Southern blot were prepared by using a Roche DIG labelling kit (Roche) according to the instructions.

2.3. Construction of suicide vector containing fapS-disrupted sequences

To generate the non-polar mutation in the gene encoding FAP of *M. smegmatis* (named as *fapS*), suicide vector containing DNA fragment flanking the *fapS* gene and kanamycin resistance cassette was constructed. A 3.0 kb fragments both upstream and downstream to *fapS* gene was amplified from *M. smegmatis* mc^2155 genomic DNA using the following two pairs of primers, for upstream; US1 (including *Hind* III site) and UA1 (including *Xba* I site); for downstream DS1 (including *Xba*I site) and DA1 (including *Kpn* I site). The PCR products were digested with restriction enzymes and cloned into *Hind* III–*Xba* I and *Xba* I–*Kpn* I site of pUC19 to give pUDFAP. The kanamycin resistance cassette, a selective marker of integration, was excised from pMV261 with *Hind* III and *Spe* I and cloned into corresponding sites of pBluescript II SK (+). The resulting plasmid was digested with *Kpn* I and *Xba* I, and derived fragment was cloned into the corresponding sites of pUC19. The 1.1 kb *Hind* III fragment harboring kanamycin resistance cassette was then cloned into pUDFAP to create the plasmid, pUDFAPKm, carrying the disrupted sequences.

2.4. Generation of the fapS-disrupted strain

The two-step recombination was conducted to disrupt the *fapS* gene located on the *M. smegmatis* chromosome. The suicide vector pUDFAPKm was introduced into the *M. smegmatis* mc^2155 by electroporation, and single crossover strains (plasmid-integrated transformants) were selected on the 7H10 plate containing 25 μg/ml kanamycin. A kanamycin-resistant colony was subjected to repeated cultivation in the 7H9 medium without kanamycin to excise the pUC19 region and kanamycin resistant cassette through the second crossover event. After several subcultures, the kanamycin-sensitive clones were selected and their genomic DNA was subjected to Southern blot and PCR analysis using the primers U1 and D1 to confirm the disruption of the *fapS* gene.

2.5. Construction of the FAP expression vectors for complementation

The *fapS* (FAP-S) gene and its homologous gene of FAP-B (*M. bovis* BCG), FAP-L (*M. leprae*) and FAP-A (*M. avium*) were used to construct the expression vector for complementation of *fapS*-mutant. Four genes were amplified from each genomic DNA with following primers; S1 (including *Hind* III site and start codon) and S2 (including *Cla* I site and stop codon) for *fapS*, B1 (including *Bam*H I site and start codon) and B2 (including *Eco*R I site and stop codon) for FAP-B, L1 (including *Bam*H I site and start codon) and L2 (including *Pst* I site and stop codon) for FAP-L, and A1 (including *Eco*R I site and start codon) and A2 (including *Cla* I site and stop codon) for FAP-A. The PCR products were digested with each restriction enzyme and cloned into the corresponding sites of pMV261. The expression cassette, consisting of *hsp60* promoter, the FAP gene and transcriptional terminator, were excised with *Xba* I and *Nhe* I and inserted into *Xba*I site of pMV306kan (kindly gifted from Dr. W.R. Jacobs, Jr.), a site-specific integrating mycobacterial vector, to give complementation vector pFAPS, pFAPB, pFAPL and pFAPA, respectively. These vectors were introduced into the *fapS* mutant by electroporation. Transformants were selected on the 7H10 plate containing 25 μg/ml kanamycin.

2.6. Morphological observation

The wild type, mutant strain and its complemented strains were cultured for two days in 2 ml of Middlebrook 7H9 broth. Aliquots of cultured broth were stained by Ziehl-Neelsen method and the bacteria was morphologically examined under light microscopy BX60 (Olympus). The images were acquired using a microscope digital camera DP50 (Olympus) and Viewfinder Lite software.

2.7. Assessment of cell surface hydrophobicity

The hydrophobicity of the bacteria was assessed by measuring the adherence of bacteria to hydrocarbons, *n*-hexadecane and xylene, as previously described with some modification [11,18]. Briefly, the 0.5 ml of *n*-hexadecane or xylene was added to the test tube containing 2.0 ml of phosphate buffered saline (PBS)-washed bacterial suspension, of which OD_{650} titer was adjusted at 1.0, and vigorously mixed using vortex for 1 min. After standing at room temperature for 20 min, the absorbance (OD_{650}) of the lower aqueous phase was measured. The hydrophobicity of bacteria was evaluated by calculating the reduction of absorbance values. The hydrophobic bacteria are assumed to remain in upper phase because of adhesion to hydrocarbons.

Congo red binding assay was also conducted to measure the bacterial hydrophobicity [11,19]. The bacteria was cultured for two days in 5 ml of Middlebrook 7H9 broth supplemented with the Congo red (100 μg/ml). The cells recovered from cultured broth were washed with PBS to eliminate unbound Congo red completely, and then resuspended in 0.2 ml of acetone. After gently shaking for 2 h, cells were pelleted by centrifugation and the concentration of Congo red released from bacteria into supernatant was measured spectrophotometrically

at 488 nm. The relative binding index was defined as the OD_{488} of acetone extract divided by OD_{650} of the original broth after certain period of cultivation.

2.8. Isolation and analysis of lipids

Total lipids were extracted from 50 ml of bacterial cell with $CHCl_3/CH_3OH$ (2:1, v/v) for several hours at room temperature. The lipid extracts from the organic phase were separated from the aqueous phase and evaporated to dryness. For analysis of GPLs, the lipids extracts were subjected to mild alkaline hydrolysis as previously described [20]. Total lipids and GPLs were analyzed by thin layer chromatography (TLC) on silica gel 60 plates (Merck) using $CHCl_3/CH_3OH/H_2O$ (30:8:1, v/v/v) and $CHCl_3/CH_3OH$ (9:1, v/v) as solvent respectively and visualized by spraying with a solution of 10% H_2SO_4 and subsequent charing.

2.9. Statistical analysis

Students' *t* test was applied to demonstrate statistically significant differences.

3. Results

3.1. Sequence analysis of fapS gene

The gene data base analysis of FAP-L (ML2055) and FAP-B (AF013569) indicated that the putative ORF of FAP, designated as *fapS*, was located between 1,810,474 and 1,811,535 bases of contig 3310 of the unfinished *M. smegmatis* genome database of in the Institute for Genomic Research (http://www.tigr.org). As shown in Fig. 1, the BLAST search analysis of the amino acids sequences of various FAP indicated that the homology of FAP-S with FAP-B (AF013569), FAP-A (U53585) and FAP-L (ML2055) was 51%, 48% and 47%, respectively. The homology was confirmed by FASTA program. We deduced the fibronectin-binding region of *M. smegmatis* FAP by homology analysis to that of *M. avium* and *M. bovis* BCG which were previously described [21].

3.2. Disruption of fapS gene

For the functional analysis of the FAP in *M. smegmatis*, the *fapS* gene was disrupted by the homologous recombination using the suicide vector. From the kanamycin-resistant isolates, MF96 was isolated as the putative mutant (Fig. 2). Southern blot analysis was performed on *Eco*R I-digested chromosomal DNA from wild type *M. smegmatis* and mutant strain MF96 to reveal the target gene disruption. Two different probes corresponding to the sequences in upstream (P1) and downstream (P2) of the *fapS* region were constructed (Fig. 2(a)). The probes P1 and P2 hybridized to the predicted 2.4 kb and 2.8 kb *Eco*RI fragments of the wild type strain, respectively, while both probes hybridized to sole 4.2 kb fragment in the strain MF96. The *fapS* gene disruption was further confirmed by using PCR analysis,

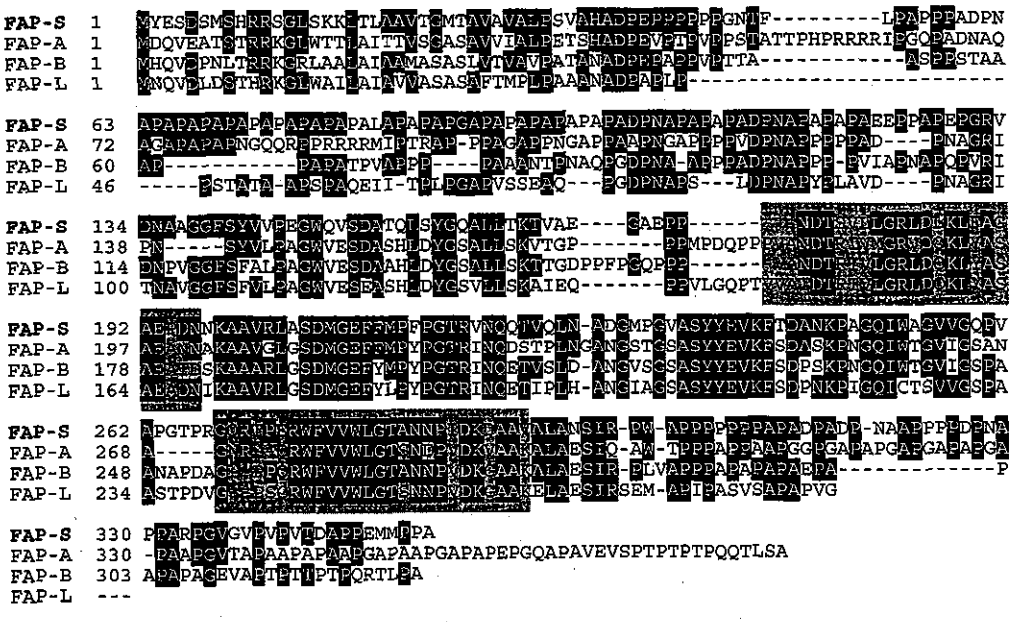

Fig. 1. Alignment of deduced amino acid sequences of *fapS* gene product (FAP-S) with FAP-A (*M. avium*), FAP-B (*M. bovis* BCG) and FAP-L (*M. leprae*). The amino acids identical to those of FAP-S are indicated by black boxes. The putative sequences of fibronectin-binding region are shown in gray background.

