Antigenic and Structural Similarities between *Mycobacterium tuberculosis* 50- to 55-Kilodalton and *Mycobacterium bovis* BCG 45- to 47-Kilodalton Antigens

CLARA ESPITIA,^{1,2*} RAFAELA ESPINOSA,² RAFAEL SAAVEDRA,² RAUL MANCILLA,²
FELIX ROMAIN,³ ANNE LAQUEYRERIE,³ AND CARLOS MORENO¹

Tuberculosis and Related Infections Unit, Medical Research Council Clinical Sciences Centre, Royal Postgraduate Medical School, Hammersmith Hospital, London W12 0HS, United Kingdom¹; Departamento de Inmunología, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, C.P. 04510, Mexico City, Mexico²; and Unité de Physiopathologie de L'Infection, Institut Pasteur, 75724 Paris Cédex 15, France³

Received 27 June 1994/Returned for modification 8 September 1994/Accepted 15 November 1994

The relationship between *Mycobacterium tuberculosis* 50- to 55-kDa protein and *Mycobacterium bovis* BCG 45- to 47-kDa antigen was examined by using immunological and biochemical criteria. Reciprocal cross-reactivity with a rabbit polyclonal antiserum against the *M. bovis* BCG protein and with a monoclonal antibody raised against the *M. tuberculosis* antigen was observed. The epitope recognized by this antibody was apparently present only in proteins of *M. tuberculosis* and *M. bovis* BCG among the 11 mycobacterial species tested. The amino-terminal sequences and total amino acid contents of these proteins showed strong similarities. Both antigens are glycoproteins as assessed by binding of concanavalin A, labeling of carbohydrate moieties with biotin-hydrazide, and digestion of carbohydrates with jack bean α -D-mannosidase, which produced a reduction of the molecular weights of the proteins and totally eliminated concanavalin A binding. Both *M. tuberculosis* and *M. bovis* BCG proteins are secreted, since they were found mainly in the culture medium. Analysis of *M. tuberculosis* 50- to 55-kDa antigen by two-dimensional gel electrophoresis showed at least seven different components, as previously described for the *M. bovis* BCG antigen. Solid-phase immunoassays showed that the purified *M. tuberculosis* 50- to 55-kDa protein was recognized by serum specimens from 70% of individuals with pulmonary tuberculosis from a total of 77 Mexican patients examined.

Identification and characterization of mycobacterial antigens are critical for evaluation of their role in diagnosis, vaccination, and pathogenesis of mycobacterial diseases, such as tuberculosis and leprosy (1). Particular attention has recently been given to bacterial antigens secreted into the culture medium, since they seem to play an important role in the development of protective immunity to mycobacteria (17, 19). It has been documented that *Mycobacterium tuberculosis* proteins found predominantly in culture supernatants induce strong humoral and cellular immune responses (2, 4, 10, 25, 27). Secreted proteins, such as the 30- to 31-, 24-, 22-, 19-, and 12-kDa antigens, have been found to be shared by *M. tuberculosis* and *Mycobacterium bovis* BCG (5).

An immunogenic protein complex of 45 to 47 kDa from culture supernatants of *M. bovis* BCG has been found to be a dominant immunogen after immunization of guinea pigs with live but not dead BCG organisms (22). As reported here, this antigen has been found to cross-react with a 50- to 55-kDa *M. tuberculosis* protein antigen described previously (3). In this communication, we describe immunological and biochemical similarities found between *M. tuberculosis* 50- to 55-kDa and *M. bovis* BCG 45- to 47-kDa antigen complexes.

MATERIALS AND METHODS

Bacteria and antigens. Some of the mycobacterial soluble extracts (SE) used in this study were obtained by sonication of mycobacteria killed by gamma irradiation (10). They are part of a collection of extracts produced by the Tu-

berculosis and Related Infections Unit, Medical Research Council Clinical Sciences Centre (London, United Kingdom). The series included *Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycobacterium fortuitum*, *Mycobacterium scrofulaceum*, *Mycobacterium phlei*, *Mycobacterium leprae*, and *Mycobacterium paratuberculosis*.

