Differences in serological responses to specific glycopeptidolipid-core and common lipid antigens in patients with pulmonary disease due to *Mycobacterium tuberculosis* and *Mycobacterium avium* complex

Yukiko Fujita,¹ Takeshi Doi,¹ Ryoji Maekura,² Masami Ito² and Ikuya Yano¹

¹Japan BCG Central Laboratory, 3-1-5 Matsuyama, Kiyose-shi, Tokyo 204-0022, Japan

²Toneyama National Hospital, 5-1-1 Toneyama, Toyonaka-shi, Osaka 560-0045, Japan

Correspondence

Yukiko Fujita

y-fujita@bcg.gr.jp

Disease due to the *Mycobacterium avium* complex (MAC) is one of the most important opportunistic pulmonary infections. Since the clinical features of MAC pulmonary disease and tuberculosis (TB) resemble each other, and the former is often difficult to treat with chemotherapy, early differential diagnosis is desirable. The humoral immune responses to both diseases were compared by a unique multiple-antigen ELISA using mycobacterial species-common and species-specific lipid antigens, including glycopeptidolipid (GPL)-core. The results were assessed for two patient groups hospitalized and diagnosed clinically as having TB or MAC pulmonary disease. Diverse IgG antibody responsiveness was demonstrated against five lipid antigens: (1) monoacyl phosphatidylinositol dimannoside (Ac-PIM₂), (2) cord factor (trehalose 6,6'-dimycolate) (TDM-T) and (3) trehalose monomycolate from *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) (TMM-T), and (4) trehalose monomycolate (TMM-M) and (5) GPL-core from MAC. Anti-GPL-core IgG antibody was critical, and detected only in the primary and the secondary MAC diseases with high positivity, up to 88.4 %. However, IgG antibodies against Ac-PIM₂, TDM-T and TMM-T were elevated in both TB and MAC patients. Anti-TMM-M IgG antibody was also elevated in MAC disease preferentially, with a positive rate of 89.9 %, and therefore, it was also useful for the diagnosis of the disease. IgG antibody levels were increased at the early stages of the disease and declined in parallel to the decrease of bacterial burden to near the normal healthy control level, when the anti-mycobacterial chemotherapy was completed successfully. Unexpectedly, about 25 % of hospitalized TB patient sera were anti-GPL-core IgG antibody positive, although the specificity of GPL-core was sufficiently high (95.8 % negative in healthy controls), suggesting that a considerable number of cases of latent co-infection with MAC may exist in TB patients. Taken together, the combination of multiple-antigen ELISA using mycobacterial lipids, including GPL-core and TMM-M, gives good discrimination between healthy controls and sera from patients with TB or MAC disease, although for accurate diagnosis of TB more specific antigen(s) are needed.

Received 14 March 2005

Accepted 15 October 2005

INTRODUCTION

Mycobacterium avium complex (MAC) is a prominent opportunistic pathogen, with links to HIV infection and anti-mycobacterial-drug-resistant tuberculosis (TB) (Anonymous, 1979; Falkinham, 1996). Originally, MAC organisms were recognized as avian pathogens distributed widely in natural environments, such as soil or water (Falkinham,

1996). Also, recently, infectious diseases with MAC have been increasingly recognized not only in domestic animals such as swine (Ikawa *et al.*, 1989), but also in human beings (Falkinham, 1996; Kuth *et al.*, 1995; Ottenhoff *et al.*, 2002), although direct airborne infection from human to human has not been reported. More recently, serious MAC disease has become widely recognized (Ottenhoff *et al.*, 2002); some strains or serotypes show high resistance to anti-mycobacterial chemotherapy and the disease often shows poor prognosis after treatment (Wallace *et al.*, 1997). Although some MAC pulmonary disease shows characteristic disseminated lesions or lymphadenitis (Kuth *et al.*,

Abbreviations: Ac-PIM₂, monoacyl phosphatidylinositol dimannoside; BCG, Bacillus Calmette-Guérin; GPL, glycopeptidolipid; MAC, *Mycobacterium avium* complex; TB, tuberculosis; TDM, trehalose 6,6'-dimycolate; TMM, trehalose monomycolate.

1995), most resembles TB and a simple and reliable differential diagnostic tool is required. MAC infection can be diagnosed by culture, and DNA-amplification techniques are also available (MacKellar, 1976; Saito *et al.*, 1990; Wallace *et al.*, 1997). However, for smear-negative and culture-negative or PCR-negative cases, a serological tool would be useful. The practical use of T cell-specific protein antigen for skin testing to differentiate TB and MAC disease has been precluded because of high cross-reactivity (von Reyn *et al.*, 1993).

Previously, we have reported that MAC patient IgG antibodies were more reactive against cord factor (trehalose 6,6'-dimycolate; TDM) from MAC than against that from *Mycobacterium tuberculosis*, whereas TB patient sera were more reactive against TDM from *M. tuberculosis* than against that from MAC (Enomoto *et al.*, 1998; Pan *et al.*, 1999). These results suggest that the subclass structure of the mycoloyl moiety of TDM and trehalose monomycolate (TMM) can be recognized by TB or MAC patient IgG antibodies, respectively. We considered that the use of these species-specific glycolipid antigens in ELISA might provide a diagnostic tool for TB or MAC disease. Nonetheless, TDM and TMM from *M. tuberculosis* and MAC share multiple subclasses of mycolic acids (alpha-, methoxy- and keto-mycolates in *M. tuberculosis*, with alpha-, keto- and wax ester-mycolates in MAC), and therefore, cross-reactivity was inevitable.

