こととした。ハンドブックにはハンセン病の再発と皮膚病に気軽に対応できる皮膚科医の一覧表を掲載した。

2010 年には 4 名の新規ハンセン病患者がいた。4 名の新規患者については主治医に対して、実際の検査の実技指導、治療の指導を行い、ハンセン病を確実に診療できる体制を確立した。

顧みられない熱帯病 (neglected tropical diseases)としてハンセン病と共に挙げられているブルーリ潰瘍についても検討・研究を行った。すなわち、両者は末梢神経症状を呈すること、潰瘍をおこすこと、熱帯地域に多いことなどから極めて類似した所見を示すので鑑別が困難である。日本においてもブルーリ潰瘍が現在まで 19 例報告されており、ハンセン病との鑑別に困難をきたし、両疾患の異同が問題になっている。両疾患の臨床的、菌学的な検討を行ない、臨床現場で鑑別がスムーズにできるように症例検討を行った。

D. 考察

ハンセン病患者が減少し、診療する機会が減少し、教育を受けていない、一度も診療機会がない皮膚科医が大多数を占めるようになっている。またハンセン病とブルーリ潰瘍の鑑別ができない場合もおきている。また、ハンセン病の歴史やハンセン病回復者の心情なども理解できていない。それらを解決するために、講習会を開催し、意識向上に努めた。皮膚科医は知識吸収の意欲はあり、講習会には 20 名の皮膚科医が参集した。講習会を実りあるものにするためにハンセン病回復者の方、4 名にも参加いただき、彼らの現状などについて講演いただいた。今後も講習会を通じて学習意欲を持続させるために、年に一回程度の継続した教育機会を設けることが必要である。

ハンセン病回復者を一般医療機関に受診させる(インテグレーション)事は難しいが、一歩でもそれに近づける努力は必要である。そのため、気軽に相談できる皮膚科医名簿を更新し、ハンセン病療養所退所者等ハンドブックを作成し配布した。これらの皮膚科医を起点として他の診療科などに受診できることを期待したい。また、ハンセン病回復者などから生の声を聞いて、患者と医師とのあるべき関係を構築することも大事である。

ハンセン病の新規患者は減少しているが、外国人患者については鑑別にハンセン病が入っているので、診断に迷うことは多くないようである。一方、日本人患者については、ハンセン病とブルーリ潰瘍や他の皮膚病との鑑別は難しく、診断が遅れる場合がある。数年に1名程度は日本人新規患者も登録されることがあり、必ず鑑別に「ハンセン病」を入れることが必要である。2010 年は 4 名の新規患者が登録されたが、2 名のフィリピン人と、ブラジル人とミャンマー人が各1人であった。

E. 結論

ハンセン病診療を皮膚科医が主体的に実施するためのネットワーク作りは、まだ始まったばかりであるが、皮膚科医の教育、ハンセン病回復者の一般医療機関への受診の動きを、引き続き行うことが重要である。

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H. 知的財産権の出願・登録状況

なし

Ⅲ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

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Ⅳ. 研究成果の刊行物・別刷

Ⓒ *M. avium* 感染症

Rx 処方例　下記1），2）のいずれかを用いる．

1）クラリス錠（200 mg）　2-3錠　分2-3 49，およびリファジンカプセル（150 mg）　3カプセル　分1　朝食前

2）クラリス錠（200 mg）　2-3錠　分2-3 49，およびシプロキサン錠（200 mg）　2-4錠　分2

また，温熱療法は無効とされる．治療困難例も多い．

ハンセン病
Hansen's disease, leprosy

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病態と診断

ハンセン病は，らい菌感染によって主に皮膚と末梢神経が侵される疾患である．

診断は①知覚低下を伴う皮疹，②末梢神経の知覚麻痺・肥厚・運動障害，③らい菌検出，④病理組織所見の4項目を総合して行う．

生体のらい菌に対する特異的免疫能の差によって臨床，病理，神経障害などの病像が異なる．皮膚スメア検査，または病理での抗酸菌染色のいずれかでらい菌を検出できる多菌型（multibacillary：MB）と，らい菌を検出しにくい少菌型（paucibacillary：PB）に分類して治療するのが簡単である．

