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Original article

Contribution of GM-CSF on the enhancement of the T cell-stimulating activity of macrophages

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Abstract

Mycobacterium leprae is an intracellular parasitic organism that multiplies in macrophages (MØ). It inhibits the fusion of mycobacterial phagosome with lysosome and induces interleukin (IL)-10 production from macrophages. However, macrophages are heterogenous in various aspects. We examined macrophages that differentiated from monocytes using either recombinant (r) granulocyte-MØ colony-stimulating factor (GM-CSF) (these MØ are named as GM-MØ) or rMØ colony-stimulating factor (M-CSF) (cells named as M-MØ) in terms of the T cell-stimulating activity. Although both macrophages phagocytosed the mycobacteria equally, GM-MØ infected with *M. leprae* and subsequently treated with IFN-γ- and CD40 ligand (L) stimulated T cells to produce interferon-gamma (IFN-γ), but M-MØ lacked the ability to stimulate T cells. While M-MØ mounted a massive IL-10 production, GM-MØ did not produce the cytokine on infection with *M. leprae*. *M. leprae*-infected, IFN-γ- and CD40L-treated GM-MØ expressed a higher level of HLA-DR and CD86 Ags than those of M-MØ, and expressed one of the dominant antigenic molecules of *M. leprae*, Major Membrane Protein-II on their surface. These results indicate that GM-CSF, but not M-CSF, contributes to the up-regulation of the T cell-stimulating activity of *M. leprae*-infected macrophages.

Keywords: Macrophage; *M. leprae*; GM-CSF; IFN-γ

1. Introduction

Mycobacterium leprae (*M. leprae*), a causative agent of human leprosy, is a representative parasitic pathogen that induces skin lesions and chronic progressive peripheral nerve injury, leading to systemic deformity [1,2]. Leprosy represents a clinical spectrum, in which clinical manifestations are associated with different levels of immune responses to *M. leprae* infection [3]. One representative type is a tuberculoid leprosy, in which patients exhibit innate and adaptive immunities to *M. leprae* and manifest a localized form of the disease with granuloma formation in infected tissues [4–6]. For the activation of an adaptive immunity, dendritic cells (DC) derived from inflammatory monocytes, play a central role [7,8]; and, in *in vitro* experiments, both $CD4^+$ and $CD8^+$ T cells are activated by DC infected with *M. leprae*, and these DC expressed Major Membrane Protein-II (MMP-II) as a dominant antigenic molecule [9,10]. Another representative manifestation is lepromatous leprosy, in which patients show reduced levels of host defense associated immunities and manifest a disseminated form of the disease with a broad spread of foamy MØ, in which an abundance of bacilli are usually involved [11,12]. *M. leprae* resides in the phagosome in MØ and replicates there without being digested by lysosomal enzymes [13]. Furthermore, *M. leprae* stimulates MØ to produce IL-10 [5,6] and suppresses the DC-mediated Ag-specific

Abbreviations: Ag, antigen; APC, Ag-presenting cells; BCG, *Mycobacterium bovis* BCG; DC, dendritic cells; GFP-BCG, BCG expressing GFP; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN-γ, interferon-gamma; IL, interleukin; L, ligand; mAb, monoclonal antibody; MØ, macrophages; M-CSF, MØ colony-stimulating factor; *M. leprae*, *Mycobacterium leprae*; MMP-II, Major Membrane Protein-II; PBMC, peripheral blood mononuclear cells; r, recombinant.

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adaptive immunity [14,15]. These observations may indicate that the induction of intracellular processing of *M. leprae* and that of expression of molecules, such as MMP-II could lead to the activation of IFN-γ producing type 1 $CD4^+$ T cells. Another important element that should be considered for the full activation of T cells is the suppression of IL-10 production from *M. leprae*-infected MØ.

So far, a variety of methods and tools, including cytokines, have been used for the differentiation of MØ from human peripheral monocytes *in vitro* [16–18]. One representative MØ can be differentiated by using M-CSF, termed M-MØ, and another by using GM-CSF, termed GM-MØ. Both MØ represent different functions on infection with mycobacteria. However, much remains not fully understood with regard to *M. leprae* infection and the T cell-stimulating activity of these MØ.

In this report, we analyzed the characteristics of *M. leprae*-infected GM-MØ and M-MØ, and tried to develop immunological methods to enhance the MØ-mediated host defense activities against the bacteria.

2. Materials and methods

2.1. Preparation of cells and bacteria

Peripheral blood was obtained from healthy PPD-positive individuals under informed consent. We are aware that PPD-negative individuals would help to provide more information for our study; however, in Japan, most healthy individuals are PPD-positive, because *Mycobacterium bovis* BCG vaccination is compulsory for children (0–4 years old). Moreover, PPD-negative individuals in the Japanese population are those who do not respond to BCG vaccination, and therefore, it is likely that they suffer from some immune insufficiency. Therefore, these individuals cannot be used as controls for our experiments. Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Paque Plus (Pharmacia, Uppsala, Sweden) and cryopreserved in liquid nitrogen until use, as previously described [19]. For preparation of peripheral monocytes, $CD3^+$ T cells were removed from either freshly isolated heparinized blood, or cryopreserved PBMC using immunomagnetic beads coated with anti-CD3 monoclonal antibody (mAb) (Dynabeads 450, Dynal, Oslo, Norway). The $CD3^-$ PBMC fraction was plated on collagen-coated plates and the non-plastic adherent cells were removed by extensive washing. The remaining adherent cells were used as monocytes [19]. MØ were differentiated by culturing monocytes in the presence of 20% fetal calf serum and either rM-CSF (R and D Systems, Abingdon, UK) (M-MØ) or rGM-CSF (Pepro Tech EC LTD, London, UK) (GM-MØ) [20]. Both GM-MØ and M-MØ were pulsed with *M. leprae*, treated with an optimal dose of IFN-γ on day 3 of culture, further treated with CD40L on day 4, and were used as a stimulator of T cells on day 5 [21]. *M. leprae* (Thai-53) was isolated from the footpads of BALB/c-*nu/nu* mice [22]. The isolated bacteria were counted by Shepard's method [22]. Killed *M. leprae* was prepared by heating the bacteria at 60 °C for 18 h. BCG (Pasteur strain) was cultured *in vitro* using Middlebrook 7H9 broth supplemented with 0.05% Tween 80 and albumin-dextrose-catalase. BCG expressing GFP was constructed as follows. The GFP sequence was amplified from pEGFP-1 vector (CLONTECH, Palo Alto, CA), and cloned into pMV261 [23]. Transformants were selected on a 7H10 plate containing 25 μg/ml kanamycin. The phagocytosis of BCG by GM-MØ and M-MØ after culture was determined using FACScalibur (Becton Dickinson Immunocytometry System, San Jose, CA). The multiplicity of infection (MOI) was determined based on the assumption that MØ were equally susceptible to infection with *M. leprae* [24].

2.2. Analysis of cell surface antigen (Ag)

The expression of cell surface Ag on MØ was analyzed using FACScalibur. Dead cells were eliminated from the analysis by staining with propidium iodide (Sigma Chemical Co., St. Louis, MO) and 1×10^4 live cells were analyzed. For analysis of cell surface Ag, the following mAbs were used: FITC-conjugated mAb against HLA-ABC (G46-2.6, PharMingen, San Diego, CA), HLA-DR (L243, PharMingen), CD14 (M5E2, BD Biosciences, San Jose, CA), TLR2 (TL2.3, Serotec, Oxford, UK), TLR4 (HTA125, Santa Cruz Biotech, Santa Cruz, CA), CD209 (DCN46, PharMingen), CD86 (FUN-1, PharMingen), and CD40 (5C3, PharMingen).

The expression of MMP-II, which is one of the dominant antigenic molecules of *M. leprae* [9] on *M. leprae*-infected MØ was determined using the mAb (IgM, kappa) against MMP-II, followed by FITC-conjugated anti-mouse Igs Ab (Tago-immunologicals, Camarillo, CA).

2.3. APC function of M. leprae-infected MØ

The ability of *M. leprae*-infected MØ to stimulate T cells was assessed using an autologous MØ-T cell co-culture as previously described [24,25]. Freshly thawed PBMC were depleted of $CD56^+$, MHC class II^+ and $CD8^+$ cells by using magnetic beads coated with mAb to CD56, MHC class II and CD8 Ags (Dynabeads 450; Dynal) [25]. The purity of $CD4^+$ T cells was more than 98% as assessed by FACS analyses. The purified responder cells (1×10^5 per well) were plated in 96-well round-bottom tissue culture plates and MØ were added to give the indicated MØ:$CD4^+$ T cell ratio. Supernatants of MØ-T cell co-cultures were collected on day 4 and the concentration of cytokines was determined.

2.4. Cytokine production

Levels of the following cytokines were measured; IFN-γ produced by $CD4^+$ T cells, IL-10, IL-1β, TNFα and IL-12p40 produced by MØ stimulated for 24 h with *M. leprae*. The concentrations of these cytokines were quantified using the enzyme assay kits, Opt EIA Human ELISA Set (BD PharMingen International).

2.5. Statistical analysis

Student's *t*-test was applied to determine the statistical differences.

3. Results

3.1. Characteristics of MØ differentiated from monocytes using GM-CSF

MØ were differentiated from monocytes using either GM-CSF (GM-MØ) or M-CSF (M-MØ). To characterize these two types of MØ, surface markers expressed on GM-MØ and M-MØ were analyzed using the monocytes obtained from the same donor by flow cytometry (Fig. 1). MHC class I (HLA-ABC) and class II (HLA-DR) Ags were similarly expressed on GM-MØ and M-MØ, but the expression of CD14 Ag was significantly reduced in GM-MØ. While the expression level of TLR2, CD209, CD40 and TLR4 Ags was not different between GM-MØ and M-MØ, the expression of CD86 was significantly higher on GM-MØ than M-MØ. Then, we examined the phagocytic capacity of GM-MØ and M-MØ by using BCG expressing GFP (GFP-BCG), since *M. leprae* cannot be cultured *in vitro* or express GFP. The percentage of MØ expressing GFP after co-culture of MØ with GFP-BCG was similar between GM-MØ and M-MØ (Fig. 2). These results indicate that GM-MØ and M-MØ differed in the expression of some surface markers, but they similarly phagocytosed the mycobacteria.

