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Generation of Monoclonal Antibodies to the Specific Sugar Epitopes of *Mycobacterium avium* Complex Serovars

BECKY RIVOIRE,[1] BARBARA J. RANCHOFF,[1] DELPHI CHATTERJEE,[1] HARVEY GAYLORD,[1] ANNA Y. TSANG,[2] AREND H. J. KOLK,[3] GERALD O. ASPINALL,[4] AND PATRICK J. BRENNAN[1]*

Department of Microbiology, Colorado State University, Fort Collins, Colorado 80523[1]; Department of Medicine, National Jewish Center for Immunology and Respiratory Medicine, Denver, Colorado 80206[2]; Laboratory of Tropical Hygiene, Royal Tropical Institute, Amsterdam, The Netherlands[3]; and Department of Chemistry, York University, North York, Ontario M3J 1P3, Canada[4]

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Monoclonal antibodies have been generated to the unique distal sugar epitopes on the oligosaccharide haptens of the glycopeptidolipid antigens of clinically prominent members of the *Mycobacterium avium* serocomplex. Thus, antibodies are described that recognize the distal *O*-acetyl-α-L-rhamnopyranosyl residue of the specific glycopeptidolipid of *M. avium* serovar 1, the 4-*O*-acetyl-2,3-di-*O*-methyl-α-L-fucopyranose of serovar 2, the 4-*O*-methyl-α-L-rhamnopyranosyl-(1→4)-2-*O*-methyl-α-L-fucopyranosyl unit of serovar 4, the 4,6-(1′-carboxyethylidene)-3-*O*-methyl-β-D-glucopyranosyl unit of serovar 8 [and the 4,6-(1′-carboxyethylidene)-β-D-glucopyranosyl residue of serovar 21], and the 4-*O*-acetyl-2,3-di-*O*-methyl-α-L-fucopyranosyl-(1→4)-β-D-glucuronopyranosyl unit of serovar 9. Epitope definition was arrived at through use of the pure, chemically defined glycopeptidolipid antigens and neoglycoproteins containing the chemically synthesized distal sugars of some select serovars. These monoclonal antibodies combined with the already published information on the structure of the antigen determinants and the tools used to arrive at these structures provide powerful means for fundamental studies on the role of these antigens in immunopathogenesis and for the precise mapping of the epidemiology of opportunistic infections caused by *M. avium*.

The recent resurgent interest in members of the *Mycobacterium avium* complex arises from their occurrence as opportunistic pathogens in many persons with acquired immunodeficiency syndrome (AIDS) (40), although these atypical mycobacteria have long been associated with pulmonary and other organ infections (38). Over the past 10 years, we have been defining in precise detail the surface antigens that differentiate the 28 serovars that comprise *M. avium* and *M. intracellulare* (the *M. avium* complex) and the three serovars that comprise *M. scrofulaceum* (3). These antigens are glycopeptidolipids related to mycoside C and, accordingly, are composed of a glycosylated lipopeptide core, fatty acyl-D-phenylalanine-D-*allo*threonine-D-alanine-L-alaninyl-*O*-(3,4-di-*O*-methyl-α-L-rhamnopyranoside), to which a haptenic oligosaccharide is linked at the threonine substituent (see Fig. 3); this oligosaccharide unit is the source of the dominant immunogenicity of members of the *M. avium* complex, i.e., their characteristic agglutinability and type specificity (5). While most of our published work is on the chemistry and antigenicity of these unusual oligosaccharides (2, 4, 7, 9, 11, 12, 28, 29), we have also been applying the methods developed in the course of these studies to the identification and differentiation of *M. avium* complex serovars obtained from individuals with other underlying medical problems (6, 37, 39). In this report, we describe the preparation of monoclonal antibodies to the unique sugar epitopes of the prominent *M. avium* complex serovars and identification of the specificities of these antibodies.

(Preliminary accounts of some of this work have been published elsewhere. [B. J. Ranchoff, S. D. Buzzell, and P. J. Brennan, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, U2, p. 119, and B. J. Ranchoff, M. S. thesis, Colorado State University, 1987]).

* Corresponding author.

MATERIALS AND METHODS

Mycobacteria. All serovars of the *M. avium* complex were from the authenticated collection described previously (37). *M. avium* complex serovar 1, strain 11907-300; *M. avium* complex serovar 2, strain 14141-1395; *M. avium* complex serovar 4, strain 13528-1079; *M. avium* complex serovar 8, strain SJB no. 2; and *M. avium* complex serovar 9, strain Watson, were the major immunogens. Bacterial cultures were grown in 7H11 medium (Difco Laboratories, Detroit, Mich.) supplemented with oleic acid-albumin-dextrose-catalase (16) (Pasco Labs, Wheatridge, Colo.) before use.

Preparation of antibodies and relevant protocols. (i) Immunization of mice. Several different immunization procedures were used, depending on the serovar, the laboratory, and the investigator. For example, in the case of *M. avium* complex serovar 9, BALB/c mice, 6 to 8 weeks old, were primed intraperitoneally with whole washed heat-killed bacteria (50 μg in 50 μl of sterile saline) in a suspension with an equal volume of incomplete Freund adjuvant. On days 14 and 21 after priming, identical boosts were given. At about day 20, the mice were bled and individual sera were tested in enzyme-linked immunosorbent assays (ELISA) for reactivity against an unfractionated preparation of the glycopeptidolipids (GPLs) from serovar 9 (GPL-9). The animal presenting the highest antibody response was given a final intravenous injection of 100 μg of lyophilized bacteria suspended in phosphate-buffered saline (PBS) (0.1 ml). On the other hand, in order to ensure serovar specificity for the antibodies to *M. avium* serovar 4, mice were immunized subcutaneously with an emulsion of 0.1 ml of Freund complete adjuvant and 0.1 ml of PBS containing 100 μg of the serovar 4-specific neoglycoprotein, 4-*O*-methyl-α-L-rhamnopyranosyl(1→4)-2-*O*-methyl-α-L-fucopyranosyl-oxynonanoyl-bovine serum albumin (BSA) (neoantigen 4) (Fig. 1). A subsequent intraperitoneal injection 2 weeks later con-

sisted of 300 μg of whole washed *M. avium* complex serovar 4 cells in Freund incomplete adjuvant followed 1 month later with a third intraperitoneal boost consisting of 100 μg of unfractionated serovar 4 GPLs and 20 μg of the neoantigen 4. A blood sample was tested by ELISA 7 to 10 days posttertiary boost, and a final injection was given intravenously 7 days postbleeding with 40 μg of unfractionated GPLs in PBS. In the case of *M. avium* complex serovars 1, 2, and 8, the typical initial immunogen consisted of 150 μg of whole cells combined with 300 μg of whole unfractionated lipid emulsified in 0.1 ml of PBS. This suspension was administered intravenously six times, 3 days apart. A blood sample was tested 2 days after the sixth injection. The mouse responding with the highest titer was given a final intravenous boost. The antibodies produced by A.H.J.K. in the Amsterdam laboratory arose from animals immunized with whole bacilli as described previously (26).

(ii) Myeloma and hybridoma cell culture. Three days after the final injections, mice were exsanguinated and sacrificed, and the spleens were used as donors of immune lymphocytes in the fusion protocol. The nonsecreting B-cell line SP2/oAg14 (Vector Borne Disease Center, Centers for Disease Control, Fort Collins, Colo.) was maintained at 37°C in either Roswell Park Memorial Institute (RPMI) 1640 medium or Dulbecco modified Eagle medium (K. C. Biological, Inc., Lenexa, Kans.) supplemented with 10% total bovine serum (Flow Laboratories, McLean, Va.), L-glutamine, sodium pyruvate, nonessential amino acids, 50 μg of streptomycin per ml, and 50 IU of penicillin per ml (complete medium; all supplements were obtained from K. C. Biological). The SP2/o myelomas were diluted from log-phase cultures 3 days before fusion to yield 1×10^5 to 7×10^5 cells per ml in log-phase growth on the day of fusion. Mouse spleen cells were prepared in Hanks balanced salt solution (K. C. Biological) by teasing the cells into a single-cell suspension. These cells were used without lysis of red cells or enrichment for any lymphocyte population.

(iii) Cell fusion and selection of hybrids. Splenocytes and SP2/o cells at a 4:1 ratio were washed three times in incomplete RPMI 1640 medium (i.e., 50 IU of penicillin per ml, 50 μg of streptomycin per ml, and 0.29 mg of L-glutamine per ml). After being washed, cells (1.0 ml per 1.6×10^8 splenocyte cells) were fused with 50% polyethylene glycol (4,000 molecular weight) (VWR Scientific, Denver, Colo.) in incomplete RPMI medium (18, 20, 24). Polyethylene glycol was added slowly while the solution was stirred gently over a 37°C water bath for a duration of 1 min. Stirring continued for an additional min and was followed by a 10% addition of a calculated volume (10 ml per 1.6×10^8 spleen cells) of completed RPMI while being stirred gently for 1 min. A further 10% of the volume was added over the next minute, followed by 30 and 50% in subsequent minutes. In our experience, this gradual, gentle addition is crucial to the success of healthy hybridomas. Cells were centrifuged for 5 min at low speed. The pellet was loosened with gentle tapping and gentle pipetting with a precalculated volume of complete RPMI (22 ml per 1.6×10^8 spleen cells). Fused cells were plated at 0.1 ml per well into 96-well polystyrene plates.

