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Production and Characterization of Monoclonal Antibodies against Specific Serotypes of *Mycobacterium avium* and the *Mycobacterium avium-Mycobacterium intracellulare-Mycobacterium scrofulaceum* Complex

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Serotype-specific and *Mycobacterium avium-Mycobacterium intracellulare-Mycobacterium scrofulaceum* complex (MAIS complex)-specific monoclonal antibodies (MAbs) were prepared. A series of MAbs were obtained, five specific for serotype 2, three specific for serotype 4, eight against a strain of serotype 19, two specific for the MAIS complex, and two against a common glycolipid shared by all the mycobacteria tested so far. The serotype-specific and the MAIS complex-specific MAbs reacted in immunofluorescence with intact mycobacteria and in enzyme-linked immunosorbent assay and immuno-thin-layer chromatography with lipid extracts of mycobacteria. The two MAbs against a common mycobacterial glycolipid reacted only in lipid enzyme-linked immunosorbent assay and immuno-thin-layer chromatography. All MAbs were directed against glycopeptidolipids (GPLs), except for four MAbs against proteins of serotype 19. The serotype- and MAIS complex-specific epitopes on GPLs are exposed on the mycobacterial cell wall, in contrast with the common mycobacterial glycolipid, which is probably located inside the cell wall. The serotype-specific MAbs reacted with native as well as deacetylated GPLs, in contrast with the MAIS complex-specific MAbs, which reacted only with native GPLs. The MAbs will be useful for the identification of MAIS complex and *M. avium* serotypes 2 and 4 and a strain of serotype 19, GPL analyses with immuno-thin-layer chromatography, and the localization of GPL epitopes in mycobacteria.

Mycobacterium avium-M. intracellulare-M. scrofulaceum complex (MAIS complex) is among the most common nontubercular mycobacteria recovered from clinical specimens (23). These mycobacteria commonly cause tuberculosislike diseases in humans. The emergence of both tuberculosis and nontubercular mycobacterial infections as a common cause of opportunistic infections in patients with acquired immune deficiency syndrome has led to a resurgence of interest in this field. These patients often have disseminated infections with one or more serotypes of the MAIS complex.

The differentiation between MAIS complex and *M. tuberculosis* is of clinical importance, since infections with MAIS complex strains are difficult to treat because of resistance to many antibiotics and chemotherapeutics, whereas tuberculosis can be cured with a number of standard medications (25). Identification of serotypes of the MAIS complex is important both for epidemiological studies and the development of more effective drug therapy.

The MAIS complex can be differentiated by seroagglutination (21), enzyme-linked immunosorbent assay (ELISA) (24), or thin-layer chromatography (TLC) of the type-specific antigen (18, 22). It is difficult to distinguish the separate members of the MAIS complex by serology because of cross-reactions within the complex (21). The methods of serotyping depend on the surface-located polar mycoside C glycopeptidolipid (GPL) antigen (16). Brennan and colleagues have recently elucidated the type-specific structure of the oligosaccharide of serotype 4. They found that the serotype-specific antigens were alkali stable and used TLC of the alkali-stable lipid extracts for serotype identification (16).

We report here the production and characterization of serotype-specific and MAIS complex-specific monoclonal antibodies (MAbs). These will provide a useful tool for the rapid diagnosis of MAIS complex infections, the study of the epidemiology of these infections, the localization of GPLs in the mycobacterial cell wall, the elucidation of B-cell epitopes on the GPLs, and the further analysis of the complex oligosaccharides among the serotypes.

A preliminary account of some of this work has been reported previously (13).

MATERIALS AND METHODS

Mycobacteria. Strains of 15 different species of mycobacteria were obtained from D. G. Groothuis, National Institute for Public Health, Bilthoven, The Netherlands, and from F. Portaels, Institute for Tropical Hygiene, Antwerp, Belgium. The bacteria were cultured on Löwenstein Jensen medium and subcultured every 2 months.

Strains identified as belonging to *M. avium* were obtained from the collection of Schaefer (20). In addition, we obtained mycobacterial isolates belonging to the MAIS complex from F. Portaels, Institute for Tropical Hygiene, Antwerp, Belgium; J. E. Landheer, St. Antonius Hospital Nieuwegein, Utrecht, The Netherlands; J. Kazda, Forschungsinstitut Borstel, Borstel, Federal Republic of Germany; and P. J. Rietra and R. van Ketel, Academic Medical Center, Laboratory of Medical Microbiology, University of Amsterdam, The Netherlands (Table 1).

