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Generation and Characterization of Monoclonal Antibodies to 28-, 35-, and 65-Kilodalton Proteins of *Mycobacterium tuberculosis*

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Three monoclonal antibodies (H60.15, H61.3, and H105.10) directed to protein antigens of *Mycobacterium tuberculosis* were obtained and characterized. H60.15 recognizes a protein with a molecular mass of 28 kilodaltons (kDa) with broad cross-reactivity on a panel of 12 species and strains of mycobacteria. H61.3 reacts with a 35-kDa protein present in *M. tuberculosis*, *Mycobacterium bovis* BCG, and *M. africanum*. On the basis of the antigen molecular masses and competition experiments with other monoclonal antibodies, H60.15 and H61.3 seem to be the first described monoclonal antibodies to these *M. tuberculosis* proteins. H105.10 binds to the cross-reactive 65-kDa protein present in mycobacteria. Epitope mapping of H105.10 was performed by using the *M. leprae* DNA sublibrary available in bacteriophage λgt11 for this antigen and revealed that its epitope resides in the region from amino acids 20 to 54. The 28-, 35-, and 65-kDa antigens isolated by immunoblotting and presented on nitrocellulose to pleural effusion T cells from tuberculosis patients induced a proliferative response, indicating the presence of T-cell epitopes. These observations indicate that two protein antigens should be added to the list of antigens detectable in *M. tuberculosis* by monoclonal antibodies. The common feature of such proteins, the elicitation of an immune response of limited or broad cross-reactivity for mycobacteria, encourages the search for their role in the pathogenesis of mycobacterioses.

Tuberculosis and leprosy, diseases caused by mycobacteria, represent important subjects of multidisciplinary investigation owing to the urgent need for rapid and reliable diagnostic tests and effective vaccines for disease control, mainly in developing countries.

A large number of monoclonal antibodies (MAbs) have been raised that react with *Mycobacterium tuberculosis* antigens. Proteins with molecular masses of 71, 65, 38, 23, 19, 14, and 12 kilodaltons (kDa) have been defined in this way. An international workshop was held to compare and standardize these reagents (H. D. Engers and workshop participants, Letter, Infect. Immun. **51**:718–720, 1986). The more-specific MAb-defined epitopes known to date are present not only in *M. tuberculosis* preparations but also in preparations of a limited number of closely related species (*M. bovis* and *M. africanum*) that constitute the so-called *M. tuberculosis* complex. Some of these reagents have been used to create new serodiagnostic assays for tuberculosis (12, 14) and to screen genomic DNA libraries of *M. tuberculosis* in bacteriophage λgt11 (22, 26). The recombinant antigens isolated by genetic engineering have been used to define the reactivity of specific T-cell clones derived from tuberculosis patients (20).

It is probable that the immune response to chronic infections due to complex microorganisms such as *M. tuberculosis* involves more antigenic determinants than those defined by the MAbs reported thus far (19). Moreover, still-undetected antigens and epitopes could well contain species-specific and/or protective *M. tuberculosis* determinants or could represent molecules which are possible targets for drugs. Strategies to obtain MAbs to such antigens can be devised.

We describe here the specificity of three novel MAbs obtained after a long mouse immunization regimen by using live *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) and killed *M. tuberculosis*. Two of the MAbs are of broad cross-reactivity on 12 strains of mycobacteria, whereas the third, H61.3, recognizes only mycobacteria of the *M. tuberculosis* complex. Direct comparison with well-characterized MAbs indicates that two of the antigens, of 28 (MAb H60.15) and 35 kDa (MAb H61.3), have not been previously defined by use of the reported anti-*M. tuberculosis* MAbs, whereas MAb H105.10 recognizes the 65-kDa protein, of which at least 14 different epitopes have already been defined by MAbs (2).

Since protection from mycobacterial infection is a function of the cell-mediated immune response (3), it is of interest to determine whether the antigens defined by MAbs contain epitopes recognized by T lymphocytes. The recent development of a proliferative-T-cell assay using nitrocellulose-bound antigens for screening polypeptides with defined molecular weight (25) provides the possibility of direct identification of antigens recognized by human T lymphocytes. We describe here its application to the identification of *M. tuberculosis* antigens recognized by murine MAbs and involved in the human T-cell response.

MATERIALS AND METHODS

Mycobacterium and antigen preparations. Live lyophilized BCG vaccine was purchased from Istituto Berna, Como, Italy. Lyophilized *M. tuberculosis* H37Ra and *M. butyricum* (*M. smegmatis*) were purchased from Difco (catalog no.

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3114-33-8 and 0640-33-7, respectively; DID Italia, Milan, Italy). *Escherichia coli* Y1090 was grown in LB medium.

To obtain sonically treated material, bacteria were suspended at 10 mg/ml, dialyzed against phosphate-buffered saline (PBS) for 48 h, and sonicated by 15-s pulses at 15-s intervals on ice for a total of 5 min. Less than 5% of the bacilli were found to be intact by microscopy after such treatment. The sonically treated material was then centrifuged at 600 × *g* for 10 min, and the supernatant was designated sonic extract and used as indicated. In some experiments, a fraction designated cytoplasm preparation was prepared by centrifugation of sonic extracts at 16,000 × *g* for 20 min followed by filtration of the supernatant through 0.2-μm-pore-size membrane filters (Millipore Corp.). The pellet was resuspended in PBS and designated the cell wall preparation.