More recently, we demonstrated that an ELISA with a cocktail of serotype-specific glycopeptidolipids (GPLs) of MAC showed improved characteristics for detecting MAC disease (Kitada *et al.*, 2002). We have also reported that the use of β -eliminated GPL-core as serotype common antigen for the detection of IgM and IgA antibodies of MAC patient sera gave a good discrimination between MAC disease and colonization (Kitada *et al.*, 2005). However, MAC disease may also occur as a co-infection with TB, and such co-infections may be missed due to TB smear or culture positivity. Finally, the incidence of MAC infection and environmental exposure differs considerably according to the district of the country (Saito *et al.*, 1998; Tomioka *et al.*, 1991) and environmental conditions (Kamala *et al.*, 1994, 1996). Therefore, a precise and simple diagnostic tool for TB, MAC disease or co-infection would be useful. This paper describes a multiple-antigen ELISA using GPL-core and TMM-M from MAC that shows discrimination between healthy controls and sera from patients with TB or MAC disease.

METHODS

Serum samples and patients. Serum samples of 105 TB and 69 MAC patients were obtained from Toneyama National Hospital, Osaka, Japan. TB and MAC patients were diagnosed at the first visit clinically, including chest X-ray examination, and based on smear staining and culture test results. For the TB patients with smear-negative and culture-negative results (53/105), a PCR test was performed, and PCR-positive cases (13/53) were diagnosed as active

TB. The PCR-negative patients (40/53) who had a history of pulmonary TB in the past 5 years were diagnosed by clinical symptoms, chest X-ray examination and tuberculin skin test (TST)-positive results. For the MAC patients, three culture-positive results were required to get results by DNA-DNA hybridization test. For the donated serum samples, informed consents were obtained from the individual patients.

Selection of healthy control subjects and determination of cut-off values for ELISA. To select the healthy control subjects, we carefully chose individuals who received a health examination with chest X-ray examination. Among these, 70% of individuals were BCG (*Bacillus Calmette-Guérin*) vaccinated at a young age (<12 years old). However, the IgG antibody titres against mycobacterial lipid antigens were not elevated after BCG vaccination, although TST results were positive. Thus, a co-relationship between IgG antibody titre elevation and the TST results has not been observed. Therefore, we simply determined that the normal range for IgG antibody titres was lower than the mean Δ absorbance value +2 SD of the healthy control.

The control samples consisted of 48 sera from HIV-negative healthy adult individuals with no history of TB or other mycobacterial infectious disease in their families and with TST results that were positive and negative.

Antigens. Lipid antigens were isolated from heat killed *Mycobacterium bovis* BCG Tokyo 172 and MAC serotype 4. Lipids were extracted from the packed cells with a chloroform/methanol 2:1 (v/v) mixture and the solvent was evaporated off with a rotary evaporator. First, they were separated by solvent fractionation into acetone-soluble, methanol-soluble or tetrahydrofuran-soluble fractions, and then further separated by TLC on silica gel (UNIPATE; Analtech) with the solvent system of chloroform/methanol/water 65:25:4 (v/v) or 90:10:1 (v/v) to isolate the lipid antigens. MAC-specific GPL-core was purified from the acetone-soluble alkali-stable lipid fraction as follows. Extracted lipids from MAC serotype 4 were hydrolysed with 0.2 M sodium hydroxide in methanol to remove alkali-labile lipid. GPLs were separated by TLC with the solvent system of chloroform/methanol/water 30:8:1 (v/v). After that, GPLs were β -eliminated with 1 M sodium hydroxide and sodium borohydride, and the resultant GPL-core was purified by TLC until a single spot was obtained. All antigenic lipids were identified by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Each of the components, such as sugars or fatty acids, was analysed by GC/MS after hydrolysis or methanolysis of the original lipid, respectively.

Based on a preliminary test using an ELISA of the mycobacterial lipid antigens (data not shown), we carefully selected five lipid antigens, monoacyl phosphatidylinositol dimannoside (Ac-PIM₂), TDM-T, TMM-T, TMM-M and GPL-core, for the diagnosis of active mycobacteriosis, including TB and MAC disease.

Multiple-antigen ELISA microplate system. Ac-PIM₂ (2.0 μ g), TMM-T (1.0 μ g), TMM-M (1.0 μ g) and GPL-core (0.4 μ g) were dissolved in ethanol (50 μ l per well), while TDM-T (0.2 μ g) was dissolved in n-hexane (50 μ l per well). Each antigen was deposited in a polystyrene microplate well (Nunc-Immunoplate; Nalge Nunc International). After that, the plates were allowed to dry at room temperature overnight. Plates were either used immediately or sealed in aluminium foil and stored at 4 °C. Using identical samples, no difference was observed in results between plates used immediately after preparation and those stored for a month (data not shown). All chemicals used were purchased from Wako Pure Chemical Industries. For multiple-antigen ELISA, non-specific binding was blocked using 150 μ l per well 0.05% Tween 20 in PBS (PBS-T) adjusted to pH 7.4 with 5 M NaOH, and incubated for 10 min at

room temperature. The plates were then washed three times with 250 μ l PBS-T per well. Samples tested for antibodies from serum were diluted 1:201, with PBS-T. The diluted sample (50 μ l per well) was added to each well, and the plate was incubated for 1 hr. Horseradish peroxidase (HRP)-goat anti-human IgG (H+L) (Zymed) diluted 1:500 in PBS-T was used as a secondary antibody. After incubation for 1 h, the substrate, *O*-phenylenediamine (Sigma) (1 mg ml⁻¹) in citrate buffer containing 0.06% H₂O₂ was added. The reaction was stopped with 0.5 M H₂SO₄, and absorption was measured in a microplate reader (NPR-A4i; Tosoh; dual wavelength operation mode) at 492 nm with the background absorption measured at a wavelength of 600 nm; the final absorbance value was calculated as $A_{492} - A_{600}$. Each incubation was performed at room temperature, and after each step of the procedure the plates were washed three times with PBS-T.

