Production, Characterization, and Species Specificity of Five Monoclonal Antibodies to Mycobacterium tuberculosis

GURURAJ V. KADIVAL† and SOTIROS D. CHAPARAS*

Laboratory of Mycobacteria and Cellular Immunology, Division of Bacterial Products, Office of Biologics Research and Review, Center for Drugs and Biologics, Food and Drug Administration, Bethesda, Maryland 20892

Received 29 May 1986/Accepted 15 September 1986

The production and characterization of five monoclonal antibodies to Mycobacterium tuberculosis are described. Specificity of the monoclonal antibodies was tested against other mycobacterial species by an enzyme-linked immunosorbent assay and immunobLOTS. HGT 3a, an immunoglobulin M (IgM) antibody, recognizes a molecule of 38,000 molecular weight present only in the tuberculin complex of M. tuberculosis and Mycobacterium bovis BCG. HGT 6, an IgG1 antibody, recognized two molecules with molecular weights of 43,000 and 45,000 and showed limited cross-reactivity. Three other antibodies, HGT 1, HGT 2, and HGT 4, all belonging to the IgG1 type, recognized multiple bands and showed broad reactivity among all mycobacterial antigens tested, Escherichia coli, and Nocardia asteroides.

MATERIALS AND METHODS

Antigens. Antigens were prepared from mycobacterial species obtained from the Trudeau Mycobacterial Collection (currently housed at the American Type Culture Collection, Rockville, Md.). The antigens belonged to the following species (Trudeau Mycobacterial Collection numbers are given in parentheses): M. tuberculosis H37Rv (102) and Erdman (107), M. bovis BCG (405), M. intracellulare (1403; serovar MAIS 14), M. avium (716, serovar MAIS 1), M. kansasi (1284), M. scrofulaceum (1323), M. vaccae (1526), M. smegmatis (1546), and M. phlei (1523). Organisms were grown in Long synthetic medium (14) and were harvested after complete pellicle formation or as young actively growing cultures which varied from 2 to 6 weeks depending on the species. The organisms were sonicated and centrifuged to obtain antigens as described earlier (3, 4). Antigens were also obtained from Escherichia coli and Nocardia asteroides by sonication and centrifugation. The protein concentration of the antigens was measured by the method of Lowry et al. (15).

Hybridoma procedure. Female BALB/c mice that were 6 to 8 weeks old were used for immunization. A 1-ml amount of dry M. tuberculosis cells (cells autoclaved at 100°C and dried at 37°C) suspended in 0.25 ml of normal saline was mixed with an equal volume of a 35:65 mixture of Aldrich A (Sigma Chemical Co., St. Louis, Mo.) and Drakeol 6V (Panreco Inc., Butler, Pa.). A 0.5-ml volume of the emulsion was injected intraperitoneally into mice. Secondary immunization was carried out with intraperitoneal injection of 100 μg of sonically treated antigen every 2 weeks. A minimum of three such injections was given. Mice were boosted with 50 μg of antigen in 0.1 ml of saline: 3 days later their spleens were removed, and the spleen cells were fused with Sp2/O plasmacytoma cells by using polyethylene glycol 1500 (Malinkrodt, Inc., Paris, Ky.) and standard procedures (2).

The enzyme-linked immunosorbent assay (ELISA) procedure described below was used to screen culture supernatants for the antibody. Cells from wells whose media showed reactivity with sonically treated M. tuberculosis antigen were selected and cloned by the limiting dilution technique at 0.5 and 1.0 cell per well. These were further recloned at 0.3 cell per well at least two more times. Selected clones were then expanded in culture flasks. Finally, 10⁶ cells were injected intraperitoneally into mice primed with pristane (Sigma) to obtain ascites fluid.