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TABLE 1. Origins of MAIS complex mycobacteria

Serotype	Source of supply[a] (strain code origin[b])	Source of isolate	Our code
MAIS complex			
1	A (19076 Marks, 16909-3380)	Patient	avi 16, 17
1/2	C (myc10343)	Patient	avi 68
2	A (myc3875)	Patient	avi 1
2	A (Sheard, NZ2437, V431, Vet1387)	Patient	avi 18, 19, 20, 21
2	B (8063)	Patient	avi 2
2	B (11106 from armadillo 189)	Animal	avi 8
2/3	D (myc8945, avi 5)	AIDS Patient	avi 3, 5
3	A (Ben Kova, Cardiff 6195)	Patient	avi 22, 23
4	A (V429, Cheltenham 13528-1701, Tudor-sat582)	Patient	avi 29, 30, 31
4	D (myc8946, myc8455, myc8895, myc9592)	AIDS Patient	avi 4, 6, 7, 13
4	D (myc9306)	Patient	avi 11
5	A (5433-1693, 4443-1237)	Patient	avi 32, 33
6	A (Susook, V441, Sweatman)	Patient	avi 34, 35, 36
7	A (V450, 32114 Perth, Manten157)	Patient	avi 37, 38, 39
8	A (S.S.C.222, J 1868)	Patient	avi 40, 41
8	D (myc9469, myc9591)	AIDS Patient	avi 12, 14
9	A (6450-204, 17584-286)	Patient	avi 60, 61
10	A (Borne Iowa, 290-152)	Patient	avi 24, 25
11	A (V455, Zemenskosva, 14186-1424)	Patient	avi 26, 27, 28
12	A (P42, Hatcher, Howell)	Patient	avi 45, 46, 47
13	A (Orcie Rogers, Chance b, Lynn b)	Patient	avi 42, 43, 44
14	A (Clark, Edgar, P39)	Patient	avi 48, 49
15	A (Simpson, Dent)	Patient	avi 62, 63
16	A (Jandle, Mark Robert)	Patient	avi 58, 59
16	C (myc3798, myc5089)	Patient	avi 10, 67
16	B (myc6997; other codes, NCTC 10682 and ATCC 15985)	Patient	int 1
17	A (Connell, P54)	Patient	avi 52, 53
18	A (Melnick, Peter, 2219 Altman)	Patient	avi 50, 51
19	A (Huntly, Claude, Darden)	Patient	avi 54, 55
19	C (myc5654)	Patient	avi 66
19	E (N 9666 originally from Schaefer)	Patient	int 2
19	F (236/85)	Patient	int 4
19	E (MI19, from a soil sample from Bombay)	Soil	int 3
20	A (Findly at 545A, Newberry)	Patient	avi 56, 57
MAIS complex, nontypeable	D (myc9590)	Patient	avi 15
	B (43 from armadillo 193)	Animal	avi 9
	B (11108, 11067 from armadillo 189)	Animal	mai 1, 2
	B (44, 47, 49 from armadillo 193)	Animal	mai 3, 4, 5
M. scrofulaceum	A (myc3442)	Patient	scr 1
	B (1182)	Patient	scr 2
	B (11107, 11109, 11125 from armadillo 189)	Animal	scr 3, 4, 5
	B (45, 46 from armadilo 193)	Animal	scr 6, 7

[a] A, D. G. Groothuis; B, F. Portaels; C, J. E. Landheer; D, R. van Ketel; E, J. Kazda; F, K. H. Schröder, Forschungsinstitut Borstel.
[b] Code of original supplier.

Mycobacteria were grown in Sauton liquid medium, composed of 2 mM Mg_2SO_4, 10 mM citric acid, 2.5 mM K_2HPO_4, 30 mM asparagine, 0.1 mM ferric ammonium citrate, and 830 mM glycerol (final pH 7.4), as shaking cultures at 37°C, using a Brunswick gyratory shaker (New Brunswick Scientific Co., Edison, N.J.) with 100 rotations per min. Bacteria were harvested after 7 to 10 weeks during the late-logarithmic phase, killed by treatment for 15 min at 80°C, then centrifuged for 30 min at 16,000 × *g* and 4°C, washed twice with distilled water, and finally lyophilized.

Seroagglutination. The slide agglutination method was used for the serological identification of the MAIS complex strains (4).

Preparation of MAbs. Two kinds of protocols were used for immunization of BALB/c mice. For fusion 75, the mice were immunized with a mixture of seven heat-killed strains of *M. avium* belonging to serotypes 2 and 4, isolated from seven patients, five of whom had acquired immune deficiency syndrome. The mice were injected according to the following protocol: day 0, 5×10^7 intact mycobacteria emulsified in incomplete Freund adjuvant (IFA), given half intraperitoneally and half subcutaneously; day 14, 5×10^7 intact mycobacteria without adjuvant, given half intraperitoneally and half subcutaneously; 4 days before fusion an intravenous booster injection of 2.5×10^8 intact mycobacteria was given. For fusion 85, 100 μg (dry weight) of the supernatant (20,000 × *g*) of the same seven sonicated *M. avium* strains used in fusion 75 were injected intraperitoneally on days 0 (in IFA) and 14 (without IFA); 4 days before the fusion, 5×10^8 intact mycobacteria were given intravenously. For fusion 108, 100 μg (dry weight) of the supernatant (20,000 × *g*) of a sonicated strain of *M. intracellulare* serovar 19 (our code int 3 in Table 1) were injected intraperitoneally on days 0 (in IFA) and 14 (without IFA). An

intravenous booster injection consisting of 700 μg (dry weight) of the same sonicated mycobacteria was given 4 days before the fusion.