Data analysis. A positive response of serum IgG antibodies against Ac-PIM₂, TDM-T, TMM-T, TMM-M and GPL-core was recognized as a Δ absorbance value exceeding the mean healthy control Δ absorbance value + 2 SD, and we defined the Δ absorbance value as the test absorbance value minus the low control titre. Low control titre was defined as the absorbance value of antigen uncoated plate with each patient's serum. Statistical analysis was done according to Mann-Whitney U test.

RESULTS

Structures of mycobacterial lipid antigens used for ELISA

Most pathogenic mycobacteria contain unique phosphatidylinositol mannosides possessing different numbers of mannose residues and fatty acyl moieties. Among them, Ac-PIM₂ is one of the most abundant phospholipid antigens in mycobacteria. MALDI-TOF mass spectra showed that Ac-PIM₂ (Fig. 1a), derived from *M. bovis* BCG Tokyo 172, possessed C_{16:0} and branched chain C₁₉ (tuberculostearic)

fatty acids. TMM-T (Fig. 1c) derived from *M. bovis* BCG Tokyo 172 was a TMM possessing one molecule of α -, methoxy- or keto-mycolic acid from C₇₆ to C₉₁ (the latter two subclasses predominated), while TDM-T (Fig. 1b), from the same strain, possessed two molecules of mycolic acid from among the above subclasses or molecular species, thus making a complex mixture of the trehalose diesters with the molecular mass ranging from 2660 to 2920 Da. TMM-M (Fig. 1c) from MAC serotype 4 had a distinctive mass spectrometric pattern, showing the existence of a characteristic C₈₂ or longer wax ester-mycolic acid subclass instead of the methoxy-mycolic acid of *M. bovis* BCG Tokyo 172. Furthermore, MAC possesses a serotype-specific GPL whose lipopeptide core structure is shared among serotypes (Brennan & Nikaido, 1995). Based on the precise analysis by MALDI-TOF mass spectrometry of molecular mass, carbohydrate component, fatty acyl residue and peptide moiety, the main molecular species of the GPL-core (Fig. 1d) from MAC serotype 4 consisted of 3-*O*-methyl-C_{32:1} fatty acyl-D-phenylalanyl-L-threonyl-D-alanyl-L-alaninol to which 3,4-di-*O*-methylrhamnose was attached, but that was lacking a serotype-specific carbohydrate chain.

Thus, TDM-T and TMM (-T and -M) were partially species-specific, while Ac-PIM₂ was essentially a common antigen in mycobacterial species, and GPL-core was a MAC-specific antigen.

TDM from *M. bovis* Tokyo 172 possessed the same subclasses of mycolic acid as those of *M. tuberculosis*, and further the preparation or purification of lipid antigens from *M. bovis* BCG Tokyo 172 is much easier than those from *M. tuberculosis*, therefore, in the present study, we used TDM and TMM from *M. bovis* BCG Tokyo 172 (TDM-T and TMM-T).

Fig. 1. Structure of mycobacterial lipid antigens for multiple-antigen ELISA (a) Ac-PIM₂ containing tuberculostearic acid (R₁) and palmitic acid (R₂ and R₃), (b) TDM-T containing two molecules (R₁ and R₂) of mycolic acids from α -, methoxy- or keto-mycolic acid, (c) TMM-T containing one molecule (R₁) of mycolic acid from α -, methoxy- or keto-mycolic acid or TMM-M containing one molecule (R₁) of mycolic acid from α -, keto- or wax ester-mycolic acid and (d) GPL-core containing 3-hydroxy or 3-methoxy fatty acid with a carbon chain longer than 30 (R).

Dose-dependent response curves of antigen amount and antibody dilutions

First, we determined the most appropriate amount of each of the five lipid antigens and antibody dilutions for ELISA, according to dose-response curves using an appropriate titre of TB and MAC patient sera (Fig. 2). Since in cases of lipid molecule suspension, the micelle forms and the critical micelle concentration (cmc) of each lipid antigen differs, which is crucial for ELISA tests, we carefully selected the solvent and determined the antigen concentrations and antibody dilutions. Each dose-response curve showed a characteristic profile. In Ac-PIM₂, dose-response curves reached a maximum at 2.0 µg per well. In the case of TDM-T, the curves reached a plateau at 0.2 µg per well in the ELISA, while TMM-T and TMM-M showed double sigmoidal curves and reached a plateau at 1.0 µg per well. The curve of GPL-core reached a plateau at 0.4 µg per well. Based on both the above cmc and antigenic reactivity against healthy control sera, we determined each antigenic concentration as 2.0, 0.2, 1.0, 1.0 and 0.4 µg per well for Ac-PIM₂, TDM-T, TMM-T, TMM-M and GPL-core, respectively.

IgG antibody response pattern and diversity in TB and MAC patient sera

IgG antibody responses against the five lipid antigens were extremely diverse. In TB patients, IgG antibody titres against Ac-PIM₂, TDM-T and/or TMM-T, but not GPL-core, were elevated, and anti-TMM-T IgG antibody titres were higher than anti-TMM-M IgG antibody titres in general. In contrast, in MAC patients, IgG antibodies against TMM-M or GPL-core, or both, were positive and the titres to TMM-M were generally higher than those to TMM-T. Although the IgG antibody positive rate of each single antigen was not satisfactory for the diagnosis of active TB or MAC disease, the overall positive rate, when any one or more IgG antibodies scored positive, was useful for the diagnosis of active diseases. Fig. 3 illustrates the diversity of serum IgG antibody responses in TB (Fig. 3a, b, c, d) and MAC (Fig. 3e, f, g) patient sera. TB patient sera were highly reactive against TDM-T and/or TMM-T, but not against TMM-M and GPL-core. A majority of TB patient sera were also reactive against Ac-PIM₂ (Fig. 3c, d). IgG antibody response patterns of the TB patient sera were grouped into five to seven types reactive

Fig. 2. Dose-response curves of mycobacterial lipid antigens for multiple-antigen ELISA. Dose responsiveness (absorbance = $A_{492} - A_{600}$) of IgG antibodies of TB or MAC patient sera was plotted (serum dilution: $\diamond$, 1:100; $\square$, 1:160; $\triangle$, 1:200; $\times$, 1:400). Ac-PIM₂ showed clear sigmoidal curves at higher concentrations, due to the lower hydrophobicity. TDM-T showed typical dose-dependent curves at concentrations of 0.05–1.0 µg per well due to the higher hydrophobicity, while TMM-T and TMM-M showed characteristic double sigmoidal curves at concentrations of 0.1–1.6 µg per well. Antigen concentrations close to the plateau were selected for IgG antibody detection in ELISA.

