indicated that the secretion of an immunodominant Ag of *M. leprae* in phagosomes of APCs of host is a useful way to inhibit the growth of *M. leprae* through the activation of T cells by delivering the antigenic determinants on APCs. This point was also revealed in other intracellular infection systems such as *Mycobacterium tuberculosis*, in which the secretion of Ag85 complex, one of the immunogenic molecules of *M. tuberculosis*, from vaccinated BCG was revealed to be effective in inhibiting the replication of *M. tuberculosis* challenged subsequently (16). Although the mechanisms involved have not been fully clarified, the activation of $CD8^+$ T cells seems to be induced by Ag85 protein secreted from BCG (16).

In general, the most efficient immunological means of activating naive $CD8^+$ T cells using mycobacteria, including BCG, is to upregulate the activity of DC to cross-present mycobacteria-derived Ags to the $CD8^+$ T cells. In this respect, an active inducer of cross-presenting activity in APCs is heat shock protein (HSP)70 (17, 18). HSP70 may be closely associated with host defenses against intracellular pathogens such as mycobacteria (19, 20).

In this study, in the search for another tool capable of stimulating naive $CD8^+$ T cells efficiently, we newly constructed a rBCG having an extrachromosomal BCG-derived HSP70 gene linked to the gene encoding MMP-II of *M. leprae* (BCG-70M), and evaluated its immunostimulatory activities. The BCG-70M secreted the HSP70-MMP-II fusion protein in vitro, and DC infected with BCG-70M more efficiently activated not only naive $CD8^+$ T cells by cross-presentation, but also naive $CD4^+$ T cells. Furthermore, BCG-70M produced memory T cells, of both $CD4^+$ and $CD8^+$ subsets in mice, capable of responding to MMP-II.

Materials and Methods

Preparation of cells and Ags

Peripheral blood was obtained from healthy purified protein derivative-positive individuals under informed consent using a double-blind system. In Japan, a BCG vaccination is compulsory for children (0~4 years old). PBMCs were isolated using Ficoll-Paque PLUS (Pharmacia) and cryopreserved in liquid nitrogen until used, as described previously (21). 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 mAb (Dynabeads 450; Dynal Biotech). The $CD3^-$ PBMC fraction was plated on collagen-coated plates, and the nonplastic-adherent cells were removed by extensive washing. The remaining adherent cells were used as monocytes (22). Monocyte-derived DC were differentiated, as described previously (21, 23). Briefly, monocytes were cultured in the presence of 50 ng of rGM-CSF (PeproTech) and 10 ng of rIL-4 (PeproTech) per ml (23). On day 4 of culture, immature DC were infected with rBCG at an indicated multiplicity of infection (MOI) and, on day 6 of culture, DC were used for further analyses of surface Ag and for mixed lymphocyte assays. The rMMP-II protein and BCG-derived cytosolic protein (BCC) were produced, as described previously (6, 24).

Vector construction and preparation of rBCG

For the preparation of rBCG that secretes HSP70-MMP-II fusion protein, a plasmid pMV-70M was constructed having a hygromycin resistance gene and origins of replication for *Escherichia coli* and mycobacteria. Briefly, the genomic DNA from BCG substrain Tokyo or *M. leprae* strain Thai-53 was purified by proteinase K digestion and phenol-chloroform extraction. The oligonucleotide primers for the amplication of the *hsp70* gene were FMb70Bal (5′-aaaTGGCCAtggctcgtgcggtcggg-3′; capital letters indicate a *BalI* site) and RMb70Eco (5′-aaaGAATTCcttggcctcccggccg-3′; capital letters indicate an *EcoRI* site). The primers for the Ag85B signal sequence of BCG were FMbAg85Bal (5′-tttTGGCCAtgacagacgtgagccgaaa-3′; capital letters indicate a *BalI* site) and RMbAg85 Eco120 (5′-aaaGAATTCcgcgcccgcggttgcc-3′; capital letters indicate an *EcoRI* site). The MMP-II sequence from *M. leprae* genomic DNA was amplified with FMMPEco4 (5′-aaaGAATTCcaaggtgatccggatgt-3′; capital letters indicate an *EcoRI* site) and RMMP Sal (5′-tgaGTCGACttaactcggcggccggga-3′; capital letters indicate a *SalI* site). The amplified products were digested with appropriate restriction enzymes and cloned into a *BalI-SalI*-digested parental pMV261 plasmid. For replacing the kanamycin resistance gene with a hygromycin resistance cassette, the *XbaI-NheI* fragment from pYUB854 (25) was cloned into *SpeI-NheI*-digested plasmids.

