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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

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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-PIM_2), (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-PIM_2, 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.

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-PIM_2, 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 Δabsorbance 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 (UNIPLATE; 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-PIM_2), 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-PIM_2 (2·0 μg), TMM-T (1·0 μg), TMM-M (1·0 μg) and GPL-core (0·4 μg) were dissolved in ethanol (50 μl per well), while TDM-T (0·2 μg) was dissolved in n-hexane (50 μl 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 μl 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 µl PBS-T per well. Samples tested for antibodies from serum were diluted 1:201, with PBS-T. The diluted sample (50 µl 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% H_2O_2 was added. The reaction was stopped with 0·5 M H_2SO_4, 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-PIM_2, TDM-T, TMM-T, TMM-M and GPL-core was recognized as a Δabsorbance value exceeding the mean healthy control Δabsorbance value + 2 SD, and we defined the Δabsorbance 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-PIM_2 is one of the most abundant phospholipid antigens in mycobacteria. MALDI-TOF mass spectra showed that Ac-PIM_2 (Fig. 1a), derived from *M. bovis* BCG Tokyo 172, possessed $C_{16:0}$ and branched chain C_{19} (tuberculostearic) fatty acids. TMM-T (Fig. 1c) derived from *M. bovis* BCG Tokyo 172 was a TMM possessing one molecule of alpha-, methoxy- or keto-mycolic acid from C_{76} to C_{91} (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 C_{82} 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-PIM_2 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).

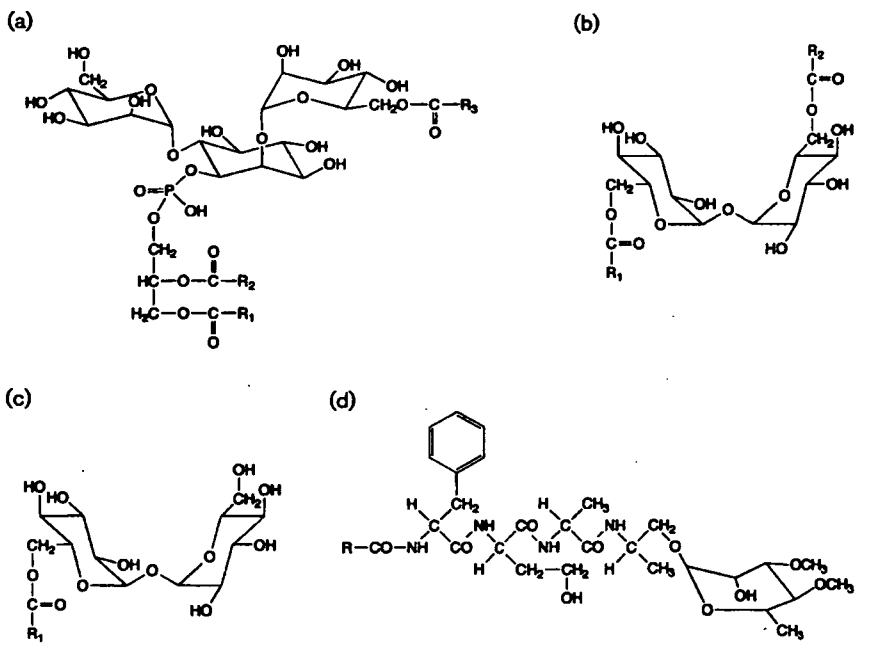

Fig. 1. Structure of mycobacterial lipid antigens for multiple-antigen ELISA (a) Ac-PIM_2 containing tuberculostearic acid (R_1) and palmitic acid (R_2 and R_3), (b) TDM-T containing two molecules (R_1 and R_2) of mycolic acids from alpha-, methoxy- or keto-mycolic acid, (c) TMM-T containing one molecule (R_1) of mycolic acid from alpha-, methoxy- or keto-mycolic acid or TMM-M containing one molecule (R_1) of mycolic acid from alpha-, 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-PIM_2, 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-PIM_2, 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-PIM_2, 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-PIM_2 (Fig. 3c, d). IgG antibody response patterns of the TB patient sera were grouped into five to seven types reactive

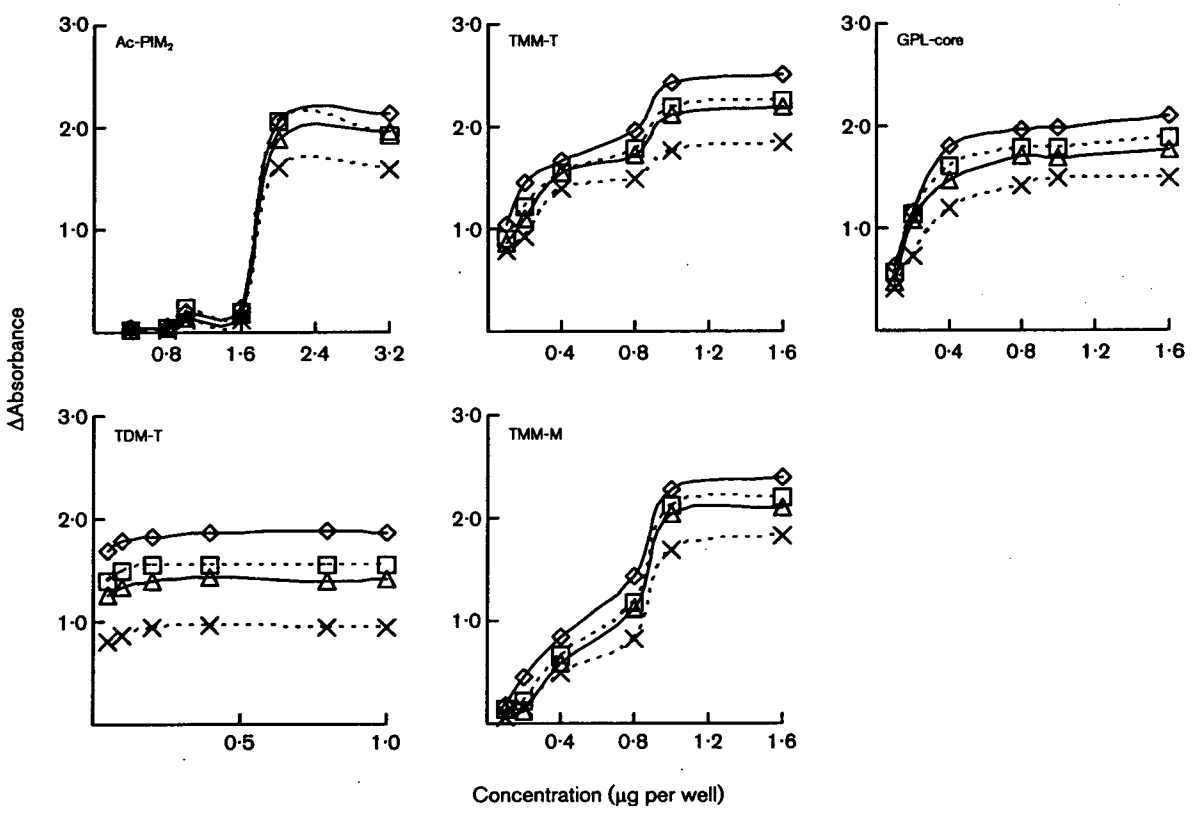

Fig. 2. Dose-response curves of mycobacterial lipid antigens for multiple-antigen ELISA. Dose responsiveness (absorbance = $A_{492}-A_{600}$) of IgG antibodies of TB or MAC patient sera was plotted (serum dilution: ◇, 1:100; □, 1:160; △, 1:200; X, 1:400). Ac-PIM_2 showed clear sigmoidal curves at higher 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.