Mycobacterium smegmatis and *Mycobacterium vaccae* were cultured for 4 days on Middlebrook 7H9 medium (Difco Laboratories, Detroit, Mich.) supplemented with glucose (2%, wt/vol). *M. tuberculosis* H37Rv and *M. bovis* BCG were cultured on Proskauer and Beck synthetic medium for 6 and 4 weeks, respectively. Their culture filtrates (CF) and SE were obtained as described elsewhere (2, 10). *Escherichia coli* YM109 was grown on Luria-Bertani medium, and an SE was produced by disruption of the bacteria by shaking for 5 min with glass beads.

Purification of proteins. An enriched fraction of 50- to 55-kDa protein from *M. tuberculosis* was obtained after acid alcohol fractionation of the CF as described elsewhere (3, 23). Proteins from this fraction were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto nitrocellulose membranes (0.45- μ m pore size; Millipore Corp., Bedford, Mass.), and the 50- to 55-kDa protein was purified by being eluted from the membrane by incubation with 40% acetonitrile in 0.1 M ammonium acetate (pH 8.9) for 5 h at 37°C as described by Parekh et al. (20). *M. bovis* BCG 45- to 47-kDa antigen was purified from culture supernatants by the sequential use of gel filtration, DEAE column chromatography, and reverse-phase high-pressure liquid chromatography as described elsewhere (22).

Human sera. Serum specimens from 77 individuals with pulmonary tuberculosis were obtained from various medical centers in Mexico City, Mexico. Diagnosis was established by smear and/or culture of sputum. Control serum samples were obtained from 92 healthy Mexican adults.

Rabbit sera. Serum against the 50- to 55-kDa *M. tuberculosis* protein was obtained from New Zealand White rabbits immunized subcutaneously on days 0 and 8 with 200 μ g of purified protein in incomplete Freund's adjuvant. They were given intraperitoneal (i.p.) booster injections of 100 μ g of purified protein 2 weeks later and every 2 weeks thereafter for 2 months. Rabbit polyclonal antiserum against the *M. bovis* BCG 45- to 47-kDa complex was raised as described elsewhere (22).

MAb. Female BALB/c mice were immunized i.p. with 200 μ g of purified 50- to 55-kDa antigen from *M. tuberculosis* in incomplete Freund's adjuvant on day 0 and were given booster injections on days 3 (100 μ g i.p.) and 18 (50 μ g i.p.). On days 445 and 450 (7 and 2 days before fusion, respectively), the mice were given booster injections of 10 μ g i.p. and 100 μ g intravenously, respectively, of the protein in saline solution. Spleen cells were fused with the SP2/0-Ag14 myeloma

* Corresponding author. Present address: Departamento de Inmunología, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Apartado postal 70228, C.P. 04510, Mexico City, Mexico. Phone: (525) 5503982. Fax: (525) 5500048.

line in the presence of polyethylene glycol 3550 (Sigma Chemical Co., St. Louis, Mo.) and then cultured under standard conditions (6). Antibody activity in hybridoma supernatants was detected by using purified 50- to 55-kDa antigen in an enzyme-linked immunosorbent assay (ELISA). Hybridomas from positive wells were cloned twice by limiting dilutions and expanded. Although 14 clones were positive by ELISA, only one was positive by Western blotting (immunoblotting) using *M. tuberculosis* CF. This monoclonal antibody (MAb), designated 6A3, was isotyped by immunoblotting using a panel of heavy-chain-specific anti-mouse immunoglobulins (Southern Biotechnology Associates, Inc., Birmingham, Ala.) and found to be immunoglobulin G1.

