Specificity of Mycobacterium tuberculosis Antigen 5 Determined with Mouse Monoclonal Antibodies

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Four mouse monoclonal antibodies have been developed which react with Mycobacterium tuberculosis antigen 5. Each of these monoclonal antibodies has been used to prepare immunoabsorbents, and antigen has been isolated from unheated M. tuberculosis H37Ra culture filtrate by affinity chromatography with these absorbents. Antigen thus obtained was found to be similar in tubulin reactivity and in enzyme-linked immunosorbent assays to antigen 5 isolated from polyvalent immunoabsorbents, and the protein yields from the monoclonal immunoabsorbents were similar to those from polyvalent absorbents. Antigen recovered from monoclonal absorbents cross-reacted with antigens of Mycobacterium kansasi in delayed skin tests. Immunoelectrophoresis demonstrated that the major component of the antigens eluted from the monoclonal immunoabsorbents was mycobacterial arabinomannan. Antigen 5 was not identified in the eluates by immunoelectrophoresis. These observations support the hypothesis that antigen 5 and M. tuberculosis arabinomannan contain a single major shared epitope.

Abstract

Antigen 5 is a well-characterized protein antigen of Mycobacterium tuberculosis (7-9, 13). When studied by immunoprecipitation techniques using hyperimmune goat antiserum, it is limited to M. tuberculosis and Mycobacterium bovis among 14 mycobacterial species studied (9). In our experience, it is the only major antigen readily identified by immunoelectrophoresis which is thus limited and to which reactivity cannot be removed from polyvalent hyperimmune antiserum by absorption with other species of mycobacteria. Paradoxically, it is apparently no more specific than tuberculin purified protein derivative (PPD) when used to skin test human populations sensitized to M. tuberculosis and other mycobacteria (8). Moreover, the serum of many patients with disease due to other mycobacteria contains immunoglobulin G (IgG) antibody to M. tuberculosis antigen 5 recognizable by enzyme-linked immunosorbent assay (ELISA) (1). To cast further light on the antigenic specificity of antigen 5, several mouse hybridoma monoclonal antibodies reactive with antigen 5 have been produced. Their reactivities have been studied, and they have been used to isolate antigen by immunoabsorbent affinity chromatography. The several antigenic products thus prepared have been studied and compared with antigen 5 prepared from immunoabsorbents made with polyclonal goat antibody.

MATERIALS AND METHODS

Development of monoclonal antibodies. Female 10- to 12-week-old BALB/c mice were injected intraperitoneally with 50 µg of unheated, lyophilized M. tuberculosis H37Ra culture filtrate solids (10) in 0.5 ml of an emulsion containing equal volumes of Freund incomplete adjuvant (Difco Laboratories, Detroit, Mich.) to which 10 mg of washed, heat-killed, dried M. tuberculosis H37Ra cells had been added per ml. Four weeks later these animals were boosted intraperitoneally with 200 µg of the same non-dialyzable culture filtrate solids in isotonic saline. Several attempts to immunize mice with soluble purified M. tuberculosis antigen 5 did not yield mice with measurable serum antibody to antigen 5.

At 3 or 4 days after the soluble culture filtrate injection, mice were sacrificed, and single-cell suspensions from the spleens were fused with P3/NS1/1-Ag4-1 mouse myeloma cells at a ratio of 1 myeloma cell to 10 mouse spleen cells. The fusion was performed using 50% polyethylene glycol-1500, and the hybrids were grown in hypoxanthine-, aminopterin-, and thymidine-containing medium at 37°C in a 5% CO2 atmosphere as previously described (16). After sufficient growth had occurred, supernatants were removed and screened for anti-mycobacterial reactivity by ELISA. Hybridic reactive with M. tuberculosis antigen 5 but minimally reactive with Mycobacterium intracellulare, Mycobacterium kansasi, Mycobacterium scrofulaceum, and Mycobacterium gordonae were cloned once by limiting dilution and grown as ascites-producing tumors in the peritoneal cavities of pristane-primed mice. The immunoglobulin classes of the monoclonal antibodies were determined by immunodiffusion of cell culture supernatants concentrated 10-fold by lyophilization against specific sheep anti-mouse immunoglobulin antisera (New England Nuclear Corp., Boston, Mass.).

