tially clarified by the GPL. We propose that the lipid components in the cell envelope are important for MAIC infection and that the structure modification must be taken into account. These findings shed light on the better understanding of the structure-function relationships of GPLs and may open a new avenue for the prevention of MAIC infections.

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Apoptosis-Inducing Activity of Clofazimine in Macrophages[▽]

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Clofazimine is a riminophenazine compound which has been used for the treatment of leprosy since the 1960s. Although the drug is effective in the management of leprosy reactions because of its anti-inflammatory activity, the mechanism leading to the cessation of inflammation is not well understood. In the present study, it was shown that clofazimine exhibits apoptosis-inducing activity in macrophages. When human monocyte-derived macrophages were cultured *in vitro* in the presence of clofazimine, the cells exhibited a marked decrease in metabolic activity and showed shrinkage in cell size, indicating cell death. Nuclear condensation and fragmentation were also observed by Giemsa and Hoechst 33248 stains. The endonuclease inhibitor $ZnCl_2$ inhibited the clofazimine-induced cell death. Significant enhancement of caspase-3 activity was observed in clofazimine-treated macrophages and THP-1 cells. Collectively, these results suggest the apoptosis-inducing activity of clofazimine in macrophages, which may also be responsible for the antibacterial properties of clofazimine.

Clofazimine (B663) is a phenazine iminoquinone derivative, specifically, a riminophenazine dye with the empirical formula $C_{27}H_{22}C_{12}$. In the 1950s, Barry et al. synthesized a large number of compounds by progressive chemical alteration of the anilinoaposafranine molecule, several of which showed antituberculous activity both *in vitro* and in experimental animals (1). Of these compounds, clofazimine (or Lamprene or B663) was found to be highly active against mycobacteria with the least toxicity. Chang (4) observed the antibacterial activity of clofazimine against *Mycobacterium lepraemurium* at about the same time as its anti-*M. leprae* activity was reported by Browne (2) and Browne and Hogenzeil (3). Later, after the introduction of the mouse footpad method of Shepard and Chang (22), its antibacterial activity against *M. leprae* was demonstrated (18).

Clofazimine has bifunctional activity: antibacterial and anti-inflammatory. It was used in the treatment of leprosy for its antibacterial action against *M. leprae*. Later, it was also found to possess an anti-inflammatory action which makes it a very useful drug in the treatment of acute reactions, including erythema nodosum leprosum (ENL), neuritis, iritis, etc., although its mechanism of action is unknown (2). *In vitro* studies on the effect of clofazimine on immune cells have been conducted. Clofazimine increases superoxide anion production and degranulation by stimulated neutrophils. and tumor necrosis factor alpha (TNF-α) potentiates this enhancement (15). The mechanism underlying this pro-oxidative effect seems to involve stimulation of phospholipase A2 (PLA2) activity with subsequent accumulation of arachidonic acid and lysophospholipids, which act as second messengers to activate oxidase (10). In addition, a number of reports have demonstrated the effects of clofazimine that might predict increased immune activity. Lysosomal enzyme activity of cultured macrophages was upregulated by clofazimine (21). Peripheral blood monocytes from healthy volunteers have been demonstrated to exhibit increased major histocompatibility complex class II expression following incubation with clofazimine (25). Increased oxygen uptake during phagocytosis was observed in neutrophils derived from patients with pyoderma gangrenosum during clofazimine therapy (5). Suppressor T-cell activity was decreased in mycobacteria-infected mice during clofazimine treatment (26). However, the mechanisms underlying the anti-inflammatory action of clofazimine are still unclear.

In the present study, we examined the effect of clofazimine on macrophages and found that the drug possessed apoptosis-inducing activity.

MATERIALS AND METHODS

Drug and chemicals. Clofazimine (Sigma-Aldrich Co., St. Louis, MO), rifampin (catalog no. R3501; Sigma-Aldrich Co.), and dapsone (DDS; Biomol Research Inc., Butler Pike Plymouth Meeting, PA) were dissolved in dimethyl sulfoxide (DMSO) and stored at −30°C until use. Ampicillin was obtained from Sigma-Aldrich Co.

Culture of human macrophages and isolation of bacilli. Human peripheral blood was obtained under informed consent from healthy individuals. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque Plus (GE Healthcare Life Sciences, Buckinghamshire, United Kingdom) gradient centrifugation (12). The cells were suspended in AIM-V medium (Gibco BRL, Invitrogen Corp., Carlsbad, CA), and 1×10^6 PBMCs were cultured in a well of a 24-well tissue culture plate (Falcon; Becton Dickinson Labware, Becton Dickinson and Company, Franklin Lakes, NJ) containing 13-mm round coverslips (Nunc Thermanox coverslips; Nalge Nunc, Thermo Scientific, Rochester, NY) at 37°C in a 5% CO_2 incubator for adherence of monocytes. After 1 h incubation, the coverslips were washed with Hanks' balanced salt solution (HBSS; Sigma-Aldrich Co.) to remove nonadherent cells. The monocytes on the coverslips were cultured in a new 24-well plate containing RPMI 1640 medium (Sigma-Aldrich Co.) supplemented with 25 mM HEPES, 10% fetal bovine serum (FBS; Bio Whittaker Co., Walkersville, MD), 2 mM L-glutamine, and 100 μg/ml ampicillin (RPMI-10F) in the presence of 40 ng/ml of granulocyte-macrophage colony-stimulating factor (R&D Systems, Minneapolis, MN). After 10 days, the cells were differentiated into macrophages and used for experiments. In some experiments, PBMCs were cultured in 35-mm cell culture dishes (Corning Inc., Corning, NY) for adherence, and adherent monocytes were cultured for 10 days. Human monocytic leukemia cell line THP-1 was maintained in RPMI 1640 medium containing 15% fetal bovine serum.

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M. leprae (Thai-53 strain) was isolated from the footpads of BALB/c *nu/nu* mice that had been inoculated with *M. leprae* 8 months prior to isolation, and the bacillary number was enumerated according to the method of Shepard and Chang (22).

Light and phase-contrast microscopy. Macrophages on the coverslip were fixed with absolute methanol, followed by performing Giemsa stain (Wako Co., Japan). After they were mounted on a glass slide, the cells were observed under a light microscope (Optiphot-2; Nikon Co., Tokyo, Japan). Photographs were taken with a digital camera (Nikon F70s). Macrophages in 35-mm dishes were incubated in the presence of clofazimine and observed under a phase-contrast microscope (Olympus CKX41 with ×10- and ×20-objective lenses). Photographs were taken with an Olympus DP50 system. Image acquisition and data processing were done using the DP controller software.

Fluorescence microscopy. Fluorescence staining for DNA was employed. Macrophages were cultured in an 8-well chamber slide (Lab-Tek II chamber slide system; Nalge Nunc). The cells were incubated in the presence of clofazimine and subsequently fixed with 2.5% glutaraldehyde in phosphate-buffered saline (PBS). Hoechst 33342 dye (Sigma-Aldrich Co.) in PBS was added to the wells at a final concentration of 10 μM, and the slide was incubated for 1 h at 37°C. The cells on the slide were observed under a fluorescence microscope (Olympus BX60 with a ×40-objective lens) equipped with an Olympus DP50 system. The digital images were processed with DP controller software.

Determination of cell death. Cell viability was determined using the colorimetric method (Cell Titer 96 aqueous nonradioactive cell proliferation assay; Promega Corp., Madison, WI). Briefly, cells in a 24-well plate were incubated in the presence of clofazimine in phenol red-free RPMI 1640 medium containing 10% FBS, followed by addition of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt/phenazine methosulfate solution (formazan reagent). After 1 h incubation at 37°C, an aliquot of medium was transferred into a well of a 96-well plate, and the developed color was measured by a microplate reader at 490 nm. In addition, the activity of lactate dehydrogenase (LDH), released from dead cells into culture supernatants, was measured by a colorimetric assay (Cytotox 96 nonradioactive cytotoxicity assay; Promega Corp.). The color that developed in a sample incubated with LDH substrate was measured by a microplate reader at 490 nm (Vmax; Molecular Devices Corp., Sunnyvale, CA).

DNA electrophoresis. THP-1 cells or macrophages were harvested from the culture, and DNA was purified by a spin column method (E.Z.N.A. tissue DNA kits; Omega Bio-Tek, Norcross, GA). Briefly, 5×10^6 cells incubated in the presence of clofazimine were harvested, centrifuged at 2,000 rpm for 5 min, washed once with PBS, and resuspended in PBS. Protease was added, the mixture was heated at 65°C for 5 min, and buffer BL was added. After the mixture was heated at 70°C for 10 min, ethanol was further added. The mixture was applied to a HiBind spin column and centrifuged. DNA bound to the column was finally eluted, and the DNA preparation was subjected to electrophoresis in a 1% agarose gel, followed by ethidium bromide staining, and DNA was visualized by UV transillumination.

Western blotting. THP-1 cells or macrophages incubated with clofazimine were washed once with PBS(−) and lysed in lysis buffer (CelLytic-M; Sigma-Aldrich Co.) containing 2 protease inhibitor cocktails (phosphatase inhibitor cocktail 1 and phosphatase inhibitor cocktail 2; Sigma-Aldrich Co.). In the case of clofazimine-treated adherent macrophages, the cells were scraped off the dishes with a rubber policeman. The lysates were incubated for 10 min on ice and centrifuged at 13,000 rpm for 5 min. The protein concentration was determined. Ten micrograms of total protein was loaded onto an SDS-PAGE gel. After running the electrophoresis, the proteins in the gel were transferred onto an Immobilon PSQ membrane (Millipore Corporation, Billerica, MA). After washing with Tris-buffered saline (2.42 g Tris base and 8 g NaCl per 1 liter, pH 7.6) containing 0.05% Tween 20 (TBS-T), the membrane was blocked with 5% skim milk (Amersham ECL Plus Western blotting reagent pack; GE Healthcare Life Sciences, Amersham Place, Buckinghamshire, United Kingdom) for 1 h at room temperature. The membrane was washed 3 times with TBS-T and incubated overnight with 1:3,000-diluted primary anticleaved caspase-3 antibody (cleaved caspase antibody sampler kit; Cell Signaling Technology Inc., Danvers, MA). The membrane was then incubated with 1:10,000-diluted horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature. Finally, proteins were detected by incubating the membrane with HRP substrate (Immobilon Western chemiluminescent HRP substrate; Millipore Corporation), and the membrane was exposed to X-ray film (Amersham Hyperfilm ECL; GE Healthcare). For reprobing of the membrane, the membrane was washed with TBS-T and incubated with stripping buffer (Restore Plus Western blot stripping buffer; Pierce, IL). After the membrane was blocked, it was used again for probing different antibodies, such as cleaved caspase-9 and poly(ADP-ribose) polymerase (PARP) antibodies (cleaved caspase antibody sampler kit; Cell Signaling Technology Inc.) and beta-actin antibody (Cell Signaling Technology Inc.).

Colorimetric caspase assay. Colorimetric substrates for caspases were used to determine caspase-3 activity (colorimetric caspase assay kits; Biovision Research Products, CA) in lysates of cells incubated in the presence of clofazimine. Briefly, 5×10^6 cells were pelleted and lysed with chilled lysis buffer. After centrifugation, the supernatant was transferred to a new tube, and reaction buffer and a substrate for caspase-3, Asp-Glu-Val-Asp–*p*-nitroanilide, were added to the tube. After incubation for 2 h at 37°C, the samples were transferred into a well of a 384-well plate and read by a plate reader at 405 nm (Infinite F200; Tecan Systems Inc., San Jose, CA). The background reading was obtained by subtracting the reading for the reaction buffer from the reading for the lysate samples.

PGE_2 assay. The amount of prostaglandin E_2 (PGE_2) in the culture supernatants was measured by enzyme-linked immunosorbent assay (catalog no. 514010; Cayman Chemical Co., MI).

RESULTS

Morphological changes observed after treatment with clofazimine. Macrophages differentiated from human monocytes were incubated in the presence of 10 μg/ml of clofazimine for 20 h. The change in cell morphology was observed under a phase-contrast microscope. As shown in Fig. 1B, in the presence of clofazimine, the cells exhibited shrinkage in cell size and membrane blebbing. The death of more than 80% of cells was observed (Fig. 1B). As a control, Fig. 1A shows the normal morphology of macrophages. By Giemsa stain, too, these clofazimine-treated cells exhibited shrinkage in cell size, accompanied by the appearance of fragmented smaller nuclei (arrow in Fig. 1D), suggesting the apoptotic nature of the cells. Nontreated macrophages showed intact nuclei (arrow in Fig. 1C). Again, the change of nuclear structure was confirmed by Hoechst dye staining. Under a fluorescence microscope, nuclear condensation and membrane blebbing were observed in the clofazimine-treated cells (Fig. 1F and G), in contrast to normal cells, which showed intact nuclei (Fig. 1E). Similar fragmentation or condensation of chromatin was observed in THP-1 cells (data not shown). Such morphological changes were not observed in THP-1 cells treated with rifampin or dapsone at a concentration up to 50 μg/ml. Also, DMSO, which was used as a solvent for clofazimine at a concentration of 0.2%, had no effect on cell morphology or cell functions (negative control).