The spleen cells were fused with NS 1 mouse myeloma cells in a ratio of 4:1 by using standard techniques (5, 6, 23a). Peritoneal macrophages of nonimmunized BALB/c mice were used as feeder cells. The ELISA described below was used to screen culture supernatants. Each supernatant was tested for antibody reactivity with different mycobacterial species to identify the most selective antibody-producing hybridomas. Hybridomas showing positive reactions of the desired specificity were cloned by using the limiting-dilution technique at a density of 1 cell per well and recloned twice at a density of 1 cell per three wells. Selected clones were then grown in bulk and injected intraperitoneally into pristane-primed BALB/c mice to obtain ascites. Both culture supernatants and ascites were used in subsequent testing of the MAbs. The immunoglobulin subclasses of the MAbs were determined by lipid ELISA or Western (immuno-) blot with subclass-specific antisera (Serotec, Bicester, England).

Preparation of GPLs. One hundred milligrams of freeze-dried *M. avium* isolates coded avi 1 to 7 and 10 (Table 1) with serotypes 2, 2/3 (a strain belonging to both serotypes 2 and 3), 4, and 16 was extracted with 4 ml of chloroform-methanol (2:1, vol/vol) for 18 h at 50°C, as described by McNeil et al. (16). Part of the lipid fraction was deacetylated with 0.2 M NaOH in methanol for 30 min at 37°C, neutralized with glacial acetic acid, evaporated, and subjected to a biphasic wash (2). Native and deacetylated lipids were stored at −70°C in chloroform containing 0.005% butylated hydroxytoluene as an antioxidant. For immuno-TLC (ITLC) the antioxidant was omitted, since it inhibits the substrate conversion at the solvent front of the TLC plates, interfering with possible reactions close to the front.

ELISA with lipid extracts of mycobacteria. Lipid extracts of 2 mg of freeze-dried bacteria prepared as described above were suspended in 5 ml of phosphate-buffered saline (PBS) by sonication for 2 min. Polyvinyl chloride microdilution plates (Titertek Immuno Assay plates; Flow Laboratories, Inc., McLean, Va.) were coated overnight at 37°C with 50 μl of this suspension per well (this corresponds with the lipid extract of 2×10^8 bacteria per well).

Nonspecific binding sites were blocked by incubation with 1% bovine serum albumin in PBS at 150 μl per well for 1 h at 37°C. After the plates were washed four times with PBS, ascites diluted 1:1,000 with 1% bovine serum albumin in PBS were added to each well and incubated for 1 h at 37°C. The plates were washed four times with PBS and incubated for 1 h at 37°C with 100 μl of horseradish peroxidase-labeled goat anti-mouse heavy- and light-chain immunoglobulin G (IgG) (Pasteur Institute, Paris, France) per well. After the plates were washed four times with PBS, 100 μl of tetramethylbenzidine (0.24 mg/ml) (Sigma Chemical Co., St. Louis, Mo.)–0.015% hydrogen peroxide in 0.05 M citrate–0.1 M K_2HPO_4 buffer (pH 5.0) was added (TMB substrate solution) to each well and incubated for 1 h at room temperature. The plates were read with a Titertek ELISA reader at 405 nm (A_{405}). Absorbance was transferred to matrix readings: matrix 1 corresponds to an A_{405} between 0.2 and 0.4, matrix 2 corresponds to an A_{405} between 0.4 and 0.6, matrix 3 corresponds to an A_{405} between 0.6 and 0.8, etc.

ELISA with intact mycobacteria. Polystyrene microdilution plates (Costar, Cambridge, Mass.) were coated with 2×10^8 mycobacteria per well in 0.05 M sodium bicarbonate buffer, pH 9.6 (100 μl per well), overnight at 37°C. The plates were washed and further treated in the same way as described above for ELISA with lipid extracts except that PBS–0.05% Tween 20 was used for the washing solution.

Immunofluorescence technique (IFT) on mycobacteria. Mycobacteria were grown on Löwenstein-Jensen medium for 8 or more weeks, scraped from the surface of the medium, suspended in distilled water, killed by heating for 5 min at 80°C, suspended by sonication for 2.5 s, and diluted to 10^8 bacteria per ml in water. Ten microliters of the bacillus suspension was placed on each spot of a microscope slide as previously described (11) and dried under a current of warm air at 30°C. A drop of culture supernatant was layered on each spot of the slide and incubated at 37°C in a moist chamber for 60 min, followed by washing and incubation with fluorescein-conjugated sheep anti-mouse heavy- and light-chain IgG (TAGO, Copenhagen, Denmark) as previously described (11). As controls, irrelevant MAbs of all different isotypes were used. All tests were performed in triplicate and read in a blind manner.