Fig. 3. Different patterns of IgG antibody responses in TB and MAC patient sera. (a–d) TB patient sera, no case responded against GPL-core: (a) reactive to TDM-T, (b) reactive to TMM-T and TMM-M, (c) reactive to Ac-PIM₂ and (d) reactive to Ac-PIM₂, TDM-T and TMM-T. (a, b) Smear-positive, and (c, d) smear-negative. (e–g) MAC patient sera, all cases responded against TMM-M and GPL-core. (e, f) Smear positive and (g) smear negative. Absorbance = $A_{492} - A_{600}$. Bars with stripes, Ac-PIM₂; black bars, TDM-T; white bars, TMM-T; bars with dots, TMM-M; grey bars, GPL-core.

to Ac-PIM₂, TDM-T, TMM-T or any of two or three lipid antigens depending on the individual background of the patient. However, MAC patient sera were highly reactive to TMM-M and/or GPL-core. Some MAC patient sera were highly reactive against Ac-PIM₂, a mycobacterial common antigen (Fig. 3f).

Distribution of anti-lipid antigen IgG antibody titres

Fig. 4 shows scatter plots of the IgG antibody titres against 5 lipid antigens in 105 TB and 69 MAC patients, and in 48 healthy controls. The serum IgG antibody titres against TDM-T did not differ significantly between TB and MAC patients, while those against TMM-M and GPL-core were significantly higher ($P > 0.01$) in the MAC patient group than in the TB patient group. The mean Δ absorbance values of healthy control sera against five lipid antigens were markedly lower compared with both TB and MAC patient sera. These results showed that GPL-core and TMM-M are MAC-specific antigens, and are potentially useful for the diagnosis of MAC disease. However, TB patient sera were partially cross-reactive against TMM-M, and MAC patient sera were partially cross-reactive against TMM-T.

Sensitivity and specificity of multiple-antigen ELISA

Table 1 shows the sensitivity and specificity of multiple-antigen ELISA with MAC-specific GPL-core, TMM-M and

the other three mycobacterial lipid antigens in the patient groups. MAC patient sera showed a distinctively higher positive rate against GPL-core (88.4%) and TMM-M (89.9%) than TB patient sera, and also higher mean Δ absorbance values against GPL-core (1.380) and TMM-M (1.003). However, the IgG antibody response against GPL-core was low in TB patient sera. In summary, MAC disease was recognized by multiple-antigen ELISA including MAC-specific GPL-core and TMM-M with a high sensitivity of 97.1%. However, it was particularly noted that 24.8% of TB patients diagnosed clinically showed anti-GPL-core IgG antibody positivity, raising the possibility that exposure to or latent co-infection with MAC in TB patients existed more frequently than expected, since the specificity of GPL-core was 95.8% in healthy controls. For the discrimination of healthy control versus TB + MAC disease, the specificity, positive predictive value (PPV) of each lipid antigen ELISA and the cumulative sensitivity are sufficiently high, although the negative predictive value of each individual lipid antigen was not satisfactory. However, for the discrimination of healthy control + TB versus MAC disease, GPL-core and an additional combination of TMM-M ELISA were effective, although the specificity and PPV of each lipid antigen were low. Taken together, for a more precise and differential diagnosis of TB and MAC disease, further combinations of highly specific antigen reactive to TB, but not to MAC patient sera would be necessary.

Fig. 4. Scattergrams of IgG antibody titres of TB patient sera ($n=105$), MAC patient sera ($n=69$) and healthy control sera ($n=48$). TDM-T, TDM from *M. bovis* BCG Tokyo; TMM-T, TMM from *M. bovis* BCG Tokyo; TMM-M, TMM from MAC serotype 4; GPL-core, β -eliminated GPL-core from MAC serotype 4. Horizontal lines indicate the mean IgG antibody values for the TB patient group (Ac-PIM₂, 0.961; TDM-T, 0.630; TMM-T, 0.373; TMM-M, 0.404; GPL-core, 0.344) and the MAC patient group (Ac-PIM₂, 1.216; TDM-T, 0.775; TMM-T, 0.576; TMM-M, 1.003; GPL-core, 1.380), and for the healthy control group indicates the cut-off values (Ac-PIM₂, 0.350; TDM-T, 0.146; TMM-T, 0.152; TMM-M, 0.181; GPL-core, 0.326). Absorbance = $A_{492} - A_{600}$.

Time-course changes of IgG antibody levels during anti-mycobacterial chemotherapy

Since it is important to know the efficacy of anti-mycobacterial chemotherapy and the prognosis of the disease, we have estimated the IgG antibody titres against lipid antigens throughout the disease progression. As shown in Fig. 5(a, b, c, d), IgG antibody titres of TB patient sera were elevated against at least one or more lipid antigens besides TMM-M and GPL-core at the early stage of the disease. When anti-TB chemotherapy was successful, 2–6 weeks after starting, levels decreased sharply near to the normal healthy control level in parallel with the eradication of tubercle bacilli in the sputum. In a striking case of MDR-TB co-infected with MAC, anti-TMM-M and anti-GPL-core IgG antibodies elevated sharply and in parallel, reciprocal to the decline of anti-TDM-T, anti-TMM-T and anti-Ac-PIM₂ IgG antibodies (Fig. 5e).