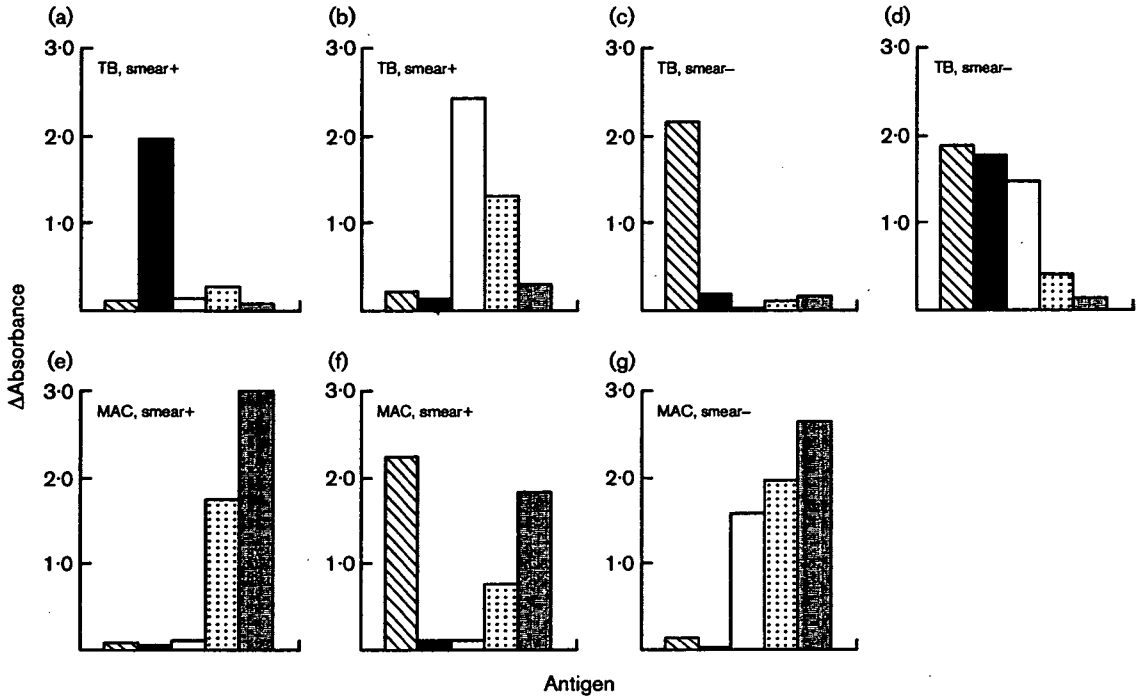

Fig. 3. Different patterns of IgG antibody responses in TB and MAC patient sera. (a–d) TB patient sera, no case responded against GPL-core: (a) reactive to TDM-T, (b) reactive to TMM-T and TMM-M, (c) reactive to Ac-PIM_2 and (d) reactive to Ac-PIM_2, TDM-T and TMM-T. (a, b) Smear-positive, and (c, d) 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-PIM_2; black bars, TDM-T; white bars, TMM-T; bars with dots; TMM-M; grey bars, GPL-core.

to Ac-PIM_2, 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-PIM_2, 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 ($P > 0{\cdot}01$) in the MAC patient group than in the TB patient group. The mean Δabsorbance 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 Δabsorbance 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.

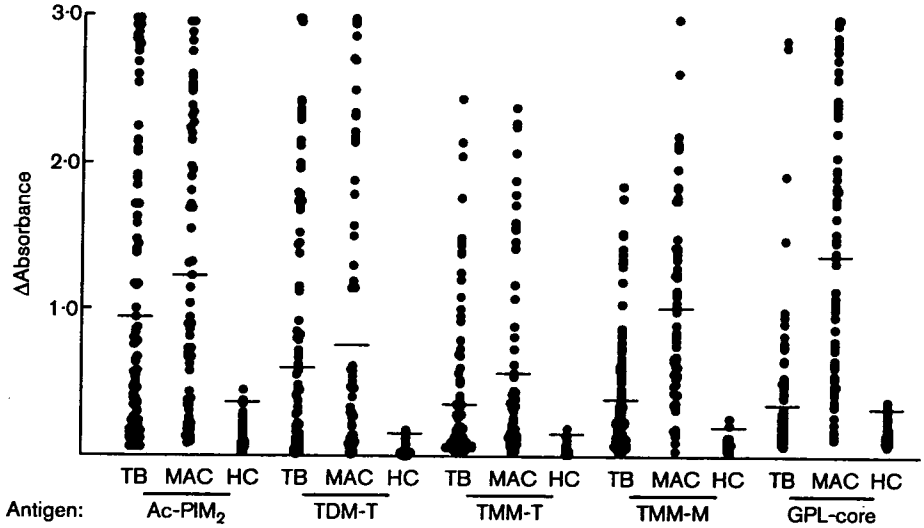

Fig. 4. Scattergrams of IgG antibody titres of TB patient sera ($n=105$), MAC patient sera ($n=69$) and healthy control sera ($n=48$). TDM-T, TDM from *M. bovis* BCG Tokyo; TMM-T, TMM from *M. bovis* BCG Tokyo; TMM-M, TMM from MAC serotype 4; GPL-core, β-eliminated GPL-core from MAC serotype 4. Horizontal lines indicate the mean IgG antibody values for the TB patient group (Ac-PIM_2, 0·961; TDM-T, 0·630; TMM-T, 0·373; TMM-M, 0·404; GPL-core, 0·344) and the MAC patient group (Ac-PIM_2, 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-PIM_2, 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-PIM_2 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-PIM_2 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-PIM_2 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-PIM_2 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

