.057)	0.309	0.380	0.190	0.339	0.396	0.510
P4	0.308 (0.080)	0.457	0.404	0.374	0.468	0.548	0.707
P5	0.200 (0.049)	0.288	0.416	0.175	0.298	0.347	0.444
P6	0.277 (0.086)	0.292	0.341	0.269	0.448	0.534	0.705
P7	0.134 (0.031)	0.176	0.228	0.147	0.197	0.228	0.291

[a] OD_{492}, optical density at 492 nm.
[b] Cutoff values for each antigen represent the means of the values for the control group plus 2, 3, and 5 standard deviations for cutoffs 1 through 3, respectively.

specificity and sensitivity of a diagnostic ELISA for tuberculosis (8), with MTP40-derived peptides as antigens, is necessary.

Another relevant finding of this study was the relatively high proportion of PBL proliferative responses to MTP40-derived peptides in some of the groups of patients studied. In general, all the peptides, especially those close to the C terminus of the MTP40 protein (P4 to P7), showed the highest recognition by T cells from HH individuals and patients undergoing recovery by antibiotic therapy (BK−). This recognition contrasts with the one obtained from both severely ill patients (BK+) and normal donors (Fig. 2).

The low T-cell responsiveness to the MTP40 antigen in the BK+ group of patients could possibly be explained as the result of massive in vivo stimulation of T cells by excess antigen. Alternatively, antigen-specific T cells may have homed into the *M. tuberculosis*-infected tissues and are therefore underrepresented in the PBL pool at the acute stages of the disease. It is interesting that the frequency and intensity of lymphoproliferative responses to the MTP40-derived peptides are higher in the group of tuberculous patients that became BK− after pharmacological intervention. Again, a longitudinal study with selected groups of tuberculous patients should be critical in establishing whether the individual lymphoproliferative responses to certain MTP40-derived peptides can be correlated to the course and recovery of the disease.

The immunodominance of certain peptides, such as P7, at the T-cell level is intriguing. Assuming that the distribution of human leukocyte antigen haplotypes among the patients in this study is similar to that of the general population, our results seem to indicate that P7 is being presented to human T cells in the context of more than one human leukocyte antigen molecule. In this respect, T-cell epitopes displaying degenerate interaction with several human and mouse major histocompatibility complex class II molecules, referred to as universal epitopes (4), have been described for the malarial CS protein and the tetanus toxin (14, 37). Such universal epitopes could be ideal candidates in the design of subunit vaccines.

The apparent switch from a humoral to a cellular response (Fig. 4) is in accordance with the idea recently proposed by David (9), which states that antibodies to immunodominant tuberculosis antigens might play an important role at the beginning of the infection and that adequate replacement of the humoral system- by the cell-mediated immune response to those antigens might provide resistance to mycobacterial infection.

Finally, nearly half of the individuals belonging to the normal donor group had been previously vaccinated with BCG, but neither T cells nor serum antibodies of these persons recognize the MTP40-derived peptides. This indicates that immunological memory for MTP40 was not induced by immunization with BCG, providing indirect proof of the absence of the *mtp40* gene product in *M. bovis* BCG, as we had previously suspected.

We conclude that MTP40, a protein apparently present exclusively in *M. tuberculosis*, is a prominent target of human B- and T-cell-mediated immune responses in human beings afflicted with tuberculosis. The MTP40 antigen as a whole or some of the peptides derived from it and described in this study are possible candidates for the development of

TABLE 5. Statistical analysis of the cellular immune response

Antigen	Mean SI						
	Control (SD)	BK+	BK−	HH	Cutoff[a]		
					1	2	3
P1	0.945 (0.197)	1.038	1.141	2.104	1.339	1.537	1.931
P2	1.135 (0.332)	1.328	2.126	4.348	1.799	2.131	2.795
P3	1.268 (0.577)	1.100	1.991	3.042	2.421	2.998	4.151
P4	0.996 (0.248)	1.008	1.551	3.003	1.491	1.739	2.235
P5	1.014 (0.243)	1.123	1.584	3.042	1.501	1.744	2.231
P6	1.013 (0.336)	1.030	2.918	4.021	1.685	2.022	2.694
P7	1.012 (0.174)	1.112	1.639	3.286	1.360	1.535	1.883
$H_{37}R_v$	1.485 (0.776)	3.060	5.852	5.331	3.037	3.814	5.367

[a] Cutoff values for each antigen represent the means of the values for the control group plus 2, 3, and 5 standard deviations for cutoffs 1 through 3, respectively.

specific diagnostic tests. Moreover, a detailed evaluation of the protein's role in protective immunity should help determine whether it or peptides derived from it may become candidate subunit vaccines.