TLC and ITLC. Lipid extracts of mycobacteria were partly purified by silicic acid-Celite (2:1) column chromatography (1, 3, 17), applied on thin-layer plates (HP-TLC Alufolien Kiesel 60; E. Merck AG, Darmstadt, Federal Republic of Germany). Solvents were chloroform-methanol-water, 60:15:2 or 65:25:4. Lipids were visualized by being sprayed with 0.1% orcinol in 40% H_2SO_4 and heating for 10 min at 105°C. For ITLC, water-resistant silica plates (K6 TLC plates, 10 by 20 cm; Whatman Ltd., Maidstone, Kent, England) were used (15, 19). After chromatography, plates were first soaked in PBS for a few seconds and then soaked in 15 ml of 1% bovine serum albumin in PBS for 15 min at room temperature. Plates were blotted with tissue paper and incubated with 15 ml of 1/1,000-diluted MAb for 1 h at room temperature. The plates were washed five times with PBS for 30 min, blotted with tissue paper, and incubated with goat anti-mouse heavy- and light-chain IgG horseradish peroxidase conjugate diluted 1:1,500 in 1% bovine serum albumin in PBS. Plates were washed four times with PBS, and for the fifth wash distilled water was used. The plates were blotted with tissue paper and incubated for 10 min with TMB substrate solution to which 4.5 mM dioctyl sodium sulfosuccinate was added. After being washed with water, plates were dried with a cold hair dryer and photographed or photocopied.

Western blot analysis. Mycobacterial sonicates (*M. intracellulare* serotype 19 [our code int 3], *M. intracellulare* serotype 19 [our code int 1], *M. avium* serotype 2 [our code avi 1; Table 1], *M. tuberculosis*, *M. scrofulaceum*, *M. gastri*, *M. flavescens*, *M. kansasii*, *M. duvalii*, *M. fortuitum*, *M. nonchromogenicum*, *M. smegmatis*, *M. xenopi*, and *M. leprae*) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (14). The separated antigens were electrophoretically transferred to 0.2-μm nitrocellulose sheets (Schleicher & Schuell, Dassel, Federal Republic of Germany) in a buffer containing 25 mM Tris, 192 mM glycine (pH 8.5), and 20% methanol, using a Transblot apparatus (Bio-Rad Laboratories, Richmond, Calif.). After electrophoretic transfer at 25 V and room temperature for 18 h, the nitrocellulose sheets were incubated in 0.5% Tween 20 in PBS, pH 7.4 (PBST).

Ascites (175 μl) diluted 1:1,000 in PBST were applied on the sheets in small lanes by using the Miniblotter (Immunitics, Cambridge, Mass.) and incubated for 1 h at room temperature with gentle agitation. After the lanes were washed with PBST, the Miniblotter was disassembled and the sheets were washed five times in PBST for 30 min with gentle agitation. The membranes were incubated for 1 h with

TABLE 2. Characterization of MAbs against serotypes of MAIS complex in IFT

MAbs	Reactivity with serotype[a] (no. positive)										Antigen detected[b]
	1 (n = 2)	1/2	2 (n = 7)	2/3 (n = 2)	3 (n = 2)	4 (n = 8)	19 (n = 6)	MAI (n = 6)	scr (n = 7)	myc (n = 18)	
Fusion 75											
F75-1, IgM	2+ (2)	3+	3+ (7)	2+ (2)	3+ (2)	2+ (8)	3+ (6)	2+ (6)	3+ (7)	2+ (11)	NC
F75-2, IgM	2+ (1)	—	2+ (3)	3+ (1)	—	—	—	—	—	—	GPL
F75-4, IgM	—	—	2+ (4)	3+ (1)	—	—	—	—	—	3+ (1)	GPL
F75-5, IgM	—	—	2+ (4)	3+ (1)	2+ (1)	—	—	—	—	3+ (1)	GPL
F75-7, IgM	—	—	2+ (5)	3+ (1)	2+ (1)	—	—	—	—	—	GPL
F75-9, IgM	—	—	—	3+ (1)	—	—	—	—	—	—	GPL
F75-10, IgM	—	—	—	—	—	—	—	—	—	2+ (3)	GPL
F75-11, IgM	—	—	2+ (2)	—	—	—	—	—	—	2+ (3)	GPL
F75-13, IgM	—	—	2+ (2)	3+ (1)	2+ (1)	—	—	—	—	3+ (1)	GPL
F75-14, IgM	—	—	3+ (5)	3+ (1)	2+ (1)	—	—	—	—	—	GPL
Fusion 85											
F85-2, IgG3	3+ (2)	2+	2+ (4)	2+ (1)	3+ (2)	3+ (7)	2+ (3)	3+ (3)	3+ (7)	—	GPL
F85-3, IgG3	—	—	3+ (1)	—	—	3+ (8)	—	—	—	—	GPL
F85-5, IgM	—	—	—	—	—	3+ (8)	—	—	—	—	GPL
F85-6, IgG1	—	—	3+ (1)	—	—	3+ (8)	—	—	—	—	GPL
F85-10, IgG3	3+ (2)	2+	2+ (4)	2+ (1)	3+ (2)	3+ (7)	2+ (3)	3+ (3)	3+ (7)	—	GPL
Fusion 108											
F108-1, IgG2b	—	—	—	—	—	—	3+ (1)[c]	—	—	—	GPL
F108-7, IgM	—	—	—	—	—	—	—	—	—	—	36K
F108-9, IgM	—	—	—	—	—	—	—	—	—	—	31K
F108-10, IgG2b	—	—	—	—	—	—	3+ (1)[c]	—	—	—	GPL
F108-14, IgG2a	—	—	—	—	—	—	—	—	—	—	27/25K
F108-17, IgM	—	—	—	—	—	—	3+ (1)[c]	—	—	—	GPL
F108-18, IgG2a	—	—	—	—	—	—	—	—	—	—	31/28K
F108-21, IgM	—	—	—	—	—	—	3+ (1)[c]	—	—	—	GPL

[a] All tested strains (see Table 1) of serotypes 5 to 9, 12 to 18, and 20 reacted only with MAbs F75-1, F85-2, and F85-10; all strains of serotypes 10 and 11 reacted only with F75-1.

