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Immunological Analysis of the Components of the Antigen Complex A60 of *Mycobacterium bovis* BCG

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The antigen complex A60 of *Mycobacterium bovis* BCG was analyzed by different immunological techniques to assess its relevance to tuberculosis and the involvement of its components in the immune reactions elicited in humans by tuberculous infection. A60 is composed of about 30 components, of which 8 were identified by available monoclonal antibodies (lipoarabinomannan, a glycolipid, and proteins of 65, 40, 38, 35, 19, and 14 kDa). The majority (87.5%) of anti-mycobacterial antibodies in sera from tuberculosis patients was directed against A60. Western blot (immunoblot) analysis indicated that the majority of the highly antigenic proteins present in mycobacterial homogenates were components of the A60 complex. A small percentage (7.8%) of A60 epitopes proved to be species specific. Thus, A60 proteins of 66, 41, 38, 37, 35, 34, 32, and 22 kDa were found to contain B-cell epitopes specific for *M. bovis* and not shared by *Mycobacterium leprae* or *Mycobacterium avium*.

Mycobacteria contain numerous immunologically active substances, which play a dominant role in mycobacterial diseases. One group of mycobacterial antigens that has been the object of extensive investigation is the thermostable macromolecular antigen complexes (TMA), which are present in all mycobacteria. The best-known members of this group are A60 of *Mycobacterium bovis* and *Mycobacterium tuberculosis* (9, 31, 34, 42), A7 of *Mycobacterium leprae* (33, 40), and A36 of *Mycobacterium paratuberculosis* (19, 26, 30). The presence of TMA on the cell surface was shown by agglutination of bacilli with anti-TMA (34) and by immunoelectron microscopy (19). A procedure for purification of TMA from mycobacterial soluble sonic extract has been described (12). Although TMA appear as a single precipitinogen line, they are complex antigens which contain polysaccharides, proteins, and lipids (5, 22). TMA are powerful immunogens, eliciting humoral (11) and cellular (3, 6, 11) immune reactions, and represent the main active components of sensitins (25, 26, 31), mycobacterial cell extracts used for cutaneous testing for mycobacterioses. Accordingly, A60-based serological assays (2, 10) and skin tests (3) have been developed for use in tuberculosis, and an enzyme-linked immunosorbent assay (ELISA) for A36 has been used for the diagnosis of paratuberculosis (19).

One aim of the present work was to evaluate the immunological relevance of the A60 complex to tuberculosis by measuring the fraction of antimycobacterial antibodies directed against A60 and other cytoplasmic *M. bovis* BCG antigens. A60 was dissected and its antigenic components were identified by using both the available monoclonal antibodies and sera from tuberculosis patients. Finally, the occurrence in A60 proteins of species-specific B-cell epitopes was assessed, to identify possible candidates for a species-specific ELISA to be used in diagnostic assays for tuberculosis.

MATERIALS AND METHODS

Bacteria. *M. bovis* BCG was the Calmette-Guérin strain (Pasteur Institute, Paris, France). The following strains of the *Mycobacterium avium-Mycobacterium intracellulare-Mycobacterium scrofulaceum* (MAIS group) were used: MAIS-10708, MAIS-10719, *M. avium* D4 (F. Portaels, Institute of Tropical Medicine, Antwerp, Belgium), and *M. scrofulaceum* 82/549 (M. Weckx, Pasteur Institute, Brussels, Belgium). An *M. leprae* preparation purified by the procedure of P. Draper was provided by the World Health Organization (WHO, Geneva, Switzerland).

Antigen preparation. A60 was obtained by French press-disrupted *M. bovis* BCG; it was purified from soluble sonic extract by exclusion gel chromatography on Sepharose 6B (Pharmacia, Uppsala, Sweden) as described before (12–14). The purity of the A60 preparation was analyzed by two-dimensional crossed immunoelectrophoresis on agarose gels, with rabbit anti-BCG immunoglobulin (Ig) (Dako, Copenhagen, Denmark) in second-dimension runs (9). A60 protein content was spectrophotometrically measured by the procedure of Bradford (4).

Preparation of antibodies. Sera from patients with tuberculosis were prepared by centrifugation of blood samples (Pneumology Department, Mt. Godinne Clinics, Namur, Belgium). Five sera, which were pooled and used in both ELISAs and Western blots (immunoblots), were from human immunodeficiency virus-negative postprimary tuberculosis cases receiving chemotherapy. Polyclonal antimycobacterial sera were obtained from hyperimmunized rabbits (10 μg of A60 proteins from *M. bovis* BCG in 500 μl of buffered saline emulsified with an equal volume of incomplete Freund's adjuvant, with weekly repeated subcutaneous injections).

