Serodiagnosis of Pulmonary Disease Due to *Mycobacterium avium* Complex with an Enzyme Immunoassay that Uses a Mixture of Glycopeptidolipid Antigens

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It is difficult to distinguish pulmonary disease due to *Mycobacterium avium* complex (MAC) from that due to other mycobacteria, such as *Mycobacterium tuberculosis* and *Mycobacterium kansasii*. We developed an enzyme immunoassay (EIA) for diagnosis of MAC pulmonary diseases that uses glycopeptidolipid (GPL) antigens specific for MAC, and we used it for diagnosis in immunocompetent patients. The mean optical densities (± standard deviation) of serum immunoglobulin G antibodies to GPLs in patients with MAC disease, MAC colonization, *M. kansasii* disease, and tuberculosis and in healthy subjects were 0.778 ± 0.784, 0.042 ± 0.035, 0.059 ± 0.035, 0.071 ± 0.035, and 0.030 ± 0.027, respectively. A significant increase in the level of anti-GPL antibodies was detected in patients with MAC disease. The level of anti-GPL antibodies reflected disease activity, because the level was decreased in culture-negative patients who had conversion of culture results. When a cutoff level of seropositivity (0.119) was defined, the sensitivity of EIA for diagnosis of MAC disease was 92.3%, and the specificity was 96.7%. Measurement of serum anti-GPL antibodies is useful for both the diagnosis of and assessment of activity in MAC disease.

*Mycobacterium avium* and *Mycobacterium intracellularare* are closely related and are commonly grouped as “*M. avium* complex” (MAC). Organisms of this complex are ubiquitous in nature and have been isolated from water, soil, plants, house dust, and other environmental sources [1]. MAC infections have become increasingly common, and MAC is the most common cause of disease in humans due to nontuberculous mycobacteria (NTM), whereas *Mycobacterium kansasii* ranks second among NTM as a cause of disease in humans. These organisms have low pathogenicity, and single positive specimens with low numbers of organisms are obtained not infrequently from individuals who do not have apparent disease. This complicates the interpretation of culture results, especially for cultures of sputum and other respiratory-tract specimens [2]. Thus, physicians frequently face diagnostic difficulties.

MAC causes a chronic, slowly progressive pulmonary infection that resembles tuberculosis (TB) in immunocompetent patients [2]. The diagnosis of lung disease caused by MAC is made on the basis of a combination of clinical, radiographic, and microbiological criteria and the exclusion of other diseases that can resemble...
the condition [3]. The diagnosis rests on recovery of the pathogen from cultures, although sputum cultures that are positive for MAC do not in themselves prove infection, because MAC may exist as saprophytes in the airway or as environmental contaminants [1]. The culture-based system of radiometry and fluorometry allows the detection of mycobacterial growth at an early stage of infection (<7 days for NTM). Limitations of the system include the inability to observe colony morphology, difficulty in recognizing mixed cultures, difficulty in recognizing overgrowth due to contamination, cost, and radioisotope disposal. The rapid identification of MAC is possible with use of DNA hybridization, nucleic-acid amplification, or high-pressure liquid chromatography [2]. The use of molecular technology has shortened the time to identification of mycobacteria from several weeks to as little as 1 day. The detection sensitivity is ~95% for smear-positive specimens, but it decreases to as low as 50%–60% for smear-negative specimens. The overall sensitivity for detection of NTM varies from 70% to 100%, with specificity of >98%. However, the inability to distinguish between live and dead organisms precludes use of nucleic-acid amplification for therapeutic monitoring [4].