SDS-PAGE and Western blotting. Electrophoresis in 12% polyacrylamide gels containing SDS and subsequent immunoblotting procedures were carried out by standard methods (11, 24). Samples (10 µg each) of SE from 11 different mycobacteria and CF only from *M. tuberculosis*, *M. bovis* BCG, *M. paratuberculosis*, *M. smegmatis*, and *M. vaccae* were run in polyacrylamide gels. For antigen detection, proteins were transferred to nitrocellulose membranes and incubated with MAb 6A3 and with rabbit polyclonal antiserum. For carbohydrate detection, purified proteins from both *M. tuberculosis* and *M. bovis* BCG were stained with concanavalin A (ConA)-peroxidase (Sigma) after blotting, and purified *M. tuberculosis* 50- to 55-kDa protein and the fraction enriched in 50- to 55-kDa protein were labeled in solution with biotin-hydrazide (Calbiochem Co., San Diego, Calif.) after oxidation of oligosaccharide moieties with sodium metaperiodate (18). After being labeled, electrophoresed proteins were transferred to nitrocellulose membranes and stained with streptavidin-peroxidase (Sigma). The same basic procedure was used for processing and staining membranes for antibodies, ConA, and streptavidin. Nonspecific binding was blocked by incubating blots with 3% (wt/vol) bovine serum albumin in phosphate-buffered saline (PBS) containing Tween 20 (0.3%, vol/vol); after repeated washes with PBS-Tween 20, the membranes were incubated for 2 h at room temperature with either MAb 6A3 diluted 1/100 or polyclonal rabbit anti-*M. bovis* BCG 45- to 47-kDa protein (1/1,000). After repeated washes with PBS-Tween 20, the membranes were incubated with the following peroxidase conjugates: anti-mouse immunoglobulin G1 diluted 1/1,000, anti-rabbit immunoglobulin G (Sigma) diluted 1/10,000, ConA (2.5 µg/ml), and streptavidin diluted 1/1,000 in PBS-Tween 20. After incubation for 1 h at room temperature, the blots were stained for peroxidase activity by adding 3,3'-diaminobenzidine HCl and hydrogen peroxide in PBS.

For chemical analysis, the antigens were transferred to polyvinylidene difluoride nylon membranes (Applied Biosystems, Calif.), and after a brief staining with Coomassie blue, the 50- and 55-kDa bands were cut out separately. N-terminal sequencing and total amino acid analysis (14) of each band were done as described below. Two-dimensional PAGE gels were run as follows. Urea was added to 10 µg of enriched 50- to 55-kDa-protein fraction or 5 µg of biotin-labeled enriched 50- to 55-kDa-protein fraction, to a final concentration of 9 M, and then lysis buffer containing urea and Nonidet P-40 was added as described by O'Farrell (16). Samples were separated initially by isoelectric focusing in tube gels containing 4% ampholytes in the pH range 3.5 to 5.0 (Pharmacia, Uppsala, Sweden) and then by SDS-PAGE in the second dimension as described above. Proteins were transferred to nitrocellulose membranes, and antigens were stained by using MAb 6A3, ConA-peroxidase, and streptavidin-peroxidase.

Digestion of glycoproteins with α -D-mannosidase. One microgram each of purified *M. tuberculosis* 50- to 55-kDa and *M. bovis* BCG 45- to 47-kDa antigen complexes, 1 µg of biotin-labeled 50- to 55-kDa protein, and 10 µg of enriched 50- to 55-kDa-protein fraction were digested for 1 h at 37°C with jack bean α -D-mannosidase (EC 3.21.2.4) (Sigma) in 0.1 M sodium acetate buffer (pH 5), with 15 U of the enzyme per mg of protein or with 0.5 U of α -D-mannosidase from almonds per mg of protein (Sigma). Sample controls without enzyme were also included. A sample of digested, enriched 50- to 55-kDa-protein fraction was also labeled with biotin-hydrazide after dialysis against 0.1 M sodium acetate buffer, pH 5.5. The digestion reactions were stopped by the addition of SDS-PAGE sample buffer (62 mM Tris [pH 6.8], 2% SDS, 0.7% 2-mercaptoethanol) and boiled for 3 min at 100°C or by addition of isoelectric focusing sample buffer (9.5 M urea, 2% Nonidet P-40, 2% ampholytes, 5% 2-mercaptoethanol). Thereafter, proteins were subjected to SDS-PAGE or two-dimensional PAGE, transferred to nitrocellulose membranes, and incubated with the MAb, ConA-peroxidase, or streptavidin-peroxidase as described above.