ELISA. ELISA for assessing the antibody content of cell culture supernatant fluids and ascitic fluids was performed as previously described (1). Polyvinyl microtiter plates (Dynatech Laboratories, Alexandria, Va.) were sensitized with antigen 5 at 5 µg/ml or with culture filtrate at 10 µg/ml from M. intracellulare, M. kansasi, M. scrofulaceum, and M. gordonae. Goat anti-mouse immunoglobulin conjugated to alkaline phosphates (New England Nuclear or Tago, Inc., Burlingame, Calif.) was used in this assay. Plates were read in a microELISA plate reader (Dynatech). Because a positive reference mouse antibody was not available, the highest serum dilution giving a reading of 0.06 optical density units at 410 nm was accepted as the endpoint. Endpoints of human sera were determined by comparison with a positive reference serum as previously described (1). Data were expressed as the means of replicate titrations.

Immunoadsorbent affinity chromatography. Immunoadsorbent affinity chromatography was performed as previously described (4, 7). The polyclonal but apparently monospecific goat antiserum used for the preparation of antigen 5 in this study was the same as the one used for all of our previously

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published work with antigen 5. For the preparation of immunoabsorbents from ascitic fluids, the globulin was precipitated at 50% saturation with ammonium sulfate. For the preparation of immunoabsorbents from cell culture supernatants, 50 ml of supernatant was lyophilized and reconstituted to a volume of 5 ml. The globulin was then precipitated with ammonium sulfate at 50% saturation. For the preparation of immunoabsorbents, monoclonal antibody globulins of each immunoglobulin class have been used successfully in concentrations of 0.6 to 6.0 mg/ml of activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden). The studies reported here were performed with 0.6 mg/ml. Polyclonal goat immunoabsorbents were prepared using 5.0 mg immunoglobulin per ml of activated Sepharose 4B (Pharmacia) as previously described (6, 7). Protein was determined by the method of Lowry and his co-workers (18) after concentration by lyophilization if necessary.

Skin testing of guinea pigs. Delayed skin tests in guinea pigs were performed as previously described (9, 21). Groups of six guinea pigs were sensitized with heat-killed cells of M. tuberculosis H37Ra or M. kansasii incorporated in incomplete Freund adjuvant (Difco). Reference tuberculin PPD (PPD-S) of M. tuberculosis and PPD of M. kansasii (PPD-Y) were kindly supplied by Robert C. Good, Centers for Disease Control, Atlanta, Ga. They were used as supplied at a concentration of 1 µg/ml in buffered saline without Tween-80. All antigen 5 preparations used for skin testing were prepared in phosphate-buffered saline containing 0.0005% Tween-80 (17) at a concentration of 5 µg/ml, providing a skin test dose of 0.5 µg, the dose previously found to be bioequivalent to 0.1 µg of PPD-S (13). All injection sites were randomized separately for each animal, and readings were made without knowledge of the randomization schemes. Analysis of variance was used to estimate the statistical significance of skin test results.

Immunoprecipitation. Immunelectrophoresis for the identification of M. tuberculosis antigens was performed as previously described (5, 11, 15). Lot 002A polyvalent reference antiserum (11) was employed. Arabinomannan used in immunelectrophoresis was purified by concanavalin A affinity chromatography as described previously (3).

RESULTS

Monoclonal antibodies. Three hybridoma lines producing monoclonal antibodies were obtained as the result of our initial cell fusion and designated TB5-1, TB5-2, and TB5-20. The monoclonal antibodies which they produced were, respectively, IgM, IgG, and IgG2b immunoglobulins. In a later fusion an additional cell line was obtained. Because this cell line was suspected of containing two clones, it was subjected to a second and third limiting dilution, and the subclone selected for propagation was designated TB-C-1. The immunoglobulin class of this antibody was IgM. Cell lines TB5-1, TG5-2, and TB5-20 were studied as cell culture supernatants and were also implanted in mouse peritoneal cavities and studied as antibody-containing ascitic fluids. Cell line TB-C-1 was studied as cell culture supernatant only.