Cell suspensions of a panel of 11 strains and species of mycobacteria, cultured as described previously (26), were kindly made available by C. Grossinsky (Albert Einstein College of Medicine, Bronx, N.Y.). To test differential binding of MAbs to these strains and to strain H37Ra (Difco), bacteria were homogenized as described previously (26). Briefly, 1 ml of acid-washed glass beads was added to 0.2 ml of pelleted bacteria (which had been heated previously for 20 min at 70°C), and the mixture was homogenized for 2 min in a Braun rotary homogenizer. Preparations of disrupted mycobacteria were then centrifuged at 600 × *g* for 10 min, and the supernatant was used to coat polyvinyl chloride (PVC) microtiter plates.

Mice, immunizations, and hybridomas. BALB/c mice were injected subcutaneously five times at 20-day intervals with approximately 25 μl of an emulsion containing 0.5 mg of lyophilized live BCG vaccine per ml and 2.5 mg of *M. tuberculosis* per ml of incomplete Freund adjuvant (Behring, Scoppito, Italy). Four days after the sixth injection of emulsion (intraperitoneal), one of the mice was sacrificed and 17.5×10^7 spleen cells were fused with 3.5×10^7 P3X63Ag8U.1 myeloma cells as previously described (5). After fusion, cells were suspended in selective HAT (hypoxanthine-aminopterin-thymidine)-containing medium and seeded in 144 wells (six 24-well trays) at 0.25×10^6 myeloma cells per well.

Screening of hybridoma supernatants was performed by indirect radioimmunobinding (RIA) on *M. tuberculosis* H37Ra sonic extract in multiwell plastic plates as described below. Cloning of positive hybridomas in soft agar, production of ascitic fluid, purification of antibodies from ascites, and radioiodination by the chloramine T method of Greenwood et al. (11) were performed as previously reported (5).

RIA assays. Protein in sonic extracts and in preparations of disrupted mycobacteria was adjusted to 1.0 mg/ml and distributed in 50-μl aliquots to coat 96-well PVC microtiter plates (catalogue no. 77-172-05; Flow Laboratories, Inc., McLean, Va.). The preparations in the plates were treated with antigen for 18 h and washed three times in PBS containing 0.5% bovine serum albumin, and residual protein-reactive sites were saturated for 2 h with 5% bovine serum albumin in PBS-NaN_3 and either frozen at −90°C for later use or processed. After 6 h of incubation with hybridoma supernatants or diluted ascites, the plates were washed four times and reacted for 18 h with ^{125}I-radioiodinated goat anti-mouse immunoglobulin G (IgG) affinity-purified antibodies prepared as previously described (4). After six washes with PBS, the wells were cut with incandescent steel wire and counted for bound radioactivity. Binding was expressed as the binding index: bound radioactivity divided by the background radioactivity value of non-antigen-coated wells.

Competition experiments were performed as described by Hewitt et al. (12), with minor modifications. Briefly, microtiter plates were coated with 100 μl of strain H37Ra sonic extract at 0.5 mg/ml in PBS for 2 h at 22°C. Reactive sites were blocked with 3% bovine serum albumin for 20 h at 4°C. Serially diluted competitor MAb samples were added in triplicate in 50-μl aliquots and left for 4 h at 22°C. A 10-μl portion of chloramine T-iodinated purified MAb (2×10^4 cpm per well) was added, and the sample and the iodinated MAb were thoroughly mixed. The plates were incubated for 20 h at 4°C, washed, and counted for bound radioactivity.

MAbs to *M. tuberculosis* used for competition experiments, SAI.D2D1 (IT 17), WTB-71 (IT 23), WTB-72 (IT 15), and WTB-78 (IT 13), have been already defined and are listed in an international workshop report (Engers et al., Letter). PTF 22.1 is an anti-human lymphocyte antigen A,B monomorphic determinant characterized previously (6).

SDS-PAGE and immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in slab gels in a discontinuous Tris buffer system (17). Sonic extracts of mycobacteria at a protein concentration of 1 mg/ml were added with sample buffer and loaded onto the gels.

In some experiments, before gel electrophoresis, mycobacterial sonic extracts (5 mg of protein per ml) were added with proteinase K (catalog no. 24568; E. Merck AG, Darmstadt, Federal Republic of Germany) to a final concentration of 0.1 mg/ml and agitated at 4°C for 15 min. SDS was then added to a final concentration of 3%, and the samples were boiled for 3 min. After electrophoresis, proteins were transferred to nitrocellulose paper (catalog no. BA85; Schleicher & Schuell, Inc., Keene, N.H.) by the method of Towbin et al. (23) in a Bio-Rad Laboratories Trans-Blot apparatus. Blotting was performed for 2 h at 4°C at 400 mA in Tris-glycine-methanol buffer, pH 8.5. After reactive sites were blocked with 30% fetal calf serum in PBS for 30 min, 5-mm-wide strips were cut out of the nitrocellulose paper and each strip was reacted with MAb culture supernatant in a separate tube for 18 h at 4°C. Bands formed by immunocomplexes were evidenced by adding goat anti-mouse IgG affinity-purified antibodies coupled to horseradish peroxidase (catalog no. P-8375; Sigma Chemical Co., St. Louis, Mo.) by the method of Farr and Nakane (10). *O*-Dianisidine (Sigma; catalog no. D-9143) dissolved in methanol was used as the chromogenic substrate. In some experiments, a biotin-avidin-based ABC kit (PK 4002; Vector Laboratories, Burlingame, Calif.) was used as the developing system. In each experiment, molecular weight standards (molecular weight markers kit, catalog no. MW-SDS-70L; Sigma) were also subjected to blotting after SDS-PAGE and were stained with amido black. In the experiments involving T-cell cultures, the blotted nitrocellulose strips were saturated and cut at the level of proteins recognized by the three MAbs. Several small disks with a diameter corresponding to that of the culture microtiter well were prepared and inserted on the bottom of each well of the plate.

