Serodiagnostic Contributions of Antibody Titers against Mycobacterial Lipid Antigens in *Mycobacterium avium* Complex Pulmonary Disease

Tomoyasu Nishimura,1,3 Naoki Hasegawa,1 Yukiko Fujita,1 Ikuya Yano,2 and Akitoshi Ishizaka1

1Division of Pulmonary Medicine, Department of Medicine, Keio University School of Medicine, Shinjuku-ku, and 2Japan BCG Central Laboratory, Kiyose-shi, Tokyo, and 3Department of Internal Medicine, Sano kohsei General Hospital, Sano-shi, Tochigi, Japan

**Background.** Although the incidence of pulmonary tuberculosis is decreasing, the number of immunocompetent patients with *Mycobacterium avium* complex (MAC) pulmonary disease is steadily increasing. Therefore, albeit not contagious, MAC pulmonary disease needs to be diagnosed rapidly and accurately. We examined the serodiagnostic contributions of serum immunoglobulin G antibody titers against the species-specific and -common mycobacterial lipid antigens in the diagnosis of MAC pulmonary disease.

**Methods.** Serum samples were obtained from 65 patients with MAC pulmonary disease, 15 patients with suspected MAC disease, 25 patients with pulmonary tuberculosis, 10 patients with *Mycobacterium kansasii* disease, and 100 healthy volunteers (control subjects). We measured the serum immunoglobulin G antibody titers against trehalose monomycolate (TMM-M) and apolar-glycopeptidolipid (GPL), lipid antigens extracted from MAC.

**Results.** In patients with MAC pulmonary disease, the antibody titers against TMM-M and apolar-GPL were significantly higher than those in the other patient groups or in the control subjects. By receiver operator characteristic curve analysis, an optical density of 0.27, corresponding to the optimal cutoff antibody titer against TMM-M, was associated with a sensitivity of 89.2% and a specificity of 97.0%, and an optical density of 0.33, corresponding to the optimal cutoff antibody titer against apolar-GPL, was associated with a sensitivity of 89.2% and a specificity of 94.0%.

**Conclusions.** Measurements of antibody titers against TMM-M and apolar-GPL would be useful in the diagnosis of MAC pulmonary disease and in the differential diagnosis of mycobacterial pulmonary disease.

The unique clinical manifestations of pulmonary infections due to *Mycobacterium avium* complex (MAC), an opportunistic pathogen widely distributed in soil and water worldwide, have recently drawn attention [1, 2]. With an increasing number of immunocompetent patients presenting with MAC pulmonary disease, an accurate and early diagnosis has become particularly important. However, because of the radiological similarity between MAC disease and other mycobacterial pulmonary disease, such as pulmonary tuberculosis or *Mycobacterium kansasii* disease, an accurate diagnosis might be challenging. In some cases, the disease presentation does not fulfill the diagnostic criteria of either the American Thoracic Society or the Infectious Diseases Society of America guidelines, although it is consistent with specific clinical and radiological characteristics of MAC pulmonary disease. Furthermore, a clear distinction between MAC pulmonary disease and pulmonary tuberculosis is essential, and new diagnostic methods are needed to increase the diagnostic accuracy, with a view to choosing the most effective treatment.

Several recent studies have shown the usefulness of serodiagnosis of MAC pulmonary disease. Kitada et al [3–6] used the measurements of serum immunoglobulin (Ig) A antibody titers against core-glycopeptidolipid (GPL). Fujita et al [7] reported that the humoral immune responses measured by multiple-antigen en-
zyme-linked immunosorbent assay (ELISA), using (a) mycobacterial lipid antigens, phosphatidylinositol dimannoside (PL-2), trehalose 6,6'-dimycolate, and trehalose monomycolate from *Mycobacterium bovis* bacillus Calmette-Guérin and (b) trehalose monomycolate (TMM-M) and core-GPL from MAC, might reliably distinguish healthy control subjects from patients with pulmonary tuberculosis or MAC disease.

In the present study, we purified apolar-GPL, a species-specific and serotype-common antigen, from heat-killed cells of MAC, which possesses 3,4-di O-methyl rhamnose and 6-deoxytalose as a MAC common carbohydrate epitope. Matsunaga et al [8] observed an IgG response to apolar-GPL in MAC-infected guinea pigs. We report here the contribution of serum IgG antibody titers against TMM-M and apolar-GPL in the diagnosis of MAC pulmonary disease versus pulmonary disease due to *M. kansasii* or *Mycobacterium tuberculosis*.

