production of proinflammatory cytokines, such as IL-12 and IFN-γ [6–8]. TDM has also been indirectly implicated in the inhibition of phagosome–lysosome fusion, because it inhibits fusion between phospholipid vesicles in vitro [9]. Collectively, these support a role for TDM not only in macrophage activation, but also in mycobacterial survival within infected macrophages, perhaps as a result of phagosome arrest [10]. Thus, TDM participates in the pathogenicity of *M. tuberculosis*, such as the ability to escape killing by ingested macrophages and induce DTH.

DTH is defined as an immune response in which T cell-dependent macrophage activation and inflammation cause tissue damage. DTH is a common accompaniment of protective cell-mediated immunity against intracellular pathogens including *M. tuberculosis* [11]. The expression of DTH and cell-mediated immunity is dependent on the macrophage–cytokine-type 1 helper T (Th1) lymphocyte axis. Cytokines, IL-12 and IFN-γ play a critical role in the process and IL-12-activated signal transducer and activator of transcription (STAT) 4 are required for the development of fully functional Th1 cells. A transcription factor called T-bet that is induced by IFN-γ also plays an essential role in Th1 cell development. Similarly, IL-4-activated STAT6 is essential for the differentiation of Th2 cells [12,13]. STAT4 is required for promoting Th1 development and for defense against mycobacterial infection with *Mycobacterium avium* [14], *Mycobacterium leprae* [15], and *M. tuberculosis* [16].

To clarify host responses to mycobacterial TDM, including development of DTH and granulomas in association with STAT4 activation, we have analyzed footpad reaction, histopathology and cytokine profile of experimental granulomatous lesions using mice lacking STAT4 protein.

## 2. Results

### 2.1. Decreased DTH responses to TDM in mice genetically deficient STAT4 protein

DTH response to TDM was significantly decreased in STAT4-deficient mice immunized with Freund's complete adjuvant (FCA) (1.78 mm ± 0.25), when compared to preimmunized BALB/c mice (2.46 mm ± 0.32) (Fig. 1). However, mice challenged with Freund's incomplete adjuvant (FIA) revealed very mild swelling and no difference was observed in preimmunized BALB/c (0.23 mm ± 0.14) and STAT4-deficient mice (0.23 mm ± 0.09). Histopathologically, mononuclear cells were infiltrated markedly in the dermis, epidermis and perivascular region in BALB/c mice challenged with TDM, whereas mild to moderate infiltration was observed in STAT4-deficient mice (Fig. 2). BALB/c and STAT4-deficient mice challenged with FIA showed negligible tissue response. Thus, DTH response to TDM was depressed in STAT4-deficient mice.

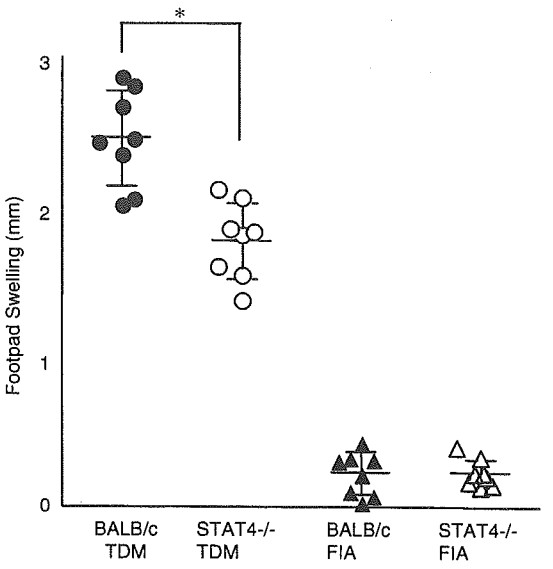


Fig. 1. Footpad DTH responses in BALB/c and STAT4-deficient mice. Mice were immunized with Freund's complete adjuvant (FCA). Eight days after immunization, 20 μl of either TDM (1 mg/ml) or Freund's incomplete adjuvant (FIA) was injected into the hind footpad. The difference of footpad thickness at 24 h after and just before injection was measured. Data represent the mean swelling (mm) ± SD compiled from eight mice per group. The asterisk indicates $P < 0.01$ when compared to BALB/c mice.

### 2.2. Semiquantitative analysis of granulomatous inflammation of the lungs

It has been demonstrated that w/o/w micelles containing TDM are trapped in alveolar vessels and induce granulomatous inflammation in the lungs [4,5]. To clarify the role of STAT4 in the development of granulomatous inflammation by TDM challenge, 100 μg of TDM was injected intravenously to either BALB/c or STAT4-deficient mice preimmunized with FCA. A significant increase in lung indices and granuloma areas was found in immunized BALB/c mice 3–7 days after the challenge with TDM, compared with STAT4-deficient mice (Fig. 3). A marked increase was found within 3 days, reached the maximum by day 7 in BALB/c mice and by day 3 in STAT4-deficient mice, and gradually declined thereafter. Challenge with w/o/w vehicles alone resulted in the negligible response and no significant difference was observed in BALB/c and STAT4-deficient mice.

### 2.3. Histopathologic features of the lungs

In both BALB/c and STAT4-deficient mice preimmunized with FCA, mild infiltration of inflammatory cells in alveolar and perivascular areas was seen 1 day after TDM challenge. Three to seven days after the challenge,

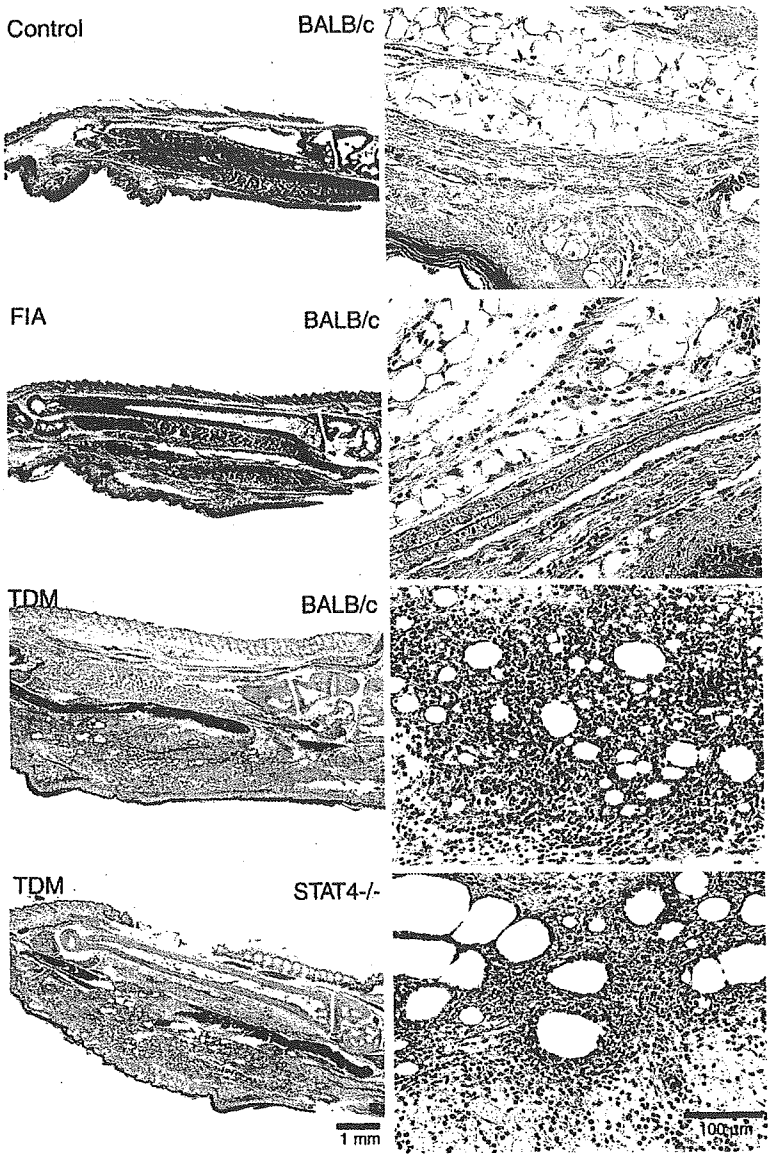


Fig. 2. Histopathologic features of footpads challenged with antigens. Eight days after immunization with FCA, 20 μl of either TDM (1 mg/ml) or FIA was injected into the hind footpad. FCA-preimmunized mice without footpad challenge served as controls. Mononuclear cells were infiltrated markedly in the dermis, epidermis and perivascular region in BALB/c mice challenged with TDM, whereas mild to moderate infiltration was observed in STAT4-deficient mice by TDM challenge.

both BALB/c and STAT4-deficient mice showed randomly distributed, well-organized granulomas composed of epithelioid macrophages and lymphocytes (Fig. 4). The lesion was more prominent in BALB/c mice than STAT4-deficient mice. The lesions were subsided thereafter and diminished 14 days after the challenge. In contrast, the challenge with w/o/w vehicles alone did not lead to the development of granulomatous inflammation (data not shown). These findings were consistent with semiquantitative analyses of granulomatous inflammation (Fig. 3).

### 2.4. Protein expression of cytokines and chemokine in the lungs

We next examined the protein expression of proinflammatory cytokines and chemokine in the lungs of BALB/c and STAT4-deficient mice challenged with TDM (Fig. 5), because TDM challenge resulted in granulomatous inflammation (Figs. 3 and 4). In both BALB/c and STAT4-deficient mice, the protein expression of proinflammatory cytokines and cc-chemokine, such as IL-1β, tumor necrosis factor (TNF)-α, and MIP-1α, was detectable within 1 day,

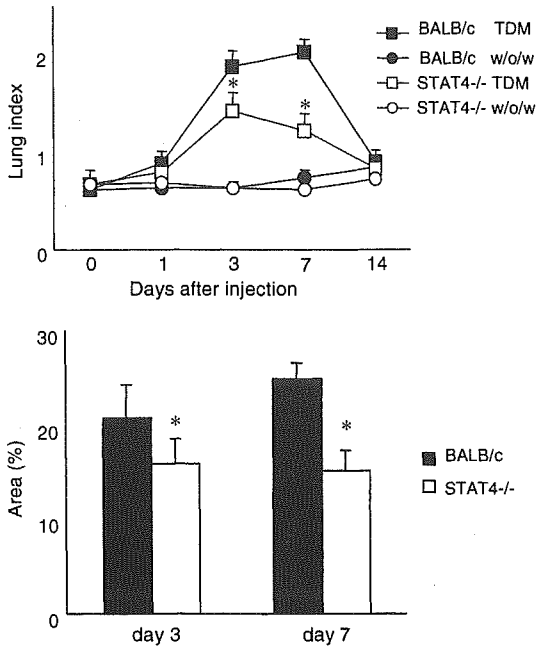


Fig. 3. Semiquantitative analysis of granulomatous inflammation of the lungs. One hundred microgram of TDM was injected intravenously to both BALB/c and STAT4-deficient mice preimmunized with FCA. As controls, w/o/w micelles without TDM were used. The lung index was calculated as lung weight × 100/body weight. The areas of granulomatous inflammation were measured using the image analysis program, and quantitated as a percentage value for the areas of lesional lungs. Data represent the mean ± SD compiled from three to four mice per group. The asterisks indicate $P < 0.05$ compared to preimmunized BALB/c mice challenged with TDM.

and reached a peak by 3 days after the challenge. A rapid decrease in such cytokines and chemokine was observed 7–14 days after the challenge. The similar kinetics and amount of cytokines and chemokine were observed in both BALB/c and STAT4-deficient mice. However, no detectable proinflammatory cytokines and chemokine were observed in the lung extract prepared from preimmunized BALB/c and STAT4-deficient mice challenged with w/o/w vehicles alone. Thus, the profile of proinflammatory cytokines and chemokine reflected the histopathologic feature of granulomatous inflammation. In contrast, IL-12 p70 and IFN-γ were detectable only 3 days following TDM challenge in both BALB/c and STAT4-deficient mice and rapidly declined thereafter. Although there was no significant difference of IL-12 p70 expression between BALB/c and STAT4-deficient mice, IFN-γ expression was significantly reduced in STAT4-deficient mice, when compared to BALB/c mice (Fig. 5). We could not detect IL-4, a Th2 cell-derived cytokine, in both BALB/c and STAT4-deficient mice throughout the course of these experiments (data not shown). Thus, TDM could favor the dominance of Th1 response characterized by the expression of IL-12 and IFN-γ.

### *2.5. Expression of Th1 cytokines by lymph node cells stimulated with TDM in vitro*

To confirm the polarized Th1 response induced by TDM in vivo, we examined protein expression of IL-12 p40, IFN-γ, and IL-4 by lymph node cells from mice stimulated with TDM in vitro. Axillary lymph node cells were prepared 8 days following the immunization with FCA. A significant and similar amount of IL-12 p40 was produced by cells of both BALB/c and STAT4-deficient mice and reached the peak at day 3, whereas IFN-γ was only produced by those of BALB/c mice (Fig. 6). However, IL-4 was undetectable in the culture supernatant of lymph node cells stimulated with TDM prepared from both BALB/c and STAT4-deficient mice. As controls, α-galactosylceramide (α-GC) and sulfolipid (SL) were used. α-GC is an antigen for CD1d-restricted NKT cells [17] and could be induced by both Th1 and Th2 cell-derived cytokines [18]. SL is a mycobacterial cell surface component that is unable to induce granulomatous lesions in vivo [5]. Lymph node cells stimulated with α-GC produced both IFN-γ and IL-4, but not IL-12 p40, in a time-dependent manner. IL-4 production by cells from BALB/c mice was significantly increased when compared to STAT4-deficient mice. IFN-γ and IL-4 were not produced by lymph node cells stimulated with SL. In contrast, IL-12 p40 was produced by cells stimulated with SL of STAT4-deficient mice, but not BALB/c mice.

## 3. Discussion

In the present study, we have demonstrated for the first time that mycobacterial TDM selectively induces the Th1 response through the STAT4 signaling pathway, because mice lacking STAT4 protein significantly reduced to develop DTH (Figs. 1 and 2), hypersensitivity granulomas (Figs. 3 and 4), and Th1 cytokine responses (Figs. 5 and 6), when compared to BALB/c mice. Thus, the present study clarifies the molecular pathogenesis of mycobacterial infection, in which the development of Th1 responses plays a central role.

