Characterization of *Mycobacterium tuberculosis* Antigen 5 Epitopes by Using a Panel of 19 Monoclonal Antibodies

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Antigen 5 is a 35,000-dalton protein which has been purified from culture filtrates of *Mycobacterium tuberculosis* and shown by immunoprecipitation to be restricted in distribution to *M. tuberculosis* and *M. bovis* among 14 mycobacterial species studied. We raised 19 antigen 5-reactive monoclonal antibodies and used them to characterize epitopes of this antigen by enzyme-linked immunosorbent assay and immunoabsorbent affinity chromatography. Fifteen monoclonal antibodies, all immunoglobulin M (IgM), cross-reacted in two major patterns with culture filtrates from five species of mycobacteria and with purified mycobacterial arabinomannan and arabinogalactan. All 15 monoclonal antibodies also reacted with *M. tuberculosis* antigen 6. Immunoabsorbent affinity columns prepared with these antibodies yielded principally arabinomannan and arabinogalactan. Four monoclonal antibodies, three IgG2a and one IgM, reacted by enzyme-linked immunosorbent assay with antigens 5 and 6 exclusively and not with mycobacterial culture filtrates or polysaccharides. All four monoclonal antibodies yielded antigen 5 and small amounts of antigen 6 when used for immunoabsorbent affinity chromatography. We conclude from these studies that antigen 5 has two nonspecific epitopes, possibly carbohydrate in nature, and one apparent species-specific epitope which is shared with antigen 6.

Antigen 5 is a well-characterized 35,000-dalton protein of *Mycobacterium tuberculosis* (7). When studied by immunoprecipitation with goat hyperimmune antiserum, this antigen is restricted to *M. tuberculosis* and *M. bovis* and is not shared by other less pathogenic or nonpathogenic mycobacteria (9). Despite this observation, antigen 5 is no more specific for eliciting skin-test reactions than the crude mycobacterial antigen preparation, purified protein derivative (8). Some persons infected with other mycobacteria (which do not contain antigen 5) produce antibodies that are reactive with purified antigen 5 by enzyme-linked immunosorbent assay (ELISA) (1). These findings suggest that the antigen 5 molecule contains both species-restricted epitopes and epitopes which cross-react with those of other mycobacterial antigens. Identification of species-restricted epitopes might lead to a more specific skin-test antigen(s) and more specific serodiagnostic tests for tuberculosis.

In this investigation, 19 antigen 5-reactive monoclonal antibodies were produced and used to characterize epitopes on this molecule. We identified two cross-reactive epitopes (probably carbohydrates) which are shared by antigen 5 and the mycobacterial polysaccharides arabinomannan and arabinogalactan, as well as an apparently restricted epitope shared by antigens 5 and 6. In addition, we produced monoclonal immunoabsorbents which could be used to purify antigen 5.

MATERIALS AND METHODS

Culture filtrates and purified antigens. Culture filtrates of *M. tuberculosis* H₃₇Ra (TMCC 201), *M. intracellulare* serotype 5 (TMCC 1464), *M. scrofulaceum* Gause (TMCC 1316), *M. kansasii* Forbes (TMCC 1201), and *M. gordonae* P-15 (TMCC 1324) were prepared as previously described (10, 13). *M. tuberculosis* arabinomannan and arabinogalactan were purified from culture filtrates by concanavalin

A affinity chromatography, and *M. tuberculosis* antigen 5 was prepared by immunoabsorbent affinity chromatography as previously described (6, 7). Antigen 6 was purified from culture filtrates of *M. tuberculosis* by using immunoabsorbents prepared from a goat antiserum principally reactive with this protein. The tuberculin purified protein derivative used in this study was prepared by ammonium sulfate precipitation from *M. tuberculosis* culture filtrate as previously described (23). Protein concentration of antigens and other solutions was estimated by the method of Lowry et al. (22), and the carbohydrate concentration of polysaccharides was expressed as total pentoses measured by the orcinol reaction, with an arabinose standard (15).