In cases of MAC disease, as shown in Fig. 6(a, b, d), IgG antibodies against TMM-M and GPL-core were generally elevated, and in some cases anti-Ac-PIM₂ and anti-TDM-T IgG antibodies were also elevated. In addition, IgG antibody titres against lipid antigens decreased, when the chemotherapy was successful. However, compared with the cases of TB, it took longer for levels to decline near to the normal healthy control level (Fig. 6d). One case, Fig. 6(c), showed a retarded elevation of IgG antibodies against four antigens, including GPL-core, whereas anti-Ac-PIM₂ IgG antibody

had been elevated earlier on. In another particular case, shown in Fig. 6(e), a smear-positive MAC patient showed an acute increase in anti-TDM-T, anti-Ac-PIM₂ and anti-TMM-T IgG antibodies, and a transient decrease in anti-GPL-core IgG antibody, and immediate recoveries. Based on the above data, estimation of IgG antibody levels of GPL-core and TMM-M may be useful for prospective diagnosis of the MAC disease.

Effect of smear positivity of acid-fast bacilli on the IgG antibody responses at the first examination

Since, in cases of active TB, patient serum IgG antibody levels against lipid antigens varied greatly and the titres changed in parallel with the levels of bacteria excreted (Fujita *et al.*, 2005), we assessed this relationship in MAC patients. In such cases, no clear relationship between IgG antibody positivity and smear test results was demonstrable (data not shown). However, it was noted that the IgG antibody positive rate (sensitivity) of anti-TMM-M and/or anti-GPL-core was relatively high in the smear-negative MAC cases while the positivity to other antigens was extremely diverse. This may be due to the stage of the disease when the patients first visited the hospital. In MAC patients, irrespective of smear-positive or -negative status, IgG antibody titres against lipid antigens were already elevated, since the humoral immune response proceeded rapidly without distinctive clinical symptoms.

Table 1. Comparison of positive rate and cumulative sensitivity of multiple-antigen ELISA in the *M. tuberculosis* and MAC-infected patient groups

PPV, positive predictive value; NPV, negative predictive value.

Antigen	Positivity and sensitivity	TB*	MAC†	Healthy control vs TB+MAC			Healthy control+TB vs MAC		
				Specificity	PPV	NPV	Specificity	PPV	NPV
Ac-PIM ₂	Positive/total	62/105	50/69	—	—	—	—	—	—
	Sensitivity (%)	59.0	72.5	91.7	96.6	41.5	56.9	43.1	82.1
	ΔAbsorbance (mean ± SD)	0.961 ± 0.983	1.216 ± 0.943	—	—	—	—	—	—
TDM-T	Positive/total	53/105	39/69	—	—	—	—	—	—
	Sensitivity (%)	50.5	56.5	95.8	97.9	35.9	64.1	41.5	76.6
	ΔAbsorbance (mean ± SD)	0.630 ± 0.831	0.775 ± 0.955	—	—	—	—	—	—
TMM-T	Positive/total	48/105	42/69	—	—	—	—	—	—
	Sensitivity (%)	45.7	60.9	97.9	98.9	35.9	68.0	46.2	79.4
	ΔAbsorbance (mean ± SD)	0.373 ± 0.522	0.576 ± 0.969	—	—	—	—	—	—
TMM-M	Positive/total	67/105	62/69	—	—	—	—	—	—
	Sensitivity (%)	63.8	89.9	93.8	97.7	50.0	54.2	47.0	92.2
	ΔAbsorbance (mean ± SD)	0.404 ± 0.413	1.003 ± 0.651	—	—	—	—	—	—
GPL-core	Positive/total	26/105	61/69	—	—	—	—	—	—
	Sensitivity (%)	24.8	88.4	95.8	97.8	34.6	81.7	68.5	94.0
	ΔAbsorbance (mean ± SD)	0.344 ± 0.446	1.380 ± 0.915	—	—	—	—	—	—
Total‡	Positive/total	89/105	67/69	—	—	—	—	—	—
	Sensitivity (%)	84.8	97.1	81.3	94.5	68.4	35.9	40.6	96.5

*Smear-positive or smear-negative, and culture-positive (PCR or DNA–DNA hybridization) TB.

†Smear-positive or smear-negative, and culture-positive (PCR or DNA–DNA hybridization) MAC disease.

‡Serologically positive when IgG antibody titre against at least one or more antigen(s) was positive.

DISCUSSION

Recently, the increasing recognition of non-tuberculous mycobacterial diseases has been apparent and the need for differential diagnosis of TB and MAC, *Mycobacterium kansasii* or other slow-growing mycobacteria substantial. We have reported recently that single infection or co-infection with some particular serotypes of MAC often show a very poor prognosis, and therefore, early diagnosis is desirable (Maekura *et al.*, 2005). To date, a rapid antibody detection system to diagnose the disease and the particular mycobacterial species involved, other than TB, has not been reported. We have previously established an ELISA test using cord factor as antigen to detect anti-cord factor antibodies in TB patient sera (He *et al.*, 1991; Kawamura *et al.*, 1997; Maekura *et al.*, 1993, 2001, 2003). These tests are straightforward, reproducible and applicable to the diagnosis of non-pulmonary tuberculous disease such as colonic TB (Kashima *et al.*, 1995) or uveitis (Sakai *et al.*, 2001). While these ELISA tests were useful for the recognition of active disease, including TB and non-TB mycobacteriosis, they were not useful for differentiating between mycobacterial species.