Monoclonal antibodies were provided by D. Chatterjee (Colorado State University, Fort Collins), G. Damiani (Biochemistry Department, University of Genoa, Italy), and WHO (United Nations Development Program/World Bank/WHO Special Programme for Research and Training in Tropical Diseases).

Dot blots and Western blots. For dot blots, A60 and other antigens (1 μg of protein per sample) were spotted on nitrocellulose membranes (BA85; Macherey-Nagel, Dueren, Germany). Polyclonal antisera (10^{-1} to 10^{-3} dilutions with buffered saline) or monoclonal antibodies (10^{-3} dilution for ascites and 10^{-1} dilution for culture supernatants) were placed onto the spots for 20 h of incubation at room temperature in the presence of phenylmethylsulfonyl fluoride, a protease inhibitor. Bound Ig was identified by using either peroxidase-

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TABLE 1. Properties of monoclonal antibodies and their reactivity with A60 components

Monoclonal antibody	Antigen recognized[a]			Species specificity[b]	A60 reactivity	
	Nature	Size (kDa)	Origin		Intensity[c]	Lane[d]
IT1	Protein	14	*M. tuberculosis*	+	+	
IT10	Protein	19	*M. tuberculosis*	–	++	8
H60.15	Protein	28	*M. tuberculosis*	+	–	
H613	Protein	35	*M. tuberculosis*	–	++	7
IT15	Protein	38	*M. tuberculosis*	+	+	
IT23	Protein	38	*M. tuberculosis*	+	++	6
CS35	LAM	39	*M. tuberculosis*	–	++	9
IT7	Protein	40	*M. tuberculosis*	–	++	5
MC5802	PGL		*M. leprae*	+	–	
MC0313	Glycolipid	4.5–6	*M. leprae*	–	+	
MC5041	Protein	28	*M. leprae*	–	–	
MC3607	Protein	35	*M. leprae*	–	–	
MC0445	Protein	65	*M. leprae*	–	+	
MC4220	Protein	65	*M. leprae*	–	++	4
MC5205	Protein	65	*M. leprae*	–	++	3

[a] For a description, see references 15, 20, 21, 38, and 41. PGL, phenolic glycolipid.
[b] +, specific; –, cross-reactions within genus *Mycobacterium*.
[c] A60 reactivity on dot-blot only (+) or on both dot-blot and Western blot (++).
[d] Western blots showing A60 proteins stained by monoclonal antibodies are in Fig. 1.

labeled protein A or peroxidase-labeled anti-rabbit Ig antibodies (Dako).

For Western blot analysis, A60 and *M. bovis* BCG soluble sonic extract samples were fractionated by electrophoresis on 10% polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS-PAGE), in parallel with molecular weight markers (Sigma, St. Louis, Mo.). Electrophoresed components were electrophoretically transferred to nitrocellulose membranes (BA85; Macherey-Nagel) in a transblot unit (217 Multiphor 2; LKB, Uppsala, Sweden). Membranes washed with TBST buffer (0.5 M NaCl, 0.023 M Tris-HCl [pH 7.5] containing 1% [wt/vol] gelatin and 0.05% [vol/vol] Tween 20) were incubated for 3 h with primary antibodies (diluted in TBST) and for 2 h with secondary IgG (1:400 dilution of peroxidase-labeled anti-rabbit IgG [Dako]) and washed with TBST before being stained with α-chloronaphthol (Bio-Rad, Richmond, Calif.) in the presence of hydrogen peroxide. The entire procedure has been described in a previous work (19).

Immunoassay for determination of antimycobacterial Ig. Multiwell microtiter plates (Microwell Module; Nunc) were coated either with purified TMA antigens or with bacterial soluble sonic extracts (0.5 μg of soluble proteins per 100 μl of 0.05 M sodium carbonate buffer [pH 9.6] per well). Air-dried wells were saturated with bovine serum albumin (BSA), 0.1% (wt/vol) in 0.15 M NaCl for 1 h at 37°C. Increasing dilutions of serum to be tested in 0.15 M NaCl–0.005% Tween 80–0.02 M sodium phosphate buffer (pH 7.2) (PBST buffer) were added at 100 μl per well for 1 h at 37°C, optimal dilutions being identified by checkerboard titration. Horseradish peroxidase-labeled anti-human IgG (Bio-Rad) was added at 100 μl of 1:400-diluted IgG in PBST per well for 1 h at 37°C. Excess reagent was removed by five buffer washes. After incubation with the peroxidase reagent (100 μl of a 17 mM sodium citrate buffer [pH 6.3] containing 0.2% [wt/vol] *O*-phenylenediamine and 0.015% [vol/vol] H_2O_2 per well) for 30 min at 37°C in the dark, the reaction was stopped with 100 μl of 2 M H_2SO_4 per well, and samples were spectrometrically measured (plate reader SLT 210; Kontron Analytical). Results were recorded as A_{492} values.