3.2. Effect of M. leprae infection to GM-MØ on the T cell-stimulating activity

Since *M. leprae* is an intracellular parasitic bacterium and is hardly digested with lysosomal enzyme in MØ unless MØ are activated [26], we analyzed the T cell-stimulating activity of *M. leprae*-infected GM-MØ and M-MØ (Table 1). When M-MØ were infected with up to MOI 80 of *M. leprae* and treated with IFN-γ and CD40L, they did not stimulate $CD4^+$ T cells to secrete a significant dose of IFN-γ. In contrast to M-MØ, when *M. leprae*-infected, IFN-γ- and CD40L-treated GM-MØ were used as Ag-presenting cells (APC), T cells produced significant levels of IFN-γ in a manner dependent on the dose of *M. leprae*. Since GM-MØ express CD40, and are activated by IFN-γ, we examined the effect of treatment with IFN-γ and CD40L on the T cell-stimulating activity of *M. leprae*-infected GM-MØ (Table 2). While IFN-γ production from $CD4^+$ T cells was not significantly induced by GM-MØ untreated or treated with either IFN-γ or CD40L, the cytokine production was significantly enhanced by the treatment of GM-MØ with

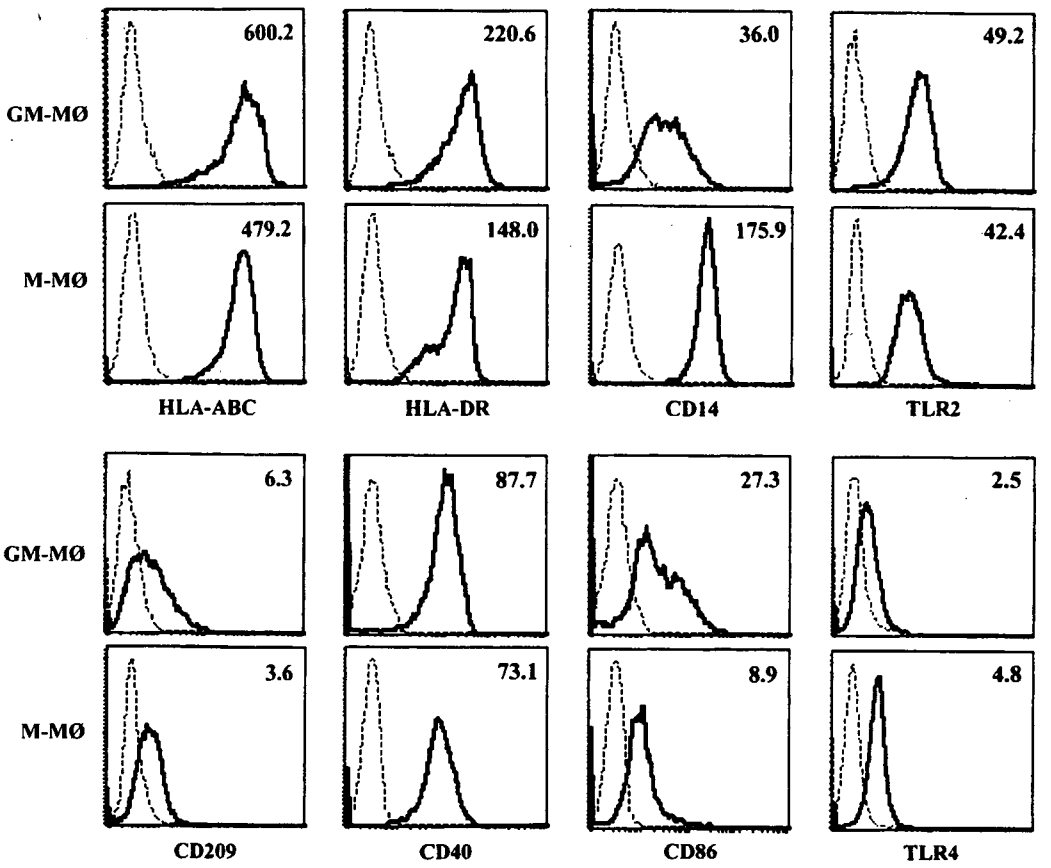

Fig. 1. Phenotype of GM-MØ and M-MØ differentiated from monocytes. Plastic adherent monocytes were differentiated into MØ by 3 days culture with either rGM-CSF or rM-CSF. Dashed lines, isotype-matched control IgG; solid lines, mAb staining. The number represents the difference in mean fluorescence intensity between dashed and solid lines. Representatives of three independent experiments are shown.

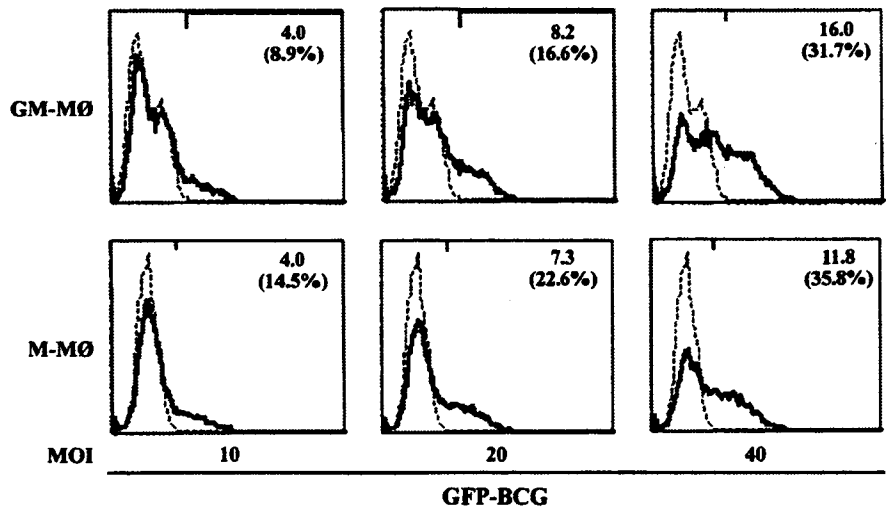

Fig. 2. Phagocytic activity of GM-MØ and M-MØ. Plastic adherent monocytes were differentiated into MØ by 3 days culture with either rGM-CSF or rM-CSF. For analysis of the phagocytic activity of MØ, GM-MØ and M-MØ were pulsed with BCG expressing GFP and assessed on day 4 of culture. Dashed lines, unpulsed cells; solid lines, GFP-BCG pulsed cells. The number represents the difference in mean fluorescence intensity between the dashed and solid lines. The number in parenthesis indicates the percent GFP-positive cell number. Representatives of three independent experiments are shown.

both IFN-γ and CD40L. Then, we compared the T cell-stimulating activity of live and heat-killed *M. leprae* (Table 3). Both forms of *M. leprae* stimulated $CD4^+$ T cells when pulsed to GM-MØ, but the heat-killed *M. leprae* more efficiently induced T cell activation than live bacteria. When we examined the effect of heat-killed *M. leprae* on M-MØ, they did not stimulate $CD4^+$ T cells significantly, even when IFN-γ and CD40L were administered (data not shown). Also note that, when GM-MØ and monocyte-derived DC were compared in terms of their T cell-stimulating activity, GM-MØ were less efficient in this respect (data not shown).

3.3. Factors associated with the enhancement of the T cell-stimulating activity of GM-MØ

Various factors may be responsible for enhancing the T cell-stimulating activity of APC. When we examined the expression of APC associated molecules on *M. leprae*-infected MØ (Fig. 3), the expression of HLA-DR and CD86 on GM-MØ was higher than on M-MØ, although there was no significant difference in the expression of HLA-ABC between GM-MØ and M-MØ. The cytokines produced from APC should also be considered to be another important factor that should be monitored and MØ produce a variety of cytokines, including IL-10, IL-1β, TNFα and IL-12 [6,11,27]. IL-10 was efficiently produced from M-MØ by stimulation with *M. leprae*, but it was hardly produced from GM-MØ (Fig. 4a). When macrophages were differentiated by using both GM-CSF and M-CSF, the function of GM-CSF was dominant and, the production of IL-10 was suppressed (Fig. 4a). Similarly to the production of IL-10, IL-1β was more efficiently produced from M-MØ than GM-MØ (Fig. 4b). In contrast, TNFα, which is important for granuloma formation, was more efficiently produced from GM-MØ (Fig. 4c). However, there was no significant difference in the production of IL-12p40 between GM-MØ and M-MØ (Fig. 4d). Finally, we assessed whether *M. leprae*-infected GM-MØ expressed dominant antigenic molecules of *M. leprae* on the surface (Fig. 5). To this end, we examined the expression of MMP-II on GM-MØ and M-MØ. No apparent expression of

Table 1
T cell-stimulating activity of *M. leprae*-infected GM-MØ and M-MØ[a]

Stimulator of $CD4^+$ T cells	*M. leprae* infection of macrophages (MOI)	IFN-γ (pg/ml) production by $CD4^+$ T cells after stimulation with macrophages at ratio (T:MØ)		
		2:1	4:1	8:1
GM-MØ	0	$0.6 \pm 0.2^{*,\dagger}$	$0.5 \pm 0.1^{\ddagger,\S}$	$1.4 \pm 0.2^{\P,\|}$
	40	$38.1 \pm 3.8^{*}$	$34.2 \pm 2.3^{\dagger}$	$23.4 \pm 3.8^{\P}$
	80	$230.7 \pm 21.4^{\dagger}$	$120.5 \pm 16.9^{\S}$	$74.7 \pm 6.8^{\|}$
M-MØ	0	0.9 ± 0.1	3.1 ± 1.2	13.9 ± 2.2
	40	0.9 ± 0.1	2.6 ± 1.3	12.2 ± 3.1
	80	11.8 ± 0.3	17.5 ± 2.1	12.2 ± 2.9

$^{*}p < 0.005$; $^{\dagger}p < 0.005$; $^{\ddagger}p < 0.01$; $^{\S}p < 0.01$; $^{\P}p < 0.01$; $^{\|}p < 0.005$.