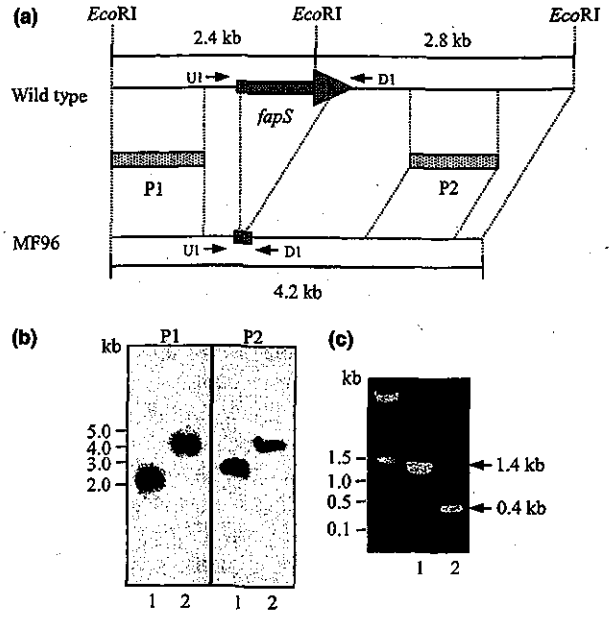

Fig. 2. Disruption of *fapS* gene. (a) Schematic diagram of the *fapS* region on the chromosome of wild type *M. smegmatis* (mc^{2}155) and the mutant strain MF96. The position of the two hybridization probes, P1 and P2, are also shown. The arrows indicate the primers (U1, D1) used for PCR analysis. (b) Southern blot analysis of wild type (lane 1) and strain MF96 (lane 2). Chromosomal DNA was digested with *Eco*R I and hybridized with probe P1 and P2. C, PCR analysis of wild type (lane 1) and the strain MF96 (lane 2) using the primers indicated above.

in which the primers (U1 and D1) were constructed outside the *fapS* gene. As expected, while 1.4 kb fragment was amplified from wild type, 0.4 kb fragment was revealed by strain MF96. The size difference between the strains (1.0 kb) indicated that most of *fapS* gene was deleted.

3.3. *Morphology of* fapS *mutant*

The *fapS* mutant, cultured in liquid medium, showed a morphological change. Observation by the light microscopy revealed that the strain MF96 bacteria clumped together (Fig. 3A(b)), whereas most of wild type cells grew as a single cell and distributed equally in the field (Fig. 3A(a)). The strain MF96 cells quickly settled down to the bottom of the tube, whereas wild type remained dispersed in the broth (Fig. 3B). However, aggregation of strain MF96 was not attributable to the growth rate, moreover no differences were observed between MF96 and wild type strain in the colony morphology (data not shown). In order to confirm that the enhanced aggregation of strain MF96 was induced by the disruption of *fapS* gene, *fapS* gene was complementarily introduced into the *fapS* mutant (Fig. 4). The strain MF96 was transformed with the vectors containing each expression cassette of the FAP from

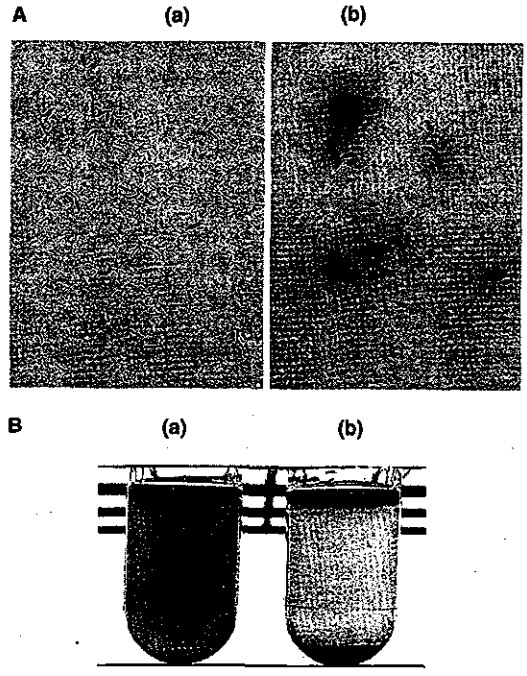

Fig. 3. Morphological observation of wild type (a) and mutant strain MF96 (b). (A) Light microscopic observation of cultured broth diluted 1:100 in PBS containing 0.2% skim milk followed by Ziehl-Neelsen staining. Magnification, 1000×. (B) Cultured broths left for 1 h without shaking.

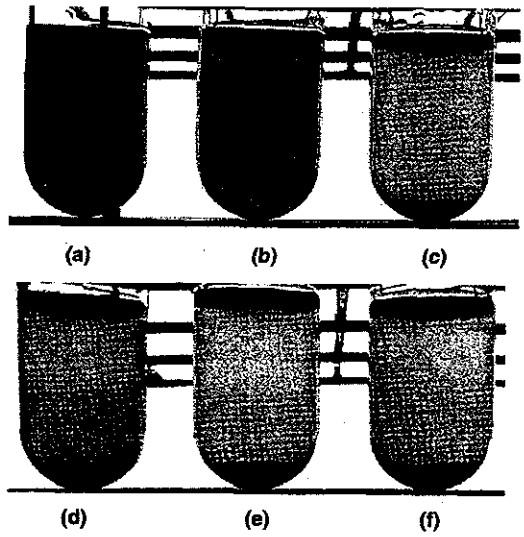

Fig. 4. Complementation experiment. Observation of wild type (a) and mutant strain MF96 transformed with pFAPS (b), pMV306kan (c), pFAPL (d), pFAPB (e) and pFAPA (f). All cultured broths were left for 1 h without shaking.

M. smegmatis, *M. leprae*, *M. bovis* BCG and *M. avium*, and their expressions in the complemented MF96 strains were confirmed by Western blot analysis of cell wall fractions obtained by disrupting the cell wall using

Mini-BeadBeater. The level of FAP expression was similar among the complemented strains (data not shown). The monoclonal Ab used in the Western blot solely reacted with cell wall fraction. The strain MF96 lost the property to aggregate each other by transformation with parent *M. smegmatis*-derived FAP gene (pFAPS). However, the strains transformed with other mycobacterial FAP expression vectors did not reverse the aggregation property, and they were similar to the strain MF96 which were transformed with a control vector (pMV306kan). Therefore, the FAP from other mycobacteria could not supplement the aggregation-inhibiting function of *M. smegmatis*-derived FAP, in spite of FAP genes being complementarily introduced. These results demonstrated that the bacterial aggregation induced in the strain MF96 was caused by the disruption of *fapS*.

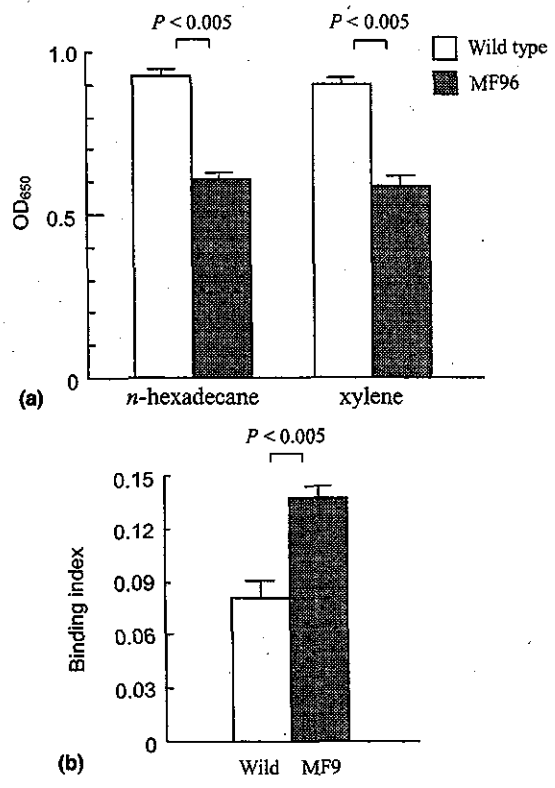

Fig. 5. (a) The cell surface hydrophobicity based on the degree of adherence to two hydrocarbons (*n*-hexadecane and xylene), assessed by the reduction in absorbance of aqueous phase separated from hydrocarbon mixture including cells of wild type (open square) and mutant strain MF96 (filled square). (b) Congo red binding of wild type (open square) and mutant strain MF96 (filled square). The binding index is derived from OD_{488} of acetone extract divided by OD_{650} of cultured broth. Representative of more than three separated experiments are shown. Assays were done in triplicate, and the results are expressed as means ± SD.

3.4. Cell surface properties of fapS mutant

It might be reasonable to speculate that *fapS* gene disruption induces the change of bacterial surface properties. Therefore, hydrophobicity and Congo red binding ability of strain MF96 were assessed. The hydrophobicity assay was basically assessed by measuring the degree of adherence of bacteria to hydrophobic molecules, such as *n*-hexadecane and xylene (Fig. 5(a)). The OD_{650} of aqueous phase in the strain MF96 was significantly lower than that in wild type *M. smegmatis*. Similar results were obtained in both assays using *n*-hexadecane and xylene (Fig. 5(a)). These results suggested that strain MF96 adhered more easily to hydrophobic molecule and remained in the upper hydrophobic phase. Furthermore, the hydrophobicity of strain MF96 was assessed using Congo red binding assay (Fig. 5(b)). The concentration of Congo red bound to bacterial surface and subsequently solubilized was significantly higher in strain MF96 than that in the wild type (Fig. 5(b)).

Next, the role of FAP in the bacterial attachment to host cells (J774 cells) was examined by Ziehl Neelsen staining method. There was no significant difference in the number of attached or intracellular bacteria between

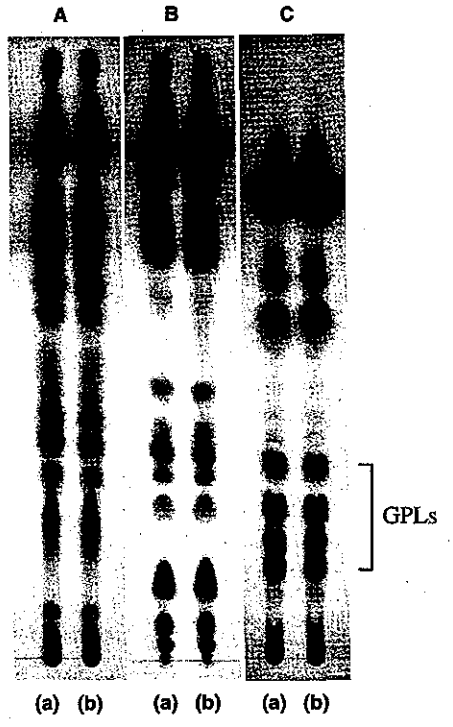

Fig. 6. Comparison of the lipid composition of wild type (a) and mutant strain MF96 (b). (A) Total lipids developed with $CHCl_3/CH_3OH$ (9:1, v/v). (B) Total lipids developed with $CHCl_3/CH_3OH/H_2O$ (30:8:1, v/v/v). (C) Total lipids subjected to mild alkaline hydrolysis and developed with $CHCl_3/CH_3OH$ (9:1, v/v). GPLs, glycopeptidolipids.

the fibronectin-opsonized wild and MF96 mutant strains (data not shown).

3.5. Lipid analysis

In order to find out whether the *fap* gene disruption induces the alteration of cell wall lipid composition of *M. smegmatis* and causes aggregation of the mutant strain, TLC analysis using different solvent systems on total lipids from wild type and mutant strain was carried out. The TLC profiles of both strains were very similar to each other, and no detectable alterations were observed in the total lipid content as well as GPLs (Fig. 6).