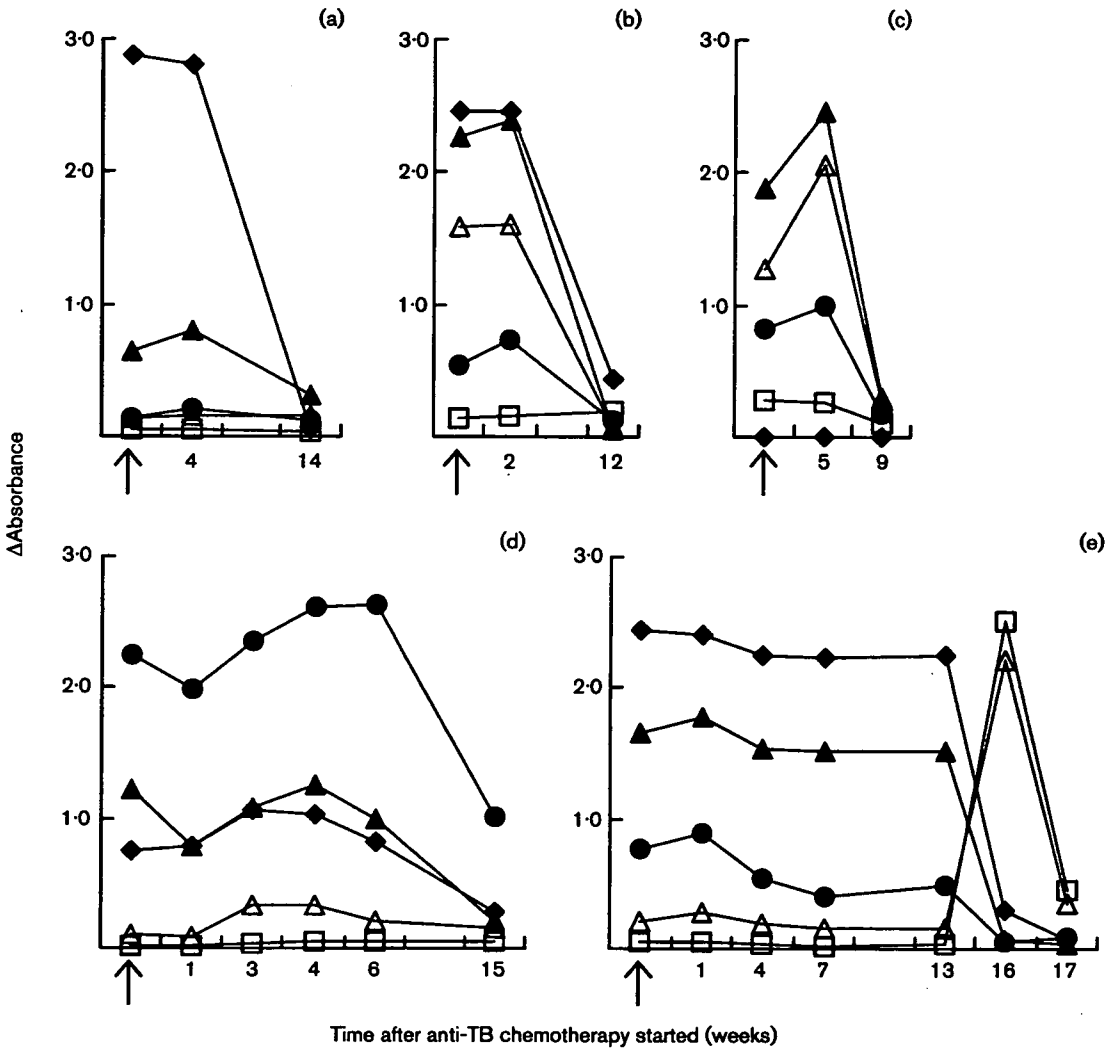

Fig. 5. Time-course changes of IgG antibody levels in TB patient sera. The arrow shows the initiation point of anti-TB chemotherapy. For all five cases the patients were culture positive. (a) Smear-positive (G+9) patient. Anti-TDM-T and anti-TMM-T IgG antibodies were initially elevated and decreased until near the normal healthy control level after 14 weeks anti-TB chemotherapy. Anti-Ac-PIM_2, 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-PIM_2 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-PIM_2 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-PIM_2, 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-PIM_2 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-PIM_2; ◆, TDM-T; ▲, TMM-T; △, TMM-M; □, GPL-core.

the IgG antibody in TB and MAC patient sera. Since Ac-PIM_2 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-PIM_2 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

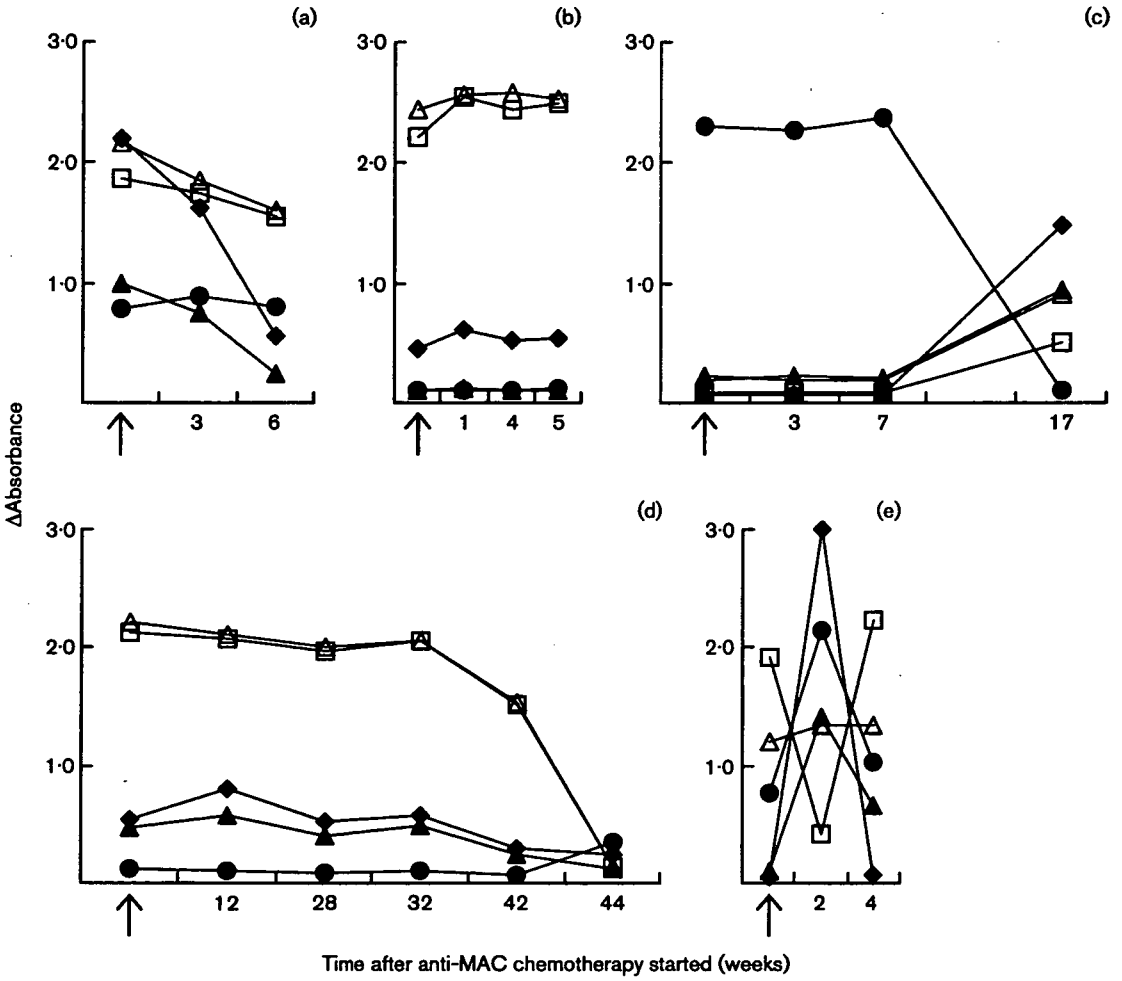

Fig. 6. Time-course changes of IgG antibody levels in MAC patient sera. The arrow shows the initiation point of anti-MAC (anti-TB in the case of c) chemotherapy. For all five cases the patients were culture positive. (a) Smear-positive (G+5) patient. Anti-TDM-T, anti-TMM-M and anti-GPL-core IgG antibodies were highly elevated, and anti-TMM-T and anti-Ac-PIM_2 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-PIM_2 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-PIM_2 IgG antibody showed a high level, but not the other antibodies. After 7 to 17 weeks, anti-Ac-PIM_2 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-PIM_2 and anti-TMM-T IgG antibodies were elevated after 2 weeks and decreased after 4 weeks. Absorbance = $A_{492}-A_{600}$. ●, Ac-PIM_2; ◆, 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.

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Clinical evaluation of serodiagnosis of active tuberculosis by multiple-antigen ELISA using lipids from *Mycobacterium bovis* BCG Tokyo 172

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Abstract

The humoral immune responses of 69 active tuberculosis (TB) patients against three major mycobacterial lipid antigens, monoacyl phosphatidylinositol dimannoside (Ac-PIM_2), trehalose 6,6'-dimycolate (TDM-T) and trehalose 6-monomycolate (TMM-T) from *Mycobacterium bovis* BCG Tokyo 172, were examined by ELISA. IgG antibodies from active TB patients were reactive against each of the three lipid antigens (Ac-PIM_2, TDM-T and TMM-T), giving positive results of 42.0–59.4%. If tests were combined and an overall positive was scored when any of the three tests was positive, sensitivity was 71.0%, showing better discrimination between patients and normal subjects. Although this value is not satisfactory for the clinical diagnosis of active TB, it is still higher than values for Determinar TBGL (56.5%) and MycoDot (31.9%) test results, both of which are commercially available. IgG antibody responses to particular lipid antigens in an individual patient differed diversely between patients. Positive IgG antibody rates and IgG antibody levels to lipid antigens were mostly paralleled by the amount of mycobacteria excreted and by the severity of pathological lesions (size and cavity formation). Serologically positive responsiveness was shown in 60.0% of tuberculin skin test (TST)-negative TB patients. Furthermore, seropositivity for multiple-antigen ELISA in active TB patients was demonstrated in other possible immune-suppressed cases, such as senile, diabetes mellitus and fulminant TB patients. Therefore, in contrast to tests based on cellular immune responses such as the TST, the humoral immune responses of TB patients against mycobacterial lipid antigens were disease-specific and were shown to be useful for the early diagnosis of active TB disease in conjunction with smear and cultivation tests, even if cellular immune responses are decreased.