ELISA. Each well of a 96-well Immulon 1 plate (Dyneatech Laboratories, Inc., Alexandria, Va.) was coated with 0.1 ml of a 15-μg/ml solution of M. tuberculosis sonically treated antigen in 0.1 M Tris hydrochloride buffer, pH 8.5, and incubated overnight at 28°C. Plates were washed three times with saline-Brij 35 (0.1%) and incubated for 1 h with a 1:10 dilution of culture supernatants. Phosphate-buffered saline (0.01 M) with 0.1% Brij 35 was used to make all dilutions. The plates were washed, and 0.1 ml of a 1:1,000 dilution of sheep anti-mouse immunoglobulin G (Ig-G)-alkaline phosphatase conjugate (Sigma) was added. After a 1-h incubation, the plates were again washed, and 0.1 ml of a 1-mg/ml solution of p-nitrophenyl disodium phosphate substrate (Sigma) in 1 M Tris hydrochloride buffer (pH 9.6)–3 mM MgCl₂ was added. Optical density was measured in a Dynatech ELISA reader after 30 min. For specificity studies, sonically treated antigens obtained from M. bovis BCG, M. intracellulare, M. avium, M. kansasii, M. scrofulaceum, or M. vaccae were used instead of M. tuberculosis.

Characterization of immunoglobulin class and subclass. The immunoglobulin subclass of the MAbs was characterized by ELISA. The procedure was similar to the ELISA described above. In brief, sonically treated M. tuberculosis antigen was coated onto Dynatech plates as described above and...
TABLE 1. Immunoglobulin subclass and molecular weight of antigens recognized by MAb

<table>
<thead>
<tr>
<th>MAb</th>
<th>Immunoglobulin subclass</th>
<th>Mol wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>HGT 1</td>
<td>IgG1</td>
<td>45,000-60,000</td>
</tr>
<tr>
<td>HGT 2</td>
<td>IgG1</td>
<td>45,000-60,000</td>
</tr>
<tr>
<td>HGT 3a</td>
<td>IgM</td>
<td>38,000</td>
</tr>
<tr>
<td>HGT 4</td>
<td>IgG1</td>
<td>28,000-60,000</td>
</tr>
<tr>
<td>HGT 6</td>
<td>IgG1</td>
<td>43,000-45,000</td>
</tr>
</tbody>
</table>

reacted with all the MAbs. Each MAb was then reacted with anti-mouse IgG1, IgG2a, IgG2b, IgM, IgA, and IgE conjugated to alkaline phosphatase (Southern Biotechnology Association, Birmingham, Ala.). After a 2-h incubation, the plates were washed, and color was developed by the addition of p-nitrophenyl disodium phosphate substrate. Optical density was measured in a Dynatech ELISA reader. One of the MAbs, HGT 3a, reacted only with anti-IgM, indicating that it is an IgM antibody, while the others reacted only with anti-IgG1.

SDS-PAGE and immunoblotting. Antigens were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 5 to 20% gradient gel under reducing conditions in a discontinuous buffer system (11). Molecular weight standards (Sigma) were included. Resolved antigens were electrophoretically transferred to nitrocellulose paper (pore size, 0.45 μm) (BA 85; Schleicher & Schuell, Inc., Keene, N.H.) in a 25 mM Tris–192 mM glycine–20% methanol buffer (pH 9.0) at 10 V/cm for 3 h. The paper was washed in phosphate-buffered saline containing 2% Tween 20 for 30 min to block remaining binding sites. A strip of nitrocellulose containing the molecular weight standards was stained with a 0.1% solution of amido black in 45% methanol–10% acetic acid for 15 min and destained in a solution containing 90% methanol and 10% acetic acid. Nitrocellulose paper (0.5-cm strips) with resolved antigens was incubated with a 1:100 dilution of culture supernatant or a 1:2,000 to 1:10,000 dilution of ascites fluid in phosphate-buffered saline–2% Tween 20 for 2 h. After three washings with phosphate-buffered saline–2% Tween 20, the strips were further incubated with a 1:1,000 dilution of affinity-purified anti-mouse IgG–horseradish peroxidase conjugate (Bio-Rad Laboratories, Richmond, Calif.) and incubated for 2 h. After three washings the strips were developed with a mixture of 80 μg of diocetyl sulfosuccinate (Sigma), 10 ml of ethanol, 24 mg of 3,3′,5,5′-tetramethylbenzidine (Sigma), 20 μl of 30% hydrogen peroxide (Fisher Scientific Co., Fairlawn, N.J.), and 30 ml of citrate-phosphate buffer, pH 5.0.

RESULTS

Five MAb s designated as HGT 1, HGT 2, HGT 3a, HGT 4, and HGT 6 are described. One of the MAb s was an IgM, and the others were of subclass IgG1 (Table 1). The molecular weights of the antigens which reacted with the MAb s are shown in Table 1. The specificity of the MAb s was determined both by ELISA and immunobLOTS.