Disease progression to active tuberculosis is dependent on the interplay between bacterial and host factors. The major lipid component of mycobacterial cell wall is mycolic acids, α-branched β-hydroxy long-chain fatty acids (C78–C90). Strains of *M. tuberculosis* have TDM associated with mycolic acids, a surface glycolipid [10]. The pathogenicity of *M. tuberculosis* is related to its ability to induce cell-mediated immunity/delayed-type hypersensitivity. Granulomatous inflammation can be broadly classified as either a hypersensitivity (immunologic, T cell-dependent) type or a foreign-body (non-immunologic, T cell-independent) type [2,11,19]. Our previous studies have demonstrated that mechanisms of granulomatous inflammation in tuberculosis are composed of both foreign-body and hypersensitivity types, because such inflammation induced by either

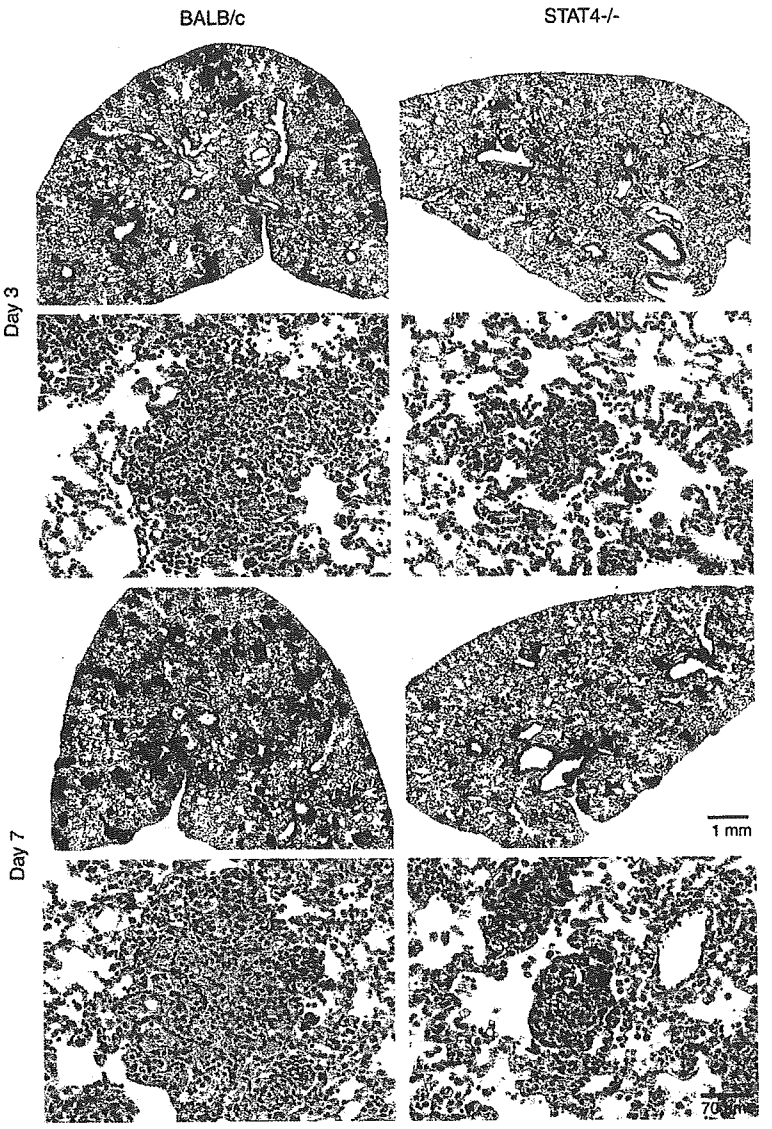


Fig. 4. Histopathologic features of the lungs. Eight days after immunization with FCA, BALB/c or STAT4-deficient mice were injected intravenously with 100 μg of TDM in 100 μl of w/o/w emulsion. Three to seven days after the challenge, both BALB/c and STAT4-deficient mice showed randomly distributed, well-organized granulomas composed of epithelioid macrophages, and lymphocytes. The lesion was more prominent in BALB/c mice than STAT4-deficient mice.

*Mycobacterium bovis* BCG or TDM of *M. tuberculosis* in mice was developed regardless of preimmunization with BCG or *M. tuberculosis*, although augmented lesions were observed in preimmunized mice [4,20].

We have reported previously that hypersensitivity granulomas induced in preimmunized mice were larger than the lesions induced in unimmunized mice and immunized mice challenged with irrelevant antigens [20–22]. The hypersensitivity lesions are augmented by the result from the amplification of specific Th1 immune responses [11,19]. The defect in the IL-12–IFN-γ axis is critical for defense against mycobacterial infection and associated with impaired development of granulomas [23–25]. In the present study, TDM challenge could induce granulomatous inflammation in mice, although BALB/c mice produced more prominent lesions than STAT4-deficient mice (Figs. 3 and 4). It is likely that granulomatous inflammation in STAT4-deficient mice is a foreign-body type lesion, because such mice are depressed to mount the Th1-mediated immune response, including DTH and cell-mediated immunity [13].

In contrast, a similar amount of proinflammatory cytokines (TNF-α and IL-1β) and chemokine (MIP-1α) was detected in granulomatous lesions of BALB/c

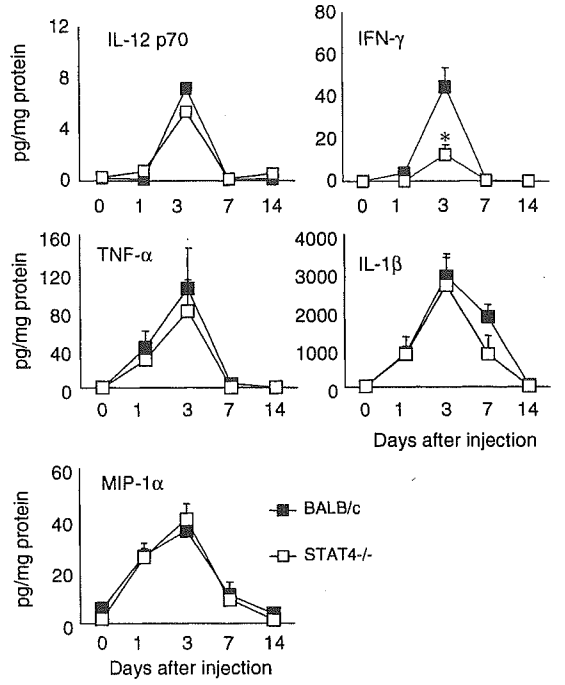


Fig. 5. Protein expression of cytokines and chemokine in the lungs. Data represent the mean ± SD compiled from three to four mice per group. The asterisk indicates $P<0.05$ compared to preimmunized BALB/c mice challenged with TDM.

and STAT4-deficient mice, because these cytokines play a key role in the expression of both foreign-body and hypersensitivity granulomas [4,19,20,26] and they were regulated by nuclear factor κB that is independent on the STAT4 signaling pathway [27,28]. Indeed it has been demonstrated that STAT4-deficient mice can produce a comparable amount of proinflammatory cytokines and chemokines from wild-type mice [29].

It is clear that Th1 responses participate in the development of hypersensitivity granulomas induced by TDM in preimmunized BALB/c mice, because STAT4-deficient mice significantly reduced the expression of DTH, granulomatous inflammation, and Th1 cytokine (IFN-γ) response in vivo and in vitro. The mild and lesional expression of IFN-γ in STAT4-deficient mice (Fig. 5) may represent the STAT4-independent pathway of IFN-γ production [30–32]. It should be noted that there was no significant difference in the level of IL-12 p70 in BALB/c and STAT4-deficient mice challenged with TDM (Fig. 5). In addition, we demonstrated that lymph node cells stimulated with TDM in STAT4-deficient mice significantly reduced production of IFN-γ than those of BALB/c mice, although those from both BALB/c and STAT4-deficient mice produced IL-12 p40, but not IL-4, in a similar manner (Fig. 6). The Th1 cytokine response induced by mycobacterial TDM is mediated through the STAT4 signaling pathway. The production of IFN-γ from lymph nodes cells is TDM-specific, because other stimulants, such as SL and α-GC, showed different patterns of cytokine production. These may be explained by the fact that IL-12 acts the upstream of STAT4 expression in the IL-12-STAT4 cascade/signaling pathway [13].

T, NK and NKT cells are considered to be important as a source of IFN-γ [33,34]. In the present study, we found that the time-kinetics of IFN-γ production from lymph nodes cells stimulated with TDM peaked at day 3 was different from that produced by NKT cells stimulated with α-GC peaked at day 5 (Fig. 6). It has been reported that NK cells induce IFN-γ within 2 days when mouse splenocytes were stimulated with viable *M. bovis* BCG [35]. Taken together with our previous study that TDM induces foreign-body and hypersensitivity granulomas which are mediated through T cells using euthymic and athymic nude mice [4], these results suggest that the induction of IFN-γ by TDM may be dependent on specific T cells. It is an unsolved issue of interaction between TDM and host cells. The receptor molecules related to TDM in humans and mice, and the antigenic epitope of TDM (mycolic acid or trehalose) have not yet been identified, although mycobacterial lipids containing mycolic acid are recognized by CD1b-restricted T cells in humans [36]. Further study will be required to clarify the issue.

In the present study, we have clarified the mechanism of TDM-stimulated expression of IL-12 p70 in vivo, an active form of IL-12, as a Th1 cytokine, although the previous study demonstrated that TDM could induce IL-12 p40 mRNA expression [37]. CD1d-restricted NKT cells stimulated with α-GC produce both IFN-γ and IL-4, and the signaling pathway may be different from the STAT4 pathway, because IL-12 p40 was undetectable by stimulation with α-GC. It has been shown that activation of murine NKT cells by α-GC in vivo results in a Th2 pattern in conventional adaptive immune responses [38,39]. It is interested in the decreased protein expression of IL-4 by lymph node cells stimulated with α-GC in STAT4-deficient mice when compare to those of BALB/c mice (Fig. 6), although the precise mechanism of the decreased expression is not yet elucidated. Further study on the biological effects of SL and α-GC will be required.

Our previous studies have demonstrated that mycobacterial TDM can induce apoptosis [5] and angiogenesis [40]. Taken together, TDM is a pleiotropic molecule against the host and participates in the pathogenesis.

## 4. Materials and methods

### *4.1. Animals*

STAT4-deficient mice with BALB/c background were purchased from The Jackson Laboratory (Bar Harbor, Me.) and bred and maintained. Female STAT4-deficient and BALB/c mice of 6–8-weeks-old were used in all

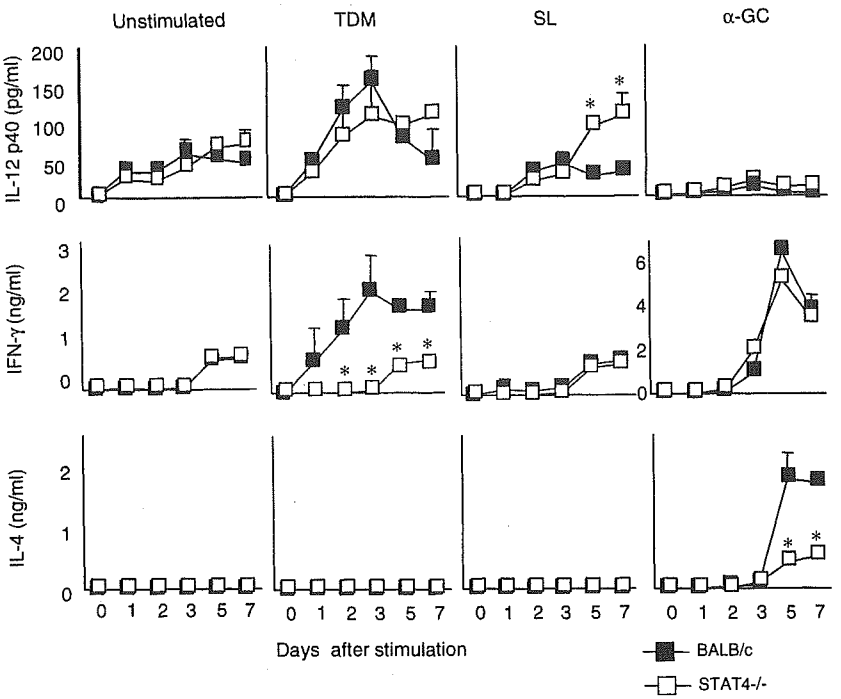


Fig. 6. Expression of Th1 cytokines by lymph node cells stimulated with TDM in vitro. Axillary lymph node cells were prepared 8 days after the immunization with FCA. Cells ($5\times10^6$ cells/ml) were cultured in the presence of 10 μg/well TDM, 10 μg/well SL, and 50 ng/ml α-GC. Results are expressed as mean ± SD compiled from three separate experiments of three to four mice per condition. The asterisk indicates $P<0.05$, compared to preimmunized BALB/c mice challenged with TDM.

experiments. Experiments were conducted according to the standard guidelines for animal experiments of Osaka City University Graduate School of Medicine.

### 4.2. Mycobacterial glycolipids and KRN7000

*M. tuberculosis* Aoyama B was cultivated in Sauton medium for 5–6 weeks at 37 °C. Mycobacteria were autoclaved, disrupted ultrasonically and then suspended in chloroform–methanol (4:1, 3:1, 2:1, vol%) to extract lipids. The chloroform layer was collected and dried. TDM was partially purified by precipitation with acetone, chloroform–methanol (1:2, vol%) and tetrahydrofuran–methanol (1:2, vol%), and follows to passage through a column of silica gel (Wakogel C-200; Wako Pure Chemical, Osaka, Japan) with chloroform–methanol (8:2, vol%). The purity of TDM was checked as a single spot on thin-layer chromatography (TLC). Purified TDM contained less than 6 pg of lipopolysaccharide and 1 ng of protein per 1 mg TDM, as determined by a limulus test (Endospecy, Seikagaku Co., Tokyo, Japan) for lipopolysaccharide and a protein assay kit (Bio-Rad Protein Assay, Bio-Rad, Hercules, CA) for protein. SL was separated by preparative TLC of silica gel (Uniplate; 20×20 cm, 250 μm; Analtech, Inc., Newark, DE). KRN7000, synthetic α-GC, was kindly provided by the Pharmaceutical Research Laboratory (Kirin Brewery, Gunma, Japan) [17].