[b] NC, Not characterized; 36K, 31K, 27/25K, and 31/28K, reactions in Western blot with proteins of molecular weights of 36,000, 31,000, 27,000 and 25,000, and 31,000 and 28,000, respectively.

[c] Only int 3 of serotype 19 (see Table 1) was positive with these MAbs of fusion 108. 1/2, strain belonging to both serotypes 1 and 2; 2/3, strains belonging to both serotypes 2 and 3; MAI, untypeable MAIS strains; scr, *M. scrofulaceum*; myc, *M. tuberculosis*, *M. africanum*, *M. bovis*, *M. bovis* BCG, *M. fortuitum*, *M. kansasii*, *M. malmoense*, *M. marinum*, *M. ulcerans*, *M. xenopi*, *M. flavescens*, *M. vaccae*, *M. gastrii*, *M. nonchromogenicum*, *M. smegmatis*, *M. terrae*, *M. leprae*, and armadillo-derived mycobacteria. 1+, 2+, and 3+, increasing strengths of reactions in IFT; —, no reaction.

horseradish peroxidase-conjugated sheep anti-mouse heavy- and light-chain IgG (Pasteur Institute) diluted 1:1,500 in PBST, using a roller bottle incubator. After being washed five times for 30 min with PBST, the membranes were incubated with TMB substrate solution with dioctyl sodium sulfosuccinate, as described for ITLC. The reaction was stopped after 5 to 10 min by rinsing the blots with distilled water. The blots were dried between filter paper and stored in the dark.

RESULTS

In fusion 75, hybridomas were selected on the basis of a strong positive reaction in ELISA with the target antigen *M. avium* serotype 2 (avi 1) and a negative reaction with *M. tuberculosis* H37Rv. Of the 590 antibody-producing wells, 69 met these criteria. We assumed that the MAbs would be species specific. They turned out to be serotype specific (Table 2). Seven MAbs (F75-2, F75-4, F75-5, F75-7, F75-9, F75-13, and F75-14) reacted in IFT with most of the strains of serotype 2 and one strain of serotype 2/3 and 3. One MAb, F75-1, reacted with almost all mycobacteria tested in IFT, but no reaction was found in Western blots. The antigen detected by this MAb was not further characterized.

For fusion 85, hybridomas were selected on the basis of a strong positive reaction in ELISA with two strains of serotype 4 (avi 4, avi 7) and a strain (not used for immunization) of *M. intracellulare* serotype 16 (int 1) and a negative reaction with *M. tuberculosis*. Of 78 antibody-producing wells, 52 were both serotype 4 or 16 positive and *M. tuberculosis* negative. Species- as well as serotype-specific MAbs were obtained (Table 2). F85-2 and F85-10 were MAIS complex specific, with some exceptions; no reaction was found with some strains belonging to serotypes 2, 4, 5, 6, 7, and 19, some untypeable MAIS complex strains, and all strains of serotypes 10 and 11. Of 82 MAIS complex strains tested, 61 were positive with these MAbs.

For fusion 108, we intended to prepare MAbs against the *M. intracellulare* serotype 19 strain int 3 (Table 1) since none of the specific MAbs of fusions 75 and 85 reacted with this strain. This strain of serotype 19 enhanced the pathogenicity of *M. leprae* when they were inoculated together into the footpads of nude mice (7).

Hybridomas were selected on the basis of a strong positive reaction in ELISA with the strain int 3 or scr 1 and a negative reaction with *M. tuberculosis*. Of the 102 antibody-producing wells, 86 met these criteria.

Four MAbs (F108-1, F108-10, F108-17, and F108-21) reacted both in lipid ELISA and with intact bacteria. These MAbs were strictly strain specific and reacted only with the strain int 3 of serotype 19 used for immunization. Four MAbs reacted in Western blots. MAb F108-14 reacted with epitopes on a 27,000-molecular-weight protein (27K protein)