Fusion cultures in wells were fed with 0.1 ml of complete medium containing hypoxanthine (1×10^{-4} mM), aminopterin (4×10^{-7} mM), and thymidine (1.6×10^{-5} mM) on days 2, 3, 4, and 5 after fusion; screening for antibody generally commenced on day 10. Cloning of positive cultures was done on 24-well plates in complete medium containing hypoxanthine and thymidine only. Limiting dilution of cells onto 96-well thymocyte feeder layers (5×10^5 thymocyte cells per 100 μl of complete medium) was monitored by growth and rescreening for antibody production.

(iv) ELISA. Assays of monoclonal antibodies or of whole serum (mouse or rabbit) were performed in a variety of configurations on polystyrene microtiter plates (Dynatech Laboratories, Chantilly, Va.) or as nitrocellulose-based dot ELISA. For polystyrene plates, glycolipids were coated in the wells by evaporation of a sonicated suspension of glycolipids in absolute ethanol (39). For unfractionated lipid preparations, a total of 2.5 μg in 50 μl was plated on each well; individual purified glycolipids were plated at 0.5 μg per well in 50 μl or at the concentrations shown in the figure legends. Unbound sites on the polystyrene were blocked with 0.05% polyoxyethylene sorbitan monolaurate (Tween 80; Sigma Chemical Co., St. Louis, Mo.) in PBS (PBS-Tween) for 2 min or with 1% BSA (Fraction V; Miles Scientific, Naperville, Ill.) in PBS-Tween for 1 h. Antibody was incubated in the well for at least 1 h after block. Hybridoma supernatants were diluted in SP2/o culture supernatant; rabbit serum was diluted in 10% normal goat serum in PBS-Tween. Unbound antibody was removed with PBS, and the second antibody of horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit immunoglobulin, reactive with immunoglobulin G (IgG), IgM, and IgA classes of immunoglobulin (Cappel Laboratories, West Chester, Pa.) was added. The second antibody was diluted in PBS-Tween or 10% normal goat serum in PBS-Tween; incubation time was 20 min. After washing, peroxidase was detected by addition of 0.4 mg of *o*-phenylenediamine (Sigma) per ml and 0.012% H_2O_2 in citrate phosphate buffer, pH 5. Color development was stopped by addition of 50 μl of 2.5 N H_2SO_4. Optical density at 490 nm was read by using a spectrophotometer (Dynatech Industries, Inc., McLean, Va.) for microtiter plates.

Typing of mouse monoclonal antibody heavy chains was done by a variation of the assay described above. The second antibody in this case consisted of a panel of biotinylated rabbit antiserum rendered specific for mouse immunoglobulin heavy chains (γ_1, γ_{2a}, γ_{2b}, γ_3, or μ chains), which was followed by horseradish peroxidase-conjugated streptavidin.

(v) Inhibition assays. Inhibition assays were performed in the plate ELISA system by preincubating the monoclonal antibody or rabbit antiserum at a predetermined dilution with different amounts of test inhibitors before dispensing the antibody into quadruplicate wells on antigen-coated plates. The preincubations were carried out in 10% normal goat serum in PBS–0.1% Tween for 16 h at 4°C. Inhibitor concentrations are expressed as micrograms of inhibitor per well.

(vi) Dot blot and thin-layer chromatography (TLC) immunoassays. Immunoassays on nitrocellulose sheets (Trans-Blot sheets; Bio-Rad Laboratories, Richmond, Calif.) were performed in a manner analogous to plate assays, with the following changes. Total lipids were suspended in PBS by using a Bransonic 12 pan sonicator (Branson Sonic Power Co., Shelton, Conn.) at 50 μg/ml (purified glycolipids at 10 μg/ml), and 100 μl was applied as a spot on nitrocellulose sheets by using a manifold (Mini Blot; Schleicher & Schuell, Inc., Keene, N.H.). The sheet was then blocked with 2% polyvinyl pyrrolidone (PVP-40; Sigma) in PBS for at least 2 h. The sheet was cut into strips, and each strip was immersed in antibody in the form of either murine hybridoma culture fluid or rabbit antiserum. After 1 to 2 h of incubation, strips were washed in a large excess of PBS–0.1% Tween for

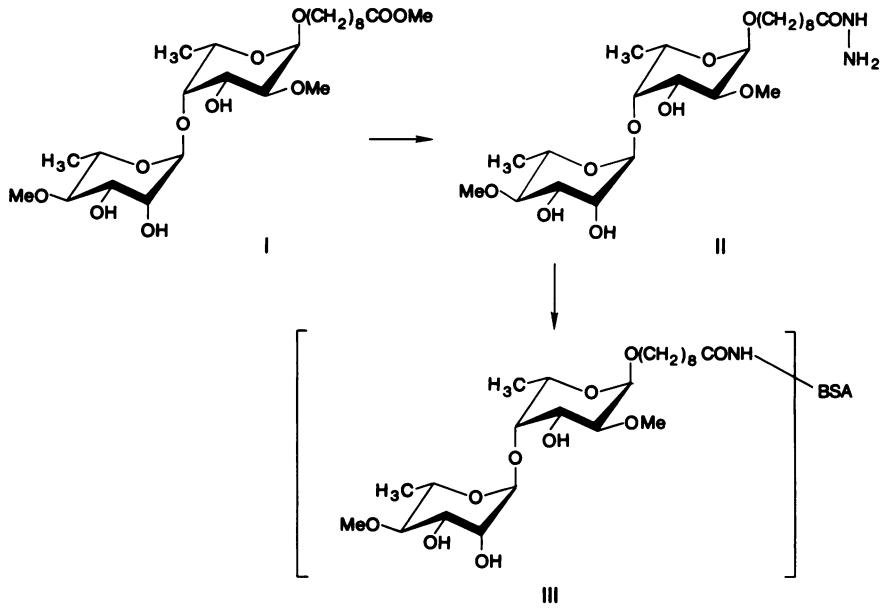


FIG. 1. Terminal stage of synthesis of the *M. avium* complex serovar 4-specific neoglycoprotein *O*-(4-*O*-methyl-α-L-rhamnopyranosyl)-(1→4)-*O*-(2,-*O*-methyl-α-L-fucopyranosyl)-(1→9)-oxynonanoyl-BSA.

10 min and immersed in second antibody-horseradish peroxidase for 20 min. After a second 10-min wash, the strips were immersed for color development. To each 10 ml of PBS was added 6.0 mg of 4-chloro-1-naphthol color reagent (Bio-Rad) in 2 ml of CH_3OH and 40 μl of 30% H_2O_2. The development of dark purple spots was stopped by transferring the nitrocellulose to water. Immunoassays on TLC plates were conducted as described by Minnikin et al. (30).

Preparation of GPL antigens. Lipids were extracted from dry bacterial harvests with $CHCl_3$-CH_3OH (2:1 [vol/vol]) at 50°C (5). Solvent was removed from extracts by flash evaporation, and the ensuing residue was partitioned between the two phases arising from a mixture of $CHCl_3$-CH_3OH-H_2O (4:2:1 [vol/vol/vol]). The lipids in the lower $CHCl_3$ phase are referred to as total or whole lipid. All of the *M. avium* complex GPLs are alkali stable, although the glycosyl substituents may be acetylated to various extents (5, 17); accordingly, when lipid preparations are treated with base to destroy nonspecific acylglycerols, the native GPLs, if acetylated, become deacetylated (5). Nevertheless, for ease of purification, the total lipid preparations were usually treated with 0.2 N NaOH in CH_3OH for 30 min at 37°C and washed. The product is referred to as unfractionated GPLs. Procedures for the purification of the individual specific GPLs from individual serovars have been described previously (11, 12, 29). All purification procedures involved silica gel column chromatography in $CHCl_3$ with increasing concentrations of CH_3OH, depending on the relative polarity of the specific GPL, followed by preparative TLC. In addition, the naturally acetylated GPLs were purified as described previously (29) from total lipid preparations that had not been subjected to alkali treatment. The monoacetylated GPL-9 (GPL-9I) from *M. avium* complex serovar 9 was an exception in that some of it survived mild alkali treatment; it was finally purified by preparative TLC in $CHCl_3$-CH_3OH-H_2O (65:25:4 [vol/vol/vol]).