[a] $CD4^+$ T cells (1×10^5/well) were stimulated for 4 days with autologous GM-MØ or M-MØ at the indicated dose of macrophage. GM-MØ or M-MØ were pulsed with *M. leprae* and IFN-γ (100 IU/ml) on day 3, treated with CD40L (1 μg/ml) on day 4, and were used as APC on day 5. Representatives of three separate experiments are shown. Assays were done in triplicate, and results are expressed as mean ± SD. Groups with identical symbols were compared using Student's *t*-test.

Table 2
Contribution of IFN-γ and CD40L on T cell-stimulating activity of GM-MØ[a]

M. Leprae infection of GM-MØ (MOI: 80)	Treatment of *M. leprae*-infected GM-MØ with		IFN-γ (pg/ml) production by $CD4^+$ T cells after stimulation with GM-MØ at ratio (T:MØ)		
	IFN-γ (100 IU/ml)	CD40L (1.0 μg/ml)	2:1	4:1	8:1
−	+	+	2.3 ± 0.3	0.1 ± 0.2	0.8 ± 0.5
+	−	−	4.0 ± 1.1*	5.5 ± 1.9[§]	6.0 ± 2.1[#]
+	−	+	21.4 ± 3.1[†]	22.7 ± 4.0[¶]	14.8 ± 2.2**
+	+	−	20.3 ± 1.7[‡]	15.9 ± 1.3[‖]	10.7 ± 2.3[††]
+	+	+	226.1 ± 20.9*,†,‡	107.8 ± 13.7[§,¶,‖]	94.8 ± 9.7[#,**,††]

*$p < 0.005$; [†]$p < 0.005$; [‡]$p < 0.005$; [§]$p < 0.005$; [¶]$p < 0.005$; [‖]$p < 0.01$; [#]$p < 0.005$; **$p < 0.005$; [††]$p < 0.005$.

[a] $CD4^+$ T cells (1×10^5/well) were stimulated for 4 days with autologous GM-MØ at the indicated dose of macrophage. GM-MØ were pulsed with *M. leprae* and IFN-γ (100 IU/ml) on day 3, treated with CD40L (1 μg/ml) on day 4, and were used as APC on day 5. Representatives of three separate experiments are shown. Assays were done in triplicate, and the results are expressed as the mean ± SD. Groups with identical symbols were compared using Student's *t*-test.

MMP-II was observed on M-MØ, but, on GM-MØ, significant expression of MMP-II was induced. The expression was dependent on the dose of *M. leprae* (Fig. 5). However, the MMP-II expression on *M. leprae*-infected GM-MØ required both IFN-γ and CD40L, and apparent expression was not induced by sole treatment of macrophages with either IFN-γ or CD40L (data not shown).

4. Discussion

In order to avoid the intracellular multiplication and intercellular spread of *M. leprae*, the activation of adaptive immunity, especially of IFN-γ-producing type 1 T cells, plays an important role [5,6]. In fact, paucibacillary (tuberculoid) leprosy patients activate $CD4^+$ T cells through DC, although the bacteria cannot be eliminated completely [8,28]. The *M. leprae*-infected DC digest the bacteria and express dominant antigenic molecules for the efficient IFN-γ production from T cells [9]. In contrast, multibacillary (lepromatous) leprosy patients retain a large number of *M. leprae* in their MØ, and concordantly induce reduced levels or completely lack the ability to effectively stimulate T cells [11,12]. Since tissue resident MØ are heterogenous with regard to functional aspects [17,29], we assessed two different types of MØ: GM-MØ and M-MØ, and found that GM-MØ, but not M-MØ, stimulated T cells. GM-MØ were generated from monocytes using cytokine GM-CSF whilst M-MØ were produced using M-CSF. Although there were some differences in the expression levels of MHC class I, II, CD14 and CD209 Ags on GM-MØ and M-MØ, both forms were equally susceptible to mycobacteria as far as phagocytosis of BCG-GFP was examined. However, there was a striking difference between *M. leprae*-infected GM-MØ and M-MØ in the expression of antigenic molecules; only GM-MØ expressed MMP-II, which is one of the dominant antigenic molecules capable of stimulating T cells in *M. leprae*-infected individuals. The induction of MMP-II expression on GM-MØ requires not only GM-CSF, but also the co-stimulation of MØ with IFN-γ and CD40L. In case of *M. leprae*-infected DC, the phagosomal bacteria could be processed by lysosomal enzymes, and MMP-II expression was observed on DC [9]. The MMP-II expression observed on GM-MØ may indicate that at least some intracellular *M. leprae* were processed. However, the processing of *M. leprae* by GM-MØ still seemed partial, since the heat-killed *M. leprae* induced T cell activation more vigorously than live bacteria, and *M. leprae*-infected DC stimulated T cells more efficiently than GM-MØ, although other factors, such as an induction of IL-12, cannot be ruled out completely. The cell wall architecture including surface-exposed molecules, of heat-killed mycobacteria is globally altered [29,30], resulting in the exudation of some soluble antigenic molecules which may be feasibly digested in macrophages (unpublished observation). Therefore, T cells are more efficiently activated by heat-killed bacteria than by live bacteria.

Table 3
Comparison of T cell-stimulating activity of live and heat-inactivated *M. leprae*[a]

M. leprae pulsed on GM-MØ (MOI)	IFN-γ (pg/ml) production by $CD4^+$ T cells after stimulation with GM-MØ at ratio (T:MØ)		
	2:1	4:1	8:1
None	2.3 ± 1.1	2.1 ± 1.2	2.4 ± 0.9
HK (40)	406.5 ± 49.3*	157.3 ± 20.1[‡]	75.4 ± 6.8[¶]
HK (80)	399.8 ± 33.2[†]	187.7 ± 17.8[§]	106.9 ± 11.2[‖]
Live (40)	101.5 ± 8.8*	30.2 ± 4.6[‡]	3.2 ± 1.9[¶]
Live (80)	152.0 ± 12.7[†]	82.9 ± 7.4[§]	32.7 ± 2.8[‖]

*$p < 0.01$; [†]$p < 0.005$; [‡]$p < 0.005$; [§]$p < 0.005$; [¶]$p < 0.005$; [‖]$p < 0.005$.

[a] $CD4^+$ T cells (1×10^5/well) were stimulated for 4 days with autologous GM-MØ at the indicated dose of macrophage. GM-MØ were pulsed with either heat-killed (HK) or live *M. leprae* and IFN-γ (100 IU/ml) on day 3, treated with CD40L (1 μg/ml) on day 4, and were used as APC on day 5. Representatives of three separate experiments are shown. Assays were done in triplicate, and the results are expressed as the mean ± SD. Groups with identical symbols were compared using Student's *t*-test.

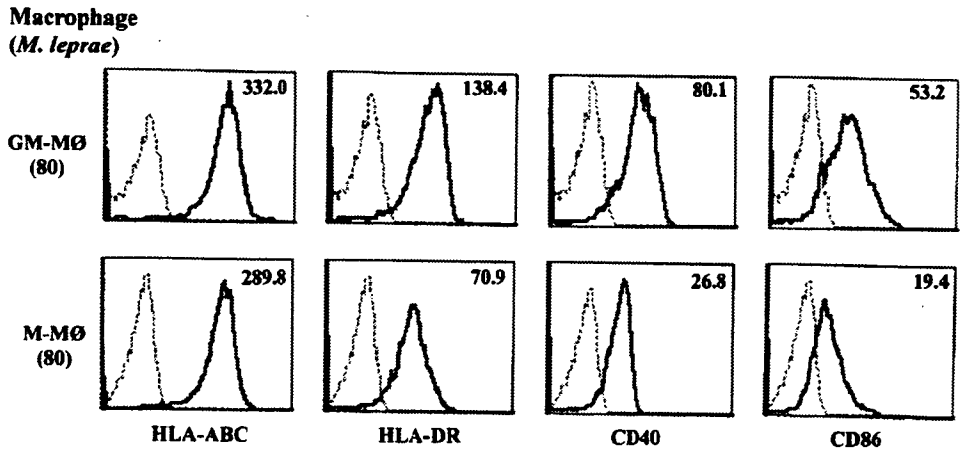

Fig. 3. Phenotype of *M. leprae*-infected GM-MØ and M-MØ. GM-MØ and M-MØ differentiated from monocytes by 3 days culture with rGM-CSF or rM-CSF were infected with *M. leprae*, treated with IFN-γ (100 IU/ml) on day 3, and further treated with CD40L (1 μg/ml) on day 4 of culture. On day 5, the phenotype of GM-MØ and M-MØ was analyzed. Dashed lines, isotype-matched control IgG; solid lines, mAb staining. The number represents the difference in mean fluorescence intensity between dashed and solid lines. Representatives of three independent experiments are shown.