ACKNOWLEDGMENTS

We thank Pedro Romero for valuable discussion and critical reading of the manuscript, Luis E. Sarmiento and Arnoldo Barbosa for help in the statistical analysis, Mario Posada for assistance provided in writing the manuscript, the staff of the Ambulatory Service of Neumology at the Hospital Santa Clara in Bogotá Colombia, especially Carlos Torres, and Juan Silva of the Colombian Ministry of Public Health.

This research has been supported by the Presidency and the Public Health Ministry of Colombia and the German Leprosy Relief Association.

REFERENCES

1. **Andersen, A. B., and E. B. Hansen.** 1989. Structure and mapping of the antigenic domains of protein antigen b, a 38,000-molecular-weight protein of *Mycobacterium tuberculosis*. Infect. Immun. **57**:2481–2488.
2. **Ashbridge, K. R., R. J. Booth, J. D. Watson, and R. B. Lathigra.** 1989. Nucleotide sequence of the 19 kDa antigen gene from *Mycobacterium tuberculosis*. Nucleic Acids Res. **17**:1249.
3. **Assad, F., I. Azuma, T. M. Buchanan, F. M. Collins, R. Curtiss, J. R. David, P. Draper, T. Godal, M. Goren, B. W. Janicki, S. H. E. Kaufmann, D. A. Mitchinson, A. Pio, and G. Torrigiani.** 1983. Plan of action for research in the immunology of tuberculosis: memorandum from a WHO meeting. Bull. W.H.O. **61**: 779–785.
4. **Bordignon, P., A. Tan, A. Termijtelen, S. Demotz, G. Corradin, and A. Lanzavecchia.** 1989. Universally immunogenic T cell epitopes: promiscuous binding to human MHC class II and promiscuous recognition by T cells. Eur. J. Immunol. **19**:2237–2242.
5. **Borremans, M., L. De Wit, G. Volckaert, S. Ooms, J. De Bruyn, K. Huygen, J. Van Vooren, M. Stelandre, R. Verhofstadt, and J. Content.** 1989. Cloning, sequence determination, and expression of a 32-kilodalton protein gene of *Mycobacterium tuberculosis*. Infect. Immun. **57**:3123–3130.
6. **Chan, S. L., Z. Reggiando, T. M. Daniel, D. J. Girling, and D. A. Mitchinson.** Am. Rev. Respir. Dis., in press.
7. **Crowle, A. J.** 1988. Immunization against tuberculosis: what kind of vaccine? Infect. Immun. **56**:2769–2773.
8. **Daniel, T. M., and S. M. Debanne.** 1987. The serodiagnosis of tuberculosis and other mycobacterial diseases by enzyme-linked immunosorbent assay. Am. Rev. Respir. Dis. **135**:1137–1151.
9. **David, H. L.** 1990. The spectrum of tuberculosis and leprosy: what can be the significance of specific humoral response? Res. Microbiol. **141**:191–205.
10. **Forget, A., J. C. Benoit, R. Turcotte, and N. Gusewchartand.** 1976. Enhancement activity of anti-mycobacterial sera in experimental *Mycobacterium bovis* (BCG) infection in mice. Infect. Immun. **13**:1301–1306.
11. **Garsia, R. J., L. Hellquist, R. Booth, A. Rathford, W. Britton, L. Atsbury, R. Trent, and A. Basten.** 1989. Homology of the 70-kilodalton antigens from *Mycobacterium leprae* and *Mycobacterium bovis* with the *Mycobacterium tuberculosis* 71-kilodalton antigen and with the conserved heat shock protein 70 of eucaryotes. Infect. Immun. **57**:204–212.
12. **Good, R. C.** 1989. Serological methods for diagnosing tuberculosis. Ann. Intern. Med. **110**:97–98.
13. **Han, H., and S. H. E. Kaufmann.** 1981. The role of cell-mediated immunity in bacterial infections. Rev. Infect. Dis. **3**:1221–1250.