Amino-terminal sequence and amino acid composition. Amino-terminal sequences were determined by automatic Edman degradation by using an Applied Biosystems 470A sequenator and working on pieces of polyvinylidene difluoride sheets containing electrotransferred proteins. Total amino acid composition was determined with a Beckman Autoanalyzer. These procedures were performed at the Biopolymer Synthesis and Analysis Unit, Department of Biochemistry, Nottingham University, Nottingham, United Kingdom.

ELISA. An ELISA was carried out with Immunolon II microtiter plates (Dynatech Industries, McLean, Va.) as described previously (2). Briefly, wells were coated with 100 µl of antigen (50 µg/ml), and after a blocking and washing, 50 µl of serum diluted 1/1,000 in PBS was added and the mixture was incubated for 1 h at 37°C. Then protein A labeled with horseradish peroxidase (Sigma) was added, and the color reaction was developed with orthophenylenediamine. The reaction was stopped by addition of 1 N sulfuric acid, and the optical density at 492 nm was read.

FIG. 1. Cross-reactivity is shown by immunoblotting with a rabbit polyclonal antiserum against the *M. bovis* BCG protein complex (A) and with MAb 6A3 against the *M. tuberculosis* protein complex (B). For panel A: lane 1, *M. tuberculosis* CF; lane 2, *M. tuberculosis* SE; lane 3, *M. bovis* BCG CF; lane 4, *M. bovis* BCG SE; lane 5, purified *M. tuberculosis* 50- to 55-kDa protein; lane 6, purified *M. bovis* BCG 45- to 47-kDa protein. For panel B: lane 1, *M. tuberculosis* H37Rv CF; lane 2, *M. tuberculosis* H37Rv SE; lane 3, *M. bovis* BCG CF; lane 4, *M. bovis* BCG SE; lane 5, *M. avium* SE; lane 6, *M. intracellulare* SE; lane 7, *M. fortuitum* SE; lane 8, *M. scrofulaceum* SE; lane 9, *M. phlei* SE; lane 10, *M. leprae*; lane 11, *M. paratuberculosis* CF; lane 12, *M. paratuberculosis* SE; lane 13, *M. smegmatis* CF; lane 14, *M. smegmatis* SE; lane 15, *M. vaccae* CF; lane 16, *M. vaccae* SE; lane 17, *E. coli*. Positions of molecular mass markers are shown on the left.

RESULTS

Antigenic cross-reactivity. Cross-reactivity between the *M. tuberculosis* and the *M. bovis* BCG antigen complexes was established when proteins from CF and SE and purified proteins from both mycobacteria were tested by immunoblotting with the polyclonal antiserum against *M. tuberculosis* 50- to 55-kDa protein (not shown) and *M. bovis* BCG 45- to 47-kDa antigen and with MAb 6A3 against *M. tuberculosis* 50- to 55-kDa antigen. With the MAb only, the doublet band was seen in both *M. tuberculosis* and *M. bovis* BCG extracts. The polyclonal antiserum identified several less prominent bands of higher and lower molecular weights (Fig. 1). In addition, the interspecies specificity of MAb 6A3 was examined by immunoblotting using extracts of 11 different mycobacterial species. Only in SE and CF of *M. tuberculosis* and *M. bovis* BCG was the complex identified. No reactivity with *E. coli* was detected (Fig. 1B).

ConA reactivity of purified antigens and partial deglycosylation with jack bean α -D-mannosidase. To determine whether *M. bovis* BCG 45- to 47-kDa protein bound ConA, as previously described for *M. tuberculosis* 50- to 55-kDa protein (10), purified 50- to 55- and 45- to 47-kDa proteins were incubated with ConA-peroxidase after transfer to nitrocellulose membranes. Positive staining of both doublets was observed (Fig. 2A). In addition, carbohydrates present in the purified proteins

FIG. 2. (A) ConA reactivity of the purified, electroblotted proteins. Lane 1, *M. tuberculosis* protein; lane 2, *M. bovis* BCG protein. (B) Effects of digestion of purified glycoproteins with jack bean α -D-mannosidase. Lanes 1 and 2, untreated and treated *M. tuberculosis* glycoproteins, respectively; lanes 3 and 4, untreated and treated *M. bovis* BCG glycoproteins, respectively. The immunoblot in panel A was developed with MAb 6A3. Positions of molecular mass markers are shown on the left.