**METHODS**

**Study groups.** The study groups (Table 1) included 65 patients with MAC pulmonary disease, 15 patients with suspected MAC disease, 25 patients with pulmonary tuberculosis, and 10 patients with *M. kansasii* pulmonary disease who were admitted to the Keio University hospital from October 2002 through December 2006 and 100 healthy volunteers. After the patients and healthy volunteers had agreed to participate in the study and had granted informed consent, blood specimens were collected. Patients with a history of tuberculosis or human immunodeficiency virus (HIV) infection or who had received chemotherapy for malignant disorders or treatment with prednisone at a dosage of ≥10 mg/day for >1 month were excluded from the study. The patients were not treated for mycobacterial disease at the time of collection of the serum samples.

The diagnoses of MAC and *M. kansasii* pulmonary disease were based on the guidelines issued by the American Thoracic Society [1]. Cultures were performed using the Bactec MGIT 960 Mycobacterial Detection System (Becton Dickinson Microbiology Systems), including ≥1 egg-based solid slant. The colonies formed on each culture were identified using an Accuprobe culture confirmation kit (Gen-Probe), the Amplicor polymerase chain reaction assay (Roche Diagnostic System), and the DNA-DNA hybridization method (Kyokuto Pharmaceutical). The 65 organisms isolated in specimens obtained from patients with MAC pulmonary disease included *M. avium* (62) and *Mycobacterium intracellulare* (3). Patients who fulfilled the clinical and radiographic but not the microbiological criteria formulated in the American Thoracic Society guidelines [1] were classified as having suspected MAC pulmonary disease.

The healthy volunteers were health care workers of the Keio University hospital who had no history of mycobacterial disease and agreed to participate in the control group. This study was in compliance with the Declaration of Helsinki and was approved by the institutional ethics review committee for human research of the Keio University School of Medicine and hospital.

**Antigens.** TMM-M, apolar-GPL, and PL-2, a mycobacterial common antigen, were isolated as described in a previous report [7]. Briefly, the lipids were extracted from the packed cells with a chloroform–methanol (vol/vol, 2:1) mixture, and the solvent was evaporated with a rotary evaporator. The lipids were separated by solvent fractionation into acetone soluble, methanol-soluble, or tetrahydrofuran-soluble fractions and were further separated by thin-layer chromatography on silica gel Uniplates (Analtech) with a solvent system of chloroform-methanol-water (vol/vol/vol, 65:25:4 or 90:10:1) to isolate the lipid antigens. PL-2, TMM-M, and apolar-GPL were identified by matrix-assisted laser desorption ionization–time of flight mass spectrometry. Each component, such as sugars or fatty acids, was analyzed by gas chromatography and/or mass spectrometry. On the basis of preliminary tests, using ELISA of the mycobacterial lipid antigens (data not shown), we selected PL-2, TMM-M, and apolar-GPL for the diagnosis of MAC disease.

**ELISA microplate system for titers of IgG antibody against PL-2, TMM-M, and apolar-GPL.** IgG antibody titers against PL-2, TMM-M, and apolar-GPL, determined by an ELISA microplate system, were measured as described in a previous report [7]. TMM-M was dissolved in n-hexane (0.4 μg of antigen in 50 μL of n-hexane per well), and PL-2 and apolar-GPL were dissolved in an n-hexane–ethanol mixture (vol/vol, 9:1; 0.2 μg of antigen in 50 μL of n-hexane–ethanol mixture per well).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patients with MAC disease (n = 65)</th>
<th>Patients with suspected MAC disease (n = 15)</th>
<th>Patients with pulmonary tuberculosis (n = 25)</th>
<th>Patients with <em>Mycobacterium kansasii</em> disease (n = 10)</th>
<th>Healthy control subjects (n = 100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>63.2 ± 14.5</td>
<td>65.7 ± 8.1</td>
<td>34.2 ± 17.9a</td>
<td>48.8 ± 13.4</td>
<td>37.2 ± 10.7a</td>
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<tr>
<td>Male:female sex ratio</td>
<td>14:51</td>
<td>6:9</td>
<td>16:9a</td>
<td>10:0b</td>
<td>36:62</td>
</tr>
</tbody>
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**NOTE.** MAC, *Mycobacterium avium* complex; SD, standard deviation.

