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Implications of Antibodies to Pyruvylated Glucose in Healthy Populations for Mycobacterioses and Other Infectious Diseases

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Members of the *Mycobacterium avium-Mycobacterium intracellulare* (MAI) complex are typeable because each serovar is characterized by its own specific antigenic glycolipid. By means of an enzyme-linked immunosorbent assay, we studied serum specimens obtained from 148 healthy college students for antibodies to these glycopeptidolipids. Ninety-two (61.5%) of the serum specimens were positive to the specific glycolipid antigen from MAI serovar 8. In a study of a pediatric population, antibodies appeared to develop during adolescence. Individuals with overt mycobacterial disease had a significantly lower incidence (tuberculosis patients, 34.5%; leprosy patients, 25%). We found a lower incidence of positive results in a survey of 96 Japanese serum specimens (29.1%), but the results from a survey of sera obtained from Bombay, India, indicated a large degree of reactivity (55.5%). Antibodies to other MAI serovars (serovars 2, 4, and 11) were not found, except antibodies to MAI serovar 21 were seen in the same individuals with antibodies to serovar 8. The dominant epitope of the MAI serovar 8-specific glycopeptidolipid is a terminal pyruvylated 3-*O*-methylglucose residue [4,6-(1′-carboxyethylidene)-3-*O*-methyl-α-D-glucopyranosyl] unit, whereas that of the MAI serovar 21 has the same terminal pyruvylated glucose devoid of the 3-methoxy group. Thus the antibodies appear specific for the pyruvylated glucose. It is unclear whether the high prevalence of antibodies to these epitopes reflects a high incidence of subclinical colonization or infection with certain MAI serovars or whether they are acquired through contact with some other related antigen source.

Serology, widely used in medicine to detect infection and survey the experience of a population with infectious agents, has been applied to such mycobacterial diseases as tuberculosis and leprosy. However, few investigators have examined antibodies to the 28 serovars of the *Mycobacterium avium-Mycobacterium intracellulare* (MAI) complex. The most notable of these studies was a survey of healthy individuals as controls in which whole *M. avium* cells were used as a solid-phase antigen in a study of acquired immune deficiency syndrome and hairy-cell leukemia patients (42).

MAI complex serovars appear widely distributed in the United States (12), being frequently found in water, soil, air, and dust samples (5, 15). MAI serovar 8 of the complex is the most common MAI organism isolated from patients with nontuberculous mycobacteria (14, 26). Although serovar 4 is more commonly isolated from individuals with underlying acquired immune deficiency syndrome (14, 23, 27), serovar 8 is also commonly found (14, 23, 27). Members of the MAI complex are easily differentiated because each serovar is characterized by its own set of immunogenic glycopeptidolipids (GPLs), often called polar C-mycosides (3). These are composed of a lipopeptidyl-*O*-(3,4-di-*O*-methyl-α-L-rhamnopyranoside) core which is common to all serovars and a variable oligosaccharide haptenic unit which is attached to the threonine substituent of the lipopeptide. The attached oligosaccharide moiety is composed of a common internal disaccharide residue [L-rhamnopyranosyl-(α1→2)-6-deoxytalose] and a distal segment which is unique to the individual serovar and accounts for the serologic specificity (3).

Preliminary studies designed to search for mycobacterial antibodies in Crohn's disease found that both patients and controls often showed reactivity to the GPL of MAI serovar 8. Accordingly, a population survey was conducted to find the frequency of antibodies to the GPL of MAI serovar 8 in several healthy and diseased populations.

MATERIALS AND METHODS

Antigens. Preparations of specific GPLs followed previously described procedures of this laboratory (2, 4). The known structures of the GPLs used are shown in Fig. 1 (4, 30).

A semisynthetic serovar 8 neoantigen containing the synthetic 4,6-*O*-(1′-carboxyethylidene)-3-*O*-methyl-β-D-glucopyranose attached to bovine serum albumin via an allyl linkage was kindly supplied by Gerald O. Aspinall of York University, Downsview, Ontario, Canada (Fig. 1) (30).