4. Discussion

The FAP is involved in the attachment and internalization of mycobacteria to host cells. However, little is known about physiological role of FAP, such as how FAP is involved in bacterial interaction with the host cells. In this study, we undertook a gene disruption approach to construct the gene mutant of *M. smegmatis* to characterize the function of FAP

The FAP disruptant showed increased hydrophobic properties and a change in morphology which caused an enhanced bacterial aggregation. Previously, it has been reported that the absence or poor content of some glycolipids was induced by the reduction of biosynthetic enzyme activities and this caused an aggregation of bacteria along with the changes in the cell surface properties [10,11,22]. Also, the other family of fibronectin-binding protein, such as antigen 85 complex in *M. tuberculosis*, possess mycolyltransferase activity required for maintaining the integrity of the mycobacterial cell envelope [23–25]. However, comparative TLC analysis on total lipids extracts in different solvent system revealed no significant differences in the lipid profile between wild type and the FAP mutant (Fig. 6). Although the mycobacterial proteins, PE_PGRS and HBHA, are known to promote aggregation of cells [26,27], there is no report indicating that the absence of such a protein component would directly lead to the more aggregated phenotype. Thus, the FAP seems not to have influence on the biosynthesis of cell wall lipid, but show an enhanced aggregation of the *M. smegmatis*.

Analysis of the amino acid sequences of FAP showed that the hydrophobic amino acids, alanine and proline, are relatively rich compared to the other mycobacterial proteins [21,28], and these amino acids are notably present in the N-terminal region of FAP (Fig. 1). These facts may indicate that FAP is an amphiphilic molecule consisting of both hydrophobic and hydrophilic part similar to glycolipid. Alanine and proline rich region of FAP may correspond to acyl chains of glycolipid such as polyacyltrehalose and GPLs which are inserted into the hydrophobic core composing mycolic acid chain [10,11]. FAP mutant, generated by gene disruption, showed increased surface hydrophobicity as observed by enhanced adherence to hydrocarbons (Fig. 5). The increased hydrophobicity may be due to the exposure of hydrophobic lipid molecules which are otherwise located inside the cell wall in the wild type strain and FAP may have functioned as the amphiphilic layer with the hydrophilic proteins on the surface. Therefore, the increased hydrophobicity of the mutant could lead to the aggregation of bacteria.

The analysis of the deduced amino acid sequences of *fapS* gene product indicated that its homology with the FAP of other mycobacteria was around 50%, although the deduced fibronectin-binding region of the FAP was conserved in various mycobacteria including *M. avium*, *M. bovis* BCG and *M. leprae* (Fig. 1) [21,28]. The morphology of the bacteria was not recovered when the genes encoding FAP of *M. bovis* BCG, *M . leprae* and *M. avium* were introduced into the mutant strain, while the complementation with *M. smegmatis*-derived *fapS* reverse the aggregation property (Fig. 4). The possible explanation for non-recovery of the morphology of the bacteria is due to (1) low homology except the fibronectin-binding domain which is highly conserved between the FAP homologues, (2) interaction of FAP with other specific components in the cell wall of *M. smegmatis*. It has been reported that FAP locates in the cell wall fraction of mycobacteria [2]. However, its precise location in the cell wall is different in each mycobacteria [1,3,5]. In fact, in contrast to *M. bovis* BCG, the FAP of *M. smegmatis* does not appear on the bacterial surface as far as examined by fluorescence-activated cell sorting (FACS) using FAP monoclonal antibody which can detect the *M. smegmatis*-derived FAP by Western blot analysis (data not shown). This fact might be another reason why FAPs from other mycobacteria failed to reverse the aggregation property of MF96 mutant strain. Furthermore, it may be able to explain why there is no significant difference of host cell attachment activity between wild type and MF96 mutant strains.

Taken together, our study indicates that the FAP possesses novel function which are indispensable to prevent the bacterial aggregation that might contribute to the understanding of the mycobacterial biogenesis.

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Upregulation of T-Cell-Stimulating Activity of Mycobacteria-Infected Macrophages

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Abstract

Macrophages are one of the most abundant host cells to come in contact with mycobacteria. However, the infected macrophages less efficiently stimulate autologous T cells *in vitro*. We investigated the effect of the induction of phenotypic change of macrophages on the host cell activities by using *Mycobacterium leprae* as a pathogen. The treatment of macrophages with interferon-γ (IFN-γ), GM-CSF and interleukin-4 deprived macrophages of CD14 antigen expression but instead provided them with CD1a, CD83 and enhanced CD86 antigen expression. These phenotypic features resembled those of monocyte-derived dendritic cells (DC). These macrophage-derived DC-like cells (MACDC) stimulated autologous $CD4^+$ and $CD8^+$ T cells when infected with *M. leprae*. Further enhancement of the antigen-presenting function and CD1a expression of macrophages was observed when treated with IFN-γ. The *M. leprae*-infected and -treated macrophages expressed bacterial cell membrane-derived antigens on the surface and were efficiently cytolysed by the cell membrane antigen-specific $CD8^+$ cytotoxic T lymphocytes (CTL). These results suggest that the induction of phenotypic changes in macrophages can lead to the upregulation of host defence activity against *M. leprae*.

Introduction

The extent of mycobacterial spread is closely associated with the disease severity and is controlled by bacterial antigen-specific cell-mediated immunity. The activation of $CD4^+$ and $CD8^+$ T-cell subsets is induced by cell–cell contact with antigen-presenting cells (APC), which is therefore the most critical host defence component against mycobacterial infection [1–3]. The mycobacteria reside in phagosomes of macrophages but restrict the capacity of the phagosomes to fuse with late endosomes/lysosomes [4] and avoid getting being processed. These observations suggest that mycobacteria-infected macrophage less efficiently express the bacterial antigens on their surface. Furthermore, macrophages generally lack the activity to load peptide derived from exogenous protein or bacterial protein to major histocompatibility complex (MHC) class I molecules. Therefore, it is assumed that macrophages are resistant to MHC-restricted-killing activities of cells such as CTL allowing mycobacteria to reside and hide in macrophages.

Here, we tried to upregulate the APC function of macrophages in order to enhance the host defence activity. Leprosy, caused by *Mycobacterium leprae* infection, provides a useful model to evaluate immunoregulatory mechanism against an intracellular pathogen, because the disease extent is closely associated with the potential of host defence activity. *M. leprae* preferentially infects macrophages and Schwann cells [5–7] and induces clinical manifestation mainly in skin and peripheral nerves. However, the disease shows broad spectrum with the various skin manifestations from single lesion (tuberculoid leprosy) to almost entirely disseminated one (lepromatous leprosy). The mechanism that produces this broad spectrum involves the extent of cell-mediated immune response to *M. leprae*. We previously reported that macrophages phagocytosed *M. leprae*, but their antigens are not fully expressed on the surface of macrophages, which results in reduction or avoidance of their contact with T cells [8]. In addition to macrophages, monocyte-derived dendritic cells (DC) are well-characterized subset of professional APC, capable of stimulating both naïve and memory type autologous $CD4^+$ and $CD8^+$ T cells [9–11]. We also demonstrated that, in contrast to macrophages, the DC expressed *M. leprae*-derived antigens on the surface and stimulated

both T-cell subsets to produce interferon-γ (IFN-γ) which is a representative type 1 cytokine and is considered to be associated with the killing of intracellular mycobacteria [2, 12]. Thus, DC can evoke the activation of type-1 T cells and control the multiplication of *M. leprae* [8, 13–15].

Macrophages are the most abundant primary cell type to come in contact with *M. leprae* and are frequently seen in inflamed tissues. These macrophages are thought to play a role in initial antimycobacterial immune responses for the better control of bacterial spread. Therefore, we tried to induce phenotypic changes in *M. leprae*-infected macrophages to DC-like cells and examined their antigen-presenting activities such as stimulation and differentiation of T cells into type-1 cells. Furthermore, we examined their susceptibility to killing activities of *M. leprae*-derived antigen-specific CTL.

Materials and methods

Preparation of cells and bacteria. Peripheral blood was obtained under informed consent from healthy, but PPD-positive, individuals. Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll–Paque Plus (Pharmacia, Uppsala, Sweden) and cryopreserved in liquid nitrogen until use, as previously described [16]. Macrophages were differentiated by culturing plastic adherent $CD14^+$ monocytes with RPMI 1640 medium containing M-CSF (R&D Systems, Minneapolis, MN, USA) [17]. For preparation of the monocytes, $CD3^+$ T cells were removed from either freshly isolated heparinized blood or cryopreserved PBMC using immunomagnetic beads coated with anti-CD3 monoclonal antibody (Dynabeads 450, Dynal, Oslo, Norway), and the plastic adherent cells were used as monocytes [16]. Monocyte-derived DC were differentiated from the monocytes [16, 18]. Briefly, the monocytes were cultured for 5 days in the presence of 50 ng of recombinant GM-CSF (Pepro Tech EC LTD, London, UK) and 10 ng of rIL-4 (Pepro Tech) per ml. rGM-CSF and rIL-4 were supplied every 2 days. Macrophage-derived DC (MACDC) were differentiated from macrophages as follows. Macrophages were treated with 300 U/ml of IFN-γ during last 24 h of 3-day culture. On day 3, macrophages were washed, and the media were replaced with media containing rGM-CSF and rIL-4. The production of MACDC was conducted by using the same protocol to that of DC. In some cases, *M. leprae*-infected or -uninfected macrophages, MACDC and DC were further treated with indicated doses of maturation and activation factors for DC including CD40 ligand (L) (Pepro Tech) or lipopolysaccharide (LPS) (*Escherichia coli* 0111: B4, Difco Laboratories, Detroit, MI, USA).

M. leprae (Thai-53) was obtained from footpads of BALB/*c-nu/nu* mice. The isolated bacteria were counted by Shepard's method [19] and were frozen at −80 °C until use. The viability of *M. leprae* was assessed by using fluorescent diacetate/ethidium bromide test [20]. The macrophages and DC were infected with *M. leprae* by coculturing at indicated multiplicity of infection (MOI). The MOI was determined upon an assumption that all macrophages and DC were susceptible to infection with *M. leprae*. The macrophages and DC were previously shown to be similarly susceptible to the bacterial infection *in vitro* [8].

Analysis of cell-surface and intracellular antigens. The expression of cell-surface antigens on macrophages, MACDC and DC was analysed using FACScalibur (Becton Dickinson Immunocytometry System, San Jose, CA, USA). About 1×10^4 live cells were analysed. For analysis of cell-surface antigens, the following monoclonal antibodies were used: FITC-conjugated monoclonal antibodies against HLA-ABC (G46-2.6, BD Biosciences, San Jose, CA, USA), HLA-DR (L243), CD14 (Leu-M3, Becton Dickinson) and CD86 (FUN-1, BD Biosciences) and phycoerythrin-labelled monoclonal antibodies against CD83 (HB15a, Immunotech, Marseille, France). Murine-unlabelled monoclonal antibody to CD1a (NA1/34, Serotec, Oxford, UK) was also used and was visualized by FITC-labelled goat $F(ab')_2$ antimouse immunoglobulin G (IgG) (Tago-immunologicals, Camarillo, CA, USA). Purified rabbit polyclonal antibodies (pAbs) to cell-wall proteins, cell membrane fractions and cytosol fractions of *M. leprae* each depleted of lipoarabinomannan (provided by Drs J. Spencer and P. J. Brennan, Colorado State University) were used. The details of the preparation are available at http://www.cvmbs.colostate.edu/mip/leprosy. Among the *M. leprae*-derived fractions, membrane fraction was the most antigenic in terms of activation of adaptive immunity [21]. We also used pooled sera from 10 untreated lepromatous leprosy patients who were classified according to clinical criteria (WHO) based on skin smears (given by Dr H. Minagawa, Leprosy Research Center, Tokyo, Japan) [8]. FITC-conjugated murine antihuman Ig (Tago-immunologicals) was used as secondary antibody for detection. The optimal concentrations of monoclonal antibodies, pAbs and patient's pooled sera were determined in advance.