Antibody preabsorption. In some experiments, cross-reactive Ig was removed by incubation of antiserum for 18 h at 4°C with either purified TMA preparations or whole bacterial sonicates. Human sera properly diluted in NPTP buffer (0.15 M NaCl, 0.02 M K_2HPO_4 [pH 7.5] containing 0.05% [vol/vol] Tween 20, 10 mM phenylmethylsulfonyl fluoride, and 1% [wt/vol] gelatin) were incubated overnight at 4°C in the presence of either A60 (0.5 mg of protein per ml of diluted serum) or mycobacterial sonicates (5 mg [dry weight] per ml of diluted serum). The efficiency of preabsorption was controlled by dot-blot for Western blot analysis and by ELISA for preparations used in ELISAs.

RESULTS

Identification of A60 components by using monoclonal antibodies. Some of the A60 components might correspond to those already found by others with antimycobacterial monoclonal antibodies (15, 20, 21, 38, 41). This possibility was tested by dot-spot analysis of A60 with 15 monoclonal antibodies directed against proteins and glycolipids. In our screening, 11 of the tested monoclonal antibodies bound to A60, recognizing different components of this complex: 2 of them recognized glycolipids, and 9 bound to proteins (Table 1).

A more precise identification of the components of A60 was obtained by dissociation of the complex and fractionation by polyacrylamide gel electrophoresis. After electrophoretic transfer to nitrocellulose membranes, A60 components were tested with the monoclonal antibodies mentioned above. Four single bands corresponding to 19, 35, 38, and 40 kDa were recognized (Fig. 1, lanes 5 to 8). Monoclonal antibodies directed against the 65-kDa protein of *M. leprae* (a cross-reacting protein) recognized several bands (Fig. 1, lanes 3 and 4). Antilipoarabinomannan (anti-LAM) monoclonal antibodies yielded a smear in the 35- to 40-kDa region (Fig. 1, lane 9).

These data show that A60 contains several antigenic determinants that react with the available monoclonal antibodies. The 19-, 35-, 38-, 40-, and 65-kDa proteins and LAM were identified by Western blot. In addition, the 14-kDa protein and the 4.5- to 6-kDa glycolipid were identified by dot spot.

Quantification of A60 immunodominance. TMA complexes were shown by previous work to be immunodominant in mycobacterioses (19, 32, 34). To quantitate this immunodomi-

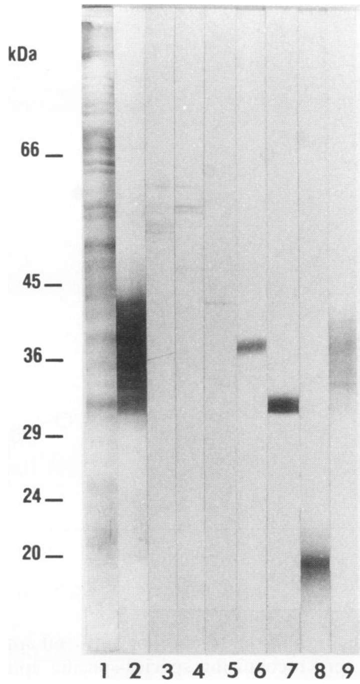

FIG. 1. Recognition of different A60 components by monoclonal antibodies. A60 components from *M. bovis* BCG soluble sonic extract were fractionated by SDS-PAGE and blotted onto nitrocellulose membranes, which were incubated with anti-A60 rabbit antiserum (lane 2) and monoclonal antibodies MC5205, MC4220, IT7, IT23, H613, IT10, and CS35 (lanes 3 to 9, respectively). A60 components (lane 1) were stained with colloidal gold.