治療方針

神経障害などの後遺症を残さず，耐性菌を生じさせないために早期診断，早期治療，定期内服を心がける．神経痛，皮疹の増悪や再発，発熱などを認めた場合はらい反応を鑑別し，直ちに治療を行う．

ハンセン病は治癒する病気であるが，治療終了後も皮疹の再燃，らい反応，神経障害などのフォローのため数年間は定期的に通院させる．

治療はWHOの推奨する多剤併用療法（multidrug therapy：MDT）を修飾して治療する．日本ハンセン病学会は治療指針を作成し公開している．

Ⓐ 多菌型（MB）

Rx 処方例　下記1）-3）を併用する．

1）リファジンカプセル（150 mg）　4カプセル　分1　朝食前　月1回（2年間）

2）レクチゾール錠（25 mg）　4錠　分2　朝・夕食後　毎日（2年間）

3）ランプレンカプセル（50 mg）　1カプセル　分1　朝食後　毎日（2年間）

2年後，臨床症状や皮膚スメア検査によって治療の見直しを行う．特に臨床症状の沈静化を治療終了の目標にする．

Ⓑ 少菌型（PB）

Rx 処方例　下記の1）と2）を併用する．可能な限り3）を追加する．

1）リファジンカプセル（150 mg）　4カプセル　分1　朝食前　月1回（6か月間）

2）レクチゾール錠（25 mg）　4錠　分2　朝・夕食後　毎日（6か月間）

3）ランプレンカプセル（50 mg）　1カプセル　分1　朝食後　毎日（6か月間）

Ⓒ らい反応

Rx 処方例　上記AないしBの内服を継続しながら下記の1）と2）を追加する．

1）ランプレンカプセル（50 mg）　1カプセル　分1　朝食後　毎日

2）プレドニン錠（5 mg）　6-15錠　分3　食後　毎日（症状を改善させてから漸減，健胃薬を併用）

患者説明のポイント

- ハンセン病は単なる感染症であり，特別視するものではないことを説明する．
- 確実に内服することで後遺症なく治癒する病気であることを十分説明する．
- ランプレンは可逆的な色素沈着を起こすことを説明する．
- 治療中に皮疹や全身状態が悪化する場合は速やかに主治医に連絡する（らい反応の疑い）．

皮膚糸状菌症（白癬）
tinea, dermatophytosis

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病態と診断

Ⓐ 病態

皮膚糸状菌症（白癬）は，皮膚糸状菌が皮膚の角層や皮膚付属器に寄生して生じる疾患である．菌の寄生部位により浅在性と深在性に分類されるが，ほとんどの例が浅在性である．角層は主にケラチンという強固な蛋白質で構成されているが，これを分解できる皮膚糸状菌は角層を栄養として発育する．

病型分類は部位により行われ，菌が頭部の毛髪に寄生するものは頭部白癬，生毛部に寄生するものは体部白癬，手掌に寄生するものは手白癬，足底や趾間に寄生するものは足白癬，爪に寄生するものは爪白癬とよばれる．一般に足白癬は水虫，体部白癬は

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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

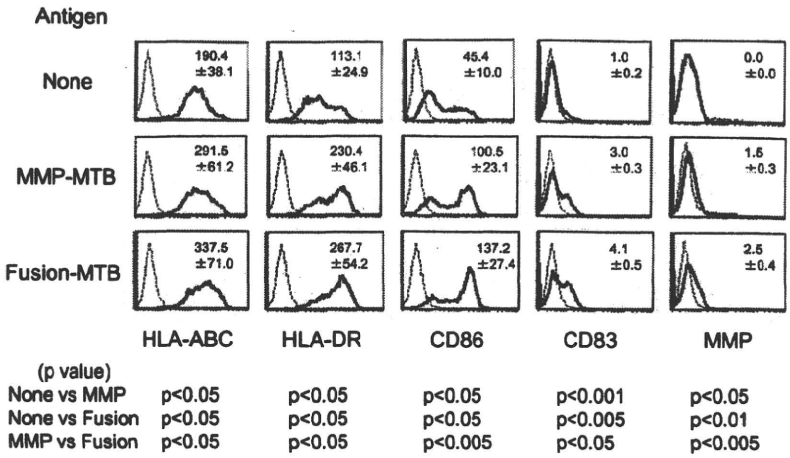

FIG. 1. Expression of APC-associated molecules and MMP on DC by stimulation with recombinant proteins. Immature DC obtained from monocytes in the presence of rGM-CSF and rIL-4 were pulsed with either MMP-MTB or Fusion-MTB at 10 μg/ml on day 4 of culture. The DC were gated and analyzed on day 6 after 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-