The development of rapid, simple, and accurate methods to detect MAC disease is needed to control the disease. For this purpose, serodiagnosis that differentiates among TB, M. kansasii disease, and MAC disease is essential, because MAC (but not the majority of strains of M. tuberculosis and M. kansasii) is resistant to most available antituberculous drugs [1]. We have reported elsewhere that measurement of levels of serum antibodies against lipoarabinomannan [5], trehalose dimycolate/cord factor [6], and glycolipid antigen [7, 8] of tubercle bacilli is useful for diagnosis of TB. These measurements, however, cannot differentiate between TB and NTM infection, because antibodies to trehalose dimycolate and glycolipids are immunologically cross-reactive in mycobacteria [9]. In the present study, we developed an EIA for diagnosis of MAC disease that uses the glycopeptidolipids (GPLs) that are the major and specific cell-surface antigens of the MAC and Mycobacterium scrofulaceum group [9], and we conducted a study that involved immunocompetent patients, most of whom had pulmonary disease due to MAC, but not severely immunosuppressed patients with HIV infection.

**SUBJECTS, MATERIALS, AND METHODS**

**Study subjects.** Serum samples were obtained from patients with pulmonary disease due to NTM (MAC and M. kansasii), patients with pulmonary TB, individuals with MAC colonization, and healthy subjects (table 1). Isolates of mycobacteria recovered from culture were identified by biochemical analyses and DNA probes. MAC (in 78 subjects [30 were male and 48 were female]) included M. avium (in 36 subjects [12 were male and 24 were female]), M. intracellulare (in 14 subjects [4 were male and 10 were female]), and unclassified strains of MAC (in 28 subjects [14 were male and 14 were female]) by DNA probes (AccuProbe Culture Identification Tests; Gen-Probe). Unclassified MAC strains reacted with AccuProbe MAC but not with M. avium and M. intracellulare. Healthy subjects had no history of mycobacterial disease. MAC disease was diagnosed according to the criteria of the American Thoracic Society [2]. Colonization with MAC was diagnosed if there was a single positive sputum culture, especially if the culture yielded a small number of organisms, but a single positive sputum culture was not sufficient for diagnosis of MAC disease according to the criteria of the American Thoracic Society. All subjects who were tested for HIV were seronegative. Serum samples used in our study were obtained from all patients before the commencement of antimicrobial therapy. In addition, serum samples obtained from 10 patients with MAC disease were obtained sequentially before the commencement of and after the completion of chemotherapy.

Informed consent was obtained from all study subjects, and we followed the human experimentation guidelines of the US
Department of Health and Human Services (Washington, D.C.), Osaka City University Graduate School of Medicine, and Toneyama National Hospital Human Investigation Committee (Osaka, Japan).

**Preparation of GPLs from MAC.** Eleven reference strains of MAC were obtained from the American Type Culture Collection (ATCC): serotypes 1 (ATCC 15769), 4 (ATCC 35767), 6 (ATCC 35773), 7 (ATCC 35847), 8 (ATCC 35771), 9 (ATCC 35774), 12 (ATCC 35762), 13 (ATCC 35769), 14 (ATCC 35761), 16 (ATCC 13950), and 20 (ATCC 35764) [10]. Serotypes 1, 4, 6, 8, and 9 were *M. avium*, and serotypes 7, 12, 13, 14, 16, and 20 were *M. intracellularare*, as assessed by DNA probes [11]. In accordance with a method described elsewhere [9, 12], we prepared GPLs of MAC in a reproducible fashion. In brief, strains of MAC were cultured for 3 weeks at 37°C in aerated Middlebrook 7H9 broth (Difco Laboratories) supplemented with albumin dextrose catalase (Difco). After culturing, mycobacteria were autoclaved, centrifuged for 5 min at 2400 g, and lyophilized. Lyophilized cells were extracted with chloroform-methanol (2/1 vol/vol). The extracts were hydrolyzed with 0.2 N sodium hydroxide/methanol to remove alkali-labile lipids. Alkali-stable lipids were dissolved in CHCl₃ and applied to a silica-gel column (Analtech). GPLs were eluted sequentially with CH₃OH (2%–60%)/CHCl₃. GPLs were purified repeatedly by thin-layer chromatography (TLC) of silica gel G (Uniplate; Analtech) and developed with the solvent system chloroform/methanol/distilled water (30/8/1 vol/vol/vol) until a single spot was obtained.