Cell death-inducing activity of clofazimine determined by colorimetric assay. Cell death was determined by a biochemical analysis using a colorimetric method. The conversion of the tetrazolium compound into soluble formazan is accomplished by metabolically active cells. When higher concentrations up to 10 μg/ml of clofazimine were employed in macrophage cultures, decreased color intensity of soluble formazan was observed, indicating cell death (Fig. 2A). Cell death was also observed in THP-1 cells (Fig. 2B). Hansen's disease is caused by infection of macrophages with *M. leprae*; therefore, we are curious to know whether *M. leprae* infection affects the cell death-inducing activity of clofazimine. When we infected macrophages with *M. leprae* at a multiplicity of infection (MOI) of 10 or 30, we found no significant difference in the induction of cell death in the presence of 10 μg/ml clofazimine, indicating that the bacilli did not inhibit or enhance clofazimine-induced cell death (Fig. 2C). Another method of determining cell death is by measurement of LDH release from

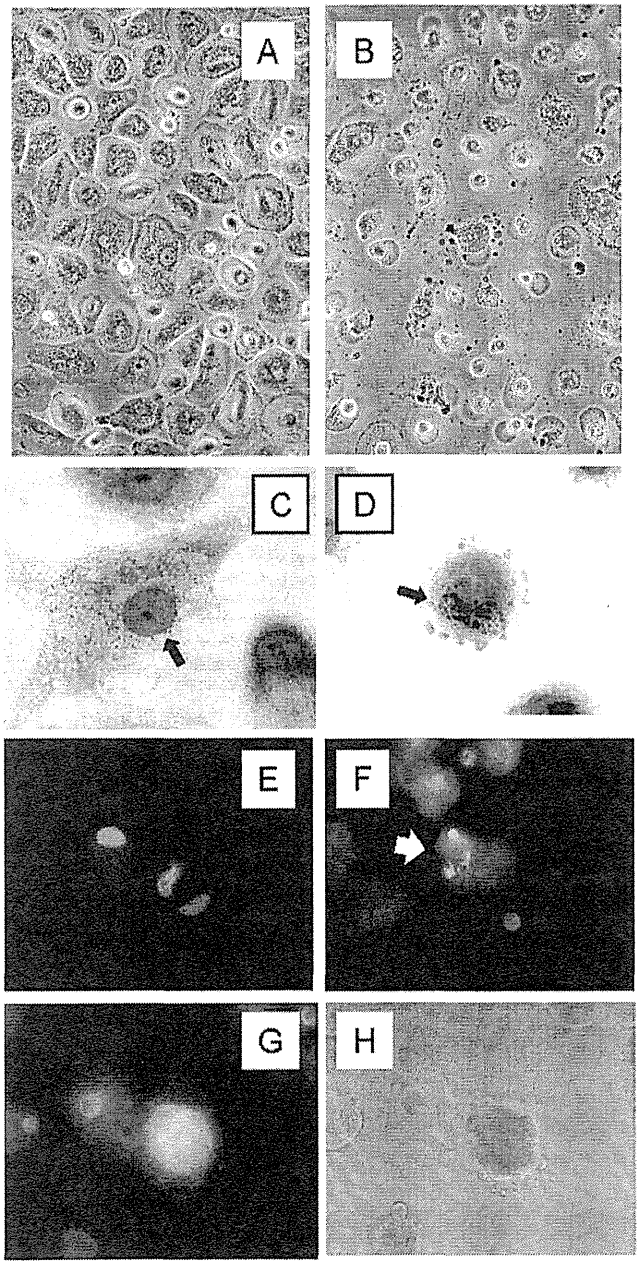

FIG. 1. Cell death induced in macrophages by clofazimine. Photographs were taken under a phase-contrast microscope (A and B) with a ×20-objective lens. (B) Human monocyte-derived macrophages were incubated in the presence of 10 μg/ml of clofazimine for 20 h. (A) Cells cultured in the absence of clofazimine showed normal morphology. Giemsa stain of clofazimine-treated macrophages was also performed (C and D). Human monocyte-derived macrophages were incubated in the presence of 10 μg/ml of clofazimine (D) or in the absence of clofazimine (C) for 24 h. Photographs were taken under a light microscope with a ×100-objective lens. Fragmentation of the nucleus was significant in the clofazimine-treated cells (arrow in panel D), in contrast to the intact morphology of the nucleus in normal cells (arrow in panel C). Nuclear condensation and fragmentation of clofazimine-treated macrophages were also confirmed under a fluorescence microscope (E to G). Macrophages were incubated in the presence of 10 μg/ml of clofazimine, followed by fixation and stained with a nucleus-staining dye, Hoechst 33342. The cells were observed under a fluorescence microscope (×40-objective lens). Cells cultured without clofazimine (E), clofazimine-treated cells (F and G), and a phase-contrast image of panel G (H) are shown.

dead cells. As shown in Fig. 3, more LDH release was observed in the manner dependent on the concentration of clofazimine.

Clofazimine treatment induces DNA ladder formation in macrophages. We examined the condition of DNA in clofazimine-treated THP-1 cells. Agarose gel electrophoresis showed fragmentation of DNA into integer multiples of 180 bp, a so-called DNA ladder (Fig. 4A), suggesting that DNA endonuclease was activated by clofazimine treatment. Therefore, we examined the effect of one of the apoptosis inhibitors, $ZnCl_2$, which is known to possess suppressing activity for endonuclease, and found that clofazimine-induced DNA fragmentation in THP-1 cells was completely blocked by $ZnCl_2$ treatment even at a low concentration of 0.25 mM $ZnCl_2$ (Fig. 4B), although it is still not clear whether $ZnCl_2$ can directly block the activity of clofazimine. Moreover, it was evident that neither cell death nor DNA fragmentation was induced by other antileprosy drugs, such as DDS or rifampin (Fig. 4C).

Clofazimine-induced cell death is mediated by activation of caspase-3. Caspases are known to be central regulators of apoptotic cell death, and caspase-3, which locates downstream of the caspase pathway, is one of the key executioners of apoptosis. Upon apoptotic stimulation, caspases are cleaved into active fragments. Figure 5 shows a Western blot analysis of extracts from THP-1 cells and macrophages cultured in the presence of clofazimine. Enhanced expression of cleaved caspase-3 was detected in cells (Fig. 5A and B). In addition, caspase-9 was also cleaved. A DNA-repairing enzyme, PARP, which is cleaved by caspase-3, was significantly activated in clofazimine-treated THP-1 cells (Fig. 5A). We next measured the caspase activity by colorimetric assay (Fig. 5C). The induction of caspase-3 by clofazimine was significantly high in macrophages as well as THP-1 cells.

Clofazimine enhanced PGE_2 production in *M. leprae*-infected macrophages. Monocyte-derived macrophages were preincubated in the presence of clofazimine for 4 h, followed by replenishment with *M. leprae*-containing medium for 20 h. The culture supernatants were collected, and the PGE_2 concentration was measured. As shown in Fig. 6, clofazimine clearly enhanced PGE_2 production in macrophages.

DISCUSSION

Riminophenazines are structurally phenazine compounds which were derived from lichens historically and were targeted for treatment of tuberculosis. The first clinically developed phenazine compound was clofazimine, whose activity has been extended to other mycobacterial diseases (1, 17). In test animals, the drug was found to inhibit the growth of mycobacteria *in vivo*, as well as *in vitro* (22), but the molecular mechanism of clofazimine in inducing anti-*M. leprae* activity is still not yet clear.

In the present study, it was found that both human monocyte-derived macrophages and THP-1 cells exhibited marked decreases in their metabolic activity in the presence of 10 μg/ml clofazimine. Under a phase-contrast microscope, 80% of the cells showed irregular morphology with shrinkage in cell size, and by a precise time course study, it was revealed that the morphological changes were evident from 6 h incubation with clofazimine. From this early time point, the cell body began to shrink, accompanied by membrane blebbing, which was also

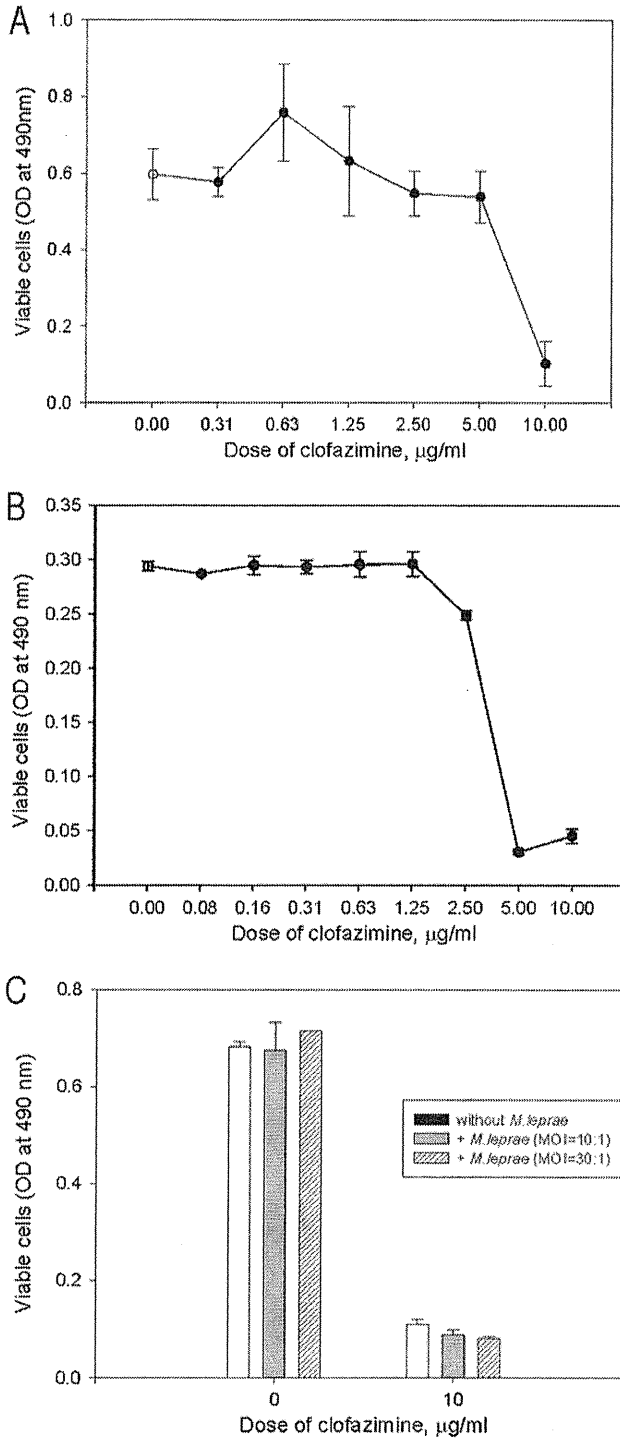

FIG. 2. Clofazimine-induced cell death in macrophages and THP-1 cells. Human monocyte-derived macrophages (A) and THP-1 cells (B) were incubated with various concentrations of clofazimine for 24 h, followed by determination of viable cells by the Cell Titer 96 cell proliferation assay. The results are representative of three independent cell culture tests. The cell death-inducing effect of clofazimine in the presence of *M. leprae* was also examined. Monocyte-derived macrophages were infected with *M. leprae* at an MOI of 10 or 30 per cell for 24 h. The infected cells were further incubated with 10 μg/ml clofazimine for another 24 h, followed by determination of viable cells by Cell Titer 96 cell proliferation assay (C). The results are representative of three independent cell culture tests. OD, optical density.

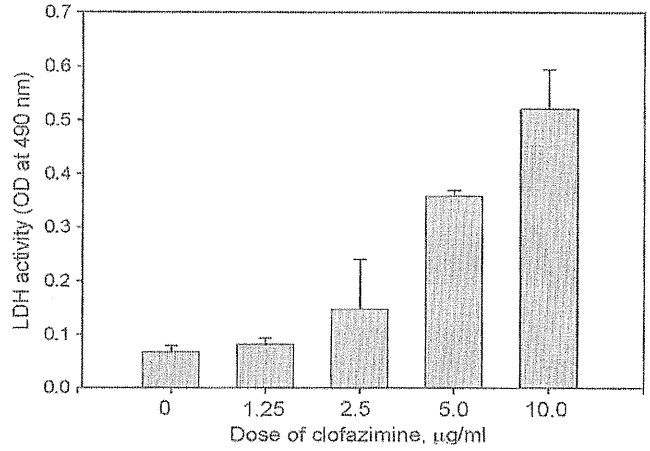

FIG. 3. LDH release from clofazimine-treated macrophages. Human monocyte-derived macrophages were incubated in the presence of the indicated concentrations of clofazimine for 24 h, and the LDH activity was measured. The results were obtained from triplicate cultures and are representative of three independent cell culture tests. OD, optical density.

evident from Giemsa stain and Hoechst staining of the nuclei (Fig. 1). Interestingly, the dose of clofazimine (10 μg/ml) required to cause cell death was equivalent to the dose required to exhibit anti-*M. leprae* activity *in vitro* by radiorespirometry (data not shown), the dose of which is in concordance with the dose required to kill *M. leprae* reported by Franzblau and O'Sullivan (7). Moreover, in our study, at 5-μg/ml concentrations of clofazimine, *M. leprae* viability was lowered in *in vitro* experiments with *M. leprae*-infected macrophages, and with this dose, *M. leprae* was found not to inhibit clofazimine-induced cell death. Therefore, clofazimine might inhibit mycobacterial growth through an alternative way by inducing apoptosis of host cells. Although the concentration of clofazimine in sera of patients taking regular doses of the drug is as low as 1 to 2 μg/ml, fat-soluble clofazimine readily accumulates in cells. In one patient, 7 months treatment with clofazimine (200 mg/day) resulted in accumulation of needle-shaped crystal inclusions in his alveolar macrophages (20). In another report, clofazimine-induced crystal-storing histiocytosis was observed in a leprosy patient (23). So, we are of the opinion that in some cells, the concentration of clofazimine is higher (10 to 20 μg/ml) than in others, so we have used a concentration of 10 μg/ml for our experiments.

