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Enzyme-Linked Immunosorbent Assay of Glycolipid Antigens for Identification of Mycobacteria

DONNA L. YANAGIHARA,[1] VIRGINIA L. BARR,[2] CATHY V. KNISLEY,[1] ANNA Y. TSANG,[2] J. KENNETH McCLATCHY,[2] AND PATRICK J. BRENNAN[1]*

Department of Microbiology, Colorado State University, Fort Collins, Colorado 80523,[1] and Department of Clinical Laboratories, National Jewish Hospital and Research Center, Denver, Colorado 80206[2]

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Enzyme-linked immunosorbent assays which are based on species- or type-specific glycolipid antigens and in which rabbit antisera are prepared with homologous strains are capable of distinguishing among serological variants of the *Mycobacterium avium-Mycobacterium intracellulare-Mycobacterium scrofulaceum* complex, *Mycobacterium chelonei* subspecies *chelonei* and *abscessus*, *Mycobacterium simiae* I and II, *Mycobacterium kansasii*, *Mycobacterium szulgai*, *Mycobacterium xenopi*, and *Mycobacterium fortuitum* biovariant *peregrinum*. The immunoreactive glycolipids can be divided into two classes. Those resistant to alkali, the C-mycoside glycopeptidolipids, are present in the *M. avium-M. intracellulare-M. scrofulaceum*, the *M. chelonei* subspecies *chelonei* and *abscessus*, and the *M. simiae* I and II complexes and in *M. fortuitum* biovariant *peregrinum*. The alkali-labile glycolipid antigens, the lipooligosaccharides, are present in *M. kansasii*, *M. szulgai*, and *M. xenopi*. In one study, the combination of enzyme-linked immunosorbent assay and alkaline susceptibility was compared with seroagglutination in the identification of 60 clinical isolates of nontuberculous mycobacteria: 45 showed perfect concordance, 9 could be narrowed to one, two, or three possibilities, and the rest did not correspond. In a second study involving 43 clinical isolates that were untypable by seroagglutination or were autoagglutinable, the results of enzyme-linked immunosorbent assay and thin-layer chromatography of glycolipid antigens were compared: 21 showed clear concordance. The results demonstrate that enzyme-linked immunosorbent assay is particularly useful in assessing the antigenicity of lipids, and sensitivity, ease, and rapidity recommend it as an adjunct to seroagglutination and thin-layer chromatography for the identification of nontuberculous mycobacteria.

For the past few years, workers in our laboratories have determined the structures of the specific serotyping antigens of the clinically important mycobacteria. In all species examined to date, the specific antigens were found to be glycolipid in nature: those from the *Mycobacterium avium-Mycobacterium intracellulare-Mycobacterium scrofulaceum* (MAIS) complex are C-mycoside glycopeptidolipids (2, 3), those from *Mycobacterium kansasii* are trehalose-containing lipooligosaccharides (8), and those from *Mycobacterium leprae* are triglycosylphenolic diacylphthiocerols (6, 7, 9). For these studies, lipid from different species was extracted and fractionated, and the fractions were examined for serological activity with rabbit antiserum raised against the homologous strain of bacteria. Immunoreactive substances were then purified and the chemical structures were determined.

In recent studies, knowledge of the chemical structures of the specific antigens has been applied to the rapid detection and identification of nontuberculous mycobacteria of clinical importance. For this purpose the mycobacteria may be divided into two groups: those with alkali-stable glycolipids and those with alkali-labile glycolipids (4). In the species with alkali-stable glycolipids, the C-mycosides are characterized by a fatty acyl function that is amide linked to the amino terminal of the peptide moiety, and therefore, the glycolipids survive alkalinolysis. This property has been used to aid in the identification of human isolates of the MAIS complex on the basis of thin-layer chromatography (TLC) profiles of the alkali-stable lipid extracts (4, 5, 14). The lipooligosaccharide antigens are alkali labile and show distinct TLC profiles only before alkali treatment (4).