BCG substrain Tokyo was cultured in vitro using Middlebrook 7H9 broth (BD Biosciences) supplemented with 0.05% Tween 80 and 10% albumin-dextrose-catalase (BD Biosciences) or Sauton medium containing 0.05% Tween 80. Expression vectors were introduced into BCG by electroporation (26). Transformants were selected on Middlebrook 7H10 agar (BD Biosciences) plates. The BCG containing pMV-HSP70-MMP-II as an extrachromosomal plasmid is referred to as BCG-70M, and that containing pMV-261 is referred to as BCG-261H (BCG vector control). rBCGs were grown to a log phase, and stored at 10^8 CFU/ml at −80°C. Before the infection of DC, BCGs were counted by the colony assay method. There was no significant difference in growth in vitro between BCG-261H and BCG-70M.

Expression of the fusion protein HSP70-MMP-II

To verify the secretion of MMP-II and HSP70 from BCG-70M, the culture supernatant of BCG-70M, cultured for 20 days in Sauton medium, was collected, and concentrated using the Labscale TFF system (Millipore), after the supernatant was depleted of the cells by centrifugation. rMMP-II protein was used as a control for Western blotting. SDS-PAGE and electroblotting were conducted using standard methods (27). Western blotting was performed, as follows: a membrane having the transferred protein was blocked in 5% skim milk and then incubated with anti-MMP-II mAb 202-3 (IgG2a) or anti-mycobacterial HSP70 mAb (HyTest), which is not cross-reactive to mammalian HSP70 homologues. An alkaline-phosphatase-conjugated anti-mouse IgG Ab (BioSource International) was used as the secondary Ab. Color development was performed using NBT/5-bromo-4-chloro-3-indolyl phosphate detection reagent (Calbiochem).

Analysis of cell surface Ag

The expression of cell surface Ag on DC was analyzed using FACS Calibur. Dead cells were eliminated from the analysis based on staining with propidium iodide (Sigma-Aldrich), and 1×10^4 live cells were analyzed. For the analysis of the cell surface Ag, the following mAbs were used: a FITC-conjugated mAb against HLA-ABC (G46-2.6; BD Pharmingen), HLA-DR (L243; BD Biosciences), CD86 (FUN-1; BD Biosciences), CD83 (HB15a; Immunotech), and CD62L (Dreg 56; BD Biosciences), and a PE-conjugated mAb to CD8 (RPA-T8; BD Biosciences).

The expression of MMP-II on rBCG-infected DC was determined using a mAb (M270-13, IgM, κ) against MMP-II, which probably detects MMP-II in a complex with MHC molecules on the surface of DC (7), followed by a FITC-conjugated anti-mouse Igs Ab (Tago-immunologicals). For the inhibition of the intracellular processing of phagocytosed bacteria, DC were treated with 50 μM chloroquine (Sigma-Aldrich) for 2 h, washed, subsequently infected with BCG, and subjected to analyses of MMP-II surface expression. The intracellular production of perforin was assessed, as follows: unseparated naive T cells were stimulated with rBCG-infected DC for 5 days, and $CD8^+$ T cells were surface stained with a PE-labeled mAb to CD8, and fixed in 2% formaldehyde. Subsequently, the cells were permeabilized using permeabilizing solution (BD Biosciences), and stained with a FITC-conjugated mAb to perforin (δG9; BD Biosciences).