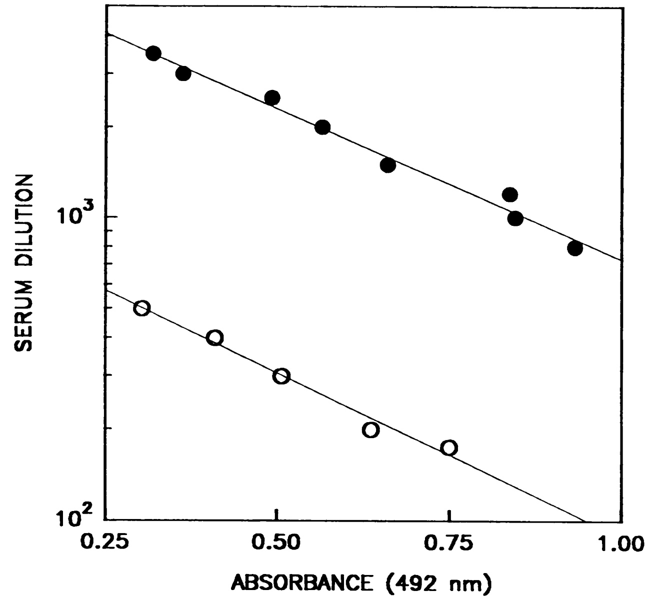

FIG. 2. Evaluation of the fraction of antimycobacterial antibodies in tuberculosis patient sera directed against the A60 complex. Increasing dilutions of a pool of five tuberculosis patient sera either preabsorbed (○) or not (●) on A60 were added to microtitration plates coated with *M. bovis* BCG soluble sonic extract (1 μg of protein per well). After the plates were washed, peroxidase-labeled anti-human Ig and then the peroxidase substrate were added, and the A_{492} was read (the ELISA immunoassay is detailed in the text).

nance, we measured the proportion of antimycobacterial antibodies present in tuberculous patient sera which were directed against this complex. For this purpose, a pool of patient sera was preabsorbed or not on A60; we then evaluated the concentrations of these paired samples that yielded equivalent optical density values in an ELISA with *M. bovis* BCG homogenate as the reagent. The ratio between the concentrations of antimycobacterial Ig in paired samples would thus be a function of the antibodies neutralized in the preabsorption step and yield an evaluation of A60 immunodominance compared with that of total antigens from the *M. bovis* BCG homogenate. Two straight lines of equal slope were obtained when non-preabsorbed serum was diluted eightfold with respect to the same serum preabsorbed with A60 (Fig. 2). This indicates that the preabsorption step neutralized about 85% of the antimycobacterial antibodies present in tuberculosis patient sera.

Among the *M. bovis* BCG components which are best recognized by tuberculous patient sera, A60 and non-A60 components were identified by Western blotting. A60 and *M. bovis* BCG soluble sonic extract were fractionated by SDS-PAGE, transferred to a nitrocellulose membrane, and incubated with a pool of patient sera preabsorbed or not with A60. As shown in Fig. 3, a small proportion of the proteins in A60 (lane e) and in *M. bovis* BCG soluble sonic extract (lane a) were recognized by patient sera (lanes b and d), most of which were located within the 25- to 40-kDa region. This very region was no longer recognized when serum was preabsorbed on A60 (Fig. 3, lane c). A few other proteins located within the 40- to 70-kDa region are recognized by tuberculous patient sera even after preabsorption with A60 (Fig. 3, lane c). These data indicate that many of the *M. bovis* BCG components best recognized by tuberculous patient sera are present in A60.

Identification of A60 species-specific epitopes. TMA-based immunoassays have high sensitivity but lack species specificity. The occurrence in A60 of species-specific epitopes was estimated as follows. A pool of tuberculosis patient sera was preabsorbed or not on an *M. avium* sonicate. The ratio between the concentrations of preabsorbed and non-preabsorbed serum that yielded equivalent optical density readings in an A60-based ELISA provides an evaluation of relevant *M. bovis* BCG epitopes not shared by *M. avium*. By this procedure, equivalent optical density values were obtained when non-preabsorbed tuberculosis serum was diluted 11.5-fold with respect to serum preabsorbed on *M. avium* (Fig. 4). This indicates that 8.5% of the anti-A60 Ig in tuberculosis serum is directed against A60 components that are not present in *M. avium*.

To identify the A60 proteins carrying species-specific epitopes, A60 was fractionated by polyacrylamide gel electrophoresis. After electrophoretic transfer to nitrocellulose membranes, separated A60 components were immunoblotted with a pool of tuberculosis patient sera preabsorbed with sonicates of either *M. leprae* (Fig. 5, lane C) or different strains of MAIS (Fig. 5, lanes D, E, F, and G) (reference non-preabsorbed tuberculosis serum is in lane B). Some eight proteins of 66, 41, 38, 37, 35, 34, 32, and 22 kDa (lanes C to G) proved to have epitopes specific for *M. bovis* not shared by *M. leprae* or *M. avium*.