More recently, we have used a cocktail antigen from MAC serotype-specific GPL including a representative 11 serotypes (serotype 1, 4, 6, 7, 8, 9, 12, 13, 14, 16 and 20). This test showed an excellent sensitivity in serodiagnosis of MAC disease (a positive rate of 92.3%), and the differential diagnosis of TB and MAC disease seemed to be promising (Kitada *et al.*, 2002). However, such antigen preparation requires great labour and high cost, and furthermore, the antigenic epitope(s) recognized by IgG antibody in MAC patient sera are more complicated. In other studies, it has been reported that some isolated colonies from MAC patient specimens lacked antigenic carbohydrate epitopes entirely and it was impossible to determine serotypes based on the biochemical analysis of GPL (Chan *et al.*, 1990). Therefore, more recently, we have investigated MAC species-specific and serotype-common GPL-core, which is liberated from the complete serotype-specific GPL antigens by β -elimination and purification (Kitada *et al.*, 2005).

In the present study, we used multiple combinations of species-specific and species-common lipid antigens to detect

Fig. 5. Time-course changes of IgG antibody levels in TB patient sera. The arrow shows the initiation point of anti-TB chemotherapy. For all five cases the patients were culture positive. (a) Smear-positive (G+9) patient. Anti-TDM-T and anti-TMM-T IgG antibodies were initially elevated and decreased until near the normal healthy control level after 14 weeks anti-TB chemotherapy. Anti-Ac-PIM₂, anti-TMM-M and anti-GPL-core IgG antibodies were negative throughout the disease progression. (b) Smear-positive (G+8) patient. Anti-TDM-T, anti-TMM-T, anti-TMM-M and anti-Ac-PIM₂ IgG antibodies were initially elevated and decreased until near to the normal healthy control level after 12 weeks anti-TB chemotherapy. Anti-GPL-core IgG antibody was negative throughout the disease progression. (c) Smear-positive (G+9) patient. Anti-TMM-T, anti-TMM-M and anti-Ac-PIM₂ IgG antibodies were elevated after anti-TB chemotherapy started and then decreased until near to the normal healthy control level after 9 weeks. Anti-GPL-core and anti-TDM-T IgG antibodies were negative throughout the disease progression. (d) Smear-negative patient. After anti-TB chemotherapy started, the smear-negative and culture-positive status continued for 6 weeks, and a high level of IgG antibody against Ac-PIM₂, TMM-T and TDM-T continued for 6 weeks then decreased. Anti-GPL-core IgG antibody was negative throughout the disease progression. (e) Smear-positive (G+8) patient. After anti-TB chemotherapy started, smear-positive, culture-positive status and the elevation of IgG antibodies against TDM-T, TMM-T and Ac-PIM₂ continued for 13 weeks, at which time MAC was first isolated and anti-GPL-core and anti-TMM-M IgG antibodies elevated sharply; a case of typical microbial substitution by MAC. Absorbance = $A_{492} - A_{600}$. ●, Ac-PIM₂; ◆, TDM-T; ▲, TMM-T; △, TMM-M; □, GPL-core.

the IgG antibody in TB and MAC patient sera. Since Ac-PIM₂ is a common mycobacterial lipid antigen anchored in cell membranes, we expected high positivity in both TB and MAC patient sera. However, the positive rate to Ac-PIM₂ was at best up to 59.0 % in active TB and 72.5 % in active

MAC patient sera, indicating the individual diversity in responsiveness.

Using our multiple-antigen ELISA we obtained up to 97.1 % for our MAC patient group. Even though the GPL-core is

Fig. 6. Time-course changes of IgG antibody levels in MAC patient sera. The arrow shows the initiation point of anti-MAC (anti-TB in the case of c) chemotherapy. For all five cases the patients were culture positive. (a) Smear-positive (G+5) patient. Anti-TDM-T, anti-TMM-M and anti-GPL-core IgG antibodies were highly elevated, and anti-TMM-T and anti-Ac-PIM₂ IgG antibodies moderately elevated, and decreased after 6 weeks anti-MAC chemotherapy. (b) Smear-positive (G+3) patient. Anti-TMM-M and anti-GPL-core IgG antibodies were highly elevated and showed a continuously high level for 5 weeks after anti-MAC chemotherapy. Anti-Ac-PIM₂ and anti-TMM-T IgG antibodies were negative. (c) Smear-positive (G+9) and culture-positive (*M. tuberculosis*) patient initially. Up to 7 weeks after anti-TB chemotherapy started, anti-Ac-PIM₂ IgG antibody showed a high level, but not the other antibodies. After 7 to 17 weeks, anti-Ac-PIM₂ IgG antibody declined and anti-TDM-T, anti-TMM-T, anti-TMM-M and anti-GPL-core IgG antibodies were elevated acutely; MAC was isolated at the 17th week. (d) Smear-negative patient. Anti-TMM-M and anti-GPL-core IgG antibodies were highly elevated continuously for at least 32 weeks after anti-MAC chemotherapy started. Other IgG antibodies showed low levels. At 44 weeks all anti-lipid antigen IgG antibodies declined to near the normal healthy control level. (e) Smear-positive (G+2) patient. Anti-GPL-core IgG antibodies were elevated initially, decreased transiently after 2 weeks, and increased again after 4 weeks. In contrast, anti-TDM-T, anti-Ac-PIM₂ and anti-TMM-T IgG antibodies were elevated after 2 weeks and decreased after 4 weeks. Absorbance = $A_{492} - A_{600}$. ●, Ac-PIM₂; ◆, TDM-T; ▲, TMM-T; △, TMM-M; □, GPL-core.

specific to MAC, many positive responses were demonstrated in TB patients. The positivity rate to GPL-core among TB patients differed (4–30 %) between the hospitals (data not shown). These unexpected results lead us to suggest that TB patients diagnosed clinically or bacteriologically may have latent subclinical infection or co-infection with MAC. Indeed, it has been reported recently that a significant proportion (7–12 %) of healthy adults have been

infected subclinically with MAC, as assessed by delayed-type hypersensitivity to *M. avium* sensitin (Kardjito *et al.*, 1982), although we have not yet examined the rate of subclinical infection with MAC in Japan. Only 4.2 % of our healthy control subjects showed anti-GPL-core IgG antibody positivity. Therefore, TB co-infected with MAC might have been misdiagnosed bacteriologically or serologically. In order to establish strict discrimination among TB, MAC disease or

co-infection with TB and MAC disease, a more suitable combination of TB-specific antigen ELISA, bacteriological examination and serological test is desirable. In the present study, we used GPL-core and TMM-M as MAC-specific antigens. For the serological diagnosis of TB, we have reported that the multiple combinations of six antigens gave useful sensitivity overall, although any single antigen did not (Fujita *et al.*, 2005).