Synthesis of neoantigens. (i) Synthesis of a neoantigen containing the specific terminal glycobiose unit of the *M. avium* serovar 4-specific GPL (disaccharide-containing neoantigen-4) (Fig. 1). Previously, we described the structure of the precise haptenic tetrasaccharide of *M. avium* serovar 4 as 4-*O*-methyl-α-L-rhamnopyranose-(1→4)-2-*O*-methyl-α-L-fucopyranose-(1→3)-α-L-rhamnopyranose-(1→2)-6-deoxytalose (29). Since the reducing-end disaccharide [α-L-rhamnopyranose-(1→2)-6-deoxytalose] is common to all serovars, we concluded that the specific antigen determinant on GPL-4 is inherent to the nonreducing-end disaccharide, 4-*O*-methyl-α-L-rhamnopyranosyl-(1→4)-2-*O*-α-L-methyl-fucopyranose. Our approach to the synthesis of this specific terminal glycobiose unit involved a block synthesis; the disaccharide unit was then elaborated on the 8-methoxycarbonyloctyl aglycon, a strategy used previously in preparing the *M. leprae*-specific neoantigens (13, 14); because of the sensitivity of the spacer arm to various reaction conditions, a block synthesis approach facilitated the stability of the aglycon. In the terminal stages of this synthesis, the 8-methoxycarbonyloctyl *O*-(4-*O*-methyl-α-L-rhamnopyranosyl)-(1→4)-2-*O*-methyl-α-L-fucopyranoside (compound I in Fig. 1) was converted to its hydrazide (compound II in Fig. 1) as described previously (13, 14); freeze-dried hydrazide was then converted to its acyl azide and immediately coupled to the carrier protein BSA (13). The resulting neoglycoconjugate was dialyzed and purified by gel filtration on Sephadex G-75 (13). An incorporation of 30 mol of carbohydrate per mol of BSA was achieved. Full details of the synthesis of this neoantigen will be described separately.

(ii) Synthesis of a neoantigen containing the terminal monosaccharide of the *M. avium* serovar 4-specific GPL (monosaccharide-containing neoantigen-4). Allyl 2,3-*O*-isopropylidene-4-*O*-methyl-α-L-rhamnopyranoside was synthesized (21), purified by column chromatography, and hydrolyzed to give allyl-4-*O*-methyl-α-L-rhamnopyranoside. By the procedure of Bernstein and Hall (1), the appropriate glycoconjugate was prepared from the allyl glycoside and BSA. The neoglycoconjugate was purified by gel filtration on Sephadex G-25 (13). Fractions containing both carbohydrate and protein were pooled, dialyzed, and freeze-dried to give the conjugate. The conjugate contained 28 mol of carbohy-

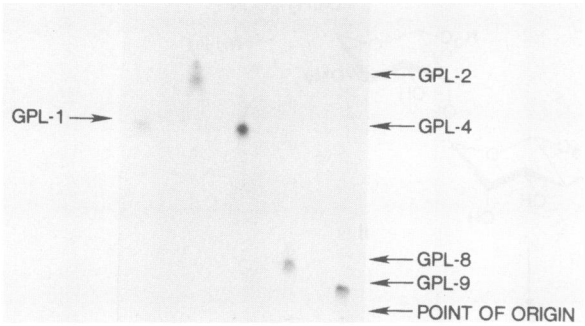


FIG. 2. Thin-layer chromatogram of the specific GPLs from serovars 1 (GPL-1), 2 (GPL-2), 4 (GPL-4), 8 (GPL-8), and 9 (GPL-9II). Solvent, $CHCl_3$-CH_3OH-H_2O (65:20:3 [vol/vol/vol]). Spray, 10% H_2SO_4 with heating.

drate (13). Full details of this conjugation procedure and of this neoantigen synthesis will be described separately.

(iii) Synthesis of neoantigens containing the terminal monosaccharide or the monoacetylated terminal monosaccharide of the *M. avium* serovar 9-specific GPL (acetylated or nonacetylated neoantigen-9). The starting point for these syntheses was the allyl 2,3-di-*O*-methyl-α-L-fucopyranoside; the full details of its synthesis have been described by Takeo et al. (36). In addition, a portion of this material was acetylated by using pyridine and acetic anhydride. Both of these monosaccharide derivatives were converted to their corresponding neoglycoconjugates by using the method described above.

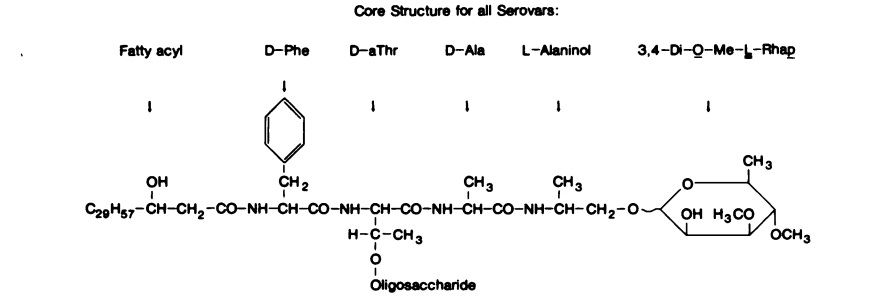
(iv) Synthesis of a neoantigen containing the terminal pyruvylated, 3-*O*-methyl-glucose unit of *M. avium* serovar 8 (neoantigen-8), i.e., allyl 4,6-*O*-(1′-carboxyethylidene)-β-D-glucopyranoside. Allyl 2,4,6-tri-*O*-acetyl-3-*O*-methyl-β-D-glucopyranoside was synthesized by Koenigs-Knorr condensation of 1,2,4,6-tetra-*O*-acetyl-3-*O*-methyl-D-glucose with allyl alcohol (21). The allyl group was protected by epoxidation, forming epoxypropyl glycoside using *m*-chloroperoxybenzoic acid. Ketalization using 3,4-dimethoxyacetophenone gave the 4,6-*O*-ketal which upon RuO_4 oxidation gave its 4,6-*O*-(1′-carboxymethylethylidene) derivative (10). The allyl glycoside needed for the preparation of the glycoconjugate was regenerated from the epoxypropyl glycoside by using 3-methyl-2-selenoxobenzthiazole. Transesterification of this product gave the title compound (i.e., the allyl glycoside). The detailed synthesis of this particular epitope will be published elsewhere.

RESULTS

Distribution of *M. avium* serovars in acquired immunodeficiency syndrome patients. The results of the application of a combination of seroagglutination of whole cells (35), ELISA (39), and comparative TLC of the extracted GPL antigens (6) to over 1,000 *M. avium* complex isolates from acquired immunodeficiency syndrome patients has shown that serovars 1, 2, 4, 8, and 9 predominate (results not shown). This and other corresponding information (22, 29) prompted us to prepare monoclonal antibodies to the type-specific GPL antigens of these particular serovars in order to facilitate their identification.

Figure 2 shows a thin-layer chromatogram of the purified GPL typing antigens from these predominant serovars. The different polarities of the purified GPLs reflect the marked differences in the structures of the terminal segments of the oligosaccharide haptens. The structures of the GPL antigens of serovars 1, 2, 4, 8, and 9 are summarized in Fig. 3; we have recently published the structures of the oligosaccharide haptens of 12 of the 28 known *M. avium* complex serovars (2, 3). It has been reasoned (3) that since the lipopeptide core and the reducing-end disaccharide of the oligosaccharide segments of the GPL antigens are invariant from one serovar to another, then the nonreducing-end segment of the variable oligosaccharide must contain the respective antigen determinants. We hoped to match the nonreducing, distal, type-specific segments of the oligosaccharide haptens with corresponding antibodies and thereby provide the means for optimal differentiation and identification of clinically significant *M. avium* complex isolates.

Monoclonal antibodies to the sugar determinants of the prominent *M. avium* serovars. Cell lines secreting antibodies to these particular serovars were raised in many separate



Serovar No.	Structure of Oligosaccharide Hapten
1	L-Rhap-(α1→2)-6d-L-Tal
2	2,3-Di-O-Me-L-Fucp-(α1→3)-L-Rhap-(α1→2) 6d-L-Tal
4	4-O-Me-L-Rhap (α1→4)-2-O-Me-L-Fucp-(α1→3)-L-Rhap-(α1→2)-6d-L-Tal
8	4,6-(1′-Carboxyethylidene)-3-O-Me-D-Glcp-(β1→3)-L-Rhap-(α1→2)-6d-L-Tal
9	2,3-Di-O-Me-L-Fucp-(α1→4)-D-GlcAp-(β1→4)-2,3-Di-O-Me-L-Fucp-(α1→3)-L-Rhap-(α1→2)-6d-L-Tal

FIG. 3. Structures of the glycopeptidolipid antigens from clinically prominent members of the *M. avium* complex.