Determination of the epitope of MAb H105.10. The epitope of MAb H105.10 was determined by the use of λgt11 lysogenic *E. coli* clones as previously described (16). Briefly, cell lysates of selected lysogenic clones from the sublibrary of the 65,000-molecular-weight-*M. leprae*-protein gene obtained by Mehra et al. (18) were subjected to SDS-PAGE and blotting. The blots were reacted with H105.10 ascites at a 1:1,000 dilution, and immunocomplex formation was evi-

denced by immunoperoxidase-coupled second antibody. Two of the recombinant clones used (Y3143 and Y3150) were from an *M. tuberculosis* DNA library (13).

Preparation of lymphocytes and proliferation assay. Fresh and cryopreserved cells from the ascitic pleural effusions of two patients, an 18-year-old male and a 21-year-old female, with primary tuberculous infections (with pleurisy) and with positive skin tests to purified protein derivative (PPD) were used. After centrifugation on a discontinuous density gradient on Ficoll-Hypaque, the cells were suspended in RPMI 1640 medium supplemented with 10% heat-inactivated pooled human serum, 2 mM glutamine, and 100 U of penicillin and 100 μg of streptomycin per ml. More than 80% of the cells were T lymphocytes, as determined by immunofluorescence using anti-CD3 antibodies. These cell populations contained a high precursor frequency of mycobacterium-specific T cells, as determined by limiting-dilution analysis (1/1,000 and 1/2,000).

Cells (10^6/ml) were cultured in 96-well flat-bottom microtiter plates for 6 days with different concentrations of PPD (Serum Institute, Copenhagen, Denmark) and H37Ra (Difco). Solid-phase antigen was prepared in two ways: by dotting different concentrations of PPD or H37Ra on small nitrocellulose disks and by blotting H37Ra extracts on nitrocellulose strips as described above. After 5 days of incubation, the cultures were resuspended and the nitrocellulose disks were discarded prior to the addition of radioactive [^{3}H]thymidine (1 μCi; Amity-PG, Milano, Italy). The cultures were incubated for 16 h and then automatically harvested onto glass fiber filters. Proliferation, as correlated with thymidine incorporation, was measured by liquid scintillation spectroscopy. The results are expressed as the mean counts per minute ± standard deviation of the mean of duplicate or triplicate cultures.

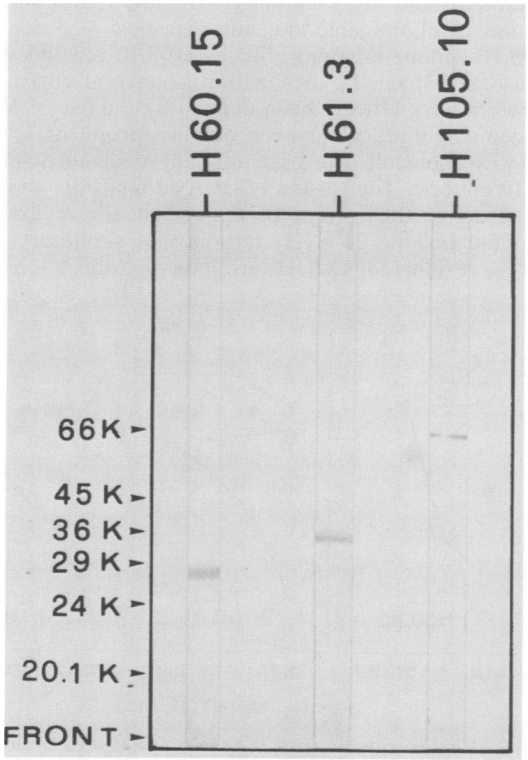

FIG. 1. Immunoblot analysis of the proteins recognized by MAbs. A sonic extract of *M. tuberculosis* H37Ra was fractionated by SDS-PAGE (10% acrylamide), electrophoretically transferred to nitrocellulose paper, and processed for immunoblotting as reported in the text. Nitrocellulose strips were incubated for 18 h at 4°C with the indicated MAb culture supernatants, washed, and reacted with horseradish peroxidase-coupled goat anti-mouse IgG, followed by incubation with *O*-dianisidine and H_2O_2. Migrations of molecular weight standards, run in separate lanes, blotted, and stained with amido black, are indicated on the left.

TABLE 1. Characteristics of antigens recognized by anti-*M. tuberculosis* MAbs

MAb	Band molecular mass (kDA)	Strain specificity[a]	Distribution[b]	
			Cytoplasm	Cell wall
H60.15	28	Broad cross-reactivity	1.79	2.73
H61.3	35	*M. tuberculosis* complex +, *M. smegmatis* −	1.69	4.99
H105.10	65	Broad cross-reactivity	1.77	4.20

[a] Tested by SDS-PAGE followed by immunoblotting on *M. tuberculosis*, *M. bovis* BCG, and *M. smegmatis*. Protease treatment, performed as described in the text, indicated proteinase sensitivity for all three MAbs.