Ottenhoff et al. have also reported that GM-CSF up-regulates the T cell-stimulating activity of MØ, but not M-CSF, and mycobacteria-infected GM-MØ promoted the type 1 cell-mediated immunity against pathogens [31]. Our observations are in line with their data and provide ways to enhance the cell-mediated immunity, especially in cases progressing towards lepromatous leprosy. To facilitate the T cell activation and MMP-II expression, it was required to use

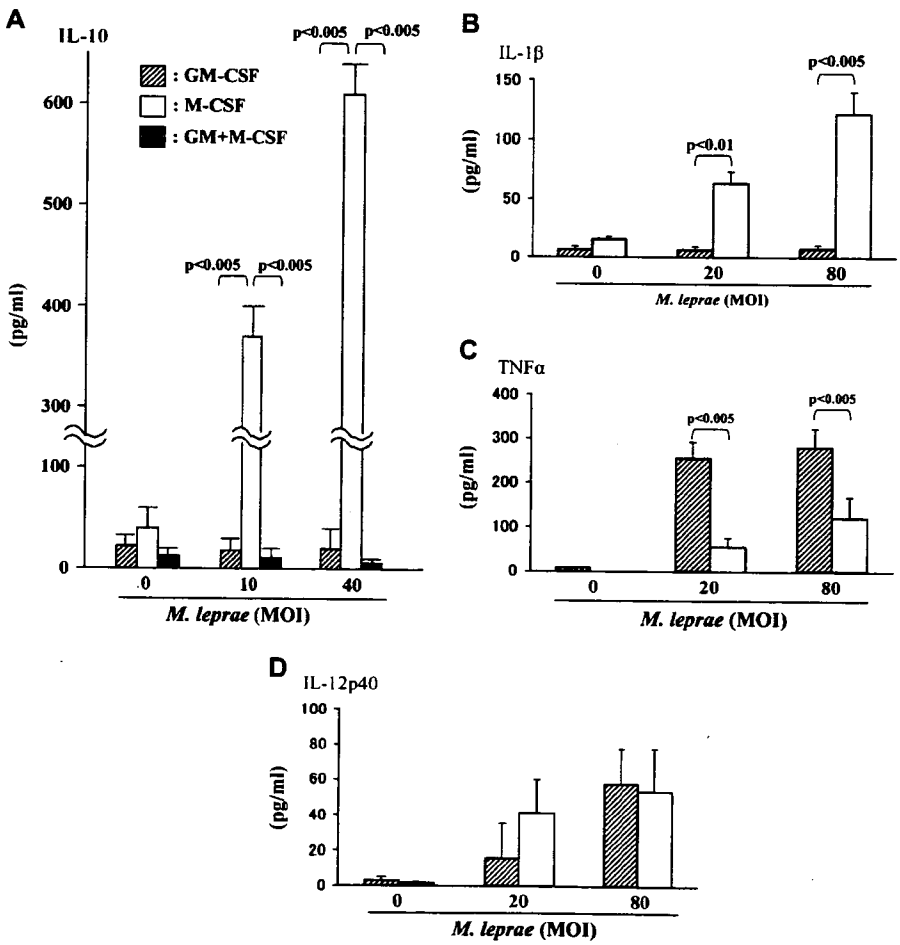

Fig. 4. Cytokine production from GM-MØ and M-MØ. MØ were differentiated by 3 days culture with rGM-CSF, rM-CSF or rGM-CSF + rM-CSF, and were stimulated with *M. leprae* for 24 h. The cytokines: (a) IL-10; (b) IL-1β; (c) TNFα; and (d) IL-12p40 were measured by ELISA. Representatives of three independent experiments are shown.

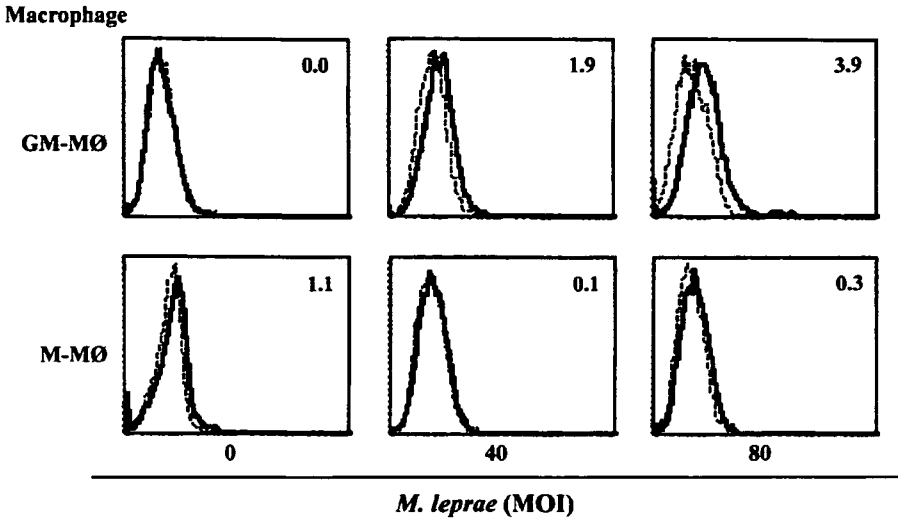

Fig. 5. Expression of MMP-II on the surface of GM-MØ and M-MØ. GM-MØ and M-MØ were differentiated from monocytes by 3 days culture with either rGM-CSF or rM-CSF, respectively. These macrophages were infected with an indicated dose of *M. leprae* and treated with IFN-γ (100 IU/ml) on day 3 of monocyte culture, further treated with CD40L (1 μg/ml) on day 4, and were analyzed for MMP-II expression on day 5. Dashed lines, control IgM; solid lines, MMP-II mAb staining. The number represents the difference in mean fluorescence intensity between dashed and solid lines. Representatives of three independent experiments are shown.

both IFN-γ and CD40L, in addition to the lineage-determining cytokine GM-CSF. IFN-γ and CD40L are probably required to compensate for the lower antigenic characteristics of *M. leprae* [24]. Studies using other mycobacteria, such as *M. bovis* BCG, may provide further useful information. Although IFN-γ is known to activate MØ for bacterial digestion and to induce IL-12p35 gene transcription [26,31], it remains to be determined if CD40L treatment on GM-MØ furthers the intracellular processing of phagosomal bacteria or whether conditioning of GM-MØ through CD40-CD40L interaction, such as in DC [32], is required for T cell stimulation. In addition to MMP-II expression, there were some differences in the phenotypic features of *M. leprae*-infected GM-MØ and M-MØ. A higher level of HLA-DR and CD86 Ags was expressed on the GM-MØ infected with *M. leprae* and co-stimulated, than on similarly treated M-MØ. The mechanism, leading to the enhanced Ag expression, especially of Ag processing by IFN-γ, has not been clearly demonstrated and remains to be elucidated, but GM-CSF, IFN-γ and CD40L seem to at least partially co-ordinate and induce the higher expression of HLA-DR and CD86. These phenotypic differences between *M. leprae*-infected GM-MØ and M-MØ again contribute to the differences in T cell stimulatory activity.

Another peculiar difference observed between the two types of MØ, GM-MØ and M-MØ, was the cytokines that they induced. IL-10 suppresses DC-dependent as well as DC-independent T cell activation [14,15,33], and creates a situation in which *M. leprae* can feasibly parasitize in the cells. While M-MØ secreted a large amount of IL-10, GM-MØ completely lacked in the production of cytokine upon stimulation with *M. leprae* (Fig. 4) or lipopolysaccharide (data not shown). Furthermore, the presence of GM-CSF diminished the IL-10 production from M-MØ by *M. leprae*. Thus, treatment of monocytes with GM-CSF can wipe off the favorable conditions for *M. leprae* survival. On the other hand, GM-MØ produced a higher level of TNFα than M-MØ. TNFα plays an important role in the granuloma formation, and TNFα is an important mediator of host defense activity in MØ, in mycobacterial lesions [34,35]. The treatment of monocyte with GM-CSF would be beneficial for MØ-mediated host defense in this respect. These observations were consistent with the previous findings that IL-10-deficient mice display increased anti-mycobacterial immunity with concordant higher levels of TNFα and a lower bacterial burden [36]. Our previous studies show that T cells from lepromatous leprosy can mount a significant production of IFN-γ by appropriate stimulation [10], therefore, the present studies may provide useful information for the development of immunotherapeutic tools, such as endogenous or exogenous treatment of macrophages with GM-CSF, and thus prevent the dissemination of *M. leprae*.

In this study, we analyzed the two types of MØ with regard to T cell-stimulating activity, and found that GM-CSF and co-stimulators enhance the host defense activity of *M. leprae*-infected MØ.

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Evaluation of major membrane protein-II as a tool for serodiagnosis of leprosy

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Abstract

As serodiagnosis is the easiest way of diagnosing a disease, the utility of *Mycobacterium leprae*-derived major membrane protein-II (MMP-II), one of the immuno-dominant antigens, in the serodiagnosis of leprosy was examined. The percent positivity by an enzyme-linked immunosorbent assay for anti-MMP-II antibody was 82.4% for multi-bacillary leprosy, and the specificity of the test was 90.1%. For pauci-bacillary leprosy where cell-mediated immunity predominates, 39.0% showed positive results. These percentage values were significantly higher than these values obtained for existing phenolic glycolipid-I based methods, suggesting that MMP-II antibody detection would facilitate the diagnosis of leprosy.