We have also reported that IgG antibody titres against lipid antigen in TB patient sera varied greatly with the stage of the disease (Fujita *et al.*, 2005). This differs from the delayed-type hypersensitivity skin test with purified protein derivative due to the cellular immune response, which shows sustained positivity. In MAC disease, after the initiation of anti-mycobacterial chemotherapy, IgG antibody titres against MAC-specific lipid antigens showed some trends consistent with response to chemotherapy. Our MAC patient with multi-drug-resistant MAC disease showed longer-lasting high levels of anti-GPL-core and anti-TMM-M IgG antibodies. These characteristics may be useful for monitoring active MAC disease.

It has been reported that exposure to MAC or GPL causes immunosuppression (Horgen *et al.*, 2000; Tassell *et al.*, 1992) and can induce a decline in BCG-vaccine efficacy (Anonymous, 1979; Fine *et al.*, 2001; Palmer & Long, 1966). A simple, reliable diagnostic test for MAC infection could shed further light on these important features.

In conclusion, we have shown that MAC infection with or without TB can be recognized serologically using MAC-specific GPL-core and TMM-M antigens. Furthermore, we suggest that co-infection with MAC may be relatively frequent in our TB patients.

REFERENCES

- Anonymous (1979).** Trial of BCG vaccines in south India for tuberculosis prevention: first report-tuberculosis prevention trial. *Bull W H O* **57**, 819–827.
- Brennan, P. J. & Nikaido, H. (1995).** The envelope of mycobacteria. *Annu Rev Biochem* **64**, 29–63.
- Chan, S. L., Reggiardo, Z., Daniel, T. M., Girling, D. J. & Mitchison, D. A. (1990).** Serodiagnosis of tuberculosis using an ELISA with antigen 5 and a hemagglutination assay with glycolipid antigens. Results in patients with newly diagnosed pulmonary tuberculosis ranging in extent of disease from minimal to extensive. *Am Rev Respir Dis* **142**, 385–389.
- Enomoto, K., Oka, S., Fujiwara, N., Okamoto, T., Okuda, Y., Maekura, R., Kuroki, T. & Yano, I. (1998).** Rapid serodiagnosis of *Mycobacterium avium-intracellulare* complex infection by ELISA with cord factor (trehalose 6, 6'-dimycolate), and serotyping using the glycopeptidolipid antigen. *Microbiol Immunol* **42**, 689–696.
- Falkinham, J. O., III (1996).** Epidemiology of infection by non-tuberculous mycobacteria. *Clin Microbiol Rev* **9**, 177–215.
- Fine, P. E., Floyd, S., Stanford, J. L. & 7 other authors (2001).** Environmental mycobacteria in northern Malawi: implications for the epidemiology of tuberculosis and leprosy. *Epidemiol Infect* **126**, 379–387.
- Fujita, Y., Doi, T., Sato, K. & Yano, I. (2005).** Diverse humoral immune responses and changes in IgG antibody levels against mycobacterial lipid antigens in active tuberculosis. *Microbiology* **151**, 2065–2074.
- He, H., Oka, S., Han, Y. K., Yamamura, Y., Kusunose, E., Kusunose, M. & Yano, I. (1991).** Rapid serodiagnosis of human mycobacteriosis by ELISA using cord factor (trehalose-6,6'-dimycolate) purified from *Mycobacterium tuberculosis* as antigen. *FEMS Microbiol Immunol* **3**, 201–204.
- Horgen, L., Barrow, E. L., Barrow, W. W. & Rastogi, N. (2000).** Exposure of human peripheral blood mononuclear cells to total lipids and serovar-specific glycopeptidolipids from *Mycobacterium avium* serovars 4 and 8 results in inhibition of TH1-type responses. *Microb Pathog* **29**, 9–16.
- Ikawa, H., Oka, S., Murakami, H., Hayashi, A. & Yano, I. (1989).** Rapid identification of serotypes of *Mycobacterium avium*-*M. intracellulare* complex by using infected swine sera and reference antigenic glycolipids. *J Clin Microbiol* **27**, 2552–2558.
- Kamala, T., Paramasivan, C. N., Herbert, D., Venkatesan, P. & Prabhakar, R. (1994).** Isolation and identification of environmental Mycobacteria in the *Mycobacterium bovis* BCG trial area of South India. *Appl Environ Microbiol* **60**, 2180–2183.
- Kamala, T., Paramasivan, C. N., Herbert, D., Venkatesan, P. & Prabhakar, R. (1996).** Immune response & modulation of immune response induced in the guinea-pigs by *Mycobacterium avium* complex (MAC) & *M. fortuitum* complex isolates from different sources in the south Indian BCG trial area. *Indian J Med Res* **103**, 201–211.
- Kardjito, T., Handoyo, I. & Grange, J. M. (1982).** Diagnosis of active tuberculosis by immunological methods. 1. The effect of tuberculin reactivity and previous BCG vaccination on the antibody levels determined by ELISA. *Tubercle* **63**, 269–274.
- Kashima, K., Oka, S., Tabata, A., Yasuda, K., Kitano, A., Kobayashi, K. & Yano, I. (1995).** Detection of anti-cord factor antibodies in intestinal tuberculosis for its differential diagnosis from Crohn's disease and ulcerative colitis. *Dig Dis Sci* **40**, 2630–2634.
- Kawamura, M., Sueshige, N., Imayoshi, K., Yano, I., Maekura, R. & Kohno, H. (1997).** Enzyme immunoassay to detect antituberculous glycolipid antigen (anti-TBGL antigen) antibodies in serum for diagnosis of tuberculosis. *J Clin Lab Anal* **11**, 140–145.
- Kitada, S., Maekura, R., Toyoshima, N., Fujiwara, N., Yano, I., Ogura, T., Ito, M. & Kobayashi, K. (2002).** Serodiagnosis of pulmonary disease due to *Mycobacterium avium* complex with an enzyme immunoassay that uses a mixture of glycopeptidolipid antigens. *Clin Infect Dis* **35**, 1328–1335.
- Kitada, S., Maekura, R., Toyoshima, N., Naka, T., Fujiwara, N., Kobayashi, M., Yano, I., Ito, M. & Kobayashi, K. (2005).** Use of glycopeptidolipid core antigen for serodiagnosis of *Mycobacterium avium* complex pulmonary disease in immunocompetent patients. *Clin Diagn Lab Immunol* **12**, 44–51.
- Kuth, G., Lamprecht, J. & Haase, G. (1995).** Cervical lymphadenitis due to mycobacteria other than tuberculosis: an emerging problem in children? *ORL J Otorhinolaryngol Relat Spec* **57**, 36–38.
- MacKellar, A. (1976).** Diagnosis and management of atypical mycobacterial lymphadenitis in children. *J Pediatr Surg* **11**, 85–89.
- Maekura, R., Nakagawa, M., Nakamura, Y. & 9 other authors (1993).** Clinical evaluation of rapid serodiagnosis of pulmonary tuberculosis by ELISA with cord factor (trehalose-6,6'-dimycolate) as antigen purified from *Mycobacterium tuberculosis*. *Am Rev Respir Dis* **148**, 997–1001.
- Maekura, R., Okuda, Y., Nakagawa, M. & 10 other authors (2001).** Clinical evaluation of anti-tuberculous glycolipid immunoglobulin G antibody assay for rapid serodiagnosis of pulmonary tuberculosis. *J Clin Microbiol* **39**, 3603–3608.