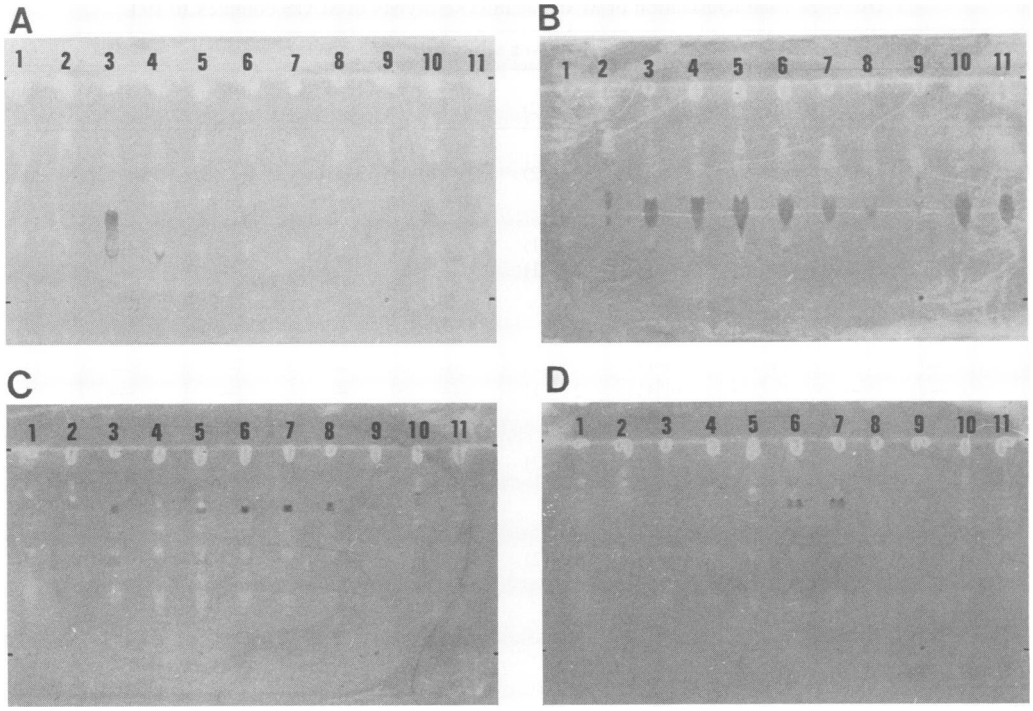

FIG. 1. ITLC of native lipids of *M. avium*. Samples of each *M. avium* lipid fraction in chloroform were applied to silica plates and chromatographed in chloroform-methanol-water (65:25:4). The origins (bottom of plates) and solvent fronts are indicated (-). Lanes: 1, *M. avium* serotype 2 (strain avi 1); 2, *M. avium* serotype 2 (strain avi 2); 3, *M. avium* serotype 2/3 (strain avi 3); 4, *M. avium* serotype 2/3 (strain avi 5); 5, *M. avium* serotype 4 (strain avi 4); 6, *M. avium* serotype 4 (strain avi 6); 7, *M. avium* serotype 4 (strain avi 7); 8, *M. avium* serotype 16 (strain avi 10); 9, MAIS complex untypeable strain (strain avi 15); 10, *M. intracellulare* serotype 16 (strain int 1); 11, *M. intracellulare* serotype 19 (strain int 3). The plates were immunostained with the following MAbs: (A) MAb F75-9 specific for *M. avium* 3 serotype 2/3; (B) MAb F75-11 reactive with a common mycobacterial glycolipid; (C) MAb F85-2 reactive with MAIS complex-specific glycolipid; (D) MAb F85-6 specific for serotype 4.

and a 25K protein of the strain int 3 and showed weak cross-reactivity in Western blots with *M. tuberculosis*, *M. intracellulare* strain int 1, *M. xenopi*, and *M. leprae* and no reaction with the nine other mycobacterial species tested (see Materials and Methods) (data not shown). MAb F108-7 reacted in a Western blot with a 36K protein of strain int 3 and showed weak cross-reactivity in Western blots with *M. tuberculosis*, *M. leprae*, *M. duvalii*, and *M. avium* and no reaction with the nine other mycobacterial species tested (see Materials and Methods). F108-9 and F108-18 reacted in Western blots with a 31K protein and a 31 and 28K protein of strain int 3, respectively. These MAbs were broadly cross-reactive; 11 of the 14 mycobacterial species were positive.

The glycolipids recognized by each MAb were identified by ITLC (Fig. 1). The serotype-specific, MAIS complex-specific, and common mycobacterial lipid epitopes were localized on different lipids (Fig. 1A, B, and C). Table 3 shows the reactivities of 16 MAbs from fusions 75, 85, and 108 in lipid ELISA, ITLC, and IFT with some strains belonging to the serotypes 2, 2/3, 4, 16, 19, *M. scrofulaceum*, and *M. tuberculosis*. The fact that a considerable number of

TABLE 3. Reactivities of MAbs against GPLs in lipid ELISA, ITLC, and IFT with MAIS complex mycobacteria

MAb[a]	Reactivity (ELISA/ITLC/IFT) with strain[b] (serotype):								
	avi 1 (2)	avi 2 (2)	avi 3 (2/3)	avi 5 (2/3)	avi 4 (4)	int 1 (16)	int 3 (19)	scr 1	tub 1
F75-2	9/–/2	6/–2	9/2/3	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–
F75-9	1/–/–	3/–/–	9/3/3	1/–/–	1/–/–	1/–/–	1/–/–	1/–/–	1/NT/–
F75-10	3/1/–	9/2/–	5/3/–	5/3/–	7/3/–	3/3/–	3/3/–	8/2/–	3/1/2
F85-2	–/–/2	4/–/2	1/2/2	–/–/–	7/2/3	–/1/3	–/–/–	6/1/3	–/NT/–
F85-3	–/–/–	2/–/–	1/–/–	–/–/–	8/2/3	–/–/–	–/–/–	–/–/–	–/–/–
F85-5	9/–/–	9/–/–	5/–/–	–/–/–	7/2/3	1/1/–	–/–/–	1/–/–	–/–/–
F108-1	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	9/3/3	–/–/–	–/–/–
F108-17	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–	9/–/3	–/–/–	–/–/–

[a] Almost the same reactions were found with MAbs F75-5, F75-7, and F75-14 as with MAb F75-2; with MAb 75-11 as with MAb F75-10; with MAb F85-10 as with MAb F85-2; with MAb F85-6 as with MAb F85-3; with MAb F108-10 as with MAb F108-1; and with MAb F108-21 as with MAb F108-17.