Monoclonal antibodies. BALB/c mice (Jackson Laboratory, Bar Harbor, Maine) were immunized by intraperitoneal injection of 0.2 ml of an emulsion containing equal parts of *M. tuberculosis* culture filtrate (10 mg/ml) and incomplete Freund adjuvant (Difco Laboratories, Detroit, Mich.) to which 10 mg of washed, autoclaved *M. tuberculosis* H₃₇Ra cells per ml was or was not added. Animals were boosted subcutaneously with 0.2 ml of aqueous culture filtrate (0.5 mg/ml) at 4 and 6 weeks after primary immunization. A final injection of 0.4 mg of the same culture filtrate was given intraperitoneally 10 weeks after the initial immunization, and spleens were harvested 4 days later. Some animals were immunized intraperitoneally exclusively with purified antigen 5, first with adjuvant and then every other week with 0.2 ml of a 5-mg/ml saline solution. The animals were then left unstimulated for 4 to 6 weeks, boosted with purified antigen 5, and sacrificed 4 days later. Spleen cells were mixed with P3/NS1/1-Ag4-1 BALB/c myeloma cells (20) at a ratio of 3:5 and fused with polyethylene glycol (M_r , 4,000; Matheson Scientific, Inc., Elk Grove Village, Ill.) (14). Hybrid cells were plated and grown in Dulbecco modified Eagle medium (M. A. Bioproducts, Walkersville, Md.) containing 20% heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, Utah) supplemented with L-glutamine, HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer (M. A. Bioproducts), gentamicin, 2-mercaptoethanol, hypo-

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xanthine, aminopterin, and thymidine (all from Sigma Chemical Co., St. Louis, Mo.). Supernatant fluids from growing clones were screened for reactivity by ELISA as described below. Positive clones were expanded and subjected to three limiting dilutions to assure monoclonality. Subcloned hybridoma cells were then injected intraperitoneally (10^7 cells per mouse) into mice treated with 2,6,10,14-tetramethylpentadecane (Pristane; Aldrich Chemical Co., Inc., Milwaukee, Wis.), and ascitic fluids were collected several weeks later (24). The immunoglobulin class of monoclonal antibodies was determined on 10-fold-concentrated culture supernatant by two-dimensional immunodiffusion with specific antisera (Southern Biotech, Birmingham, Ala.).

ELISA. The ELISA was performed as described previously (13). Controls on each plate included a standard cell culture supernatant known to react positively and a color standard with an optical absorbancy of 0.24 when read in an automated plate reader set to read the ratio of 405/630 nm. Plates were read when the positive standard cell supernatant solution well gave a color intensity equal to that of the color standard. Wells with readings equal to or greater than three times the readings obtained in negative control wells were considered positive, and the highest dilution with a positive result was taken as the endpoint.

Immunoabsorbent affinity chromatography. Immunoabsorbent affinity chromatography was performed as previously described (6, 13). Immunoglobulin was precipitated from cell culture supernatants or from mouse ascitic fluid with ammonium sulfate at 50% saturation at 4°C and used in a concentration of 0.5 mg/ml of activated Sepharose 4B. *M. tuberculosis* culture filtrate was added to the columns, and bound antigens were eluted with 4 M urea. Eluates were concentrated by lyophilization and suspended in phosphate buffer. Protein concentration was determined as before.

Immunoelectrophoretic analysis of eluted antigens. Immunoelectrophoresis was used as described by Janicki et al. (19) and Daniel et al. (12). The numerical antigen nomenclature introduced by these investigators was used throughout this work. Modifications of immunoelectrophoresis which combine immunodiffusion with immunoelectrophoresis (30) and which use modified patterns (5) were used for the identification of individual precipitin arcs.

RESULTS

Production of monoclonal antibodies. Nineteen monoclonal antibodies that reacted with both crude culture filtrate and antigen 5 were produced and are presented in this paper (Table 1). Ten monoclonal-antibody-producing hybridoma lines, designated TB-C-1 through TB-C-10, were obtained from fusions performed with spleen cells from animals immunized with culture filtrate antigens emulsified with Freund adjuvant containing whole mycobacterial cells (Table 1). All of the monoclonal antibodies produced by these cell lines were immunoglobulin M (IgM). One of these monoclonal antibodies, designated TB-C-1, was described in detail previously (13). Seven monoclonal antibody-producing hybridoma lines were obtained from fusions performed with spleen cells from animals immunized with culture filtrate antigens emulsified with incomplete Freund adjuvant not containing mycobacterial cells. Three of these monoclonal antibodies, designated TB-C-13, TB-C-14, and TB-C-15 (Table 1), were IgG2a. Four monoclonal antibodies, designated TB-C-11, TB-C-12, TB-C-16, and TB-C-17, were IgM. Two monoclonal antibody-producing hybridoma lines were obtained from fusions performed with spleen cells