Introduction

Leprosy represents a broad-spectrum disease caused by *Mycobacterium leprae*, with lepromatous leprosy at one pole and tuberculoid leprosy at the other end of the pole, depending on the clinical manifestation, which is an ultimate effect of the immunity of the host (Ridley & Jopling, 1996). In all forms of the disease, *M. leprae* induces skin lesions and a chronic progressive peripheral nerve injury, to a lesser or greater extent, which leads to systemic deformity (Stoner, 1979; Job, 1989). Therefore, early detection of *M. leprae* infection is the key to avoiding deformities. The diagnosis of leprosy is based on microscopic detection of acid-fast bacteria (AFB) in skin smears or biopsies, along with clinical and histopathological evaluation. Acid-fast staining requires at least a thousand organisms per gram of tissue for reliable detection (Shepard & McRae, 1968), resulting in an extremely low sensitivity, especially for the tuberculoid form of the disease, where AFB are rare or absent. Recently, real-time PCR-based methods have been developed (Martinez *et al.*, 2006), but the sensitivity of the test for clinical specimens is still problematic. In developing countries, where leprosy is endemic, diagnosis still relies on clinical observations and easy inexpensive tests. Serodiagnosis is the most easy and tangible way of diagnosing a disease. For leprosy, the only antigen currently used is phenolic glycolipid-I (PGL-I), which is supposed to be *M. leprae* specific. Since the discovery of PGL-I in 1981 by Hunter and Brennan, considerable progress has been made in the development of serological tools (Hunter & Brennan, 1981). In this process, simple user-friendly assays such as Serodia leprae®, a simple lateral flow test, and dipstick assays, based on PGL-I antigen, have been developed to detect leprosy patients in leprosy-endemic areas (Izumi *et al.*, 1990; Buhrer-Sekula *et al.*, 2003). However, these tests seem to be insufficient to detect both multi-bacillary and pauci-bacillary patients, as well as for early diagnosis, and have not been used as widely as would be expected, in the field situations. Therefore, there is a need to look for more sensitive

antigens. To date, various antigens of *M. leprae* have been studied (Hunter *et al.*, 1990), but due to a lack of either specificity or sensitivity, their use has been limited. Major membrane protein-II (MMP-II, #ML2038c, gene name bfrA, also known as bacterioferritin) had been identified previously from the cell membrane fraction of *M. leprae* as an antigenic molecule capable of activating both antigen-presenting cells and T cells (Pessolani *et al.*, 1994; Maeda *et al.*, 2005). These findings prompted examination of the role of MMP-II in the humoral responses of patients. Here, MMP-II was expressed and purified in *Escherichia coli* and the use of MMP-II as an antigen for the serodiagnosis of leprosy was evaluated.

Materials and methods

Study population

Sera were obtained with informed consent from healthy individuals, leprosy patients, and tuberculosis patients from Japan. The samples were frozen at $-30\,^{\circ}C$ before use. The population studied included multi-bacillary ($n=74$) and pauci-bacillary ($n=77$) leprosy patients, either treated or untreated, from the National Sanatorium Oshimaseishoen, and tuberculosis patients ($n=55$) from Fukujuji Hospital. Classification of leprosy was performed according to WHO recommendations. The home page is available at http://www.who.int/lep/classification/en/index.html. Individuals who have not been vaccinated with *Mycobacterium bovis* bacillus Calmette–Guerin (BCG) are unavailable in Japan, due to compulsory vaccination at least once in childhood. Therefore, sera from BCG-vaccinated healthy volunteers ($n=81$) residing in Japan were used as negative controls in the enzyme-linked immunosorbent assay (ELISA) to determine the cut-off value for positivity. The age and sex of normal individuals may not be fully matched with those of patients because some of the details of leprosy patients as well as normal individuals are unknown. This study was approved by the ethics committee of the National Institute of Infectious Diseases, Tokyo.

Expression and purification of protein

The MMP-II gene (ML2038c, *bfr*A) was expressed in *E. coli* as a fusion construct using a pMAL-c2X expression vector (New England BioLabs). The protein was affinity purified to almost homogeneity using an amylose column (data not shown), and used as the antigen for the detection of anti-MMP-II antibody levels in the leprosy patients. The synthetic bovine serum albumin (BSA)-conjugated trisaccharide-phenyl propionate (NTP-BSA) for the detection of PGL-I antibodies was kindly provided by Dr T. Fujiwara, Institute for Natural Sciences, Nara University. The procedure for synthesis of the antigen is described elsewhere (Fujiwara *et al.*, 1984).

Assay method for the detection of antibodies

The ELISA for the detection of anti-MMP-II immunoglobulin G (IgG) antibodies or anti-PGL-I IgM antibodies was performed as described previously, with modifications (Izumi *et al.*, 1990). Ninety-six well plates (Immunosorb, Nunc) were coated overnight, with MMP-II at a concentration of $2\,\mu g\,mL^{-1}$ in 0.1 M carbonate buffer (pH 9.5). After blocking with 2% skim milk, the plates were washed with phosphate-buffered saline containing 0.1% Tween 20 (PBST), and human sera (normal or patient's sera) diluted 100-fold were added and incubated at 37 °C for 2 h. After washing with PBST, biotinylated anti-human IgG (Vector Laboratories) was added at a concentration of $0.5\,\mu g\,mL^{-1}$ and incubated for 1 h. The plates were incubated with reagents from a Vectastain ABC Kit (Vector Laboratories) for 30 min. After further washing with PBST, a substrate solution consisting of $0.2\,mg\,mL^{-1}$ of 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 0.02% H_2O_2 in 0.1 M citrate buffer was added until a blue color developed and the OD was measured at 405 nm using a spectrophotometer. Plate-to-plate variations in OD readings were controlled using a common standard serum with an OD value of 0.350. For detecting anti-PGL-I antibody, NTP-BSA was coated at a concentration of $0.5\,\mu g\,mL^{-1}$, and the same procedure used to detect anti-MMP-II antibody was followed, except that the secondary antibody used was biotinylated anti-human IgM at a concentration of $1\,\mu g\,mL^{-1}$. The volume of all solutions used in the 96-well plate was $100\,\mu L\,well^{-1}$.

Statistical analyses

The data were analyzed using MEDCALC software (MedCalc, Belgium). A receiver operator characteristics (ROC) curve was drawn to calculate the cut-off levels. The McNemar test was applied (MMP-II vs. PGL-I test) to determine the P value. The P value of < 0.05 was considered to be statistically significant. The κ value was calculated to determine the agreement between the two tests.

Results and discussion

MMP-II (bfrA) has been previously identified as one of the components of *M. leprae* capable of stimulating $CD4^+$ and $CD8^+$ T cells (Maeda *et al.*, 2005). In multi-bacillary leprosy, *M. leprae* is widely disseminated and abundant antibody production is observed due to polyclonal B cell activation, while in pauci-bacillary leprosy, the bacilli are usually localized in skin lesions and type 1 T cells are predominantly activated, and so the level of antibodies to *M. leprae* antigens is usually low. However, preliminary experiments showed that the pooled pauci-bacillary leprosy sera reacted to MMP-II on polyvinylidene difluoride (PVDF) membranes.

Table 1. Positivity rates of MMP-II and PGL-I tests in various groups of subjects by an enzyme-linked immunosorbent assay

	MMP-II				PGL-I						
	Tested	Positive	%	95% CI	Tested	Positive	%	95% CI	McNemar test (MMP-II vs. PGL)	Rate difference (95% CI)	Inter-rater agreement (κ)
Multi-bacillary leprosy	74	61	82.4	71.8–90.3	74	51	68.9	57.1–79.2	$P=0.063$	13.5% (−0.7 to 24.2)	0.14
Pauci-bacillary leprosy	77	30	39	28.8–50.1	77	15	19.5	12.2–29.7	$P=0.007$	19.5% (5.4 to 28.9)	0.189
Tuberculosis	55	9	16.4	8.9–28.3	ND	ND	ND	ND	–	–	–
Healthy subjects	81	8	9.9	5.1–18.3	81	8	9.9	5.1–18.3	–	–	–

ND, not detected.

Therefore, a systematic study was conducted, measuring the anti-MMP-II antibody IgG levels in the leprosy patients' sera. The study population consisted of multi-bacillary leprosy, pauci-bacillary leprosy, tuberculosis patients, and normal healthy BCG-vaccinated volunteers from Japan. Some of the leprosy patients were already under treatment, so that all patients were not active leprosy patients. The cut-off value of OD 0.130 was defined by an ROC curve analysis (MEDCALC software) using the OD titers from 81 normal individuals and 74 multi-bacillary leprosy patients. Using this cut-off value, it was observed that 61 (82.4%, 95% CI; 71.8–90.3) out of 74 multi-bacillary patients had positive results, and 30 (39.0%, 95% CI; 28.8–50.1) out of 77 pauci-bacillary patients had positive titers (Table 1). The only serological test for leprosy that is currently available is the detection of antibodies to PGL-I of *M. leprae*. The haptenic trisaccharide of PGL-I is known to be *M. leprae* specific, and this trisaccharide unit could be chemically synthesized (Fujiwara *et al.*, 1987). Several reports show the performance of PGL-I for serodiagnosis (Agis *et al.*, 1988; Cho *et al.*, 1991). When the anti-PGL-I IgM antibody levels were examined in the same Japanese leprosy patients by ELISA, it was found that only 68.9% (95% CI; 57.1–79.2) of multi-bacillary patients ($n=74$) and 19.5% (95% CI; 12.2–29.7) of pauci-bacillary patients ($n=77$) showed positive values (Table 1). These percentages were far lower than expected, which may be due to the influence of chemotherapy. However, the percent positivity for anti-MMP-II antibodies was significantly higher than that for anti-PGL-I antibodies of the same sera ($P=0.0008$, McNemar test, $n=152$) when both multi-bacillary and pauci-bacillary leprosy were considered together. When multi-bacillary sera were taken separately, the significance of the MMP-II test was statistically marginal compared with that of the PGL-I test ($P=0.06$), but there was a significant difference ($P=0.007$) between the two tests for pauci-bacillary leprosy (Table 1). The agreement between these two tests was low (κ value for multi-bacillary leprosy: 0.140, κ value for pauci-bacillary leprosy: 0.189), so that when positive titers for either or both PGL-I and MMP-II were considered, the percent positivity increased to 91.9% in multi-bacillary patients and 48.7% in pauci-bacillary patients. The specificity of both the MMP-II and PGL-I tests was 90.1%.