Up to now the specific glycolipid antigens have been used only for the chemical-based identification of mycobacteria. Serological identification has been difficult to implement, owing to the inherent hydrophobic nature of the antigens. In the present work, an enzyme-linked immunosorbent assay (ELISA) was developed and was found to be suitable for the detection of the specific glycolipids of members of the MAIS complex and other mycobacteria. Emphasis was placed on members of the MAIS group of nontuberculous mycobacteria since they are widely distributed in nature and are frequent opportunistic invaders of human hosts (16).

MATERIALS AND METHODS

Glycolipid antigens. Serovars (serotypes or immunotypes) of *M. avium*, *M. intracellulare*, and *M. scrofulaceum* were obtained from the authenticated collection maintained at National Jewish Hospital (14). The identity of each isolate had been established by seroagglutination and confirmed by TLC (14). Other mycobacteria were obtained from the Trudeau Mycobacterial Culture Collection, also maintained at National Jewish Hospital.

Mycobacteria were grown on 7H11 agar at 37°C (3). Rapidly growing strains were harvested within 7 to 10 days, and slow growers were harvested after 3 weeks in phenolized phosphate-buffered saline as described previously (14). The harvested cells were autoclaved at 20 lb/in^2 and 121°C for 30 min and then centrifuged at 1,500 × *g* for 20 min. The supernatant was decanted, and the cells were dried overnight in a P_2O_5-containing desiccator under vacuum. Lipid was extracted from the dry cells by sonication in $CHCl_3$-

* Corresponding author.

TABLE 1. Details of strains and antisera used for development of the ELISA based on glycolipid antigens of the MAIS complex

Serovar	Strain	Amt (μg) of glycolipid per well	Serum no.	Cross-absorption[a]	Serum dilution
1	11907-300	12.5	1299	−	1/600
2	14141-1395	7.5	1093	+ (1)	1/300
3	128 Germany	2.5	1003	+ (2)	1/800
4	13528-1079	7.5	1297	−	1/300
5	25546-759	12.5	1155	−	1/300
6	34540 Wales	7.5	451	−	1/600
7	P49	1.0	1094	+ (12)	1/300
8	SJB2	7.5	1240	−	1/300
9	Watson	5.0	1130	−	1/300
10	IIIa TMC 1461	12.5	1235	+ (11)	1/300
11	IIIb TMC 1462	12.5	1293	−	1/300
12	Wood Duck	12.5	1301	+ (13)	1/400
13	TMC 1466	12.5	1156	+ (12)	1/200
14	P39	1.0	806	+ (15)	1/300
15	Simpson	7.5	1238	+ (14)	1/300
16	Yandle	10.0	1295	+ (17)	1/300
17	P54	12.5	1908	+ (16)	1/300
18	Melnick 1158	12.5	1234	+ (2)	1/300
19	Darden	7.5	1909	−	1/300
20	TMC 1419	12.5	1239	−	1/400
21	2293	7.5	1406	+ (8)	1/200
22	10409	2.5	1161	−	1/600
23	CDC 1214	12.5	1097	−	1/400
24	12645	10.0	1098	+ (23)	1/200
25	72-888	7.5	1449	−	1/300
26	MacKenzie	2.5	1001	−	1/200
27	Lane 3081	7.5	1236	−	1/200
28	9055 Matthews	12.5	800	−	1/300
41	Bridge	7.5	1100	+ (20)	1/300
42	CDC 1198	2.5	431	−	1/300
43	Brooks	12.5	1505	−	1/300

[a] Cross-reacting antibodies were agglutinated by the serovar in parentheses, as described by Schaefer (11).

CH_3OH (2:1, vol/vol) for 10 to 15 s and heating in a 50°C water bath for 18 h (5). The $CHCl_3$-CH_3OH extracts were used directly or were treated with mild alkali (5).