ELISA results for specificity are described in Table 2.

TABLE 2. Reactions of MAb s with M. tuberculosis and other mycobacterial antigens as studied by ELISA

<table>
<thead>
<tr>
<th>Antigens from:</th>
<th>MAb reactiona</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HGT 1</td>
</tr>
<tr>
<td>M. tuberculosis</td>
<td>+++</td>
</tr>
<tr>
<td>M. bovis</td>
<td>±</td>
</tr>
<tr>
<td>M. intracellulare</td>
<td>±</td>
</tr>
<tr>
<td>M. avium</td>
<td>±</td>
</tr>
<tr>
<td>M. kansasii</td>
<td>±</td>
</tr>
<tr>
<td>M. scrofulaceum</td>
<td>+</td>
</tr>
<tr>
<td>M. vaccae</td>
<td>–</td>
</tr>
</tbody>
</table>

a Results are the mean of five experiments.

b ELISA reactions were scored as follows: +++ (0.5 to 1.0), strongly positive; + + (0.25 to 0.5), moderately strong; + (0.1 to 0.25), positive; ± (0.05 to 0.1), borderline; – (0 to 0.05), negative. Numbers in parentheses indicate the range of optical density values.

FIG. 1. Coomassie blue stain of SDS-PAGE-separated antigens. Lanes: 0, molecular weight standards (from top to bottom, β-galactosidase [116,000], phosphorylase B [97,400], bovine albumin [66,000], egg albumin [45,000], carbonic anhydrase [29,000], trypsin inhibitor [20,100], and α-lactalbumin [14,200]); 1, M. tuberculosis H37Rv; 2, M. tuberculosis Erdman; 3, M. bovis BCG; 4, M. intracellulare; 5, M. avium; 6, M. kansasii; 7, M. scrofulaceum; 8, M. vaccae; 9, M. smegmatis; 10, M. phlei; 11, E. coli; 12, N. asteroides.

FIG. 2. Immunoblot studies of five MAb s with sonically treated M. tuberculosis antigen. Lanes: 0, molecular weight standards (amido black stained); 1, HGT 1; 2, HGT 2; 3, HGT 3a; 4, HGT 4; 5, HGT 6.

FIG. 4. Immunoblot studies of HGT 3a with 12 antigens. Lanes are as in the legend to Fig. 3.

Sonically treated antigens were derived from *M. tuberculosis*, *M. bovis* BCG, *M. intracellulare*, *M. avium*, *M. kansasi*, *M. scrofulaceum*, and *M. vaccae*. *A$_{410}$* readings were obtained with a Dynatech ELISA reader and scored as described in Table 2, footnote b. HGT 1 and HGT 2 reacted strongly with *M. scrofulaceum*, gave borderline reactions with *M. intracellulare* and *M. avium*, and were negative with *M. bovis* BCG. HGT 3a showed a positive reaction with *M. tuberculosis* and negative reactions with all the others. HGT 4 showed strong reactions with *M. tuberculosis*, *M. bovis* BCG, and *M. scrofulaceum* and a positive reaction with *M. kansasi*. HGT 6 showed moderately strong reactions with *M. tuberculosis* and *M. avium*, positive reactions with *M. intracellulare*, *M. scrofulaceum*, and *M. kansasi*, and a negative reaction with *M. bovis* BCG. None of the MAbs showed a positive reaction with *M. vaccae*.

FIG. 5. Immunoblot studies of HGT 4 with 12 antigens. Lanes are as in the legend to Fig. 3.

FIG. 6. Immunoblot studies of HGT 6 with 12 antigens. Lanes are as in the legend to Fig. 3.

Figure 1 shows the Coomassie blue stain of SDS-PAGE separation of 10 mycobacterial, *E. coli*, and *N. asteroides* antigens. A large number of antigens were resolved with a 5 to 20% gradient gel. This pattern served as the basis for determining the specificity of the five MAbs.