[b] See Table 1 for details of source of supply, strain codes, and origins. Increasing strengths of reactions in lipid ELISA are indicated by 1 to 9 and for ITLC and IFT by 1, 2, and 3. –, No reaction. tub 1, *M. tuberculosis* 1 isolated from a tuberculosis patient, used as a control; NT, not tested.

[c] The same reactions were found with avi 6 and avi 7, both belonging to serotype 4.

TABLE 4. Reactivities of MAbs with native and deacetylated lipid fractions of *M. avium* serotypes in lipid ELISA

MAb	Reactivity[a] (native/deacetylated) with strain (serotype):			
	avi 1 (2)	avi 3 (2/3)	avi 4 (4)	avi 7 (4)
F75-7	4/5	5/8	–/–	–/–
F75-11	7/7	7/8	7/8	7/7
F85-2	–/–	1/–	7/–	7/–
F85-10	–/–	3/–	7/–	8/–
F85-3	–/–	–/–	8/8	7/7
F85-6	–/–	–/–	6/6	7/7

[a] Increasing strengths of reactions are indicated by 1 to 9. –, No reaction.

antibodies gave negative reactions in some or all of the tests indicates that the positive results are not due to nonspecific reactions. MAbs F75-2, F75-5, F75-7, and F75-14 reacted in lipid ELISA and IFT with both strains of serotype 2 and with one strain of serotype 2/3, whereas in ITLC only one strain of serotype 2/3 reacted. MAb F75-9 reacted in all three tests with one strain of serotype 2/3 and in lipid ELISA also with one strain of serotype 2 (avi 2). MAbs F75-10 and F75-11 reacted with all the mycobacterial strains tested in the lipid ELISA and ITLC, including *M. tuberculosis*, but were negative in IFT with the exception of *M. tuberculosis*. MAbs F85-2 and F85-10 reacted with almost all strains of the MAIS complex except for avi 5 belonging to serotype 2/3 and int 3 belonging to serotype 19, and both MAbs failed to react with avi 1 and avi 2 in ITLC (Table 3). MAbs F85-3, F85-5, and F85-6 reacted in all three tests almost exclusively with the strains avi 4, avi 6, and avi 7, all belonging to serotype 4. All MAbs described in Table 3 were negative in the three tests with *Escherichia coli* and *Nocardia asteroides* except for F75-10 and F75-11, which reacted in lipid ELISA and ITLC with *N. asteroides*.

The strain-specific MAbs F108-1 and F108-10 reacted in all three tests, whereas F108-17 and F108-21 were positive in lipid ELISA and IFT and negative in ITLC. This lack of reaction in ITLC may be caused by the differences in isotype of the MAbs.

The reactivities of MAbs with native and deacetylated lipid fractions of some *M. avium* serotypes were investigated by ELISA (Table 4). The serotype-specific MAbs F75-7, F85-3, and F85-6 reacted with both native and deacylated lipid fractions of the specific serotypes. The MAb F75-11 reacted with the native and deacetylated lipid fractions of all the strains tested. F85-2 and F85-10 were reactive only with the native lipid fractions.

DISCUSSION

From these results, we conclude that serotype-specific and species-specific MAbs can be produced by immunization with a mixture of *M. avium* serotypes. Using the selection procedure outlined here, we have obtained several families of MAbs which are MAIS complex specific (MAbs F85-2 and F85-10), serotype specific (MAbs F75-2, F75-4, F75-5, F75-9, F75-13, and F75-14), and strain specific (MAbs F108-1, -10, -17, and -21) and which react with a common glycolipid (MAbs F75-10 and F75-11). The selection procedure was essential for the specificity of the hybridomas obtained. *M. avium* serotype-specific MAbs were obtained by selection in fusion 75 with only one serotype. By selection with a strain not used for immunization (int 1, serotype 16) in fusion 85, MAIS complex-specific MAbs F85-2 and F85-10 were found, in addition to serotype 4-specific MAbs (Tables 2 and 3). Strain-specific MAbs were selected in fusion 108 against a strain of *M. intracellulare* serotype 19 (int 3). Despite selection with a strain of *M. scrofulaceum*, which was not used for immunization of the mice, no serotype- or MAIS complex-specific MAbs could be selected.

MAbs F75-2 and F85-2 reacted in IFT with only three and four of the seven strains of serotype 2, respectively, indicating that there is considerable heterogeneity in serotype 2 (Table 2). One strain serotyped as 2/3 is different from the rest of the MAIS complex, since this strain reacted only with the very broadly reactive MAbs F75-1, F75-10, and F75-11 and not with the MAIS complex-specific or serotype-specific MAbs (Tables 2 and 3). The strains belonging to serotypes 10 and 11 differ from the rest of the MAIS complex, since no reaction was found with either the serotype-specific or the MAIS complex-specific MAbs.