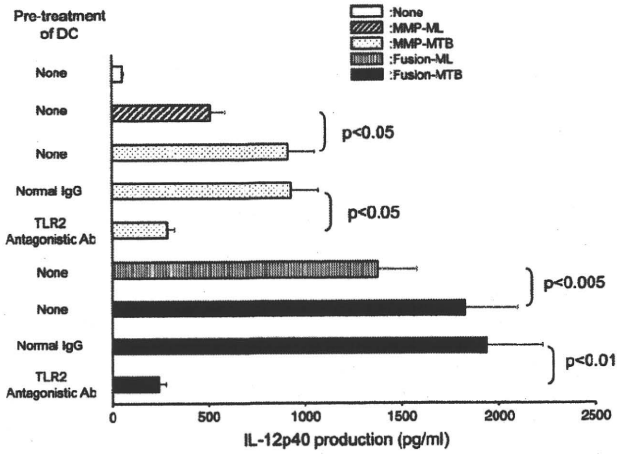

FIG. 2. IL-12p40 production by DC stimulated with recombinant proteins. Monocyte-derived DC from 5 days of culture in the presence of rGM-CSF and rIL-4 were stimulated with the indicated recombinant protein at 10 μg/ml for 24 h. In some cases, immature DC were pretreated with normal murine IgG or TLR2-antagonistic 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.

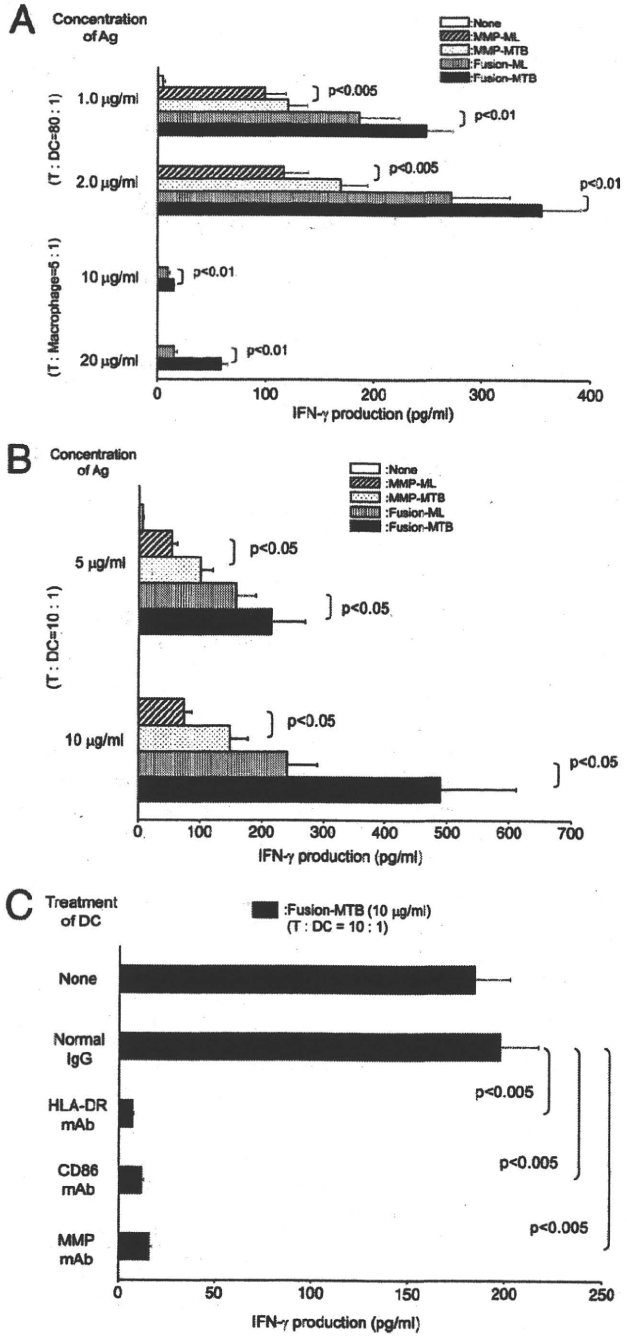

FIG. 3. (A) IFN-γ production by memory-type $CD4^+$ T cells stimulated with recombinant proteins. Monocyte-derived DC or macrophages were pulsed with the indicated recombinant protein at the indicated concentration and used to stimulate memory-type $CD4^+$ T cells in a 4-day culture. Responder $CD4^+$ 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).