Assessment of APC functions of macrophages, MACDC and DC. The ability of macrophages, MACDC and DC infected with *M. leprae* to stimulate autologous T cells was assessed using an autologous stimulator T-cell-mixed reaction as previously described [8, 18]. The stimulators such as macrophages, MACDC and DC were treated with 50 μg/ml of mitomycin C, washed extensively to remove extracellular bacteria and were used as a stimulator. $CD4^+$ and $CD8^+$ T cells purified using immunomagnetic beads coated with monoclonal antibodies to CD8 and CD4, respectively, were used as a responder population. Responder cells (1×10^5/well) were plated in 96-well, round-bottom tissue culture plates, and stimulators were added to give an indicated stimulator responder T-cell

ratio. The T-cell proliferation during the last 10 h of a 4-day culture in the presence of 4% heat-inactivated human serum was quantified by incubating the cells with 1 μCi/well of [^{3}H]-thymidine.

Assessment of cytokine production. Levels of the following cytokines were measured: IFN-γ and IL-10 produced by $CD4^+$ and $CD8^+$ T cells stimulated for 4 days with macrophages, MACDC or DC and IL-12 p70 produced by *M. leprae*-infected or -uninfected macrophages and MACDC upon a stimulation with soluble form CD40L for 24 h. The concentrations of IFN-γ, IL-12 p70 and IL-10 were quantified using OptEIA ELISA Set available from BD Biosciences.

Assessment of cytotoxic activity of $CD8^+$ T cells. The susceptibility of *M. leprae*-infected macrophages and MACDC to the cytokilling activity of $CD8^+$ T cells was evaluated. Ten thousand macrophages and MACDC, either uninfected or infected with *M. leprae* at MOI 5, were used as a target cell. As an effector population, $CD8^+$ CTL activated by stimulation with autologous DC, which were pulsed with 15 μg/ml of *M. leprae*-derived cell membrane fractions, were used. The targeT cells were cocultured for 5 h with the effector cells at various effector/target cell ratios. The supernatant was collected for lactate dehydrogenase (LDH) release assay. The concentration of LDH released by target cell death was measured according to the instructions of the assay kit (Cyto Tox 96® Non-Radioactive Cytotoxicity Assay, Promega, Madison, WI, USA). The percent specific killing was calculated as follows:

$$\frac{\text{LDH (test sample)} - \text{LDH (spontaneous effector cell level)} - \text{LDH (spontaneous target cell level)}}{\text{LDH (total target cell lysis level)} - \text{LDH (spontaneous target cell level)}}$$

Statistical analysis. Student's *t*-test was applied to demonstrate statistically significant differences.

Results

Upregulation of APC function of *M. leprae*-infected macrophages

Phenotypic characterization of MACDC was carried out by analyzing the surface expression of various molecules on macrophages and MACDC (Fig. 1). There were no

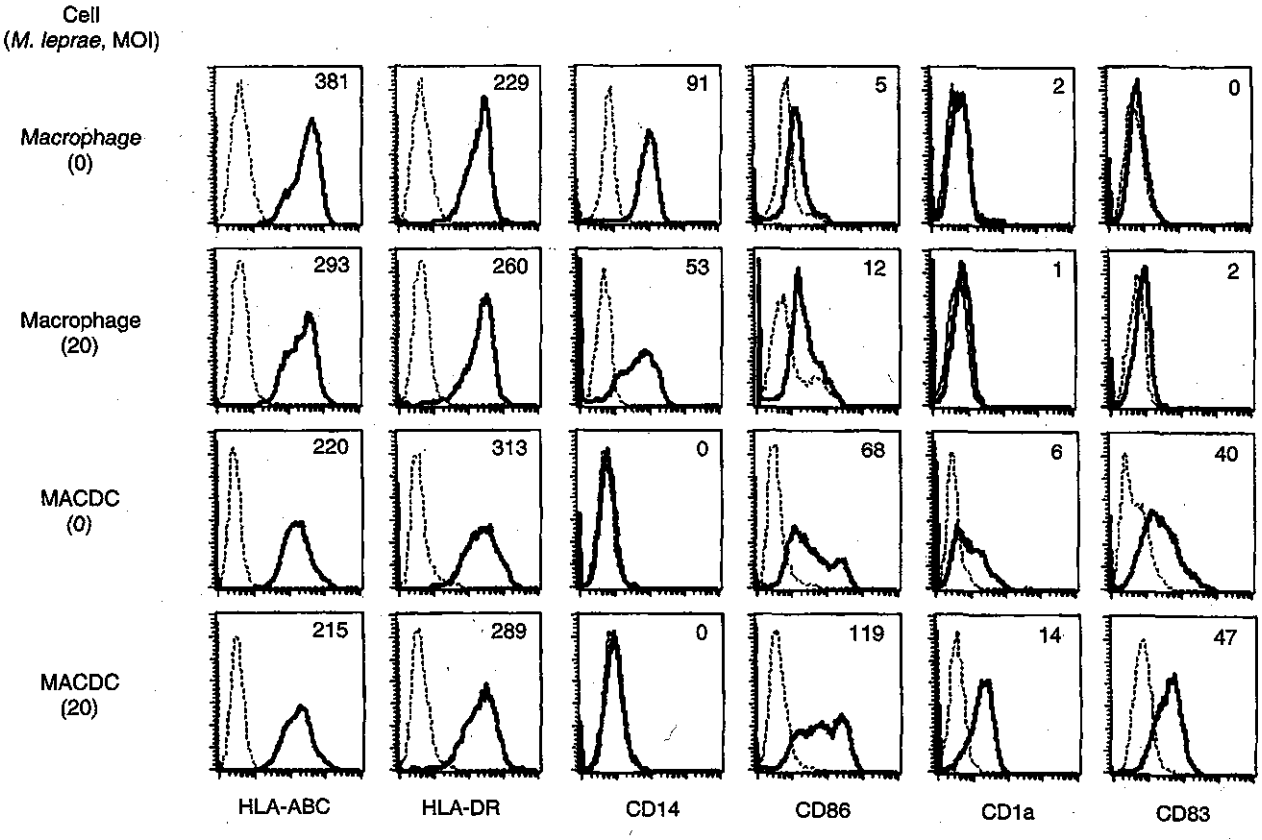

Figure 1 Expression of various molecules on macrophages and macrophage-derived dendritic cell-like cells (MACDC) either uninfected or infected with *Mycobacterium leprae* [multiplicity of infection (MOI) 20]. Both macrophages and MACDC were infected with *M. leprae* for 5 days. The number represents the difference in mean fluorescence intensity between dotted and solid lines. A representative of three independent experiments is shown. Dotted line, control monoclonal antibody; solid line, monoclonal antibody.

apparent difference in the expression of HLA-ABC and HLA-DR antigens between uninfected macrophages and MACDC. Also, no significant upregulation of expression of these molecules was induced by *M. leprae* infection. While macrophages expressed CD14 antigen, the surface CD14 expression on MACDC was completely lost. On the contrary, the expression of CD86 antigen on MACDC was higher than that on macrophages. The expression of CD1a and CD83 antigens which is DC-specific marker was induced on MACDC, both of which level was further upregulated by *M. leprae* infection. These results indicated that MACDC showed phenotypic characteristics similar to monocyte-derived DC, and MACDC were at least partially activated by *M. leprae* infection. Further characterization of MACDC was conducted by comparing the APC function of MACDC with that of monocyte-derived DC (Table 1). Both DC and MACDC induced proliferation of autologous $CD4^+$ and $CD8^+$ T cells in a bacterial dose-dependent manner, but a higher T-cell proliferation was induced by MACDC rather than DC. More than 75% of the MACDC-induced T-cell proliferation was suppressed by monoclonal antibody to MHC or CD86 antigens (data not shown). Previously, we showed that monocyte-derived DC were resistant to exogenous factors such as LPS and CD40L in terms of APC function [8], but using MACDC, both factors upregulated the T-cell-stimulating function of *M. leprae*-infected, but not uninfected, MACDC (Table 2). The proliferation of both $CD4^+$ and $CD8^+$ T-cell subsets were upregulated in a manner dependent on the dose of *M. leprae*.

Table 1 Proliferative response of autologous T cells to *Mycobacterium leprae*-infected macrophage-derived dendritic cell (DC)-like cells (MACDC)*

		CD4		CD8	
M. leprae (MOI)	Stimulator T/DC	20	40†	10†	20
0	DC	1.9 ± 0.6	1.6 ± 0.5	1.8 ± 0.4	1.5 ± 0.3
	MACDC	2.1 ± 0.6	1.7 ± 0.5	1.7 ± 0.4	1.3 ± 0.2
5	DC	3.8 ± 0.9‡	2.7 ± 0.9‡	3.7 ± 1.2‡	2.6 ± 0.7‡
	MACDC	14.6 ± 2.3‡	8.0 ± 2.0‡	13.9 ± 3.3‡	10.6 ± 2.8‡
20	DC	6.9 ± 1.2‡	7.6 ± 1.3‡	10.1 ± 2.1§	8.1 ± 2.0‡
	MACDC	22.3 ± 5.2‡	17.9 ± 5.0‡	21.6 ± 4.1§	18.2 ± 4.3‡

*The responder $CD4^+$ and $CD8^+$ T cells (1×10^5/well) were stimulated for 4 days with an indicated dose of autologous monocyte-derived DC (DC) or MACDC. MACDC were differentiated from macrophages, either uninfected or infected for 5 days with an indicated dose of *M. leprae*, by using IFN-γ, rGM-CSF and rIL-4. The proliferation of responder cells was quantified by an incorporation of [^{3}H]-thymidine. Representative of three separate experiments is shown. Assays were done in triplicate, and results are expressed as mean ± SD.
†[^{3}H]-thymidine uptake ($\times 10^3$ cpm).
‡$P < 0.05$.
§$P < 0.01$.