[b] Determined by RIA on cell wall and cytoplasmic preparations of strain H37Ra. Values for antigen distribution are expressed as binding index (bound radioactivity value divided by the background radioactivity value of non-antigen-coated microtiter wells). Standard error was within ±5%. Background radioactivity was 113, 146, and 190 cpm for radioiodinated MAbs H60.15, H61.3, and H105.10, respectively. The protocol followed allows considerable cross-contamination between cytoplasmic and cell wall components. However, since the binding index to the cytoplasmic preparation is very similar for the three MAbs, we are confident that the binding to the cell wall preparation reflects a real antigen distribution.

RESULTS

Hybridoma production. Immunization of mice and cell fusion were performed as described in Materials and Methods.

Vigorous cell growth was observed in 137 of 144 wells, and the corresponding supernatants were screened for the presence of antibodies to *M. tuberculosis* H37Ra by solid-phase RIA. Eighteen supernatants bound sonicated H37Ra preparations from two to five times over background, and hybridomas in the corresponding wells were cloned in soft agar. Upon rescreening by RIA, one of the clones from each hybridoma was chosen on the basis of strong reactivity with H37Ra and grown to mass culture.

Characterization of the recognized antigens. Since MAbs reacting with denatured proteins (i.e., subjected to SDS and boiling) are likely to recognize sequential epitopes, we tried to select for such MAbs by a second screening performed on sonicated H37Ra preparations after SDS-PAGE and immunoblotting. Of the 18 clone supernatants tested in immunoblotting, 7 bound discrete bands.

The molecular masses of the recognized bands were estimated for two groups of three MAbs each. MAbs from clones H9.19, H54.6, and H60.15 reacted with a major band of 28 kDa. MAbs from clones H1.17, H28.15, and H61.3 stained a major band of 35 kDa. Bands of lower molecular mass and of variable intensity among different experiments were also observed. Clone H105.10 bound a single band of 65 kDa. MAbs H60.15, H61.3, and H105.10 were chosen for further characterization on the basis of stronger reactivity; their immunoblotting profiles are shown in Fig. 1. Their isotypes were determined to be IgG1, IgG2b, and IgG1, respectively. Table 1 summarizes the characteristics of the antigens recognized. Treatment of *M. tuberculosis* H37Ra sonic extract with a broad-specificity proteolytic enzyme,

TABLE 2. Specificity of MAbs

Incubation mixture	Mycobacterial species and strain(s)	MAb binding index value[a]		
		H60.15	H61.3	H105.10
Culture supernatants	*M. tuberculosis* H37Ra[b]	2.86	4.50	5.90
	M. tuberculosis H37Rv	1.12	3.22	1.54
	M. tuberculosis Erdman	1.04	2.18	1.45
	M. bovis	1.13	4.36	1.95
	M. bovis BCG	1.22	3.86	1.95
	M. africanum	1.59	4.54	1.81
	M. marinum	1.59	1.50	1.68
	M. haemophilus	1.31	1.18	2.36
	FMR 51[c]	1.27	1.31	1.40
	M. smegmatis	1.31	1.27	2.22
	M. avium	1.97	2.09	2.00
	M. intracellularis	1.45	1.45	1.81
Ascites[d]	*M. tuberculosis* H37Ra[b]	7.4	3.0	5.4
	M. bovis BCG	5.1	3.3	7.5
	M. leprae	6.3	1.2	5.9

[a] Radioactivity incorporation in the absence of disrupted preparations was 440 cpm. Samples were run in triplicate. Standard errors were within ±5% and were omitted from the table.
[b] Sonicated preparation from lyophilized strain H37Ra bacteria (Difco).
[c] Mycobacterial isolate from a leprosy lesion.
[d] Experiments were performed by using ascitic fluid diluted 1:100 in culture medium; bacteria were sonicated as described in the text.

proteinase K, caused the disappearance of the bands after immunoblotting for all three MAbs. Diminished binding, from 12 to 35% that of the untreated control, was also observed in RIA after treatment of the sonic extract with proteinase K before coating of microtiter plates (data not shown). This indicates the proteinaceous nature of at least part of the three recognized epitopes.

Experiments involving binding of MAbs to the cytoplasmic preparation and to the cell wall preparation of sonic extracts of strain H37Ra indicated that H60.15 was present in both the cytoplasmic and cell wall preparations, whereas H61.3 and H105.10 recognized antigens associated mostly with the cell wall (Table 1).