Normally, cells undergo distinct morphological changes when they progress through either necrosis or apoptosis. Necrosis occurs when cells are exposed to an extreme variance from physiological conditions, resulting in damage to the plasma membrane. As such, necrosis is characterized by cell swelling and disruption of cellular organelles, with little change in the chromatin initially. In contrast, apoptotic cells shrink in size, undergo membrane blebbing, and exhibit marked alterations in their chromatin structure at an early stage under normal physiological conditions. As mentioned earlier, treatment with clofazimine resulted in highly condensed chromatin within the nucleus and membrane blebbing, indicating macrophages undergoing apoptosis. To confirm this, DNA from clofazimine-treated THP-1 cells was examined. Fragmented DNA was

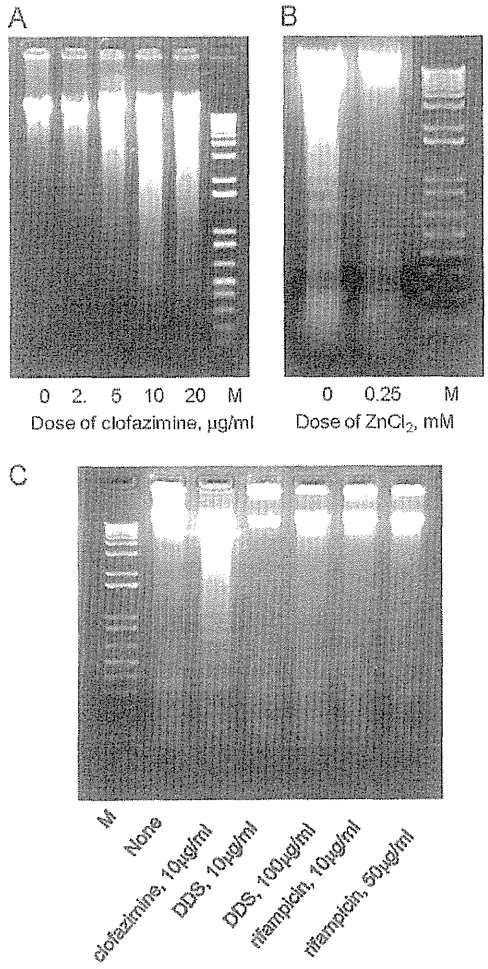

FIG. 4. DNA ladder formation in clofazimine-treated THP-1 cells and effects of other antileprosy drugs on DNA ladder formation. (A) THP-1 cells were incubated in the presence of the indicated concentrations of clofazimine for 4 h, followed by purification of DNA for agarose gel electrophoresis. An ethidium bromide-stained agarose gel is shown. (B) An endonuclease inhibitor, $ZnCl_2$, was examined for its effect on clofazimine-induced ladder formation. THP-1 cells were incubated in the presence of 10 μg/ml clofazimine and $ZnCl_2$ for 4 h. DNA was purified for electrophoresis. (C) THP-1 cells were incubated in the presence of clofazimine, DDS, and rifampin for 4 h, followed by purification of DNA. An ethidium bromide-stained agarose gel is shown. Lanes M, molecular weight marker.

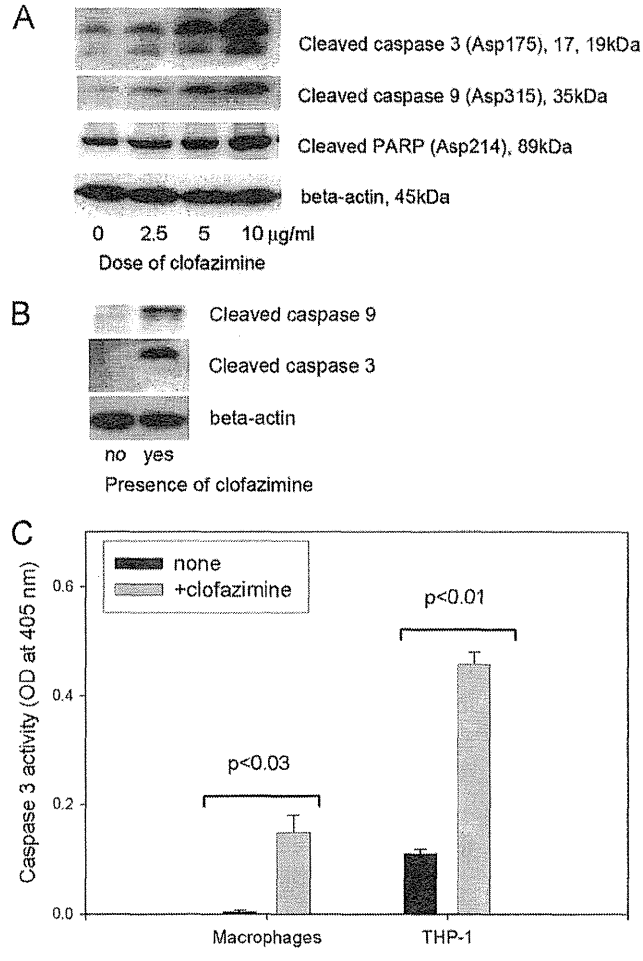

FIG. 5. Expression of caspase in clofazimine-treated THP-1 cells and macrophages. THP-1 cells were incubated in the presence of the indicated concentrations of clofazimine for 6 h, and cell lysates were processed for detection of cleaved caspase-3, caspase-9, and PARP by Western blotting (A). Similarly, monocyte-derived macrophages were incubated in the presence of 10 μg/ml of clofazimine, and the cell lysates were examined for cleaved caspase-3 and caspase-9 expression (B). The caspase activity in clofazimine-treated macrophages and THP-1 cells was analyzed. Macrophages were incubated in the presence of 10 μg/ml of clofazimine for 6 h, and the caspase-3 activity in the cell lysates was determined by colorimetric assay (C). The results are representative of three independent cell culture tests.

demonstrated, suggesting that DNA endonuclease was activated causing apoptosis.

We observed that *M. leprae* by itself does not induce apoptosis of human cells. Similarly, infection of mouse macrophages with viable *M. leprae* was shown not to induce apoptosis (11). Although apoptosis is induced when macrophages infected with *M. leprae* are treated with clofazimine, the host cell viability does not change significantly in the presence of *M. leprae*. Nevertheless, the viability of *M. leprae* in macrophages was significantly lower in clofazimine-treated cells than infected cells not treated with clofazimine (data not shown). Therefore, we can speculate that clofazimine induces apoptosis of *M. leprae*-infected macrophages, which in turn inhibits *M. leprae* growth.

Consequently, we investigated the pathways involved in the execution of macrophage apoptosis (6, 14). We observed enhanced expression of cleaved caspase-3, caspase-9, and PARP following clofazimine treatment in THP-1 cells (Fig. 5A). Colorimetric assay also indicated enhanced caspase-3 activity in both macrophages and THP-1 cells treated with clofazimine (Fig. 5C), suggesting the involvement of caspases in clofazimine-induced apoptosis.

Apoptosis has been shown to be effective in therapy of chronic inflammatory diseases (16). An immunomodulatory drug, thalidomide, is used for treatment of ENL in leprosy patients, and its anti-inflammatory activity is believed to be through the downregulation of production of the proinflammatory cytokine TNF-α (19). Gockel et al. showed that thalidomide induces apoptosis in human monocytes (8). Clofazimine

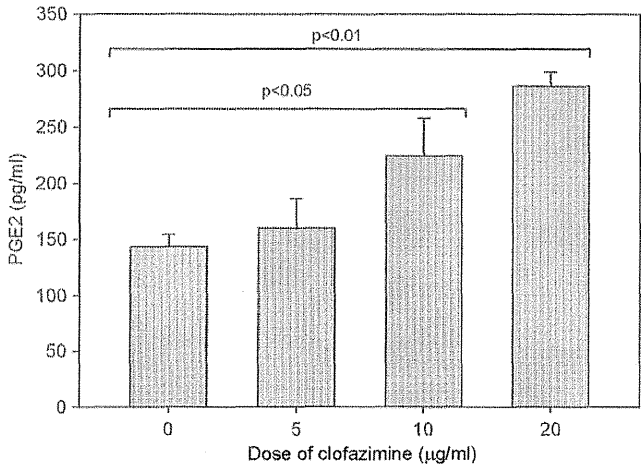

FIG. 6. Enhancement of PGE_2 production by clofazimine in *M. leprae*-infected macrophages. Macrophages were preincubated in the presence of the indicated doses of clofazimine for 4 h, followed by infection with *M. leprae* (5×10^6/well), and the culture was continued for another 20 h. The amount of PGE_2 in the culture supernatants was measured. The results are representative of three independent cell culture tests.

is known to have a favorable influence on the reversal reaction in human leprosy (2). Browne and Hogenzeil found that clofazimine controlled persistent exacerbations in patients who were corticosteroid dependent for controlling the passing reactions, including ENL, and suggested that the drug may exert a suppressive effect on the development of acute exacerbation in lepromatous leprosy (3). These observations have been followed by those of later workers, and one of the special indications for use of clofazimine is the presence of acute reactions or a chronic recurrent reaction in lepromatous leprosy. These clinical data suggest that the mechanisms underlying the action of the drug in these leprosy patients mainly seem to be anti-inflammatory, although there is no direct evidence. Macrophages are capable of elaborating a series of biochemical products with potent immunomodulatory activities. We have observed enhancement of the production of PGE_2, when macrophages were pretreated with clofazimine. PGE_2 is released from arachidonic acid by PLA2. The enzyme is reported to be stimulated in clofazimine-treated neutrophils (10). With respect to B-cell function, prostaglandins of the E series (PGE) inhibit both B-cell proliferation and the generation of antibody-forming cells, and also, B-cell tolerance is induced by PGE_2 (9, 24). T-cell proliferation is also suppressed by PGE_2 (13). The reaction to leprosy involves antibody (ENL caused by immune complex) and cells (delayed-type hypersensitivity mediated mainly by T cells). The mechanisms underlying the immunomodulatory role of clofazimine are still not clear, but the present study clarifies certain aspects. Apoptosis induced in macrophages might explain the anti-inflammatory activities of clofazimine *in vivo*.

In conclusion, our findings suggest that clofazimine induced apoptosis of macrophages through the activation of caspases. The data indicate that the action of clofazimine in leprosy patients may be at least partially mediated by apoptosis.

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MAJOR ARTICLE

Analysis of Drug-Resistant Strains of *Mycobacterium leprae* in an Endemic Area of Vietnam

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(See brief report by Ramien and Wong, e133–e135.)

Background. Multidrug therapy has effectively reduced the number of leprosy cases in the world. However, the rate of reduction has decelerated over the years, giving early detection of *Mycobacterium leprae* and epidemiological study of relapse renewed relevance in attempts to eliminate the disease.

Methods. A molecular epidemiological survey for drug-resistant *M. leprae* was conducted in the central and highland regions of Vietnam. A total of 423 samples taken from patients, including 83 patients with new cases, 321 patients receiving treatment, and 19 patients with relapse, were studied for detection of *M. leprae* with mutations relating to drug resistance by sequencing the drug resistance determining region of the *folP1*, *rpoB*, and *gyrA* genes, which are responsible for dapsone, rifampicin, and ofloxacin resistance, respectively.

Results. Nineteen mutations were found in the *folP1* gene samples, and no mutations relating to drug resistance were found in either the *rpoB* or *gyrA* genes. Samples from patients with relapse showed *folP1* mutation rates as high as 57%, and the mutation rates in samples from new and recent cases were <10%. Patients with relapse who had histories of treatment with dapsone monotherapy showed high mutation rates (78%), compared with patients with relapse who had previously only received multidrug therapy (33%).

Conclusions. Our study indicated high rates of dapsone resistance in patients with relapse, compared with patients with new and recent cases of leprosy. Moreover, it was observed that many of the patients with relapse who had dapsone-resistant mutations had histories of treatment with dapsone monotherapy.

Leprosy is a chronic infectious disease caused by infection with *Mycobacterium leprae*. The present strategy for leprosy control is based on the multidrug therapy (MDT), recommended by the World Health Organization (WHO) [1], which has successfully reduced the number of leprosy cases in the world. However, transition in the number of registered cases and new cases amounting to ~210,000 and ~250,000, respectively, has almost come to a standstill [2]. Drug-resistant strains were first found in 1964, 1976, and 1997 [3–5]. MDT was designed to prevent the emergence and spread of drug-resistant strains. However, a strain showing resistance to both dapsone and rifampicin was reported in 1993 [6], and at present, there are further reports indicating the emergence of *M. leprae* strains resistant to multiple drugs [5, 7]. At present, the rapid detection and control of such drug-resistant strains is essential in countries approaching leprosy elimination levels, such as Vietnam.

MDT has been quite successful in Vietnam, and elimination of leprosy (prevalence rate, < 1/10,000 population) was achieved on the national level in 1995 [8]. The prevalence rate per 10,000 population in 2006

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was .07 [8, 9]. However, the majority of patients with leprosy are found in the central and highland regions of Vietnam [10], consisting of 11 provinces, including 4 provinces in the highland region and 7 provinces in the delta region. In 2005, the number of patients with leprosy was 236, spread through 4 provinces of the highland region; the prevalence rate of newly detected cases was 3.5 cases/10,000 population, although the overall prevalence rate was .25 cases/100,000 population on the national level. The rate of newly detected cases in the 7 delta region provinces was 1.38 cases/10,000 population [8, 9]. These cases not only present the danger of being possible infectious sources for leprosy but also harbor the risk of developing into relapse cases. However, little is known regarding the effects of drug-resistant *M. leprae* in patients with leprosy, especially in cases of relapse.