Keywords: disease-specific humoral response; lipid antigen; tuberculosis serodiagnosis.

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Introduction

Diagnosis of tuberculosis (TB) has so far been performed by clinical examination and bacteriological tests based on the detection of acid-fast bacilli by smear test or cultivation. Recently, a polymerase chain reaction (PCR) or nucleic acid amplification (NAA) technique has been introduced. However, the sensitivity of smear testing is low, cultivation takes a long time and NAA techniques are expensive. The tuberculin skin test (TST) is highly sensitive and useful as a criterion for delayed hypersensitivity based on the cellular immune response to infection with *Mycobacterium tuberculosis* (*M. tuberculosis*). However, the TST is not applicable for the differential diagnosis of active TB disease or latent infection, or of TB or another mycobacterial infection, including BCG vaccination. Since only 5–10% of cases infected with TB develop the disease, the most desirable clinical tool is a diagnostic test to decide whether anti-TB chemotherapy or chemoprophylaxis should be started immediately or successfully completed. A serological test based on the humoral immune response is simple, economical, non-invasive and disease-related, in contrast to the TST, and it is applicable at the first clinic visit for smear-negative (or -positive), culture-negative (or -positive) patients suspected of having TB. To date, many investigations or trials have been reported on the serodiagnosis of TB using crude cell sonicates (1, 2), culture filtrates (3, 4), purified protein antigens (5, 6) and cell wall lipids (7) as antigens. Despite this, the practical use of serodiagnosis for active TB has not been widely introduced. The reason seems to be that the reliability of the current serological tests is not yet satisfactory, as review articles have pointed out (8–10). We have previously reported that the IgG antibodies of hospitalized patients against cord factor (trehalose 6,6'-dimycolate), a cell surface glycolipid antigen in *M. tuberculosis*, can be detected by ELISA with high sensitivity and specificity for smear-positive and -negative active TB (11–13). For a clinically reliable test to diagnose active TB, higher sensitivity and specificity and easy preparation of the test kit are necessary. Recent papers have reported that the sensitivity of serodiagnostic tests in smear-negative or smear- and culture-negative TB cases is equal to or rather higher than that of NAA tests (14, 15). Furthermore, the relationship between serological test results and the clinical background, such as antigenic stimulation, severity of the pathological lesions, immunological states and progression of the disease, should be clarified. We have examined humoral immune responses in suspect TB patients by

ELISA against various lipid antigens, including the representative phospholipids and glycolipids from *M. bovis* BCG Tokyo 172. Results revealed that the IgG antibody responses of individual TB patient sera were extremely heterogeneous, depending on the degree of antigen stimulation (amount of bacilli excreted), the stage of progression of the disease, and the individual or genetic background of the patient. The purpose of this investigation was to improve the sensitivity of IgG antibody detection for the diagnosis of active TB in the early stages of the disease, and to reveal the relationship between IgG antibody responsiveness against lipid antigens and various clinical background features of the patients.

Materials and methods

Serum samples and patients

A total of 69 serum samples were collected from patients aged from 20 to 92 years attending the Fukujuji Hospital, Kiyose, Tokyo, after informed consent was obtained from each patient. Patients were diagnosed with or suspected as having TB based on clinical symptoms and chest X-ray examination, with 55 smear-positive and 14 smear-negative. The detailed clinical background of each patient is shown in Table 1.

Selection of healthy control subjects and determination of cut-off values for ELISA

To select healthy control subjects, we chose individuals on the basis of a health check and chest X-ray. Of these, 70% of individuals were BCG vaccinated at a young age (<12 years of age). However, IgG antibody titers against mycobacterial lipid antigens were not elevated after BCG vaccination, although TST results were positive. Thus, no relationship between elevation of IgG antibody titers and the TST results was observed. Therefore, we simply determined that the normal range for IgG antibody titers was lower than the mean ΔOD value+2SD for healthy controls.

The control subjects consisted of 48 sera from HIV-negative healthy adult individuals with no history of TB in their families, with either a positive or negative TST result.

Antigens

Lipid antigens were isolated from heat-killed *M. bovis* BCG Tokyo 172. Lipids were fully extracted from the packed cells with a chloroform/methanol (2:1, v/v) mixture and the organic solvent layers were combined and evaporated off. The crude lipids were first separated by solvent fractionation using a modification of the method of Anderson (16). Briefly, chloroform/methanol-soluble lipids were first acetone-precipitated to remove neutral lipids and acetone-soluble glycolipids, and then methanol-soluble phospholipids were obtained from the resultant acetone precipitate. Mycoloyl glycolipids were fractionated as the chloroform-soluble lipid fraction. Each fraction was further separated by thin-layer chromatography on silica gel (Uniplate, Analtech, Newark, NJ, USA) with a chloroform/methanol/water (65:25:4, by vol.) solvent system for phospholipids, and chloroform/methanol/acetone/acetic acid (90:10:6:1, by vol.) or chloroform/methanol/water (90:10:1, by vol.) for mycoloyl glycolipids. Antigenic lipids were purified until a single spot was obtained by repeated thin-layer chromatography, as shown in Figure 1, and their structures were identified by mass spectrometric analysis. Components such as sugars and fatty acids were analyzed by gas chromatography/mass spectrometry (GC/MS) after hydrolysis or methanolysis of the original lipids, respectively, and the molecular weight of the intact lipids was determined by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) MS. Prior to selecting multiple antigens for serodiagnosis of active TB, we tested the positive rate for more than 20 antigens isolated from *M. tuberculosis*, *M. bovis* BCG and *M. avium* complex against sera from 40 hospitalized patients. Based on these preliminary ELISA results, we selected three lipid antigens from *M. bovis* BCG Tokyo 172, monoacyl phosphatidylinositol dimannoside (Ac-PIM_2), trehalose 6,6'-dimycolate (TDM-T) and trehalose 6-monomycolate (TMM-T), for the diagnosis of active TB. Ac-PIM_2 is a common phospholipid antigen among mycobacterial species. Most IgG antibodies from TB patient sera were reactive in the early stages of the disease, although the positive rate for single use of this antigen is not sufficient. TDM and TMM are species-specific antigens, both of which share antigenic epitopes between *M. tuberculosis* and *M. bovis* BCG Tokyo 172. Anti-TDM and anti-TMM IgG antibodies are also disease-specific and parallel the progression of the disease. Since the antibody responsiveness pattern differed between patients, we considered the most useful combination of antigens.