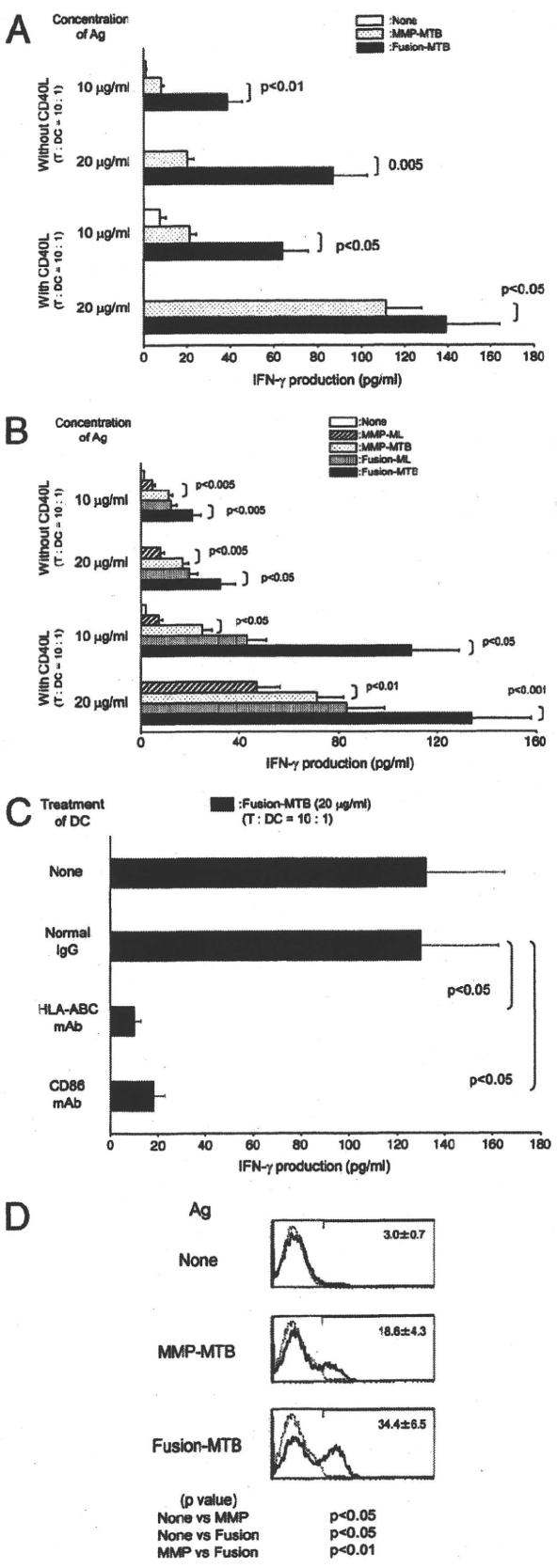

FIG. 4. (A) IFN-γ production by memory-type $CD8^+$ T cells by stimulation with recombinant protein. Monocyte-derived DC were pulsed with MMP-MTB or Fusion-MTB at 10 or 20 μg/ml, costimulated with or without CD40L (1.0 μg/ml), and used to stimulate memory-type $CD8^+$ T cells in a 4-day culture. Responder $CD8^+$ 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.