Species specificity. For initial characterization of species specificity, H60.15, H61.3, and H105.10 were tested in parallel for reactivity with sonic extracts of *M. tuberculosis* H37Ra, *M. bovis* BCG Danish, *M. smegmatis*, and *E. coli* Y1090 after SDS-PAGE and immunoblotting. In this analysis, none of the MAbs reacted with *E. coli* sonic extracts; H60.15 and H105.10 reacted with the same bands on all three mycobacterial preparations tested, whereas MAb H61.3 reacted with the same bands in *M. tuberculosis* H37Ra and BCG Danish preparations but did not react with the *M. smegmatis* sonic extract. To evaluate further the strain specificity of the epitopes, we tested by indirect RIA the reactivity of H60.15, H61.3, and H105.10 with disrupted or sonicated cell preparations of the different species and strains of mycobacteria reported in Table 2. All three MAb culture supernatants bound to the H37Ra strain more than to the other strains. H60.15 and H105.10 bound to all the other mycobacteria tested to the same extent. H61.3 bound preferentially (from two to four times over background) to *M. bovis*, *M. bovis* BCG, *M. tuberculosis* Erdman, *M. africanum*, and *M. tuberculosis* H37Rv but bound to a lesser extent to the other strains and species. When tested in the form of diluted (1:100) ascites and on sonicated mycobacterium preparations, high binding indexes were evident on all three strains tested for the MAbs H60.15 and H105.10, whereas MAb H61.3 again bound only to the *M. tuberculosis*-complex mycobacteria and not to *M. leprae*.

Taken together with the immunoblotting profiles on sonic extracts of different mycobacteria, these data indicate an *M. tuberculosis*-complex reactivity for the epitope recognized by MAb H61.3 and a broad cross-reactivity among mycobacteria for the epitopes recognized by MAbs H60.15 and H105.10.

Competition experiments. Since all three MAbs strongly recognized epitopes present in sonic extracts of strain H37Ra, we used this strain to investigate whether the new MAbs presented a cross-reactivity for the same epitopes as did MAbs described by other investigators and whether they recognized proteins with similar characteristics. Inhibition experiments were performed on PVC-immobilized sonic extracts, as described in Materials and Methods. The results are reported in Fig. 2. Binding of the radioiodinated MAbs was inhibited to a great extent by the autologous unlabeled antibody. Radioiodinated MAb H60.15 (28-kDa antigen) was not found to compete significantly with MAb SAI.D2D1 (IT 17; 23-kDa antigen) or WTB-78 (IT 13; 55 to 65 kDa). Binding of radioiodinated MAb H61.3 was not inhibited by MAb WTB-71 (IT 23) or WTB-72 (IT 15), both of which recognized epitopes on a 38-kDa, *M. tuberculosis*-complex-specific antigen. Radioiodinated H105.10 (65 kDa) was not observed to compete significantly with IT 13 (55- to 65-kDa antigen) or with IT 17. MAb PTF 22.1 anti-human lymphocyte antigen A,B did not inhibit the binding of any of the three MAbs. This result indicates that those MAbs recognize epitopes different from those recognized by previously described MAbs and suggests that H60.15 and H61.3 may well recognize novel antigenic molecular species.

H105.10 epitope mapping. Since H105.10 reacted with a 65-kDa band, since the localization of several epitopes on this protein have already been defined by the use of MAbs, and since a sublibrary has been obtained from the DNA of the 65-kDa molecule, we used this approach to define the H105.10 epitope. The results of such an analysis, shown in Table 3, define the epitope as residing in the region from amino acid residue 20 (NH_2 terminus of sublibrary clone Y3243) to residue 54 (COOH terminus of clone Y3223).

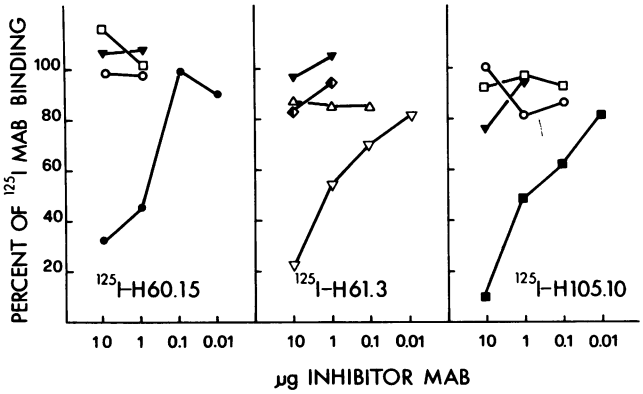

FIG. 2. Epitope specificity of MAbs as investigated by competition assays. Binding of ^{125}I-labeled MAbs H60.15, H61.3, and H105.10 to H37Ra sonic extract-coated microtiter wells was inhibited in the presence of the indicated final concentrations of unlabeled MAbs. Values are expressed as percentages of the maximum binding obtained in the absence of competitor MAb. Each datum point represents the mean from three wells; the standard deviation was within ±4%. The unlabeled competitor MAbs tested were: ●, H60.15; ▽, H61.3; ■, H105.10; ○, IT 13 (WTB-78); △, IT 15 (WTB-72); □, IT 17 (SAI.D2D1); ◇, IT 23 (WTB-71); ▼, PTF 22.1 anti-human lymphocyte antigen class I MAb.

Identification of T-cell epitopes. The presence of T-cell epitopes on the proteins defined by the new MAbs was determined by a lymphocyte proliferation assay. For this purpose, ascitic effusions of two patients with tuberculous pleuritis were used. This cell population was particularly appropriate because it contained *M. tuberculosis*-specific T cells at high frequency, as determined by limiting dilution analysis (1/1,000 and 1/2,000). Ascitic T cells proliferate in the presence of PPD in either soluble or nitrocellulose-bound form, as shown in experiment 1 of Table 4. As described previously for the T-cell response to viral antigen (25), a higher concentration of a solid-phase antigen was required to stimulate a response equivalent to that obtained with soluble antigen. No proliferation was observed in the absence of antigen, and the nitrocellulose itself was neither toxic nor mitogenic.