TABLE 1. ELISA results obtained with monoclonal antibody-containing cell culture supernatants tested against mycobacterial antigens

Antigen	Mean optical density for ^a :																		
	TB-C-1	TB-C-2	TB-C-3	TB-C-4	TB-C-5	TB-C-6	TB-C-7	TB-C-8	TB-C-9	TB-C-10	TB-C-11	TB-C-12	TB-C-13	TB-C-14	TB-C-15	TB-C-16	TB-C-17	TB-C-18	TB-C-19
Culture filtrates from:																			
<i>M. tuberculosis</i>	0.82	0.54	0.21	0.15	0.76	0.17	0.67	0.59	0.22	0.21	0.67	0.29	0.05	0.03	0.03	0.43	0.21	0.03	0.22
<i>M. intracellulare</i>	0.17	0.30	0.11	0.13	0.61	0.17	0.53	0.51	0.14	0.18	0.42	0.23	0.04	0.03	0.03	0.39	0.24	0.08	0.20
<i>M. kansasii</i>	0.22	0.34	0.12	0.08	0.57	0.19	0.54	0.47	0.13	0.18	0.57	0.24	0.04	0.04	0.03	0.38	0.25	0.09	0.19
<i>M. scrofulaceum</i>	0.28	0.26	0.12	0.13	0.54	0.22	0.64	0.51	0.14	0.25	0.49	0.25	0.05	0.04	0.02	0.41	0.23	0.09	0.18
<i>M. goodii</i>	0.17	0.30	0.09	0.07	0.73	0.23	0.65	0.53	0.16	0.29	0.50	0.24	0.06	0.03	0.02	0.42	0.27	0.15	0.18
Purified antigens																			
5	0.79	0.60	0.42	0.26	0.62	0.26	0.79	0.46	0.56	0.64	0.60	0.26	0.23 ^b	0.21 ^b	0.11 ^b	0.40	0.44	0.30	0.32
6	0.80	0.59	0.38	0.28	0.55	0.31	0.76	0.48	0.61	0.75	0.61	0.31	0.19 ^b	0.12 ^b	0.09 ^b	0.42	0.43	0.56	0.38
PPD ^c	0.58	0.39	0.14	0.12	0.66	0.27	0.57	0.47	0.36	0.36	0.50	0.23	0.05	0.03	0.03	0.40	0.19		
Arabinomannan	0.24	0.29	0.17	0.20	0.41	0.28	0.54	0.51	0.15	0.16	0.49	0.26	0.04	0.03	0.03	0.34	0.10	0.05	0.17
Arabinogalactan	0.28	0.23	0.17	0.18	0.54	0.23	0.48	0.44	0.19	0.11	0.55	0.34	0.04	0.02	0.03	0.34	0.12	0.06	0.16

^a Monoclonal antibody-containing cell culture supernatants were obtained from hybridoma lines derived from mice immunized with culture filtrate in an emulsion containing whole mycobacterial cells (monoclonal antibodies TB-C-1 through TB-C-10) or from mice immunized without the emulsion (monoclonal antibodies TB-C-11 through TB-C-19).

^b Because prozones were observed with these monoclonal antibodies, values for TB-C-13, TB-C-14, and TB-C-15 against antigens 5 and 6 are reported at a dilution of 1:80.

^c PPD, Purified protein derivative.

from animals immunized with purified *M. tuberculosis* antigen 5. One line, designated TB-C-18, produced IgA antibody. The other line, designated TB-C-19, produced IgM antibody.