ELISA. Absolute ethanol in the amounts described below was added to lipid preparations which were sonicated in a sonic bath (Ultramet III; Buehler Ltd., Evanston, Ill.) until they appeared homogeneous. A 50-μl sample of the lipid suspension was applied to flat-bottomed wells of 96-well polystyrene microtiter plates (Linbro enzyme immunoassay plates; Flow Laboratories, Inc., McLean, Va.). Plates were dried overnight, followed by a 10-min block with phosphate-buffered saline (0.09 M Na_2HPO_4, 0.02 M NaH_2PO_4, 0.01 M NaCl, pH 7.4) containing 0.1% Tween 80. Rabbit antiserum (50 μl) against homologous strains of mycobacteria (11, 14), diluted in phosphate-buffered saline–Tween 80, was added to each well. Plates were incubated for 30 min in a humid chamber at room temperature. Serum was aspirated and plates were washed four times, 5 min per wash, with phosphate-buffered saline. Goat anti-rabbit immunoglobulin G peroxidase-linked conjugate (Cooperbiomedical, Inc., Malvern, Pa.) in phosphate-buffered saline–Tween 80 (50 μl, diluted 1:1,000) was added to each well, followed by 45 min of incubation. Four additional 5-min washes with phosphate-buffered saline followed. Substrate mixture (50 μl; 20 mg of *o*-phenylenediamine dihydrochloride and 20 μl of 30% H_2O_2 in 50 ml of citrate phosphate buffer [24.3 mM citric acid, 51.4 mM Na_2HPO_4, pH 5.0]) was distributed into wells, and the

TABLE 2. Application of ELISA to unknown clinical isolates of the MAIS complex

Isolated no.	Identification according to:	
	Seroagglutination[a]	ELISA
2-20	4	—[b]
3-15	16	16
3-22	8	8, 21, or 22
3-24	8	8
3-27	8	8
3-34	43	43
3-43	9	9
3-45	8	8
4-8	6	43
4-9	43	—
4-9a	43	43
4-10	12	12
4-14	4	4
4-18	42	42
4-19	4	3
4-22	9	9
4-27	14	14
4-27a	1	1
4-29	1	1, 3, or 6
4-33	4	4
4-42	2	2 or 9
4-43	2	2
4-46	2	2
4-47	2	2
4-48	2	2 or 3
4-50	2	2
4-51	2	2
4-52	2	2
4-53	2	2
4-54	2	2
4-57	9	2
4-60	4	4 or 8
4-62	2	2 or 3
4-65	8	8
4-66	2	2
4-68	4	4
4-71	4	4
4-72	4	4
4-75	14	14
4-84	8	8
4-88	12	12
4-93	16	16
4-94	12	12
5-6	1	3
5-20	42	42
5-34	14	6, 5, or 3
5-35	6	6
5-49	6	6
5-53	4	4
5-54	4	4
5-56	25	25
6-5	25	25
6-12	8	8
6-38	9	2 or 9
6-43	8	8
6-48	4	4
6-52	4	4
7-10	8	8
7-17	8	8
8-32	8	6 or 8

[a] The same antisera were used for seroagglutination and ELISA.
[b] —, No reaction.