FIG. 3. Two-dimensional PAGE analysis of the 50- to 55-kDa *M. tuberculosis* protein-enriched fraction and effect of α -D-mannosidase treatment. Blots were developed with MAb 6A3 (A), with ConA-peroxidase (B), and with streptavidin-peroxidase (C). Arrows indicate carbohydrate moieties of the 50- to 55-kDa protein. The additional spots most likely indicate labeling of mannosidase or of other contaminant glycoproteins. Positions of molecular mass markers are shown on the left.

were digested with jack bean α -D-mannosidase, which produced a reduction in molecular mass of about 1.5 to 2 kDa for each of the doublet bands, as shown in Fig. 2B. When the purified *M. tuberculosis* 50- to 55-kDa protein was labeled with biotin-hydrazide after periodate oxidation and then digested with jack bean α -D-mannosidase, a smaller reduction (0.5 to 1 kDa) in molecular mass was also observed (not shown). Treatment with α -D-mannosidase from almonds (Sigma) did not affect ConA reactivity or SDS-PAGE migration (results not shown). Two-dimensional PAGE revealed an increase in the number of isoforms after digestion of the proteins with jack bean α -D-mannosidase, as detected with the MAb (Fig. 3A) and with streptavidin-peroxidase after digestion of the previously carbohydrate-labeled glycoproteins (Fig. 3C). In contrast, digestion of the proteins with jack bean α -D-mannosidase destroyed the ConA reactivity (Fig. 3B).

Composition of amino acids and amino-terminal sequencing. Total amino acid analyses of the two major components of *M. tuberculosis* 50- to 55-kDa protein and 45- to 47-kDa protein from BCG gave very similar results (Table 1). Proline and alanine were the predominant amino acids in both proteins. Histidine, tyrosine, and methionine were absent or present in <1% concentrations in the two proteins. The amino-terminal sequences showed a high degree of similarity between the two proteins, with minor substitutions (D for A at position 1 and VA for PP at positions 9 and 10) (Table 2).

The antibody response to the isolated 50- to 55-kDa antigen in pulmonary tuberculosis patients as analyzed by ELISA. Studies of the antibody response were performed with serum samples from 77 tuberculosis patients and 92 serum samples from healthy individuals which served as a control group (Fig.

4). For the patients, the mean optical density $\pm$ standard deviation was 1.064 ± 0.621 , and for healthy individuals it was 0.096 ± 0.128 . Antibody levels in sera of diseased individuals were significantly higher than those in control sera ($P < 0.001$). When a cutoff point of 2 standard deviations above the mean value for control sera was established, 70% of the serum specimens from tuberculosis patients and 2% of the specimens from healthy subjects were considered positive.

TABLE 1. Total amino acid composition of *M. tuberculosis* 50- to 55-kDa protein and *M. bovis* BCG 45- to 47-kDa protein

Amino acid	Mol% in:		
	45- to 47-kDa protein	50-kDa component	55-kDa component
Asx	2.5	4.0	10.6
Glx	3.3	7.6	6.0
Ser	5.3	5.5	5.0
Gly	11.5	18.2	7.4
His	0.0	1.2	0.4
Arg	2.3	2.6	2.5
Thr	5.7	4.0	5.4
Ala	19.3	16.8	19.2
Pro	21.2	17.8	21.9
Tyr	0.9	0.7	2.2
Val	7.9	5.0	5.8
Met	0.6	0.0	0.5
Ile	4.4	5.2	2.3
Leu	8.0	6.9	4.7
Phe	4.7	2.1	2.2
Lys	2.2	2.4	2.9

TABLE 2. Comparison of the proline-rich amino-terminal ends of different mycobacterial strains