When the anti-MMP-II IgG levels were measured in the normal individuals (BCG-vaccinated), a low level of sero-positivity was found, 9.9% (95% CI; 5.1–18.3) ($n=81$). As the amino acid homology between MMP-II protein from *M. leprae* and its homologue in *M. bovis* BCG is 90.6%, it was expected that a higher percentage of normal individuals would be positive. But this result was to the contrary and advantageous to the assay system. Also, tuberculosis patients showed a low positive result of 16.4% (95% CI; 9.9–28.3) ($n=55$). As, the genes encoding MMP-II were conserved between *M. leprae* and *M. tuberculosis*, the low positivity in tuberculosis was unexpected. The exact reason for the low positivity in tuberculosis patients is not clear, but might be due to slight conformational differences in the antibody recognition site on MMP-II between *M. leprae* and *M. tuberculosis*. More intensive studies need to be carried out using active tuberculosis patients and native MMP-II protein derived from *M. tuberculosis* to fully realize the significance of MMP-II homologue in the detection of tuberculosis.

There is only one report showing that the sera from leprosy patients had a higher IgG titer to MMP-II, regardless of the clinical type of leprosy (Deshpande *et al.*, 1995). However, their study was carried out with a limited number of individuals ($n=10$ in each group), and so, here, a more systematic study has been conducted for evaluating the expediency of measuring anti-MMP-II antibody levels. In addition, when monitoring of patients on multidrug treatment was conducted ($n=4$) for a period of 2 years, there was a definite decline in the MMP-II antibody levels after chemotherapy (data not shown). Further intensive studies have to be carried out with a larger cohort of patients to fully realize the significance of MMP-II in the monitoring of chemotherapy.

This report showed that MMP-II could contribute to the sero-detection of multi-bacillary as well as pauci-bacillary leprosy patients. Further study will be pursued to evaluate its efficacy for serodiagnosis in developing countries and for the development of user-friendly tools.

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Identification of trehalose dimycolate (cord factor) in *Mycobacterium leprae*

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Abstract Glycolipids of *Mycobacterium leprae* obtained from armadillo tissue nodules infected with the bacteria were analyzed. Mass spectrometric analysis of the glycolipids indicated the presence of trehalose 6,6′-dimycolate (TDM) together with trehalose 6-monomycolate (TMM) and phenolic glycolipid-I (PGL-I). The analysis showed that *M. leprae*-derived TDM and TMM possessed both α- and keto-mycolates centering at C78 in the former and at C81 or 83 in the latter subclasses, respectively. For the first time, MALDI-TOF mass analyses showed the presence of TDM in *M. leprae*.

Keywords: Trehalose 6,6′-dimycolate; Trehalose 6-monomycolate; Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; Thin-layer chromatography; *Mycobacterium leprae*

1. Introduction

Mycolic acids and mycolyl glycolipids are unique and ubiquitous components of mycobacterial cell envelopes. Among such components, trehalose 6,6′-dimycolate (TDM) was first isolated as cord factor from highly virulent *Mycobacterium tuberculosis* showing cord-like growth on the surface culture in liquid media [1–3]. TDM of *M. tuberculosis* was recognized as one of the virulence factors capable of inhibiting fusion of phagosome with lysosome in infected macrophage [4]. However, on the other hand, the TDM was considered to be associated with host defence against the mycobacterial infection since it induced immune responses such as type 1 T cell activation and the formation of granuloma in the mycobacteria-infected lesion [5–7]. Wang et al. have reported that a high proportion of patients infected with *Mycobacterium leprae* possess IgG antibody against TDM of unknown origin as well as trehalose 6-monomycolate (TMM), a biosynthetic precursor of TDM [8]. These observations suggest the possible existence of both TDM and TMM in *M. leprae*. Previously, TDM has been isolated from almost all species of culturable mycobacteria [9,10], and also TMM was isolated from *M. leprae*, however, the search for TDM in *M. leprae* has been unsuccessful [11]. The possible reason for not being able to identify TDM, may be due to (1) inadequate supply of *M. leprae*, (2) negligible amount of the product, (3) technically inefficient to identify TDM. Recent development of newer techniques such as MALDI-TOF mass spectrometry has enabled us to identify even several pg amounts of products. Therefore, the present study was designed as an attempt to directly detect TDM in *M. leprae*, by use of newer technologies. In the process, higher amounts of *M. leprae* phenolic glycolipid-I (PGL-I) was obtained which was analyzed by MALDI-TOF mass spectrometry.

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Abbreviations: TDM, trehalose 6,6′-dimycolate; TMM, trehalose 6-monomycolate; *M. leprae*, *Mycobacterium leprae*; *M. tuberculosis*, *Mycobacterium tuberculosis*; *M. bovis*, *Mycobacterium bovis*; TLC, thin-layer chromatography; MALDI-TOF mass, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

2. Materials and methods

2.1. Sources for extraction of glycolipids

M. tuberculosis Aoyama B (ATCC 31726) and *Mycobacterium bovis* BCG Connaught (ATCC 35745) were grown at 37 °C on Sauton's medium for four weeks as surface pellicles until early stationary phase. Cultivated mycobacterial strains were used for extraction of glycolipids. Because *M. leprae* cannot be cultivated in any artificial media, armadillo tissue nodules infected with *M. leprae* (Thai 53 strain) were used for the extraction of glycolipids.

2.2. Extraction of glycolipids and mycolic acid methyl esters

Glycolipids were extracted according to the methods described previously [12]. In brief, bacterial culture or tissues infected with *M. leprae* [13] were autoclaved at 121 °C for 15 min and collected by centrifugation. Lipids were extracted from homogenized tissue with 20 volumes of chloroform/methanol (2:1, v/v) three times with vigorous grinding. The two phases were separated in a funnel, the lower organic phase was collected, and the solvent was evaporated from the organic phase. The total lipids were separated by solvent fractionation and tetrahydrofuran-soluble fraction was further separated by thin-layer chromatography (TLC) on silica gel plates (Uniplate; Analtech Inc. Newark, DE) with the solvent system of chloroform/methanol/water (90:10:1, by vol.) or chloroform/methanol/acetone/acetic acid (90:10:6:1, by vol.). Glycolipid spots were visualized with a 9 M H_2SO_4 spray followed by charring at 200 °C for 10 min or with iodine vapor for preparative purposes.

To determine the subclass composition of the mycolic acids in each mycobacterial TMM and TDM, mycolic acid methyl esters were prepared by alkaline hydrolysis of glycolipids. The glycolipids were hydrolyzed with 1.25 M NaOH in 90% methanol at 70 °C for 1 h and the resultant mycolic acids were then extracted with *n*-hexane after acidification with HCl, followed by methylation with benzene/methanol/H_2SO_4 (10:20:1, by vol.) [14]. Mycolic acid methyl esters from each

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glycolipid were fully separated into subclasses by TLC with the solvent system of benzene in a draft chamber under reduced pressure.

2.3. Mass spectrometry analysis

Analysis by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF mass) was carried out on a Voyager DE-STR (Applied Biosystems, Tokyo, Japan) with pulsed UV light (337 nm) from an N_2 laser, essentially according to the method reported previously [14]. TDM and TMM were analyzed in the reflectron mode by the instrument operated at 20 kV in the positive ion mode. The 2,5-dihydroxybenzoic acid (2,5-DHB) matrix was used at a concentration of 10 mg/ml in chloroform/methanol (2:1 v/v). Typically, 5 μl of TDM or TMM samples (5 μg) in chloroform/methanol (2:1 v/v) solution and 5 μl of the matrix solution were mixed, and 1.5 μl of the mixture was applied on a sample plate. An external mass spectrum calibration was performed using calibration mixture 2 of the Sequazyme Peptide Mass Standards kit (Applied Biosystems), including known peptide standards in a mass range from 1290 to 5700 Da. The molecular mass of mycobacterial TMM, TDM, and PGL-I was determined based on the quasimolecular mass ions $[M+Na]^+$ by the reflectron mode. In general, nominal number of atomic mass is used for calculation of number of molecular mass. However, there is a slight difference between nominal mass number and accurate mass number read from spectrometry [15]. For instance, when both of the two molecules of mycocerosates ($R_1$and R_2) in PGL-I are C32, the nominal mass number $[M+Na]^+$ of PGL-I ($C_{124}H_{232}O_{19}$) is $[(C \times 124) + (H \times 232) + (O \times 19) + (Na \times 1)] = 2047$ (2024 + 23), but the accurate number is 2050.15 $[(12.0107 \times 124) + (1.00794 \times 232) + (15.9994 \times 19) + 22.9898]$. The nominal mass numbers are given in the text.

3. Results

3.1. TLC analysis of mycolic acids methyl ester

To determine the subclass composition of the mycolic acids in each mycobacterial TMM and/or TDM, mycolic acid methyl esters were analyzed by TLC. The TLC analysis indicated that fatty acid methyl esters had two spots corresponding to either α- and keto-mycolic acid (Fig. 1A). The same two spots pattern was observed for *M. bovis* BCG Connaught (BCG-C) and *M. leprae* while three spots were detected for *M. tuberculosis* corresponding to α-, methoxy-, and keto-mycolic acid [16,17]. The bottom spot in ML lane could be cholesterol from armadillo's tissues (data not shown).