### 4.3. Immunization and footpad assays for DTH

Mice were immunized by subcutaneous injection of 100 μl of oil-in-water (o/w) emulsion containing FCA into the inguinal region, front footpad, and base of the tail. FCA emulsion was prepared by the mixture of PBS and FCA (1:1, by vol.) that contained heat-killed *M. tuberculosis* Aoyama B in FIA at a concentration of 2 mg/ml [20]. Mice immunized with FIA emulsion were served as controls. Eight days after immunization, mice were challenged with 20 μl of TDM (1 mg/ml) in the form of o/w emulsion containing FIA into the hind footpads. Eight mice were used for each group. Triplicate measurements of footpad thickness were performed with an engineer's micrometer (Mitsutoyo Co., Kanagawa, Japan) before and 24 h after the challenge [4,20]. The difference between the measurements was calculated and expressed as the mean ± standard deviation (SD) in millimeters.

### 4.4. Induction of pulmonary granulomas

To prepare 100 μl of sample, 100 μg of purified TDM was dissolved in 3.2 μl FIA (Difco Laboratories, Detroit, MI) in a teflon grinder. After adding 3.2 μl of 0.1 M phosphate-buffered saline (PBS), a water-in-oil emulsion was made. Then 93.6 μl of saline containing 0.2% Tween 80

was added at the final concentration (3.2%) of FIA, and water-in-oil-in-water (w/o/w) emulsion was made by mixing [4,40]. As controls, w/o/w micelles without TDM were used. Eight days after immunization, mice were injected intravenously with 100 μg of TDM in 100 μl of w/o/w emulsion or w/o/w micelles alone. To exclude the possibility of boosting with repeated TDM exposures, we used two different sets of mice for DTH footpad assays and induction of lung granulomas throughout the study. Previous reports have indicated that a considerable proportion of the increase in lung weight as a consequence of granulomatous inflammation is due to an increase of cellularity in the organ [19,41,42]. The lung index was calculated as follows: lung index = lung weight (g) × 100/ body weight (g). The areas of granulomatous inflammation were measured using the image analysis program (Scion Image, Scion Corp., Frederick, MD). The level of granulomatous inflammation was quantitated by determining the lesional areas within each section that showed a pixel density greater than a threshold value, and was indicated as a percentage value for the areas of lesional lungs.

### 4.5. Histopathology

Routine paraffin-embedded, hematoxylin and eosin-stained sections were prepared from the left lobe of lungs. Footpads were removed 24 h after the challenge with TDM and fixed with 10% formalin. Footpads were then decalcified with Plank–Rychlo's solution containing 0.3 M aluminum chloride, 3% HCl and 5% formic acid (Muto Pure Chemicals Co., Tokyo, Japan).

### 4.6. Protein expression of cytokines and chemokine in the lungs

Aqueous extracts of granuloma-bearing lungs were prepared by the method described previously [4,20]. Briefly, whole lungs (approximately 200–400 mg per lungs) were homogenized in 0.5 ml of PBS using a glass tube with a teflon pestle. Homogenized tissues were then centrifuged at 2000*g* for 30 min, and the pellet was discarded. Protein concentrations of supernatants were determined by a protein assay kit. Aqueous lung extracts contained 2–4 mg of protein per ml of PBS. The contents of cytokines and chemokine were measured by commercially available enzyme immunoassay (EIA) kits for murine IL-1β, IL-12 p40, IL-12 p70, TNF-α, IFN-γ, and macrophage inflammatory protein (MIP)-1α (Genzyme, Minneapolis, Minn.). The sensitivity was <3.0 pg/ml for IL-1β, <4.0 pg/ml for IL-12 p40, <2.0 pg/ml for IL-12 p70, <5.1 pg/ml for TNF-α, <2.0 pg/ml for IFN-γ, <1.5 pg/ml for MIP-1α, according to the manufacturer's instructions. The EIA was conducted in duplicate. The individual data were expressed as the amount per milligram protein in the lung extract.

### 4.7. Culture of lymph node cells in vitro

Eight days after the immunization, axillary lymph nodes were obtained from at least three mice per group and single cell suspensions were prepared in Hanks' balanced salt solution by gently teasing the nodes between the frosted ends of two glass slides. This procedure routinely provided single cell suspensions with 90–95% viability as assessed by trypan blue exclusion. Lymph node cells were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Life technologies, Grand island, NY). TDM or SL dissolved in *n*-hexane was dispersed into 24-well culture plates and the plates were allowed to dry overnight. α-GC dissolved with 0.5% Tween 20/PBS was added to plates. Uncoated plates were served as controls. The optimal concentration of antigens was 10 μg/well; TDM, 10 μg/well; SL, 50 ng/ml; α-GC, that were determined by a dose–responsive study. Lymph node cells ($5 \times 10^6$ cells/ml) were cultured in the plate at 37 °C for 1–7 days. The culture supernatant was assayed for cytokines and chemokine. The EIA was conducted in duplicate.

### 4.8. Statistical analyses

Data were analyzed with a Power Macintosh G3 using StatView 5.0 (SAS Institute, Inc., Cary, NC) and expressed as the mean ± SD. Data that appeared to be statistically significant were compared by an analysis of variance for comparing the means of multiple groups and were considered significant if $P$ values were less than 0.05.

## Acknowledgements

This work was supported by grants from Ministry of Health, Labour and Welfare (Research on Emerging and Re-emerging Infectious Diseases, Health Sciences Research Grants), Ministry of Education, Culture, Sports, Science and Technology, Japan Health Sciences Foundation, Inamori Foundation, Osaka City Urban Research, Osaka Tuberculosis Foundation, and The United States–Japan Cooperative Medical Science Program against Tuberculosis and Leprosy.

## References

[1] Hingley-Wilson SM, Sambandamurthy VK, Jacobs WR. Survival perspectives from the world's most successful pathogen, *Mycobacterium tuberculosis*. Nat Immunol 2003;4:949–55.
[2] Smith I. *Mycobacterium tuberculosis* pathogenesis and molecular determinants of virulence. Clin Microbiol Rev 2003;16:463–96.
[3] North RJ, Jung Y-J. Immunity to tuberculosis. Annu Rev Immunol 2004;22:599–623.

[4] Yamagami H, Matsumoto T, Fujiwara N, Arakawa T, Kaneda K, Yano I, et al. Trehalose 6,6′-dimycolate (cord factor) of *Mycobacterium tuberculosis* induces foreign-body- and hypersensitivity-type granulomas in mice. Infect Immun 2001;69:810–5.
[5] Hamasaki N, Isowa K, kamada K, Terano Y, Matsumoto T, Arakawa I, et al. In vivo administration of mycobacterial cord factor (trehalose 6,6′-dimycolate) can induce lung and liver granulomas and thymic atrophy in rabbits. Infect Immun 2000;68:3704–9.
[6] Indrigo J, Hunter Jr RL, Actor JK. Influence of trehalose 6,6′-dimycolate (TDM) during mycobacterial infection of bone marrow macrophages. Microbiology 2002;148:1991–8.
[7] Indrigo J, Hunter Jr RL, Actor JK. Cord factor trehalose 6,6′-dimycolate (TDM) mediates trafficking events during mycobacterial infection of murine macrophages. Microbiology 2003;149:2049–59.
[8] Perez RL, Roman J, Roser S, Little C, Olsen M, Indrigo J, et al. Cytokine message and protein expression during lung granuloma formation and resolution induced by the mycobacterial cord factor trehalose-6,6′-dimycolate. J Interferon Cytokine Res 2000;20: 795–804.
[9] Spargo BJ, Crowe LM, Ioneda T, Beaman BL, Crowe JH. Cord factor (α,α-trehalose 6,6′-dimycolate) inhibits fusion between phospholipid vesicles. Proc Natl Acad Sci USA 1991;88:737–40.
[10] Karakousis PC, Bishai WR, Dorman SE. *Mycobacterium tuberculosis* cell envelope lipids and the host immune response. Cell Microbiol 2004;6:105–16.
[11] Kobayashi K, Kaneda K, Kasama T. Immunopathogenesis of delayed-type hypersensitivity. Microsc Res Tech 2001;53:241–5.
[12] Alexander WS, Hilton DJ. The role of suppressors of cytokine signaling (SOCS) proteins in regulation of the immune response. Annu Rev Immunol 2004;22:503–29.
[13] O'Shea JJ, Gadina M, Schreiber RD, Ortmann RA, Cheng T, Viscornti R, et al. Cytokine signaling in 2002: new surprises in the Jak/Stat pathway. Cell 2002;109:S121–S31.
[14] Gollob JA, Veenstra KG, Jyonouchi H, Kelly AM, Ferrieri P, Paka DJ, et al. Impairment of STAT activation by IL-12 in a patient with atypical mycobacterial and staphylococcal infections. J Immunol 2000;165:4120–6.
[15] Kim J, Uyemura K, Van Dyke MK, Legaspi AJ, Rea TH, Shuai K, et al. A role for IL-12 receptor expression and signal transduction in host defense in leprosy. J Immunol 2001;167:779–86.
[16] Sugawara I, Yamada H, Mizuno S. Relative importance of STAT4 in murine tuberculosis. J Med Microbiol 2003;52:29–34.
[17] Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, et al. CD1d-restricted and TCR-mediated activation of vα14 NKT cells by glycosylceramides. Science 1997;278:1626–9.
[18] Godfrey DI, Hammond KJ, Poulton LD, Smyth MJ, Baxter AG. NKT cells: facts, functions and fallacies. Immunol Today 2000;21:573–83.
[19] Kobayashi K, Yoshida T. The immunopathogenesis of granulomatous inflammation induced by *Mycobacterium tuberculosis*. Methods 1996;9:204–14.
[20] Kobayashi K, Allred C, Castriotta R, Yoshida T. Strain variation of bacillus Calmette-Guerin-induced pulmonary granuloma formation is correlated with anergy and the local production of migration inhibition factor and interleukin 1. Am J Pathol 1985;119:223–35.
[21] Kobayashi K, Allred C, Cohen S, Yoshida T. Role of interleukin 1 in experimental pulmonary granuloma in mice. J Immunol 1985;134: 358–64.
[22] Sato IY, Kobayashi K, Kasama T, Kaga S, Kasahara K, kanemitsu H, et al. Regulation of *Mycobacterium bovis* BCG and foreign body granulomas in mice by the *Bcg* gene. Infect Immun 1990;58:1210–6.
[23] Cooper AM, Dalton DK, Stewart TA, Griffin JP, Russell DG, Orme IM. Disseminated tuberculosis in interferon γ gene-disrupted mice. J Exp Med 1993;178:2243–7.
[24] Jouanguy E, Doffinger R, Dupuis S, Pallier A, Altare F, Casanova J-H. IL-12 and IFN-γ in host defense against mycobacteria and salmonella in mice and men. Curr Opin Immunol 1999;11:346–51.
[25] Lekstrom-Himes JA, Gallin JI. Advances in immunology. Immunodeficiency diseases caused by defects in phagocytes. N Engl J Med 2000;343:1703–14.
[26] Kasahara K, Kobayashi K, Shikama Y, Yoneya I, Soezima K, Ide H, et al. Direct evidence for granuloma-inducing activity of interleukin-1. Induction of experimental pulmonary granuloma formation in mice by interleukin-1-coupled beads. Am J Pathol 1988;130:629–38.
[27] Baldwin Jr AS. The NF-κB and IκB proteins: new discoveries and insights. Annu Rev Immunol 1996;14:649–81.
[28] Yang L, Cohn L, Zhang DH, Homer R, Ray A, Ray P. Essential role of nuclear factor κB in the induction of eosinophilia in allergic airway inflammation. J Exp Med 1998;188:1739–50.
[29] Lentsch AB, Kato A, Davis B, Wang W, Chao C, Edwards MJ, et al. STAT4 and STAT6 regulate systemic inflammation and protect against lethal endotoxemia. J Clin Invest 2001;108:1475–82.
[30] Cai G, Radzanowski T, Villegas EN, Kastelein R, Hunter CA. Identification of STAT4-dependent and independent mechanisms of resistance to *Toxoplasma gondii*. J Immunol 2000;165:2619–27.
[31] Kaplan MH, Wurster AL, Grusby MJ. A signal transducer and activator of transcription (Stat)4-independent pathway for the development of T helper type 1 cells. J Exp Med 1998;188:1191–6.
[32] Stamm LM, Satoskar AA, Ghosh SK, David JR, Satoskar AR. STAT-4 mediated IL-12 signaling pathway is critical for the development of protective immunity in cutaneous leishmaniasis. Eur J Immunol 1999; 29:2524–9.
[33] Kaufmann SH. How can immunology contribute to the control of tuberculosis? Nat Rev Immunol 2001;1:20–30.
[34] Junqueira-Kipnis AP, Kipnis A, Jamieson A, Juarrero MG, Diefenbach A, Raulet DH, et al. NK cells respond to pulmonary infection with *Mycobacterium tuberculosis*, but play a minimal role in protection. J Immunol 2003;171:6039–45.
[35] Yang J, Kawamura I, Zhu H, Mitsuyama M. Involvement of natural killer cells in nitric oxide production by spleen cells after stimulation with *Mycobacterium bovis* BCG. Study of the mechanism of the different abilities of viable and killed BCG. J Immunol 1995;155: 5728–35.
[36] Dutronc Y, Porcelli SA. The CD1 family and T cell recognition of lipid antigens. Tissue Antigens 2002;60:337–53.
[37] Oswald IP, Dozois CM, Petit JF, Lemaire G. Interleukin-12 synthesis is a required step in trehalose dimycolate-induced activation of mouse peritoneal macrophages. Infect Immun 1997;65:1364–9.
[38] Burdin N, Brossay L, Kronenberg M. Immunization with alpha-galactosylceramide polarizes CD1-reactive NK T cells towards Th2 cytokine synthesis. Eur J Immunol 1999;29:2014–25.
[39] Hashimoto D, Asakura S, Miyake S, Yamamura T, Van Kaer L, Liu C, et al. Stimulation of host NKT cells by synthetic glycolipid regulates acute graft-versus-host disease by inducing Th2 polarization of donor T cells. J Immunol 2005;174:551–6.
[40] Saita N, Fujiwara N, Yano I, Soejima K, Kobayashi K. Trehalose 6,6′-dimycolate (Cord factor) of *Mycobacterium tuberculosis* induces corneal angiogenesis in rats. Infect Immun 2000;68:5991–7.
[41] Behling CA, Perez RL, Kidd MR, Staton Jr GW, Hunter RL. Induction of pulmonary granulomas, macrophage procoagulant activity, and tumor necrosis factor-alpha by trehalose glycolipids. Ann Clin Lab Sci 1993;23:256–66.
[42] Actor JK, Indrigo J, Beachdel CM, Olsen M, Wells A, Hunter Jr RL, et al. Mycobacterial glycolipid cord factor trehalose 6,6′-dimycolate causes a decrease in serum cortisol during the granulomatous response. Neuroimmunomodulation 2003;10:270–82.

CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, Jan. 2005, p. 44–51 Vol. 12, No. 1
1071-412X/05/$08.00+0 doi:10.1128/CDLI.12.1.44–51.2005


# Use of Glycopeptidolipid Core Antigen for Serodiagnosis of *Mycobacterium avium* Complex Pulmonary Disease in Immunocompetent Patients

Seigo Kitada,[1,2]* Ryoji Maekura,[2,3] Naomi Toyoshima,[3] Takashi Naka,[1] Nagatoshi Fujiwara,[1] Masami Kobayashi,[3] Ikuya Yano,[4] Masami Ito,[2] and Kazuo Kobayashi[1]

*Department of Host Defense, Osaka City University Graduate School of Medicine, Abeno-ku,[1] Toneyama Institute for Tuberculosis Research, Toyonaka-shi,[3] and Department of Internal Medicine, Toneyama National Hospital, Toyonaka-shi,[2] Osaka, and Japan BCG Laboratory, Kiyose-shi, Tokyo,[4] Japan*

Received 6 February 2004/Returned for modification 20 May 2004/Accepted 6 October 2004

**We report the development of a serodiagnostic method for *Mycobacterium avium* complex (MAC) disease with an enzyme immunoassay (EIA) with the MAC-specific glycopeptidolipid (GPL) core as the antigen. In this study, we confirmed by EIA that the GPL core antibody was in the sera of immunocompetent patients with MAC disease. The EIA for quantifying the GPL core antibody was evaluated as a clinical tool for serodiagnosis of pulmonary MAC disease. A significant increase in GPL core antibodies (immunoglobulins G, A, and M) was detected in sera of patients with MAC pulmonary diseases when they were compared to patients who were colonized with MAC, patients with *Mycobacterium kansasii* disease or tuberculosis, and healthy subjects. The sensitivities and specificities of the GPL core-based EIA for diagnosis of MAC pulmonary disease were 72.6% and 92.2%, respectively, for IgG, 92.5% and 95.1%, respectively, for IgA, and 78.3% and 91.0%, respectively, for IgM. The best sensitivity and specificity were obtained by measuring immunoglobulin A antibodies against GPL core antigen. The level of GPL core antibodies reflected disease activity, since it decreased in cured MAC patients who had responded to chemotherapy. Measurement of serum antibodies against GPL core is useful for both diagnosis and assessment of disease activity in MAC disease of the lung.**

About 10 to 20% of mycobacterial diseases are caused by nontuberculous mycobacteria. Among nontuberculous mycobacteria, *Mycobacterium avium* and *M. intracellulare* are closely related and commonly grouped to form the *M. avium* complex (MAC). The diagnosis of pulmonary MAC disease is based on a combination of clinical, radiographic, and microbiologic criteria and the exclusion of other diseases that are similar clinically (1). MAC organisms are of low pathogenicity and single positive specimens with low numbers of organisms are frequently recovered from individuals with no apparent disease. The colonization of asymptomatic individuals, the possibility of environmental contamination of specimens, and the absence of standardized skin test antigens for confirming nontuberculous mycobacterial disease all combine to complicate interpretation by physicians of diagnostic tests for nontuberculous mycobacteria. The development of a serodiagnostic test to detect MAC infection is necessary to rapidly and accurately diagnose pulmonary MAC disease.

In a previous study, we reported the characteristics of an enzyme immunoassay (EIA) for MAC pulmonary diseases with a mixture of glycopeptidolipid (GPL) antigens from 11 reference strains of MAC and applied the assay to serodiagnosis of patients with MAC disease (7). However, there are problems with the transition of the assay from a research tool to widespread clinical use. Specifically, preparation of GPL antigen of consistent quality as well as quantity from 11 reference strains of MAC is both time- and cost-consuming. Identification of a simple and stable antigen for use in serodiagnostic tests for MAC disease is necessary. In addition, the natural history of MAC lung disease is unpredictable in immunocompetent patients. Some patients are resistant to multiple drug chemotherapy and show persistent excretion of MAC organisms and a steady worsening of chest radiographic findings until death. Other patients maintain a stable clinical and radiographic picture for years (1).

We have been investigating the relationship between the serotype of MAC isolates and the long-term survival of patients with pulmonary MAC disease. However, it was difficult to accurately identify the serotypes of clinical isolates with the seroagglutination test and thin-layer chromatography (TLC). The GPLs from different clinical isolates are serologically cross-reactive and have similar $R_f$ values on TLC (17). When we used serodiagnosis to identify antibody serotypes against the different types of GPLs in some pulmonary MAC disease patients, we detected antibody against all 11 GPLs in each serum. We hypothesized that the antibody to the GPL core could be the reactive component in the sera of pulmonary MAC disease patients rather than all patients infected by every MAC serotype. Our hypothesis was supported by analysis of GPLs that shows that the fatty acyl-D-Phe-D-allo-Thr-D-Ala-L-alaninol-*O*-(3,4-di-*O*-methyl-Rha), or GPL core, is common to all serotypes (3). Because the GPL core is generally considered

* Corresponding author. Mailing address: Department of Internal Medicine, Toneyama National Hospital, 5-1-1 Toneyama, Toyonaka-shi, Osaka 560-8552, Japan. Phone: 81-6-6853-2001. Fax: 81-6-6853-3127. E-mail: kitadas@toneyama.hosp.go.jp.

TABLE 1. Characteristics of the subjects in a study of EIA for diagnosis of pulmonary MAC disease[a]

| Characteristic | Group | | | | |
|---|---|---|---|---|---|
| | MAC disease[b] | MAC colonization[c] | *M. kansasii* disease | Tuberculosis | Healthy subjects |
| No. | 106 | 11 | 30 | 77 | 126 |
| Mean age (yr) ± SD | 65.4 ± 10.4 | 60.5 ± 15.8 | 52.1 ± 12.7[d] | 50.9 ± 18.6[d] | 47.8 ± 14.3[d] |
| Age range (yr) | 37–86 | 27–80 | 29–80 | 14–83 | 26–88 |
| No. male/no. female | 40/66 | 3/8 | 28/2[d] | 66/11[d] | 70/56[d] |
| Mean duration of disease (yr) ± SD | 3.8 ± 4.3 | 0 | 0.5 ± 0.8[d] | 0.5 ± 1.1[d] | 0 |

[a] All subjects were either seronegative for HIV or had no clinical symptoms consistent with AIDS.
[b] MAC disease included *M. avium* ($n$ = 49), *M. intracellulare* ($n$ = 22), and unclassified strains of MAC ($n$ = 35).
[c] MAC colonization included *M. avium* ($n$ = 4), *M. intracellulare* ($n$ = 3), and unclassified strains of MAC ($n$ = 4).
[d] Statistically significant difference compared to patients with MAC disease ($P < 0.05$).

nonantigenic, it has received less attention than the serologically active polar GPLs that can be identified by immunoassay.

In the present study, we show that the immunodominant epitope of GPLs is the GPL core antigen and we assessed whether an EIA with GPL core antigen is a useful clinical tool for diagnosis of pulmonary MAC diseases. Because whole GPL antigens are not cross-serotypic, it is necessary to prepare a mixture of GPL antigens from different serotypes of MAC (7). By contrast, GPL core antigen is the dominant epitope and cross-reacts with serum antibodies obtained from patients with MAC disease due to different serotypes of the organisms. We have asked whether EIA serodiagnosis with GPL core antigen could differentiate pulmonary MAC disease from MAC colonization and pulmonary tuberculosis and EIA serodiagnostic results could aid in evaluating the effectiveness of the chemotherapy as well as the timing of the future cessation of treatment.

## MATERIALS AND METHODS

**Study subjects.** Sera were obtained from patients with pulmonary disease due to nontuberculous mycobacteria (MAC and *M. kansasii*), pulmonary tuberculosis, individuals with MAC colonization, and healthy subjects (Table 1). They were aliquoted into individual 1.0-ml doses in tubes, stored at −80°C until use, and thawed at room temperature just before the assay. Culture isolates of mycobacteria were identified by biochemical analyses and DNA probes. MAC ($n$ = 106) included *M. avium* ($n$ = 49), *M. intracellulare* ($n$ = 22), and unclassified strains ($n$ = 35). MAC disease was diagnosed according to the criteria of the American Thoracic Society (1). Subjects with a small amount of bacteria in a single positive sputum culture but no symptoms and normal findings on the chest computed tomograph were categorized as being colonized with MAC. Healthy subjects had no history of mycobacterial diseases. There were no subjects that were known to be positive for human immunodeficiency virus type 1 or type 2.

Twenty-seven patients had initially received combination chemotherapy for pulmonary MAC disease with clarithromycin, rifampin, and ethambutol for more than 1 year and/or streptomycin for 2 months according to the recommendation of the American Thoracic Society. Patients whose cultures converted to negative after treatment and whose sputum remained negative on culture for 6 months were categorized as cured. Patients whose cultures did not convert to negative despite treatment were classed as treatment failures. Serum specimens had been obtained sequentially before and after the chemotherapy. Informed consent was obtained from all subjects. This project was approved by the Toneyama National Hospital institutional review board for human subject experimentation and complies with international guidelines for studies involving human subjects.

**Preparation of GPL core antigen.** The 11 reference strains of MAC obtained from the American Type Culture Collection were serotypes 1 (ATCC 15769), 4 (ATCC 35767), 6 (ATCC 35773), 7 (ATCC 35847), 8 (ATCC 35771), 9 (ATCC 35774), 12 (ATCC 35762), 13 (ATCC 35768), and 14 (ATCC 35761), 16 (ATCC 13950), and 20 (ATCC 35764) (5). After culture in Middlebrook 7H9 (Difco, Detroit, Mich.) for 3 weeks, mycobacteria were autoclaved and lyophilized. Lyophilized bacteria were extracted with chloroform-methanol. Alkali-stable lipids were applied to a silica gel column (Analtech, Newark, Del.) and GPLs were eluted with methanol-chloroform. The eluted GPLs were purified repeatedly by one-dimensional thin-layer chromatography (TLC) that was developed with chloroform-methanol-distilled water until a single spot was obtained (2, 3, 7). Subsequently reductive β-elimination of GPL was used to prepare GPL core (11, 15). Briefly, purified GPL was dissolved in ethanol-sodium hydroxide-$NaBH_4$. The reaction mixture was heated, neutralized with acetic acid, and then evaporated. The organic phase was washed, and the resulting GPL core was collected. Both the purity and molecular weight of the GPL core were examined by two-dimensional TLC and by fast atom bombardment-mass spectrometry (FAB-MS) (Jeol, Tokyo, Japan).

**EIA.** EIA was done with slight modifications of the previously published method (9). Briefly, microtiter plates (Nunc Products, Roskilde, Denmark) were coated with 0.5 μg of GPLs and GPL core of *M. avium* serotype 4/well. Serum samples were diluted 40-fold with phosphate-buffered saline containing 1% bovine serum albumin. Diluted serum samples were added, followed by incubation for 1 h at 37°C. Plates were washed, then peroxidase-conjugated F(ab′)2 of goat antibody against human immunoglobulin G (IgG), IgA, or IgM (Sigma, St. Louis, Mo.) was added, and plates were incubated for 2 h at 37°C. Unbound labeled antibody was removed by washing and the substrate, *o*-phenylenediamine dihydrochloride (Sigma), was added. Following color development, the optical densities of the wells on the plates were read for absorbance at 492 nm in a reader (model 550, Bio-Rad Laboratories, Tokyo, Japan). To determine the presence of an immunodominant epitope, inhibition of EIA was done by addition of either the mixture of GPL (7) or GPL core antigen at concentrations ranging from 1 to 5 μg/well. All assays were performed in triplicate and without prior knowledge of the clinical status of the patient.

**Statistical analyses.** All data were analyzed with the statistical analysis software package StatView 5.0 (SAS Institute, Cary, N.C.). Antibody EIA titers in individual patients or patient groups were expressed as the mean ± standard deviation. Further comparisons of data from patient groups were made with analysis of variance and nonparametric analysis. Spearman's correlation coefficient by rank was used to determine the relationship between EIA titers and clinical parameters. The chi-square test was used to determine the relationship between gender and patient group. $P < 0.05$ was considered significant.

## RESULTS

**Purification of GPL core antigens from MAC strains.** One-dimensional TLC analysis of GPL antigens prepared from three serotypes (serotype 4 *M. avium*, and serotypes 16 and 20 *M. intracellulare*) of MAC reference strains showed a single spot, but each had distinct patterns (Fig. 1A), because the oligosaccharides were different in each strain. The two-dimensional TLC analysis of GPL core antigens prepared from serotype 4 (Fig. 1B), serotype 16 (Fig. 1C), and serotype 20 (Fig. 1D) exhibited a single spot that was identical among the strains. These results are consistent with the previous report that the common chemical structure of GPL is composed of C-mycoside, or GPL core (3, 7).

In a subsequent step, purified GPL cores of three MAC strains obtained by two-dimensional TLC were analyzed by

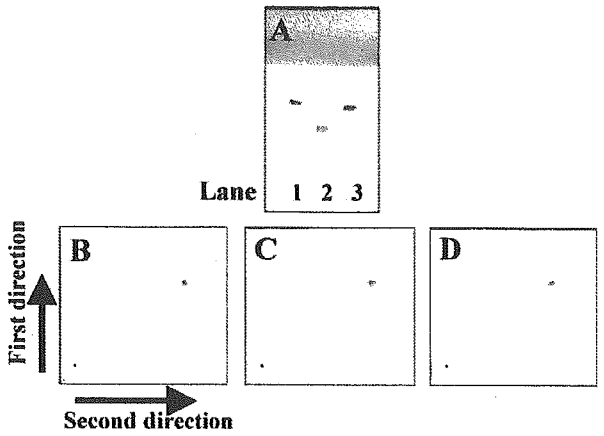


FIG. 1. One-dimensional (A) and two-dimensional TLC analysis (B to D) of GPLs and GPL core. Three reference strains of MAC were analyzed: serotypes 4 (ATCC 35767, lane 1), 16 (ATCC 13950, lane 2), and 20 (ATCC 35764, lane 3). GPLs were purified repeatedly by one-dimensional (1D) TLC developed with chloroform-methanol-distilled water until a single spot was obtained (A). Subsequently GPLs were β-eliminated to obtain GPL core, and then the purity of GPL core was examined by two-dimensional (2D) TLC (first in the vertical and second in the horizontal direction. B, serotype 4; C, serotype 16; D, serotype 20).