ELISA reactivity of monoclonal antibodies. The ELISA reactivities of the monoclonal antibodies against a panel of unheated culture filtrates and purified mycobacterial antigens are shown in Table 1. All of the monoclonal antibodies included in this report were initially selected for ELISA reactivity with antigen 5; all also reacted with antigen 6. Monoclonal antibodies TB-C-1, TB-C-2, TB-C-5, TB-C-6, TB-C-7, TB-C-8, TB-C-11, TB-C-12, TB-C-16, and TB-C-17, all of which were IgM, were broadly reactive. The other IgM monoclonal antibodies, TB-C-3, TB-C-4, TB-C-9, TB-C-10, and TB-C-19, were also reactive with culture filtrates from various species of mycobacteria but were significantly more reactive with purified antigens 5 and 6. Monoclonal antibodies TB-C-13, TB-C-14, and TB-C-15 (all IgG2a) and antibody TB-C-18 (an IgA) were reactive only with antigens 5 and 6.

Antigens recovered from monoclonal immunoabsorbents. Immunoabsorbents were prepared by using immunoglobulins precipitated with ammonium sulfate at 50% saturation from cell culture supernatants from 10 of the IgM-producing hybridoma lines, TB-C-1 through TB-C-10. These immunoabsorbents were used to purify the antigens which they bound in unheated *M. tuberculosis* culture filtrate. These elution products were characterized by immunoelectrophoresis (Table 2). Arabinomannan (antigen 1) was present in all 10 lines, and arabinogalactan (antigen 2) was present in 8 lines. Antigen 5 was not identified with certainty in any, and antigen 6 was found only in TB-C-1 eluates. Other anodal antigens were found in four eluates, and antigen 7 was the major component in each case. Eluates from TB-C-1 contained several anodal components (13).

Immunoglobulins precipitated with ammonium sulfate at 50% saturation from TB-C-13, TB-C-14, TB-C-15, TB-C-18, and TB-C-19 ascitic fluids also were used to prepare immunoabsorbents. Whole *M. tuberculosis* culture filtrate was applied to these immunoabsorbents, and the antigens bind-

FIG. 1. Identification of antigen 5 in eluate from TB-C-15 monoclonal immunoabsorbent by immunoelectrophoresis. Standard immunoelectrophoresis is shown in the upper panel; combined immunoelectrophoresis and immunodiffusion are shown in the lower panel. Polyclonal polyvalent reference antiserum was added to the bottom trough and used to develop a precipitant pattern. The upper panel shows two precipitating antigens in the eluate from the TB-C-15 column. These antigens give arcs in the positions characteristic of antigens 5 and 6. E, Wells with eluate; C, well with reference culture filtrate; 5, reaction of immunologic identity with antigen 5 of one of the components.

ing to each immunoabsorbent were recovered. Immunoelectrophoretic analysis revealed that none of the eluates from these five monoclonal immunoabsorbents contained antigens with electrophoretic mobility typical of mycobacterial polysaccharides. All five monoclonal immunoabsorbents contained antigen 5. In occasional experiments, a second anodal component was also present in the eluates. This component occupied the usual position of antigen 6. These results are shown in Table 2. A representative study identifying antigens 5 and 6 in TB-C-15 monoclonal eluate is shown in Fig. 1.

DISCUSSION

Monoclonal antibodies to antigens of *M. tuberculosis* were first described by Coates and his co-workers (3, 18, 28). Reports of additional monoclonal antibodies have appeared from a number of other laboratories (11, 13, 21, 26, 29). In June 1985 the World Health Organization sponsored a workshop at which an attempt was made to characterize 31 monoclonal antibodies submitted from nine laboratories and to compare their reactivities (16; Immunology Tuberculosis Workshop Participants, Vaccine Development Programme, Letter, Infect. Immun. 51:718-720, 1986). The workshop concluded that none of the 31 monoclonal antibodies examined was specific for an individual strain, although 6 monoclonal antibodies (including TB-C-13) were specific for the *M. tuberculosis* complex. The frequently encountered lack of species specificity of monoclonal antibodies probably means that mycobacterial antigens often contain cross-reactive epitopes and that species specificity rests with individual epitopes rather than with whole antigen molecules. Our results with a battery of monoclonal antibodies which bound to an *M. tuberculosis* species-restricted molecule, antigen 5, provide further support for this hypothesis.