Therefore, in the present study, molecular epidemiological studies on drug-resistant strains were conducted in 11 provinces primarily in the central and highland regions that represent the areas where leprosy is endemic in Vietnam.

MATERIALS AND METHODS

Sensitivity of Polymerase Chain Reaction

The number of bacilli isolated from nude mice footpads was counted using the method described by Shepard et al [11]. Serial 10-fold dilutions of the enumerated *M. leprae* bacilli were used for polymerase chain reaction (PCR) in our study.

Clinical Specimens

Samples (from slit-skin smears or punch biopsies) were taken from patients with leprosy after receipt of informed consent in primarily the central and highland regions of Vietnam (including 11 provinces: Danang, Quangnam, Quangngai, Binhdinh, Phuyen, Khanhhoa, Ninhthuan, Kontum, Gialai, Daknong, and Daklak), and the samples were classified as new (before starting MDT), recent (receiving MDT), and relapse cases. Relapse was defined as development of new skin lesions after completion of MDT and increase in bacterial index by >2 log units in any lesion.

The total of 423 samples included those from 83 patients with new cases, 321 patients with recent cases (receiving treatment), and 19 patients with relapse (collection period: March 2004–August 2009). Among 16 patients with relapse who had positive results of *M. leprae*–specific PCR, 9 cases were determined to be relapse after dapsone monotherapy (7–20 years), 3 as relapse after complete MDT, 2 as second relapse (the first after dapsone monotherapy and the second after MDT), and 2 as relapse after ofloxacin treatment. Samples were obtained from the skin lesions of patients (smear on blade or biopsy soaked in 1 mL of 70% ethanol at room temperature in the field, before being sent to Quyhoa National Leprosy & Dermato-Venereology Hospital laboratory).

DNA Extraction, Nested PCR, and Sequencing

M. leprae templates from both dilutions of *M. leprae* bacilli and slit-skin smears were prepared by treatment with lysis buffer at 60°C overnight, as described elsewhere [12]. Nested PCR amplification of the RLEP regions of *M. leprae* was performed under conditions described elsewhere with minor modifications, using the primers listed in Table 1 [13]. In brief, PCR amplification using special reagents (20 mM Tris-HCl [pH, 7.5], 8 mM magnesium chloride, 7.5 mM DTT, 2.5 mg BSA, 150 μM deoxynucleotides, 1.5 mM magnesium sulphate, and 2.5 units KOD-plus-Ver.2 DNA polymerase [Toyobo]) was performed using sample DNA as templates. Both first and second PCR conditions were as follows; strand separation at 94°C for 4 min, denaturing at 94°C for 40 s, annealing at 55°C for 1 min, and extension at 72°C for 20 s plus 1-s increment per cycle for 25 cycles. Products from the first PCR (0.5 uL) were used as templates in the second PCR. The nested PCR for DRDR was performed using the primer pairs listed in Table 1. Mutations were measured on the *folP1* gene for dapsone [14], the *rpoB* gene for rifampicin, and the *gyrA* gene for ofloxacin [15, 16]. Nested PCR conditions for drug resistance were different from that for RLEP-nested PCR. In brief, PCR amplification using standard reagents (10 mM Tris-HCl [pH, 8.3], 2 mM magnesium chloride, 250 μM dNTPs, and 2.5 units TaKaRa Ex Taq DNA polymerase [Takara shuzo]) was performed using sample genomic DNA as templates. The primer pairs used to amplify the specific drug-resistant genes are shown in Table 1. The reaction condition was 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C for 35 cycles.

The amplicons were visualized by agarose gel electrophoresis, and DNA was recovered from the gel using Mini-Elute gel extraction kits (Qiagen). The recovered DNA molecules were sequenced using the ABI Prism BigDye Terminator Cycle Sequencing kit (Perkin-Elmer Applied Biosystems) and run on an ABI Prism 3130 Genetic Analyzer (Applied Biosystems). The sequence data were analyzed by DNA analysis program Genetyx-MAC, version 15 (GENETYX), and were compared with those in the GenBank database.

RESULTS

PCR Sensitivity

Serial dilutions of the bacilli of 1×10^8–1×10^0 were prepared to determine PCR sensitivities. Genomic DNAs were extracted from the diluents with use of methods described under Materials and Methods [11]. The previously reported RLEP-nested PCR (named RLEP-L) was capable of detecting 1×10^2 bacilli in samples (Figure 1a) [13]. The newly designed RLEP-nested PCR, using K1 and K2 primers for the first PCR and LP1 and LP2 primers for the second PCR (named RLEP-K), is capable of detecting comparable counts of bacilli (Figure 1b), and RLEP-K

Table 1. Sequences of Primers Used in this Study

Name	Usage	Gene	Sequence, 5′→3′	Reference	Size, bp
K1	First PCR (F)	RLEP	CGTGGGTGTGAGGATAGTTGT-	Present study	268
K2	First PCR (R)	RLEP	GATCATCGATGCACTGTTCACT-	Present study	
LP1	First or second PCR (F)	RLEP	TGCATGTCATGGCCTTGAGG-	13	129
LP2	First or second PCR (R)	RLEP	CACCGATACCAGCGGCAGAA	13	
LP3	Second PCR (F)	RLEP	TGAGGTGTCGGCGTGGTC	13	99
LP4	Second PCR(R)	RLEP	CAGAAATGGTGCAAGGGA	13	
F1	Second PCR (F)	*folP1*	GCAGGTTATTGGGGTTTTGA	Present study	312
F2	First PCR(R)	*folP1*	CCACCAGACACATCGTTGAC	Present study	
F3	Second PCR (F)	*folP1*	CTTGATCCTGACGATGCTGT	Present study	245
F4	Second PCR(R)	*folP1*	ACATCGTTGACGATCCGTG	Present study	
F5	Sequencing primer (F)	*folP1*	ATCCTGACGATGCTGTCCA	Present study	–
F4	Sequencing primer (R)	*folP1*	ACATCGTTGACGATCCGTG	Present study	–
R1	First PCR (F)	*rpoB*	CAGACGCTGATCAATATCCGT	Present study	358
R2	First PCR (R)	*rpoB*	CAGCGGTCAAGTATTCGATC	Present study	
R3	Second PCR (F)	*rpoB*	CAATATCCGTCCGGTGGTC	Present study	337
R4	Second PCR (R)	*rpoB*	GTATTCGATCTCGTCGCTGA	Present study	
R5	Sequencing primer (F)	*rpoB*	ACGCTGATCAATATCCGTCC	Present study	–
R6	Sequencing primer (R)	*rpoB*	CGACAA TGAACCGATCAGAC	Present study	–
G1	First PCR (F)	*gyrA*	ACGCGATGAGTGTGATTGTGG	Present study	336
G2	First PCR (R)	*gyrA*	TCCCAAATAGCAACCTCACC	Present study	
G3	Second PCR(F)	*gyrA*	GATGGTCTCAAACCGGTACA	Present study	291
G4	Second PCR (R)	*gyrA*	CCCAAATAGCAACCTCACCA	Present study	
G3	Sequencing primer (F)	*gyrA*	GATGGTCTCAAACCGGTACA	Present study	–
G4	Sequencing primer (R)	*gyrA*	CCCAAATAGCAACCTCACCA	Present study	–

products are visualized more clearly with less smear bands. Therefore, the new RLEP-K system was used for detection in further experimentation with use of clinical samples.

Using DNAs extracted from the serial dilutions of *M. leprae*, we determined the sensitivity of the nested PCR for DRDRs. The limit of amplification by PCR was 1×10^3–1×10^4 bacilli (Figure 1 c–e).

RLEP-nested PCR for Clinical Samples

The PCR methods were applied on 423 clinical samples collected from areas of endemicity in Vietnam. First, we tested RLEP-K for detection of *M. leprae* after extraction of DNA from smear samples. Positive bands were obtained by gel electrophoresis using RLEP-K on 290 samples. The positivity rate was 69%. The patients supplying the 290 samples were divided into 3 categories: new, relapse, and recent cases. Positive rates of RLEP-K by category were 75%, 84%, and 66%, respectively (Table 2).

Mutations in Clinical Samples

Samples positive by RLEP-nested PCR were applied for mutation experiments on the DRDRs of *folP1*, *rpoB*, and the *gyrA* gene. Nineteen mutations were found in 187 *folP1* samples, but no mutations related to drug resistance were noted in 163 *rpoB* and 147 *gyrA* gene samples. The mutations detected on *folP1* were as follows: 6 cases of ACC to ATC in codon 53(threonine to isoleucine), 9 cases of CCC to CGC in codon 55 (proline to arginine), and 4 cases of CCC to CTC (proline to leucine). Two new cases, 8 relapse cases, and 9 recent cases had mutations on *folP1*. Mutation rates in the 3 categories were 6.1%, 57%, and 6.4%, respectively (Table 3).

Some missense mutations, of which the association with drug resistance is unknown, were detected in the *rpoB* gene from clinical samples. The mutations were detected in 7 patients at codons 517, 532, and 556. One patient with relapse showed a mutation from CAG (glutamine) to CAT (histidine) at codon 517. One new patient showed 2 mutations at codon 517 from CAG (glutamine) to CAT (histidine) and at codon 532 from GCG (alanine) to TCG (serine). Sequence electropherograms indicated double peaks of a second nucleotide at codon 556 in 3 patients categorized as having recent cases. One peak was G (identical to that of wild-type), and the other peak was T, which changed the amino acid from glycine (GGC) to valine (GTC; data not shown).

The Relation between Treatment and Drug-Resistant Mutations in Patients with Relapse

Patients with relapse were categorized into 4 groups, by treatment history (Table 4). Group 1 comprised those treated with dapsone monotherapy. Group 2 was treated with MDT for 24 months. Group 3 included patients who had received

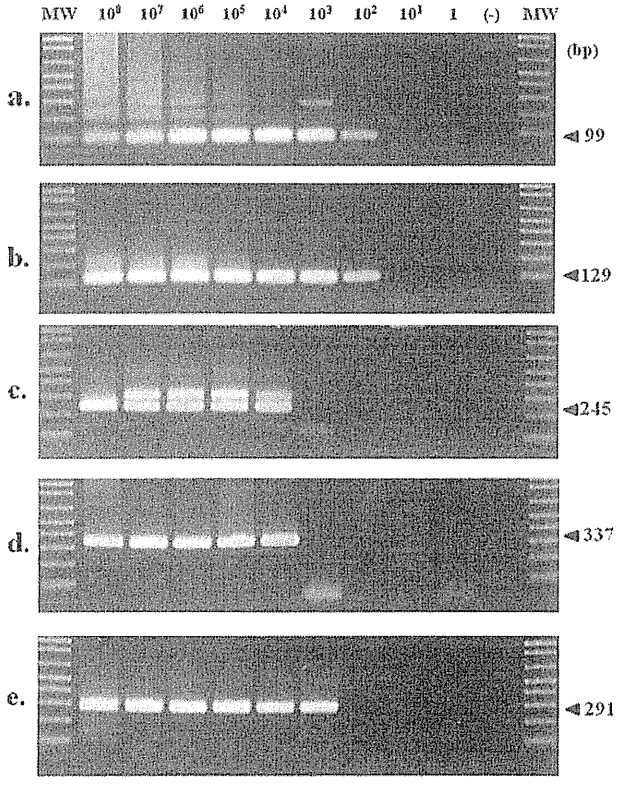

Figure 1. Sensitivity of nested polymerase chain reaction (PCR). The nested PCR products were visualized on 2 % agarose gel. A, RLEP-nested PCR (RLEP-L) using primers, LP1-LP4 (final products size, 99 bp). B, RLEP-nested PCR (RLEP-K) using primers, K1, K2, LP1, and LP2 (final products size, 129bp). C, *folP1*-nested PCR using F1-F4. D, *rpoB*-nested PCR using R1-R4. E, *gyrA*-nested PCR using G1-G4.

a diagnosis of second relapse—once after treatment with dapsone monotherapy and, subsequently, after MDT for 24 months. Group 4 was treated with ofloxacine monotherapy. Eight of the 14 patients with *folP1*-amplified relapse cases (57%) had mutations on the *folP1* gene. Seven (78%) of 9 patients with relapse who were categorized in groups 1 and 3 also had *folP1* mutations. However, 2 patients in group 4 had no mutations on any of the 3 genes.

Monitoring of Mutations in Patients

One hundred seven slit-skin smear samples from 43 patients were taken with consents at different times from each patient for monitoring mutations under treatment. Table 5 shows the difference in mutation results between 5 such patients. The other 38 patients showed no mutation during monitoring. Patients A, B, and C, who had new cases, showed a similar pattern, with no mutation at first testing and mutation in codon 53 on the *folP1* gene during MDT. However, double peaks of T and C in the second base were observed on *folP1* in the 3 patients. Patients D and E, who had relapse cases and finished dapsone monotherapy 20 years earlier, had a mutation on *folP1* in 2005 and no mutation after MDT.