Table 2 Effect of exogenous factors on the antigen-presenting cell function of macrophage-derived dendritic cell (DC)-like cells (MACDC)*

		CD4		CD8	
M. leprae (MOI)	Tested factor T/DC	20	40†	10†	20
0	None	1.0 ± 0.0	0.8 ± 0.0	1.3 ± 0.2	0.9 ± 0.1
	LPS	1.5 ± 0.3	1.2 ± 0.2	1.7 ± 0.3	1.2 ± 0.2
	CD40L	2.8 ± 0.2	1.9 ± 0.2	2.3 ± 0.4	1.9 ± 0.3
5	None	5.7 ± 0.8‡	4.5 ± 0.7‡**	4.9 ± 0.4¶	3.9 ± 0.3‡§
	LPS	16.5 ± 2.0‡	11.7 ± 2.0**	13.8 ± 0.7¶	10.9 ± 0.9‡
	CD40L	11.2 ± 0.3‡	8.3 ± 0.4‡	10.4 ± 0.7¶	7.6 ± 0.8§
20	None	9.3 ± 1.2‡	6.9 ± 0.8‡§	7.7 ± 0.6‡¶	6.4 ± 0.6‡¶
	LPS	23.3 ± 2.4‡	15.7 ± 2.1§	21.7 ± 2.0‡	16.3 ± 1.1¶
	CD40L	16.5 ± 0.5‡	12.3 ± 0.5‡	15.0 ± 0.8¶	11.9 ± 1.0‡

*The responder $CD4^+$ and $CD8^+$ T cells (1×10^5/well) were stimulated for 4 days with autologous MACDC. MACDC were differentiated from macrophages, either uninfected or infected, for 5 days with an indicated dose of *Mycobacterium leprae*, by using IFN-γ, rGM-CSF and rIL-4 and were further treated with exogenous factors including LPS (30 ng/ml) and soluble form of CD40L (1 μg/ml). The proliferation of responder cells was measured by an incorporation of [^{3}H]-thymidine. Representative of three separate experiments is shown. Assays were done in triplicate, and results are expressed as mean ± SD.
†[^{3}H]-thymidine uptake ($\times 10^3$ cpm).
‡$P < 0.005$.
§$P < 0.01$.
¶$P < 0.001$.
**$P < 0.05$.

Next, the role of exogenous IFN-γ on macrophages and its effect on MACDC production was determined (Fig. 2). While no apparent alterations were induced by 24 h IFN-γ treatment in the expression of HLA-ABC and HLA-DR antigens (data not shown), the expression of CD1a was significantly upregulated by IFN-γ treatment or by *M. leprae* infection. The highest CD1a expression on macrophages was achieved when both *M. leprae* infection and IFN-γ treatment were conducted. Similar upregulation was observed in the expression of CD86 antigen. Moreover, when the IFN-γ treatment was assessed from the functional aspect, it upregulated APC function of *M. leprae*-infected MACDC (Table 3). The IFN-γ treatment on macrophages significantly upregulated activities of MACDC to stimulate both $CD4^+$ and $CD8^+$ T-cell subsets, and 300 U/ml of IFN-γ provided the optimal upregulation. However, a sole IFN-γ treatment was not sufficient and both GM-CSF and IL-4 were required for upregulating the APC function of macrophages (data not shown).

Then, we examined whether MACDC activated type-1 T cells by measuring IFN-γ production by T cells (Table 4). While both the bacteria-infected and -uninfected macrophages, even after being stimulated with an exogenous IFN-γ, did not stimulate autologous $CD4^+$ and $CD8^+$ T cells, MACDC derived from *M. leprae*-infected macrophages did stimulate both subsets of T cells to produce IFN-γ. However, neither IL-4 nor IL-10 was produced by T cells (data not shown). The IFN-γ production by T cells stimulated with MACDC exhibited a bell-shape phenomenon. When up to MOI 20 of *M. leprae* was infected to precursor macrophages, T cells were activated in a bacterial dose-dependent manner, but when more than MOI 20 of the bacteria was infected, the APC function of MACDC decreased. Similar T-cell activation pattern was also observed with heat-killed *M. leprae* (data not shown). Furthermore, MACDC attained an ability to

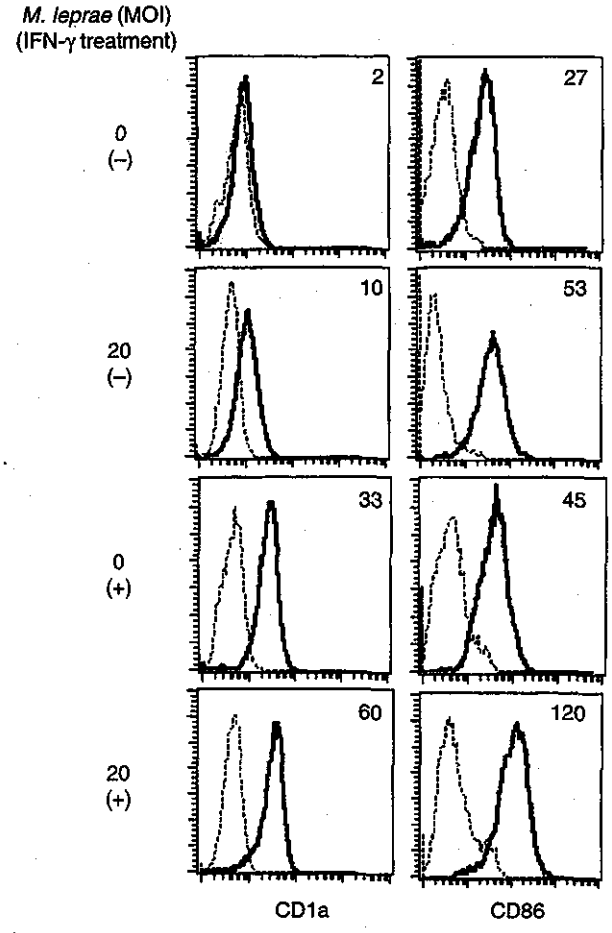

Figure 2 Expression of CD1a and CD86 antigens on macrophages. Macrophages were uninfected or infected with *Mycobacterium leprae* [multiplicity of infection (MOI 20)] on day 2 of culture and were subsequently treated with IFN-γ (300 U/ml) for 24 h on day 3 of culture. The number represents the difference in mean fluorescence intensity between dotted and solid lines. A representative of three independent experiments is shown. Dotted line, control monoclonal antibody; solid line, monoclonal antibody.

Table 3 Effect of exogenous IFN-γ on the T-cell-stimulating activity of macrophage-derived dendritic cell (DC)-like cells (MACDC)*

		CD4		CD8	
M. leprae (MOI)	IFN-γ (U/ml) T/DC	20	40†	10†	20
0	0	4.7 ± 1.2	3.2 ± 1.1	4.9 ± 2.0	3.6 ± 1.8
20	0	15.5 ± 3.0‡	11.5 ± 2.1§	14.9 ± 1.9‡§	11.4 ± 1.6§
20	100	25.7 ± 2.8‡	18.9 ± 2.7§	23.2 ± 2.2‡	18.4 ± 2.4§
20	300	33.6 ± 2.9‡	26.1 ± 3.0§	32.7 ± 3.0§	26.3 ± 3.0§
20	1000	11.1 ± 4.5	0.9 ± 0.6	11.9 ± 1.6	8.8 ± 0.7

*The responder $CD4^+$ and $CD8^+$ T cells (1×10^5/well) were stimulated for 4 days with an indicated dose of autologous MACDC. MACDC were produced from macrophages, either uninfected or infected with *Mycobacterium leprae* for 5 days at multiplicity of infection (MOI) 20 and treated with an indicated dose of IFN-γ. The proliferation of responder cells was measured by an incorporation of $[^3H]$-thymidine. Representative of three separate experiments is shown. Assays were done in triplicate, and results are expressed as mean ± SD.
†$[^3H]$-thymidine uptake ($\times 10^3$ cpm).
‡$P < 0.001$.
§$P < 0.005$.

Table 4 Production of IFN-γ by stimulated T cells and IL-12 p70 by *Mycobacterium leprae*-infected macrophage-derived dendritic cell (DC)-like cells (MACDC)*

Stimulator	M. leprae (MOI) T/DC	CD4		CD8		IL-12† (pg/ml)
		20	40‡	10‡	20	
MØ	0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	4.6 ± 0.9
	5	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	3.0 ± 1.0
	20	0.1 ± 0.0	0.1 ± 0.1	0.3 ± 0.1	0.1 ± 0.1	0.2 ± 0.0
MACDC	0	6.2 ± 0.8§	3.1 ± 0.7§¶	5.4 ± 1.0§	2.9 ± 0.8§**	11.4 ± 9.1¶**
	5	73.0 ± 2.3§	22.6 ± 0.8¶	34.3 ± 2.1§	11.1 ± 1.0§	43.6 ± 7.4**
	20	173.5 ± 6.9§	82.3 ± 2.1§	84.9 ± 3.1§	31.1 ± 1.9**	56.1 ± 8.8¶
	80	19.2 ± 1.4	9.5 ± 1.0	15.6 ± 0.9	7.7 ± 0.9	Not detected

*The responder $CD4^+$ and $CD8^+$ T cells (1×10^5/well) were stimulated for 4 days with an indicated dose of autologous macrophages or MACDC. MACDC were differentiated from macrophages, either uninfected or infected for 5 days with an indicated dose of *M. leprae*, by using IFN-γ, rGM-CSF and rIL-4. The concentration of IFN-γ produced by stimulated T cells was measured by ELISA.
†Macrophages and MACDC (2×10^5/well) were stimulated in the presence of CD40L for 24 h and the concentration of IL-12 p70 was measured. Representative of three separate experiments (IFN-γ and IL-12 production) is shown. Assays were done in triplicate, and results are expressed as mean ± SD.
‡IFN-γ (pg/ml).
§$P < 0.0005$.
¶$P < 0.0001$.
**$P < 0.001$.

produce IL-12 p70 by stimulation with CD40L. The infection of MACDC with *M. leprae* further upregulated the cytokine production. However, macrophages did not produce the cytokine by any stimuli such as CD40L or *M. leprae* infection.