a Statistically significant (*P*<.05) difference, compared with patients with MAC disease.
One antigen was deposited in a polystyrene Nunc-Immunoplate microplate well (Nalge Nunc International), and the plates were allowed to dry overnight at room temperature. The plates were used immediately or were sealed in aluminum foil and stored at 4°C. With use of identical samples, no difference was observed between the results obtained with plates used immediately and the results obtained with plates stored for 1 month (data not shown). For ELISA, nonspecific binding was blocked using 150 μL/well of 0.05% Tween 20 in tris-buffered saline (TBS-T) at a pH of 7.4 adjusted with HCl, and the plate was incubated for 10 min at room temperature. The samples tested for antibodies in serum were diluted 1:201 with TBS-T. The sample diluted to 50 μL/well was added to each well, and the plate was incubated for 1 h. Peroxidase-conjugated AffiniPure F(ab')2 Fragment Goat Anti-Human IgG (H+L) (Beckman Coulter), diluted 1:30,000 in TBS-T, was used as a secondary antibody. After incubation for 1 h, SureBlue TMB 1-Component Microwell Peroxidase Substrate (Kirkegaard & Perry Laboratories) was added. The reaction was stopped with 2 N HCl, and the absorption was measured in an NPR-A4i microplate reader (Tosoh Corporation) at 450–600 nm. The plates were incubated at room temperature and, after each procedural step, were washed 3 times with TBS-T.

Statistical methods. Comparisons of data between patients and healthy volunteers were made by analysis of variance and nonparametric tests. The optimal cutoff titers for TMM-M and apolar-GPL, associated with the highest diagnostic sensitivity and specificity to detect MAC pulmonary disease, and 95% confidence intervals (CIs) were calculated by receiver operator characteristic curve analysis, with data from all patients with MAC pulmonary disease versus data from healthy controls. Potential correlations between titers of anti–TMM-M and anti–apolar-GPL antibodies were examined by Spearman’s rank method with data from patients with MAC pulmonary disease. The data were analyzed with the GraphPad Prism software package, version 4.0 (GraphPad Software). A P value <.05 was considered to be statistically significant.

RESULTS

Demographic characteristics of the study groups. The mean age of the 65 patients with MAC pulmonary disease was 63.2 years (range, 26–91 years), and the mean duration of disease (± standard deviation) was 5.1 ± 4.7 years (range, 1 month–21 years). On average, the patients with pulmonary tuberculosis and the healthy volunteers were significantly younger than were the patients with MAC pulmonary disease (Table 1). Significant differences in sex distributions were also observed between patients with MAC pulmonary disease and patients with tuberculosis and M. kansasii disease.

Distribution of anti-lipid antigen IgG antibody titers in patients and control subjects. Figure 1 contains scatter plots of the IgG antibody titers against PL-2, TMM-M, and apolar-GPL in patients with MAC pulmonary disease, suspected MAC disease, pulmonary tuberculosis, and M. kansasii pulmonary disease and in healthy volunteers. No significant difference in serum IgG antibody titers against PL-2 was observed among the patient groups; specifically, the mean (± standard deviation) optical density was 0.51 ± 0.49 in patients with MAC pulmonary disease, 0.28 ± 0.22 in patients with suspected MAC disease, 0.28 ± 0.35 in patients with pulmonary tuberculosis, and 0.33 ± 0.36 in patients with M. kansasii pulmonary disease. However, the serum IgG antibody titers against PL-2, measured by optical density, were significantly higher (P < .05) in patients with MAC pulmonary disease (0.51 ± 0.49) than in healthy volunteers (0.24 ± 0.21). The serum IgG antibody titers against TMM-M were significantly higher in patients with MAC pulmonary disease than in all other study groups; specifically, the mean optical density was 1.30 ± 0.83 in patients with MAC pulmonary disease, 0.31 ± 0.46 in patients with suspected MAC disease, 0.16 ± 0.27 in patients with pulmonary tuberculosis, 0.08 ± 0.05 in patients with pulmonary M. kansasii, and 0.09 ± 0.14 in healthy volunteers (P < .05 for each comparison between patients with MAC pulmonary disease and all other study groups). Similarly, the serum IgG antibody titers against apolar-GPL were significantly higher in the patients with MAC pulmonary disease than in all other study groups. The mean optical density in patients with MAC pulmonary disease was 1.52 ± 0.85, compared with 0.48 ± 0.74 in patients with suspected MAC disease, 0.05 ± 0.06 in patients with pulmonary tuberculosis, 0.07 ± 0.08 in patients with M. kansasii pulmonary disease, and 0.08 ± 0.12 in healthy volunteers (P < .05 for each comparison between patients with MAC pulmonary disease and all other study groups). These results indicate that the measurements of serum IgG antibody titers against TMM-M and apolar-GPL might be useful in the diagnosis of MAC pulmonary disease, as well as for the differential diagnosis of pulmonary mycobacteriosis. We found no statistical correlation between (a) serum antibody titers against TMM-M or apolar-GPL and (b) age, disease duration, or administration of prior treatment for patients with MAC pulmonary disease and healthy control subjects.