To determine whether proteins defined by the three new MAbs contain T-cell epitope(s), experiment 2 of Table 4 was performed. Mycobacterial strain H37Ra extract was subjected to SDS-PAGE and transferred to a nitrocellulose membrane, which was then cut at the level of proteins recognized by the three MAbs, evidenced by immunoperoxidase staining in separate lanes of the same blot. It was found that 28-, 35-, and 65-kDa proteins bound to nitrocellulose have epitopes recognized by the human T-cell repertoire.

DISCUSSION

The search for *M. tuberculosis* antigens and epitopes important for immunological protection and for serodiagnosis in tuberculosis has been greatly assisted by the use of MAbs. Three MAbs directed to different protein molecules of *M. tuberculosis*, two of which present characteristics not described previously, are reported in this study.

MAb H60.15 recognizes a 28-kDa molecule that is present in several strains and species of mycobacteria. The molecule described in the literature most similar in molecular mass and strain distribution (broad) to the H60.15 antigen is detected by MAb SAI.D2D1 (IT 17), with a molecular mass of 23 kDa (Engers et al., Letter). The possibility that the two MAbs recognize the same epitope was ruled out by competition experiments.

MAb H61.3 seems to be the most interesting of the three MAbs reported here, since no *M. tuberculosis*-complex-restricted protein of 35 kDa has been detected previously by MAbs. MAbs WTB-71 and WTB-72 recognize a protein of 38 kDa, quite different in molecular mass from that recognized by MAb H61.3. Moreover, the 38-kDa protein has been purified from the soluble fraction of *M. tuberculosis* H37Rv after ultracentrifugation and filtration through a 0.45-μm-pore-size filter (24), whereas the antigen recognized by MAb H61.3 is associated mostly with the cell wall of *M. tuberculosis* H37Ra. A protein of 35 kDa recognized in sonic extracts of *M. tuberculosis* by antibodies present in the sera of tuberculosis patients has been studied recently (21). *M. tuberculosis* antigen 5, purified on conventional immunoadsorbents, presents with a molecular mass of 28,500 to 35,000 Da and a limited cross-reactivity in skin tests (7, 8). The possibility that these antigens could be related to the H61.3 antigen merits further investigation.

Minor bands of lower molecular mass were stained by both H60.15 and H61.3 in immunoblotting. The multiband appearance of MAb-defined mycobacterial antigens is a recurrent feature and has been explained in several ways (15). In the case of MAbs H60.15 and H61.3, since the intensity of staining of the lower-molecular-mass bands varied greatly among different experiments, these probably represent degradation products of the 28- and 35-kDa bands owing to either proteolysis or disruption of associated carbohydrate chains during sonication.

MAb H105.10 recognizes the 65-kDa antigen present in abundance on several species of mycobacteria (H. D. Engers and workshop participants, Letter, Infect. Immun. **48**:603–605, 1985; and Engers et al., Letter). Its reactivity with *M. leprae* (Table 2) was confirmed by epitope mapping by using the λgt11 DNA sublibrary derived for the 65-kDa antigen of *M. leprae* (18). Such analysis revealed that the epitope recognized by H105.10 is in the region of amino acids 20 to 54.

Therefore, H105.10 seems to recognize an epitope in the same region as that recognized by MAbs Y1.2 and C1.1 (18). In fact, Y1.2 and C1.1 also recognize predominantly a 65-kDa band on blots of mycobacterial lysates, whereas most of the other described anti-65-kDa MAbs stain several bands, mainly of 55 and 65 kDa. The finding that the epitope

TABLE 3. Epitope definition of MAb H105.10 by reactivity in immunoblot with lysogens prepared in *E. coli* by using λgt11 phages selected from a DNA sublibrary of the 65-kDa protein of *M. leprae*

Clone	Amino acids[a]	Reactivity with lysogens
Y3223	1–54	+
Y3203	1–138	+
Y3243	20–199	+
Y3246	100–223	−
Y3240	361–523	−
Y3189	480–541	−
Y3143[b]	c54–541	−
Y3201	269–1	−
Y3150[b]	Whole protein	+
λgt11		−

[a] Numbered starting from the first methionine in the open reading frame reported by Mehra et al. (18). This has been shown to be the N-terminal residue in the naturally occurring 65-kDa protein (9).

[b] Recombinant clone from the *M. tuberculosis* DNA library in λgt11.

TABLE 4. Proliferation of ascitic lymphocytes from tuberculosis patients in the presence of *M. tuberculosis* antigens[a]

Expt	Antigen (final concn, μg/ml)	Lymphocyte proliferation[b] in the presence of:	
		Nitrocellulose-bound antigen	Soluble antigen
1		111 ± 24	
	PPD (20)	8,457 ± 3,538	54,173 ± 2,969
	PPD (2)	6,952 ± 862	59,941 ± 892
	PPD (0.2)	1,696 ± 926	38,851 ± 3,319
2		65 ± 30	
	H37Ra (10)	23,817 ± 5,595	61,145 ± 2,164
	H37Ra (1)	ND	49,915 ± 3,675
	H37Ra (0.1)	21,384 ± 707	33,628 ± 3,206
	H37Ra, 65 kDa	9,576 ± 1,350	ND
	H37Ra, 35 kDa	12,566 ± 1,692	ND
	H37Ra, 28 kDa	7,776 ± 2,520	ND

[a] Unfractionated ascitic lymphocytes (10^6/ml) were cultured for 6 days with *M. tuberculosis* H37Ra in the form of nitrocellulose blots or soluble antigen.