Figure 2 shows immunoblot studies and comparison with molecular weight standards. *M. tuberculosis* H$_{37}$Rv sonically treated antigen was used. HGT 1 and HGT 2 recognized multiple bands in the molecular weight range of 45,000 to 60,000 and appeared to be similar, although they were derived from two different fusion experiments. HGT 3a showed a single band recognizing an antigen of 38,000 molecular weight. HGT 4 again showed multiple bands but...
was different from HGT 1 and HGT 2 in recognizing more low-molecular-weight molecules. HGT 6 recognized two major bands with molecular weights of 43,000 and 45,000.

Figures 3 to 6 show immunoblot studies of 12 antigens with HGT 1, HGT 3a, HGT 4, and HGT 6, respectively. HGT 2 is not shown since it appeared similar to HGT 1. Although ELISA results were negative for antigens of several species of mycobacteria, the immunoblot experiments of HGT 1 with SDS-PAGE-separated antigens showed reactions with all mycobacteria tested except M. vaccae. Broad cross-reactivity of HGT 1 was indicated in that E. coli and N. asteroides antigens were also recognized. HGT 3a, however, recognized a 38,000-molecular-weight antigen present in the tuberculosis complex of M. tuberculosis and M. bovis BCG. The M. bovis BCG band was much weaker than that of M. tuberculosis. HGT 4 recognized multiple bands from M. tuberculosis, M. bovis BCG, M. kansasii and N. asteroides. HGT 6 also recognized bands from all antigens except those from M. vaccae, M. phlei, and E. coli. A strip of nitrocellulose paper with resolved antigens without the first antibody or with a 1:1,000 dilution of normal mouse serum was included in each experiment as a control. No bands were visualized in the control.

DISCUSSION

Five MAbs have been described for M. tuberculosis sonically treated antigen. Cross-reactivity of these antibodies with other mycobacterial antigens and E. coli and N. asteroides have been studied by ELISA and immunoblot.

Use of ELISA and immunoblot as the two methods of screening for specificity revealed interesting aspects. ELISA tended to show the antibodies to be more specific than immunoblot. HGT 1 was shown to be negative with M. bovis BCG, M. kansasii, and M. vaccae by ELISA. Immunoblots, however, recognized weak bands from M. bovis BCG and M. kansasii. Similar results were observed with HGT 3a, HGT 4, and HGT 6. The negative values in ELISA may arise because of two factors. One is the arbitrarily chosen cutoff values which make it difficult to differentiate between weak and negative reactions. The other reason could be that all the antigens might not bind the plate template equally and thus may give false-negative results. Immunoblots, on the other hand, have many advantages. In addition to higher sensitivity than ELISA, under the conditions of SDS-PAGE the antigens are unmasked, thus making the epitopes more easily available for detection. The reasons for visualizing multiple bands with HGT 1, HGT 2, and HGT 4 could be that the epitopes recognized by these MAbs are present on a large number of antigenic molecules or that the antigens might be fragmented during sonication and are seen as multiple bands.

MAbs to M. tuberculosis, some of which appear to be similar to those described in the present study, have been described by other investigators (1, 5–8, 13, 16, 17). It has been observed that MAbs detect 12-, 14-, 19-, 38-, 40-, 65-, and 71-kilodalton antigens (World Health Organization Workshop, Letter, Infect. Immun. 51:718–720, 1986). Our HGT 3a antibody appears to be similar to TB 72 described by Coates et al. (5) and HYT 28 described by Andersen et al. (1). The immunoglobulin class, however, was different. HGT 3a is an IgM antibody, while the other two are IgG1. HGT 6, which recognizes two molecules of 43,000 and 45,000 molecular weight has not been previously described.

MAbs to M. tuberculosis have many uses. Ivanyi et al. (8) have used TB 72 in serodiagnosis. As this antibody reacts to M. bovis, serodiagnosis with this antibody in a BCG-vaccinated population needs to be fully explored. Antigen detection (9, 10) after affinity purification may be more useful in diagnosis than antibody, as a high antigen concentration indicates current infection. Young et al. (19) have used MAbs in the expression of a λ bacteriophage DNA clone of M. tuberculosis. It is important to continue and expand research on MAbs to find antibodies which react with M. tuberculosis and not with M. bovis. The isolation of antigens with MAbs may allow serodiagnosis by chromatography and DNA hybridization studies holds a greater promise in identifying small peptides active in cellular immunity in tuberculosis. Such peptides are good candidates as synthetic vaccines, as specific skin test reagents, and in serodiagnosis.

LITERATURE CITED