DISCUSSION

Mycobacteria interact with the immune system of the host through numerous antigens (7, 18). In the fight against mycobacteriosis, identification of these antigens may play an important role for both diagnosis and protection. Several mycobac-

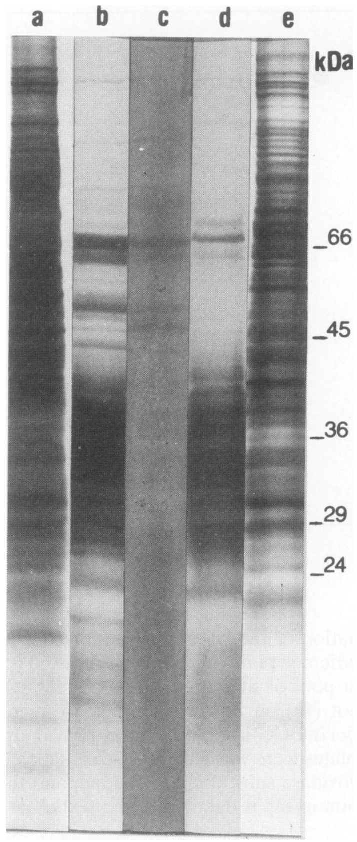

FIG. 3. Components of *M. bovis* BCG soluble sonic extract and A60 which are recognized by tuberculosis patient sera. BCG soluble sonic extract (30 μg of protein per sample) (lanes a, b, and c) and A60 (lanes d and e) were fractionated by SDS-PAGE and transblotted to nitrocellulose membranes. Components were either stained with colloidal gold (lanes a and e) or incubated with a pool of five tuberculosis patient sera, preabsorbed (lane c) or not (lanes b and d) on A60 and revealed by using peroxidase-labeled anti-human Ig secondary antibodies.

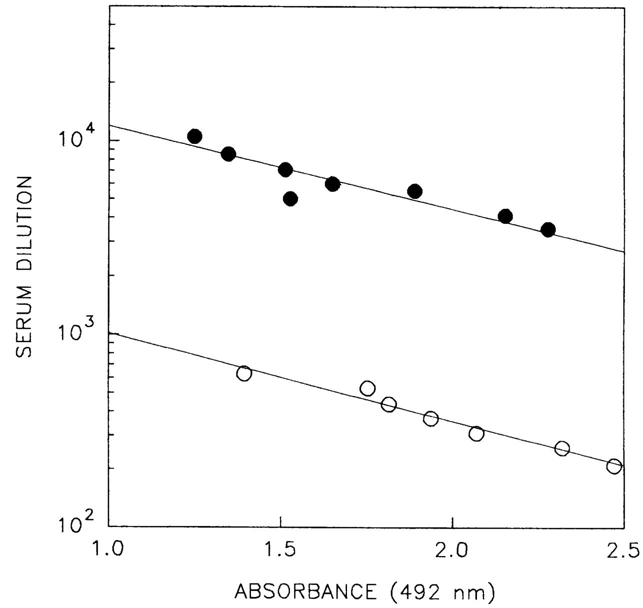

FIG. 4. Evaluation of the fraction of anti-A60 antibodies in tuberculosis patient sera recognizing species-specific epitopes. Increasing dilutions of a pool of five tuberculosis patient sera, either preabsorbed (○) or not (●) with *M. avium* sonicate, were added to microtitration plates coated with A60 (1 μg of protein per well). After the plates were washed, peroxidase-labeled anti-human Ig and then the peroxidase substrate were added, and the A_{492} was read.

terial antigens have been purified (18, 44), some of which have been proven to be of value in mycobacterial serology and cutaneous testing (16, 17, 27, 35, 37, 39). The pronounced recognition of TMA by sera from patients with leprosy (1) and tuberculosis (2, 8, 23, 24, 29, 32) suggests an immunodominant role of these complexes. In this study, the immunodominance of A60, the TMA complex of *M. bovis*, in tuberculosis has been quantitated. As shown in Fig. 2, 85% of the antimycobacterial antibodies in patient sera were directed against A60. This conclusion is in agreement with a previous estimate (34) and receives further support from the results shown in Fig. 3, indicating that the majority of *M. bovis* components that react with tuberculosis patient sera were present in the A60 complex.