[b] Proliferation, as correlated with radioactive thymidine incorporation, was determined at day 5. The results are expressed as the mean counts per minute of duplicate or triplicate cultures. H37Ra extracts were prepared and run on SDS-PAGE, and proteins were blotted as described in Materials and Methods. ND, Not done.

in the region of amino acids 20 to 54 is detectable only on the 65-kDa molecular species of the antigen suggests the presence of a preferential proteolytic target site near the NH_2 terminus of the protein.

The antigens of H60.15, H61.3, and H105.10 have been found to be associated mostly with crude cell wall fractions of *M. tuberculosis*. The H60.15 antigen seems to be present also in the soluble fraction, and an alternative explanation for this phenomenon could be the reported release of molecules from mycobacterial membranes by sonication (1).

The observed greater extent of binding of the supernatants of the three MAbs to the strain H37Ra preparation (Table 2) seems to be the result of the sonication of strain H37Ra and the disruption of the other strains, since such a difference was not observed when ascites and sonicated mycobacteria were used. This suggests a differential release of antigens by the two methods.

Screening of antibodies in patient sera for the same epitopes recognized by mouse MAb indicates that some of the known MAb-defined epitopes elicit a significant humoral immune response also in humans (12, 14). Moreover, epitopes on the same antigens of *M. tuberculosis* present in recombinant DNA libraries have been demonstrated to elicit T-cell clone responses of restricted specificity (20).

T- and B-cell epitopes need not be identical, as was found for other antigens. The development and application of SDS-PAGE and immunoblotting for screening of antigens recognized by T lymphocytes allows the identification and comparison of T- and B-cell mycobacterial epitopes. By using three MAbs, we have identified three different proteins containing T-cell epitopes, as they induce T-cell proliferation when added to ascitic cell cultures. The T-cell epitopes present in the 65-kDa antigen have been mapped previously by using recombinant antigens and synthetic peptides (16). In such a study, two regions mapping between residues 66 and 86 and between residues 391 and 413 have been shown to contain human T-cell epitopes which are distinct from the known B-cell epitopes of the 65-kDa protein. However, the selection of T-cell epitopes is a function of the major histocompatibility class II molecules present.

No information is available at present on the T-cell epitopes present in the 28- and 35-kDa *M. tuberculosis* proteins. The recent finding that recombinant proteins recognized by MAbs H60.15 and H61.3 are able to induce T-cell proliferation (D. Vismara et al., submitted for publication) indicates the possibility of defining both the T- and B-cell epitopes by DNA technology and peptide synthesis. These aspects of the reactivity of the new MAbs described here are under investigation. In any case, a better knowledge of the enzymes or proteins associated with the cell wall would be useful in understanding peculiar aspects of the biochemistry of mycobacteria. However, immunocytochemistry of cells obtained from the bronchoalveolar lavage of three tuberculous patients with H61.3 antibodies has shown that the 35-kDa protein is present inside macrophages, suggesting the relevance of this protein during *M. tuberculosis* infection (G. Barbolini et al., manuscript in preparation).

The detection by MAb of the two new epitopes of 28- and 35-kDa proteins described in this paper may be due to the immunization schedule, since in the same fusion experiment several MAbs were directed to proteins of 28 and 35 kDa. The presence of the epitopes on the protein molecules of 28 and 35 kDa with broad and limited cross-reactivity, respectively, confirms the predominance of abundant cross-reactive antigens in the humoral immune response to mycobacteria.

It has been found recently that several of the antigens recognized by an antibody in *M. leprae* and *M. tuberculosis*, including 65-kDa antigens, are highly conserved proteins with DNA and antigen homologies to heat shock or stress proteins (D. B. Young et al., Proc. Natl. Acad. Sci., in press). It will be of interest to ascertain whether the new antigens recognized here are also stress proteins and to learn whether such proteins are effective or poor immunogens for cell-mediated immunity.