3.2. MALDI-TOF-MS analysis of TMM

We separated the final solvent extracts of *M. leprae* into four fractions (M1–M4). Fig. 1B shows a thin-layer chromatogram of the TMM and TDM from *M. tuberculosis* and the solvent fractionated glycolipids from *M. leprae*. *M. leprae* exhibited bands, which were faint, but significantly reddish glycolipid-like, migrating close to bands of TDM or TMM of *M. tuberculosis*. The major bands of M2 and M4 migrated close to TDM and TMM positions of *M. tuberculosis*, respectively. Therefore, we tried to carefully analyze the bands which may correspond to TDM and TMM by mass spectrometry.

Major band in M4 in Fig. 1B was analyzed using BCG-C as a reference. The mass spectra of TMM from BCG-C showed a biphasic distribution of pseudomolecular ions, $[M+Na]^+$ (Fig. 2A). In the higher mass ranges of BCG-C TMM, dominant ions were detected at *m/z* 1555, 1583, 1597, 1611 and 1625 due to $[M+Na]^+$ of C82, C84, C85, C86 and C87 keto mycolyl TMM, and the major mass ions in the lower mass ranges were detected at *m/z* 1455, 1483, 1511 and 1539 due to $[M+Na]^+$ of α-mycolyl TMM centering at C78, respectively (Fig. 2A) [18]. On the other hand, *M. leprae* derived TMM showed in lower mass ranges at *m/z* 1427, 1455, 1483, 1511

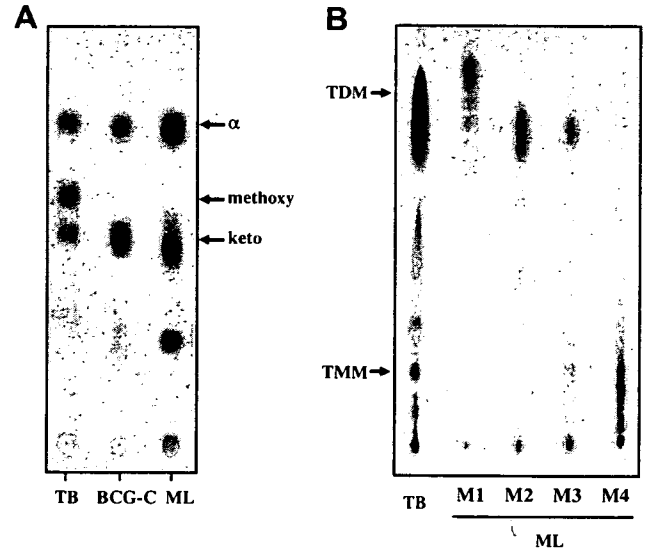

Fig. 1. Thin-layer chromatograms (TLC) of solvent extracts from tetrahydrofuran soluble fraction and mycolic acid methyl ester subclasses of *M. tuberculosis*, *M. bovis* BCG Connaught, and *M. leprae*. (A) TLC of mycolic acid methyl ester subclasses with the solvent system of benzene/methanol/H_2SO_4 (10:20:1, by vol.). Mycolic acid methyl ester subclasses of *M. tuberculosis* (TB); α-, methoxy- and keto-mycolic acid methyl esters are shown with the mycolic acid methyl ester mixture of glycolipids derived from armadillo tissue nodules infected with *M. leprae* (ML), and those of *M. bovis* BCG Connaught (BCG-C). (B) TLC of solvent extracts from *M. leprae* (ML) and *M. tuberculosis* (TB) with the solvent system of chloroform/methanol/acetone/acetic acid (90:10:6:1, by vol.). Trehalose monomycolate (TMM) and trehalose di-mycolate (TDM) bands of TB were identified previously and used as references in this TLC. Fractions 1–4 separated from the final extracts of *M. leprae* and designated M1–4.

and 1539 due to $[M+Na]^+$ of α-mycolyl TMM centering at C78 same to that of BCG-C (Fig. 2B). In the higher mass ranges, dominant ions were shifted lower and the major ions were detected at *m/z* 1541, 1569 and 1597 (Fig. 2B), indicating the major keto-mycolyl TMM consisted of C81, C83 or C85 mycolate, respectively. The molecular species of TMM from *M. leprae* and that from BCG-C are summarized in Table 1. These results indicate that *M. leprae* possess trehalose 6-monomycolate, with C78 α- and C83 keto-mycolates, as the major molecular species.

3.3. MALDI-TOF-MS analysis of TDM

TDM from BCG-C showed a diverse distribution of mass ions according to the combination of di α-, α- and keto-, and di keto-mycolic acid subclasses and each molecular species, that leads to a multiphasic distribution of mass ions due to the dominant combination of α–α, α–keto and keto–keto dimycolyl TDM (Fig. 2C). Given the small sample size, the identification of *M. leprae* TDM was achieved primarily on the thin-layer chromatographic behavior and MALDI-TOF mass analysis, in comparison with the analytical results from BCG-C possessing the same mycolic acid subclasses. Fig. 2D shows the positive MALDI-TOF mass spectra of *M. leprae* TDM (Fig. 1B, M2). In contrast to TDM from BCG-C, TDM from *M. leprae* showed a distinctive mass ion distribution shifted to lower mass ranges due to the major combinations of α–α dimycolyl TDM and α–keto dimycolyl TDM with a small shoulder due to keto–keto dimycolyl TDM. In *M. leprae* TDM, major

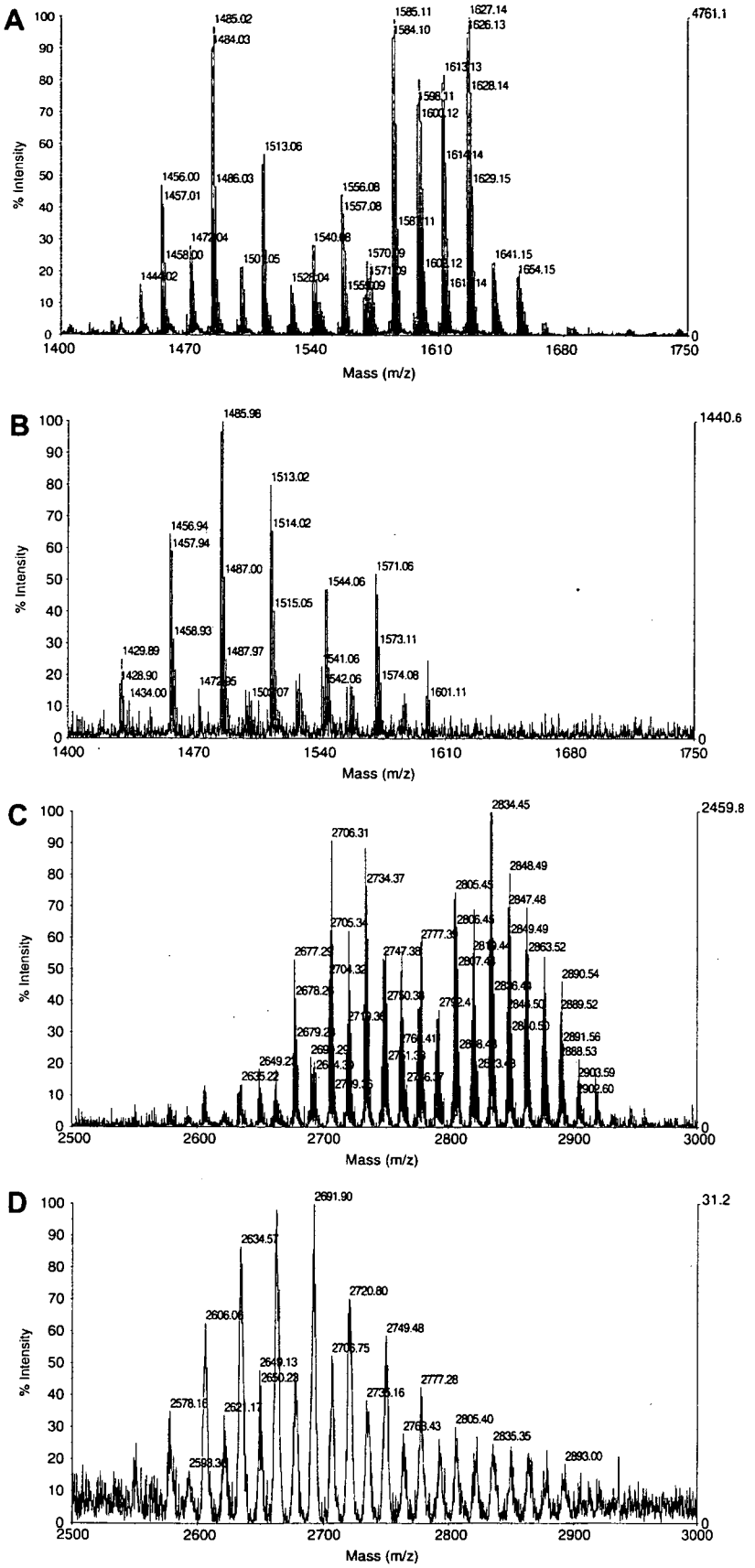

Fig. 2. MALDI-TOF-MS spectra of TMM and TDM. (A) TMM of *M. bovis* BCG Connaught, (B) TMM of *M. leprae* Thai 53, (C) TDM of *M. bovis* BCG Connaught, (D) TDM of *M. leprae* Thai 53.

Table 1
MALDI-TOF mass spectrometry data of the individual types of TMM from *M. leprae* (Thai 53) and *M. bovis* BCG (Connaught)

Species (strain)	Mycolic acid type[a]	Total carbon number of TMM mycolate																	
		74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91
M. leprae (Thai 53)	I	1427		**1455**		**1483**	1497	**1511**	1525	1539	1553								
	II			1471		1499		1527	**1541**	1555	**1569**	1583	1597						
M. bovis BCG (Connaught)	I			**1455**	1469	**1483**		**1511**		1539									
	II									**1555**	1569	**1583**	**1597**	**1611**	**1625**	1639	1653	1667	1681

[a]I; α-dicyclopropanoic or dienoic, II; keto-monocyclopropanoic or monoenoic. Major homologues are shown in bold.

mass ions due to molecular species possessing C76, C78, C80 α–α dimycolic acids were detected at *m/z* 2573, 2601 and 2629, and those possessing α–keto dimycolic acids at *m/z* 2659, 2687 and 2715, and those possessing keto–keto dimycolic acids at *m/z* 2731, 2745, 2773 and 2801, respectively. The deduced molecular species of TDM from *M. leprae* and that from BCG-C are summarized in Table 2.