Antibodies. Antibodies to several GPLs of MAI serovars were raised in New Zealand White rabbits (36). Horseradish peroxidase conjugates of goat anti-rabbit immunoglobulin G (heavy- and light-chain specific) and anti-human immunoglobulin G (Fc specific) were purchased from Organon Teknika, Malvern, Pa.

Study populations. Serum samples were obtained from 148 veterinary medical students attending Colorado State University, Fort Collins; 29 children attending a pediatric clinic and 23 healthy medical personnel from the Rhode Island Hospital, Providence; 20 tuberculous patients from a tuberculosis clinic at Roger Williams General Hospital, Providence, R.I., and 9 tuberculous patients from Leahi Hospital, Honolulu, Hawaii (samples supplied by J. Douglas); 24 leprosy patients (samples obtained from R. Gelber; 19 Crohn's disease patients attending the Gastrointestinal Clinic at Rhode Island Hospital; 96 healthy Japanese subjects (samples supplied by Yoichi Haga); and 27 healthy Indian subjects from Bombay, India (samples supplied by D. Chatterjee).

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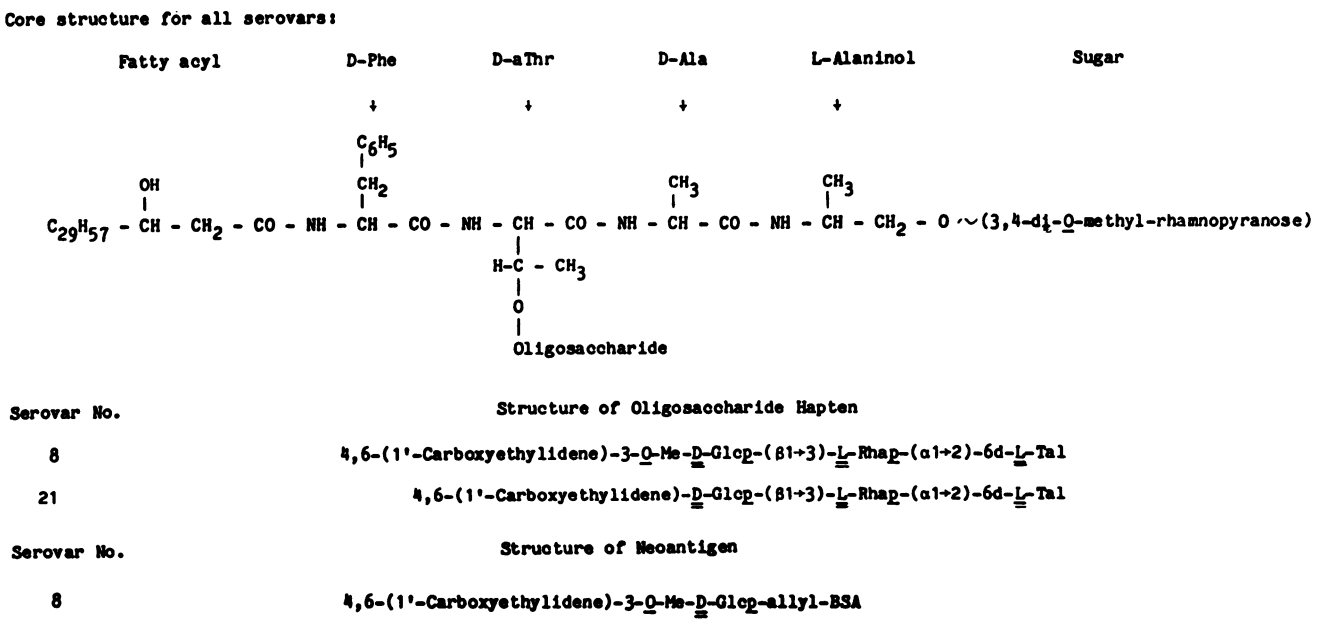

FIG. 1. Structures of the GPL antigens and neoantigens employed in this study. Abbreviations: Phe, phenylalanine; aThr, allothreonine; Ala, alanine; Me, methyl; Glcp, glucopyranose; Rhap, rhamnopyranose; 6d-L-Tal, 6-deoxy-L-talitol; BSA, bovine serum albumin.