Table 2. Polymerase Chain Reaction Positivity in New, Relapse, and Recent Cases

Case category	No.	RLEP	*folP1*	*rpoB*	*gyrA*
New	83	62 (75%)	33	39	43
Relapse	19	16 (84%)	14	15	13
Recent	321	212 (66%)	140	109	91
Total	423	290 (69%)	187 (64%)	163 (56%)	147 (51%)

DISCUSSION

The most popular PCR method for *M. leprae* detection with high sensitivity and specificity is probably the RLEP-nested PCR method, because the RLEP regions are specific for *M. leprae*, with >28 copies dispersed through the *M. leprae* genome [17]. New primers were designed for the RLEP-nested PCR in our study. This system using the new primers was termed RLEP-K. RLEP-K products appear to be a somewhat sharper and stronger band on agarose gel electrophoresis, compared with that that of previous RLEP-nested PCR (ie, RLEP-L). The RLEP-K detected *M. leprae* in 69% of the Vietnam samples. The remaining 31% of the samples were deduced as being cases either cleared of *M. leprae* by chemotherapy or those having <100 bacilli, which was below the detection limit of RLEP-K. We also designed new primers for amplification and sequencing of DRDR in the drug-resistance related genes *folP1*, *rpoB*, and *gyrA*, which were applied in examining the Vietnam samples. The mutation rates of *folP1* in new and recent cases were 6.1% and 6.4%, respectively. In contrast, the mutation rate in relapse cases was quite high, at 57%. The result indicated a strong correlation between mutation rate and relapse. Two possible reasons were conceived regarding the high positive rate of dapsone resistance in patients with relapse: (1) reinfection by the primary drug-resistant strain (7 of 8 samples indicating relapse were collected in the province in central Vietnam, which had the highest prevalence of leprosy and high rate of relapse (data not shown) and (2) reactivation of dapsone-resistant strains capable of

Table 3. Number of Mutations on *folP1*

Case category	No. of PCR-positive cases	No. of mutations (mutation ratio)	No. of mutation in mutation types
New	33	2 (6.1%)	2 (55th: CCC-CGC)
Relapse	14	8 (57%)	2 (53rd: ACC-ATC)
			3 (55th: CCC-CGC)
			3 (55th: CCC-CTC)
Recent	140	9 (6.4%)	4 (53rd: ACC-ATC)
			4 (55th: CCC-CGC)
			1 (55th: CCC-CTC)

Table 4. Mutations Noted in RLEP-Positive Relapse Cases, by Treatment Group

Group	Past treatment	No.	Mutation on *folP1*	Mutation on *rpoB*	Mutation on *gyrA*
1	DDS	7	5	0	0
2	MDT (24 months)	3	1	1[a]	0
3	DDS plus MDT (24 months)	2	2	0	0
4	OFX	2	0	0	0
All	...	14	8	1[a]	0

Abbreviations: DDS (diaminodiphenylsulfone), dapsone monotherapy; MDT,multidrug treatment; OFX, Ofloxacine monotherapy.

[a] Unknown DR mutation

persisting after chemotherapy, discussed below. Although it is still unclear whether the relapses are caused by reinfection by *M. leprae* or by reactivation of persistent *M. leprae*, close correlation between drug resistance and relapse have been recognized in several studies [18, 19].

The proportion of samples showing mutation on the *folP1* gene related to dapsone resistance was 10.2% (19 of 187) in samples from the central and highland regions of Vietnam (Table 3). Comparison with previous reports from South Korea (19.2%) indicates lower rates of relapse in these regions of Vietnam [20].

No mutation was found in the DRDR regions of *rpoB* in all samples. Mutation frequencies of the *rpoB* gene are also very low in other reports. Regarding other areas in Southeast Asia, no cases of rifampicin resistance have been detected in the Philippines, 1 (1.9%) of 54 cases in Myanmar, and 4 (3.3%) of 121 cases in Indonesia. However, in Japan, where the prevalence of leprosy is very low, the reported rate of rifampicin resistance is very high, at 29.5% (26 of 88 cases) [21]. The long-term use of drugs outside the standard MDT regimen in Japanese leprosy cases might have been instrumental in promoting this rifampicin resistance.

As such, no mutations have been found in the DRDR of the *M. leprae rpoB* gene derived from patients with leprosy, including relapse cases in Vietnam. A possible explanation for this could be the success of leprosy control in Vietnam and efficacy of properly administered MDT in which rifampicin—with its bactericidal properties—was effective in suppressing the occurrence of drug-resistant bacilli. In contrast, dapsone (not bactericidal in itself, although capable of suppressing growth), which had previously been used as monotherapy, may have enabled bacteria surviving in the patient receiving treatment to develop mutations, giving them resistance against the drug. Although occurrence of drug-resistant *M. leprae* was kept very low after application of MDT, 7 of 9 samples with drug-resistant mutations had previously been treated by dapsone monotherapy (Table 4). Jing et al [22] reported that patients with multibacillary leprosy who were retreated with MDT after dapsone monotherapy may have lower risk of early relapse while continuing to carry the risk of late relapse. Our observations suggest the possibility that efficacy of MDT may be hampered in some patients by the presence of surviving dapsone-resistant *M. leprae* in their bodies, which could develop into late relapse. Similar observations have been reported, suspecting involvement of the effects of dapsone monotherapy in patients with relapse [23].

There was no mutation in the major sites for drug resistance on the *rpoB* gene. However, we observed mutations at 3 positions, codons 517, 532, and 556, which have not been associated with rifampicin resistance. These mutations in the *rpoB* gene are a finding calling for further clarification.

Table 5. Monitoring of 5 Patients with Multibacillary Leprosy for *folP1* Mutation

Patient	Case category	Date of sample obtainment	Sample site (method of obtainment)	*folP1* mutation
A	New	2006 April 3	Abdomen (biopsy)	None[a]
		2007 January 30	Earlobe (smear)	53rd (ACC→ATC/ACC)
		2007 January 30	Abdomen (smear)	53rd (ACC→ATC/ACC)
B	New	2005 May 31	Earlobe (smear)	None
		2006 March 24	Skin (smear)	None
		2007 November 2	Skin (smear)	53rd (ACC→ATC/ACC)
C	New	2006 July 20	Skin (smear)	None
		2007 January 30	Skin (smear)	53rd (ACC→ATC/ACC)
		2007 January 30	Skin (smear)	53rd (ACC→ATC/ACC)
D	Relapse	2005 November	Earlobe (smear)	55th (CCC→CGC)
		2007 January	Skin (smear)	None
E	Relapse	2007 Jannuary 17	Arm (smear)	None
		2007 January 30	Earlobe (smear)	55th (CCC→CGC)
		2007 January 30	Arm (smear)	None

[a] ACC ATC/ACC indicates double peaks in second base at codon 53.

To reveal the possible relation between treatment and gene mutation, some patients with leprosy were monitored for gene mutations in light of drug treatments. The results showed incidence of dapsone-resistant *M. leprae* in patients receiving MDT, suggesting that some of the patients with relapse who were previously treated with dapsone monotherapy might have persistent infections with dapsone-resistant *M. leprae*. Furthermore, samples derived from different sites of lesions in the same patient sometimes showed different results (Table 5). The results suggest that we need to know the relation between the situation of patients with leprosy and drug resistance.

Overall, our study indicated a high ratio of dapsone resistance in patients with relapse, compared with the other patients with leprosy. In contrast, an unexpected outcome of our study was that we were unable to find mutations on the *rpoB* gene in patients with relapse. Moreover, it was shown that many of the patients with relapse who had dapsone-resistant mutations had histories of treatment with dapsone monotherapy. To clarify the relationship between relapse, drug resistance, and dapsone monotherapy, it might be necessary to investigate persistence of drug-resistant *M. leprae* through large-scale surveillance.

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Potential conflicts of interest. All authors: no conflicts.

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Immunostimulatory Activity of Major Membrane Protein II from *Mycobacterium tuberculosis*▽‡

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Previously, we observed that both major membrane protein II of *Mycobacterium leprae* (MMP-ML) and its fusion with *M. bovis* BCG (BCG)-derived heat shock protein 70 (HSP70) (Fusion-ML) are immunogenic and that recombinant BCG secreting either of these proteins effectively inhibits the multiplication of *M. leprae* in mice. Here, we purified *M. tuberculosis*-derived major membrane protein II (MMP-MTB) and its fusion with HSP70 (Fusion-MTB) in a lipopolysaccharide-free condition and evaluated their immunostimulatory abilities. Both MMP-MTB and Fusion-MTB activated monocyte-derived dendritic cells (DC) in terms of phenotype and interleukin-12 (IL-12) production, but Fusion-MTB more efficiently activated them than MMP-MTB did. The IL-12 production was a consequence of the ligation of those recombinant proteins with Toll-like receptor 2. The *M. tuberculosis*-derived and *M. leprae*-derived recombinant proteins activated naïve T cells of both CD4 and CD8 subsets, but *M. tuberculosis*-derived proteins were superior to *M. leprae*-derived proteins and fusion proteins were superior to MMP, regardless of the origin of the protein. Memory-type CD4$^+$ T cells obtained from BCG-vaccinated healthy individuals seem to be primed with MMP-MTB by the vaccination, and both *M. tuberculosis*-derived recombinant proteins produced perforin-producing CD8$^+$ T cells from memory-type CD8$^+$ T cells. Further, infection of DC and macrophages with *M. tuberculosis* H37Ra and H37Rv induced the expression of MMP on their surface. These results indicate that *M. tuberculosis*-derived MMP, as a sole protein or as part of a fusion protein, may be useful for developing new vaccinating agents against tuberculosis.

Tuberculosis is a chronic infectious disease caused by intracellular infection with *Mycobacterium tuberculosis* (20). It is estimated that one-third of the global population is latently infected with this inhaled pathogen, which infects primarily macrophages and dendritic cells (DC), and tuberculosis is responsible for more than two million deaths yearly worldwide (11, 34, 36). The emergence of multidrug-resistant strains of *M. tuberculosis* mandates the development of more effective preventive and therapeutic strategies, including the development of improved vaccines (48). Protective immunity against *M. tuberculosis* is conducted chiefly by adaptive cellular immune responses, and gamma interferon (IFN-γ)-producing type 1 CD4$^+$ T cells and CD8$^+$ T cells are key components of this immunity (1, 12, 16). IFN-γ produced by activated T cells is believed to be an essential element of the host defense against *M. tuberculosis* (13). Further, the contribution of CD8$^+$ T cells to protection by lysing infected cells is also important for bacterial killing (7, 19). CD8$^+$ T cells can kill *M. tuberculosis*-infected host cells via a granule-dependent mechanism involving perforin and granulysin, which has a direct antimicrobial activity (42, 49).

The only approved vaccine currently available against tuberculosis is *M. bovis* bacillus Calmette-Guérin (BCG), an attenuated strain of *M. bovis*. More than four billion doses of BCG have been administered so far, and is established as a safe vaccine (29). BCG appears to be effective at preventing diseases such as tuberculous meningitis and miliary tuberculosis in newborns and toddlers; however, it has no apparent effect on pulmonary tuberculosis in adults (3, 9). The reason why BCG cannot prevent disease development is not fully known, but one of the reasons is based on the fact that BCG has a capacity to block phagosome maturation to inhibit antigen (Ag) processing and presentation to type 1 T cells (14, 32, 38). Indeed, although *M. tuberculosis* directly delivers Ag to the major histocompatibility complex (MHC) class I processing pathway, BCG was less able to activate CD8$^+$ T cells (35, 41). Further, BCG growing in human macrophages was not recognized by immune CD4$^+$ T cells, although BCG-infected macrophages continued to express MHC class II molecules (35). These observations indicate the need for the development of a new vaccine against tuberculosis.

Various new vaccine candidates which are based on Ags that are recognized in infected individuals are currently in clinical trials, including early secretory antigenic target 6 (ESAT-6), the Ag85 family, and a polyprotein Ag, designated Mtb72F, derived from *M. tuberculosis* proteins Mtb32 and Mtb59 (1, 2, 17, 18, 37, 39). However, a fully reliable new vaccine has not been established yet.

A situation similar to that of tuberculosis can be found in leprosy, which is caused by infection with *M. leprae*, and the development of a new vaccine capable of inhibiting the multi-

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plication of *M. leprae* is highly desirable. In both tuberculosis and leprosy, the activation of T cells is induced by DC loaded with bacilli or their components, which display various antigenic molecules on their surface, including the immunodominant Ags (15, 30), although there are conflicting results indicating that *M. leprae* inhibits the activation and maturation of DC (33). We are of the opinion that future vaccines, to be successful, must (i) be highly antigenic, (ii) have the capacity to activate both naïve $CD4^+$ T cells and $CD8^+$ T cells, and (iii) have the ability to be expressed on the surface of mycobacterium-infected Ag-presenting cells (APCs) such as macrophages and DC. Previously, we identified major membrane protein (MMP; gene name, *bfrA* or ML2038) as one of the immunodominant Ags of *M. leprae* (21). *M. leprae*-derived MMP (MMP-ML) ligates Toll-like receptor 2 (TLR2) and consequently activates the NF-κB pathway of host cells (21). DC pulsed with MMP-ML activate memory-type $CD4^+$ and $CD8^+$ T cells to produce IFN-γ in an Ag-specific fashion (21, 26). Further, MMP-ML is supposed to be recognized *in vivo* by T cells of *M. leprae*-infected individuals, including paucibacillary leprosy patients (26).