**EIA.** GPLs derived from 11 strains of MAC were mixed and prepared at an ethanol concentration of 10 μg/mL. Flat-bottomed 96-well microtiter plates (Nunc-Immuno Plate I 96-F; Nunc Products) were coated with 0.5 μg/50 μL per well of GPL solution. The plate was dried at 4°C overnight, and then nonspecific binding sites on microplates were blocked for 30 min with PBS that contained 0.05% Tween 20 (PBS-T). Diluted serum samples (50 μL, 1:20–160 dilutions by PBS-T) in triplicate were added, followed by incubation for 1 h at 37°C. Plates were washed 4 times with PBS, then 50 μL per well of peroxidase-conjugated F(ab′)₂ fragment (1:5000 dilutions) of goat antibody against human IgG (Sigma Chemical) was added, and plates were incubated for 2 h at 37°C. Unbound labeled antibody was removed by washing. One hundred microliters of substrate reagent (o-phenylenediamine dihydrochloride, 1 mg/mL; Sigma) was added. The plates were incubated for 30 min at room temperature, and the reaction was stopped with 100 μL of 2 N H₂SO₄ solution. Plates were read at 492 nm in a microplate reader (Dainippon). To confirm accuracy and reproducibility, we performed inhibition of EIA by the addition of GPL antigens with a range of 0.005–5 μg per well, and we conducted 3 separate experiments in triplicate.

**Statistical analyses.** Data were analyzed with a Power Macintosh G4 using StatView, version 5.0 (SAS Institute), and the findings are expressed as mean values ± SD. Receiver-operator characteristic (ROC) curves were constructed to describe the relation between the sensitivity and specificity at varying cutoff levels. For comparison of the mean values of multiple groups, data that appeared to be statistically significant were compared by analysis of variance and nonparametric analysis, and P<.05 was considered to be statistically significant.

**RESULTS**

**Purification of GPL antigens from MAC.** The TLC pattern of GPL antigens showed a single spot (figure 1). GPLs purified from 11 serotypes of MAC reference strains exhibited a distinctive pattern by TLC. The chemical structure of GPL is composed of a common C-mycoside core, fatty acyl-d-Phe-d-allo-Thr-d-Ala-l-alaninol-O-(3,4-di-O-methyl-Rha), and the different moiety of oligosaccharide linked at the Thr substituent of the core. On the basis of the serospecific GPLs, MAC can be subdivided into 31 serotypes. They have distinct Rf values on TLC, although the chemical structures of certain serotypes have not yet been clarified. GPLs purified by TLC were analyzed by fast-atom bombardment mass-spectrometry to examine the mo-

![Origin](image.png)

**Figure 1.** Thin-layer chromatography (TLC) patterns of glycopeptido-lipids (GPLs) derived from 11 reference strains of *Mycobacterium avium* complex (MAC). GPLs were purified repeatedly by TLC of silica gel (Uniplate) developed with solvent system chloroform/methanol/distilled water (30/8/1 vol/vol/vol) until a single spot was obtained. Numbers denote serotypes of MAC. Arrows, GPLs developed by TLC.
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Figure 2. Dose-dependent inhibition according to mixture of antigens and dilution kinetics of glycopeptidolipid (GPL)-based EIA. The serum samples used in the present study were obtained from 3 patients with Mycobacterium avium complex (MAC) disease and were diluted with PBS–0.05% Tween 20. The optical density (OD) values decreased in a dose-dependent fashion with the addition of GPL antigens and the dilution of serum specimens. Similar results were obtained with the addition of GPLs that had been purified at different times.