In general there was agreement between IFT, lipid ELISA, and ELISA with intact bacteria, especially when the IFT result was strongly positive (Table 3). We conclude from this that most of the epitopes of GPLs are located on the surface of the MAIS complex mycobacterial cell wall.

MAbs F75-10 and F75-11 were selected on the basis of positive reactions in lipid ELISA with *M. avium* and *M. tuberculosis*, in order to have broadly reactive MAbs. Both MAbs reacted in lipid ELISA and ITLC with all mycobacteria, including *N. asteroides*, but in IFT only with *M. tuberculosis* and a few *M. avium* strains. These results could be explained if both MAbs react with a common GPL that is rarely, if ever, exposed on the surface of the mycobacterial cell wall. With the exception of MAbs F75-10 and F75-11, all MAbs were negative in lipid ELISA with *E. coli* and *N. asteroides*, suggesting that the MAbs so far tested were specific for mycobacteria.

It is striking that although F75-2, F75-5, F75-7, and F75-14 reacted in lipid ELISA and IFT with the two strains, avi 1 and avi 2, of serotype 2 and one strain, avi 3, of serotype 2/3, in ITLC, only avi 3 serotype 2/3 was positive (Table 3). The same strains, avi 1 and avi 2, were also negative in ITLC with MAbs F85-2 and F85-10 (Table 3). This can be explained by the instability of these lipids after TLC or by conformational changes of the lipids after TLC. The MAIS complex-specific MAbs F85-2 and F85-10 were more reactive in IFT than in ITLC or lipid ELISA with avi 1, avi 3, and int 1. It seems that these MAbs reacted better with the lipid antigens in their native environment of the cell wall. Moreover, these MAbs were negative with the deacetylated lipids (Table 4). McNeil et al. described a MAb against serotype 9 that was reactive with the native GPL but negative with the deacetylated form (16).

The GPL antigens seemed to be remarkably well expressed on the strains isolated from patients with acquired immune deficiency syndrome. The strong reactions of MAbs F85-3, F85-5, and F85-6 with strains avi 4, avi 6, and avi 7, all belonging to serotype 4, was notable. The cross-reaction of MAb F85-5 with avi 1, avi 2, and avi 3 in lipid ELISA (Table 3) may have arisen from the IgM subclass of the antibody.

The MAbs against the int 3 strain of *M. avium* serotype 19 (F108-1, F108-10, F108-17, and F108-21) are important for the detection of these mycobacteria in experimentally infected animals. This *M. intracellulare* (Table 1, serotype 19 int 3) strain has been connected with leprosy since this strain was isolated from the same soil sample from which an environmental strain of *M. leprae* was isolated (8). The role that this nonpathogenic strain of *M. intracellulare* plays in

causing leprosy in association with *M. leprae* can now be studied in detail, since specific MAbs are available for both the *M. intracellulare* isolate and *M. leprae* (9, 10, 12).

The MAIS complex-specific MAbs F85-2 and F85-10 did not react with the int 3 strain of serotype 19 (Table 3), with strains of serotypes 10 and 11 with some strains belonging to serotypes 2, 4, 5, 6, and 7, or with some untypeable MAIS complex strains (Tables 2 and 3), indicating that these strains are quite different from the rest of the MAIS complex, at least so far as the GPL composition is concerned.

The MAbs of fusion 108 that were negative in IFT reacted in Western blots (and in ELISA with sonicated mycobacteria; data not shown) with protein antigens probably located in the cytoplasm but not exposed on the surface of the intact bacteria.

The ITLC results showed that the serotype-specific, MAIS complex-specific, and common mycobacterial lipid epitopes are localized on different lipids. The serotype-specific and the MAIS complex-specific epitopes of the GPLs are located on the surface of the mycobacterial cell wall, whereas the common mycobacterial glyco(peptido) lipid(s) detected by MAbs F75-10 and F75-11 are mostly not exposed on the surface of the mycobacteria.

The localization of the GPLs or glycolipids in the cell wall and the possible role of the GPL in protecting the bacteria from the phagosome-lysosome fusion can now be studied in detail with immunoelectron microscopy. Furthermore, one can explore the host immune response against the GPLs of the MAIS complex mycobacteria by using the serotype- and MAIS complex-specific MAbs in a competition ELISA or in an ELISA using the synthesized sugar moiety of the different GPLs (B. Rivoire, B. J. Ranchoff, D. Chatterjee, H. Gaylord, A. Y. Tsang, A. H. J. Kolk, G. O. Aspinal, and P. Brennan, submitted for publication).

Rapid identification of the MAIS complex is now possible by using the MAbs described in this article with techniques of seroagglutination, immunofluorescence, lipid ELISA, or ELISA with intact bacteria. The choice depends on the preference of the individual researcher and the available experience in the laboratory.

We have evidence that these MAbs are useful for the identification of MAIS complex bacteria from clinical specimens (13; C. P. H. Verstijnen, S. Kuijper, J. Bruins, R. van Ketel, D. G. Groothuis, and A. H. J. Kolk, Abstr. Int. Congr. Trop. Med. Malaria, 1988).

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