Further, when we introduced MMP-ML with the Ag85A secretion signal of *M. tuberculosis* into BCG, the modified BCG, termed BCG-SM, secreted MMP-ML, enhanced the ability of BCG to activate naïve $CD4^+$, and further, successfully activated naïve $CD8^+$ T cells (25). Furthermore, BCG-SM at least partially inhibited the growth of *M. leprae* in C57BL/6 mice subsequently challenged by injection in the footpads (22). These observations indicate that MMP-ML could be a target molecule to be further analyzed as a vaccine candidate, and the fact that BCG-SM can activate both subsets of naïve T cells to produce IFN-γ indicates that secretion of MMP-ML, presumably in the phagosome of APCs, is a useful strategy to activate T cells (25). We sought another strategy to further enhance the T cell-stimulating activity of BCG, especially of the ability to activate IFN-γ-producing $CD8^+$ T cells quickly and strongly. To this end, we used heat shock protein 70 (HSP70) as a fusion partner (6, 10, 44, 45). The gene encoding HSP70 of BCG was directly linked with that of MMP and extrachromosomally transformed into BCG (BCG-70 M) (31). BCG-70 M secreted the HSP70-MMP fusion protein (Fusion-ML) and activated not only Ag-specific naïve $CD8^+$ T cells polyclonally but also naïve $CD4^+$ T cells strongly (31). Further, the secreted Fusion-ML protein activated DC in terms of phenotype and the production of cytokines such as interleukin-12 (IL-12) (31). Thus, the production and secretion of HSP70 in phagosomes along with MMP-ML, using BCG as a vector, seem to be effective in activating human naïve $CD8^+$ T cells. These observations led us to speculate that the use of MMP, which is commonly present in pathogenic mycobacteria, or of the HSP70-MMP fusion protein may be useful in inhibiting the multiplication of *M. tuberculosis*. However, the MMP homology between *M. leprae* and *M. tuberculosis* (MMP-MTB; gene name, *bfrA* or Rv1876) is 90.6% at the amino acid level. Therefore, in this study, we purified *M. leprae*- or *M. tuberculosis*-derived MMP and a fusion protein composed of HSP70 and *M. leprae*- or *M. tuberculosis*-derived MMP by using *M. smegmatis* and evaluated their immunostimulatory activities.

MATERIALS AND METHODS

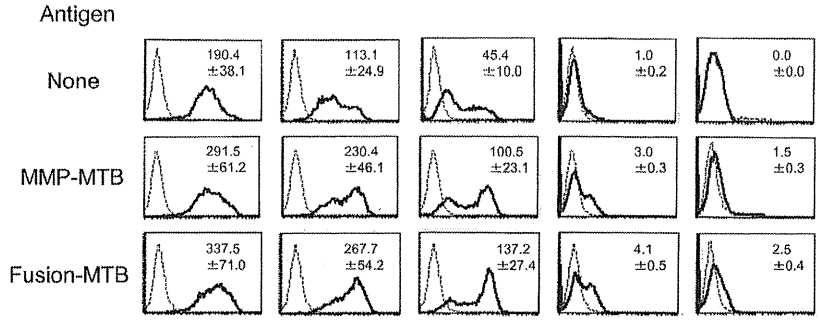
Preparation of cells and Ags. Peripheral blood was obtained from healthy, purified protein derivative-positive individuals after informed consent was obtained. In Japan, BCG vaccination is compulsory for children (0 to 4 years old). Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque Plus (Pharmacia, Uppsala, Sweden) and cryopreserved in liquid nitrogen until use, as previously described (23). The viability of T cells obtained from cryopreserved PBMCs was more than 90%, and no functional selection was induced in either monocytes or T cells by the cryopreservation of PBMCs. For the preparation of peripheral monocytes, $CD3^+$ T cells were removed from either freshly isolated heparinized blood or cryopreserved PBMCs using immunomagnetic beads coated with anti-CD3 monoclonal antibody (MAb; Dynabeads 450; Dynal Biotech, 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 (47). Monocyte-derived DC were differentiated as described previously (23, 28). Briefly, monocytes were cultured in the presence of 50 ng of recombinant granulocyte-macrophage colony-stimulating factor (rGM-CSF; PeproTech EC Ltd., London, England) and 10 ng of rIL-4 (PeproTech) per ml (28). On day 4 of culture, immature DC (purity, 70%) were pulsed with recombinant protein and on day 6 of culture, DC were used for further analyses of surface Ag and for mixed-lymphocyte assays. Macrophages were differentiated as described previously (24, 27). In brief, monocytes were cultured in the presence of 10 ng of rM-CSF (R&D Systems, Inc., Minneapolis, MN) per ml. On day 5 of culture, macrophages were pulsed with recombinant protein and on day 7 of culture, they were used for further analyses of surface Ag and for mixed-lymphocyte assays.

Preparation of *M. tuberculosis*. *M. tuberculosis* strains H37Ra and H37Rv, which were originally purchased from the American Tissue Culture Collection, were kindly donated by T. Yamazaki, National Institute of Infectious Diseases. Both H37Ra and H37Rv were cultured *in vitro* using Middlebrook 7H9 broth (BD Biosciences, San Jose, CA) supplemented with 0.05% Tween 80 and 10% albumin-dextrose-catalase (BD Biosciences). The bacilli were grown to log phase and stored at 10^8 CFU/ml at −80°C. Before the infection of DC and macrophages, H37Ra and H37Rv bacteria were counted by the colony assay method.

Construction of vectors for production of *M. leprae*- or *M. tuberculosis*-derived recombinant MMP and HSP70-MMP fusion protein (Fusion-ML) or Fusion-MTB. An *Escherichia coli-Mycobacterium* shuttle vector, pMV261 (43), was used as a parent vector. To replace the kanamycin resistance gene with a hygromycin resistance cassette, the XbaI-NheI fragment from pYUB854 (5) was cloned into SpeI-NheI-digested plasmids. The resultant vector was a hygromycin-resistant pMV261 vector, pMV261H. The acetamidase promoter was amplified from *M. smegmatis* mc^2155 genomic DNA. The primers used were F Pace Xba (5′-TTA ATC TAG AGA AGT GAC GCG GTC TCA AGC GTC-3′ [underlining indicates an XbaI site]) and R Pace Bam (5′-TTT AGG ATC CGT GGA CTC CCT TTC TCT TA-3′ [underlining indicates a BamHI site]). The *Hsp60* promoter region in pMV261H was replaced with the amplified PCR products, and the resulting vector was named p2H Pace.

We inserted an N-terminally His-tagged gene sequence encoding MMP-ML, MMP-MTB, HSP70-MMP-ML fusion (Fusion-ML), or HSP70-MMP-MTB (Fusion-MTB) into the p2H Pace vector using the In-Fusion Advantage PCR Cloning Kit (Clontech Laboratories, Inc.). Briefly, the linearized p2H Pace vector for the In-Fusion reaction was prepared by PCR with F ter (5′-TAG TTA ACT AGC GTA CGA T-3′) and R Pace H6 (5′-GTG ATG GTG GTG ATG GTG CAT GTG GAC TCC CTT TCT CTT AT-3′). PCR primers for inserts were designed that share 15 bases of homology with the sequences at the ends of linearized p2H Pace. These primers were used to amplify the insert DNAs for MMP-ML, MMP-MTB, Fusion-ML, and Fusion-MTB. The resulting PCR products were combined with the linearized vector in the In-Fusion cloning reaction and then transformed into *E. coli*. All clones was verified by sequencing.

Expression and purification of recombinant proteins in *M. smegmatis*. *M. smegmatis* mc^2155 was cultured *in vitro* using LB broth supplemented with 0.05% Tyloxapol (Sigma-Aldrich, St. Louis, MO). Expression vectors were introduced into *M. smegmatis* by electroporation (40). Transformants were selected on LB agar (BD Biosciences, San Jose, CA) plates containing 50 μg/ml hygromycin. The selected clone was grown in LB broth with 150 μg/ml hygromycin. During the logarithmic phase, acetamide was added to the culture medium at a final concentration of 0.2% (8). After an additional 16 h of culture, recombinant *M. smegmatis* was centrifuged and resuspended in lysis buffer (50 mM sodium phosphate, 300 mM NaCl, 3 M guanidine-HCl) containing proteinase inhibitor and disrupted by sonication. The suspension was centrifuged at 27,000 × *g* for 15 min. The supernatant was further filtered through a 0.45-μm filter and used as starting material. MMP-ML and MMP-MTB were purified by metal affinity



(p value)	HLA-ABC	HLA-DR	CD86	CD83	MMP
None vs MMP	p<0.05	p<0.05	p<0.05	p<0.001	p<0.05
None vs Fusion	p<0.05	p<0.05	p<0.05	p<0.005	p<0.01
MMP vs Fusion	p<0.05	p<0.05	p<0.005	p<0.05	p<0.005

FIG. 1. Expression of APC-associated molecules and MMP on DC by stimulation with recombinant proteins. Immature DC obtained from monocytes in the presence of rGM-CSF and rIL-4 were pulsed with either MMP-MTB or Fusion-MTB at 10 μg/ml on day 4 of culture. The DC were gated and analyzed on day 6 after the start of culture. Dotted lines, isotype-matched control IgG or IgM (for MMP); solid lines, the indicated test MAb. Representative results of three separate experiments are shown. The value in the top right corner of each graph is the mean fluorescence intensity of three independent experiments with a control Ig or the test MAb ± the standard deviation. Titers were statistically compared using Student's *t* test.

chromatography (TALON Metal Affinity Resins; Clontech Laboratories). Fusion-ML and Fusion-MTB were purified by two purification steps. Passage through a metal affinity column (TALON) was also used in the first step. The eluted crude proteins were applied to a HiLoad Superdex 200 pg column (GE Healthcare, Buckinghamshire, England) for further purification by gel filtration. Three major fractions were detected after the second step; one of them contained the target protein. The purified proteins (MMP-ML, MMP-MTB, Fusion-ML, and Fusion-MTB) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie brilliant blue staining (see Fig. S1 in the supplemental material).

Analysis of cell surface Ag. The expression of cell surface Ag on DC and lymphocytes was analyzed using a FACScalibur. Dead cells were eliminated from the analysis by staining with propidium iodide (Sigma-Aldrich), and 1×10^4 live cells were analyzed. For cell surface Ag analysis, fluorescein isothiocyanate (FITC)-conjugated MAbs against HLA-ABC (G46-2.6; BD Biosciences), HLA-DR (L243; BD Biosciences), CD86 (FUN-1; BD Biosciences), and CD83 (HB15a; Immunotech, Marseille, France) were used.

The expression of MMP on Ag-pulsed DC or DC infected with *M. tuberculosis* at an indicated multiplicity of infection (MOI) was determined using the MAb against MMP-ML (M270-13, IgM, kappa), which probably detects MMP complexed with MHC molecules on the surface of DC (26), followed by FITC-conjugated anti-mouse Ig Ab (Tago Immunologicals, Camarillo, CA). The intracellular production of perforin was assessed as follows. Memory-type $CD8^+$ T cells were stimulated with Ag-pulsed DC for 5 days in the presence of memory-type $CD4^+$ T cells, and $CD8^+$ T cells were surface stained with phycoerythrin-labeled MAb to CD8 and fixed in 2% formaldehyde. Subsequently, the cells were permeabilized using permeabilizing solution (BD Biosciences) and stained with an FITC-conjugated MAb to perforin (δG9; BD Biosciences) or an FITC-labeled isotype control.

APC functions of DC. The ability of Ag-pulsed DC and macrophages to stimulate T cells was assessed using an autologous APC-T cell coculture as previously described (15, 28). Purification of $CD4^+$ and $CD8^+$ T cells was conducted by using negative-isolation kits (Dynabeads 450; Dynal Biotech) (28). The purity of the $CD4^+$ and $CD8^+$ T cells was more than 95% as assessed by FACScalibur. Naïve $CD4^+$ and $CD8^+$ T cells were produced by further treatment of these T cells with MAb to CD45RO, which was followed by beads coated with MAb to goat anti-mouse IgG (Dynal Biotech). The purity of both subsets of naïve T cells was more than 97%. However, there was no contamination of memory-type T cells in the naïve T cell preparations. More than 98% of the $CD45RA^+$ T cells were positive for expression of the CCR7 molecule. Memory-type T cells were similarly produced by the treatment of cells with a MAb to CD45RA Ag. The purified responder cells (1×10^5 per well) were plated in 96-well round-bottom tissue culture plates, and DC or macrophages pulsed with Ag were added to give the indicated APC/T cell ratio. Supernatants of APC-T cell cocultures were collected on day 4, and the cytokine levels were determined. In some cases, Ag-pulsed DC were treated with MAbs to HLA-ABC (W6/32; mouse IgG2a, kappa), HLA-DR (L243; mouse IgG2a, kappa), CD86 (IT2.2; mouse IgG2b, kappa, BD Biosciences), or MMP (M270-13) or normal mouse IgG or IgM. The treatment of DC with these MAbs did not affect the viability of the DC (not shown). Also, in some cases, Ag-pulsed DC were costimulated with CD40 ligand (CD40L; 1 μg/ml; PeproTech). The optimal concentration was determined in advance.

Measurement of cytokine production. Levels of the following cytokines were measured: IFN-γ produced by $CD4^+$ and $CD8^+$ T cells and IL-12p40 produced by DC stimulated for 24 h with Ag. The concentrations of these cytokines were quantified with enzyme-linked immunosorbent assay (ELISA) kits (Opt EIA Human ELISA Set; BD Biosciences). The detection limit of the IFN-γ ELISA kit is 3.0 pg/ml.

Statistical analysis. Student's *t* test was used to determine statistically significant differences.