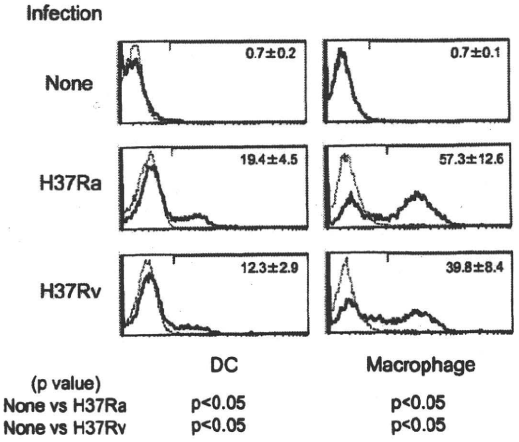

FIG. 5. Expression of MMP on DC and macrophage infected with *M. tuberculosis*. Monocyte-derived DC or macrophages were infected with either H37Ra or H37Rv at an MOI of 1.0 and cultured for another 2 days in the presence of rGM-CSF plus rIL-4 or rM-CSF, respectively. The DC and macrophages were gated 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)

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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Mutation Analysis of the *Mycobacterium leprae folP1* Gene and Dapsone Resistance[▽]

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Diaminodiphenylsulfone (dapsone) has long been used as a first-line drug worldwide for the treatment of leprosy. Diagnosis for dapsone resistance of *Mycobacterium leprae* by DNA tests would be of great clinical value, but the relationship between the nucleotide substitutions and susceptibility to dapsone must be clarified before use. In this study, we constructed recombinant strains of cultivable *Mycobacterium smegmatis* carrying the *M. leprae folP1* gene with or without a point mutation, disrupting their own *folP* gene on the chromosome. Dapsone susceptibilities of the recombinant bacteria were measured to examine influence of the mutations. Dapsone MICs for most of the strains with mutations at codon 53 or 55 of *M. leprae folP1* were 2 to 16 times as high as the MIC for the strain with the wild-type *folP1* sequence, but mutations that changed Thr to Ser at codon 53 showed somewhat lower MIC values than the wild-type sequence. Strains with mutations at codon 48 or 54 showed levels of susceptibility to dapsone comparable to the susceptibility of the strain with the wild-type sequence. This study confirmed that point mutations at codon 53 or 55 of the *M. leprae folP1* gene result in dapsone resistance.

The massive use of dapsone for treatment of leprosy led to the isolation of resistant strains of *Mycobacterium leprae* as early as 1964 (11), only a few years after discovery of the drug. Dapsone is structurally related to the sulfonamides. The mechanism of dapsone resistance in *M. leprae* is thought to be associated with dihydropteroate synthase (DHPS) in a manner similar to the mechanism of resistance to sulfonamides developed in other bacteria. The sulfonamides are structural analogs of *p*-aminobenzoate (PABA) and act as antimetabolites by competing with PABA for the active site of DHPS (4). DHPS catalyzes the reaction between dihydropteridine pyrophosphate and PABA as a part of the biosynthetic pathway leading to tetrahydrofolate (5, 12), which acts as a cofactor in the biosynthesis of purines, pyrimidines, and amino acids. Resistance to the sulfonamides has been shown to be mediated by mutations of the chromosomal *folP* gene encoding DHPS (7, 14, 15). Point mutations in the *folP1* gene have been identified in dapsone-resistant strains of *M. leprae* (9, 10, 16). Because *M. leprae* cannot be cultivated on any artificial medium and requires 13 days to double in experimentally infected mice, DNA diagnoses to detect dapsone-resistant bacteria would be highly useful. However, not all nucleotide substitutions in the *folP1* gene give rise to drug resistance. Therefore, the relationship between drug susceptibility and each nucleotide substitution observed in clinical isolates requires clarification. Dapsone-resistant *M. leprae* isolates have shown mutation at codon 53 or 55 in the *folP1* gene (6, 10, 16). Mutation at codon 48 has also been detected in our clinical specimens (unpublished data). Williams et al. have analyzed two types of mutations at codons 53 and 55 of the *M. leprae folP1* gene using a *folP*-deficient *Escherichia coli* (16). However, their analysis is as yet insufficient for direct application as molecular diagnosis for dapsone resistance.

In this study, site-directed mutagenesis techniques were used to alter the wild-type *M. leprae folP1* gene at codons shown to be mutated in clinical isolates for testing the effects of these mutations on dapsone susceptibility in a *folP*-disrupted *Mycobacterium smegmatis* host.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* DH5α cells were grown in Luria-Bertani (LB) medium. *M. smegmatis* mc^{2}155 and its transformants were grown in Middlebrook 7H9 medium (Difco, Detroit, MI) supplemented with 0.5% bovine serum albumin (fraction V), 0.2% glucose, 0.085% NaCl, 0.2% glycerol, and 0.1% Tween 80.

