Mycobacterium avium-Mycobacterium intracellulare complex was dissected and its diagnostic species-specific candidates for possible tuberculosis patients was Pasteur Institute (Paris, France). The following strains of Mycobacterium avium-Mycobacterium intracellulare-Mycobacterium africanum were used: MAIS-10708, MAIS-10719, M. avium D4 (F. Portaels, Institute of Tropical Medicine, Antwerp, Belgium), and M. scavulatum 82549 (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. Godinioh Clinical, Namur, Belgium). Five sera, which were pooled and used in both ELISAs and Western Blots (immunoblots), were obtained from human immunodeficiency virus-negative postprimary tuberculosis cases receiving chemotherapy. Polyclonal antimycobacterial sera were obtained from hyperimmunized rabbits (100 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; Machevy-Nagel, Duringen, Germany). Polyclonal antisera (10⁻¹ to 10⁻³ dilutions with buffered saline) or monoclonal antibodies (10⁻² dilution for ascites and 10⁻¹ dilution for culture supernatants) were placed onto the spots for 2 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>Nature</th>
<th>Size (kDa)</th>
<th>Origin</th>
<th>Species specificity</th>
<th>A60 reactivity</th>
<th>Intensity</th>
<th>Lane</th>
</tr>
</thead>
<tbody>
<tr>
<td>IT1</td>
<td>Protein</td>
<td>14</td>
<td>M. tuberculosis</td>
<td>+</td>
<td>+</td>
<td>8</td>
<td></td>
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<tr>
<td>IT10</td>
<td>Protein</td>
<td>19</td>
<td>M. tuberculosis</td>
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<td>+</td>
<td>5</td>
<td></td>
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<tr>
<td>H60:15</td>
<td>Protein</td>
<td>28</td>
<td>M. tuberculosis</td>
<td>+</td>
<td>+</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>H613</td>
<td>Protein</td>
<td>35</td>
<td>M. tuberculosis</td>
<td>+</td>
<td>+</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>IT5</td>
<td>Protein</td>
<td>38</td>
<td>M. tuberculosis</td>
<td>+</td>
<td>+</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>IT23</td>
<td>Protein</td>
<td>38</td>
<td>M. tuberculosis</td>
<td>+</td>
<td>+</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>CS35</td>
<td>LAM</td>
<td>39</td>
<td>M. tuberculosis</td>
<td>+</td>
<td>+</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>IT7</td>
<td>Protein</td>
<td>40</td>
<td>M. tuberculosis</td>
<td>+</td>
<td>+</td>
<td>3</td>
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</tr>
<tr>
<td>MC5802</td>
<td>PGL</td>
<td></td>
<td>M. lepra</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
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<tr>
<td>MC0313</td>
<td>Glycolipid</td>
<td>4.5-6</td>
<td>M. lepra</td>
<td>-</td>
<td>+</td>
<td></td>
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<tr>
<td>MC5041</td>
<td>Protein</td>
<td>28</td>
<td>M. lepra</td>
<td>-</td>
<td>-</td>
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<tr>
<td>MC3607</td>
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<td>M. lepra</td>
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<td>-</td>
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<tr>
<td>MC0445</td>
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</tr>
</tbody>
</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.

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 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 were 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 buffer washes. After incubation with the peroxidase reagent (100 μl of a 1:10000 serum citrate buffer [pH 6.5] containing 0.05% [vol/vol] 3,3′-diaminobenzidine 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 $A_{492}$ 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 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. lepra (a cross-reacting protein) recognized several bands (Fig. 1, lanes 3 and 4). Anti-LAM protein (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 quantify this immunodom-
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 tuberculous 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 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 tuberculous 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 immunooblotted with a pool of tuberculous 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-
Figure 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.

Figure 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 (C) 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 A492 was read.

The immunodominance of TMA complexes has prompted the development of immunoasays 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 immunoasay 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-