Mycobacterium	Protein (kDa)	N-terminal sequence	Reference(s)
<i>M. tuberculosis</i>	50-55	DPEPAPPVVAAXD	This study
<i>M. bovis</i> BCG	45-47	APEPAPPVPFAAAPPA	22
<i>M. tuberculosis</i>	41	DPAPAPPVPXTAAAPPXXAA	15, 26
	55	TPPXEXPPP	21
<i>M. leprae</i>	25	DPAPLPPSTATAPSPA	26
<i>M. smegmatis</i>	38	EPAPPA	1a

DISCUSSION

In this study, we have described the similarities found between *M. tuberculosis* 50- to 55-kDa antigen and *M. bovis* BCG 45- to 47-kDa antigen. Cross-reactivity between these antigen complexes was demonstrated with the rabbit polyclonal antiserum against the *M. bovis* BCG protein and with a MAb generated against the *M. tuberculosis* protein. The epitope recognized by MAb 6A3 was apparently present only in *M. tuberculosis* 50- to 55-kDa and *M. bovis* BCG 45- to 47-kDa proteins. This finding is not surprising in view of the close antigenic relationship between *M. tuberculosis* and *M. bovis* BCG. Moreover, the existence of cross-reactive epitopes restricted to only this species has previously been demonstrated for other antigens (9). The reactivities of the polyclonal antiserum and of MAb 6A3 with the *M. tuberculosis* 50- to 55-kDa and *M. bovis* BCG 45- to 47-kDa antigens present in CF were substantially stronger than with proteins from SE, confirming previous ob-

servations that these protein complexes are preferentially secreted into the culture medium (3, 22). In view of this observation, we cannot exclude entirely the possibility that these molecules are present in other mycobacteria, since CF of only *M. smegmatis*, *M. vaccae*, and *M. paratuberculosis* were available for study.

It has been reported by Espitia and Mancilla (3) that the *M. tuberculosis* 50- to 55-kDa protein reacts with ConA-peroxidase after SDS-PAGE and blotting onto nitrocellulose, suggesting the presence of covalently bound carbohydrate. Examination by the same procedure of the purified *M. tuberculosis* 50- to 55-kDa and *M. bovis* BCG 45- to 47-kDa antigen complexes showed ConA binding. Furthermore, digestion with jack bean α -D-mannosidase produced a reduction in the molecular weight, thus indicating the presence of α -D-mannosyl linkages in these proteins. Moreover, jack bean α -D-mannosidase digestion abolished the reactivity of the glycoproteins with ConA. This result indicates that α -linked mannosyl residues at the nonreducing end of the chain, which are susceptible to jack bean α -D-mannosidase, are binding ConA. The presence of carbohydrates was also tested by labeling *M. tuberculosis* 50- to 55-kDa protein with biotin-hydrazide. Digestion of the biotin-labeled glycoproteins with jack bean α -D-mannosidase also resulted in a reduction of the molecular weight without complete elimination of the biotin label. Furthermore, these proteins could be labeled with biotin-hydrazide after digestion with jack bean α -D-mannosidase, indicating the presence of additional carbohydrate residues different from α -linked mannosyl residues. Although digestion of the 50- to 55-kDa *M. tuberculosis* protein resulted in the liberation of free mannose, as detected amperometrically in a Dionex system (13), the small amount of material examined did not permit quantification of the mannose content (data not shown).

The failure to demonstrate any change in ConA reactivity or migration in SDS-PAGE with almond α -D-mannosidase probably reflects the low activity of this enzyme with glycoprotein substrates (12).

ConA-binding proteins of 39, 25, 22, and 19 kDa have been reported for *M. bovis* BCG (5) and a 38-kDa protein has been reported for *M. tuberculosis* (3). More recently, ConA reactivity was found in a recombinant *M. tuberculosis* 19-kDa protein expressed in *M. smegmatis* (7). Overall, there seems to be substantial evidence indicating the existence of glycoproteins in mycobacteria. However, definitive chemical proof for the existence of carbohydrate residues covalently linked to mycobacterial proteins is still missing.