TABLE 1. Summary of the approach used to generate monoclonal antibodies to the specific GPL antigens of the prominent *M. avium* complex serovars

Serovar designation	Immunogen	Cell line or antibody designation[a]	H-chain isotype[b]	Titer[c]	Reactivity pattern
1	Whole cells and whole lipid	3F6	IgG3	1:320	Whole cells of serovar 1 and acetyl-GPL-1
		4C6	IgM		
2	Whole cells	123.4	IgG2a	1:100	Whole cells of serovar 2 and acetyl-GPL-2
4	GPL-4 and neo-antigen 4	32B8, 33F7	IgG3	1:3,200	Whole cells of serovar 4, acetyl-GPL-4, and GPL-4
	Whole cells	F85-6; F85-5; F85-3	IgG3	1:8,000	Whole cells of serovar 4 and acetyl-GPL-4
8	Whole cells and whole lipid	5F7	IgG3	1:12,800	Whole cells of serovar 8, acetyl-GPL-8, and GPL-8
		2B8	IgG3	1:6,400	Whole cells of serovar 8, acetyl-GPL-8, and GPL-8
		4D7	IgG3	1:3,200	Whole cells of serovar 8, acetyl-GPL-8, GPL-8, acetyl GPL-21, and GPL-21
9	Whole cells	5210, 5214, 5510		Undiluted[d]	Whole cells of serovar 9 and acetyl-GPL-9
		2647, 2643, 2675	IgG3		
		B192, 154	IgG2b	Undiluted[d]	Whole cells of serovar 9 and acetyl-GPL-9
		1173, 1175	IgM		Highly cross-reactive for other serovars and their GPLs
		R124, 7534	IgM		
		4102, 4189	IgM		
		5839, 5892	IgM		
		481, 4811	IgM		

[a] All antibodies, except the F85 series, are available from the Colorado laboratory.
[b] Typing of mouse monoclonal antibody heavy chain was conducted by ELISA as described in Materials and Methods. In the absence of confirmatory data from immunodiffusion, isotype designations are regarded as tentative.
[c] All titers given are the ascitic fluid.
[d] Tissue culture supernatant.

fusion experiments in the Colorado and Amsterdam laboratories by using a variety of immunization strategies (Table 1). The cell lines listed in Table 1 arising from an immunization with *M. avium* serovar 9 were all independent isolates from a fusion of SP2/o with mouse spleen cells following immunization with whole killed bacteria; no added inoculation with GPL preparations was used during the immunization. Eighteen cell lines were generated, eleven of which were distinct and which secreted antibodies reactive to the unfractionated GPLs of serovar 9. Similarly, cell lines 1105.1, 1107.1, and 1206.1 (not listed in Table 1) were raised in a different fusion after immunization with whole *M. avium* serovar 8 cells and by screening against unfractionated GPLs of serovar 8; however, these latter lines were not used for precise definition of the specific epitope of serovar 8. Instead, higher-titer antibodies were obtained by intravenous immunizations with a mixture (2:1 [wt/wt]) of whole unfractionated GPLs from *M. avium* serovar 8 and whole cells, respectively (Table 1).

In the case of *M. avium* complex serovar 4, the Amsterdam laboratory was highly successful in raising serovar-specific antibodies by using whole bacilli as the immunogen as described previously (26) (Table 1). Initially, the Colorado laboratory was less successful, apparently due to use of a strain of *M. avium* serovar 4 which had a reduced content of the specific GPLs. Eventually, useful antibodies specific to the GPL of serovar 4 were raised in the Colorado laboratory by using an unfractionated preparation of the specific GPL-4 and the appropriate disaccharide-containing neoantigen-4 as the immunogens. In general, a mixture of the appropriate water-soluble neoantigens and the native GPLs were most effective in inducing a high serum titer to the respective GPLs in mice.

Identification of the epitope of serovar 9 as 4-*O*-acetyl-2,3-di-*O*-methyl-α-L-fucopyranose. Of the 18 cell lines reactive to the unfractionated GPLs of serovar 9 (Table 1), 6 secreted an IgG3 antibody, two secreted an IgG2b antibody, and the remaining 10 secreted an IgM antibody. These antibodies demonstrated varied reactions to the unfractionated GPLs of the other serovars of the *M. avium* complex (see Ranchoff, M.S. thesis, for details). However, all of the IgG2b and IgG3 antibodies listed in Table 1 were specific to the GPLs of serovar 9; none cross-reacted with the GPL antigens of the other 31 serovars (Fig. 4).

The antigen preparation used in the original screen for all of the anti-GPL-9 antibodies was unfractionated GPLs from serovar 9. The crude GPL-9 preparation was fractionated on a column of Florisil, and the partially purified lipids as they emerged from the column were subjected to TLC and ELISA against four of the anti-GPL-9 monoclonal antibodies

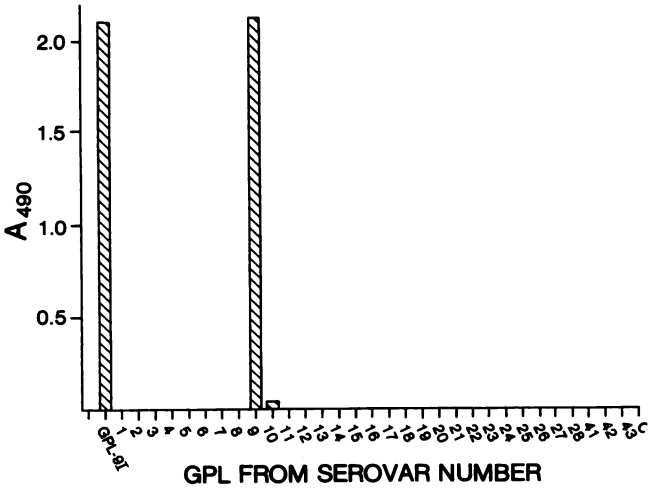


FIG. 4. Results of the reaction of the unfractionated GPLs from all 28 serovars of the *M. avium* complex and the three serovars (no. 41, 42, and 43) of *M. scrofulaceum* in plate ELISA against anti-serovar 9 antibody 5210. GPLs were suspended in C_2H_5OH at 50 μg/ml and 50 μl (2.5 μg) coated on each well. In this case, antibody was provided in the form of undiluted culture supernatants from antibody-secreting cells. GPL-9I corresponds to acetylated GPL-9I (see text). C, Control (no antigen applied to plate).

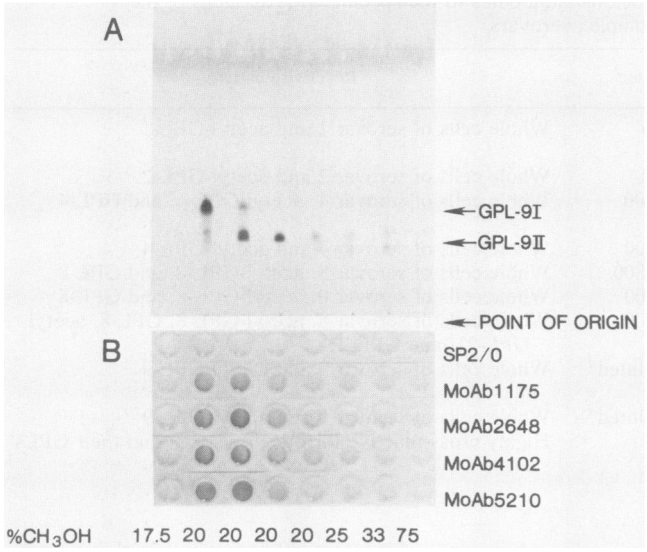


FIG. 5. Combined chromatographic fractionation and dot ELISA of specific GPL mixture from serovar 9. (A) Unfractionated GPLs were applied to a column of Florisil in $CHCl_3$ which was irrigated with increasing concentrations of CH_3OH in $CHCl_3$. A portion (0.5 μg) of the glycolipid in each eluate was applied to a TLC plate, chromatographed in $CHCl_3$-CH_3OH-H_2O (65:25:4 [vol/vol/vol]), and sprayed with 10% H_2SO_4. (B) At the same time, an equal quantity of glycolipid was applied to nitrocellulose and the dot ELISA protocol was applied by using four of the available antibodies.