All of the cell lines produced antibody reactive in ELISA with M. tuberculosis antigen 5 (Table 1). All except the very low-titer cell line TB5-1 also reacted in ELISA with unheated culture filtrates of other mycobacterial species, although in each case the titers were substantially lower than the titers with antigen 5. TB5-1, TB5-2, and TB5-20 ascitic fluids were tested by ELISA against M. tuberculosis arabinomannan. TB5-1 and TB5-2 reacted at a titer of 1:10; TB5-20 did not react at 1:10. None of the ascitic fluid antibodies precipitated with M. tuberculosis culture filtrate when used in immunelectrophoresis, and combinations of ascitic fluids TB5-1 + TB5-2, TB5-1 + TB5-20, and TB5-2 + TB5-20 did not precipitate.

Imunoabsorbent affinity chromatography. Monoclonal antibody-containing ascitic fluids from cell lines TB5-1, TB5-2, and TB5-20 were used to prepare immunoabsorbents simultaneously with polyclonal goat antibody. Cell culture supernatant from line TB-C-1 was lyophilized and reconstituted to one-tenth its original volume and similarly used to prepare immunoabsorbents simultaneously with polyclonal goat antibody. Unheated M. tuberculosis culture filtrate was applied to the immunoabsorbent columns, the columns were washed, and antigen was eluted with urea. After dialysis, the protein concentration was 9.9 to 10.8 µg/ml for the monoclonal ascitic fluid absorbents and 12.5 µg/ml for the simultaneously prepared polyclonal goat immunoabsorbents. It was 6.1 µg/ml for the TB-C-1 cell culture supernatant immunoabsorbent. These values are all within the usual range obtained in our laboratory.

Characterization of antigens eluted from immunoabsorbents. The eluates from the TB5-1, TB5-2, and TB5-20 ascitic fluid immunoabsorbents were lyophilized and reconstituted at a protein concentration of 2.0 mg/ml. At this concentration immunelectrophoresis with polyvalent hyperimmune goat antiserum yielded single precipitin arcs for each eluate which were similar in appearance and displayed cathodal migration rather than the anodal migration characteristic of antigen 5 (Fig. 1). By a modified Osserman technique, as described previously (5), these arcs were shown to give precipitation reactions of complete identity with M. tuberculosis arabinomannan and not with antigen 5.

The eluates from the ascitic fluid monoclonal immunoabsorbents and from a goat polyclonal immunoabsorbent were used to sensitize microtiter plates for ELISA at protein concentrations of 5 µg/ml. All yielded plates which were satisfactorily sensitized. Titration of high- and low-titer human sera and a hyperimmune goat antiserum (Table 2) yielded similar titers with all of the eluates.

The eluates from the ascitic fluid monoclonal absorbents and from the polyclonal goat immunoabsorbent were all adjusted to a concentration of 5 µg/ml and used to skin test guinea pigs sensitized to M. tuberculosis or M. kansasii. These animals were also skin tested with PPD-S and PPD-Y (Table 3). All of the eluates elicited delayed skin test reactions in both sets of animals. The reactions to antigen recovered from the immunoabsorbent prepared from cell line TB5-20 were significantly smaller than the those to TB5-1 and TB5-2 antigens, but there were no other significant differences among the sizes of reactions elicited by the four absorbent eluates. Reactions elicited with antigen eluted from the goat antibody absorbent did not differ from those elicited with the monoclonal eluates. In no case was there a significant difference between the reactions in M. tuberculosis- and M. kansasii-sensitized animals.

DISCUSSION

We were able to use all four of the monoclonal antibodies, including antibodies of both IgM and IgG classes, for antigen purification by our previously described technique of immunoabsorbent affinity chromatography (6). We were able to use antibody from both ascitic fluids and cell culture supernatants. The products recovered from the three ascitic fluid monoclonal immunoabsorbents were all similar and identified with arabinomannan but not antigen 5. However,
the ascitic fluids from which the immunoabsorbsents were prepared reacted in ELISA with antigen 5 but not with arabinomannan. Since, as seen in Table 2, these elution products were capable of sensitizing ELISA plates and since purified arabinomannan is also capable of sensitizing ELISA plates (R. G. Benjamin, S. M. Debanne, and T. M. Daniel, unpublished data), we conclude that the epitope on arabinomannan with which our antigen 5-reactive monoclonal antibodies react is not sterically accessible when arabinomannan is fixed to ELISA plates. Since the TB5-1, TB5-2, and TB5-20 elution products were all identical in every aspect studied and since immune precipitation could not be obtained with combinations of these monoclonal antibodies, we believe that they all react with the same epitope and that it is a single epitope on those molecules on which it is present. Our ELISA and immunoelectrophoresis data suggest strongly that this single epitope is present on both *M. tuberculosis* antigen 5 and arabinomannan. Assuming that skin test reactions are directed against the same epitope, our skin test data (Table 3) indicate that animals sensitized with *M. kansasii* develop cellular hypersensitivity to it. However, our ELISA data (Table 1) indicate that this epitope is no more than a minor constituent of culture filtrates of *M. intracellulare*, *M. kansasii*, *M. scrofulaceum*, and *M. gordonae*. Using immunoelectrophoresis, we were not able to identify antigen 5 in our elution products, and the single precipitin arc obtained did not identify with antigen 5. We conclude that the conditions of immunoabsorbent affinity chromatography which we employed (3) preferentially yielded arabinomannan.