Serum specimens of 23 of the veterinary students were examined concurrently in the enzyme-linked immunosorbent assay (ELISA) against the GPLs of MAI serovars 2, 4, 8, 11, and 21.

All sera were stored at −20°C until use (1 day to 4 years).

ELISA. The ELISA was performed by using a modification of the method of Engvall and Perlmann (11). Briefly, antigens were suspended in absolute alcohol (10 μg/ml), sonicated, and applied in 50-μl samples to a 96-well polystyrene, U-bottom microdilution plate (Dynatech Laboratories, Inc., Alexandria, Va.). After drying overnight, the plates were blocked for 1 h with 0.1 M phosphate-buffered saline containing 0.1% Tween 80 (Fisher Scientific, Fairlawn, N.J.). After removing the phosphate-buffered saline-Tween block, 50 μl of serum in a 1:100 dilution was applied for 1 h at room temperature. (Rabbit anti-*M. avium* serovar 8 was applied at a 1:160 dilution.) After a washing, goat anti-human or anti-rabbit immunoglobulin G, diluted 1:1,000, was added and incubated for 1 h. The plates were again washed, and 50 μl of *O*-phenyldiamine (Sigma Chemical Co., St. Louis, Mo.) in citrate-phosphate buffer (40 μg/100 ml) with 30% hydrogen peroxide (40 μl) was added. The plates were incubated in the dark for 20 min before the reaction was terminated by the addition of 2.5 N sulfuric acid. The optical density at 490 nm (OD_{490}) was measured with an automatic ELISA reader (Dynatech). Run concurrently were appropriate controls, including known positive and negative sera, wells without antigen, and wells without serum. In studies using rabbit antisera, normal rabbit serum was used as a control. An OD of at least 0.2 U greater than those of both the antigen- and antibody-free wells was considered positive. All samples were performed in duplicate or triplicate.

Several modifications of the ELISA protocol were employed when the semisynthetic MAI serovar 8 antigen was used. This neoantigen was applied to plates in a carbonate-bicarbonate buffer (pH 9.6) at 10, 1, 0.1, and 0.01 μg/ml. Wells containing only bovine serum albumin in similar concentrations were also used. The antigens were incubated overnight on plates at 37°C in a humid environment. Blocking was carried out by using phosphate-buffered saline containing 2% (wt/vol) polyvinylpyrrolidone-40 (Sigma). The remaining steps of the assay were performed as described above.

Chi-square analysis was used to compare the results of the different populations.

RESULTS

Initially a group of eight healthy laboratory personnel and a similar number of patients with Crohn's disease were tested during a study of the reaction of Crohn's disease patient sera to mycobacterial antigens. Among the antigen preparations examined was a lipid extract of MAI serovar 8. Serum specimens from seven of eight controls and six of eight patients with Crohn's disease reacted at serum dilutions of 1:100 to the unfractionated lipid antigen (10 μg/ml). Similar results were obtained when the same sera were tested against the specific GPL antigen of serovar 8. Two positive serum specimens were tested at various dilutions with differing specific GPL antigen concentrations. Serum diluted at 1:100 detected as little as 1 μg of the native glycolipid antigen of MAI serovar 8 per ml, and dilutions of 1:1,000 detected 10 μg of this same antigen per ml. Three serum specimens that had reacted to the native antigen from MAI serovar 8 reacted to as little as 0.1 μg of the serovar 8 neoantigen per ml at a dilution of 1:100; two serum specimens also reacted at a dilution of 1:1,000. None showed any response to the bovine serum albumin carrier protein alone.