Site-directed mutagenesis. The wild-type *M. leprae folP1* gene was amplified by PCR from *M. leprae* Thai-53 and cloned into pMV261. Site-directed mutagenesis was performed using PCR with KOD DNA polymerase (Toyobo, Osaka, Japan) and the primers listed in Table 2. PCR products were purified and phosphorylated with T4 kinase and ATP and then ligated to become circular. The ligation mixture was used to transform *E. coli* DH5α, and kanamycin-resistant colonies were isolated. Plasmids were extracted from the transformants, and the mutated sequences were confirmed by sequencing. Mutations introduced in the *M. leprae folP1* gene are shown in Fig. 1A.

Disruption of the *folP* gene on the *M. smegmatis* chromosome. *M. smegmatis* mc^{2}155 cells were transformed with plasmids carrying the *M. leprae folP1* with or without a point mutation. Recombinants were selected on LB medium containing kanamycin. Allelic exchange mutants were constructed by the temperature-sensitive mycobacteriophage method (3). Using the *M. smegmatis* mc^{2}155 genome sequence (accession number CP000480), the upstream and downstream flanking DNA sequences were used to generate a deletion mutation in the *folP* gene (MSMEG_6103). In order to disrupt the *folP* gene, DNA segments from 736 bp upstream through 286 bp downstream of the initiation codon of *M. smegmatis folP* and from 198 bp upstream through 832 bp downstream of the termination codon were cloned directionally into the cosmid vector pYUB854, which contains a *res-hyg-res* cassette and a *cos* sequence for lambda phage assembly. Plasmids thus produced were digested with PacI and ligated to the PH101 genomic DNA excised from the phasmid phAE87 by PacI digestion. The ligated DNA was packaged using GigaPackIII Gold Packaging Extract (Stratagene, La Jolla, CA), and the resultant mixture was used for transduction of *E. coli* STBL2 (Life Technologies, Carlsbad, CA) to yield cosmid DNA. After *E.*

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
E. coli		
DH5α	Cloning host	
STBL2	Cloning host	
C600 Δ*folP*::Km^r	*folP* mutant	7
M. smegmatis mc^{2}155		
Plasmids		
pYUB854	Cosmid vector	3
phAE87	Phasmid vector carrying full length DNA of mycobacteriophage PH101	3
pMV261	*E. coli*-mycobacteria shuttle plasmid vector (multicopy in mycobacteria)	13
pNN301[a]	pMV361-type integrative vector (single copy in mycobacteria)	13; this study

[a] pNN301 has an *int-attP* fragment of mycobacteriophage L5 instead of *oriM*.

coli was transduced and the transductants were plated on hygromycin-containing medium, phasmid DNA was prepared from the pooled antibiotic-resistant transductants and electroporated into *M. smegmatis* mc^{2}155. Bacterial cells were incubated at 30°C to produce the recombinant phage. The *M. smegmatis* transformant carrying the *M. leprae folP1* gene was infected by the produced temperature-sensitive phage at 37°C for allelic exchange, and kanamycin- and hygromycin-resistant colonies were isolated. Two colonies for each point mutation were subjected to subsequent tests.

Dapsone susceptibility testing. The MIC values for *M. smegmatis* recombinant clones were determined by culture on Middlebrook 7H10 agar plates containing 2-fold serial dilutions of dapsone (0.25 to 64 μg/ml). The MIC value for each strain was defined as the lowest concentration of dapsone needed to inhibit bacterial growth.

RESULTS

Construction of recombinant *M. smegmatis* strains. We prepared plasmids with point mutations in the *M. leprae folP1* gene. Each plasmid has 1 of 21 single point mutations at codon 48, 53, 54, or 55 (Fig. 1A). The first or second nucleotide at each codon was replaced by another nucleotide to change the amino acid residue. Mutated sequences were confirmed by sequencing. Plasmids carrying the *M. leprae folP1* with or without a point mutation were individually introduced into *M. smegmatis*. The *M. smegmatis* transformants were subjected to allelic exchange to disrupt the *folP* gene on their own chromosome (Fig. 1B). PCR analysis confirmed that the *folP* sequences in the recombinant strains were replaced by hygromycin resistance gene sequences (Fig. 2). Isolation of a *folP*-disrupted *M. smegmatis* strain carrying the *M. leprae folP1* with mutation 48-4 (mutation 4 at codon 48) was unsuccessful. All the strains except for the strains with mutation 48-5 or 53-4 showed comparable growth rates. The strains with mutation 48-5 or 53-4 grew a little more slowly than the strain with the wild-type sequence. These two mutations may reduce DHPS activity.