Susceptibility of MACDC to CTL-killing activity

We examined the expression of *M. leprae*-derived antigens on the MACDC by using leprosy patient sera and pAbs to subcellular components of the bacteria, as it has been reported that leprosy sera detected *M. leprae*-derived antigens on the bacteria-infected DC [8]. *M. leprae*-infected MACDC expressed molecules which reacted with sera obtained from leprosy patients (Fig. 3). Furthermore, these mycobacteria-infected MACDC were also positively stained with pAb to cell membrane fraction of *M. leprae* but did not react to pAbs against cell wall or cytosol fractions. These results may suggest that MACDC expressed cell membrane components on the surface. In order to clarify the significance of expression of membrane components, we assessed whether *M. leprae*-infected MACDC could be more efficiently killed by *M. leprae* cell membrane-specific CTL than the bacteria-infected macrophages (Fig. 4). In a previous report, we showed that $CD8^+$ T cells, stimulated *in vitro* with *M. leprae*-derived cell membrane fraction-pulsed DC, produced intracellular perforin [21]. In this experiment, we used these perforin-producing $CD8^+$ T cells as an effector population. While $CD8^+$ T cells stimulated with DC unpulsed with any specific antigens did not kill either macrophages or MACDC regardless of the bacterial infection (data not shown), $CD8^+$ T cells stimulated with cell membrane-pulsed DC did kill *M. leprae*-infected, but not uninfected, target cells. More than 50% of *M. leprae*-infected MACDC were killed

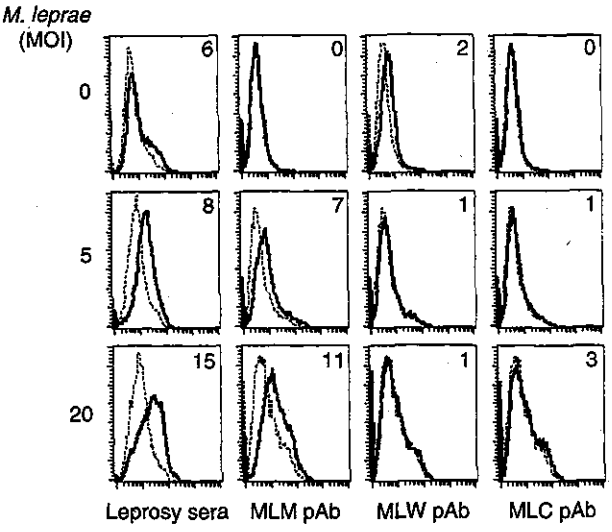

Figure 3 Expression of *Mycobacterium leprae*-derived molecules on macrophage-derived dendritic cell-like cells (MACDC). MACDC were differentiated from macrophages either uninfected or infected with an indicated dose of *M. leprae* for 5 days and were stained with lepromatous leprosy patients' sera and polyclonal antibody (pAb) to *M. leprae* subcellular fractions. The number represents the difference in mean fluorescence intensity between dotted and solid lines. A representative of three independent experiments is shown. MLC, *M. leprae* cytosol fraction; MLM, *M. leprae* cell membrane fraction; MLW, *M. leprae* cell-wall fraction. Dotted line, normal rabbit immunoglobulin G; solid line, pAb.

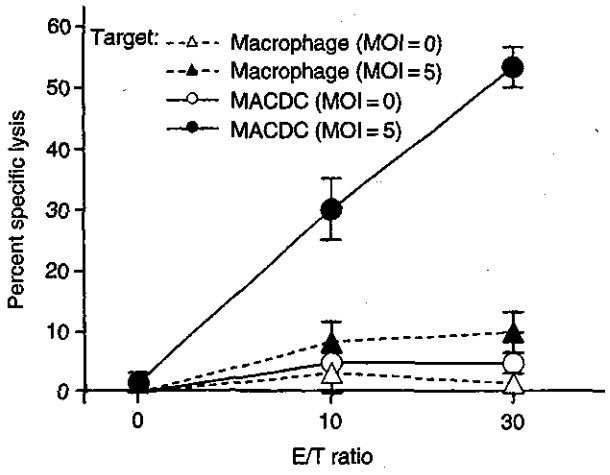

Figure 4 Susceptibility of *Mycobacterium leprae*-infected macrophage-derived dendritic cell-like cells (MACDC) to *M. leprae*-derived membrane antigen-specific CTL. *M. leprae*-derived membrane antigen-specific $CD8^+$ CTL were differentiated from $CD8^+$ T cells by stimulation with the antigen-pulsed autologous mature DC as described previously [21] and were used as effector cells. Macrophages and MACDC either uninfected or infected with *M. leprae* (MOI 5) for 5 days were cocultured with effector cells for 5 h at the indicated E/T ratio. lactate dehydrogenase released by cells were measured. The mean ± SD of triplicate assay and a representative of three independent experiments is shown.

at the E/T ratio of 30, and these MACDC were more efficiently killed than the bacteria-infected macrophages.

Discussion

We tried to induce phenotypic change in mycobacteria-infected macrophages and examined its effect on the host defence activity such as antigen-presenting capacity and the sensitivity to $CD8^+$ CTL. It is known that *M. leprae*-infected macrophages produce IL-10 and stimulate T cell less efficiently [22], and in our hands too, the infected macrophages did not stimulate T cells vigorously, even after being treated with exogenous IFN-γ (Table 4). We have previously demonstrated that monocyte-derived DC also exhibited similar *in vitro* susceptibility to *M. leprae* infection, but in contrast to macrophages, the DC stimulated autologous T cells [8]. However, when the DC were infected with a low number of bacteria, they did not vigorously stimulate T cells, and additional stimulation by bacterial subcellular components was necessary for induction of significant T-cell activation [21]. The exact reason for the less efficient T-cell-activating ability of *M. leprae* is not fully uncovered but might be due to scarcity of antigens on the surface of *M. leprae* as a consequence of large number of pseudogenes in their genome [23]. On the other hand, from the aspect of host defence, these observations indicate the necessity to recruit professional APC, which can initiate T-cell responses by responding to a small number of the bacteria.

In animal models, *M. tuberculosis* induces disease similar to human tuberculosis [24–27], in which the bacteria infect both macrophages and DC [1, 14, 25, 28]. Although these APC are found in the lesion microenvironment, macrophages and DC seem to respond differently following infection. Macrophages produce IL-10 upon an infection with the mycobacterium, and the secreted IL-10 lead naïve T cells to unresponsiveness against the bacterial antigens, although they promote the formation of tuberculous granuloma by residing in close opposition with activated T cells [29]. However, they produce no detectable level of IL-12. In contrast to macrophages, DC initiate both type-1 $CD4^+$ and $CD8^+$ T-cell activation and further act as a primary producer of IL-12 p70 following mycobacterial infection. The DC-mediated IL-12 triggers rapid differentiation of both T-cell subsets into type-1 T cells which cognately interact with DC. These T cells produce IFN-γ, which in turn, contributes to induce mycobacteriocidal action to APC such as macrophages [14, 29, 30]. Considering these facts, we tried to induce phenotypic changes in mycobacteria-infected macrophages, so that T cells could be stimulated. On treatment with rIFN-γ, rGM-CSF and rIL-4, *M. leprae*-infected macrophages were phenotypically transformed to DC-like cells (MACDC). These cells expressed CD1a and CD83 antigens but lacked the expression of CD14 and produced IL-12 and induced responses, such as proliferation and IFN-γ production, of both $CD4^+$ and $CD8^+$ T-cell subsets. These results are partly supported by previous report that Th1-polarizing potential was observed when macrophages, not infected with any bacteria, were treated with GM-CSF and IL-4 [31]. Although, monocyte-derived DC required higher dose of bacterial infection for T-cell activation, MACDC showed a distinctive feature, in that they initiated T-cell proliferation more efficiently than the DC with rather small number of *M. leprae* (Table 1). Therefore, it may be reasoned out that MACDC and DC contribute distinctively to the host defence; the former might be more important in the microenvironment where a small number of *M. leprae* exist. This hypothesis might be associated with the previous finding that $CD1a^+$ and $CD83^+$ cells were enrolled in tuberculoid leprosy lesion [22], although it was not clarified whether they originated from macrophages or monocytes.

IFN-γ contributed to the efficient development of MACDC by upregulating the expression of CD1a and CD86 molecules on macrophages (Fig. 2). In murine system, IFN-γ is known to be associated with production of the reactive nitrogen intermediates which can directly kill intracellular mycobacteria [32]. Although there is no definite evidence suggesting the association of such intermediate products with mycobacterial killing in human, IFN-γ can activate macrophages and kill the intracellular

bacteria. In this study, we showed that IFN-γ did not directly endow macrophages with T-cell-stimulating activity but contribute to efficient differentiation of macrophages to MACDC by upregulating the expression of CD86 and CD1a molecules (Fig. 2). Furthermore, the IFN-γ obviously upregulated T-cell-activating ability of MACDC (Table 3).

Another peculiar feature of *M. leprae*-infected MACDC is that they showed an enhanced susceptibility to killing activity of *M. leprae* cell membrane antigen-specific $CD8^+$ CTL, although the identification of immunodominant antigenic determinants remains unknown. When compared to *M. leprae*-infected macrophages, the *M. leprae*-infected MACDC were more efficiently killed by CTL. It is interesting to note that *M. leprae*-infected MACDC expressed antigens reactive to pAb to cell membrane components, but not antigens recognized by pAb to cell wall or cytosol components. In addition, our previous data showed that cell membrane was the most efficient antigen among *M. leprae* subcellular components for the activation of $CD8^+$ CTL [21]. When we compared *M. leprae*-infected MACDC and the macrophages, there were no difference in the expression of MHC class I and class II antigens, but only the former expressed *M. leprae*-derived antigens on the surface. Therefore, the expression of cell membrane antigens on MACDC might be closely associated with the enhanced susceptibility to killing activity.

Taken together, an induction of phenotypic change on *M. leprae*-infected macrophages resulted in enhanced type-1 T-cell-stimulating ability and an upregulated susceptibility to CTL activity. These observations may be further useful for developing immunotherapeutic tools against intracellular pathogens which threaten humans worldwide.

Acknowledgments

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Role of the polypeptide region of a 33 kDa mycobacterial lipoprotein for efficient IL-12 production

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Abstract

Mycobacterium leprae lipoprotein, LpK, induced IL-12 production from human monocytes. To determine the components essential for cytokine production and the relative role of lipidation in the activation process, we produced lipidated and non-lipidated truncated forms of LpK. While 0.5 nM of lipidated LpK-a having N-terminal 60 amino acids of LpK produced more than 700 pg/ml IL-12 p40, the non-lipidated LpK-b having the same amino acids as that of LpK-a required more than 20 nM of the protein to produce an equivalent dose of cytokine. Truncated protein having the C-terminal 192 amino acids of LpK did not induce any cytokine production. Fifty nanomolar of the synthetic lipopeptide of LpK produced only about 200 pg/ml IL-12. Among the truncated LpK, only LpK-a and lipopeptide stimulated NF-kB-dependent reporter activity in TLR-2 transfectant. However, when monocytes were stimulated with lipopeptide in the presence of non-lipidated protein, they produced IL-12 synergistically. Therefore, both peptide regions of LpK and lipid residues are necessary for efficient IL-12 production.

Keywords: Lipoprotein; IL-12; Mycobacteria; TLR-2

1. Introduction

Bacterial lipoproteins, containing *N*-acyldiglyceride-cysteine residues at their amino termini, have been well studied in gram-positive and gram-negative bacteria [1,2]. Acylation of the amino group of cysteine in the consensus lipid-binding sequence takes place by attachment of the diacylglycerol moiety in a thioether linkage and subsequent cleavage of the proprotein by a specific signal peptidase. One of the functional characteristics of such acylated proteins is the production of interleukin-12 (IL-12)[1] from host antigen (Ag)-presenting cells (APCs). Lipoproteins stimulated APCs and these APCs in turn activated both type 1 $CD4^+$ and $CD8^+$ T cells, to produce interferon-γ (IFN-γ), which endows bacteriocidal activities to APCs mainly macrophages. Therefore, lipoproteins play a central role as an inducer of host defense activities to control the growth of intracellular parasitic bacteria such as mycobacteria. Such lipoproteins were isolated from *Mycobacterium tuberculosis*, of which the 19- and 38-kDa proteins have been reported to be capable of activating both innate and adaptive immunity [3–5]. There are only a few reports of lipoprotein from other mycobacterial species, but recently we have identified a novel 33 kDa lipoprotein, LpK, from *Mycobacterium leprae* [6].