Because the preceding results suggested that a widespread antibody directed against the epitope of the GPL of MAI serovar 8, 4,6-*O*-(1'-carboxyethylidene)-3-*O*-methyl-β-D-glycopyranosyl, existed in healthy individuals, we examined larger population groups, including 148 consecutively numbered serum specimens obtained previously from veterinary students (Table 1). At a 1:100 dilution, 92 (61.5%) of the serum specimens examined had positive responses, as determined by OD_{490} values; 18% had readings of 0.2 to 0.5, 24% had readings of 0.5 to 1.0, 9% had readings of 1 to 1.5,

TABLE 1. Results of serological survey for antibodies to MAI complex serover 8

Source of serum	*n*	No. positive[a]	% positive	*P* value[b]
CSU veterinary students	148	92	61.5	
Rhode Island subjects	23	12	52.2	NS
Pediatric patients of age (yr):	29	7	24.1	<0.001
Less than 1	7	1	14.2	<0.02
1–5	10	1	10.0	<0.01
6–10	5	2	40.0	NS
11–15	5	1	20.0	<0.10
16–20	2	2	100	NS
Tuberculosis patients	29	10	34.5	<0.02
Leprosy patients	24	6	25.0	<0.001
Crohn's disease patients	19	12	63.2	NS
Japanese subjects	96	27	28.1	<0.001
Indian subjects (Bombay)	27	15	55.5	NS

[a] OD more than 0.2 U greater than those of antigen-free and antibody-free wells.

[b] All results compared with those of Colorado State University veterinary students. NS, Not significant.

6% had readings of 1.5 to 2.0, and 5% had readings above 2.0. Although titers were not usually determined for the sera in the analysis described above, one sample had an OD_{490} value greater than 0.2 even when the serum was diluted 1:20,000.

Twenty-three serum specimens from randomly selected healthy veterinary students were examined for antibodies to type-specific GPL of MAI serovars 2, 4, and 11 and serovar 21, which is similar to serovar 8 but lacks the 3-*O*-methyl group (Fig. 1). Reactions were considered positive if the OD_{490} was more than 0.20 U greater than those of antigen-free and antibody-free wells. Antibodies were not found to serovar 2, 4, or 11 in this population, but 18 of 23 (78.2%) samples reacted with serovar 21. Sera reacting to serovar 21 also reacted to the serovar 8 GPL (19 of 23 samples; 82.6% concurrence) (Fig. 2). The OD_{490} readings for the two serovars were quite similar.

Since most of the sera were from individuals who resided in the western United States and who, as veterinary students, may have had contact with animals as potential reservoirs of MAI complex, we examined 23 serum specimens obtained from residents of the eastern United States. Of the 23 serum specimens, 12 (52%) were positive.

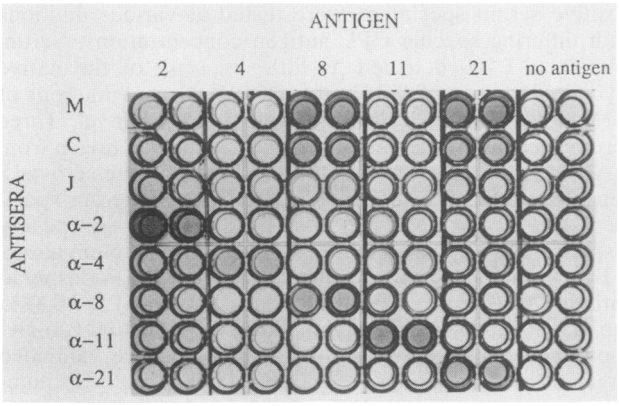

FIG. 2. Microdilution plates showing the reactions of three healthy control serum specimens and rabbit antisera (α) to MAI serovars 2, 4, 8, 11, and 21 to the GPLs of serovars 2, 4, 8, 11, and 21. M, C, and J, Healthy control sera. (All sera tested at 1:100 except for anti-serovar 8, which tested at 1:160.)