Dapsone susceptibility. Dapsone susceptibilities of the recombinant *M. smegmatis* strains were tested. As shown in Fig. 3, the MIC of dapsone for recombinant *M. smegmatis* carrying the wild-type *M. leprae folP1* gene was 0.5 μg/ml. MIC values for most of the strains with mutations at codon 53 or 55 were 2 to 16 times as high as the MIC for the strain with the wild-type sequence. Interestingly, two strains with alterations

TABLE 2. Primers used in this study

Primer	Sequence[a]	Application
MLFPWTF	GCGAATTCGTGAGTTTGGCGCCAGTGCA	Cloning of *M. leprae folP1*, forward
MLFPWTR	GCAAGCTTTCAGCCATCACATCTAACCT	Cloning of *M. leprae folP1*, reverse
MSFPUF	GCAGGCCTGTATCCTCATCCCGGACAGC	*folP* disruption, upstream forward
MSFPUR	GCTCTAGATGGTGTCGATGCTGATCGTG	*folP* disruption, upstream reverse
MSFPDF	GCAGATCTCGCAAACGTTTCCTCGGTAC	*folP* disruption, downstream forward
MSFPDR	GCACTAGTACTGGTCGATCTCCGACAGC	*folP* disruption, downstream reverse
MSFPF	TCACCGAGTACGGCATGAGC	Detection of *folP* disruption, forward
MSFPR	TAGAGCGCATGGATCAGCAG	Detection of *folP* disruption, reverse
MLFPR1	CGATTCGCCACCGACGTCGAC	Introduction of point mutations for codons 53, 54, and 55
MLFPR2	GTCGACAATCGCCGCGCCTT	Introduction of point mutations for codon 48
MLFP48-1	ATCGGTGGCGAATCGACCCG	Introduction of point mutation 48-1
MLFP48-2	CTCGGTGGCGAATCGACCCG	Introduction of point mutation 48-2
MLFP48-3	TTCGGTGGCGAATCGACCCG	Introduction of point mutation 48-3
MLFP48-4	GACGGTGGCGAATCGACCCG	Introduction of point mutation 48-4
MLFP48-5	GCCGGTGGCGAATCGACCCG	Introduction of point mutation 48-5
MLFP48-6	GGCGGTGGCGAATCGACCCG	Introduction of point mutation 48-6
MLFP53-1	GCCCGGCCCGGTGCCATTAG	Introduction of point mutation 53-1
MLFP53-2	ATCCGGCCCGGTGCCATTAG	Introduction of point mutation 53-2
MLFP53-3	TCCCGGCCCGGTGCCATTAG	Introduction of point mutation 53-3
MLFP53-4	CCCCGGCCCGGTGCCATTAG	Introduction of point mutation 53-4
MLFP53-5	AACCGGCCCGGTGCCATTAG	Introduction of point mutation 53-5
MLFP53-6	AGCCGGCCCGGTGCCATTAG	Introduction of point mutation 53-6
MLFP54-1	ACCAGGCCCGGTGCCATTAG	Introduction of point mutation 54-1
MLFP54-2	ACCCGGCCCGGTGCCATTAG	Introduction of point mutation 54-2
MLFP54-3	ACCTGGCCCGGTGCCATTAG	Introduction of point mutation 54-3
MLFP55-1	ACCCGGACCGGTGCCATTAG	Introduction of point mutation 55-1
MLFP55-2	ACCCGGGCCGGTGCCATTAG	Introduction of point mutation 55-2
MLFP55-3	ACCCGGTCCGGTGCCATTAG	Introduction of point mutation 55-3
MLFP55-4	ACCCGGCACGGTGCCATTAG	Introduction of point mutation 55-4
MLFP55-5	ACCCGGCGCGGTGCCATTAG	Introduction of point mutation 55-5
MLFP55-6	ACCCGGCTCGGTGCCATTAG	Introduction of point mutation 55-6