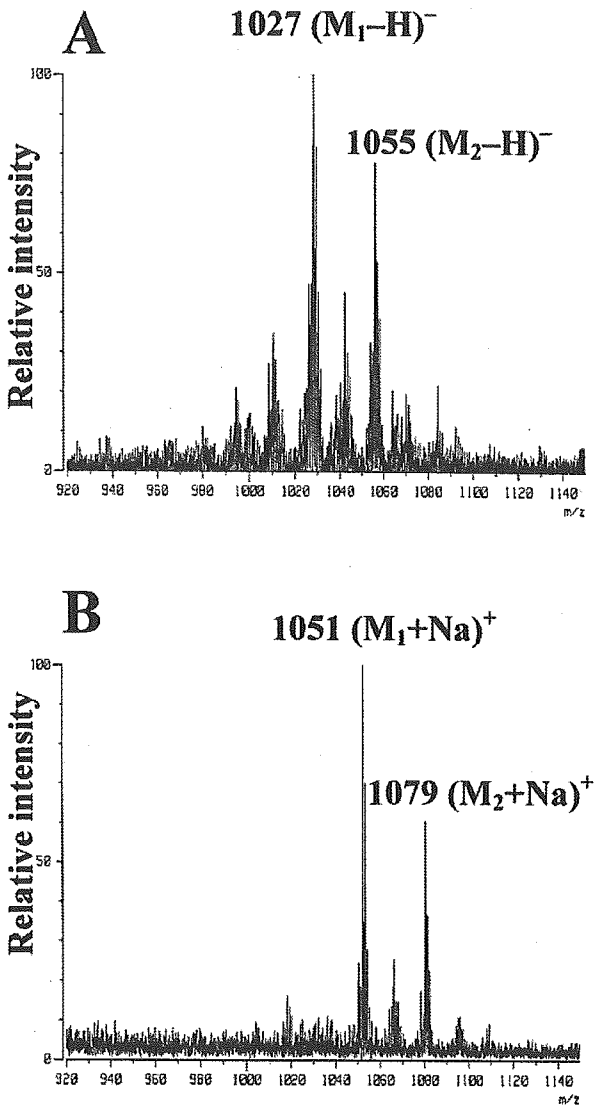


FIG. 2. FAB-MS of purified GPL core. GPL core of serotype 4 showed a main peak at an *m/z* of 1,027, corresponding to the (M-H)- ion in negative-ion mode (A) and an *m/z* of 1,051 corresponding to the (M+Na)+ in positive-ion mode (B). Based on these results, the molecular weight of GPL core of MAC serotype 4 was calculated to be 1,028.

FAB-MS to determine the molecular weight. The GPL core of serotype 4 showed a main peak at a *m/z* of 1,027 corresponding to the $(M-H)^-$ ion in negative-ion mode (Fig. 2A) and an *m/z* of 1051 corresponding to the $(M+Na)^+$ in positive-ion mode (Fig. 2B). Based on these results, the molecular weight of the GPL core of serotype 4 was calculated to be 1,028, which was consistent with the previous report (4). Similar results were obtained with serotypes 16 and 20 (data not shown).

**Development of the EIA.** We identified sera from pulmonary MAC disease patients with antibody against all 11 GPLs (Fig. 3). The titers of GPL antibody were reduced to background levels by adding GPL core antigens (5 μg/ml) to the assay for antibodies to every GPL, with the exceptions shown in Fig. 3. Antibodies to serotypes 1, 6, and 14 in patient A, serotype 1 in patient B, and serotypes 1, 14, and 20 in patient C were still detectable after adding core antigen. We considered the possibility that there was antibody against GPL core in the sera of pulmonary MAC disease patients and that the remaining antibodies after addition of the GPL core antigens might be to the serotype-specific oligosaccharide polar GPL antigens. We also examined the antigen specificity of the EIA by adding concentrations of GPL core antigens ranging from 1 to 5 μg to the assay for antibodies to either a mixture of GPLs prepared from 11 reference strains of MAC (five strains of *M. avium* and six strains of *M. intracellulare*) (7) or GPL core from MAC serotype 4 *M. avium*.

Sera were collected from five patients with pulmonary MAC disease (two patients with *M. avium* and three patients with *M. intracellulare*). Addition of GPL core inhibited the optical density levels in the EIA for IgG (Fig. 4A and 4D), IgA (Fig. 4B and 4E), and IgM (Fig. 4C and 4F) antibodies against both GPLs (Fig. 4A to 4C) and GPL core antigens (Fig. 4D to 4F) in a dose-dependent fashion (Fig. 4). Similar results were obtained by the addition of GPL core antigens purified from other serotypes of MAC, such as serotypes 16 and 20 (data not shown). These results point to the GPL core of MAC as having the immunodominant epitope of GPLs.

**Levels of anti-GPL core IgG, IgA, and IgM antibodies in serum samples obtained from study subjects.** The levels of IgG, IgA, and IgM antibodies against GPL core antigen was summarized in Table 2. IgG, IgA, and IgM antibody levels were significantly elevated in MAC disease patients but not in other patient groups ($P < 0.0001$). In the group of MAC disease patients, relationships between GPL core antibody levels of IgG and IgM and IgA and IgM were significant ($P <$

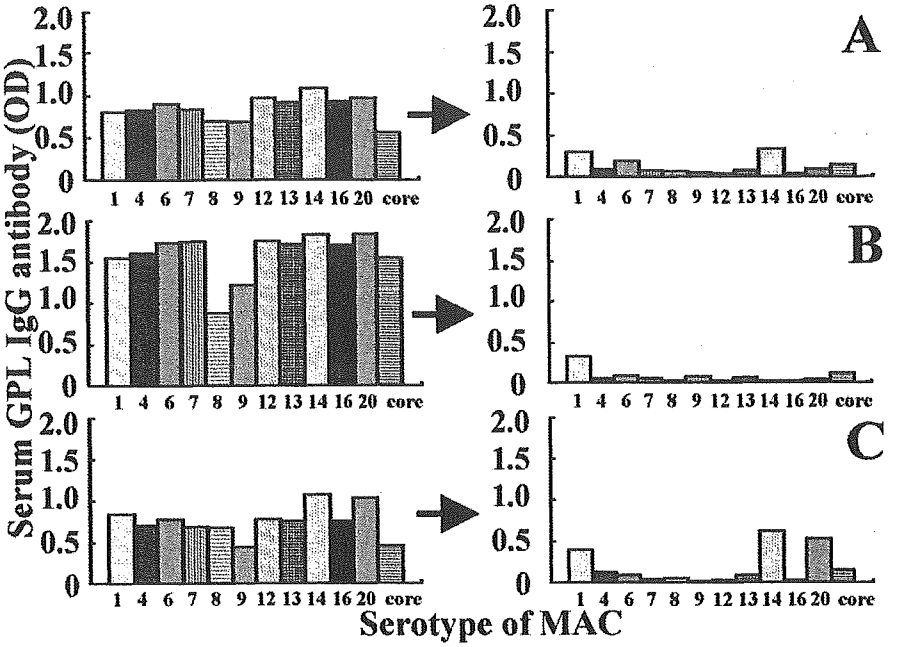


FIG. 3. Antibody titers to 11 serotypes of MAC and GPL core antigen in sera from three patients with MAC disease before and after adsorption with GPL core antigen. The serum titers before adsorption are shown in the left panel. After adsorption with 5 μg of GPL core antigen per ml, the titers of GPL antibody were reduced to background levels in most samples, with the exception of patient C, as shown in this figure. The titers of the three serum samples after adsorption are shown in the right panel.

0.05) but not IgG and IgA. No relationships were found between antibody levels and clinical parameters, including erythrocyte sedimentation rate, C-reactive protein, and the number of colonies after cultures, with the single exception of IgM antibody levels and erythrocyte sedimentation rate ($P < 0.05$).

**Sensitivity and specificity of GPL core-based EIA for diagnosis of MAC disease.** The cutoff levels, as defined with receiver operator characteristic (ROC) curves, were 0.064 for GPL core IgG, 0.072 for IgA, and 0.312 for IgM. The sensitivity and specificity of GPL core-based EIA for diagnosis of MAC pulmonary disease were 72.6% and 92.2%, respectively, for IgG, 92.5% and 95.1%, respectively, for IgA, and 78.3% and 91.0%, respectively, for IgM (Table 3). The best sensitivity and specificity for the diagnosis of MAC pulmonary disease was obtained by measuring IgA antibodies against GPL core. With national surveillance data for Japan, the prevalence of MAC pulmonary disease was estimated to be 2.45 cases per 100,000 population (13). The predictive values for positive results [true positive/(true positive + false positive)] and negative results [true negative/(true negative + false negative)] were 80.2% and 88.6%, respectively, (corrected values, 0.023% and 99.99%, respectively, by the prevalence rate) for IgG, 89.1% and 96.7%, respectively, (corrected values, 0.046% and 99.99%, respectively, by the prevalence rate) for IgA, and 79.0% and 90.6%, respectively, (corrected values, 0.021% and 99.99%, respectively, by the prevalence rate) for IgM.

**Disease activity and level of GPL core antibodies.** The levels of GPL core IgA antibody were quantified before implementation and after completion of antimicrobial chemotherapy for 27 patients with pulmonary MAC disease. The patients were divided into two groups according to the outcome of chemotherapy. There were 14 patients in the cured group and 13 patients in the treatment failure group. The GPL core IgA antibody levels before chemotherapy were the same in both groups. To determine whether the level of GPL core antibodies reflected MAC disease activity, we compared antibody levels sequentially in each patient before and after chemotherapy. In the cured patient group, the mean level of antibody before chemotherapy was 0.546 ± 0.369. After chemotherapy the mean level of antibody was 0.289 ± 0.293. In the treatment failure group, the mean level of antibody before chemotherapy was 0.704 ± 0.550 and after chemotherapy was 0.767 ± 0.429.

The mean IgA antibody titers in the cured group significantly decreased after chemotherapy ($P < 0.001$) but did not change in the treatment failure group (Fig. 5A). In the treatment failure group, a 61-year-old female patient had undergone lobectomy after chemotherapy. Her GPL core IgA antibody titer decreased rapidly after the surgery, and her sputum specimens converted to negative (Fig. 5B). No statistically significant differences were found between the cured and treatment failure groups for age, gender, duration of treatment, and timing of serum collection. Similar results were observed in changes of IgG and IgM antibodies to GPL core antigen before and after chemotherapy in both the cured ($P < 0.001$: IgG and IgM) and treatment failure groups (IgG, $P = 0.25$; IgM, $P = 0.55$).

## DISCUSSION

In Japan, there are more than 30,000 new cases of mycobacterial diseases every year (incidence rate per 100,000, 25.8) and

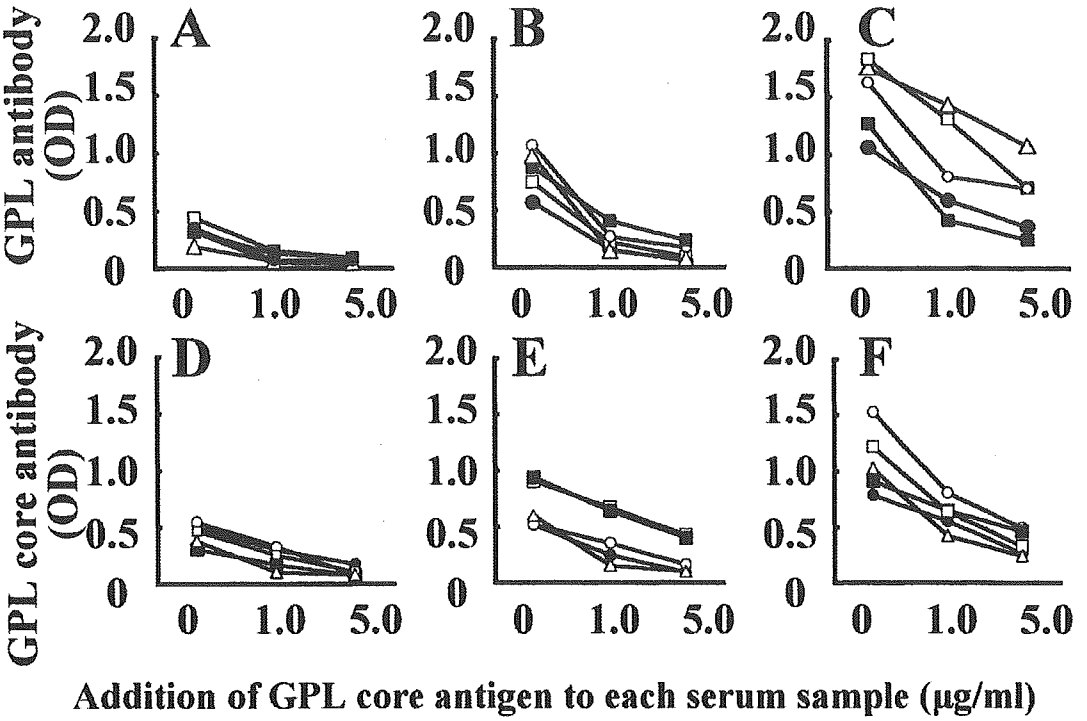


FIG. 4. Antigen specificity and recognition of an immunodominant epitope for GPLs. GPL core concentrations ranging from 1 to 5 μg/ml were added to the EIA with a mixture of GPLs prepared from 11 reference strains of MAC and GPL core of a serotype 4 strain. Sera were from five patients with MAC pulmonary disease, patient A (○), patient B (●), patient C (□), patient D (■), and patient E (△). Addition of GPL core antigen inhibited the level of optical density (OD) by IgG (panels A and D), IgA (panels B and E), and IgM (panels C and F) antibodies against GPLs (panels A, B, and C) and GPL core (panels D, E, and F) in a dose-dependent fashion.

about 20% of them in clinical practice are caused by nontuberculous mycobacteria such as MAC and *M. kansasii*. Of theses, 70% were MAC and 20% were *M. kansasii* (13). Because there are more than 500 patients with pulmonary disease due to nontuberculous mycobacteria in our hospital specializing in chest diseases and our previous study has demonstrated the availability of EIA with a mixture of GPL antigens for diagnosis of MAC pulmonary disease, we could conduct the present study.