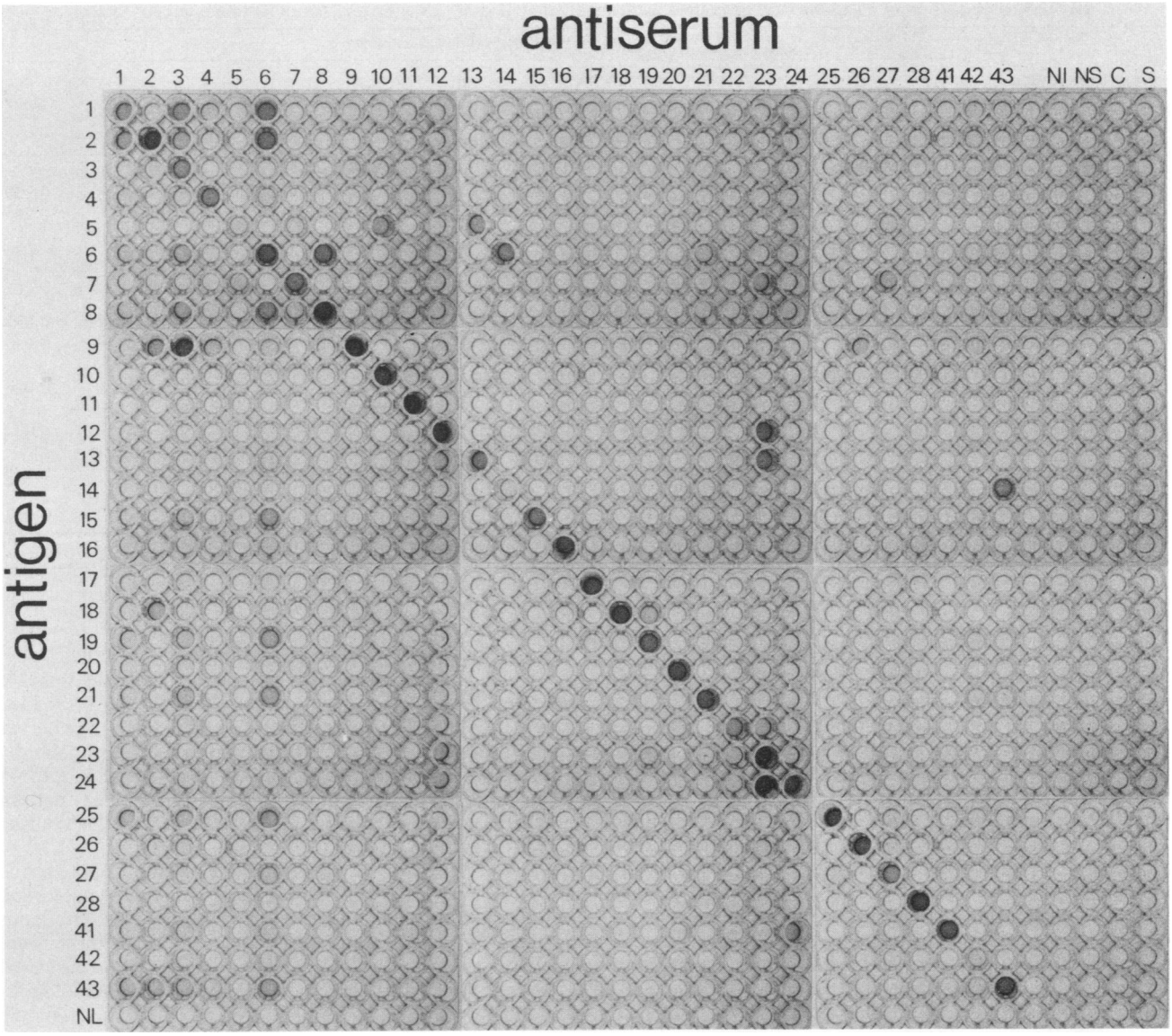

FIG. 1. Composite photograph of the reaction between the C-mycoside glycopeptidolipid antigens from MAIS complex serovars and rabbit antisera raised against the homologous bacteria. Experimental conditions are described in Table 1 and in the text. NI, Nonimmune serum; NS, no serum; C, conjugate; S, substrate; NL, no lipid.

plate was incubated in the dark for 30 min. The reaction was stopped by the addition of 50 μl of 2.5 N H_2SO_4, and the color absorption was read at 488 nm on a Micro-ELISA Reader (Dynatech Laboratories, Inc., Alexandria, Va.).

Other procedures. Methods for identification of clinical isolates by seroagglutination and TLC have been described previously (14).