The data in Fig. 1 and Table 1 show recognition of the LAM and a glycolipid and of the 65-, 40-, 38-, 35-, 19-, and 14-kDa A60 proteins by previously described monoclonal antibodies. Monoclonal antibody IT1, directed against the 14-kDa protein, recognized A60 in a dot-blot test (Table 1) but not in the Western blot (Fig. 1), suggesting the presence, in the 14-kDa protein, of a structural B-cell epitope that is recognized in the native state but not in a denatured state. The occurrence of the 14-kDa protein in A60 was independently confirmed by others (36) with a monoclonal antibody different from the one that we used. Monoclonal antibodies MCO445, MC5205, and MC4220, which recognize different epitopes of the 65-kDa heat shock protein, recognized multiple bands of 44 to 57 kDa in A60 Western blots (Fig. 1, lanes 3 to 5). This observation was made independently by others (43) and attributed to degradation by proteases. Interestingly, only one band, corresponding to the 65-kDa protein, was recognized by the three above mentioned monoclonal antibodies in a Western blot of *M. paratuberculosis* TMA proteins (19). Note that several proteins which were shown in this study to be A60 components (e.g., the 65-, 38-, 35-, and 14-kDa proteins) have been localized to the periphery of mycobacterial cells by other studies (44).

The immunodominance of TMA complexes has prompted the development of immunoassays endowed with high sensitivity but lacking species specificity (for a review, see reference 10) because of the strong cross-reactions among TMA (19, 34). This difficulty could be circumvented by using TMA species-specific epitopes. According to our data, species-specific epitopes of A60 proteins were recognized by 8.5% of the anti-A60 Ig present in tuberculosis patient sera (Fig. 4), which represents a substantial fraction (7%) of the total antimycobacterial antibodies in these sera. Among the A60 proteins that are recognized by monoclonal antibodies (Fig. 1) and have species-specific epitopes (Fig. 5), we find the 38-kDa protein, previously described as being specific for the *M. tuberculosis-M. bovis-M. africanum* group (35). A species-specific immunoassay based on the 38-kDa protein has already been developed (35), although its sensitivity could be improved. The A60 proteins identified herein, carrying species-specific epitopes, could be used to increase the sensitivity of this test. Some of the genes coding for proteins endowed with the specific epitopes, which are described in the present work, are pres-

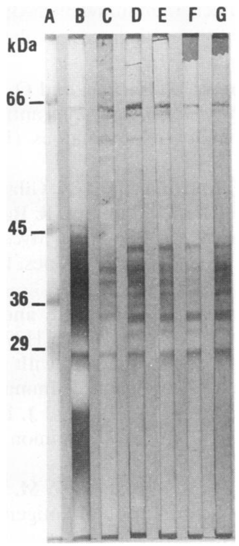

FIG. 5. Identification of the A60 proteins carrying species-specific epitopes. A60 proteins were electrophoretically fractionated and blotted to nitrocellulose membranes. Western blots were incubated with a pool of tuberculosis patients' sera either untreated (lane B) or preabsorbed on sonicates of *M. leprae* (C), *M. avium* (D), *M. scrofulaceum* (E), MAIS 10708 (F), or MAIS 10719 (G) and revealed with peroxidase-labeled Ig. Molecular size standards are in lane A.

ently being cloned, to obtain the large-scale reagent production needed for medical applications.