14. **Ho, P. C., D. Mutch, K. D. Winkel, A. J. Saul, G. L. Jones, T. J. Doran, and M. R. Christine.** 1990. Identification of two promiscuous T cell epitopes from tetanus toxin. Eur. J. Immunol. **20**:477–483.
15. **Houghten, R. A.** 1985. General method for the rapid solid phase synthesis for a large number of peptides. Specificity of antigen-antibody interaction at the level of individual amino acids. Proc. Natl. Acad. Sci. USA **82**:5131–5135.
16. **Ivanyi, J., G. H. Bothamley, and T. S. Jacket.** 1988. Immunodiagnostic assays for tuberculosis and leprosy. Br. Med. Bull. **44**:635–644.
17. **Kadival, G. V., S. D. Chaparas, and D. Hussong.** 1987. Characterization of serological and cell-mediated reactivity of a 38-kDa antigen isolated from *Mycobacterium tuberculosis*. J. Immunol. **139**:2247–2251.
18. **Kalish, S. B., R. C. Radin, J. P. Phair, D. Levitz, C. R. Zeiss, and E. Metzger.** 1983. Use of an enzyme-linked immunosorbent assay technique in the differential diagnosis of active pulmonary tuberculosis in humans. J. Infect. Dis. **47**:523–530.
19. **Kaufmann, S. H. E.** 1988. Immunity against intracellular bacteria: biological effector functions and antigen specificity of T lymphocytes. Curr. Top. Microbiol. Immunol. **138**:141–176.
20. **Kaufmann, S. H. E., and I. Flesch.** 1986. Function and antigen recognition pattern of L3T4$^+$ T-cell clones from *Mycobacterium tuberculosis*-immune mice. Infect. Immun. **54**:291–296.
21. **Kingston, A. E., P. R. Salgame, N. A. Mitchison, and J. M. Colston.** 1987. Immunological activity of a 14-kilodalton recombinant protein of *Mycobacterium tuberculosis* H37Rv. Infect. Immun. **55**:3149–3154.
22. **Lamb, J. R., J. Ivanyi, A. D. M. Rees, J. B. Rothbard, K. Howland, R. A. Young, and D. B. Young.** 1987. Mapping of T cell epitopes using recombinant antigens and synthetic peptides. EMBO J. **6**:1245–1249.
23. **Lamb, J. R., R. Lathigra, J. B. Rothbard, D. Sweetser, R. A. Young, J. Ivanyi, and D. B. Young.** 1989. Identification of mycobacterial antigens recognized by T lymphocytes. Rev. Infect. Dis. **11**:443–447.
24. **Lamb, J. R., A. D. M. Rees, V. Bal, H. Ikeda, D. Wilkinson, R. R. P. de Vries, and J. D. Rothbard.** 1988. Prediction and identification of an HLA-DR-restricted T cell determinant in the 19-kDa protein of *Mycobacterium tuberculosis*. Eur. J. Immunol. **18**:973–976.
25. **Merrifield, R. B.** 1963. Peptide synthesis. I. The synthesis of a tetrapeptide. J. Am. Chem. Soc. **85**:2149–2154.
26. **Munk, M. E., B. Shoel, and S. H. E. Kaufmann.** 1988. T cell responses of normal individuals toward recombinant protein antigens of *Mycobacterium tuberculosis*. Eur. J. Immunol. **18**:1835–1838.
27. **Oftung, F., A. S. Mustafa, R. Husson, R. A. Young, and T. Godal.** 1987. Human T cell clones recognize two abundant *M. tuberculosis* protein antigens expressed in *E. coli*. J. Immunol. **138**:927–931.
28. **Oftung, F., A. S. Mustafa, T. M. Shinnick, R. A. Houghten, G. Kvalheim, M. Degre, K. E. A. Lundin, and T. Godal.** 1988. Epitopes of the *Mycobacterium tuberculosis* 65-kilodalton protein antigen as recognized by human T cells. J. Immunol. **141**:2749–2754.

28a. **Parra, C. A., et al.** Submitted for publication.