Mycobacterium leprae induce a chronic infectious disease, termed leprosy which has been characterized by

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[1] *Abbreviations used:* IL-12, interleukin-12; M., *Mycobacterium*; Ag, antigen; APC, antigen-presenting cell; IFN, interferon; PVDF, polyvinyl difluoride; PBMC, peripheral blood mononuclear cell; LPS, lipopolysaccharide; PG, peptidoglycan; TLR, toll-like receptor; DCs, dendritic cells.

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progressive peripheral nerve injury and skin lesions [7]. One representative spectrum of the disease is a paucibacillary form of leprosy, in which the disease lesion is localized. The localization of the lesion is a consequence of the suppression of bacterial spread and, in this process, IL-12 producing APCs seem to play a central role in activating innate and type 1 cellular immunity [8–11]. Since the newly identified lipoprotein LpK was found to be capable of inducing IL-12 production in human peripheral monocytes, it can be predicted that LpK is one of the antigens in *M. leprae* with the potential to contribute to the host defense against leprosy.

Although it may be assumed that the immuno-dominant region of the lipoprotein is the lipid region, the immuno-dominant region of LpK in terms of IL-12 production has not been studied, and it remains to be clarified whether the acylated lipopeptide region alone could represent the immuno-stimulatory domain of the lipoprotein.

In this study, we expressed various forms of truncated LpK, assessed its IL-12 producing activity and attempted to clarify the role of peptide lipidation in the context of cytokine production.

2. Materials and methods

2.1. Bacterial strains, plasmids, and lipopeptides

Escherichia coli DH5α strain (Toyobo, Tokyo, Japan) was used for all cloning and recombinant expression experiments. The plasmids used for the expression in *E. coli* were pGEM-T Easy Vector (Promega, Madison, WI), and pGFPuv (Clontech, Palo Alto, CA). Clones were selected on Luria–Bertani medium agar plates (1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 1.5% agar) or broth supplemented with ampicillin at 100 μg/ml. All other chemicals were purchased from Wako Chemicals (Richmond,VA), Sigma–Aldrich (St. Louis, MO) or Amersham–Pharmacia (Piscataway, NJ). The LpK lipopeptide containing the N-terminal 12 amino acids of LpK was synthesized by Bachem AG (Germany). The structure of the lipopeptide is as follows: Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Leu-Pro-Asp-Trp-Leu-Ser-Gly-Phe-Leu-Thr-Gly-Gly-OH. The corresponding unlipidated LpK peptide containing only the N-terminal 12 amino acids was also synthesized.

2.2. Cloning and sequencing of the truncated forms of the lpk gene

To clone the *lpk* gene, the DNA of interest was amplified by PCR by taking the genomic DNA from *M. leprae* (Thai-53 strain) as a template for PCR, and the expressed LpK lipoprotein was purified as previously described [6]. The primers used for the amplification of the gene coding protein constructs in Fig. 1 were as follows: For LpK-a, the sense primer 5′ACATGCA TGCCCTGGTGTTGGTCCTGTGG3′ (a-s) and the antisense primer 5′CGGAATTCTTAGTGATGGTGA TGGTGATGGCCTGCCCGCTGCCG3′ (a-as) were used. For LpK-b amplification, primers 5′ACATGCA TGCCCTGTTGCCTGATTGGTTGT3′ (b-s) and the antisense primer a-as were utilized. Similarly, for LpK-c, the sense primer used was a-s and antisense 5′GGAA TTCTTAGTGATGGTGATGGTGATGGCTAAGCT TAGTGATCC3′ (c-as), for LpK-d, primers used were b-s and c-as. LpK-e utilized the sense primer 5′ACAT

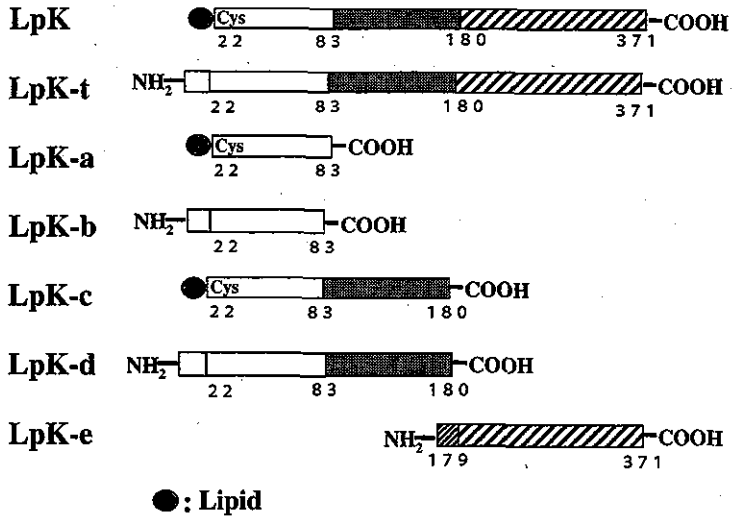

Fig. 1. Schematic representation of the constructs of LpK and truncated LpK. The lipidated constructs are LpK, LpK-a, and LpK-c. Non-lipidated constructs include LpK-t, -b, -d, and -e. The thatched region indicates the C-terminal half of the LpK protein. Numbering shows the position of the amino acid of LpK in the prolipoprotein form.

GCATGCCCTTAGCGAGCGTACTGA3′ and the previously described antisense primer for LpK amplification [6]. For LpK-t, the sense primer a-s and the same antisense primer for LpK amplification was used. All antisense primers contained the histidine tag coding sequence at the C-terminus of the protein for easy protein detection. The gene was first cloned into pGEM-T Easy Vector (Promega), and further inserted into the expression vector. All other genetic manipulations were done according to established cloning techniques [12]. All lipidated and non-lipidated *lpk* genes were expressed in *E. coli*. Restriction enzymes were purchased from New England Biolabs (Beverly, MA), Takara Shuzo (Shiga, Japan) or Toyobo (Osaka, Japan) and used according to the manufacturer's specifications. For DNA sequencing, plasmid DNA samples were purified using a Qiagen MiniPrep Kit (Qiagen, Valencia, CA). DNA sequence analysis was performed on an ABI Prism Genetic Analyser (PE Biosystems, Foster City, CA) using the dideoxy dye termination PCR method.

2.3. Detection of the expressed proteins and protein purification

Escherichia coli transformants were lysed in 6 M urea, 0.5% CHAPS, and 1 mM DTT containing 50 mM Tris–Cl and run on a 12% SDS–polyacrylamide gel [13]. The resolved proteins were transferred to a polyvinyl difluoride (PVDF) membrane (Millipore, Bedford, MA). The proteins were then detected using penta-His mAb (Qiagen), and color developed with 5-bromo,4-chloro, 3-indoylphosphate/nitroblue tetrazolium (BCIP/NBT). The overexpressed protein was also gel filtrated through a HiLoad 26/60 Superdex 75 prep grade column (Amersham–Pharmacia), using buffer containing 6 M urea, 50 mM Tris–Cl (pH 8.0), and 0.1% CHAPS at a flow rate of 2 ml/min. After collecting around 30 fractions, SDS–polyacrylamide gel electrophoresis was performed; the proteins were stained either by Silver Stain 'Daiichi' (Dai-ichi Pure Chemicals, Tokyo, Japan) or Coomassie blue brilliant stain. Western blotting was performed using a penta-His mAb. The fraction containing the desired protein was used for further evaluation. By SDS–PAGE of the protein and further staining with a silver stain, no apparent contamination of *E. coli*-derivatives was observed. The concentrations of LpK and its mutant proteins were determined using a Bio-Rad Protein Assay kit according to the manufacturer's instructions.

2.4. Measurement of IL-12 production by human PBMC

Human PBMCs from healthy individuals were isolated on Ficoll–Paque Plus (Amersham–Pharmacia, Upsala, Sweden) and cultured for 1 h in 10 cm dishes. The non-adherent cells were removed by washing several times with RPMI 1640 (Sigma) containing 2% FCS. By flow cytometric analyses, among the plastic adherent cells, 95–98% of the cells were CD14 positive. T cells and B cells constituted less than 1% and $CD1a^+$ dendritic cells constituted less than 0.1% of the adherent cell population. These adherent cells were then detached and cultured in triplicate in 96-well plates (10^5 cells/well) with purified lipoproteins at various concentrations. Twenty to twenty-four hours later, the culture supernatants were collected and assayed for human IL-12 p40 production using an OptEIA Set (Pharmingen, SanDiego, CA). The amount of lipopolysaccharide (LPS) in the purified lipoprotein was measured quantitatively with a Limulus Amoebocyte Lysate assay (Whittaker Bioproducts, Walkersville, MD) and found to be <10 pg/μg protein, an amount that did not stimulate IL-12. Also, the contribution of $CD1a^+$ dendritic cells within the plastic adherent cells in the IL-12 production was examined. No significant difference in the cytokine production was observed by depleting the $CD1a^+$ cells using immunomagnetic beads (Dynabeads 450, Dynal, Oslo, Norway).

2.5. Cell transfection and luciferase assay

Human embryonic kidney cells (HEK293) were obtained from the American Type Cell Culture Collection (Manassas, VA). Cells were cultured in DMEM supplemented with 10% FCS, 50 mg/ml penicillin/streptomycin, and non-essential amino acids (Invitrogen, Carlsbad, CA), at 37 °C in a humidified incubator of 5% CO_2. The cDNA of human Toll-like receptor 2 (TLR) was PCR-amplified using a human spleen cDNA library (BD Biosciences, San Jose, CA) and inserted into pCIneo (Promega, Madison, WI). HEK293 cells (2×10^4) were transiently transfected with a mixture of plasmids: 200 ng pCIneo hTLR2, 25 ng p5× NF-kB-luc (Stratagene, La Jolla, CA), and 10 ng pRL-TK-*Renilla* luciferase plasmid (Promega) using the FuGENE 6 reagent (Roche molecular Biochemicals, Indianapolis, IN), as previously described [14]. Thirty-six hours after transfection, cells were treated with or without various amounts of LpK and its truncated forms, or peptidoglycan (PG) as positive control (for TLR2-dependent luciferase activity) for further 6 h. The cells were lysed in 70 μl of 1× passive lysis buffer (Promega) and luciferase activity in 10 μl of the cell lysate was measured using Promega Dual-Luciferase Reporter Assay System according to the protocol provided by the manufacturer. Data were expressed as fold induction relative to the activity of *Renilla* luciferase, which is an internal control for transfection efficiency.

2.6. Statistical evaluation

The Student's *t* test was applied to reveal statistically significant differences.