[a] Restriction sites are underlined

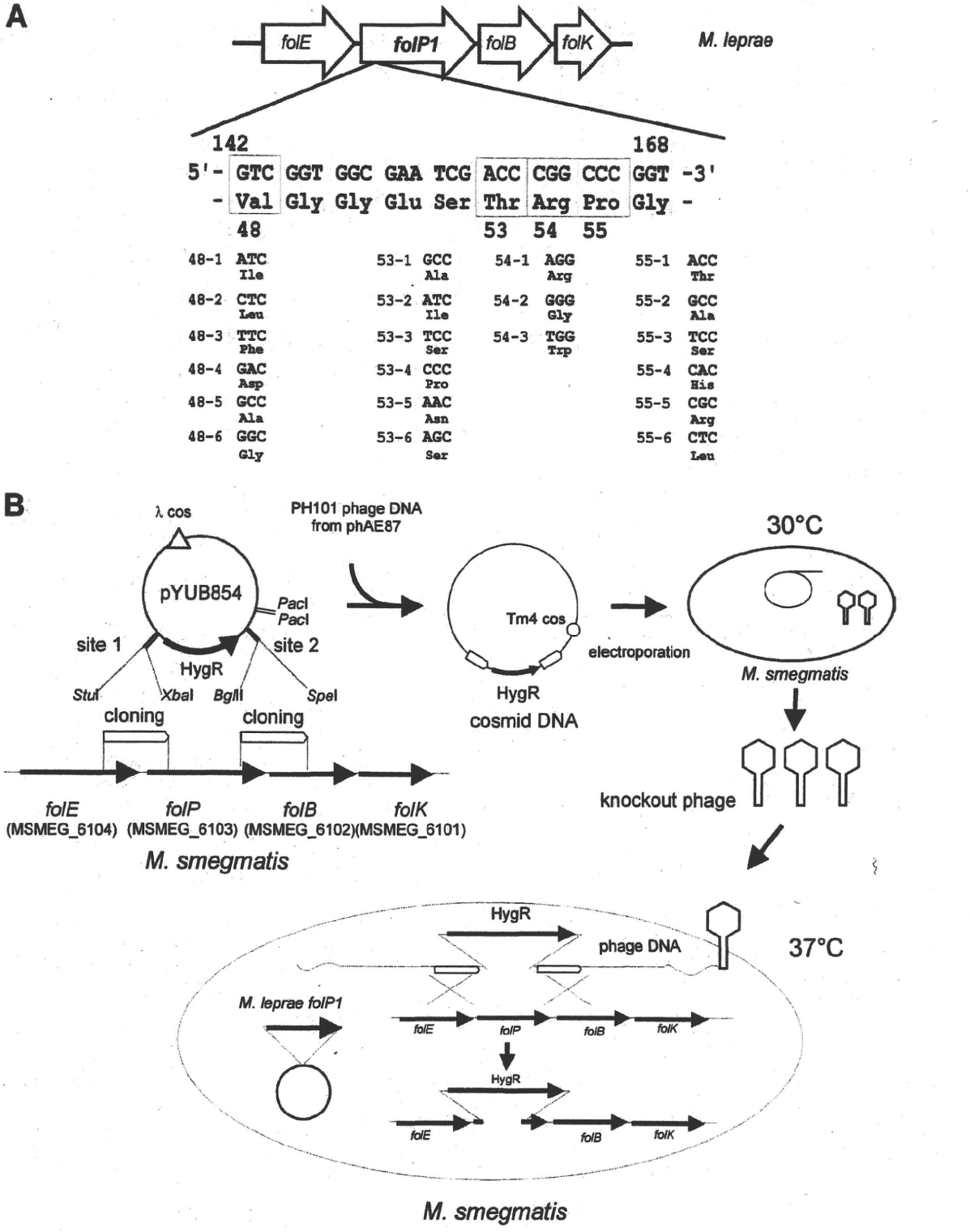

FIG. 1. Construction of recombinant *M. smegmatis* strains for dapsone susceptibility testing. (A) Point mutations introduced in the *M. leprae folP1* gene. Single nucleotide substitutions introduced in the *M. leprae folP1* at codons 48, 53, 54, and 55 are shown. Deduced amino acid residues are shown below the triplets. (B) Construction of *M. smegmatis* recombinants by allelic exchange.