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 (lipopolysaccharide, 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 (h) 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 sensitis (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 anticytobacterial antibodies directed against A60 and other cytoplasmic *M. bovis* BCG antigens. A60 was dissected and its antigenic components were identified by being 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. Godinée 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 anticytobacterial 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, Duren, 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-
TABLE 1. Properties of monoclonal antibodies and their reactivity with A60 components

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>Antigen recognized†</th>
<th>Species specificity‡</th>
<th>A60 reactivity</th>
<th>Intensity§</th>
<th>Lane¶</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nature</td>
<td>Size (kDa)</td>
<td>Origin</td>
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<tr>
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<td>8</td>
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<tr>
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<td>6</td>
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<td>MC5802</td>
<td>PGI</td>
<td></td>
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<tr>
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<tr>
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<td>65</td>
<td>M. lepra</td>
<td>++</td>
<td>3</td>
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</table>

† For a description, see references 15, 20, 21, 38, and 41. PGL, phenolic glycolipid.
‡ +, specific; –, cross-reactions within genus Mycobacterium.
§ A60 reactivity on dot-blot only (+) or on both dot-blot and Western blot (± ±).
¶ 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).

Immunoblot assay 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] H2O2 per well) for 30 min at 37°C in the dark, the reaction was stopped with 100 µl of 2 M H2SO4 per well, and samples were spectrometrically measured (plate reader SLT 210; Kontral Analytical). Results were recorded as A492 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 K2HPO4 [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-
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 c) and 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-
tuberculosis 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-
ently being cloned, to obtain the large-scale reagent production needed for medical applications.

REFERENCES