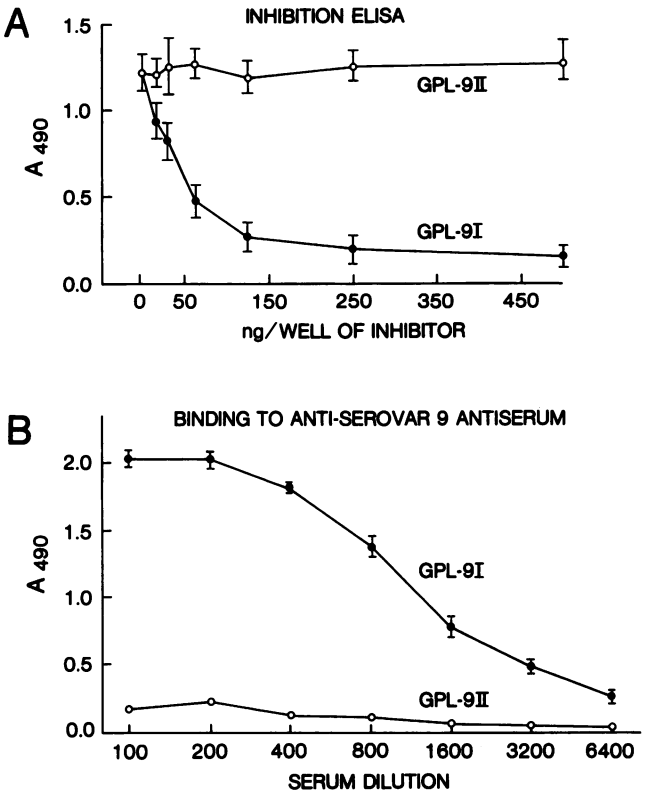


FIG. 6. Relative antigenic activities of GPL-9I and GPL-9II in ELISA. (A) Inhibition ELISA using monoclonal antibody 5210. The data demonstrate that the binding of plate-bound GPL-9I to the antibody can be inhibited by preincubation of antibody and GPL-9I. ●, Titration curve when GPL-9I was used as the inhibitor; ○, titration curve when GPL-9II was included. Data points represent the absorbance obtained due to uninhibited antibody binding to 5 ng of GPL-9I per well. (B) Direct binding to anti-serovar 9 rabbit antibodies. Rabbit antiserum specific for serovar 9, prepared as described previously (35), was serially diluted and tested in ELISA against either GPL-9I or GPL-9II (5 ng/well). Other conditions are described in the text.

(Fig. 5). Two GPLs, GPL-9I and GPL-9II, were at once obvious on the thin-layer plates (Fig. 5A), and it seemed from their reactions with the antibodies that only the faster-migrating GPL-9I was antigenic (Fig. 5B); note how the later 20% CH_3OH eluates, those which contained primarily GPL-9II, barely reacted with the antibodies. Indeed, when GPL-9I and GPL-9II were fully purified by preparative TLC and reacted against this set of monoclonal antibodies in plate ELISA, only GPL-9I was shown to be reactive (Table 2). The results of inhibition ELISA in which monoclonal antibody 5210 was preincubated with either GPL-9I or GPL-9II prior to reaction with GPL-9I are shown in Fig. 6A. The decrease in ELISA absorbance which occurred after preincubation of the antibody with GPL-9I indicates that the antibody had bound to GPL-9I during the preincubation, and the unchanging absorbance readings after preincubation of antibody with GPL-9II demonstrated that the antibodies did not bind to GPL-9II during the preincubation since they were still capable of reaction with the antigen on the ELISA plate. Polyclonal rabbit sera raised to whole cells of *M. avium* serovar 9 demonstrated an identical reactivity pattern (Fig. 6B).

TABLE 2. ELISA reactivity of anti-serovar 9 monoclonal antibodies against GPL-9I and GPL-9II

Antibody[a]	A_{490} with antigen[b]:	
	GPL-9I	GPL-9II
SP2/0	0.009 ± 0.006	0.015 ± 0.004
1175	1.159 ± 0.121	0.068 ± 0.004
2648	0.599 ± 0.130	0.023 ± 0.004
4102	1.040 ± 0.050	0.038 ± 0.013
5210	0.785 ± 0.142	0.060 ± 0.009

[a] Each antibody was diluted 1:16.
[b] Both antigens were applied at a level of 5 ng per ELISA well.

Recently, using GPL-9II as the source of the oligosaccharide hapten, Chatterjee et al. (11) established the structure of the oligosaccharide segment of the specific GPL of serovar 9 (Fig. 3), and we had assumed that the distal 2,3-di-*O*-Me-L-Fuc*p*(α1→4)-D-GlcA*p* unit is the specific epitope of serovar 9. However, now that we have found that GPL-9II is inactive, earlier speculation of ours (5, 11) seemed important, i.e., that GPL-9I may be a minor, acetylated form of GPL-II which, due perhaps to the particular location of the *O*-acetyl function(s), survived the mild alkali treatment applied in the work up of the crude GPLs. Accordingly, the 360 MHz ^1H NMR spectra of GPL-9I and GPL-9II were compared (Fig. 7). Only the spectrum of GPL-9I contained the signal (δ 2.18) of an acetyl function (Fig. 7B), a signal which was absent from GPL-9II (Fig. 7A). Two-dimensional ^1H NMR located this single acetyl function on the C-4 of the nonreducing-end 2,3-di-*O*-Me-fucose unit (results not shown). Furthermore, the approach described in detail by Chatterjee et al. (11) established that the oligosaccharide unit of GPL-9I was identical to that of GPL-9II. Accordingly, the

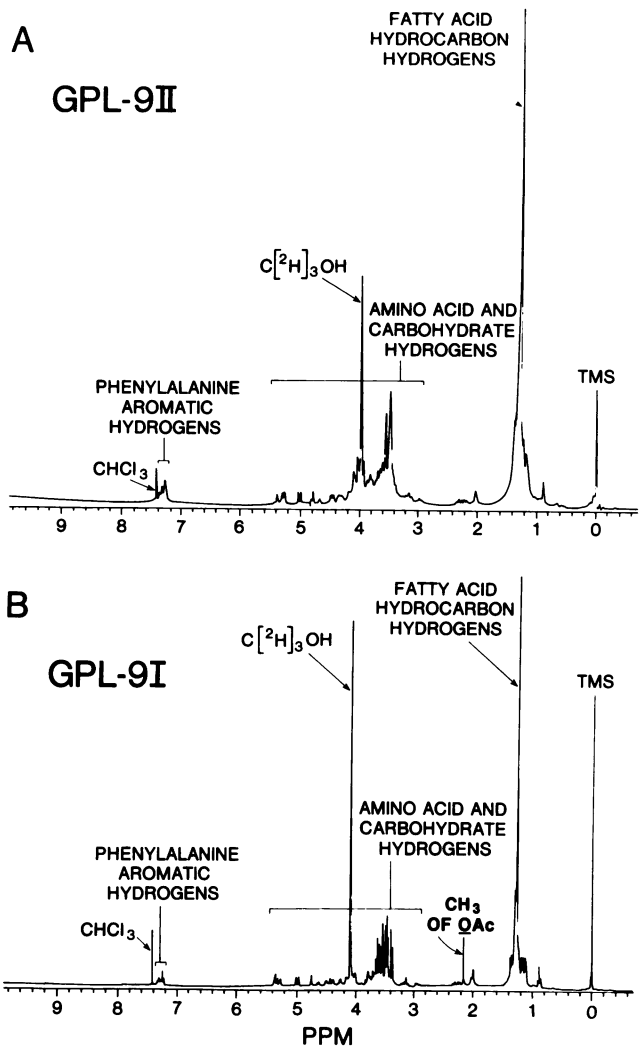


FIG. 7. Comparison of the 360 MHz ^{1}H NMR spectra of GPL-9II (A) and GPL-9I (B). See reference 11 for further details.

structure of the entire oligosaccharide hapten of GPL-9I is as shown in Fig. 8.

Thus, a combination of conventional and correlation (COSY) ^{1}H NMR spectroscopy combined with the availability of an array of monoclonal antibodies and the fortunate

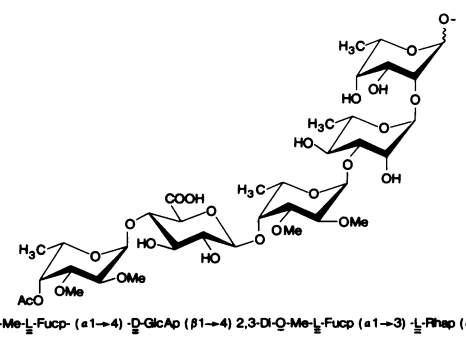


FIG. 8. Complete structure of the oligosaccharide hapten of the antigenic GPL-9I. Structure was determined by using data reported elsewhere (11) and in the text.

event that some of the *O*-acetylated GPL-9 survived alkaline treatment allowed us to establish that the presence of the terminal 4-*O*-acetyl function was crucial to antigenicity. In order to finally settle the issue of the exact dimensions of the relevant epitope, the reactivities of the two synthetic serovar 9-specific neoantigens, both generated in the Ontario laboratory, were examined. Each contained the proper nonreducing-end sugar but one, representing GPL-9II, was unsubstituted, and the other, representing the epitope of GPL-9I, was acetylated at the 4-OH position (the structures of these two neoantigens are shown in Fig. 9). Stock solutions of each of the neoantigens were prepared such that each contained 300 μg of carbohydrate per ml of buffer and were then diluted. Triplicates of each neoantigen dilution were reacted with a uniform dilution of monoclonal antibody 5210 in plate ELISA; BSA was used as an antigen control. Only the 4-*O*-acetyl-2,3-di-*O*-Me-α-L-fucopyranosyl-allyl-linked neoantigen reacted (Fig. 9). Thus, the results were decisive in support of the evidence that antibody reactivity to GPL-9I was due to the presence of a single 4-*O*-acetyl function on the terminal 2,3-di-*O*-methyl fucose of the oligosaccharide, and lack of this acetyl group resulted in no recognition by the antibody. The plate ELISA was repeated with all 18 monoclonal antibodies raised to *M. avium* serovar 9. Regardless of heavy-chain subclass, all demonstrated the identical reactivity pattern seen with monoclonal antibody 5210. Anti-serovar 9 polyclonal rabbit serum yielded identical results. Thus, the epitope recognized by all 18 monoclonal antibody cell lines produced to *M. avium* complex serovar 9 is the 4-*O*-acetyl-2,3-di-*O*-methyl-α-L-fucopyranoside.