RESULTS AND DISCUSSION

ELISA and glycolipid antigens of MAIS complex serovars. Strains and antisera used in this study are listed in Table 1. All were from a recently established, authenticated collection (14). Alkali treatment of total lipid extract from MAIS complex serovars resulted in degradation of glycerides, and the resulting lipid preparation contained only the serologically active glycopeptidolipids, unreactive fatty acids, and some nonsaponifiable lipids (3). Thus, alkali treatment reduced the possibility of nonspecific reactions. Throughout this study, alkali-stable whole-lipid preparations were used instead of pure glycolipid antigens, which are more difficult to derive. The amounts used in the assays shown in Fig. 1 are listed in Table 1. Subsequent work, however, led to the development of one standard antigen concentration (8.4 μg/ml) with a variable antibody dilution for purposes of identifying unknown clinical isolates. The cross-reactive patterns described below remained the same at this concentration.

The reactions shown in Fig. 1 represent one of eight complete assays in which glycolipids from the authenticated serovars were used. The combinations with good specificity or sizable cross-reactivity are demonstrated. In other assays, the absolute color development often varied. However, both specific and cross-reacting pairs always behaved consistently, indicating that cross-reactivities were absolute. Antiserum to serovar 3 reacted strongly with antigens of serovars 3 and 9 but also with antigens of serovars 1, 2, 6, 8,

TABLE 3. Application of ELISA to the glycolipid antigens of some non-MAIS complex, nontuberculous mycobacteria

Solid-phase glycolipid antigen[a]	Activity (A_{488}) of following antiserum[b]:									
	M. simiae			*M. chelonei* subsp.		*M. kansasii* (1160)	*M. xenopi* (1657)	*M. szulgai* (1409)	Nonimmune	None
	I		II (1128)	*abscessus* (315)	*chelonei* (314)					
	432	1101								
M. simiae I										
1595	**1.558**	**1.999**	0.089	0.102	0.164	0.049	0.181	0.205	0.069	0.150
7729	**1.193**	**1.999**	0.075	0.103	0.252	0.143	0.311	0.146	0.093	0.265
M. simiae II W-58	0.141	0.358	**1.419**	0.086	0.314	0.076	0.117	0.168	0.073	0.027
M. chelonei subsp.										
abscessus TMC 1543	0.263	0.158	0.097	**1.999**	**1.916**	0.087	0.179	0.174	0.046	0.031
chelonei TMC 1544	0.170	0.078	0.096	**0.415**	**0.443**	0.167	0.106	0.246	0.085	0.046
M. kansasii CAP E-17	0.116	0.062	0.122	0.046	0.064	**0.942**	0.228	0.147	0.031	0.028
M. xenopi TMC 1482	0.112	0.088	0.142	0.156	0.282	0.097	**1.045**	0.143	0.129	0.082
M. szulgai 1878-9	0.091	0.040	0.034	0.029	0.035	0.064	0.049	**1.121**	0.046	0.140
None	0.190	0.127	0.202	0.191	0.092	0.195	0.053	0.256	0.177	0.058

[a] Amount of glycolipid added to each well was 12.5 μg (strains W-58 and 1543) or 25 μg (strains 1595, 7729, 1544, CAP E-17, 1482, and 1878).
[b] Numerical designations given to the sera are shown. All serum dilutions were 1:300 except those for *M. simiae* II (1:160), *M. xenopi* (1:160), and *M. szulgai* (1:100).

15, 19, 21, 25, and 43. The glycolipid from serovar 3 did not react with antiserum to serovar 9, although the glycolipid from serovar 9 did react with antisera to serovars 2, 3, 4, 6, 26, and 42. Figure 1 and other data indicate that the antisera and glycolipids of serovars 12 and 23 are extensively cross-reactive. The reactivity patterns shown in Fig. 1 extended to other lots of antiserum and glycolipid.

ELISA based on the glycolipid antigens established new cross-reacting relationships which generally do not correspond to those established by Schaefer (12) and Meissner and Anz (10), who used whole-cell agglutination. One reason for the absence of conformity may be due to the extraction of shared glycolipid antigens, perhaps cryptic in the intact cell. Indeed, examination of published TLC profiles of the glycolipid antigens shows evidence in several instances of common, minor, presumed antigenic glycolipids (4, 14). For instance, the glycolipid pattern of serovar 3 always shows a minor component that is chromatographically indistinguishable from antigen II of serovar 9 (3, 14).