The chemical structure of GPL is composed of a common GPL core, fatty acyl-D-Phe-D-allo-Thr-D-Ala-L-alaninol-*O*-(3,4-di-*O*-methyl-Rha), with the different oligosaccharide (polar GPL) moieties linked at the Thr substituent of the core. GPLs are the major cell surface antigens of slowly growing mycobacteria, such as MAC and *M. scrofulaceum*. There are 31 distinct GPL serotypes. In the present study, we report detection by EIA of an antibody against the purified MAC GPL core in sera of patients with pulmonary MAC disease. The GPL antibody levels were reduced in a dose-dependent fashion when different concentrations of GPL core were incorporated into the EIA. These results show that GPL core, the common component of all GPLs, has an immunodominant epitope in MAC strains. Although the present study has demonstrated that GPL core is the dominant epitope, there is a possibility that other components of GPLs, including oligosaccharide and oligosaccharide-GPL complex and whole GPLs, possess antigenicity. Indeed, serum antibodies to whole GPL antigen were incompletely adsorbed with GPL core with the serum of the

TABLE 2. Serum antibodies against GPL core antigen in patients with lung disease

| Group | Titer[a] | | | | | |
|---|---|---|---|---|---|---|
| | IgG | | IgA | | IgM | |
| | Mean ± SD | 95% CI | Mean ± SD | 95% CI | Mean ± SD | 95% CI |
| MAC disease | 0.281 ± 0.346 | 0.017–1.042 | 0.435 ± 0.385 | 0.051–1.599 | 0.714 ± 0.492 | 0.133–2.720 |
| MAC colonization | 0.026 ± 0.027* | 0.005–0.104 | 0.021 ± 0.049* | 0.006–0.143 | 0.164 ± 0.115* | 0.038–0.472 |
| *M. kansasii* disease | 0.039 ± 0.015* | 0.008–0.144 | 0.043 ± 0.054* | 0.010–0.120 | 0.159 ± 0.131* | 0.028–0.508 |
| Tuberculosis | 0.047 ± 0.031* | 0.021–0.068 | 0.030 ± 0.018* | 0.012–0.065 | 0.156 ± 0.113* | 0.040–0.409 |
| Healthy subjects | 0.040 ± 0.028* | 0.018–0.074 | 0.031 ± 0.042* | 0.008–0.077 | 0.164 ± 0.092* | 0.031–0.570 |

[a] CI, confidence interval. *, statistically significant difference compared to patients with MAC disease ($P < 0.0001$).

TABLE 3. Sensitivity and specificity of EIA for serodiagnosis of pulmonary MAC disease

| Group | No. | IgG | | IgA | | IgM | |
|---|---|---|---|---|---|---|---|
| | | Sensitivity (no. of seropositives) | Specificity (no. of seronegatives) | Sensitivity (no. of seropositives) | Specificity (no. of seronegatives) | Sensitivity (no. of seropositives) | Specificity (no. of seronegatives) |
| MAC disease | 106 | 72.6 (77) | 27.4 (29) | 92.5 (98) | 7.5 (8) | 78.3 (83) | 21.7 (23) |
| MAC colonization | 11 | 9.1 (1) | 90.9 (10) | 9.1 (1) | 90.9 (10) | 18.2 (2) | 81.8 (9) |
| *M. kansasii* disease | 30 | 6.7 (2) | 93.3 (28) | 10.0 (3) | 90.0 (27) | 16.7 (5) | 83.3 (25) |
| Tuberculosis | 77 | 10.4 (8) | 89.6 (69) | 5.2 (4) | 94.8 (73) | 5.2 (4) | 94.8 (73) |
| Healthy subjects | 126 | 6.3 (8) | 93.7 (118) | 3.2 (4) | 96.8 (122) | 8.7 (11) | 91.3 (115) |

patient C (Fig. 3). Serotype-specific antisera were produced from rabbits by repeated immunizations with MAC (14). Thus, both the individual variability of humans and the species specificity in the immune response to antigens should be noted.

The recognition that there was an immunodominant antigenic epitope on the GPL core has minimized some of the problems of antigen preparation from an array of MAC strains. The GPL core based EIA described in this study had both high sensitivity and specificity when used to assay MAC-specific IgG, IgA, and IgM, and results were comparable to those previously obtained with mixtures of GPL antigens from 11 reference strains of MAC (9). It was likely that antibodies against both GPL core and polar GPL were being detected in the EIA when mixtures of GPL antigens were used since the addition of GPL core antigens reduced levels of GPL antibodies without reducing levels of GPL antibodies to serotype-specific oligosaccharide antigens. For the diagnosis of MAC pulmonary disease, the best sensitivity and specificity were obtained by measuring IgA antibodies against GPL core. The values for IgG antibodies against a mixture of GPL antigens were almost the same as IgA antibodies against GPL core. However both the ease and low cost of antigen preparation make the EIA for IgA with GPL core antigen a practical tool. Future studies would also benefit from the standardization of serum samples, as well as the methods for rapid and reliable serodiagnosis of MAC disease.

We previously reported the development of a rapid diagnostic EIA for tuberculosis, which is specific for antibodies to tuberculous glycolipid (9, 10). It was recently reported that the combination of lipoarabinomannan polysaccharide antigen, antigen-60, and tuberculous glycolipid appear to be the best choices as antigens for the serodiagnosis of tuberculosis (12). When we combined the results of three serodiagnostic tests which used lipoarabinomannan, antigen-60 and tuberculous glycolipid as antigens, the nontuberculous mycobacteria patients could not be differentiated serologically from tuberculosis patients because tuberculous glycolipid, lipoarabinomannan polysaccharide, and antigen-60 are common cell wall components of all acid-fast organisms, such as mycobacteria and nocardiae.

Nontuberculous mycobacteria cause a chronic, slowly progressive pulmonary infection resembling tuberculosis in immunocompetent hosts. MAC ranks first and *M. kansasii* ranks second among causes of human nontuberculous mycobacterial lung disease (6). We have used GPLs as an antigen for the differential serodiagnosis of pulmonary MAC disease and tuberculosis. This is feasible because GPLs are the major cell surface antigens of slowly growing mycobacteria, such as MAC and *M. scrofulaceum* (3). By contrast, *M. kansasii* and *M. tuberculosis* complex, including bacille Calmette-Guérin (BCG), do not have GPLs in their cell walls (3). In this study the low positive rate (Table 3) and low levels of serum GPL core antibodies in *M. kansasii* and tuberculosis patients confirmed that serodiagnosis by EIA with GPL core antigen could differentiate pulmonary MAC disease from both pulmonary tuberculosis and pulmonary nontuberculous mycobacterial disease caused by *M. kansasii*. Rapidly growing mycobacteria such as *M. chelonae* and *M. fortuitum* also have GPLs as major cell surface components but rarely cause pulmonary nontuberculous mycobacteria disease in humans (6).

MAC organisms are ubiquitous in nature. They have been isolated from water, soil, plants, house dust, and other environmental sources (6) and asymptomatic colonization of human hosts can occur. In this study, GPL core-based EIA could exclude MAC colonization as well as tuberculosis and *M. kansasii* disease because sera of individuals with MAC colonization have both a low positive rate and low antibody levels (Table 2, Table 3).

Despite the fact that most Japanese people (≥90%) have been given BCG (16), the rate of seropositivity for GPL core antibodies in healthy subjects is low (3.2 to 8.7%). One explanation is that GPL core-based EIA for MAC disease is not affected by prior vaccination with BCG, because GPLs are not present in *M. tuberculosis* complex (3). Although *M. tuberculosis* complex and *M. kansasii* do not have GPLs, a low positive rate (Table 3) and low levels of serum GPL antibodies in *M. kansasii* (6.7 to 16.7%), tuberculosis (5.2 to 10.4%), and healthy subjects (3.2 to 8.7%) could be detected. It is possible that latent subclinical infection with MAC leads to false-positive results since 7 to 12% of adults show evidence of subclinical MAC infection when tested for delayed-type skin reactivity to *M. avium* sensitin (18).

In our study, we were unable to determine the rate of subclinical infection with MAC because an appropriate test is not yet available in Japan. However, there are several possible explanations for the low levels of antibody against GPL core antigen. First, a low positive rate is directly related to the cutoff values defined by with the ROC curves. Second, subclinical infections with other nontuberculous mycobacteria, such as *M. chelonae* and/or *M. fortuitum*, must be excluded because such organisms possess GPL antigens in their cell surface (8), which may induce antibody production in the host. Third, a follow up of this antibody levels in these individuals needs to be done looking for individual variability according to the time. There are several possible explanations for the few false-negative patients (29 for IgG, 8 for IgA, and 23 for IgM); presence of

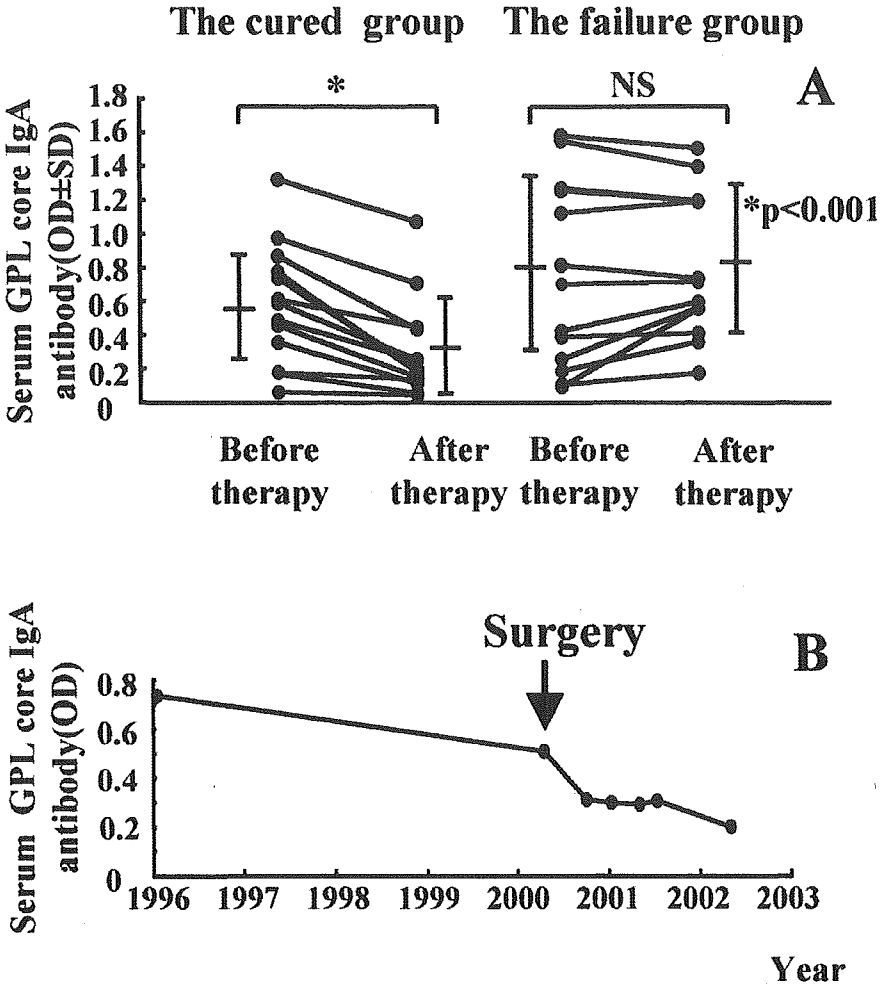


FIG. 5. Disease activity and level of antibodies to MAC GPL core. The levels of serum IgA antibodies to GPL core are shown before and after the completion of antimycobacterial chemotherapy for both the cured (14 MAC patients) and the failure groups (13 MAC patients). In the cured group, the culture results indicated conversion from positive to negative after successful chemotherapy, and in the failure group, the culture results indicated no conversion to negative despite treatment. All results are expressed as individual data (■), and the bars show the mean ± standard deviation for each group. The optical density levels decreased significantly (*, $P < 0.001$) in the cured group of patients but were not changed ($P = 0.381$) in the treatment failure group. The changes of serum IgA levels of to GPL core in a 61-year-old female MAC patient who had undergone lobectomy is shown in B. IgA levels decreased rapidly after the surgery, and sputum cultures converted from positive to negative.

circulating immune complexes, excess of GPL core antigens relative to antibodies, very low bacterial load, and recently diagnosed disease.

By obtaining EIA data before and after successful antimicrobial chemotherapy or surgery, we could demonstrate that the level of GPL core antibodies reflected disease activity of MAC infection. This result is consistent with our previous study on the level of GPL antibodies in MAC disease (7) and show that a merit of the assay is the ability to monitor disease activity. This finding is important because there is, as yet, no consensus as to when to discontinue chemotherapy for MAC disease (1). To validate the use of the EIA as an appropriate clinical tool for monitoring disease and scheduling treatment, data must be obtained in prospective, large-scale studies of active MAC disease.

The high sensitivity and specificity, combined with the simplicity, safety, and rapidity of obtaining results, point to the possibility that new avenues for serodiagnosis of MAC disease will become available with the introduction of the GPL core-based EIA. Results of serodiagnosis by EIA with MAC-specific GPL core antigen, when used in combination with acid-fast staining of sputum and culture confirmation and analyzed together with clinical, radiographic, and microbiologic criteria, may be a powerful tool for diagnosing MAC disease.

ACKNOWLEDGMENTS

This work was supported by grants from the Ministry of Health, Labor, and Welfare (Research on Emerging and Re-emerging Infectious Diseases, Health Sciences Research Grants), Ministry of the Environment (Global Environment Research Fund), Ministry of Ed-

ucation, Culture, Sports, Science and Technology, Osaka City University (Urban Research Project), the United States-Japan Cooperative Medical Science Program against Tuberculosis and Leprosy, and the Grant-in-aid for Community Health and Medical Care from Ichou Association for Promotion of Medical Science.