REFERENCES

1. **Axelsen, N., M. Harboe, O. Closs, and T. Godal.** 1974. BCG antibody profiles in tuberculoid and lepromatous leprosy. Infect. Immun. **9:**952–958.
2. **Baelden, M. C., B. Vanderelst, M. Dieng, J. Prignot, and C. Cocito.** 1990. Serological analysis of human tuberculosis by an ELISA with mycobacterial antigen 60. Scand. J. Infect. Dis. **22:**63–73.
3. **Benoit, C., A. Beschin, M. Desmecht, P. Dekeyser, and C. Cocito.** 1989. Delayed hypersensitivity reactions by the mycobacterial antigen A60, and cutaneous testing in tuberculosis. Med. Microbiol. Immunol. **178:**105–112.
4. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
5. **Bruneteau, M., J. Perret, F. Vanlinden, G. Michel, and C. Cocito.** 1992. Composition and immunogenicity of the polysaccharide components of the thermostable macromolecular antigen group of mycobacterial antigens. Med. Microbiol. Immunol. **181:**13–23.
6. **Carlucci, S., A. Beschin, L. Tuosto, F. Ameglio, G. M. Gandolfo, C. Cocito, F. Fiorucci, C. Saltini, and E. Piccolella.** 1993. Mycobacterial antigen complex A60-specific T-cell repertoire during the course of pulmonary tuberculosis. Infect. Immun. **61:**439–447.
7. **Chaparas, S. D.** 1982. The immunology of mycobacterial infection. Crit. Rev. Microbiol. **9:**139–197.
8. **Charpin, D., H. Herbault, M. J. Gevaudan, M. Saadjian, P. De Micco, A. Arnaud, D. Vervloet, and J. Charpin.** 1990. Value of ELISA using A60 antigen in the diagnosis of active pulmonary tuberculosis. Am. Rev. Respir. Dis. **142:**380–384.
9. **Closs, O., M. Harboe, N. X. Axelsen, K. Bunch-Christensen, and M. Magnusson.** 1980. The antigens of *Mycobacterium bovis* strain BCG, studied by crossed immunoelectrophoresis: a reference system. Scand. J. Immunol. **12:**249–263.
10. **Cocito, C.** 1991. Properties of the mycobacterial antigen complex A60 and its application to the diagnosis and prognosis of tuberculosis. Chest **100:**1687–1693.
11. **Cocito, C., M. C. Baelden, and C. Benoit.** 1987. Immunological properties of antigen 60 of BCG: induction of humoral and cellular immune reactions. Scand. J. Immunol. **25:**579–585.
12. **Cocito, C., and F. Vanlinden.** 1986. Preparation and properties of antigen 60 from *Mycobacterium bovis* BCG. Clin. Exp. Immunol. **66:**262–272.
13. **Cocito, C., and F. Vanlinden.** 1988. Metabolism of the TMA group of antigens during the growth cycle of mycobacteria. Med. Microbiol. Immunol. **177:**357–367.
14. **Cocito, C., and F. Vanlinden.** 1988. Subcellular localisation and sedimentation behaviour of antigen 60 from *Mycobacterium bovis* BCG. Med. Microbiol. Immunol. **177:**15–25.
15. **Damiani, G., A. Biano, A. Beltrame, D. Vismara, M. F. Mezzopreti, V. Colizzi, D. B. Young, and B. R. Bloom.** 1988. Generation and characterization of monoclonal antibodies to 28-, 35-, and 65-kilodalton proteins of *Mycobacterium tuberculosis*. Infect. Immun. **56:**1281–1287.
16. **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.
17. **Daniel, T. M., S. M. Debanne, and F. Vanderkuyp.** 1985. Elisa using *M. tuberculosis* antigen 5 of PPD for the serodiagnosis of tuberculosis. Chest **88:**388–392.
18. **Daniel, T. M., and B. W. Janicki.** 1978. Mycobacterial antigens: a review of their isolation, chemistry, and immunological properties. Microbiol. Rev. **42:**84–113.
19. **De Kesel, M., P. Gilot, M. Coene, and C. Cocito.** 1992. Composition and immunological properties of the protein fraction of A36, a major antigen complex of *Mycobacterium paratuberculosis*. Scand. J. Immunol. **36:**201–212.
20. **Engers, H. D., M. Abe, B. R. Bloom, V. Mehra, W. Britton, T. M. Buchanan, S. K. Khanolkar, D. B. Young, O. Closs, T. Gillis, M. Harboe, J. Ivanyi, A. H. J. Kolk, and C. C. Shepard.** 1985. Results of a World Health Organization-sponsored workshop on monoclonal antibodies to *Mycobacterium leprae*. Infect. Immun. **48:**603–605.
21. **Engers, H. D., V. Houba, J. Bennedsen, T. M. Buchanan, S. D. Chaparas, G. Kadival, O. Closs, J. R. David, J. D. A. van Embden, T. Godal, S. A. Mustafa, J. Ivanyi, D. B. Young, S. H. E. Kaufmann, A. G. Khomenko, A. H. J. Kolk, M. Kubin, J. A. Louis, P. Minden, T. M. Shinnick, L. Trnka, and R. A. Young.** 1986. Results of a World Health Organization-sponsored workshop to characterize antigens recognized by mycobacteria-specific monoclonal antibodies. Infect. Immun. **51:**718–720.
22. **Fabre, I., O. L'Homme, M. Bruneteau, G. Michel, and C. Cocito.** 1986. Chemical composition of antigen 60 from *Mycobacterium bovis* BCG. Scand. J. Immunol. **24:**591–602.
23. **Fadda, G., R. Grillo, F. Ginesu, L. Santoru, S. Zanetti, and G. Dettori.** 1992. Serodiagnosis and follow up of patients with pulmonary tuberculosis by enzyme-linked immunosorbent assay. Eur. J. Epidemiol. **8:**81–87.
24. **Gevaudan, M. J., C. Bollet, D. Charpin, M. N. Mallet, and P. De Micco.** 1992. Serological response of tuberculosis patients to antigen 60 of BCG. Eur. J. Epidemiol. **8:**666–676.
25. **Gilot, P., and C. Cocito.** 1993. Comparative analysis of three sensitins used in cutaneous testing for tuberculosis and paratuberculosis in cattle. FEMS Microbiol. Lett. **110:**307–312.
26. **Gilot, P., M. De Kesel, M. Coene, and C. Cocito.** 1992. Induction of cellular immune reactions by A36, an antigen complex of *Mycobacterium paratuberculosis*: comparison of A36 and johnin components. Scand. J. Immunol. **36:**811–821.
27. **Gilot, P., M. De Kesel, L. Machtelinckx, M. Coene, and C. Cocito.** 1993. Isolation and sequencing of the gene coding for an antigenic 34-kilodalton protein of *Mycobacterium paratuberculosis*. J. Bacteriol. **175:**4930–4935.
28. **Grange, J. M.** 1984. The humoral immune response in tuberculosis: its nature, biological role and diagnostic usefulness. Adv. Tuberc. Res. **21:**1–78.
29. **Gulletta, E., M. Del Pezzo, A. Sanduzzi, F. Bariffi, and I. Covelli.** 1988. Serodiagnosis survey of tuberculosis by a new Elisa method. Eur. J. Epidemiol. **4:**331–334.
30. **Gunnarson, E., and F. Fodstad.** 1979. Analysis of antigens in *Mycobacterium paratuberculosis*. Acta Vet. Scand. **20:**200–215.
31. **Harboe, M.** 1981. Antigens of PPD, old tuberculin and autoclaved *M. bovis* BCG studied by crossed immunoelectrophoresis. Am.