Absorbance values in these assays are dependent upon several conditions. First, sera and conjugate tend to decrease in reactivity over an extended period of time and hence diminish absorbance readings. The pH of each buffer used in washing, diluting conjugate, and preparing substrate is of critical importance; slight deviations in pH, especially that of the citrate phosphate buffer, showed marked decrease in speed and degree of color change. Slight variations in incubation periods and times of washing also have an additive effect. This problem can be compensated for by use of a set standard for each ELISA performed; the corrected absorbance for each sample is then calculatable (15). Furthermore, since similar cross-reactive patterns are apparent, this may be used for identification.

The results of a blind study in which glycolipid from clinical isolates was used as the solid-phase antigen are shown in Table 2. Of 60 isolates that had been typed by seroagglutination, 45 were correctly identified by ELISA. A further nine could be narrowed to one of two or three possibilities, but the results on the remaining six did not correspond to those from seroagglutination. In another study, 43 clinical isolates which were untypable or autoagglutinable in seroagglutination were examined, this time by both TLC and ELISA. Of the 43 samples, 21 typed identically, whereas 10 showed conflicting results. Eight remained untypable by both methods. In the case of another three samples, ELISA provided a clear identification, but no distinct TLC pattern was seen. One isolate showed a distinct TLC pattern but no ELISA response.

Thus, a feature of ELISA in the present context is the ability to allow division of mycobacteria into typable and untypable strains; the ill-defined category "spontaneous agglutination" (12) is not applicable. Although there were incidences in which results with the various procedures did not correspond, the degree of concordance and homologous reactivity was such as to indicate that ELISA in a simple format without the need for extensive glycolipid purification and with the potential for great sensitivity is another useful technique for identifying the ubiquitous MAIS complex serovars.

ELISA and the glycolipid antigens of other mycobacteria. Details of strains and sera are described in the footnotes to Table 3. One set of figures from a large body of results, all qualitatively similar, on the application of ELISA to the intact (non-alkali-treated) lipid antigens of *M. kansasii*, *M. simiae* I and II, *M. xenopi*, *M. chelonei* subspp. *abscessus* and *chelonei*, *M. szulgai*, and *M. fortuitum* biovariant *peregrinum* are shown in Table 3. The extent of the homologous reactions are good, especially in the cases of *M. kansasii*, *M. xenopi*, and *M. szulgai*. The *M. simiae* I and II combinations are also satisfactory and support evidence for two distinct serotypes. The results on the two *M. chelonei* subspecies support recent conclusions, based on seroagglutination, absorption studies, chemical analyses, and detailed chromatography of the glycolipid antigens, that these are synonymous (13). The lipids of *M. fortuitum* biovariant *fortuitum* and *M. marinum* did not react with homologous antiserum which was known to be active in seroagglutination or with antisera to other species, and thus their overt surface immunogens

TABLE 4. Application of ELISA to the deacetylated glycolipid antigens of some non-MAIS complex, nontuberculous mycobacteria

Solid-phase glycolipid antigen[a]	Activity (A_{488}) of following antiserum[b]:							
	M. simiae			*M. chelonei* subsp.		*M. fortuitum* biovar. *peregrinum* (316)	Nonimmune	None
	I		II	*abscessus*	*chelonei*			
	1101	432						
M. simiae I								
7729	**1.999**	**0.628**	0.035	0.013	0.037	0.119	0.044	0.062
1595	**1.999**	**1.254**	0.027	0.016	0.105	0.115	0.019	0.015
M. simiae II	0.084	0.069	**1.061**	0.015	0.011	0.017	0.009	0.006
M. chelonei subsp.								
abscessus	0.046	0.189	0.074	**1.180**	1.874	1.999	0.008	0.008
chelonei	0.151	0.147	0.063	0.320	**1.054**	1.491	0.013	0.015
M. fortuitum biovar *peregrinum* TMC 1547	0.443	0.444	0.134	0.194	0.537	**1.557**	0.038	0.655
None	0.060	0.145	0.036	0.014	0.028	—[c]	0.029	0.013