The chemical structure of arabinomannan is well known (19). The arabinomannan which we used in these studies was prepared by concanavalin A-affinity chromatography (3). Its carbohydrate structure has been elucidated (12), and it contains little or no protein (3). It does not elicit significant delayed skin test reactions in sensitized guinea pigs (4). The TB5-1, TB5-2, and TB5-20 elution products contained substantial amounts of protein and did elicit delayed skin test reactions. The cathodal migration of the product was typical of purified mycobacterial arabinomannan and makes it unlikely that the protein was present as a moiety physically complexed to arabinomannan. We are forced to conclude that protein antigen 5 was present in the eluates in a form that did not permit immunoprecipitation detectable with reagent antisemur.

It has become generally accepted that polysaccharides do not elicit significant delayed skin test reactions, and it is well documented that purified arabinomannan does not (4, 19). Considering all of our data, however, we think it possible and perhaps probable that the epitope with which TB5-1, TB5-2, and TB5-20 react is the same as that which elicits delayed skin tests and is carbohydrate in nature. It may

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**TABLE 1.** ELISA titers of hybridoma cell culture supernatants and ascitic fluids against antigen 5, arabinomannan, and culture filtrates of nontuberculous mycobacteria

<table>
<thead>
<tr>
<th>Hybridoma line</th>
<th>Antigen 5 (1:2)</th>
<th>Arabinomannan (1:2)</th>
<th><em>M. intracellulare</em> (1:2)</th>
<th><em>M. kansasii</em> (1:2)</th>
<th><em>M. scrofulaceum</em> (1:2)</th>
<th><em>M. gordonae</em> (1:2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell culture supernatants</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TB5-1</td>
<td>1:8</td>
<td></td>
<td>&lt;1:2</td>
<td>&lt;1:2</td>
<td>&lt;1:2</td>
<td>&lt;1:2</td>
</tr>
<tr>
<td>TB5-2</td>
<td>1:128</td>
<td></td>
<td>&lt;1:2</td>
<td>1:2</td>
<td>&lt;1:2</td>
<td>&lt;1:2</td>
</tr>
<tr>
<td>TB5-20</td>
<td>1:1,000</td>
<td></td>
<td>1:4</td>
<td>1:64</td>
<td>1:8</td>
<td>1:4</td>
</tr>
<tr>
<td>TB-C-1</td>
<td>1:640</td>
<td></td>
<td>1:40</td>
<td>1:40</td>
<td>1:40</td>
<td>1:80</td>
</tr>
<tr>
<td>Ascitic fluids:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TB-1</td>
<td>1:10</td>
<td>1:10</td>
<td>&lt;1:10</td>
<td>&lt;1:10</td>
<td>&lt;1:10</td>
<td>&lt;1:10</td>
</tr>
<tr>
<td>TB5-2</td>
<td>1:5,600</td>
<td>1:10</td>
<td>1:10</td>
<td>1:10</td>
<td>1:10</td>
<td>1:10</td>
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<td>TB5-20</td>
<td>1:180</td>
<td>&lt;1:10</td>
<td>&lt;1:10</td>
<td>&lt;1:10</td>
<td>&lt;1:10</td>
<td>&lt;1:10</td>
</tr>
</tbody>
</table>

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**FIG. 1.** Immunoelectrophoresis with polyvalent reference antiserum (11) of antigens eluted from immunoabsorbsents prepared with monoclonal antibodies TB5-1 (top), TB5-2 (middle), and TB5-20 (bottom). Single similar precipitin arcs were obtained with each eluate, and these arcs had the cathodal electrophoretic mobility typical of mycobacterial arabinomannan. Precipitin arcs in the anodal position typical of *M. tuberculosis* antigen 5 were not observed.