RESULTS

Activation of DC by *M. tuberculosis*-derived recombinant proteins. For a recombinant protein to activate T cells, it must have the ability to activate APCs. We assessed the phenotypic change induced in DC by stimulation with MMP-MTB (gene name, Rv1876 or *bfrA*) and a fusion protein composed of BCG-derived HSP70 and MMP-MTB (Fusion-MTB) (Fig. 1). Both recombinant proteins upregulated the surface expression of HLA-ABC, HLA-DR, CD86, and CD83. However, Fusion-MTB more efficiently enhanced the expression of all of these molecules. Further, MMP-MTB- or Fusion-MTB-pulsed DC expressed molecules which react with anti-MMP-ML MAbs. Again, Fusion-MTB was more efficient than MMP-MTB in the induction of expression of the molecules. These results indicated that both recombinant proteins may have the ability to activate DC. To confirm this point, we measured the IL-12p40 production of DC by stimulation with the recombinant proteins (Fig. 2). We comparatively analyzed MMP-ML, MMP-MTB, Fusion-ML, and Fusion-MTB. All of the recombinant proteins induced the production of IL-12p40, but the levels of IL-12p40 produced by stimulation were as follows: MMP-MTB > MMP-

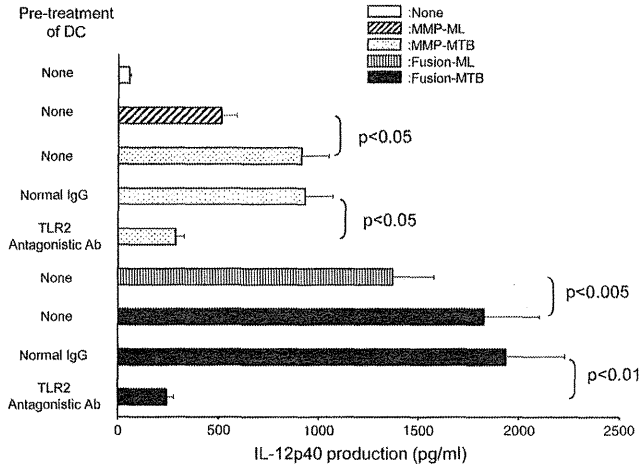


FIG. 2. IL-12p40 production by DC stimulated with recombinant proteins. Monocyte-derived DC from 5 days of culture in the presence of rGM-CSF and rIL-4 were stimulated with the indicated recombinant protein at 10 μg/ml for 24 h. In some cases, immature DC were pretreated with normal murine IgG or TLR2-antagonistic Ab (10 μg/ml) and subsequently stimulated with recombinant protein for 24 h. The concentration of IL-12p40 was determined by the ELISA method. A representative of three separate experiments is shown. Assays were performed in triplicate, and the results are expressed as the mean ± the standard deviation. Titers were statistically compared by Student's *t* test.

ML, Fusion-MTB > Fusion-ML, and Fusion-MTB > MMP-MTB. In order to reveal the mechanisms leading to the activation of DC by MMP-MTB or Fusion-MTB, we pretreated immature DC with TLR2-antagonistic Ab and subsequently stimulated the treated DC with recombinant proteins, since both MMP-ML and Fusion-ML are reported to activate the NF-κB pathway through ligation with TLR2 (21, 31). While pretreatment of DC with normal murine IgG did not affect the production of IL-12 by stimulation with recombinant proteins, the pretreatment with TLR2-antagonistic Ab significantly inhibited the cytokine production caused by MMP-MTB and Fusion-MTB (Fig. 2). Also, we tested the effect of TLR4-antagonistic Ab on IL-12 production; however, the pretreatment of DC with TLR4-antagonistic Ab did not inhibit cytokine production (not shown).

Activation of T cells by recombinant protein. The enhanced activation of DC by *M. tuberculosis*-derived MMP and fusion proteins may enable autologous T cell activation. The T cell-activating ability of the recombinant proteins was assessed chiefly by using DC as APCs. Memory-type $CD4^+$ T cells were purified from healthy, BCG-vaccinated individuals. All of the recombinant proteins activated the $CD4^+$ T cells with a small dose (~2.0 μg/ml) of Ags and a small dose of recombinant protein-pulsed DC (T cell/DC ratio, 80:1) (Fig. 3A). However, MMP-MTB and Fusion-MTB more efficiently activated the T cells than *M. leprae*-derived proteins did, and Fusion-MTB induced a significantly larger amount of IFN-γ than MMP-MTB did. When we used a higher concentration of recombinant proteins, such as 5 or 10 μg/ml, and used a higher number of DC, such as a T cell/DC ratio of 10:1, as a stimulator, similar statistically significant differences were observed (not shown). Further, only Fusion-MTB successfully activated memory-type $CD4^+$ T cells through macrophages, although a large dose of Ag and a high number of Ag-pulsed macrophages were required (T cell/macrophage ratio, 5:1) (Fig. 3A). The $CD4^+$ T cell-stimulating activity of MMP-MTB and Fusion-MTB was confirmed by using CD45RO-negative naïve $CD4^+$ T cells. All of the recombinant proteins, including MMP-ML, MMP-MTB, Fusion-ML, and Fusion-MTB, activated naïve $CD4^+$ T cells, and Fusion-MTB was the most effective (Fig. 3B). Compared to memory $CD4^+$ T cells, naïve $CD4^+$ T cells required a larger dose of Ag (~10 μg/ml) and a higher number of Ag-pulsed DC (T cell/DC ratio, 10:1) to be activated. To address the mechanisms leading to the activation of naïve $CD4^+$ T cells by Fusion-MTB, Fusion-MTB-pulsed DC were treated with MAbs against HLA-DR, CD86, and MMP-ML molecules and subsequently used to stimulate naïve $CD4^+$ T cells (Fig. 3C). IFN-γ production by these naïve $CD4^+$ T cells was significantly inhibited by the surface treatment of the DC with the MAbs, and similarly, IL-2 production by naïve $CD4^+$ T cells was inhibited (not shown). The ability of MMP-MTB and Fusion-MTB to activate memory-type $CD8^+$ T cells was then assessed (Fig. 4A). Although, in contrast to memory-type $CD4^+$ T cells, a large dose of recombinant proteins was required, both *M. tuberculosis*-derived recombinant proteins induced significant production of IFN-γ from memory-type $CD8^+$ T cells. Further, the additional treatment of Ag-pulsed DC with CD40L upregulated the production of IFN-γ by $CD8^+$ T cells. In both cases, i.e., without and with CD40L treatment, Fusion-MTB induced significantly greater IFN-γ production than MMP-MTB did. In order to confirm the $CD8^+$ T cell-stimulating abilities of both MMP-MTB and Fusion-MTB, naïve $CD8^+$ T cells were also examined as responders. In this case, purified proteins from *M. leprae* were used as a control (Fig. 4B). Both MMP-MTB and Fusion-MTB activated naïve $CD8^+$ T cells to produce IFN-γ; however, the concentration of IFN-γ released from naïve $CD8^+$ T cells was low and a cytokine concentration of less than 35 pg/ml was produced, and the concentration of IFN-γ produced from naïve $CD8^+$ T cells by stimulation with Fusion-MTB was significantly lower than that from memory $CD8^+$ T cells ($P < 0.005$). The naïve $CD8^+$ T cell-stimulating activities of the recombinant proteins were as follows: MMP-MTB > MMP-ML, Fusion-MTB > Fusion-ML, and Fusion-MTB > MMP-MTB. The IFN-γ production by naïve $CD8^+$ T cells was enhanced by the additional treatment of Ag-pulsed DC with CD40L, and the highest production of IFN-γ was achieved by Fusion-MTB; in this case, Fusion-MTB could induce an IFN-γ concentration of more than 100 pg/ml. To elucidate the mechanisms of the activation of naïve $CD8^+$ T cells by Fusion-MTB, Fusion-MTB pulsed DC were treated with MAbs to HLA-ABC and CD86 and subsequently used as a stimulator (Fig. 4C). IFN-γ production by naïve $CD8^+$ T cells was significantly inhibited by the treatment of the DC. One of the aims of $CD8^+$ T cell activation in terms of the host defense against *M. tuberculosis* is to produce cytotoxic $CD8^+$ T cells. To measure the production of cytotoxic $CD8^+$ T cells, we assessed the intracellular production of perforin in $CD8^+$ T cells which were stimulated with MMP-MTB or Fusion-MTB in the presence of $CD4^+$ T cells (Fig. 4D). Both recombinant proteins produced perforin-producing $CD8^+$ T cells, and Fusion-MTB seemed to produce them more efficiently.

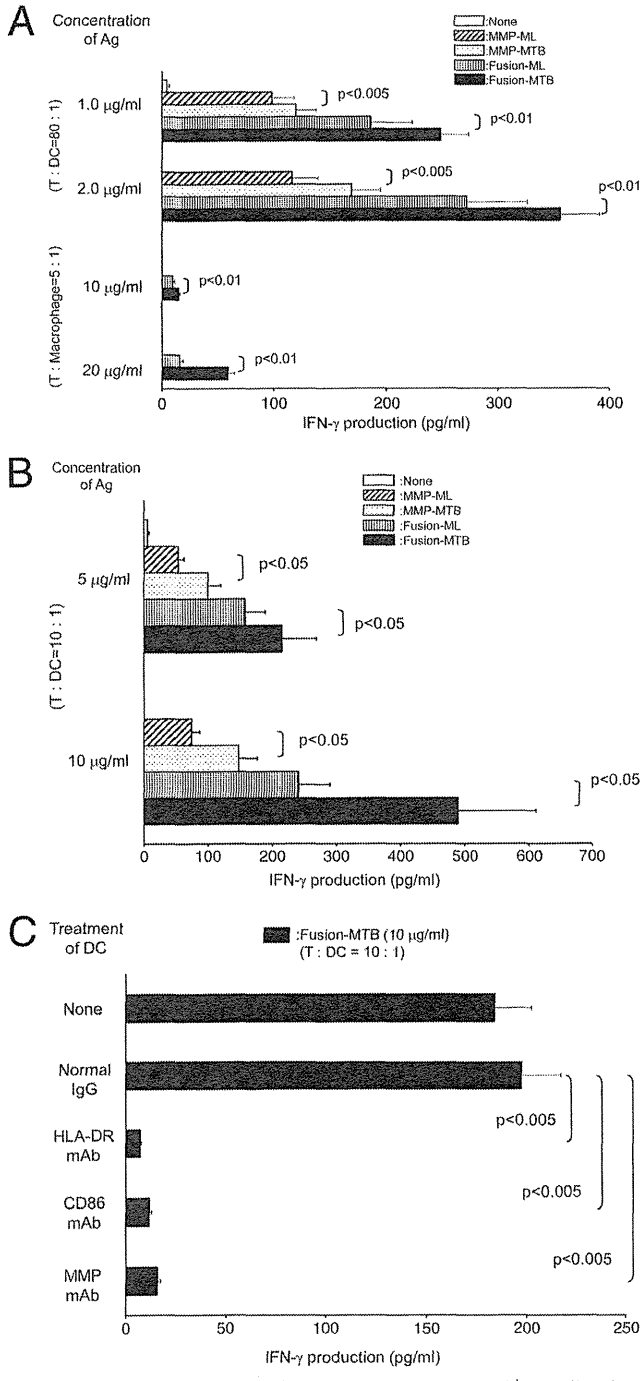


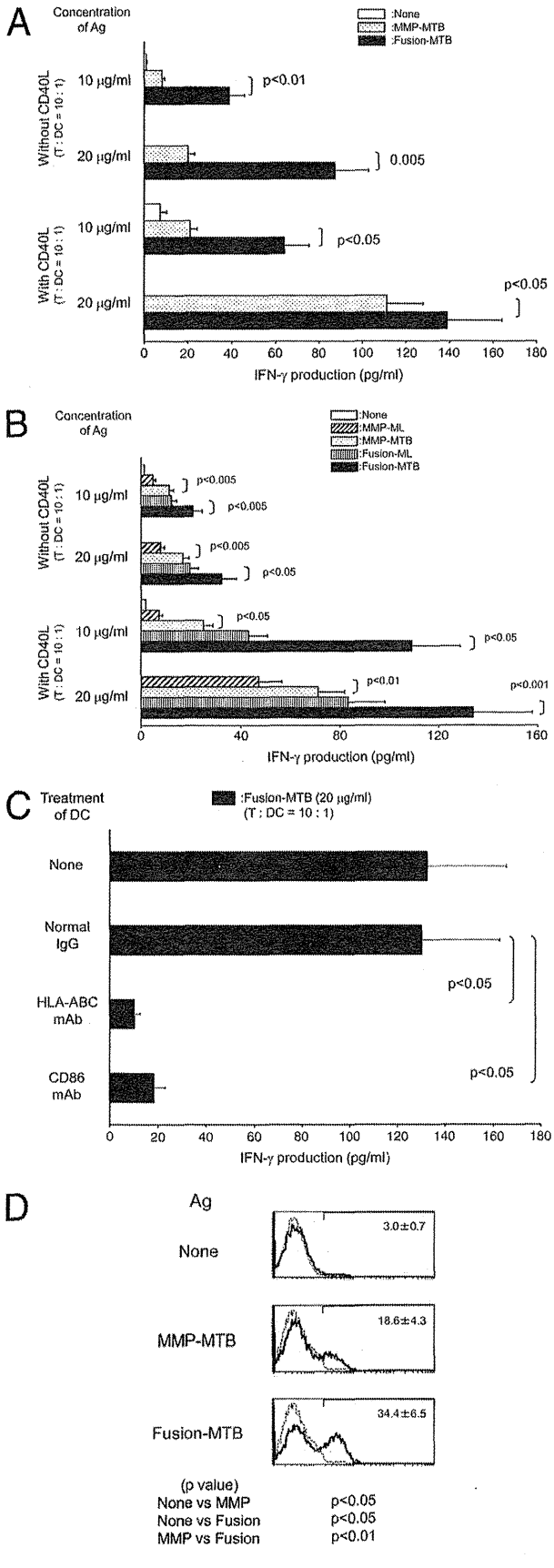
FIG. 3. (A) IFN-γ production by memory-type $CD4^+$ T cells stimulated with recombinant proteins. Monocyte-derived DC or macrophages were pulsed with the indicated recombinant protein at the indicated concentration and used to stimulate memory-type $CD4^+$ T cells in a 4-day culture. Responder $CD4^+$ T cells (1×10^5) were stimulated with the indicated dose of Ag-pulsed DC or macrophages. (B) IFN-γ production by naïve $CD4^+$ T cells by stimulation with recombinant protein. Monocyte-derived DC were pulsed with the indicated recombinant protein at 5 or 10 μg/ml and used to stimulate naïve $CD4^+$ T cells in a 4-day culture. Responder $CD4^+$ T cells (1×10^5) were stimulated with the Ag-pulsed DC at a T cell/DC ratio of 10:1. (C) Inhibition of naïve $CD4^+$ T cell activation by treatment of Ag-pulsed DC with MAb. Monocyte-derived DC were pulsed with Fusion-MTB at 10 μg/ml and subsequently treated at 10 μg/ml with MAb to HLA-DR, CD86, MMP, or normal murine IgG or IgM. These DC were used to stimulate naïve $CD4^+$ T cells (1×10^5) at a T cell/DC ratio of 10:1. IFN-γ produced from T cells was measured by the ELISA method. A representative of three separate experiments is shown. Assays were performed in triplicate, and the results are expressed as means ± standard deviations. Titers were statistically compared by Student's *t* test.