## REFERENCES

1. **American Thoracic Society.** 1997. Diagnosis and treatment of disease caused by nontuberculous mycobacteria. Medical Section of the American Lung Association. Am. J. Respir. Crit. Care Med. **156:**S1–S25.
2. **Aspinall, G. O., D. Chatterjee, and P. J. Brennan.** 1995. The variable surface glycolipids of mycobacteria: structures, synthesis of epitopes, and biological properties. Adv. Carbohydr. Chem. Biochem. **51:**169–242.
3. **Brennan, P. J., and H. Nikaido.** 1995. The envelope of mycobacteria. Annu. Rev. Biochem. **64:**29–63.
4. **Chatterjee, D., and K. H. Khoo.** 2001. The surface glycopeptidolipids of mycobacteria: structures and biological properties. Cell. Mol. Life Sci. **58:** 2018–2042.
5. **Denner, J. C., A. Y. Tsang, D. Chatterjee, and P. J. Brennan.** 1992. Comprehensive approach to identification of serovars of *Mycobacterium avium* complex. J. Clin. Microbiol. **30:**473–478.
6. **Falkinham, J. O., III.** 1996. Epidemiology of infection by nontuberculous mycobacteria. Clin. Microbiol. Rev. **9:**177–215.
7. **Kitada, S., R. Maekura, N. Toyoshima, N. Fujiwara, I. Yano, T. Ogura, M. Ito, and K. Kobayashi.** 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.
8. **Lopez-Marin, L. M., N. Gautier, M. A. Laneelle, G. Silve, and M. Daffe.** 1994. Structures of the glycopeptidolipid antigens of Mycobacterium abscessus and Mycobacterium chelonae and possible chemical basis of the serological cross-reactions in the Mycobacterium fortuitum complex. Microbiology **140:**1109–1118.
9. **Maekura, R., H. Kohno, A. Hirotani, Y. Okuda, M. Ito, T. Ogura, and I. Yano.** 2003. Prospective clinical evaluation of the serologic tuberculous glycolipid test in combination with the nucleic acid amplification test. J. Clin. Microbiol. **41:**1322–1325.
10. **Maekura, R., Y. Okuda, M. Nakagawa, T. Hiraga, S. Yokota, M. Ito, I. Yano, H. Kohno, M. Wada, C. Abe, T. Toyoda, T. Kishimoto, and T. Ogura.** 2001. Clinical evaluation of anti-tuberculous glycolipid immunoglobulin G antibody assay for rapid serodiagnosis of pulmonary tuberculosis. J. Clin. Microbiol. **39:**3603–3608.
11. **McNeil, M., A. Y. Tsang, and P. J. Brennan.** 1987. Structure and antigenicity of the specific oligosaccharide hapten from the glycopeptidolipid antigen of *Mycobacterium avium* serotype 4, the dominant *Mycobacterium* isolated from patients with acquired immune deficiency syndrome. J. Biol. Chem. **262:** 2630–2635.
12. **Okuda, Y., R. Maekura, A. Hirotani, S. Kitada, K. Yoshimura, T. Hiraga, Y. Yamamoto, M. Itou, T. Ogura, and T. Ogihara.** 2004. Rapid serodiagnosis of active pulmonary Mycobacterium tuberculosis by analysis of results from multiple antigen-specific tests. J. Clin. Microbiol. **42:**1136–1141.
13. **Sakatani, M.** 1999. Nontuberculous mycobacteriosis; the present status of epidemiology and clinical studies. Kekkaku **74:**377–384.
14. **Schaefer, W. B.** 1965. Serologic identification and classification of the atypical mycobacteria by their agglutination. Am. Rev. Respir. Dis. **92**(Suppl.)**:** 85–93.
15. **Tassell, S. K., M. Pourshafie, E. L. Wright, M. G. Richmond, and W. W. Barrow.** 1992. Modified lymphocyte response to mitogens induced by the lipopeptide fragment derived from *Mycobacterium avium* serovar-specific glycopeptidolipids. Infect. Immun. **60:**706–711.
16. **Toida, I.** 2000. Development of the *Mycobacterium bovis* BCG vaccine: review of the historical and biochemical evidence for a genealogical tree. Tuberc. Lung Dis. **80:**291.
17. **Tsang, A. Y., J. C. Denner, P. J. Brennan, and J. K. McClatchy.** 1992. Clinical and epidemiological importance of typing of *Mycobacterium avium* complex isolates. J. Clin. Microbiol. **30:**479–484.
18. **von Reyn, C. F., T. W. Barber, R. D. Arbeit, C. H. Sox, G. T. O'Connor, R. J. Brindle, C. F. Gilks, K. Hakkarainen, A. Ranki, C. Bartholomew, J. Edwards, A. N. A. Tosteson, and M. Magnusson.** 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.

JOURNAL OF CLINICAL MICROBIOLOGY, July 2005, p. 3150–3158 Vol. 43, No. 7
0095-1137/05/$08.00+0 doi:10.1128/JCM.43.7.3150–3158.2005


# Clinical and Prognostic Importance of Serotyping *Mycobacterium avium-Mycobacterium intracellulare* Complex Isolates in Human Immunodeficiency Virus-Negative Patients

Ryoji Maekura,[1]* Yoshinari Okuda,[1] Atsusi Hirotani,[1] Seigo Kitada,[1] Touru Hiraga,[1] Kenji Yoshimura,[1] Ikuya Yano,[3] Kazuo Kobayashi,[2] and Masami Ito[1]

*National Hospital Organization, Toneyama National Hospital, Toyonaka,[1] Department of Host Defense, Osaka City University Graduate School of Medicine, Osaka,[2] and Japan BCG Laboratory, Kiyose,[3] Japan*

Received 5 July 2004/Returned for modification 25 August 2004/Accepted 3 April 2005

**We studied whether the serotypes of *Mycobacterium avium-Mycobacterium intracellulare* complex (MAC) isolates determine the prognosis for pulmonary MAC disease. We prospectively monitored a cohort of 68 patients with pulmonary MAC disease for whom the serotype-specific glycopeptidolipids in isolates were identified using thin-layer chromatography and fast atom bombardment mass-spectrometry in 1990 and 1995. Serovar 4 *Mycobacterium avium* was detected in 40/68 patients (58.8%). Other serotypes were serotypes 1 (five cases), 6 (three cases), 8 (seven cases), 9 (three cases), 14 (four cases), and 16 (six cases). Patients with serovar 4 were significantly ($P < 0.01$) younger (63.0 ± 9.8 years) than patients with other serotypes (71.8 ± 10.3). Patients who failed treatment had a significantly poorer prognosis than other patients. There were no cases of MAC-related death in the cured group. Chest radiographic findings progressively worsened in 36 (90%) of patients with serotype 4, and 14/36 died from respiratory failure caused by pulmonary *Mycobacterium avium* disease. The patients with serotype 4 had a significantly poorer prognosis than patients with other serotypes. These results show that both the outcome of chemotherapy and the serotypes of MAC isolates are important for assessing the prognosis of pulmonary MAC disease.**

The prevalence of nontuberculous mycobacterial (NTM) disease, especially *Mycobacterium avium-Mycobacterium intracellulare* complex (MAC) disease, is increasing. Among NTM, *Mycobacterium avium* and *M. intracellulare* are closely related and are usually grouped together to form the MAC. MAC organisms are ubiquitous in nature and have been isolated from water, soil, plants, house dust, and other environmental sources (6, 20). Originally, MAC was been to be the etiologic agent of avian disease and also endemic disease in porcine and poultry animals. Since the 1980s, NTM disease has become recognized as an opportunistic infections in patients with advanced AIDS (16, 18, 25, 31). In Japan, however, only a few cases of NTM disease in AIDS patients have been reported, and NTM disease in the absence of AIDS predominates. In 1968 European investigators reported that *M. avium* caused refractory advanced pulmonary *M. avium* disease in immunocompetent patients (3, 11). In Japan, Yamamoto reported in 1971 that 21% of a group of 108 patients with MAC disease died within 5 years, while only 26% had inactive disease after 5 years (36). After 1971, however, the prognosis for pulmonary MAC disease tended to be neglected, since clinical symptoms and radiographic findings could be stable for years despite persistent excretion of organisms. Beginning in 1990, we observed that some patients who were resistant to multiple-drug chemotherapy showed persistent excretion of MAC bacilli and progressive worsening of chest radiographic findings until the time of death. The cases of pulmonary disease caused by MAC occurred in immunologically healthy adults and could not be regarded as opportunistic infections. However, only a few detailed studies on the clinical course of MAC cases in immunocompetent patients have been reported (14, 28, 29, 30, 33). The American Thoracic Society announced in 1997 that the natural history of MAC lung disease is unpredictable in immunocompetent patients. Specifically, some patients had a poor prognosis despite multiple-drug chemotherapy, whereas others maintained a stable clinical and radiographic picture for years (2).

Glycopeptidolipids (GPLs) are the major and specific cell surface antigens of the MAC and *Mycobacterium scrofulaceum* group, and serospecific GPLs allow subdivision into 31 distinct serotypes (21). Our previous observations documented that most patients with pulmonary serovar 4 MAC disease had worsening chest radiographic findings (23). Therefore, in 1990 we began an investigation of the relationship between the serotypes of MAC isolates and the long-term survival of patients with pulmonary MAC disease in a prospective cohort protocol.

## MATERIALS AND METHODS

**Subjects.** A total of 68 patients with pulmonary MAC disease were enrolled in December 1990 and July 1995 in a prospective cohort protocol study. Identification of the serotype-specific GPL by using thin-layer chromatography (TLC) and fast atom bombardment-mass spectrometry (FAB-MS) was required for enrollment. Patients were followed monthly for more than 5 years and underwent bacteriological examinations, including both smear and culture tests, in our hospital. Chest radiographs were taken every 3 months. Patients known to be human immunodeficiency virus (HIV) positive were excluded from enrollment. Subjects gave informed consent according to our institutional guidelines. All patients had been diagnosed according to the Japanese criteria. Pulmonary MAC disease indications were infiltrates, nodules or cavities on chest radiograms, multifocal bronchiectasis and/or multiple small nodules on computed tomography scans, and at least three positive cultures with 200 colonies or more using

* Corresponding author. Mailing address: Toneyama National Hospital, 5-1-1, Toneyama Toyonaka-City, Osaka 560-0045, Japan. Phone: 81-6-6853-2001. Fax:81-6-6853-3127. E-mail: maekurar@toneyama.hosp.go.jp.

Ogawa's egg medium for MAC during the past year. The diagnostic criteria used in this study were in accord with those recommended by the American Thoracic Society in 1997 (2).

Initial treatment consisted of a three- or four-drug regimen that included either streptomycin or ethambutol (EB), in addition to rifampin (RFP) and isoniazid. The details of the following treatment were left entirely to the judgment of the physicians. There were no differences in the drugs used for patients between the serovar 4 group and the those with other serovars. Clarithromycin (CAM) was prescribed for 20 of 40 patients with serovar 4 (50%) and for 15 of 28 patients with other serovars (53.5%).

**Causes of death.** Despite treatment, some patients deteriorated from pulmonary MAC disease and progressed to respiratory failure that necessitated long-term oxygen therapy. When patients were persistently smear and/or culture positive, the cause of death was attributed to pulmonary MAC disease. Cases of hemoptysis-caused death were also categorized as MAC related. Thirteen patients who died of causes other than pulmonary MAC disease during the follow-up period were excluded from the survival prognosis analysis. Four patients transferred to other clinics when they moved to another area. These patients were included because their survival data were available.

**Bacteriological outcome.** Patients whose culture tests converted to negative after treatment and whose sputum remained negative on culture during the follow-up period were categorized as cured. Patients whose cultures converted to negative for more than 6 months following treatment but with culture-positive sputum during the follow-up period were classified as relapsed. Patients with sputum cultures that were continuously positive despite treatment were categorized as nonresponsive.

Sputum specimens for both smear staining and culturing were obtained every month. The sputum specimens were digested and decontaminated with a solution of 2% sodium hydroxide (NaOH) and *N*-acetyl-L-cysteine and then centrifuged for 15 min at 3,000 × *g*. The supernatant was decanted, and the sediment was mixed at 1:10 (vol/vol) with sterile water. Mycobacterial cultures were performed by inoculating 0.1 ml of the processed sample onto tubes of Ogawa medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). Species of *M. avium* and *M. intracellulare* were identified with a DNA-DNA colorimetric microdilution plate hybridization kit (DDH Mycobacteria; Kyokuto Pharmaceuticals, Tokyo, Japan).

**Analysis of MAC serotype.** We had performed the identification of serotypes with MAC from patients with pulmonary MAC disease twice, in December 1990 and July 1995. Serotype-specific GPLs were identified by TLC and FAB-MS analyses (JEOL-SX-102; Nihonkohden Co., Tokyo, Japan) (10, 21, 32). The strains of acid-fast bacteria isolated from patients with pulmonary MAC disease were inoculated onto 1% Ogawa egg medium, cultured for 3 weeks, and then transferred to 7H9 broth medium at 37°C and cultured with shaking for an additional 3 weeks until the early stationary phase. The cells were harvested by centrifugation after being heat killed for 15 min by autoclaving. After alkali hydrolysis with 0.1 N NaOH in methanol, alkali-stable lipids were prepared and separated on TLC plates. Plates were charred after being sprayed with 50% $H_2SO_4$. Serotype-specific GPLs were identified by comparison of $R_f$ values and coloration with those of the reference standard. Identification of serotype-specific GPLs was confirmed by the FAB-MS analysis (Fig. 1). These analyses were done at the Department of Bacteriology of Osaka City University and without prior knowledge of the patient clinical status.

**MIC determinations.** MICs were determined by agar dilution with mycobacterium 7H11 agar supplemented with 10% Middlebrook oleic acid-albumin-dextrose-catalase enrichment. Each drug was incorporated at final concentrations of 0.05 to 100 μg/ml, along with a drug-free control. Mycobacteria were grown in 7H9 broth at 37°C and diluted with distilled water to match the turbidity of a no. 1 McFarland standard. The plates were inoculated by using a Steer's replicator with a 1/100 dilution of the suspension in distilled water. The results were read after 3 weeks of incubation at 37°C. The MIC was defined as the lowest concentration of antibiotic which completely inhibited visible bacterial growth (one colony was disregarded).

**Statistical analysis.** All data were analyzed using the statistical software package StatView 5.0 (SAS Institute, Cary, N.C.). The survival times were calculated by Kaplan-Meier analysis (19), and statistical significance was determined with the log rank (Mantel-Cox) test. Data were compared by the Student *t* and $\chi^2$ tests and are shown as means ± standard deviations.