29. **Patarroyo, M. E., C. A. Parra, C. Pinilla, P. del Portillo, M. L. Torres, P. Clavijo, L. M. Salazar, and C. Jimenez.** 1986. Immunogenetic synthetic peptides against mycobacteria of potential immunodiagnostic and immunoprophylactic value. Lepr. Rev. **57**(Suppl. 2):163–168.
30. **Patarroyo, M. E., P. Romero, M. L. Torres, P. Clavijo, A. Moreno, A. Martinez, R. Rodriguez, F. Guzman, and E. Cabezas.** 1987. Induction of protective immunity against experimental infection with malaria using synthetic peptides. Nature (London) **328**:629–632.
31. **Romero, P., J. L. Maryanski, G. Corradin, R. S Nussenzweig, V. Nussenzveig, and F. Zavala.** 1989. Cloned cytotoxic T cells recognize an epitope in the circumsporozoite protein and protect against malaria. Nature (London) **341**:323–326.
32. **Rumschlag, H. S., M. A. Yakrus, M. L. Cohen, S. E. Glickman, and R. C. Good.** 1990. Immunologic characterization of a 35-kilodalton recombinant antigen of *Mycobacterium tuberculosis*. J. Clin. Microbiol. **28**:591–595.
33. **Sada, E., L. E. Ferguson, and T. M. Daniel.** 1990. An ELISA for the serodiagnosis of tuberculosis using a 30,000-Da native

antigen of *Mycobacterium tuberculosis*. J. Infect. Dis. **160**:928–931.

34. **Shield, M. J., J. L. Stanford, and G. A. W. Rook.** 1979. The reasons for the reduction of protective efficacy of BCG in Burma. Excerpta Med. Int. Congr. Ser. **466(8)**:102–107.
35. **Shinnick, T. M.** 1987. The 65-kilodalton antigen of *Mycobacterium tuberculosis*. J. Bacteriol. **169**:1080–1088.
36. **Shinnick, T. M., B. B. Plikaytis, A. D. Hyche, R. M. van Landingham, and L. L. Walker.** 1989. The *Mycobacterium tuberculosis* BCG—a protein which has homology with the *E. coli* Gro ES protein. Nucleic Acids Res. **17**:1254.
37. **Sinigaglia, F., M. Guttinger, J. Kirgus, D. M. Doran, H. Matilde, H. Etlinger, A. Trzeciak, D. Gillessen, and J. R. L. Pink.** 1988. A malaria T-cell epitope recognized in association with most mouse and human MHC class II molecules. Nature (London) **336**:778–780.
38. **Stanford, J. L.** 1981. Immunologically important constituents of mycobacteria: antigens, p. 85–127. *In* C. Ratlidge and J. Stanford (ed.), The biology of the mycobacteria. Academic Press, Inc., New York.
39. **Strauss, E., and N. Wu.** 1980. Radioimmunoassay of the tuberculoprotein derived from *Mycobacterium tuberculosis*. Proc. Natl. Acad. Sci. USA **78**:4301–4304.
40. **Thole, J. E. R., L. F. E. M. Stabel, M. E. G. Suykerbuyk, M. Y. L. de Wit, P. R. Klatser, A. H. J. Kolk, and R. A. Hartskeerl.** 1990. A major immunogenic 36,000-molecular-weight antigen from *Mycobacterium leprae* contains an immunoreactive region of proline-rich repeats. Infect. Immun. **58**:80–87.
41. **Thole, J. E. R., W. A. van Schooten, W. J. Keulen, P. W. N. Hermans, A. A. M. Janson, R. R. P. de Vries, and J. D. A. van Embden.** 1988. Use of recombinant antigens expressed in *Escherichia coli* K-12 to map B-cell and T-cell epitopes on the immunodominant 65-kilodalton protein of *Mycobacterium bovis* BCG. Infect. Immun. **56**:1633–1640.
42. **Thompson, N. J., J. L. Glassroth, D. E. Snider, Jr., and L. S. Farer.** 1979. The booster phenomenon in serial tuberculin testing. Am. Rev. Respir. Dis. **119**:587–597.
43. **Tuberculosis Prevention Trial, Madras.** 1980. Trial of BCG vaccines in south India for tuberculosis prevention. Indian J. Med. Res. **72**(Suppl.):1–74.
44. **Vismara, D., M. F. Mezzopreti, M. S. Gilardini, P. del Porto, G. Lombardi, E. Piccolella, G. Damiani, R. Rappuoli, and V. Colizzi.** 1990. Identification of a 35-kilodalton *Mycobacterium tuberculosis* protein containing B- and T-cell epitopes. Infect. Immun. **58**:245–251.
45. **Young, D. B., L. Kent, A. Rees, J. Lamb, and J. Ivanyi.** 1986. Immunological activity of a 38-kilodalton protein purified from *Mycobacterium tuberculosis*. Infect. Immun. **54**:177–183.
46. **Young, D. B., R. Lathigra, R. Hendrix, D. Sweetser, and R. A. Young.** 1988. Stress proteins are immune targets in leprosy and tuberculosis. Proc. Natl. Acad. Sci. USA **85**:4267–4270.