Expression of MMP on APCs infected with *M. tuberculosis*. The molecule used as a vaccinating agent should be expressed on APCs infected with the pathogen. To reveal the expression of MMP on the surface of APCs, DC and macrophages were infected with *M. tuberculosis* H37Ra and H37Rv and analyzed by flow cytometry (Fig. 5). Both DC and macrophages expressed MMP molecules on their surface after infection with H37Ra and H37Rv. Expression levels seemed to be dependent on the dose of *M. tuberculosis* used for infection (not shown).

DISCUSSION

In vivo studies using various knockout mice indicate that adaptive immunities play an important role in inhibiting the multiplication of *M. tuberculosis* and that the activation of both $CD4^+$ T cells and $CD8^+$ T cells is an essential element of the control of *M. tuberculosis* infection (1, 12, 16). While $CD4^+$ T cells chiefly act in the initial phase of infection, $CD8^+$ T cells either producing IFN-γ or having cytotoxic killing activity contribute to the chronic or stationary phase of infection (7, 19, 46). Thus, the antigenic molecules which are used as an essential component of a vaccine should have the ability to activate not only naïve $CD4^+$ T cells and $CD8^+$ T cells but also APCs, including DC. So far, we have found MMP to be one of the immunodominant Ags of *M. leprae* (21) and found evidence that MMP-ML activated DC through ligation with TLR2, which resulted in the activation of the NF-κB pathway of host cells, and that DC pulsed with MMP-ML stimulated both $CD4^+$ and $CD8^+$ T cells to produce IFN-γ in an Ag-specific manner (21, 26). Further, MMP-ML is supposed to be recognized *in vivo* by both T cell subsets of *M. leprae*-infected individuals, including paucibacillary leprosy patients (26).

In addition, HSP70, one of the heat shock proteins, plays various roles in the upregulation of the ability of APCs to stimulate T cells (6, 10, 44, 45). Further, HSPs of both mammalian host cell and bacterial origins are reported to have chaperon activity (6, 44) and can effectively prime a cytolytic response (10, 45). In fact, we previously reported that HSP70 effectively induced the cross-priming of $CD8^+$ T cells through the cytosolic pathway when secreted from recombinant BCG in the phagosome of DC as part of a fusion protein (31). Also, others have reported that HSP65 activated naïve $CD8^+$ T cells and a DNA vaccine containing the *hsp65* gene inhibited the development of tuberculosis that is induced by the multiplication of subsequently challenged *M. tuberculosis* (50). Furthermore, vaccination of mice with recombinant BCG that secreted either MMP-ML or Fusion-ML, in which BCG was used as a vehicle, efficiently inhibited the multiplication of subsequently challenged *M. leprae*, although the fusion protein was more efficient in both activating naïve T cells and inhibiting *M. leprae* multiplication (22, 25, 31).



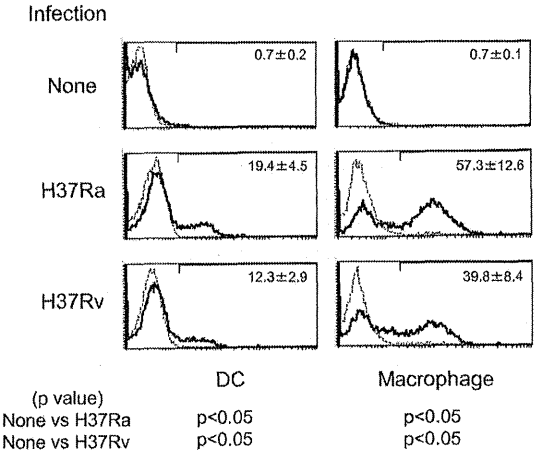


FIG. 5. Expression of MMP on DC and macrophage infected with *M. tuberculosis*. Monocyte-derived DC or macrophages were infected with either H37Ra or H37Rv at an MOI of 1.0 and cultured for another 2 days in the presence of rGM-CSF plus rIL-4 or rM-CSF, respectively. The DC and macrophages were gated and analyzed on day 5 after the start of culture. Dotted lines, control IgM; solid lines, MMP MAb. Results representative of three separate experiments are shown. The values are the mean percentages of major membrane protein II-positive DC or macrophages in three independent experiments and the standard deviations. Titers were statistically compared using Student's *t* test.

MMP is commonly expressed in both pathological mycobacteria and BCG, so that it may be that MMP-MTB plays a substantial role in inhibiting the replication of *M. tuberculosis*; however, the homology of MMP between *M. leprae* (ML2038)

FIG. 4. (A) IFN-γ production by memory-type $CD8^+$ T cells by stimulation with recombinant protein. Monocyte-derived DC were pulsed with MMP-MTB or Fusion-MTB at 10 or 20 μg/ml, costimulated with or without CD40L (1.0 μg/ml), and used to stimulate memory-type $CD8^+$ T cells in a 4-day culture. Responder $CD8^+$ T cells (1×10^5) were stimulated with the Ag-pulsed DC at a T cell/DC ratio of 10:1. (B) IFN-γ production by naïve $CD8^+$ T cells stimulated with recombinant proteins. Monocyte-derived DC were pulsed with the indicated recombinant protein at 10 or 20 μg/ml, further costimulated with or without CD40L (1.0 μg/ml), and used to stimulate naïve $CD8^+$ T cells in a 4-day culture. Responder $CD8^+$ T cells (1×10^5) were stimulated with the Ag-pulsed DC at a T cell/DC ratio of 10:1. (C) Inhibition of naïve $CD8^+$ T cell activation by treatment of Fusion-MTB-pulsed DC with MAb. Monocyte-derived DC were pulsed with MMP-MTB at 20 μg/ml, costimulated with CD40L (1.0 μg/ml), and subsequently treated at 10 μg/ml with MAb to HLA-ABC, CD86, or normal murine IgG. These DC were used to stimulate naïve $CD8^+$ T cells (1×10^5) at a T cell/DC ratio of 10:1. IFN-γ produced by T cells was measured by the ELISA method. A representative of three separate experiments is shown. Assays were performed in triplicate, and the results are expressed as means ± standard deviations. Titers were statistically compared by Student's *t* test. (D) Intracellular production of perforin by $CD8^+$ T cells. Monocyte-derived DC were pulsed at 10 μg/ml with either MMP-MTB or Fusion-MTB and cultured with unseparated memory-type T cells (T cell/DC ratio, 40:1) for 5 days. The stimulated $CD8^+$ T cells were gated and analyzed for perforin production. Values are the mean percentages of the $CD8^+$ T cell population that were perforin positive in three independent experiments and the standard deviations. Titers were statistically compared using Student's *t* test. A representative of three separate experiments is shown.

and *M. tuberculosis* (Rv1876) is only 90.6% at the amino acid level. Therefore, we assessed the immunostimulatory activity of *M. tuberculosis*-derived MMP and its fusion with BCG-derived HSP70 by using MMP-ML and Fusion-ML as controls.

As expected, MMP derived from *M. tuberculosis* activated DC in terms of phenotypic change and cytokine production, and the cytokine production was associated with the ability of MMP-MTB to ligate with TLR2. MMP-MTB-pulsed DC activated both $CD4^+$ and $CD8^+$ T cells. In this respect, only a very small amount of MMP was required to induce vigorous activation of $CD4^+$ T cells, but not $CD8^+$ T cells, obtained from BCG-vaccinated healthy donors. These results may indicate that some subsets of $CD4^+$ T cells are primed with MMP by vaccination with BCG, whose MMP is 100% homologous to that of *M. tuberculosis*, as in the case of leprosy patients whose T cells were primed by *M. leprae* infection. However, in contrast to leprosy patients, only $CD4^+$ T cells are primed with MMP by BCG vaccination, which may be linked with the fact that the parent BCG less efficiently activates naïve $CD8^+$ T cells. Activation of T cells usually depends on APCs expressing Ags, so that successful production of MMP-reactive memory-type T cells could be achieved by administration of MMP since MMP could be expressed on the surface of DC after infection with *M. tuberculosis* H37Ra and H37Rv. This speculation might be supported by our preliminary experiments in which administration of MMP-MTB to C57BL/6 mice produced memory-type splenic T cells reactive to MMP-MTB *in vitro*, which produced IFN-γ because of this stimulation.

Fourteen amino acids of *M. leprae* MMP differ from those of *M. tuberculosis* MMP, and substitutions of amino acids between these mycobacteria are known to occur randomly. However, a MAb which recognizes the epitope expressed on DC pulsed with *M. leprae*-derived MMP could also detect a peptide expressed on the surface of DC pulsed with *M. tuberculosis*-derived MMP or infected with *M. tuberculosis*. The MAb against MMP-ML inhibited the activation of naïve $CD4^+$ T cells by stimulation with MMP-MTB-pulsed DC. These observations indicated that the regions common to the MMPs of *M. leprae* and *M. tuberculosis* were chiefly used as antigenic epitopes of $CD4^+$ T cells. However, the T cell activation by *M. tuberculosis*-derived MMP and Fusion-MTB is significantly stronger than that by *M. leprae*-derived proteins. The exact mechanism leading to the difference between the T cell-stimulating activities of the MMPs derived from these two pathological mycobacterial strains remains to be elucidated, but one possibility is that some parts of *M. tuberculosis*-derived MMP other than common regions have APC-immunomodulating activities that are associated with T cell activation. In fact, *M. tuberculosis*-derived MMP more efficiently activated DC than MMP-ML did, in terms of IL-12 production. However, both MMP-ML and MMP-MTB ligate TLR2; thus, MMP-MTB may have other unknown mechanisms that can induce the activation of DC more strongly. In this respect, we assessed the IL-1β-producing ability of MMP, but there was no apparent difference between the MMPs obtained from *M. tuberculosis* and *M. leprae* (not shown). It has been reported that the replacement of one amino acid of the T cell epitope of the antigenic determinant of Ag85B of *M. tuberculosis* strongly affects its T cell-stimulating activity, i.e., the ability to induce IFN-γ production (4). Therefore, a similar change may have occurred in the MMP system, although it has not been clearly defined.

When we compared the immunostimulating activities of MMP-MTB and Fusion-MTB in terms of the activation of APC and T cells, the latter showed higher activity in the activation of both DC and $CD4^+$ and $CD8^+$ T cells. The exact mechanism of the high immunostimulating activity of the fusion protein is not fully known, but it may be associated with previous reports indicating that HSPs play a varied role in enhancing the ability of APCs to stimulate T cells (6, 10, 44, 45). In fact, the fusion protein induced the expression of higher levels of APC-associated molecules on DC than MMP did. Further, Fusion-MTB may be useful to produce cytotoxic $CD8^+$ T cells because the fusion protein efficiently produced perforin-producing $CD8^+$ T cells, although both MMP-MTB and Fusion-MTB produced cytotoxic $CD8^+$ T cells. Moreover, the fusion protein upregulated the expression of CD40 on DC (not shown) and treatment of Fusion-MTB-pulsed DC with CD40L induced the production of a larger dose of IFN-γ from both naïve $CD4^+$ T cells (not shown) and naïve $CD8^+$ T cells (Fig. 4B). These results indicate that the use of HSP70 as part of a fusion protein may make APCs susceptible to various conditioning molecules, including CD40L. This observation is in the line with the fact that only Fusion-MTB-pulsed monocyte-derived macrophages successfully activated $CD4^+$ T cells, probably MMP primed, when conditioned with CD40L.

Taken together, the data present here suggest that MMP, alone or as part of fusion protein, is highly immunogenic and may be useful for developing new vaccine against tuberculosis, at least in combination with BCG, ESAT-6, or other molecules.

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