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**TABLE 2.** ELISA titers of high- and low-titer patient sera and hyperimmune goat antisemur on microtiter plates sensitized with eluates from polyclonal goat and monoclonal ascitic fluid immunoabsorbsents

<table>
<thead>
<tr>
<th>Immunoabsorbsent</th>
<th>Low-titer human serum</th>
<th>High-titer human serum</th>
<th>Hyperimmune goat serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat Q</td>
<td>1:80</td>
<td>1:320</td>
<td>1:2,180</td>
</tr>
<tr>
<td>TB5-1</td>
<td>1:40</td>
<td>1:640</td>
<td>1:640</td>
</tr>
<tr>
<td>TB5-2</td>
<td>1:20</td>
<td>1:320</td>
<td>1:2,560</td>
</tr>
<tr>
<td>TB5-20</td>
<td>1:80</td>
<td>1:160</td>
<td>1:320</td>
</tr>
</tbody>
</table>
TABLE 3. Mean tuberculin skin test reaction diameters elicited by eluates from monoclonal immunoabsorbent affinity columns

<table>
<thead>
<tr>
<th>Antibody used for immunoabsorbent</th>
<th>M. tuberculosis</th>
<th>M. kansasii</th>
</tr>
</thead>
<tbody>
<tr>
<td>TB5-1</td>
<td>10.2 ± 0.83</td>
<td>9.1 ± 1.14</td>
</tr>
<tr>
<td>TB5-2</td>
<td>9.7 ± 1.33</td>
<td>8.6 ± 0.30</td>
</tr>
<tr>
<td>TB5-20</td>
<td>6.3 ± 1.30</td>
<td>7.8 ± 0.86</td>
</tr>
<tr>
<td>Goat Q</td>
<td>8.5 ± 0.70</td>
<td>9.2 ± 0.53</td>
</tr>
<tr>
<td>PPD-S (0.1 μg)</td>
<td>7.3 ± 0.72</td>
<td>6.0 ± 1.61</td>
</tr>
<tr>
<td>PPD-Y (0.1 μg)</td>
<td>5.3 ± 1.42</td>
<td>10.8 ± 1.13</td>
</tr>
</tbody>
</table>

* Mean ± SE in groups of six guinea pigs; 0.5 μg antigen dose.
* Reactions to eluates from TB5-20 were significantly smaller than reactions to other eluates (P < 0.05 by analysis of variance).
* Kindly supplied by Robert C. Good, Center for Disease Control, Atlanta, Ga.
* Reactions to PPD-Y were significantly smaller in M. tuberculosis animals than in M. kansasii animals (P < 0.01).

...function as a hapten on a protein carrier. The protein is probably that which we have previously identified as antigen 5 (7). Coates, Mitchison, and their associates (2, 20) have previously described seven hybridoma cell lines secreting monoclonal antibody to M. tuberculosis. One of these was obtained from a mouse immunized with whole mycobacterial cells of the H37Ra strain; the other six were obtained from mice immunized with cell pressates of the H37Rv and S1 strains. In a solid-phase radioimmunossay, the monoclonal antibody obtained after whole-cell immunization was able to distinguish between the S1 strain and four other strains of M. tuberculosis. This difference was presumed to relate to surface antigen differences, and it did not correlate with phase type. In subsequent studies (14), these monoclonal antibodies were used in a competitive inhibition radioimmunoassay to identify antibody in sera from 41 patients with pulmonary tuberculosis and 30 healthy control subjects. The monoclonal antibody which was thought to be strain specific detected antibody to M. tuberculosis in as many (49%) of the tuberculosis patients as did the other monoclonal antibodies, suggesting that the specificity of this antibody for the individual strain of M. tuberculosis did not extend to the inhibition assay with human serum. There is no reason to believe that any of the monoclonal antibodies of Coates and associates react with either antigen 5 or arabinomannan.

ACKNOWLEDGMENTS

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LITERATURE CITED