Six of the ten monoclonal antibodies which we obtained from cells of mice immunized with complete Freund adjuvant were IgM antibodies and were broadly nonspecific. They reacted primarily with mycobacterial polysaccharides. This suggests that immunization with whole mycobacterial cells preferentially leads to the development of IgM antibodies reactive with polysaccharides. The nonspecificity of these antibodies indicates a broad distribution of nonspecific epitopes, possibly carbohydrate in nature, among mycobacterial antigens and suggests that antigen 5 contains such an epitope(s).

TABLE 2. Immunoelectrophoretic identification of *M. tuberculosis* antigens in eluates from monoclonal immunoabsorbents prepared with 15 monoclonal antibodies^a

Monoclonal antibody	Ag5	Ag6	AG	AM	OAA
TB-C-1	+	+	+	+	+
TB-C-2	+	+	+	+	+
TB-C-3	-	-	-	+	-
TB-C-4	-	-	+	+	+
TB-C-5	-	-	+	+	-
TB-C-6	-	-	-	+	-
TB-C-7	-	-	+	+	+
TB-C-8	-	-	+	+	-
TB-C-9	-	-	+	+	-
TB-C-10	-	-	+	+	-
TB-C-13	+	+	-	-	-
TB-C-14	+	- ^d	-	-	-
TB-C-15	+	- ^d	-	-	-
TB-C-18	+	- ^d	-	-	-
TB-C-19	+	-	-	-	-

^a Ag5, Antigen 5; Ag6, antigen 6; Ag, arabinogalactan; AM, arabinomannan; OAA, other anodal antigens.

^b +, Found in the immunoabsorbent column eluates.

^c -, Not found in repeated experiments ($n = 3$ to 6). Molecules were identified by individual precipitin arcs after immunoelectrophoretic analysis with a standard reference serum.

^d In occasional experiments (<40%), a small amount of antigen 6 was detected.

TABLE 3. Characteristics of three epitopes of *M. tuberculosis* antigen 5 based on reactivity with 19 monoclonal antibodies

Epitope	Reacting monoclonal antibodies	Characteristics
A	TB-C-1, TB-C-2, TB-C-5, TB-C-6, TB-C-7, TB-C-8, TB-C-11, TB-C-12, TB-C-16, TB-C-17	Shared with arabinomannan and arabinogalactan. Probably carbohydrate. Not specific.
B	TB-C-3, TB-C-4, TB-C-9, TB-C-10	Shared with arabinomannan and arabinogalactan. Probably carbohydrate. Might be identical to epitope A and only reflect differing binding affinities.
C	TB-C-13, TB-C-14, TB-C-15, TB-C-18, TB-C-19	Restricted to antigens 5 and 6. Possibly species specific.

The implication that antigen 5 and arabinomannan share an epitope was a surprising finding. Arabinomannan subjected to rigorous purification by Misaki and his colleagues (27) has been characterized extensively, and the structural formula has been determined without finding any associated amino acids. Arabinomannan purified by concanavalin A as used in the present study has also been found to contain little or no protein (4). Thus, a shared peptide epitope seems unlikely. A shared carbohydrate epitope is more likely, but there is some reason to be cautious in accepting this possibility. Glycosylation of proteins is not accomplished by most bacteria. Antigen 5 has been examined without measurable carbohydrate being found (7). Moreover, antigen 5 contains no amino sugars (7), so that a site of carbohydrate attachment is not evident. However, we have observed (T. M. Daniel and P. A. Anderson, unpublished data) that antigen 5 binds with low affinity to concanavalin A-agrose, suggesting that mannose, glucose, or, possibly, arabinose is intrinsically associated with the antigen 5 molecule. Mycobacterial antigenic cross-reactivity may rest with one or two simple sugars (2). We favor the possibility that a small carbohydrate moiety, perhaps containing mannose, is responsible for the antigenic similarity between arabinomannan and antigen 5.

Four additional monoclonal antibodies were produced from the fusions of spleen cells from mice immunized with complete Freund adjuvant, and one monoclonal antibody was produced from an animal immunized with purified antigen 5 (all IgMs). These monoclonal antibodies reacted weakly with culture supernatant from various mycobacterial species but were significantly more reactive by ELISA with purified antigens 5 and 6. This finding could be explained by the presence of a second shared carbohydrate epitope most prominently displayed on antigens 5 and 6 or by similar epitopes recognized with greater affinity on antigens 5 and 6.