Rev. Respir. Dis. **124:**80–87.

32. **Harboe, M., O. Closs, B. Bjorvatn, and G. Bjune.** 1977. Antibodies against BCG antigen 60 in mycobacterial infection. Br. Med. J. **2:**430–433.
33. **Harboe, M., O. Closs, B. Bjorvatn, G. Kronvall, and H. Axelsen.** 1977. Antibody response in rabbits to immunization with *Mycobacterium leprae*. Infect. Immun. **18:**792–805.
34. **Harboe, M., O. Closs, K. Svindahl, and S. Deverill.** 1977. Production and assay of antibodies against one antigenic component of *Mycobacterium bovis* BCG. Infect. Immun. **16:**662–672.
35. **Harboe, M., and H. G. Wiker.** 1992. The 38-kDa protein of *Mycobacterium tuberculosis*: a review. J. Infect. Dis. **166:**874–884.
36. **Hubbart, R. D., C. M. Flory, F. M. Collins, and C. Cocito.** 1992. Immunization of mice with the antigen A60 of *Mycobacterium bovis* BCG. Clin. Exp. Immunol. **88:**129–131.
37. **Joint International Union Against Tuberculosis and World Health Organization Study Group.** 1992. Tuberculosis control. Tubercle **63:**157–169.
38. **Khanolkar-Young, S., A. H. J. Kolk, A. B. Andersen, J. Bennedsen, P. D. Brennan, B. Rivoire, S. Kuijper, K. P. W. J. McAdam, C. Abe, H. V. Batra, S. D. Chapparas, G. Damiani, M. Singh, and H. P. Engers.** 1992. Results of the third immunology of leprosy/immunology of tuberculosis antimycobacterial monoclonal antibody workshop. Infect. Immun. **60:**3925–3927.
39. **Krambovitis, E.** 1987. Serodiagnosis of tuberculosis in perspective. Serodiagn. Immunother. **1:**7–19.
40. **Melsom, R., B. Noees, M. Harboe, and O. Closs.** 1978. Antibody activity against *Mycobacterium leprae* antigen 7 during the first year of DIS-treatment in lepromatous (BL-LL) leprosy. Lepr. Rev. **49:**17–29.
41. **Vismara, G., M. F. Mezzopreti, M. S. Gilardini, P. Del Porto, G. Lombardi, E. Piccolella, G. Damiani, R. Rappuoli, and V. Collizi.** 1990. Identification of a 35-kilodalton *Mycobacterium tuberculosis* protein containing B- and T-cell epitopes. Infect. Immun. **58:**245–251.
42. **Wiker, H., M. Harboe, J. Bennedsen, and O. Closs.** 1988. The antigens of *Mycobacterium tuberculosis* H37Rv, studied by crossed immunoelectrophoresis. Comparison with a reference system for *Mycobacterium bovis* BCG. Scand. J. Immunol. **27:**223–239.
43. **Young, D. B., J. Ivanyi, J. H. Cox, and J. R. Lamb.** 1987. The 65 kDa antigen of mycobacteria—a common bacterial protein. Immunol. Today **8:**215–219.
44. **Young, D. B., S. H. E. Kaufman, P. W. M. Hermans, and J. E. R. Thole.** 1992. Mycobacterial protein antigens: a compilation. Mol. Microbiol. **6:**133–145.