3. Results

3.1. The role of LpK lipidation in IL-12 production

To verify the role of lipid modification of LpK in terms of IL-12 production from human monocytes, various forms of truncated LpK were produced in *E. coli*. LpK-a is a lipidated protein, while other truncated proteins (LpK-t, LpK-b, LpK-d, and LpK-e) are non-lipidated (Fig. 1). The cytokine producing ability of mature lipidated LpK was first compared with non-lipidated LpK-t having the whole conserved amino acid residues of LpK. The expression vector for LpK-t was constructed by eliminating the nucleic acids coding for the N-terminal hydrophobic region up to the cysteine residue which is acylated in LpK. While 2.5 nM LpK produced more than 1000 pg/ml IL-12 p40 in human monocytes, 2.5 nM LpK-t produced IL-12 more than 9-fold less efficiently, and it required more than 10 nM to produce an equivalent dose of the cytokine (Fig. 2). This result indicated that the presence of the N-terminal lipid entity significantly enhanced the IL-12 producing activity of LpK protein from monocytes. To confirm this point, we examined the IL-12 inducing ability of another truncated protein, LpK-a. The expression plasmid encoding *lpk-a* was constructed by taking the N-terminal 82 amino acid coding nucleic acids of *lpk* including the signal peptide region. LpK-b having no lipid residue, but only the amino acid residues of LpK-a, was also produced. The uptake of radio-labeled glycerol was used to confirm the presence of lipid modification. *E. coli* expressing LpK-a was co-cultured for 5 h in the presence of [^{14}C]glycerol and the cells were lysed. When the protein was run on an SDS–polyacrylamide gel, autoradiography showed a 12 kDa radio-labeled band, which corresponded to the predicted molecular mass (not shown). The expression of the protein at the same

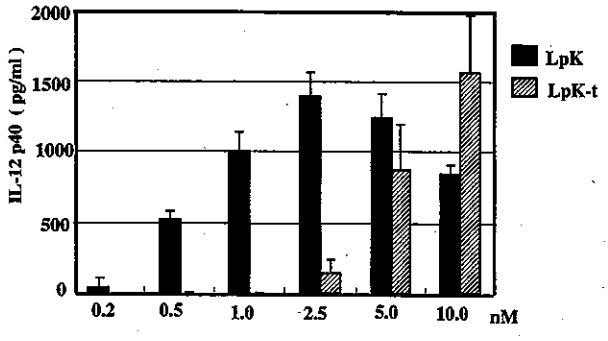

Fig. 2. LpK induces significant IL-12 production from human blood monocytes when compared to truncated LpK-t. Monocytes were isolated from healthy human blood cells as described in Section 2.4. IL-12 p40 production was measured by ELISA. The results shown are obtained from one experiment, but were consistent with three different experiments. Mean ± standard deviation of a triplicate assay is shown. By Student's *t* test, the *p* values obtained were: $p < 0.005$ for values between LpK and LpK-t at 2.5 nM and $p < 0.05$ at 5.0 nM.

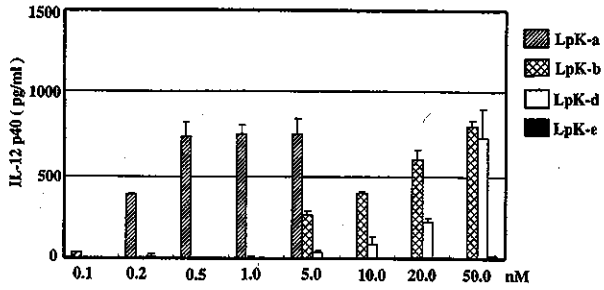

Fig. 3. Among the truncated proteins, LpK-a was the most efficient in inducing IL-12 from monocytes. Various truncated forms of LpK were expressed in *E. coli* and purified, then evaluated for their IL-12 inducing ability. Among LpK-a, -b, -d, -e, and lipidated LpK-a was the most efficient in inducing IL-12, but LpK-e did not induce cytokine production. The results were obtained from one experiment, but were consistent with three different experiments. Mean ± standard deviation of a triplicate assay is shown. By Student's *t* test, the following *p* values were obtained: $p < 0.01$ for values between LpK-a and LpK-b, LpK-a and LpK-e; $p < 0.005$ for values between LpK-a and LpK-d, LpK-b, and LpK-e; $p < 0.001$ for values between LpK-b and LpK-d; and $p < 0.05$ for values between LpK-d and LpK-e at 5.0 nM.

position was confirmed by Western blotting. Such a radio-labeled band was not observed in the case of LpK-b, which indicated that LpK-a was lipidated.

Next, we determined the ability of LpK-a to produce IL-12 in monocytes, and compared it with that of non-lipidated LpK-b. LpK-a was significantly more efficient at cytokine production than LpK-b (Fig. 3). While 0.5 nM LpK-a produced more than 700 pg/ml IL-12, non-lipidated LpK-b required more than 20 nM of protein to produce an equivalent dose of IL-12. One possible reason for the less efficient cytokine production by LpK-b is that it lacks some immuno-stimulatory domain, which may be the acylated structure itself or its contribution to the conformation of the protein. To further analyze the effect of other truncated LpK, we produced non-lipidated LpK protein, LpK-d, and LpK-e (Fig. 1). LpK-c, a lipidated protein having the N-terminal half of the LpK protein could not be expressed in *E. coli* for unknown reasons. LpK-d covers the N-terminal 158 amino acid residues of the LpK protein and LpK-e has amino acid corresponding to the C-terminal half of the LpK protein with a single overlapping amino acid with LpK-d. However, LpK-d induced IL-12 less efficiently compared to that produced by LpK-a. The IL-12 producing activity of LpK-d was comparable to that of LpK-b, but LpK-e had no such IL-12 stimulating ability (Fig. 3). These results may indicate that acylated N-terminal 60 amino acids of LpK is responsible for the strong immuno-stimulatory activity of LpK.

3.2. Immuno-stimulatory activity of synthetic lipopeptide

As shown in Fig. 3, the ability of LpK to induce IL-12 production in monocytes resides in the N-terminal

region of the protein, including the acylated portion. Therefore, we synthesized lipopeptide with N-terminal 12 amino acid residues of LpK, having the N-terminal cysteine residue conjugated to palmitoylated triacylated glycerol, which is presumed to be having the same lipid composition as that of LpK purified from *E. coli*. Fig. 4 represents the IL-12 production from monocytes by synthetic LpK lipopeptide and non-lipidated peptide having the same N-terminal 12 amino acids of the lipopeptide. It was surprising to note that, 50 nM synthetic lipopeptide produced less than 200 pg/ml IL-12. Further increasing the concentration of lipopeptide, did not result in further elevation of the level of cytokine production (not shown). The synthetic non-lipidated peptide having the same amino acids as that of the lipopeptide almost totally lacked the ability to induce IL-12 production in monocytes (Fig. 4). These results suggested that lipidation of the N-terminal peptide was necessary, but was not as efficient as LpK or LpK-a, for the production of IL-12.

3.3. Association of LpK protein and TLR-2

We examined whether NF-kB-driven luciferase activity was upregulated in TLR2 transfected HEK 293 cells by LpK and its truncated protein. PG, a well-defined TLR-2-associated Ag, was used as a positive control. Significantly higher luciferase activity was observed when the TLR-2 transfected HEK293 cells were stimulated with PG or lipidated lipoprotein including LpK and LpK-a, but not in mock transfected HEK293 cells (Fig. 5). On the contrary, no significant or no antigen-dose-dependent luciferase activity was induced by any of the non-lipidated proteins such as LpK-b, LpK-d, and LpK-e. But, significant activity was observed in cells when stimulated with synthetic lipopeptide LpK, despite lacking the ability to induce IL-12 efficiently. These results suggested that TLR-2 stimulation of LpK protein is essential, but not adequate for efficient production of the cytokine.

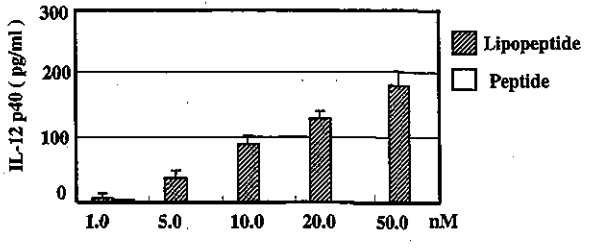

Fig. 4. The synthetic lipopeptide derived from the sequence of N-terminal LpK showed ability to induce IL-12, but to a limited extent. The same peptide sequence as that of the lipopeptide sequence without the acyl attachment did not induce any cytokine production. A representative of three independent experiments is shown. Each experiment was performed in triplicate and the mean ± standard deviation is shown. By Student's *t* test, the *p* values obtained were: $p < 0.05$ for values between lipopeptide and peptide at 5.0 nM.

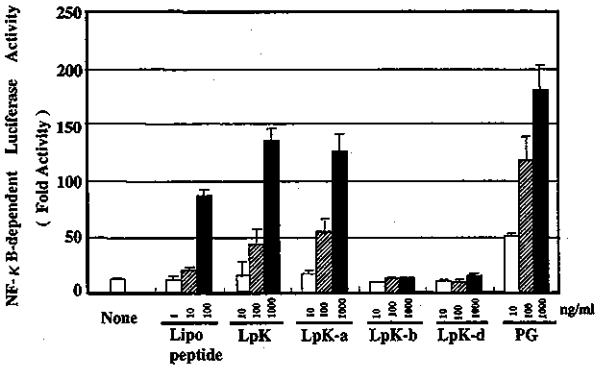

Fig. 5. Association of TLR2 and truncated LpK proteins. NF-kB-dependent reporter gene activity of the TLR2 transfectant was measured after stimulation with LpK or its truncated forms as described in Section 2.5. Peptidoglycan (PG) was used as a positive control for TLR2 dependent luciferase activity. Data are expressed as fold induction relative to the activity of *Renilla* luciferase, which is an internal control for transfection efficiency in the Dual luciferase reporter assay. The result shown is representative of three different experiments. Assays were done in triplicate and the mean ± standard deviation is shown.

3.4. The role of the non-lipidated protein component of LpK in IL-12 production

To confirm that the stimulation of monocytes with non-lipidated components of LpK protein is required for efficient IL-12 production, we stimulated monocytes with lipopeptide in the presence of various concentrations of non-lipidated proteins (Fig. 6). When monocytes were co-stimulated with lipopeptide and LpK-b or LpK-d, they produced IL-12 in a manner dependent on the concentration of truncated non-lipidated LpK proteins, although induction of IL-12 by LpK-b was a little lower in this set of experiments due to donor variations. However, the combination of lipopeptide and LpK-e or synthetic peptide did not induce any cytokine production. These results suggested that lipopeptide by itself is ineffective in producing IL-12, but is markedly synergistic with certain immuno-dominant regions of LpK, which apparently seem to correspond to the N-terminal 60 amino acid residues.

4. Discussion

The clinical manifestations of leprosy appear based on the immunological spectrum according to the level of cell-mediated immunity to *M. leprae*. Lepromatous leprosy patients manifest disseminated infection, their T cells respond weakly to the bacilli and their lesions express type 2 cytokines. In contrast, tuberculoid patients mount a strong Th1 response to *M. leprae*. When a Th1 cell-mediated immune response is generated, clinically apparent leprosy infection is localized, leading to the formation of a granuloma. For efficient induction of Th1 response, IL-12 is envisaged to play an