To determine the possible effect of aging on the incidence of antibody to the MAI serovar 8, we examined 29 children and adolescents (Table 1). Positive ELISA responses to the serovar 8 antigen were found in 1 of 7 patients (14%) under 1 year of age, 1 of 10 patients (10%) between 1 and 5 years, 2 of 5 patients (40%) between ages 5 and 10, 1 of 5 patients (20%) between the ages of 10 and 15, and in both of the adolescents.

Two groups of patients with mycobacterial disease (tuberculosis and leprosy) were also examined (Table 1). Only 10 of 29 tuberculous serum specimens (34.5%) and 6 of 24 (25%) leprosy serum specimens were positive. Serum specimens from 19 patients with Crohn's disease were examined, and 12 (63.2%) were positive. The majority of patients with leprosy or tuberculosis were foreign born (either Southeast Asians or Latin Americans), but all patients with Crohn's disease were born in the United States.

We also studied sera obtained from healthy subjects in Japan and India (Table 1). The Japanese serum specimens showed a low incidence of reactivity (27 of 96; 28.1%), while 15 of the 27 (55.5%) Indian serum specimens were positive, yielding results similar to those found in the United States population. The ELISA OD_{490} values for the Japanese sera were also low, with only 5% having readings greater than 1.0.

DISCUSSION

This study suggests that some populations have been exposed to the MAI serovar 8 or to an antigen shared with that organism. Since other MAI complex organisms are also abundant (9) but antibodies were not found to these serovars, another source of sensitization must be considered. Common epitopes are shared between mycobacteria and other antigens (35).

Pyruvic acid, linked as a cyclic acetal to the glycosyl residue of the GPL of MAI serovar 8, is a common component of extracellular bacterial polysaccharides (24, 34) and in seaweed (16) and may be an important part of the immunodominant region (13). Extracellular polysaccharides of *Xanthomonas campestris* (18) and some of the *Rhizobium* species (19) have a pyruvic acid linked as a ketal to a glycosyl residue. Because of its unique rheologic properties (20), the polysaccharide xanthan gum is produced industrially (10) for use in many foods (Kelco, Div. Merck & Co., Inc., *Xanthan Gum/Keltrol/Kelzan: a Natural Biopolysaccharide for Scientific Water Control*, 2nd ed., 1987). The widespread use of this bacterial product in foods might account for the MAI serovar 8 sensitization. However, when a number of subjects were examined for antibodies to xanthan gum, no correlation was found to the MAI serovar 8 antibodies. Sensitization could also be caused by other frequently encountered bacteria, such as *Klebsiella* species, which can contain similar antigenic configurations (8). However, this would not explain the paucity of reactions in sera from our patients with mycobacterial diseases.

Sensitization to some common pyruvylated glycosyl residue in the environment is a likely cause of the widespread antibody response to the MAI serovar 8 GPL. There are probably many environmental antigens with similar configurations which have not yet been identified. Alternatively, the antibody response which we found in the American and Indian populations might reflect previous exposure to either MAI serovar 8 or 21. Although a larger study is required, the pediatric data suggest that the antibody response is probably acquired during adolescence. Asymptomatic colonization of

the nasopharyngeal and/or intestinal surface by organisms of the MAI complex may be common (1, 32, 37). Since these organisms, so widely distributed in soil and water (5, 12), could colonize following ingestion, bacterial persistence or repeated ingestion could induce an antibody response.

MAI serovar 8 is a common environmental organism isolated from water (21, 22, 33), animals (28, 33), birds (28), insects (33), sphagnum moss (22), and dust (28). Isolation of MAI serovar 8 from patients occurs commonly (26, 41), with an increasing incidence (28, 41). This organism, frequently encountered in patients with acquired immune deficiency syndrome (14, 23, 27), has been shown in vitro to be readily taken up by intestinal mucosal cells (25).