Anti-serovar 4 monoclonal antibodies recognize the disaccharide unit 4-*O*-methyl-α-L-rhamnopyranose(1→4)2-*O*-methyl-α-L-fucopyranose. The anti-GPL-4 monoclonal antibodies arising from the immunization protocol used in the Colorado laboratory, involving a combination of the serovar-specific GPL and the corresponding neoantigen as the immunogen, were all highly specific for the homologous whole bacterium and its GPL; the pattern of reactivity shown with antibody 32B8 (Fig. 10) was also seen with 33F7 and 30B3 from the Colorado laboratory. Previously, we had shown that the characteristic GPL of *M. avium* serovar 4 (Fig. 3) also occurs in the acetylated form (29). Since then, we have demonstrated that the acetyl function is located on the reducing-end 6-deoxytalose residue, not on the nonreducing-end 4-*O*-methyl-rhamnose unit (25). The application of immunostaining to TLC plates directly (30) demonstrated that both the acetylated and nonacetylated GPL-4 were recognized equally well by monoclonal antibodies 32B8 (Fig. 11) and 33F7. Accordingly, it appeared that if the *O*-acetyl function is positioned well away from the nonreducing sugar terminus, it has no bearing on immunogenicity or antibody binding. However, the five monoclonal antibodies generated in the Amsterdam laboratory and arising from the use of whole cells as the immunogen showed a more varied reactivity pattern. Antibodies F85-5 and F85-6 were identical to 32B8 and 33F7 in their selective recognition of GPL-4 and acetylated GPL-4 among the range of *M. avium* complex GPLs. However, surprisingly, antibody F85-3 recognized only the acetylated GPL-4. On the other hand, antibody F85-2 and F85-10 reacted with a broad range of acetylated GPLs (e.g., those from serovars 1, 3, 8, 9, 15, 19, 25, etc.) and were of little practical use.

In order to identify more precisely the epitope recognized by antibodies 32B8, 33F7, F85-5, and F85-6, two neoantigens were synthesized, one containing the terminal 4-*O*-methyl-α-L-rhamnopyranose monosaccharide unit of GPL-4

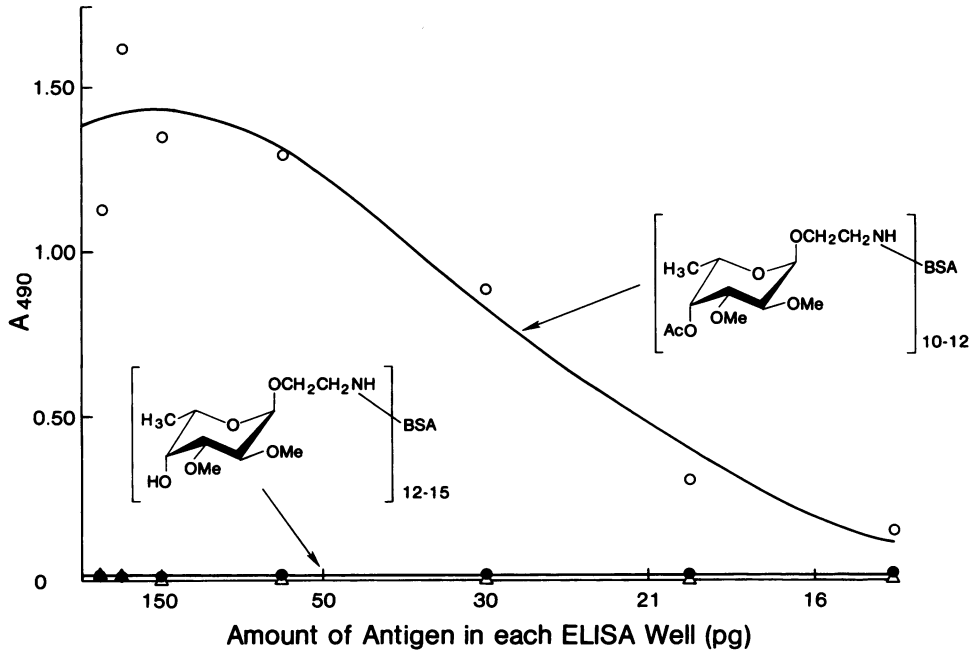


FIG. 9. Relative activities of the two neo-9-antigens against monoclonal antibody 5210 in ELISA. Details are described in the text. The antibody was in the form of undiluted culture supernatant.

and the other containing the terminal disaccharide unit (4-*O*-methyl-α-L-rhamnopyranosyl(1→4)2-*O*-methyl-α-L-fucopyranose); the former was synthesized in the Ontario laboratory by using an allyl linker arm and the latter was synthesized in the Colorado laboratory by using the oxynonanoyl link; the structures of these two neoantigens are shown as part of Fig. 12. Such neoantigens are readily electrophoresable (14). Accordingly, both were subjected to polyacrylamide gel electrophoresis, transferred to nitrocellulose, and reacted with the anti-GPL-4 monoclonal antibodies; the results from immunoblotting with 32B8 and 33F7 are shown in Fig. 13. Only the disaccharide-containing neoantigen (4-*O*-methyl-α-L-rhamnopyranosyl(1→4)2-*O*-methyl-α-L-fucopyranosyl-octyl-BSA) reacted; the 4-*O*-methyl-α-L-rhamnopyranosyl-allyl-BSA did not react (Fig. 13A and B). Antibodies F85-3 and F85-5 (from the Amsterdam laboratory) behaved in a similar fashion. However, against anti-whole serovar 4 rabbit antiserum, both neoantigens reacted readily (Fig. 13C). These conclusions were confirmed by the appropriate plate ELISA over a concentration range of each

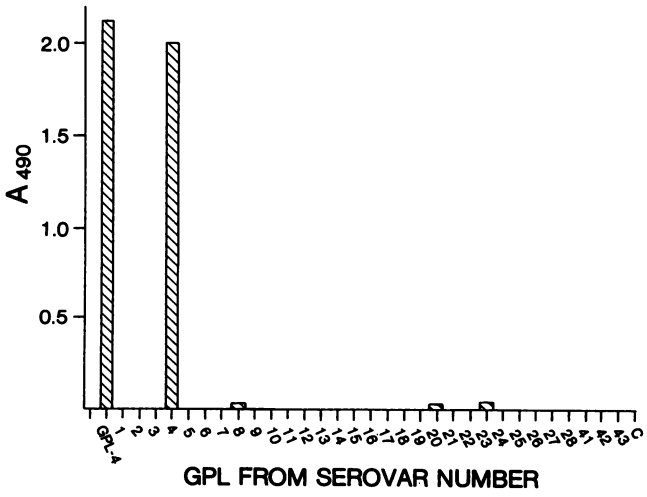


FIG. 10. Results of the reaction of the unfractionated GPLs from all 28 serovars of the *M. avium* complex and the three serovars (no. 41, 42, and 43) of *M. scrofulaceum* in plate ELISA against anti-serovar 4 antibody 32B8. Other details are provided in Fig. 4. Ascitic fluid was diluted 1:3,200.

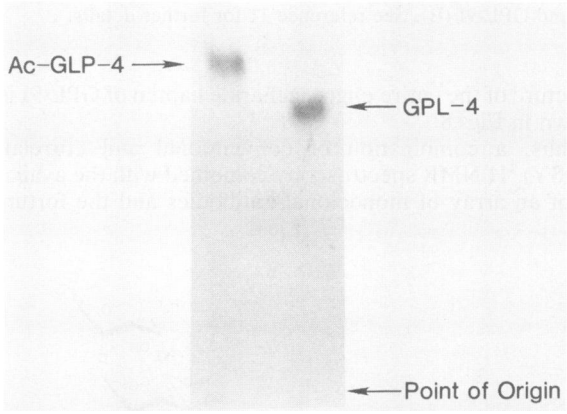


FIG. 11. TLC immunoblot of GPL-4 and acetylated GPL-4 using antibody 32B8. Samples (ca. 10 μg) of each of the glycolipids in C_2H_5OH were applied to Silica Gel G-Redi Plates (Fisher Scientific Co., Pittsburgh, Pa.), chromatographed in $CHCl_3$-CH_3OH-H_2O (30:8:1 [vol/vol/vol]), dried, and blocked for 1 h with 0.25% BSA in PBS containing 0.05% Tween 80. The plate was then exposed to the primary antibody (ascitic fluid diluted 1:400 with PBS-Tween), washed with PBS, exposed to the second antibody (peroxidase-conjugated Protein A [Zymed Laboratories, South San Francisco, Calif.] diluted 1:500 in PBS-Tween), washed, and exposed to the 4-chloro-1-naphthol substrate.