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LITERATURE CITED

1. **Brodie, A. F., and C. T. Gray.** 1957. Bacterial particles in oxidative phosphorylation. Science **125**:534–537.
2. **Buchanan, T. M., H. Nomaguchi, D. C. Anderson, R. A. Young, T. P. Gillis, W. J. Britton, J. Ivanyi, A. H. J. Kolk, O. Closs, B. R. Bloom, and V. Mehra.** 1986. Characterization of mycobacterial species specificity of 14 separate epitopes which reacted with monoclonal antibodies to the 65,000 molecular weight protein molecule of *Mycobacterium leprae*. Lepr. Rev. **57**(Suppl.):63–66.
3. **Colizzi, V.** 1984. In vivo and in vitro administration of interleukin 2-containing preparation reverses T-cell unresponsiveness in *Mycobacterium bovis* BCG-infected mice. Infect. Immun. **45**:25–28.
4. **Damiani, G., M. Frascio, U. Benatti, A. Morelli, E. Zocchi, M. Fabbi, A. Bargellesi, S. Pontremoli, and A. De Flora.** 1980. Monoclonal antibodies to human erythrocyte glucose 6-phosphate dehydrogenase. FEBS Lett. **119**:169–173.
5. **Damiani, G., E. Zocchi, M. Fabbi, A. Bargellesi, and F. Patrone.** 1983. A monoclonal antibody to platelet glycoproteins IIb and IIIa complex: its use in purifying human megakaryocytes from sternal bone marrow aspirates for immunofluorescence studies of Ia-like antigens. Exp. Hematol. **11**:169–177.
6. **Damiani, G., E. Zocchi, C. Martinoli, F. Querzola, and D. Zaccheo.** 1985. Establishment and characterization of a new human cloned myelomonocytic cell line. Exp. Hematol. **13**: 861–868.
7. **Daniel, T. M., and P. A. Anderson.** 1977. The purification of *Mycobacterium tuberculosis* antigen 5 by immunoabsorbent affinity chromatography. Am. Rev. Respir. Dis. **115**(Suppl.): 258–262.
8. **Daniel, T. M., and P. A. Anderson.** 1978. The isolation by immunoabsorbent affinity chromatography and physicochemical characterization of *Mycobacterium tuberculosis* antigen 5. Am. Rev. Respir. Dis. **117**:533–539.
9. **De Bruyn, J., R. Bosmans, M. Turneer, M. Weckx, J. Nyabenda, J.-P. Van Vooren, P. Falmagne, H. G. Wiker, and M. Harboe.** 1987. Purification, partial characterization, and identification of a skin-reactive protein antigen of *Mycobacterium bovis* BCG. Infect. Immun. **55**:245–252.
10. **Farr, A. G., and P. K. Nakane.** 1981. Immunohistochemistry with enzyme labeled antibodies: a brief review. J. Immunol. Methods **47**:129–144.
11. **Greenwood, F. C., W. M. Hunter, and J. S. Glover.** 1963. The preparation of ^{125}I labeled human growth hormone at high specific activity. Biochem. J. **89**:114–123.
12. **Hewitt, J., A. R. M. Coates, D. A. Mitchison, and J. Ivanyi.** 1982. The use of murine monoclonal antibodies without purification of antigen in the serodiagnosis of tuberculosis. J. Immunol. Methods **55**:205–211.
13. **Husson, R. N., and R. A. Young.** 1987. Genes for the major

protein antigens of *Mycobacterium tuberculosis*: the etiologic agents of tuberculosis and leprosy share an immunodominant antigen. Proc. Natl. Acad. Sci. USA **84**:1679–1683.
14. **Ivanyi, J., E. Krambovitis, and M. Keen.** 1983. Evaluation of a monoclonal antibody (TB 72)-based serological test for tuberculosis. Clin. Exp. Immunol. **54**:337–345.
15. **Ivanyi, J., J. A. Morris, and M. Keen.** 1985. Studies with monoclonal antibodies to mycobacteria, p. 59–90. *In* A. J. L. Macario and E. Conway de Macario (ed.), Monoclonal antibodies against bacteria, vol. 1. Academic Press, Inc., New York.
16. **Lamb, J. R., J. Ivanyi, A. D. Rees, J. B. Rothbard, K. Holland, R. Young, and D. B. Young.** 1987. Mapping of T cell epitopes using recombinant antigens and synthetic peptides. EMBO J. **6**:1245–1249.
17. **Maizel, J. V.** 1971. Polyacrylamide gel electrophoresis of viral proteins. Methods Virol. **5**:179–246.
18. **Mehra, V., D. Sweetser, and R. A. Young.** 1986. Efficient mapping of protein antigenic determinants. Proc. Natl. Acad. Sci. USA **83**:7013–7017.
19. **Mitchison, N. A.** 1987. The lessons to take home. Lepr. Rev. **57**(Suppl.):305–308.
20. **Oftung, F., A. S. Mustafa, R. Husson, R. A. Young, and T. Godal.** 1987. Human T cell clones recognize two abundant *Mycobacterium tuberculosis* protein antigens expressed in *Escherichia coli*. J. Immunol. **138**:927–931.
21. **Patarroyo, M. E., C. A. Parra, C. Pinilla, P. del Portillo, M. L. Torres, P. Clavijo, L. M. Salazar, and C. Jimenez.** 1986. Immunogenic synthetic peptides against mycobacteria of potential immunodiagnostic and immunoprophylactic value. Lepr. Rev. **57**(Suppl.):163–168.
22. **Thole, J. E. R., H. G. Dauwerse, P. K. Das, D. G. Groothuis, L. M. Schouls, and J. D. A. van Embden.** 1985. Cloning of *Mycobacterium bovis* BCG DNA and expression of antigens in *Escherichia coli*. Infect. Immun. **50**:800–806.
23. **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.
24. **Young, D. B., L. Kent, A. Rees, J. R. Lamb, and J. Ivanyi.** 1986. Immunological activity of a 38-kilodalton protein purified from *Mycobacterium tuberculosis*. Infect. Immun. **54**:177–183.
25. **Young, D. B., and J. R. Lamb.** 1986. T lymphocytes respond to solid-phase antigen: a novel approach to the molecular analysis of cellular immunity. Immunology **59**:167–171.
26. **Young, R. A., B. R. Bloom, C. M. Grosskinsky, J. Ivanyi, D. Thomas, and R. W. Davis.** 1985. Dissection of *Mycobacterium tuberculosis* antigens using recombinant DNA. Proc. Natl. Acad. Sci. USA **82**:2583–2587.