It is noteworthy that proline-rich N-terminal sequences have been previously found in culture supernatants of *M. tuberculosis* and *M. bovis* BCG (6, 21) (Table 2). The N terminus of the *M. tuberculosis* 41-kDa antigen (MPT32) is very similar to the N termini of the 50- to 55- and 45- to 47-kDa proteins. Moreover, a protein with similar characteristics, deduced from the DNA sequence of the cloned gene, has been found in *M. leprae*, and its secreted nature can be inferred from the 39-residue-long signal peptide at the amino terminus (26). Further analytical work and results of current research will help to establish the structural basis for the common features as well as the noted differences, such as the relative molecular weights reported for these proteins.

Finally, it is worth pointing out that antibodies to the *M. bovis* BCG 45- to 47-kDa protein were demonstrated in guinea pigs after immunization with live, but not with dead, bacteria (22), whereas antibodies to the *M. tuberculosis* counterpart were found in a large proportion (70%) of serum specimens from pulmonary tuberculosis patients in Mexico. Taken to-

FIG. 4. Serum antibody recognition of the isolated 50- to 55-kDa antigen in 77 individuals with pulmonary tuberculosis (patients) and 92 healthy individuals (control) as determined by ELISA. A threshold value of 2 standard deviations above the mean optical density of the control group was chosen (horizontal line). Each circle represents an individual serum sample tested at a 1/1,000 dilution. OD_{490 nm}, optical density at 490 nm.

gether, these observations suggest a diagnostic potential for these antigens, as well as a possible association with infection.

ACKNOWLEDGMENTS

We thank Juraj Ivanyi and Marchal Gilles for critical reading of the manuscript.

This work was in part conducted while C. Espitia was a fellow of the Commission of the European Communities (bursary "Marie Curie"), and she is the current holder of a Wellcome Trust fellowship.