3.4. MALDI-TOF-MS analysis of PGL-I

The MALDI-TOF mass spectra of M2 in Fig. 1B showed the existence of TDM as described above, however, the major cluster ions in mass spectra of M2 were observed in lower mass ranges from around *m/z* 1990 to 2100. So, we analyzed the spectra in more detail. The result indicates that the cluster ions are derived from phenolic glycolipid-I (PGL-I) (Fig. 3A). The nominal mass number of the PGL-I, which is assumed to have two molecules of C32 mycocerosates, was *m/z* 2047 (Fig. 3A and B). Thus, the deduced combination of mycocerosic acids with different carbon numbers in PGL-I are shown in Fig. 3A and the general structure of PGL-I is shown in Fig. 3B.

4. Discussion

In the present study, we have directly detected TDM and TMM from armadillo tissues infected with *M. leprae*. The presence of TMM in *M. leprae* possessing C74-82 α-mycolic acids has been reported previously [8,18], however, the existence of keto-mycolate in *M. leprae* TMM was not clear. We identified keto-mycolate clearly in *M. leprae* TDM and TMM, and the chain length of keto-mycolate in *M. leprae* was found to be shorter than those from slow-growing culturable mycobacteria such as *M. tuberculosis* and *M. bovis* BCG [19].

Previously, no detectable TDM was identified by the analysis of the lipids obtained from *M. leprae* infected armadillo [9,20]. However, in our hands, we observed a meager but significant spot on TLC from *M. leprae* extract which migrated to the position of TDM of *M. tuberculosis* (Aoyama B strain). When this spot was analyzed by mass spectrometry in reference to TDM from *M. bovis* BCG Connaught (BCG-C), TDM having α–α dimycolates were observed in the lower mass ranges than *m/z* 2673 as seen in TDM from BCG-C (Fig. 2C). Therefore, both *M. leprae* and *M. bovis*

Table 2
Most probable combination of mycolic acids constructing TDM from *M. leprae* (Thai 53) and *M. bovis* BCG (Connaught)

Mass no. of TDM (*m/z*)	*M. leprae* (Thai 53)	*M. bovis* BCG (Connaught)
2573	α76:α78	
2601	α76:α80, α78:α78	
2617	α74:k82, α76:k80, α78:k78, α80:k76	
2629	α78:α80	
2645	α74:k84, α76:k82, α78:k80, α80:k78, α82:k76	
2659	α76:k83, α78:k81	
2673	α76:k84, α78:k82, α79:k81, α80:k80, α82:k78	α76:k84, α78:k82
2687	α78:k83, α80:k81	
2701	α78:k84, α79:k83, α80:k82, α81:k81, α82:k80	α76:k86, α78:k84, α80:k82
2715	α80:k83	α76:k87, α78:k85
2729		α78:k86, α80:k84
2731	k80:k83, k81:k82	
2743		α78:k87, α80:k85
2745	k81:k83	k82:k82
2757		α80:k86
2773	k83:k83	k82:k84
2787		k82:k85
2801	k83:k85, k84:k84	k82:k86, k84:k84
2815		k82:k87, k84:k85
2829		k84:k86, k85:k85
2843		k84:k87, k85:k86
2857		k85:k87, k86:k86
2871		k86:k87
2885		k87:k87

α,α-dicyclopropanoic or dienoic; k, keto-monocyclopropanoic or monoenoic mycolic acid. Molecular ions of TDM with intensities ≥ 30% of the highest intensity observed are listed.

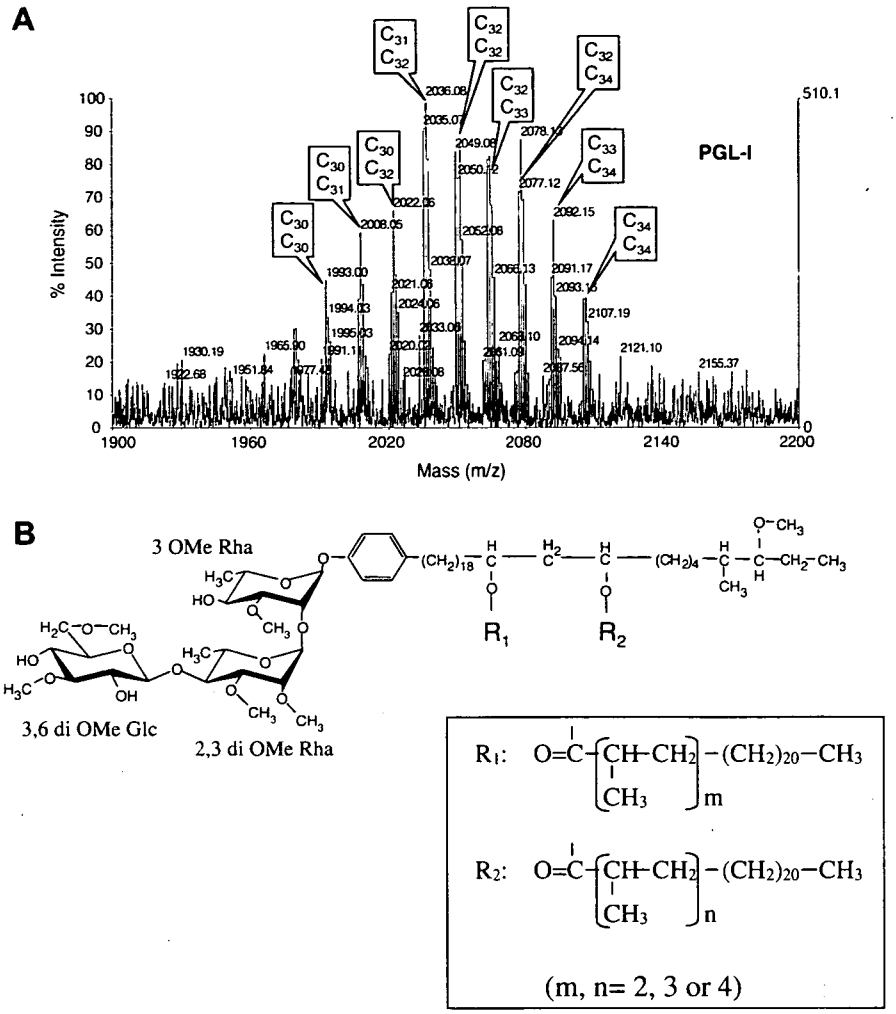

Fig. 3. MALDI-TOF mass spectra of *M. leprae* PGL-I and the deduced chemical structure. (A) The mass spectra of the major cluster ions ranging from around *m/z* 1997 (C30:C30) to 2103 (C34:C34) due to PGL-I separated by solvent fractionation, followed by TLC. C30–C34 shows carbon numbers of two sets of mycocerosate in PGL-I. (B) The chemical structure of *M. leprae* PGL-I. R indicates mycocerosate.

BCG-C can synthesize α-mycolic acids with similar sizes (C74 to C82 centering at C78), however, the subclass composition of keto-mycolate differed. *M. bovis* BCG-C synthesizes multi sub-classes of ketomycolic acids such as *cis*-monoenoic, *cis*-monocyclopropanoic, *cis*-monoenoic *cis*-monocyclopropanoic, *trans*-monoenoic and *trans*-monocyclopropanoic mycolates [21], while *M. leprae* synthesize only *cis*-monoenoic ketomycolate centering at C81 or C83 (Tables 1 and 2) [22]. Recently, the immunological role of mycolic acid has become a point of focus. The *trans*-, but not *cis*-cyclopropanation of mycolic acids in TDM induce massive inflammation in the lesion infected with *M. tuberculosis* and, in this respects, the *trans*-cyclopropane structure may be contributing to virulence [23,24]. Furthermore, recently, it was reported that the Δ*kasB* (a β-keto acyl-ACP synthase gene) mutant strain of *M. tuberculosis* synthesized shorter chain mycolate than the wild ones, and resulted in the loss of keto-mycolic acid *trans*-cyclopropanation. The *kasB* deletion induced the ability of the mutant strain to persist in infected mice for up to 600 days without causing disease [25]. Thus, the lack of *trans*-cyclopropanoic keto-mycolic acid in *M. leprae* may contribute to the low virulency in contrary to substantial quantities found in most of the slow growing pathogenic mycobacteria.

In the search for TDM, we also identified a major cluster ions in M2 fraction. Detailed analyses showed that the ions were derived from phenolic glycolipid (PGL-I), which is a major species specific glycolipid of *M. leprae*. Previously, combined GLC-mass spectroscopic analysis of the trisaccharide of PGL-I had been achieved, but TOF-mass spectrometric data have not been reported [26]. We analyzed the M1 fraction by MALDI TOF mass spectrometry and detected some clusters. Mass spectra of major clusters look like monoglycosyl phthiocerol diester and diglycosyl phthiocerol diester (data not shown), which differ from PGL-I in lacking the terminal sugars [27]. The co-existence of both PGL-I and TDM in armadillo tissue nodules infected with *M. leprae* indicates that these lipids were originated actually from *M. leprae*. Thus, this paper identifies TDM structure of *M. leprae* for the first time and shows that *M. leprae* TDM contains only α-mycolic acids and shorter chain *cis*-monoenoic keto-mycolic acids, exclusively.

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