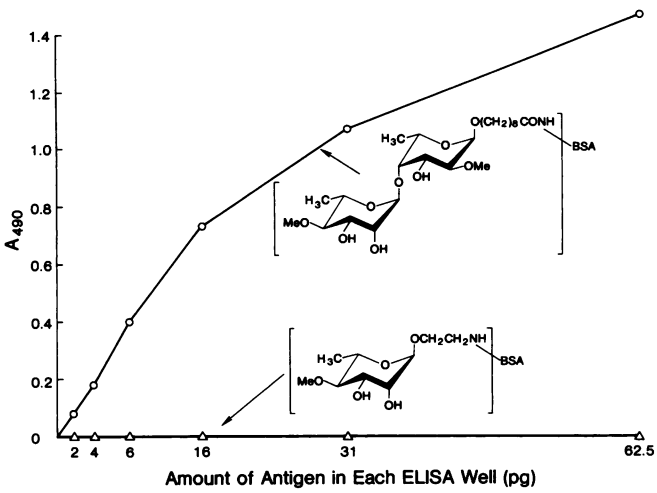


FIG. 12. Relative activities of different concentrations of the two neo-4-antigens (monosaccharide- and disaccharide-containing) against monoclonal antibody 32B8 in plate ELISA. The antibody as ascitic fluid was diluted 1:3,200. Other details are described in the text.

neoantigen (Fig. 12) and antibody. Accordingly, the penultimate sugar unit of the oligosaccharide segment of the specific GPL antigen of serovar 4 contributes to the binding of a series of murine monoclonal antibodies, in contrast to polyclonal rabbit antibodies, which require merely the single 4-*O*-methyl-α-L-rhamnopyranosyl terminal nonreducing-end sugar.

Anti-serovar 8 monoclonal antibodies recognize a pyruvylated glucosyl epitope. The most useful anti-serovar 8 mono-

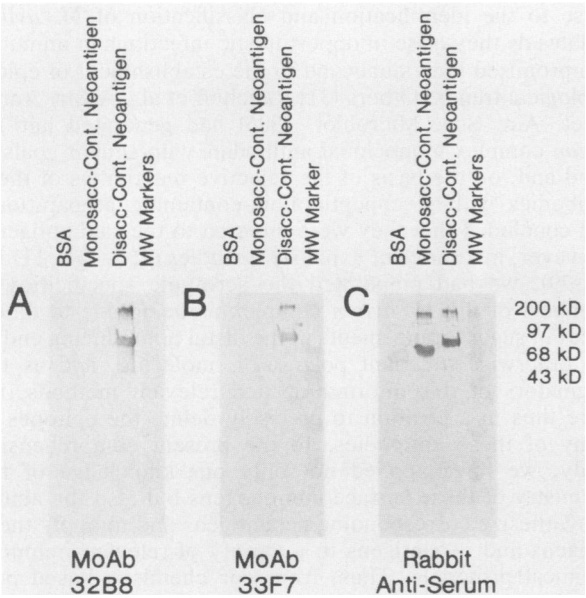


FIG. 13. Application of Western (immuno-) blotting to the two neo-4-antigens. The monosaccharide-containing neoantigen (Monosacc-Cont. Neoantigen), the disaccharide-containing neoantigen (Disacc-Cont. Neoantigen), and unsubstituted BSA (ca. 1 μg) were applied to 12.5% gels, electrophoresed, electroblotted onto nitrocullulose, blocked with 2% polyvinylpropylene-40 in PBS, and incubated with monoclonal antibody 32B8 diluted 1:1,000. Other conditions are described in the text. Molecular masses in kilodaltons (kD) are indicated at right.

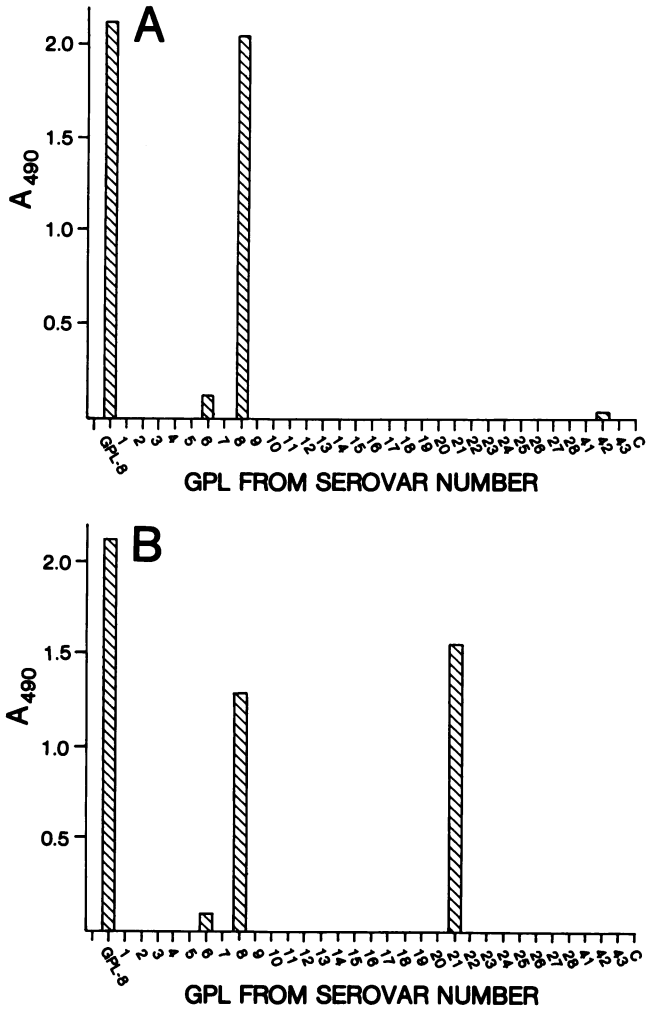


FIG. 14. Results of the reaction of the unfractionated GPLs from all 28 serovars of the *M. avium* complex and the three serovars of *M. scrofulaceum* in plate ELISA against anti-serovar 8 monoclonal antibodies 5F7 (A) and 4D7 (B).

clonal antibodies (2B8, 5F7, and 4D7) arose in the Colorado laboratory as a result of a complex immunization strategy involving whole lipid extract and whole cells (Table 1). Monoclonal antibodies 2B8 and 5F7 showed absolute specificity for the GPL of serovar 8 (Fig. 14A); the slight reactivity shown against GPL-6 is due to the contamination of some isolates of *M. avium* serovar 6 with serovar 8. However, antibody 4D7 recognized GPL-21 almost as effectively as it did GPL-8 (Fig. 14B). We have already established the structure of the terminal segment of the oligosaccharide hapten of serovar 8 as 4,6(1′-carboxyethylidene)-3-*O*-methyl-β-D-glucopyranosyl(1→3) (Fig. 3). The extensive cross-reactivity shown by antibody 4D7 for GPL-21 prompted us to examine its structure. Remarkably, it is endowed with a terminal 4,6(1′-carboxyethylidene)-β-D-glucopyranosyl(1→3) residue (D. Chatterjee and P. J. Brennan, unpublished data). Thus, the presence of a methoxy group on C-3 of the pyruvylated glucose confers absolute serovar 8 specificity on antibody 2B8, whereas antibody 4D7 shows no such stringent requirements. That the simple pyruvylated sugar 4,6(1′-carboxyethylidene)-3-*O*-methyl-β-D-glucopyranoside is indeed the epitope of antibody 2B8 was