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Levels of anti-GPL IgG antibodies in serum samples obtained from study subjects. The anti-GPL IgG antibody levels (mean OD ± SD) for subjects with MAC disease, colonization with MAC, M. kansasii disease, and TB and for healthy subjects were 0.778 ± 0.784 (95% CI, 0.063–3.582), 0.042 ± 0.035 (95% CI, 0.005–0.211), 0.059 ± 0.035 (95% CI, 0.018–0.155), 0.071 ± 0.035 (95% CI, 0.020–0.190), and 0.030 ± 0.027 (95% CI, 0.004–0.109), respectively (figure 3). These results demonstrated that there was a significant increase in serum levels of anti-GPL antibodies in patients with MAC pulmonary disease (P < .0001) but not in the other groups. We could not find a relationship between the duration of MAC disease and the level of serum anti-GPL antibodies (P = .86). No significant differences in antibody levels were seen for subjects with MAC colonization, those with M. kansasii disease, those with TB, and healthy subjects. We were unable to find a relationship between the number of colonies after culture and the level of antibodies (P = .26). A cutoff point of 0.119 was set as the level that was the best value in the relationship between sensitivity and specificity when the ROC curve was used (data not shown). We also examined the levels of serum IgA and IgM antibodies to GPLs, although they did not show good sensitivity and specificity compared with IgG antibodies (data not shown).

Disease activity and the level of anti-GPL antibodies. We next examined the levels of anti-GPL antibodies before the commencement of and after the completion of antimicrobial chemotherapy for 10 patients with MAC disease. These patients had conversion of culture results from positive to negative after they underwent successful chemotherapy, and the patients had had sputum culture–positive results for ≥6 months previously in the absence of antimycobacterial therapy. The duration of treatment for 10 patients with MAC infection who became culture negative was 1.5 ± 0.6 years, and the timing of serum collection was 2.5 ± 1.3 years after conversion. To determine whether the level of anti-GPL antibodies reflected the disease activity of MAC, we compared it sequentially in each patient before and after chemotherapy (figure 4). Before chemotherapy, the level for culture-positive patients was 0.483 ± 0.458, whereas the level for culture-negative converters was 0.264 ± 0.222. The level was decreased significantly in patients whose culture results converted from positive to negative. We performed statistical analysis of age, sex, duration of treatment, and the timing of serum sample collection for 10 patients who became culture-negative converters, because 5 patients showed a significant decrease in anti-GPL antibodies, 1 showed an in-
Figure 3. Serum IgG antibodies against a mixture of glycopeptidolipids (GPLs) derived from Mycobacterium avium complex (MAC). The serum samples were obtained from 78 patients with MAC disease, 12 with MAC colonization, 7 with Mycobacterium kansasii disease, and 72 with tuberculosis (TB) and from 131 healthy subjects (table 1). The serum samples used in the present study were all obtained from patients before the commencement of antimicrobial therapy and were diluted 40-fold with PBS–0.05% Tween 20. All results are expressed as individual data (●), and the bars indicate mean ± SD for each group. The broken line indicates the cutoff level (optical density [OD], 0.119). Statistically significant differences were observed (\*P < 0.0001).

Figure 4. Disease activity and the level of anti-glycopeptidolipid (GPL) antibodies. The levels of serum anti-GPL antibodies are shown before the commencement of and after the completion of antimicrobial chemotherapy for 10 patients with Mycobacterium avium complex (MAC) disease. The culture results for these patients converted from positive to negative (●), and bars indicate mean ± SD. The level decreased significantly in subjects who had conversion of culture results from positive to negative (\*P < .05). OD, optical density.

cress, and 4 showed no significant change (figure 4). No significant difference was found for the groups of patients.

**Sensitivity and specificity of EIA for diagnosis of MAC disease.** Seventy-two (92.3%) of 78 patients with MAC disease had positive results when the cutoff level (0.119) was defined using the ROC curve (table 2). The sensitivity and specificity of EIA for diagnosis of MAC disease were 92.3% and 96.7%, respectively. In contrast, there were low positivity rates for MAC colonization (0%), M. kansasii disease (3.3%), and TB (6.9%) and for healthy subjects (1.5%).

**DISCUSSION**

In the present study, we developed a means of serodiagnosis of MAC disease with GPL-based EIA that has high sensitivity and specificity. The level of serum anti-GPL IgG antibodies reflects disease activity.