## RESULTS

Of the 110 isolates from patients with pulmonary MAC disease that were analyzed in December 1990 and July 1995, serotype-specific GPLs of 68 isolates (62%) could be identified by TLC and FAB-MS analyses. The characteristics of the enrolled patients ($n$ = 68) with pulmonary MAC disease are shown in Table 1. Fifty patients (73.5%) had previous lung diseases. There were 42 cases of previous tuberculosis, 4 of chronic obstructive pulmonary disease, 2 of idiopathic pulmonary fibrosis, 1 of lung cancer, and 1 of pneumoconiosis. None of the 12 female patients had previous lung diseases, but they did have bronchial-ectasis lesions as shown by chest computed tomography. Six of these patients also had cavitary lesions. Five of six males without previous lung diseases had cavitary, but no bronchial-ectasis, lesions. Forty-two patients (62%) had cavitary lesions, and 6/42 also had bronchial-ectasis lesions. The lesions were moderately to far advanced in two-thirds of

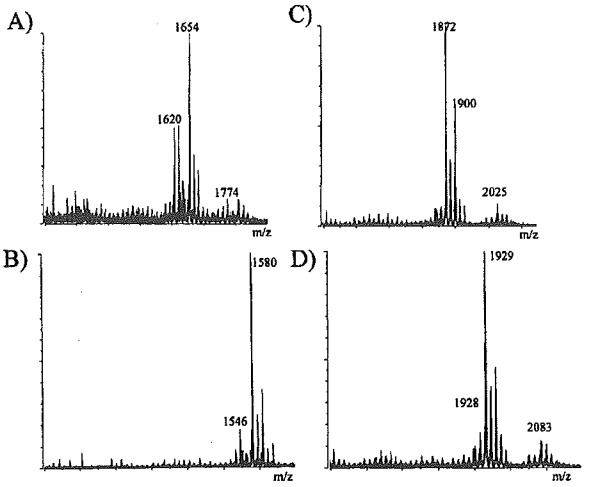


FIG. 1. Fast atom bombardment-mass spectra of (A) serovar 4, (B) serovar 8, (C) serovar 12, and (D) serovar 4 GPLs.

TABLE 1. Characteristics of enrolled patients with pulmonary MAC disease ($n$ = 68)

| Characteristic | No. of cases |
|---|---|
| Sex | |
| Male | 31 |
| Female | 37 |
| Previous lung disease | 50 |
| Cavitation | 42 |
| Bronchiectasis | 26 |
| Extent of lesion | |
| Far advanced | 9 |
| Moderately advanced | 36 |
| Minimally advanced | 23 |
| Species and serovar | |
| *M. avium* | 59 |
| 4 | 40 |
| 1 | 5 |
| 6 | 3 |
| 8 | 7 |
| 9 | 3 |
| 14 | 1 |
| *M. intracellulare* | 9 |
| 14 | 3 |
| 16 | 6 |
| Mean range age (yr) | 67 (45–91) |

TABLE 2. Follow-up results for patients with pulmonary disease caused by different MAC serovars

| Parameter | Result for patients with serovar: | |
|---|---|---|
| | 4 | Other |
| Age (yr, mean ± SD) | 63.0 ± 9.8 | 71.8 ± 10.3 |
| No. of patients | 40 | 28 |
| No. of deaths (all causes) | 21 | 13 |
| No. of MAC-caused deaths | 18 | 3 |
| Outcome of treatment (no.) | | |
| Cured | 4 | 6 |
| Relapsed | 17 | 14 |
| Nonresponsive | 17 | 6 |
| Unknown | 2 | 2 |
| Changes of radiographic findings (no.) | | |
| Worsened | 36 | 13 |
| No changes or improved | 3 | 15 |
| Unknown | 1 | 0 |

the patients when the extent was divided into three groups according to the criteria of pulmonary tuberculosis. There were no correlations between radiographic findings and serotypes.

The period from initial diagnosis to enrollment was 4.0 ± 3.2 years. Eighty-seven percent of MAC isolates from these patients were identified by DDH Mycobacteria analysis as *M. avium*. The remaining MAC isolates were identified as *M. intracellulare*. Serotypes 4 (59%), 8 (10%), and 16 (9%) predominated among enrolled patients. For eight patients serovar identification was done twice. Isolates from four cases were identified as serovar 4 both times, while isolates from four cases with other serovars initially were identified as having changed to serovar 4. Before enrollment 50/68 patients had undergone initial chemotherapy with either streptomycin or ethambutol in addition to rifampin and isoniazid. In this study, 50 patients also underwent multidrug retreatment chemotherapy that included CAM or fluoroquinolones.

We divided the subjects into the serovar 4 group and the other-serovar group, because we had recognized by retrospective analysis at the time of enrollment that most of the serovar 4 patients had progressively worsening chest radiograms. A comparison of follow-up results between the two groups is shown in Table 2. The rates of death were the same in both groups. However, the number of deaths caused by pulmonary *M. avium* disease in the serovar 4 group, despite the patients being younger, was significantly larger than that in the other-serovar group. The overall rate of sputum conversion to negative was 60.3% in this study. The rate in the serovar 4 group (52.5%) was lower than that in the other-serovar group (71.4%), despite there being no differences in the chemotherapy regimens. However, the number of relapses was similar in both groups over the long-term follow up period. The rate of nonresponsiveness to treatment in the serovar 4 group was twofold that in the other-serovar group. Ninety percent of patients with serovar 4 *M. avium* disease had worsening radiographic findings, and 17/38 cases (45%) were nonresponsive to the chemotherapy.

The course of lesion progression varied in the 21 patients who had MAC-related deaths, although they could be roughly divided into two groups. Lesions in the first category progressed to giant cavities, and the second group had an association of focal emphysematous lesions with bilateral cavitary lesions (Fig. 2 to 4). Formation of giant cavities was found in 6/21 cases of MAC-related patient deaths. Progression to bilateral cavitary lesions was found in 15 cases, including 5/6 cases that presented bronchial-ectasis lesions at enrollment.

The causes of 13 non-MAC-related deaths that were excluded from the prognostic analysis are shown in Table 3. A comparison of survival curves (Kaplan-Meier analysis) for the serovar 4 and other-serovar groups is shown in Fig. 5. The survival period for the serovar 4 group after enrollment was significantly ($P < 0.05$) shorter than that of the other-serovar group. The survival rate in the other-serovar group stopped

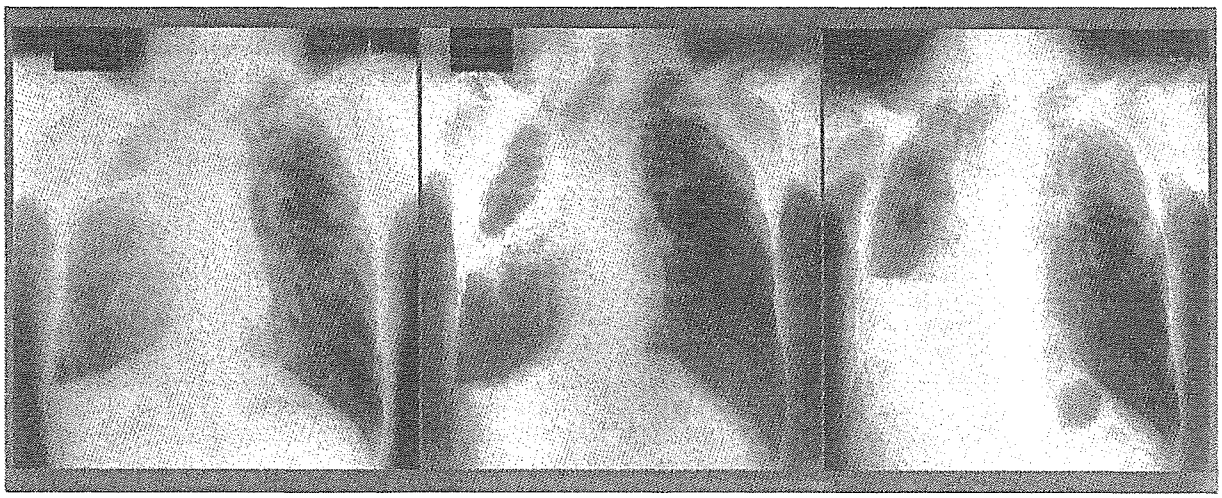

FIG. 2. Chest radiograms showing chronological changes with progression to a giant cavity lesion in a patient who died with a MAC serovar 4-associated disease. (Left) Unilateral cavitary lesion in the patient at the age of 51; (middle) enlarged unilateral cavitary lesion at the age of 55; (right) bilateral cavitary lesion and a giant cavity resembling a lung abscess at the age of 60.

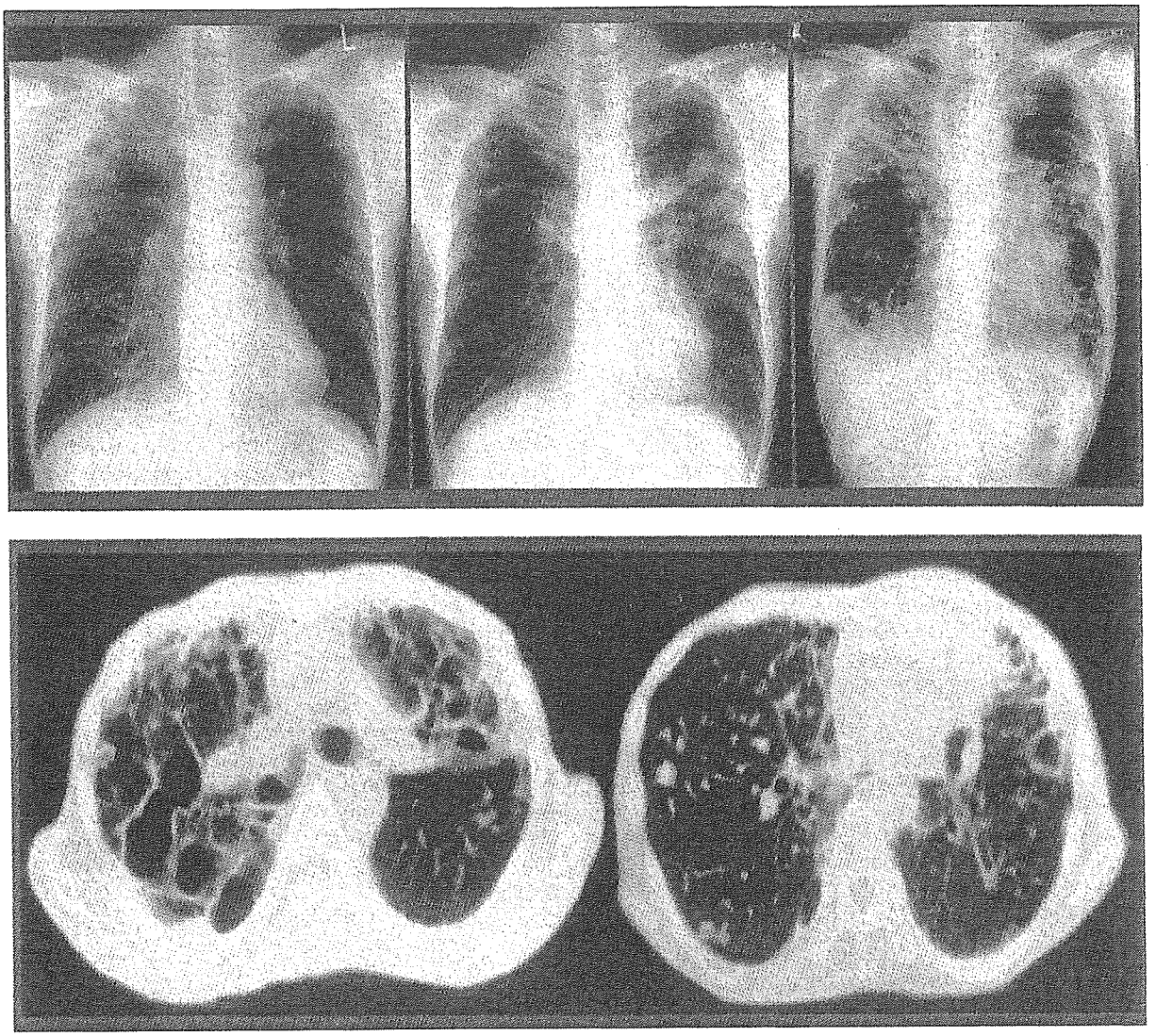

FIG. 3. Chronological changes on chest radiograms preceding the MAC serovar 4-related death of a patient whose initial radiographic finding was a unilateral cavity. (Top left) Unilateral small cavitary lesion at the age of 53; (top middle) bilateral cavitary lesions at the age of 58; (top right) many bilateral cavitary lesions with focal emphysematous and bronchiectasis lesions at the age of 65; (bottom) chest computed tomogram at the age of 63.

decreasing by 50 months after enrollment. However, the survival rate in the serovar 4 MAC group continued to decrease by 25% at 92 months and 50% at 175 months after the initial diagnosis (Fig. 6). The patients in the serovar 4 group were younger (58.1 ± 10.4 years old) than those in the other serovar group (68.3 ± 11.8 years old) at the time of initial diagnosis. These results reveal that the patients with serovar 4 disease have a poor long-term survival prognosis.

The outcome of chemotherapy also correlated significantly ($P < 0.05$) with long-term survival (Fig. 7). The prognosis of the non-treatment-responsive group was significantly ($P < 0.01$) worse than that of the relapsed group. There were no MAC-related deaths in the cured group. The prognosis of patients with far-advanced MAC disease was significantly ($P < 0.01$) worse than that of patients with less advanced lesions. However, in the long-term follow-up periods, there were deaths caused by pulmonary MAC disease in patients with only a minimally advanced lesion.

The susceptibilities of patient MAC isolates to EB, RFP, CAM, and sparofloxacin were studied in the three separate groups (*M. avium* serovar 4; *M. avium* serovars 1, 6, 8, and 9; and *M. intracellulare* serovars 14 and 16) (Table 4). The range of MICs of each drug for serovar 4 isolates varied widely. The RFP MICs that inhibited 50% (12.5 μg/ml) and 90% (50 μg/ml) of the isolates were significantly higher for serovar 4 than for the other groups. When the interpretation of MICs was done according to the achievable drug levels in serum, 67% of the isolates for EB (MICs of > 8μg/ml), 57% for RFP (MICs