REFERENCES

1. **Bulletin of the World Health Organization.** 1982. Immunological research in tuberculosis: memorandum from a W.H.O. meeting, 1982. Bull. W.H.O. **60**:723-727.
- 1a. **Espitia, C., et al.** Unpublished data.
2. **Espitia, C., I. Cervera, R. González, and R. Mancilla.** 1989. A 38-kd *Mycobacterium tuberculosis* antigen associated with infection. Its isolation and serologic evaluation. Clin. Exp. Immunol. **77**:373-377.
3. **Espitia, C., and R. Mancilla.** 1989. Identification, isolation and partial characterization of *Mycobacterium tuberculosis* glycoprotein antigens. Clin. Exp. Immunol. **77**:378-383.
4. **Espitia, C., E. Scitutto, O. Bottasso, R. González-Amaro, R. Hernández-Pando, and R. Mancilla.** 1992. High antibody levels to the mycobacterial fibronectin-binding antigen of 30-31 kD in tuberculosis and lepromatous leprosy. Clin. Exp. Immunol. **87**:362-367.
5. **Fifis, T., C. A. J. Costopoulos, A. Bacic, and P. R. Wood.** 1991. Purification and characterization of major antigens from a *Mycobacterium bovis* culture filtrate. Infect. Immun. **59**:800-807.
6. **Franssen, J. D., P. Herion, and J. Urbain.** 1981. Selection of myeloma lines suitable for hybridomas, p. 645-648. In H. Peeters (ed.), Peptides of the biological fluids, vol. 28. Pergamon Press, London.
7. **Garbe, T., D. Harris, M. Vordermeier, R. Lathigra, J. Ivanyi, and D. Young.** 1993. Expression of the *Mycobacterium tuberculosis* 19-kilodalton antigen in *Mycobacterium smegmatis*: immunological analysis and evidence of glycosylation. Infect. Immun. **61**:260-267.
8. **Harris, D. P., H. M. Vordermeier, E. Román, R. Lathigra, S. J. Brett, C. Moreno, and J. Ivanyi.** 1991. Murine T cell stimulatory peptides from the 19-kDa antigen of *Mycobacterium tuberculosis*. J. Immunol. **147**:2706-2712.
9. **Ivanyi, J., A. Morris, and M. Keen.** 1985. Studies with monoclonal antibodies to mycobacteria, p. 59-89. In A. J. L. Macario and E. Conway de Macario (ed.), Monoclonal antibodies against mycobacteria, vol. I. Academic Press, New York.
10. **Jackett, P. S., G. H. Bothamley, H. V. Batra, A. Mistry, D. B. Young, and J. Ivanyi.** 1988. Specificity of antibodies to immunodominant mycobacterial antigens in pulmonary tuberculosis. J. Clin. Microbiol. **26**:2313-2318.
11. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680-685.
12. **Lee, C.** 1972. α -Mannosidase, β -galactosidase from sweet almond emulsin. Methods Enzymol. **28**:699-702.
13. **Mark, R., R. Hardy, R. Reid Townsend, and C. Lee.** 1988. Monosaccharide analysis of glycoconjugates by anion exchange chromatography with pulsed amperometric detection. Anal. Biochem. **170**:54-62.
14. **Matsudaira, P.** 1987. Sequence from picomole quantities of proteins onto polyvinylidene difluoride membranes. J. Biol. Chem. **262**:10035-10038.
15. **Nagai, S. H., M. Wiker, M. Harbor, and M. Kinomoto.** 1991. Isolation and partial characterization of main protein antigens in the culture fluid of *Mycobacterium tuberculosis*. Infect. Immun. **59**:372-382.
16. **O'Farrell, P. H.** 1975. High-resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250**:4007-4021.
17. **Orme, I., A. P. Andersen, and H. Boom.** 1993. T cell response to *Mycobacterium tuberculosis*. J. Infect. Dis. **167**:1481-1497.
18. **O'Shannessy, D. J.** 1990. Antibodies biotinylated via sugar moieties. Methods Enzymol. **184**:163-166.
19. **Pal, P. G., and H. A. Horwitz.** 1992. Immunization with extracellular proteins of *Mycobacterium tuberculosis* induces cell-mediated immune responses and substantial protective immunity in a guinea pig model of pulmonary tuberculosis. Infect. Immun. **60**:4781-4792.
20. **Parekh, B. S., H. B. Metha, M. D. West, and R. C. Montecarlo.** 1985. Preparative elution of proteins from nitrocellulose membranes after separation by sodium dodecyl sulfate-polyacrylamide electrophoresis. Anal. Biochem. **148**:87-92.
21. **Romain, F., P. Augier, P. Pescher, and G. Marchal.** 1993. Isolation of a proline-rich mycobacterial protein eliciting delayed-type hypersensitivity reactions only in guinea pigs immunized with living mycobacteria. Proc. Natl. Acad. Sci. USA **90**:5322-5326.
22. **Romain, F., A. Laqueyrie, P. Militzre, P. Pescher, P. Chavarot, M. Lagranderie, G. Auregan, M. Gheorghiu, and G. Marchal.** 1992. Identification of a *Mycobacterium bovis* BCG 45/47-kilodalton antigenic complex, an immunodominant target for antibody response after immunization with living bacteria. Infect. Immun. **61**:742-750.
23. **Seibert, F. B.** 1949. Isolation of three different proteins and two polysaccharides from tuberculin by alcohol fractionation: their chemical and biological properties. Am. Rev. Res. Tuberc. **59**:86-101.
24. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350-4354.
25. **Vordermeier, H. M., D. P. Harris, G. Friscia, E. Román, H. M. Sourcel, C. Moreno, and J. Ivanyi.** 1991. T cell repertoire in tuberculosis: selective anergy to immunodominant epitope of 38-kDa antigen. Eur. J. Immunol. **22**:2631.
26. **Wiele, B., M. Van Agterveld, A. Janson, J. Clark-Curtiss, T. Rinke De Wit, M. Harboe, and J. Thole.** 1993. Characterization of *Mycobacterium leprae* antigen related to the secreted *Mycobacterium tuberculosis* protein MPT32. Infect. Immun. **62**:252-258.
27. **Wiker, H. G., and M. Harboe.** 1992. The antigen 85 complex: a major secretion product of *Mycobacterium tuberculosis*. Microbiol. Rev. **56**:648-661.