Multiple-antigen ELISA microplate system

Ac-PIM_2 (2.0 μg) and TMM-T (1.0 μg) were dissolved in ethanol (50 μL/well), while TDM-T (0.2 μg) was dissolved in n-hexane (50 μL/well). One antigen was deposited in a polystyrene microplate well (Nunc-Immunoplate; Nalge Nunc International, Rochester, NY, USA) and then the plates were allowed to dry at room temperature overnight. Plates were either used immediately or sealed in aluminum foil and stored at 4°C. When the same samples were measured, no difference was observed in the results between plates used immediately after preparation and those stored for 1 month (data not shown). All chemicals used were purchased from Wako Pure Chemical Industries (Tokyo, Japan). For multiple-antigen ELISA, non-specific binding was blocked using 150 μL/well of 0.05% Tween 20 in phosphate-buffered saline (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 μL/well of PBS-T. Serum samples to be tested for antibody titers were diluted 1:201 in PBS-T. Diluted sample (50 μL/well) was added to each well, and the plate was incubated for 1 h. Horseradish peroxidase (HRP)-goat anti-human IgG (H+L) (Zymed, San Francisco, CA, USA) diluted 1:500 in PBS-T was used as a secondary antibody. After incubation for 1 h, the substrate o-phenylenediamine (Sigma, St. Louis, MO, USA) (1 mg/mL) in citrate buffer containing 0.06% H_2O_2 was added. The reaction was stopped with 1 N H_2SO_4, and absorption was measured in a microplate reader (NPR-A4i; Tosoh, Tokyo, Japan) at 492–600 nm. Each incubation was performed at room temperature, and after each step of the procedure the plates were washed three times with PBS-T.

Assay of Determinar TBGL and MycoDot antibody detection systems

To evaluate the multiple-antigen ELISA comparatively, we used Determinar TBGL (Kyowa Medex, Tokyo, Japan) and MycoDot kits (Ramco, Osaka, Japan) to test the same serum samples. Both of these kits are commercially available and are licensed for clinical use. The Determinar TBGL test is a

Table 1 Results for multiple-antigen ELISA and clinical backgrounds of the 69 patients.

Serum number	Gender	Age, years	Multiple ELISA	Determinar TBGL	Myco-Dot	Smear	Culture	PCR	TST	Clinical diagnosis	Type	Comments
1	M	72	–	–	+	–	–	ND	ND	rTB	bIII3	Asthma
2	F	43	+	–	–	–	–	+	+	pTB	O	Lymphadenitis-TB
3	M	25	+	+	–	1+	+	+	+	pTB	IIII1	No BCG vaccination
4	M	51	–	–	+	2+	+	+	+	pTB	rII2	HB carrier
5	M	53	+	+	+	3+	+	+	ND	rTB	bI3	
6	M	25	+	+	–	±	+	–	+	pTB	IIII1	Pleuritis (left)
7	M	42	+	+	–	2+	+	+	+	pTB	IIII2	Hepatitis C
8	M	60	–	–	–	±	+	–	+	pTB	bIII2	
9	F	32	+	–	–	1+	+	+	+	pTB	III1	
10	M	88	+	+	–	3+	+	+	–	pTB	bIII3	Fulminant-TB
11	M	51	+	+	–	2+	+	+	+	rTB	bIII1	DM and MDR-TB
12	M	83	+	–	–	±	+	+	+	pTB	bIII1	DM-TB
13	F	25	–	–	–	–	+	+	+	pTB	rIII1	Non-sputum
14	M	48	–	–	–	±	+	+	+	pTB	bII2	Pleuritis (right)
15	M	33	–	–	–	–	–	–	+	pTB	rIII1	Pleuritis (right)
16	M	25	+	+	+	–	+	–	+	pTB	bIII1	Pleuritis (left)
17	M	85	+	+	–	2+	+	–	+	pTB	bII2	Stomach ulcer resection
18	M	37	+	–	–	–	+	+	+	pTB	rIII1	
19	M	79	+	+	+	2+	+	+	–	pTB	IIII1	Pleuritis (right), fulminant-TB
20	M	55	+	+	+	1+	–	+	+	rTB	bIII2	
21	M	35	–	–	–	–	+	–	+	pTB	bIII1	
22	M	42	+	+	–	–	–	–	+	pTB	IIII1	Pleuritis (left), *M. avium* isolated
23	M	52	+	–	–	2+	+	+	+	pTB	bIII1	Pleuritis (left)
24	F	32	+	+	–	2+	+	+	+	rTB	rIII2	
25	M	25	–	–	–	2+	+	+	+	rTB	rII2	
26	M	20	+	+	+	±	+	–	+	rTB	bII2	
27	F	31	–	+	+	3+	+	+	+	pTB	bII2	
28	M	88	–	–	–	1+	+	+	–	pTB	IIII1	Pleuritis (left)
29	M	21	–	–	–	–	+	–	+	pTB	III1	MDR-TB
30	M	20	+	+	+	±	+	–	+	rTB	bII2	
31	M	61	+	–	–	±	–	–	+	rTB	bII2	Pleuritis (right), DM and MDR-TB
32	M	45	+	+	–	3+	+	+	+	pTB	bIII2	
33	F	32	–	–	–	3+	+	+	+	pTB	bIII2	
34	F	83	–	–	+	2+	+	+	+	pTB	IIII2	
35	M	67	–	+	+	2+	+	+	+	rTB	bII1	DM-TB
36	M	37	+	+	+	–	+	–	+	rTB		Aspergillosis
37	M	62	+	+	+	2+	+	+	+	pTB	rII2	DM-TB
38	M	69	–	–	–	2+	+	+	+	pTB	rIII2	
39	F	25	+	+	–	2+	+	+	+	pTB	rII1	Hepatitis A
40	M	51	+	–	–	±	+	+	+	pTB	bII2	
41	F	32	+	+	–	3+	+	+	+	pTB	III2	
42	M	53	+	+	–	3+	+	+	+	pTB	III2	
43	M	48	+	–	+	±	+	+	+	rTB	rIII2	MDR-TB
44	M	25	+	+	+	–	+	+	+	rTB	bII2	MDR-TB
45	M	29	+	+	–	3+	+	+	+	pTB	rII2	
46	M	76	+	+	–	1+	+	+	+	pTB	bIII2	Pleuritis (left), DM-TB
47	F	21	+	+	–	1+	+	+	+	pTB	bIII3	Pleuritis (right and left)
48	M	72	+	+	–	3+	–	+	ND	pTB	bII2	Fulminant-TB
49	M	52	+	+	+	3+	+	+	+	pTB	bII3	DM-TB
50	M	48	+	+	+	2+	+	+	+	rTB	bII2	MDR-TB
51	F	21	+	+	–	1+	+	+	+	pTB	bIII3	Pleuritis (right and left)
52	M	63	+	+	+	1+	+	+	+	pTB	bIII3	Stomach resection, brain infarct
53	F	88	+	–	–	1+	+	+	+	pTB	bIII1	Pericarditis-TB

(Table 1 continued)

Serum number	Gender	Age, years	Multiple ELISA	Determinar TBGL	Myco-Dot	Smear	Culture	PCR	TST	Clinical diagnosis	Type	Comments
54	M	75	+	–	–	3+	+	+	+	pTB	bIII2	Pleuritis (right), pyothorax
55	M	38	+	+	–	3+	+	+	–	pTB	bII3	Otitis media-TB
56	M	28	+	+	+	1+	+	ND	+	pTB	bIII1	Pleuritis (right)
57	M	35	–	–	–	–	+	–	+	pTB	rIII1	
58	M	39	+	–	–	2+	+	ND	+	pTB	bII2	
59	M	46	–	–	–	2+	+	+	+	pTB	rIII2	DM-TB
60	M	59	+	+	+	3+	+	+	ND	pTB	bII3	MDR-TB
61	F	20	–	–	–	–	–	–	+	pTB	rIII1	MDR-TB
62	M	65	–	–	–	–	–	–	–	pTB		Pleuritis (low ADA)
63	M	74	+	+	–	3+	+	+	+	rTB	bI3	
64	M	92	–	–	–	1+	+	+	+	pTB	bIII2	Lung emphysema
65	M	63	+	+	–	2+	+	+	+	pTB	bII2	Pleuritis (left)
66	M	84	+	+	+	2+	+	+	ND	pTB	bII2	
67	F	90	+	–	–	2+	+	+	+	pTB	bIII1	
68	M	45	+	+	+	2+	+	ND	+	pTB	III2	MDR-TB
69	M	60	+	+	–	2+	+	+	+	rTB	rII2	MDR-TB

Type: pathological lesion by chest X-ray examination described in the text. ND, not determined; rTB, relapsed tuberculosis; pTB, primary tuberculosis; DM, diabetes mellitus; MDR, multiple drug-resistant.