[a] Amount of glycolipid added to each well was 6.25 μg for all isolates except *M. simiae* I 7729, *M. chelonei*, and *M. peregrinum* (1.25 μg).
[b] Numerical designations given to the sera are shown. All serum dilutions were 1:600 except that for anti-*M. fortuitum* biovar *peregrinum* (1:960).
[c] —, No activity detected.

might not be glycolipid. The glycolipids of *M. fortuitum* biovariant *peregrinum* were antigenic but showed considerable cross-reactivity with glycolipids from heterologous species (data not shown).

To better define the antigens responsible for these activities, we treated $CHCl_3$-CH_3OH-extracted lipids with alkali and chromatographed the products in the organic phase on thin-layer plates in $CHCl_3$-CH_3OH-H_2O (65:25:4, vol/vol/vol) and sprayed them with orcinol-sulfuric acid. The presence of alkali-stable lipids on the plates with a distinctive yellow-gold color is indicative of glycopeptidolipids (3, 5). On the other hand, the disappearance of the distinctive lipids evident in untreated extracts suggests alkali-labile, presumably lipooligosaccharide, antigens (4, 8). According to this criterion, *M. simiae* I and II, *M. chelonei* subspp. *abscessus* and *chelonei*, and *M. fortuitum* biovariant *peregrinum* contain alkali-stable glycopeptidolipid antigens; as indicated above, the glycopeptidolipid antigens of *M. chelonei* subspp. *abscessus* and *chelonei* are identical (13). When the products from the alkali treatment step were examined by ELISA, it was found that they had retained full activity (Table 4). Thus, the combination of alkalinolysis and ELISA, so suitable for MAIS complex serovars, is also applicable to *M. chelonei*, *M. simiae* I and II, and, with reservations, *M. fortuitum* biovariant *peregrinum*. The problem of extensive cross-reactivity which characterizes the glycolipid antigens and antiserum of *M. fortuitum* biovariant *peregrinum* were only partially overcome by extensive dilution (Table 4).

In contrast to these results, alkali treatment of the lipid extracts from *M. kansasii*, *M. xenopi*, and *M. szulgai* destroyed their characteristic spectra on thin-layer plates, and the specific and nonspecific serological activities on ELISA were eliminated. Thus, *M. xenopi* and *M. szulgai*, like *M. kansasii*, apparently contain lipooligosaccharide antigens.

According to the criterion defined above, the *M. simiae* and *M. chelonei* complexes and *M. fortuitum* biovariant *peregrinum*, like the MAIS complex, contain glycopeptidolipid antigens. The possibility of cross-reactivity with members of the MAIS complex was therefore explored by ELISA (data not shown). *M. simiae* II antiserum cross-reacted only with serovar 18, whereas the glycolipid from *M. simiae* II cross-reacted with antisera to serovars 1, 2, 3, 6, and 18; antisera to serovars 1, 3, and 6 were already shown to be highly reactive against several MAIS complex serovars. The observation of cross-reactivity between *M. simiae* II and serovar 18 may be significant since Boisvert and Truffort (1) noted, among other similarities, that *M. simiae* II agglutinates serovar 18 antiserum. The *M. chelonei* subsp. *abscessus* antiserum cross-reacted slightly with the glycolipid from serovar 1, whereas *M. chelonei* subsp. *chelonei* antiserum showed no cross-reactivity against any of the MAIS complex serovars. Conversely, the glycolipid from *M. chelonei* subspp. *abscessus* and *chelonei* showed cross-reactivity only with the highly reactive antisera to MAIS complex serovars 1, 3, and 6.

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