- Maekura, R., Kohno, H., Hirotani, A., Okuda, Y., Ito, M., Ogura, T. & Yano, I. (2003).** Prospective clinical evaluation of the serologic tuberculous glycolipid test in combination with the nucleic acid amplification test. *J Clin Microbiol* **41**, 1322–1325.
- Maekura, R., Okuda, Y., Hirotani, A., Kitada, S., Hiraga, T., Yoshimura, K., Yano, I., Kobayashi, K. & Ito, M. (2005).** Clinical and prognostic importance of serotyping *Mycobacterium avium-Mycobacterium intracellulare* complex isolates in human immunodeficiency virus-negative patients. *J Clin Microbiol* **43**, 3150–3158.
- Ottenhoff, T. H., Verreck, F. A., Lichtenauer-Kaligis, E. G., Hoeve, M. A., Sanal, O. & van Dissel, J. T. (2002).** Genetics, cytokines and human infectious disease: lessons from weakly pathogenic mycobacteria and salmonellae. *Nat Genet* **32**, 97–105.
- Palmer, C. E. & Long, M. W. (1966).** Effects of infection with atypical mycobacteria on BCG vaccination and tuberculosis. *Am Rev Respir Dis* **94**, 553–568.
- Pan, J., Fujiwara, N., Oka, S., Maekura, R., Ogura, T. & Yano, I. (1999).** Anti-cord factor (trehalose 6,6' dimycolate) IgG antibody in tuberculosis patients recognizes mycolic acid subclasses. *Microbiol Immunol* **43**, 863–869.
- Saito, H., Tomioka, H., Sato, K., Tasaka, H. & Dawson, D. J. (1990).** Identification of various serovar strains of *Mycobacterium avium* complex by using DNA probes specific for *Mycobacterium avium* and *Mycobacterium intracellulare*. *J Clin Microbiol* **28**, 1694–1697.
- Saito, H., Kai, M. & Kobayashi, K. (1998).** Geographical distribution of *Mycobacterium avium* complex in environment and serovars of *Mycobacterium avium* complex isolates from patients with and without AIDS. *Kekkaku* **73**, 379–383.
- Sakai, J., Matsuzawa, S., Usui, M. & Yano, I. (2001).** New diagnostic approach for ocular tuberculosis by ELISA using the cord factor as antigen. *Br J Ophthalmol* **85**, 130–133.
- Tassell, S. K., Pourshafie, M., Wright, E. L., Richmond, M. G. & Barrow, W. W. (1992).** Modified lymphocyte response to mitogens induced by the lipopeptide fragment derived from *Mycobacterium avium* serovar-specific glycopeptidolipids. *Infect Immun* **60**, 706–711.
- Tomioka, H., Sato, K., Saito, H. & Tasaka, H. (1991).** Identification of *Mycobacterium avium* and *Mycobacterium intracellulare* using three DNA probe tests, and their distributions in Japan. *Kekkaku* **66**, 739–746.
- von Reyn, C. F., Barber, T. W., Arbeit, R. D. & 10 other authors (1993).** Evidence of previous infection with *Mycobacterium avium-Mycobacterium intracellulare* complex among healthy subjects: an international study of dominant mycobacterial skin test reactions. *J Infect Dis* **168**, 1553–1558.
- Wallace, R. J., Jr, Cook, J. L., Glassroth, J., Griffith, D. E., Olivier, K. N. & Gordon, F. (1997).** Diagnosis and treatment of disease caused by nontuberculous mycobacteria: American Thoracic Society statement. *Am J Respir Crit Care Med* **156**, S1–S25.