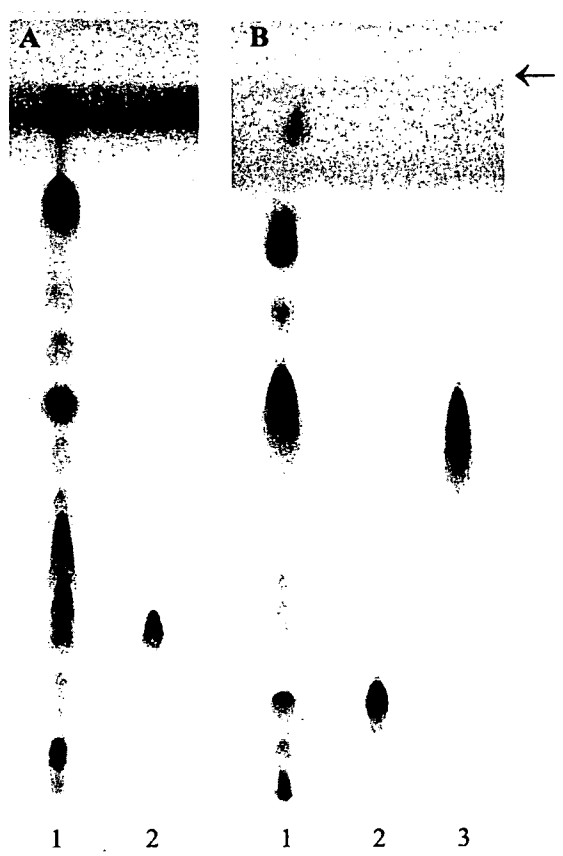

Figure 1 Thin layer chromatograms of lipids from *M. bovis* BCG Tokyo 172. (A) Lane 1, crude phospholipid fraction; lane 2, Ac-PIM_2; developing solvent, chloroform/methanol/water (65:25:4, by vol.), single development. (B) Lane 1, crude mycoloyl glycolipid fraction; lane 2, TMM-T; lane 3, TDM-T; developing solvent, chloroform/methanol/water (90:10:1, by vol.), single development. Visualization by 9 M H_2SO_4 spray, followed by heating at 200°C for 15 min. The arrow indicates the solvent front.

plate ELISA using trehalose dimycolate from *M. tuberculosis* $H_{37}Rv$ as the main antigen, while the MycoDot test involves immunoblotting using a single antigen, lipoarabinomannan (LAM). Antibody assays were performed essentially according to the manufacturers' instructions.

Classification of pathological lesions based on chest X-rays

The size and severity of pathological lesions with or without a cavity were classified based on chest X-rays, according to the Gakken classification of pulmonary tuberculosis. The extent of the disease and lesions, including exudative, caseous, fibrotic, disseminated or far-advanced mixed lesions, were classified as: 0, no lesion; I, largest lesion (larger than one side of the lung lobes); II, medium lesion (between I and III); or III, small lesion (less than one-third of one side of the lung lobes). The side of the lungs involved was classified as: r, right lobe only; l, left lobe only; or b, both lung lobes. Severity was classified as: 1, unstable lesion without a cavity confined to one-third of one lobe; 2, lesion limited to one side, with or without a cavity; or 3, lesion with a cavity larger than one lobe. For instance, bI3 indicates a large caseous lesion with large cavities over both lung lobes, and rIII1 indicates a small but unstable caseous and fibrotic lesion without a cavity confined to one-third of the right lobe.

Data analysis

The titer of serum IgG antibodies against Ac-PIM_2, TDM-T and TMM-T is expressed as the absorption index, equal to the absorption value of the test serum sample minus that of the same serum sample in wells without coated antigen.

Results

Mycobacterial lipid antigens used for ELISA

MALDI-TOF mass analysis showed that Ac-PIM_2 derived from *M. bovis* BCG Tokyo 172 possessed $C_{16:0}$ and branched-chain C_{19} (tuberculostearic) fatty acids. TMM-T derived from *M. bovis* BCG Tokyo 172 is a

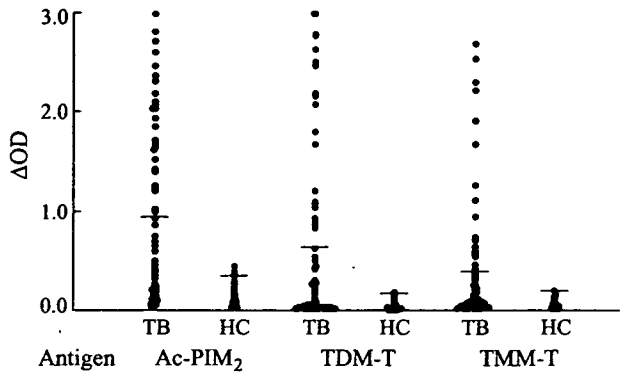

Figure 2 Scattergrams of IgG antibody titers for TB patients and healthy controls. TB, TB patient sera (n=69); HC, healthy control sera (n=48); Ac-PIM_2, monoacyl phosphatidylinositol dimannoside; TDM-T, trehalose 6,6'-dimycolate; TMM-T, trehalose 6-monomycolate. The horizontal line for TB shows the average IgG antibody value (Ac-PIM_2, 0.909; TDM-T, 0.613; and TMM-T, 0.390) and HC shows the cut-off value (Ac-PIM_2, 0.350; TDM-T, 0.146; and TMM-T, 0.152).

trehalose monomycolate possessing one molecule of α-, methoxy- or keto-mycolic acid ranging from C_{76} to C_{91} (the latter two subclasses predominated) with a molecular weight of 1100–1320 Da, while TDM-T from the same strain is a trehalose dimycolate possessing two molecules of mycolic acid from the above subclasses or molecular species, thus making a complex mixture of the trehalose diesters of mycolic acid, with the molecular weight ranging from 2660 to 2920 Da.

IgG antibody responses of 69 patients who first visited the hospital

Table 1 shows the results of IgG antibody responses against three mycobacterial lipid antigens in suspected TB patients on their first visit to the hospital and their clinical backgrounds. Most of the TST-positive or -negative patient sera were reactive against one or more lipid antigen(s), although the seropositive rate against all three antigens was only 23.2%. The distribution of IgG antibody titers for suspected TB patients and healthy controls are shown in scattergrams in Figure 2; both groups were clearly discriminated. Of the three antigens, IgG antibody levels to Ac-PIM_2 were the highest, followed by those to TDM-T and TMM-T. As shown in Table 2, patient sera against all three antigens showed considerable reactivity, with 59.4% for Ac-PIM_2, 46.4% for TDM-T and 42.0% for TMM-T. When the tests were combined and an overall positive was scored if any one or more of the tests were positive, a positive result was obtained in 71.0%. To evaluate the total (cumulative) sensitivity, we compared the positive rates with the Determinar TBGL (tuberculous glycolipid antigen) and MycoDot (lipoarabinomannan antigen) tests, both of which are commercially available. In the same group of suspected TB patients, the positive rate with the Determinar TBGL test was 56.5% and that with MycoDot test was 31.9%, lower than our multiple-antigen ELISA, indicating that the latter is superior to any single-antigen ELISA for the clinical diagnosis of TB.