Immunization of mice with purified antigen 5 yielded three IgG2a and one IgA monoclonal antibodies which appeared by ELISA to be specific for antigens 5 and 6. This finding strongly suggests that a third epitope is uniquely shared by these two proteins. Since all 19 antigen 5-reactive monoclonal antibodies also bound antigen 6, there is almost certainly major antigenic similarity between these two molecules. A description of these three epitopes, designated A, B, and C, is shown in Table 3.

Immunoabsorbent affinity chromatography with monoclonal antibodies yielded data consistent with the ELISA data in most cases. When monoclonal immunoabsorbents were used to chromatograph whole *M. tuberculosis* culture filtrate, mycobacterial polysaccharides were most frequently recovered by using the monoclonal antibodies with broad reactivity. Although the ELISA reactivity profile for monoclonal antibodies TB-C-3, TB-C-4, TB-C-9, TB-C-10, and TB-C-19 were more specific for antigen 5 by ELISA, the four monoclonal immunoabsorbents produced from animals immunized with complete Freund adjuvant (TB-C-3, TB-C-4, TB-C-9, and TB-C-10) yielded eluates very similar to those obtained with the broadly reactive monoclonal antibodies. TB-C-19, on the other hand, yielded only antigen 5. We concluded that TB-C-19 identified the same epitope (C) as TB-C-13, TB-C-14, TB-C-15, and TB-C-18 but with different affinity. It is also possible that TB-C-19 recognized a fourth epitope. This suggests that TB-C-19 identified a different epitope from the other IgM monoclonal antibodies or at least had dramatically different affinities to the same epitope. The fact that all of the other IgM monoclonal antibodies (not TB-C-19) bound antigen 5 by ELISA but that this molecule could not be identified in any of the immunoabsorbent eluates is consistent with the disparate behavior of monoclonal antibodies in different antigen binding assays observed by others (17, 25). This may be due to subtle changes in antigen confirmation produced by the different techniques or to differing binding affinities required for positive results in different assay systems.

Antigen 5 was recovered from immunoabsorbents prepared from the more specific antibodies, with occasional contamination with antigen 6. This finding agrees with the ELISA data and further supports the hypothesis that at least one major epitope is shared between mycobacterial antigens 5 and 6. The ELISA data suggest that this epitope is restricted to *M. tuberculosis*. The World Health Organization-sponsored workshop (16: Immunology of Tuberculosis Workshop Participants, Infect. Immun., 1986) reached a similar conclusion with respect to TB-C-13.

We conclude that the sharing of epitopes among mycobacterial antigens is common. Despite earlier evidence for species restriction and antigenic specificity of antigen 5 (9), it seems likely that this antigen contains at least two shared epitopes (A and B). These shared epitopes are probably carbohydrate in nature since there is a significant cross-reactivity with purified arabinomannan and arabinogalactan. A third epitope (C) was identified by monoclonal antibodies which were more restricted in their binding profile, binding only to antigens 5 and 6 by ELISA. This epitope is a likely candidate for species restriction, since the parent molecules (antigens 5 and 6) are species restricted and no cross-reactivity was observed with mycobacterial carbohydrate antigens. If a noncarbohydrate species-restricted epitope can be identified, such a reagent would not only be a better potential skin-test antigen, but these monoclonal antibodies could be used to purify this epitope after proteolytic digestion of the antigen 5 molecule. In this regard, we demonstrated that these monoclonal antibodies can be used to purify antigen molecules to high purity by immunoabsorbent column chromatography. In addition, if epitope C is a protein epitope, it may be possible to use monoclonal antibodies TB-C-13, TB-C-14, TB-C-15, TB-C-18, and TB-C-19 as probes in a molecular-biology-generated cDNA expression library. This would greatly facilitate the determination of the precise protein sequence of this epitope and its production in large quantities in vitro. All of these

techniques should be useful in further characterization and purification of epitope-containing species-specific antigen fragments.

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