Serodiagnosis of mycobacterial disease has long been the subject of investigation, because it offers a minimally invasive approach to diagnosis and uses widely available technologies [13]. Antibody responses are directed against a broad set of protein antigens of TB, responses vary among individuals, and the sensitivity of serological assays has generally been disappointing [14]. However, most experience indicates that serological testing does not offer significant advantages over more readily available diagnostic tests. A decision analysis has indicated that serodiagnosis by use of EIA does not add to the diagnostic yield in patients with TB when sputum smears are
Serovars of MAC, and anti-GPL antibodies were detected in peptidoglycolipid antigens that represented each of 15 common patients with mycobacterial disease were tested by EIA against of pulmonary disease [2, 9].

...diagnosis of pulmonary disease due to MAC, because GPLs are the major cell-surface our GPL-based EIA is specific for diagnosis of pulmonary disease needed to achieve the sensitivity and specificity of EIA. Thus, would be worthwhile to determine whether all 11 GPLs are obtain high sensitivity and specificity in the present study. It

...of seropositive results | Specificity, no. (%) of seronegative results
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MAC disease | 78 | 72 (92.3) | 6 (7.7)
MAC colonization | 12 | 0 (0) | 12 (100)
*Mycobacterium kansasii* disease | 30 | 1 (3.3) | 29 (96.7)
Tuberculosis | 72 | 5 (6.9) | 67 (93.1)
None* | 131 | 2 (1.5) | 129 (98.5)

*NOTE.* The cutoff level (optical density, 0.119) was calculated by receiver-operator characteristic curves that were constructed and used for the relation between the sensitivity and specificity. The predictive values for positive results (true positive/true positive + false positive) = 72/80 and negative results (true negative/true negative + false negative) = 237/243 were 90.0% and 97.5%, respectively.

*Healthy subjects.*

Serodiagnosis has been evaluated using EIA for antigen 5 of mycobacteria as well as a hemagglutinin assay for glycolipid antigens, and that study noted disappointing results for patients with negative smear results [16]. Although, in the future, specific antigens from mycobacteria may be identified that allow rapid and relatively easy diagnosis with use of blood samples, no serodiagnostic approach to the diagnosis of TB and MAC is currently of widespread clinical utility worldwide.

Our previous studies have demonstrated that the measurement of levels of serum antibodies against trehalose dimycolate/ cord factor [6] and glycolipid antigen [7] of tubercle bacilli is useful for diagnosis of TB. The method cannot differentiate between TB and disease due to NTM, including MAC and *M. kansasii*, because antibodies to glycolipids, such as trehalose dimycolate, are cross-reactive immunologically in mycobacteria [9]. For the present study, we developed a GPL-based EIA for diagnosis of MAC disease, because GPL antigens are specific to strains of MAC and are not encountered on any other group of mycobacteria [9]. The EIA had a high sensitivity (92.3%) and specificity (96.7%) for diagnosis of MAC disease and could discriminate between MAC disease and colonization with MAC, TB, and disease due to *M. kansasii*. We selected 11 clinically important serotypes [1] and used their GPLs to obtain high sensitivity and specificity in the present study. It would be worthwhile to determine whether all 11 GPLs are needed to achieve the sensitivity and specificity of EIA. Thus, our GPL-based EIA is specific for diagnosis of pulmonary disease due to MAC, because GPLs are the major cell-surface antigens of MAC and *M. scrofulaceum*, which is a rare cause of pulmonary disease [2, 9].