Diverse responsiveness of IgG antibody

To clarify the reason for the superiority of the multiple-antigen ELISA using mycobacterial lipid antigens, we carefully compared the IgG antibody response patterns or types of each patient serum against each of the three lipid antigens. There was extreme diversity in the patterns or types of IgG antibody responses against each lipid antigen in sera from suspected TB patients, as shown in Figure 3. Most of the smear-positive or -negative TB patient sera were more or less reactive against all the antigens (Ac-PIM_2, TDM-T and TMM-T) (Figure 3 A,B), but some were only poorly reactive or negative (Figure 3 K). Some were reactive against only mycoloylglycolipids (TDM-T and/or TMM-T; Figure 3 C,D) or phospholipid (Ac-PIM_2; Figure 3 E) and some were reactive against two of the three antigenic lipids (Ac-PIM_2 and TDM-T; Ac-PIM_2 and TMM-T; or TDM-T and TMM-T; Figure 3 F–J). The IgG antibody response pattern suggests that the diverse reactivity of sera from active TB patients against particular lipid antigens was due to the clinical background, such as the stage after onset of the disease or the individual antigen recognition mechanism regulated genetically, although this mechanism should be clarified.

Effect of amount of acid-fast bacilli excreted on IgG antibody responses

Since the IgG antibody levels in sera from active TB patients varied diversely against various mycobac-

Table 2 Sensitivity and specificity of the multiple-antigen ELISA in 69 patients.

Antigen	Multiple-antigen ELISA		Determinar TBGL	MycoDot
	Patients	Controls	Patients	Patients
	Positive/total (sensitivity, %)	Negative/total (specificity, %)	Positive/total (sensitivity, %)	Positive/total (sensitivity, %)
Ac-PIM_2	41/69 (59.4)	44/48 (91.7)		
TDM-T	32/69 (46.4)	46/48 (95.8)		
TMM-T	29/69 (42.0)	47/48 (97.9)		
Total	49/69 (71.0)	41/48 (85.4)	39/69 (56.5)	22/69 (31.9)

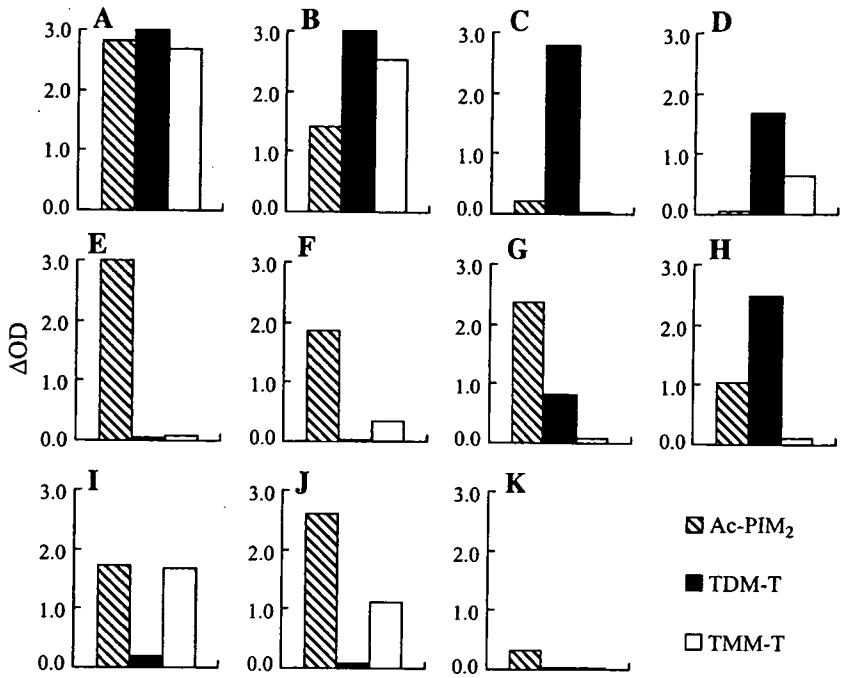

Figure 3 Diverse IgG antibody response patterns in sera from TB patients. Serum numbers: (A) 19; (B) 23; (C) 39; (D) 48; (E) 5; (F) 10; (G) 60; (H) 55; (I) 42; (J) 36; and (K) 62. Detailed clinical backgrounds are listed in Table 1. All ELISA conditions were the same and are described in the text.

terial lipid antigens, we compared the positive rate and average IgG antibody titers against each single and total (multiple) antigens, according to the degree of antigen stimulation. In general, the positive rate and average IgG antibody titers against each lipid antigen were paralleled by the amount of acid-fast bacilli excreted, based on the smear test. Patients who excreted more acid-fast bacilli in sputum on first examination clearly showed higher positive rates and higher average titers in IgG antibody responses (Table 3). Anti-TDM-T IgG antibody responses showed a marked difference in positive rate and average IgG antibody titers between smear-negative (–) and -positive (± and +1) or high smear score (+2 and +3) groups. In contrast, anti-TMM-T IgG antibody responses showed a smaller difference in positive rate and average IgG antibody titers between smear-negative (–) and -positive (± and +1) groups. These results indicate that the elevation of IgG antibody titers against mycobacterial lipid antigens, especially those against TDM-T, is strongly related to the amount of acid-fast bacteria excreted.

Effect of the extent of pathological lesions (cavity-positive or -negative) on IgG antibody responses

We compared the positive rate and average IgG antibody titers against each single and total (multiple) antigens for patient groups with different degrees of pathological lesion by chest X-ray examination. Generally, patients with larger or more severe pathological lesions showed higher positive rates and higher average titers for IgG antibody responses (Table 4). In the case of anti-TDM-T IgG antibodies, there was a marked difference in the positive rate and average IgG antibody titer between cavitary (I and II) and non-cavitary (III) groups. In the case of anti-Ac-PIM_2 and anti-TMM-T IgG antibodies, there were smaller, but

Table 3 Effect of excreting amount of acid-fast bacteria on positive rate and IgG antibody levels.

Antigen		Bacterial burden smear			
		–	± and +1	+2 and +3	Total
Ac-PIM_2	Positive/total	5/14	11/20	25/35	41/69
	Sensitivity, %	35.7	55.0	71.4	59.4
	ΔOD	0.527±0.754	0.640±0.522	1.214±0.981	
TDM-T	Positive/total	1/14	12/20	19/35	32/69
	Sensitivity, %	7.1	60.0	54.3	46.4
	ΔOD	0.052±0.109	0.473±0.542	0.857±1.068	
TMM-T	Positive/total	5/14	7/20	17/35	29/69
	Sensitivity, %	35.7	35.9	48.6	42.0
	ΔOD	0.166±0.293	0.197±0.196	0.535±0.766	
Total	Positive/total	6/14	16/20	27/35	49/69
	Sensitivity, %	42.9	80.0	77.1	71.0

ΔOD values are mean±SD.

Table 4 Effect of size and severity of pathological lesions on the positive rate and IgG antibody levels.

Antigen		Pathological lesion				
		I + bII (cavity+)	bIII (cavity−)	1 or rIII (cavity−)	Other types/ unknown	Total
Ac-PIM$_2$	Positive/total	21/30	11/20	8/16	1/3	41/69
	Sensitivity, %	70.0	55.0	50.0	33.3	59.4
	ΔOD	1.120±0.952	0.702±0.690	0.744±0.824		
TDM-T	Positive/total	19/30	10/20	2/16	1/3	32/69
	Sensitivity, %	63.3	50.0	12.5	33.3	46.4
	ΔOD	0.828±0.956	0.550±0.803	0.237±0.740		
TMM-T	Positive/total	15/30	9/20	3/16	2/3	29/69
	Sensitivity, %	50.0	45.0	18.9	66.7	42.0
	ΔOD	0.421±0.599	0.362±0.565	0.233±0.655		
Total	Positive/total	24/30	15/20	8/16	2/3	49/69
	Sensitivity, %	80.0	75.0	50.0	66.7	71.0

ΔOD values are mean±SD.