Significant numbers of MAI complex organisms have been recovered from water samples collected in various aquatic environments in the eastern United States (12) and have been found in soil samples obtained along flood plains of eastern rivers (5). The number of MAI complex organisms recovered correlated with low-pH soils. Chapman and Bernard (7) also commented on the tolerance of the MAI complex organisms to acid conditions. In the studies of Falkingham et al. (12), although fewer than half of the isolates were typeable, serovar 8 was found most frequently. Kazda has shown that recovery of this serovar (serotype Davis) was highest in acidic waters (21) and that growth stimulation occurred in sphagnum, a moss found in acidic bogs (22). Could the high incidence of serologic reactivity to this serovar reflect an increase in this environmental bacteria consequent to the gradual acidification of our environment by acid rain?

Other MAI complex organisms are encountered in patients and the environment (14), but antibodies to some of these other common serovars were not detected. Antibodies to serovar 21, a rather uncommon clinical isolate, were detected but only in those individuals who were also positive for the serovar 8 GPL. The GPL of serovar 21 differs from that of serovar 8 only in the detection of an *O*-methyl group in the C-3 position of the glucopyranose molecule (Fig. 1). Since the two proximal sugars of serovar 8, L-rhamnose and 6-deoxytalose, are similar in all MAI GPLs (4), it is unlikely the antigenic portion resides in this part of the disaccharide; patient sera likely recognized an epitope on the terminal pyruvylated monosaccharide. The presence or absence of an *O*-methyl group in the C-3 position of glucopyranose does not appear to make a difference in antigenic recognition by humans, but rabbits can usually detect the configuration (Fig. 2) (30). That the terminal pyruvylated glucopyranosyl moiety is the antigenic portion is also supported by experiments using the synthetic serovar 8 neoantigen consisting of only the terminal pyruvylated monosaccharide, which readily reacted to sera positive for MAI serovar 8 GPL.

It is unclear why the incidence of MAI serovar 8 antibodies was lower in patients with mycobacterial disease. Could the presence of one mycobacterial infection suppress infection with another mycobacterium? Could exposure to a relatively nonpathogenic mycobacterium protect against such serious mycobacterial diseases as leprosy or tuberculosis (6, 29)? Since the patients in this study with mycobacterial diseases were not born in the United States but came from Southeast Asia or Latin America, we considered whether geographic factors affected the response. The Japanese serum specimens showed a low rate of reactivity, whereas the results for Indian sera closely paralleled the American data. Sera from patients with Crohn's disease, on the other hand, showed OD_{490} values at the same level as those of the healthy veterinary students. Since the Crohn's disease patients were all American born, it was not surprising that their antibody reactivity resembled that of the healthy control group.

We are unaware of Japanese data on environmental isolations of the various MAI serovars, but some studies suggest a low isolation rate (31, 38). However, this may be more of a methodological artifact (17). Similarly, we could not find published studies on the isolation of MAI serovars from the Indian subcontinent, but data from Madras in South India from trials with a skin test (purified protein derivative-B) derived from *M. intracellulare* (Battey) strongly suggested that the population had an early and strong contact with these nontuberculous mycobacteria (39).

We can only speculate on the significance of antibodies to MAI serovar 8 or 21 in some populations. We do not know whether the antibody reflects previous experience with one or both of these serovars or whether some other common cross-reacting environmental antigen is involved. Studies on the environmental isolation rate of MAI serovars in this country and in other areas of the world are needed. If future studies show widespread contamination with one or both of these serovars, we will question whether the proliferation of the serovars is a consequence of the acidification of our environs with acid rain.

Furthermore, the widespread occurrence of antibodies to MAI serovars 8 and 21 casts doubt on the probability that it will be possible to develop reliable serologic diagnostic tests of infection with these common environmental mycobacteria. Similar conclusions were drawn by Wayne et al. (40).

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