In a study reported elsewhere, serum samples obtained from patients with mycobacterial disease were tested by EIA against peptidoglycolipid antigens that represented each of 15 common serovars of MAC, and anti-GPL antibodies were detected in 83% of patients with MAC, 57% of patients with active TB, and 14% of subjects without mycobacterial disease [17]. That level of MAC disease is consistent with our findings with regard to the serodiagnostic sensitivity of MAC (92.3%). There are several possible explanations for the few false-negative results (6 [7.7%] of 78 patients): (1) the presence of circulating immune complexes, (2) an excess of GPL antigens relative to antibodies, (3) very low bacteria load, (4) use of a reference pool of GPL antigens inappropriate for the MAC isolate, and (5) recently diagnosed disease. Our results showed a low positivity rate (table 2) of serum anti-GPL antibodies in subjects with *M. kansasii* disease (3.3%), subjects with TB (6.9%), and healthy subjects (1.5%), although the levels of antibodies (figure 3) were significantly lower than those for patients with MAC disease. Such observations have raised the possibility that latent subclinical infection with MAC leads to false-positive results. Indeed, it has been reported that a significant proportion (7%–12%) of adults have been infected subclinically with MAC, as assessed by delayed-type skin reactivity to *M. avium* sensitin [18]. Our study did not examine the rate of subclinical infection with MAC, because investigations of such skin tests have not yet been completed in Japan. Because the study population did not include patients who were known to be immunocompromised, such as those with AIDS, we are interested the genetic defect presumably associated with the IL-12–IFN-γ system that participates in the defense against mycobacterial disease [19]. Future studies need to clarify this defect in the immunocompromised population.

To develop practical serodiagnostics, our EIA used a mixture of GPL antigens (instead of each antigen) derived from 11 reference strains of MAC. Our results suggest that the mixture of GPL antigens is useful for serodiagnosis of MAC disease, excluding the condition referred to as “colonization,” and that it is helpful for discrimination of other mycobacterial diseases,
such as M. kansasii disease and TB. Because crude mycobacterial protein antigens are cross-reactive, it is difficult to discriminate between TB and diseases caused by NTM, including MAC and M. kansasii [14, 15, 17]. For serodiagnosis of MAC disease, measurement of GPL antigens may be more sensitive than measurement of protein antigens of mycobacteria. However, further studies are needed to determine whether GPLs are different molecular entities, the same molecule at different stages of maturation, or the breakdown products of the same molecule. The finding of low rates of seropositivity for healthy subjects (1.5%) suggests that GPL-based EIA for diagnosis of MAC disease is not affected by prior vaccination with bacille Calmette-Guérin (BCG), because most Japanese people (∼90%) have been vaccinated against BCG [20, 21]. The results of our study are consistent with those of a report published elsewhere, which found that GPL-based EIA is highly sensitive and specific for the serodiagnosis of M. avium infection [22], although those researchers examined infection with M. avium but not MAC disease and its disease activity. In the present study, there was no difference in the level of serum anti-GPL antibodies between cases of M. avium (n = 36) and M. intracellulare (n = 14) disease diagnosed using DNA probes (data not shown). GPL antigens are specific to the group of M. avium, M. intracellulare, and M. scrofulaceum and are not encountered in any other group of mycobacteria, including M. tuberculosis [9]. On the basis of this observation, we did not use the antigen derived from M. tuberculosis in the assay. Indeed, most patients with TB did not have serum antibodies against GPLs (figure 3).

Our study demonstrated that the level of anti-GPL antibodies reflects the disease activity of MAC infection through the result of EIA before the commencement of and after the successful completion of antimicrobial chemotherapy. This may indicate the merit of monitoring disease activity as well as the timing of cessation of treatment, because there is, as yet, no consensus with regard to the discontinuation of chemotherapy for MAC disease [2]. Before recommending use of this EIA to monitor disease or to time the cessation of treatment, further prospective, large-scale studies of active MAC disease during and after treatment are obviously needed.

Serodiagnosis by use of GPL-based EIA may open new avenues for diagnosis of MAC disease, because the overall sensitivity and specificity are relatively high, it is safe and rapid, and the results of reproducible. The serological measurement excludes sampling errors. Serodiagnosis by EIA that uses MAC-specific GPL antigens can be used in combination with culture confirmation and acid-fast staining of sputum samples. Taken together with clinical, radiographic, and microbiological criteria, GPL-based EIA may be a powerful tool for diagnosis of MAC disease.

References