Table 5 Effect of age on the positive rate and IgG antibody levels.

Antigen		Age, years				
		20–34	35–49	50–64	>65	Total
Ac-PIM$_2$	Positive/total	9/21	10/15	13/15	9/18	41/69
	Sensitivity, %	42.9	66.7	86.7	50.0	59.4
	ΔOD	0.520±0.565	0.901±0.829	1.504±0.987	0.870±0.896	
TDM-T	Positive/total	10/21	4/15	10/15	8/18	32/69
	Sensitivity, %	47.6	26.7	66.7	44.4	46.4
	ΔOD	0.545±0.827	0.286±0.625	0.784±0.949	0.704±1.011	
TMM-T	Positive/total	10/21	6/15	6/15	7/18	29/69
	Sensitivity, %	47.6	40.0	40.0	38.9	42.0
	ΔOD	0.219±0.228	0.261±0.302	0.600±0.856	0.453±0.753	
Total	Positive/total	14/21	11/15	13/15	11/18	49/69
	Sensitivity, %	66.7	73.3	86.7	61.1	71.0

ΔOD values are mean±SD.

distinctive differences in the positive rate and average IgG antibody titer between cavitary (I and II) and non-cavitary (III) groups. These results indicate that the elevation of IgG antibody titers against mycobacterial lipid antigens, especially those against TDM-T, is strongly related to the progression of active TB.

Effect of age on IgG antibody responses

We compared the positive rate and average IgG antibody titer against each single and total (multiple) antigens among patient groups with different ages (Table 5). Among four different age groups (20–34, 35–49, 50–64, and >65 years), the total (cumulative) positive rate ranged from 66.7% to 86.7%, with the 50–64 years group showing the highest rate. The group >65 years showed a 61.1% positive rate. Average IgG antibody titers for anti-Ac-PIM$_2$, anti-TDM-T and anti-TMM-T IgG antibodies were highest in the 50–64 years group, while the group >65 years also showed considerably high levels. Therefore, the multiple-antigen ELISA is useful for the diagnosis of active TB in senile patients.

IgG antibody responses of TST-negative or diabetes mellitus-TB patients against mycobacterial lipid antigens

To evaluate humoral antibody responses against mycobacterial lipid antigens in patients whose cell-mediated immunity may have been suppressed, we estimated IgG antibody titers against mycobacterial lipid antigens in TST-negative patient sera. Three of five TST-negative TB suspects who had a positive or negative smear and marked chest X-ray lesions showed a positive result in the multiple-antigen ELISA

Table 6 IgG antibody responses in TST-negative TB patients.

Serum number	IgG antibodies against			Total	TST	
	Ac-PIM$_2$	TDM-T	TMM-T		Induration	Erythema
10	+	−	+	+	0	0
19	+	+	+	+	0	0
28	−	−	−	−	0	0
55	+	+	−	+	0	17×11
62	−	−	−	−	0	4×3

Table 7 IgG antibody responses in diabetes mellitus TB patients.

Serum number	IgG antibodies against			Total	TST	
	Ac-PIM_2	TDM-T	TMM-T		Induration	Erythema
11	+	+	+	+	47×32	69×44
12	–	+	–	+	25×23	26×26
31	+	–	–	+	22×22	22×22
35	–	–	–	–	35×26	40×29
37	+	+	+	+	10×10	13×18
46	+	+	+	+	30×22	35×27
49	+	+	–	+	4×4	8×8
59	–	–	–	–	13×12	13×12

(Table 6). Furthermore, six of eight diabetes mellitus-TB patients who also had a distinctive pathological lesion showed a positive result (Table 7). Taken together, and differing from the TST based on cell-mediated immunity, humoral IgG antibody responses against mycobacterial lipid antigens seem to show greater correlation to the bacterial burden and disease progression and thus are more sensitive for the diagnosis of active TB disease.

Discussion

The immunological responses of human patients infected with *M. tuberculosis* have been extensively investigated from the aspect of cell-mediated immunity, and the TST has long been used as the most useful tool for the immunodiagnosis of TB infection. However, it has been established that only 5–10% of TB-infected individuals develop the active disease. Most young people are given the BCG vaccination before school age in Japan, and the TST cannot differentiate between latent TB infection and BCG vaccination. Furthermore, once infected with *M. tuberculosis*, TST positivity continues for a long time and therefore the test is not useful to differentiate recent infection or old TB. TST cannot differentiate the *Mycobacterium* species involved, such as *M. tuberculosis*, *M. avium* complex or BCG vaccination because of the high cross-reactivity of protein antigens, and thus it is not suitable for the diagnosis of active TB disease. Modern anti-TB chemotherapy is effective in curing the active disease if initiated properly in the early stages of the disease and chemoprophylaxis is vital for high-risk contact groups who might develop the active disease without such therapy; thus, proper diagnosis using a reliable diagnostic tool is essential. Furthermore, TST may affect the immunological status of patients when it is repeatedly applied due to the booster effect.

Studies on serodiagnosis in human patients infected with *M. tuberculosis* have long been carried out since Arloing first reported on the agglutination test in 1898 (17). However, owing to the low positive rate and low specificity of humoral responses to purified protein derivative (PPD) and other protein antigens (3, 8), the diagnostic value of such responses has not been thoroughly evaluated in the clinical field. Recent developments in serodiagnostics using multiple-protein antigens have yielded considerable improvements in sensitivity and specificity for the early diagnosis of TB disease (18, 19). However, the overall sensitivity of a multi-antigen ELISA test with 10 protein antigens (combi-10) has been reported to be at 70% at best (18).

Humoral immune responses in human TB infection against lipid antigens are poorly understood, despite the large amount and various types of lipid molecules constructed in the surfaces of mycobacterial cell walls.

In the present study, we expected that a combination of appropriate lipid antigens would give much higher sensitivity for the diagnosis of active TB in both smear-positive and -negative cases. We selected and combined three representative lipid antigens from *M. bovis* BCG Tokyo 172, and when an overall positive was scored if any one of the three tests was positive, markedly higher sensitivity was obtained, although the total (cumulative) sensitivity of IgG antibody responses did not reach 100%. However, IgG antibody positive rates for TB patient sera against the three lipid antigens in the present study showed higher values than the rates for Determinar TBGL and MycoDot tests in the same patient population.

In our previous study, we first reported that anti-*M. tuberculosis* cord factor IgG antibody titers were significantly elevated in active TB patient sera (11) and demonstrated that the detection of anti-cord factor antibody was applicable to the early diagnosis of active pulmonary TB (12), colon TB (20) and later TB uveitis (21). A TBGL (tuberculous glycolipid) antibody detection system using mainly cord factor with minor glycolipid antigens derived from *M. tuberculosis* $H_{37}Rv$ has been developed (22) and was clinically evaluated in a multicenter study to be useful for the diagnosis of TB in the early stages of the disease (23). The cord factor of *M. bovis* BCG Tokyo 172 possesses similar subclasses and molecular species of mycolic acid (α-, methoxy- and keto-mycolates) to those of *M. tuberculosis*, and the preparation of lipid antigens from *M. bovis* BCG Tokyo 172 is much easier than for those from *M. tuberculosis*. Therefore, *M. bovis* BCG Tokyo 172 cord factor (TDM-T) is useful as a diagnostic antigen for mycobacteriosis, including TB. It was especially noted that the elevation of IgG antibody titers against particular lipid antigen differed greatly and individually. Some patients showed high positivity against all three antigens generally. However,