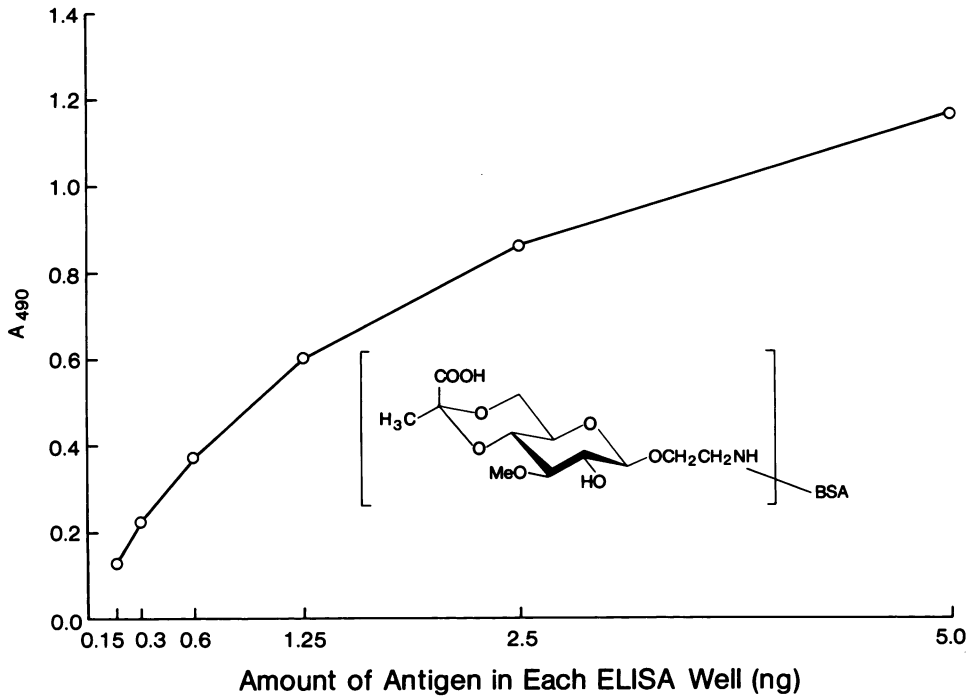


FIG. 15. Antigenic activity of different concentrations of neoantigen 8 against monoclonal antibody 2B8 in plate ELISA. Ascitic fluid was diluted 1:12,800.

demonstrated by use of the appropriate neoantigen (Fig. 15) synthesized in the Ontario laboratory. All of the available monoclonal antibodies (Table 2) and the rabbit polyclonal antibodies reacted similarly with neoantigen-8.

Monoclonal antibodies selective for the terminal glycosyl units of the specific GPLs of serovar 1 and serovar 2. Coincidentally, the simplest-named serovars also are endowed with the simplest oligosaccharide haptens (Fig. 3). Such simple haptens apparently confer relatively low specific immunogenicity on the parental GPL and the whole serovar. For instance, immunization of 10 individual mice yielded only one animal with a serum titer of 1:320 (Table 1). Nevertheless, suitably specific, albeit low-titer, monoclonal antibodies were generated in the Colorado laboratory (Table 1). However, none of these antibodies reacted with the unfractionated GPLs prepared after the usual alkali treatment. However, when the naturally acetylated unfractionated GPLs or the pure acetylated specific GPL from serovar 1 were used, they reacted with the anti-serovar 1 monoclonal antibodies. Thus, the characteristic epitope of serovar 1 must be an O-acetylated terminal α-L-rhamnopyranosyl residue. Although the α-L-rhamnopyranosyl(1→2)-linked terminal residue of GPL-1 is found in the oligosaccharide segments of the GPLs of all serovars (Fig. 3) (2), only in the case of serovar 4 is it the terminal, nonreducing-end sugar. Likewise, monoclonal antibody 123.4 of the IgG_{2a} isotype (Table 1) recognized only the acetylated specific GPL from the homologous serovar 2; it did not react with the nonacetylated specific GPL-2 nor with the acetylated or nonacetylated GPLs from other serovars (results not shown). Since the 2,3-di-*O*-methyl-L-fucopyranose(α1→3)-L-rhamnopyranose unit is present in other GPLs (e.g., GPL-9) but represents an internal diglycosyl unit in these other serovars, then the antibody must require the 2,3-di-*O*-methyl-α-L-fucopyranosyl residue at the nonreducing end, and it also must be O acetylated at the 4-OH.

DISCUSSION

The primary goal of the present work was to generate monoclonal antibodies absolutely specific for each of the major serovars of the *M. avium* complex and then to apply these to the identification and classification of *M. avium* isolates as they arise in opportunistic infections in immunocompromised individuals and to the establishment of epidemiological trends. Others (31; Ranchoff et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1986) had generated anti-*M. avium* complex monoclonal antibodies with similar goals in mind and, on the basis of the selective reactivities of these antibodies with glycopeptidolipid-containing preparations, had concluded that they were directed to the said antigens. However, in a series of exhaustive studies (2, 4, 7, 9, 11, 12, 20, 29), we had pinpointed the serotypic specificities of members of the *M. avium* complex as being due to differences in sugar arrangements at the distal nonreducing end of the otherwise invariant polar GPL molecule, and as the originators of this information and relevant methods, we were thus in a position to precisely define the epitopes of many of these antibodies. In the present comprehensive study, we have applied not only our knowledge of the chemistry of these surface immunogens but also the ability to synthesize corresponding neoantigens and to apply these antigens and neoantigens to a variety of relevant immunochemical protocols. Thus, from our chemistry-based perspective, the study represents an attempt at ultimate epitope definition and an effort to match each antibody with a chemically defined determinant. The practical applications of these antibodies will be described elsewhere. In a word, they are generally decidedly specific for the homologous, whole serovar and can be applied directly to bacterial cultures, thus allowing easy, rapid identification of at least half of the *M. avium* isolates encountered in the clinical laboratory (unpublished results). This earlier chemical ap-

proach had identified an exceptional array of novel deoxysugars, sugar acids, amido sugars, and branched-chain sugars, all occupying the distal segment of the oligosaccharide hapten and thus, presumably, the serovar-specific epitopes. Our strategy was to match these varied and unusual sugars with equally specific antibodies and thereby bypass seroagglutination with preabsorbed rabbit sera or protocols based on protracted TLC or gas chromatography of the specific GPLs, the standard means by which members of the *M. avium* complex are identified. In this we have been successful, although the enterprise was protracted and involved considerable initial frustration in raising antibodies with the requisite specificities (Ranchoff, M.S. thesis). For this reason, we also included in the study antibodies raised by other investigators, apparently with greater ease, but awaiting definition of epitope; of the many surveyed, those from the Amsterdam laboratory were of consistently high titer and, generally, high absolute specificity.

There are other more fundamental lessons to be learned from this study due to the availability of detailed knowledge of the chemistry of the appropriate surface glycolipid immunogens. These glycopeptidolipids occur in both the partially O-acetylated and completely O-deacetylated forms (5, 17). However, the positions or roles of these *O*-acetyl functions were not known in the context of the antigenicity of the parental GPLs; *O* acetylation of GPLs is clearly important in the binding of certain mycobacteriophages to the parental organism (17). Accordingly, the observation of the role of an *O*-acetyl function in antibody binding when positioned on the distal sugar is important although not unexpected. Lindberg and Hellerqvist (27) first showed the importance of *O*-acetyl groups in recognizing polyclonal antibodies to the O antigens of enteric bacteria, and the point has been repeatedly demonstrated since. Some of the anti-*M. avium* antibodies generated by Nishimori et al. (31) also showed a marked preference for GPLs isolated without the use of alkali, as described herein and elsewhere (5), and thus presumably O acetylated. The type of specificity for steric and absolute configuration, anomeric linkage, and specifically located acetoxy and methoxy functions demonstrated in this and other work (unpublished results) has also been demonstrated for the enormous collection of monoclonal antibodies raised to assorted glycosylceramides (8, 23). In this present study, we also describe murine monoclonal antibodies that selectively react with the 4,6-pyruvylated-3-*O*-methyl-D-glycosyl unit of the serovar 8-specific GPL. In an exhaustive study, Rao et al. (34) had described human monoclonal IgM proteins that distinguish between 3,4-pyruvylated-D-galactose and 4,6-pyruvylated D-glucose. Some of the antibodies described herein (especially those from the Amsterdam laboratory) that apparently require an *O*-acetyl substituent on the innermost 6-deoxytalose residue are also reminiscent of other antibodies described in the literature. Nudelman et al. (32) have described a murine monoclonal IgM antibody whose specificity is determined by the reducing-end sugar on the tetrasaccharide hapten of a ganglioside. Yet another antibody described by this group showed strict selectivity for the linkage position within an otherwise identical terminal glycobiose unit (33).

Much more fundamental information on the precise structural requirements for the binding of carbohydrate determinants to murine monoclonal antibodies will undoubtedly emerge as we continue to match the exceptional diversity of structures within the glycopeptidolipids, lipooligosaccharides, and phenolic glycolipids (3) of *Mycobacterium* spp. with corresponding monospecific antibodies. Indeed, monoclonal antibodies to the phenolic glycolipid I of *Mycobacterium leprae* (19) show an exquisite specificity for the number and the positions of the methoxy groups on the distal 3,6-di-*O*-methyl-α-D-glycopyranosyl unit (14, 15, 19). Clearly, however, for present clinical purposes the present collection of antibodies specific to the most prominent *M. avium* serovars fulfills a pressing need.

ACKNOWLEDGMENTS

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