Optimal cutoff values for serum antibody titers against TMM-M and apolar-GPL. The accuracy of measurements of serum antibody titers against TMM-M and apolar-GPL and the cutoff values associated with the highest sensitivity and specificity in patients with MAC pulmonary disease were examined by receiver operator characteristic curve analysis (Fig. 2). Analysis of the serum antibody titers against TMM-M revealed an optimal cutoff optical
Figure 1. Scattergrams of immunoglobulin (Ig) G antibody titers against phosphatidylinositol dimannoside (PL-2) (A), trehalose monomycolate (TMM-M) from *Mycobacterium avium* complex (MAC) (B), and apolar-glycopeptidolipid (GPL) from MAC (C) in serum samples obtained from 65 patients with MAC pulmonary disease ("MAC"), 15 patients with suspected MAC disease ("susp. MAC"), 25 patients with pulmonary tuberculosis ("TB"), 10 patients with *Mycobacterium kansasii* pulmonary disease ("M. kansasii"), and 100 healthy volunteers ("control"). The horizontal lines show the mean IgG antibody titers for each group. Comparisons among patients and the control group were made by analysis of variance and nonparametric analysis. *P < .05.

density of 0.33, associated with a sensitivity of 89.2% (95% CI, 79.1%–95.6%) and a specificity of 94.0% (95% CI, 87.4%–97.8%).

**Correlation between titers of anti–TMM-M and anti–apolar-GPL antibodies in patients.** We found a strong positive correlation between titers of anti–TMM-M and anti–polar-GPL antibodies ($r = 0.709; P < .001$). Although most patients with MAC pulmonary disease had high titers for both antibodies, a few patients had high titers for a single antibody (Fig. 3). MAC pulmonary disease has recently been classified into 2 distinct subtypes: fibrocavitary disease and nodular-bronchiectatic disease [2]. Although only 10 of the 65 patients with MAC pulmonary disease had fibrocavitary disease, all patients with high titers for a single antibody had nodular-bronchiectatic disease.

**DISCUSSION**

To our knowledge, this was the first study to examine the serodiagnostic contributions of the MAC-specific and serotype-common lipid antigens TMM-M and apolar-GPL in patients with MAC pulmonary disease. We chose IgG antibodies because they are the most abundant type of antibodies in plasma and play an important role in humoral immunity, and we did not study IgA and IgM.

Fujita et al [7] reported that the measurements of IgG antibody titers against multiple species-specific and serotype-common lipid antigens were useful in the diagnosis of pulmonary mycobacterial disease and, in particular, that the measurements of IgG titers against TMM-M and core-GPL were more specific in the diagnosis of MAC pulmonary disease than were those against PL-2, trehalose 6,6'-dimycolate, and trehalose monomycolate from *M. bovis* bacillus Calmette-Guérin. The serodiagnostic sensitivity and specificity for the anti–core-GPL antibody for MAC pulmonary disease were 88.4% and 81.7%, respectively. In comparison, our estimated serodiagnostic sensitivity and specificity for the anti–apolar-GPL antibody for MAC pulmonary disease are higher. We found, in an earlier, limited study, that the serodiagnostic accuracy of the IgG antibody against apolar-GPL was higher than that of the IgG antibody against core-GPL in the diagnosis of MAC pulmonary disease (authors’ unpublished data). Conventional purification techniques can isolate sufficient amounts of GPL antigens, and a core-GPL antigen obtained by $\beta$-elimination of polar-GPL...
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Figure 2. Receiver operator characteristic curves constructed using the serum antibody titers against trehalose monomycolate from *Mycobacterium avium* complex (MAC) (*A*) and apolar-glycopeptidolipid from MAC (*B*) in samples obtained from all patients with MAC pulmonary disease, compared with healthy control subjects.

exhibited antigenic properties toward IgG and IgA antibodies contained in the serum samples of patients infected with MAC. Although the core-GPL antigen lacked 6-deoxytalose at the hydroxyl group of allothreonine, apolar-GPL without β-elimination was highly immunogenic. Furthermore, the latter compound displayed a unique immunosuppressive activity in animal models and in human peripheral blood mononuclear cell systems [9, 10]. Therefore, the apolar-GPL containing 6-deoxytalose appears to be a potent T-helper 1 (Th1) suppressive and Th2 potentiating immunomodulator, which is characteristic in MAC. Our observations and previous reports suggest that apolar-GPL is a more MAC-specific antigen than is core-GPL when used for serodiagnostic purposes.

We believe that the accuracy of this assay is increased by the use of both anti-TMM-M and anti-apolar-GPL antibodies, compared with the use of anti-apolar-GPL antibody only. TMM has been reported to be a potent protein kinase C activator, causing the release of tumor necrosis factor-α in lung tissues, and a key precursor in the biosynthesis of mycoloyl glycolipids, such as trehalose 6,6'-dimycolate or cell wall–bound arabinose mycolate [11]. Because there are biochemical and structural differences between TMM-M and apolar-GPL, the measurements of antibody titers against these different antigens is useful to detect the host immune response to MAC infection. Our results demonstrated that a few patients with pulmonary tuberculosis had high titers for antibody against TMM-M. Because trehalose monomycolate from *M. tuberculosis* and MAC (ie, TMM-M) share multiple subclasses of mycolic acids, including alpha-, methoxy-, and keto-mycolates in *M. tuberculosis* with alpha-, keto-, and waxester-mycolates in MAC, the partial cross-reactivity against TMM-M of serum samples from patients with tuberculosis must be taken into consideration.

An interferon-γ–releasing assay using multiple *M. tuberculosis*–specific antigens based on cell-mediated immunity has recently been developed. Although it represents great progress in the diagnosis and epidemiological evaluation of latent tuberculosis, it might not exclude the coexistence of *M. tuberculosis* and MAC infection. Although tuberculosis and MAC pulmonary diseases share similar clinical characteristics, their immunopathological etiologies seem markedly dissimilar. In particular, *M. tuberculosis* infection induces prominent Th1-type immune responses [12], whereas MAC infection suppresses them [10, 13]. Experimental observations made in vivo have shown that the effectiveness of bacillus Calmette-Guérin vaccination is limited by pre-exposure to or preinfection with MAC strains [14, 15]. Therefore, the pre-exposure to or preinfection with MAC can down-regulate the production of interferon-γ in response to *M. tuberculosis*–specific protein antigens, although they are not produced by bacillus Calmette-Guérin. From this perspective, besides the early differential serodiag-

Figure 3. Positive correlation between titers of anti–trehalose monomycolate from *Mycobacterium avium* complex (TMM-M) and anti–apolar-glycopeptidolipid (GPL) antibodies (*r* = 0.709; *P* < .001).

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nosis of MAC and tuberculosis, it is important to confirm the coexistence of infection with both species.

It has been suggested that, in patients with bronchiectasis, the respiratory tract can be infected with MAC in the absence of tissular invasion, a condition referred to as “colonization.” In an in vitro model to study the interaction of mycobacteria with the human respiratory mucosa, Middleton et al [16] observed the multiplication of MAC in discrete colonies on the respiratory mucosal surface and its penetration of submucosal glands associated with mucosal damage. Pathological and radiological observations revealed that bronchiectasis or bronchiolitis caused by MAC infection are the result of the destruction of the bronchial structure [17–20]. Patients with suspected MAC disease who fulfill the clinical and radiographic but not the microbiological criteria formulated by the American Thoracic Society and the Infectious Diseases Society of America suggest the issue of coinfection with MAC and other organisms to be examined, such as the effects of the length of disease incubation and of anti-MAC chemotherapy on the production of antibodies.

A major limitation of our study is the small number of individuals in each group. It is necessary to study larger populations at multiple sites with various clinical and geographical settings. After temporal change in titers for antibodies against TMM-M and apolar-GPL for the patients with MAC pulmonary disease, we found that, in some patients, the titers for these antibodies correlated with disease activities assessed by clinical, microbiological, and radiographic parameters. These findings suggest that serological measurements would be useful not only to predict disease activity but also to evaluate chemotherapeutic efficacy. Therefore, clinical studies to examine these objectives should be planned.

In conclusion, the measurements of antibody titers against TMM-M and apolar-GPL would be useful in the diagnosis of MAC pulmonary disease and in the differential diagnosis of mycobacterial pulmonary disease.

**Acknowledgments**

We thank the health care workers of the Keio University hospital who volunteered for this study.

**Potential conflicts of interest.** All authors: no conflicts.

**References**