APC functions of DC

The ability of BCG-infected DC to stimulate T cells was assessed using an autologous DC-T cell coculture, as described previously (5, 23). Purification of $CD4^+$ and $CD8^+$ T cells was conducted by using negative isolation kits (Dynabeads 450; Dynal Biotech) (23). The purity of the $CD4^+$ and $CD8^+$ T cells was more than 95% when assessed using FACSCalibur. Naive $CD4^+$ and $CD8^+$ T cells were produced by further treatment of these T cells with a mAb to CD45RO, followed by beads coated with a mAb to goat anti-mouse IgGs (Dynal Biotech). The purity of both subsets of naive T cells was more than 97%. More than 98% of $CD45RA^+$ T cells expressed 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 were added to give the indicated DC:T cell ratio. Supernatants of DC-T cell cocultures were collected on day 4, and the cytokine levels were determined. In some cases, rBCG-infected DC were treated with a mAb to HLA-ABC (W6/32, mouse IgG2a, κ), HLA-DR (L243, mouse IgG2a, κ), CD86 (IT2.2, mouse IgG2b, κ; BD Biosciences), or MMP-II (M270-13), or with normal mouse IgG or IgM. The optimal concentration was determined in advance. Also, in some cases, immature DC

were treated with the indicated dose of chloroquine, brefeldin A (Sigma-Aldrich), or lactacystin (Sigma-Aldrich), and subsequently infected with BCG-70M. The optimal dose of these reagents 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-12p70, TNF-α, and IL-1β produced by DC stimulated for 24 or 48 h with rBCGs. The concentrations of these cytokines were quantified using the enzyme assay kits, Opt EIA Human ELISA Set (BD Biosciences). The murine mAb against TLR2 (clone 2392; IgG1) with antagonistic activity was obtained from Genentech. The optimal concentration of these mAbs was determined in advance.

Animal experiments

For the inoculation of mice, rBCG was cultured in Middlebrook 7H9 medium to a log phase of growth and stored at 10^8 CFU/ml at −80°C. Before the aliquots were used for inoculation, the concentration of viable bacilli was determined by plating on a Middlebrook 7H10 agar plate. Three 5-wk-old C57BL/6J mice (Japan CLEA) per group were inoculated s.c. with 0.1 ml of PBS or PBS containing 1×10^2 or 1×10^3 rBCGs. The animals were kept in specific pathogen-free conditions and supplied with sterilized food and water. Four weeks after the inoculation, the spleens were removed and the splenocytes were suspended at a concentration of 2×10^6 cells/ml in culture medium. The splenocytes were stimulated with an indicated concentration of rMMP-II, rHSP70 (HyTest), or BCC in triplicate in 96-well round-bottom microplates (14, 28). The individual culture supernatants were collected 3–4 days after the stimulation, and IFN-γ was measured using Opt EIA Mouse ELISA Set (BD Biosciences). The splenocytes obtained from C57BL/6 mice infected with rBCG were also subjected to the identification of T cell subsets responsible for IFN-γ production. The intracellular production of IFN-γ by $CD4^+$ T cells and $CD8^+$ T cells that were restimulated for 3 days in vitro with rMMP-II protein was assessed, as follows: cells were treated with Golgi Stop, and Golgi transport was inhibited for 4 h. Then the cells were surface stained with an allophycocyanin-labeled mAb to CD4 (RM4-5; BD Biosciences) and a PE-labeled mAb to CD8 (H35.17-2; BD Biosciences) in the presence of 7-aminoactinomycin D, after which the cells were washed with PBS containing 1% FCS and fixed in 1.6% formaldehyde. Subsequently, they were permeabilized using 0.1% saponin, and stained with a FITC-conjugated mAb to IFN-γ (XMG1.2; BD Biosciences) or isotype control IgG. Eight C57BL/6 mice per group were vaccinated with the indicated dose of BCG-261H or BCG-70M for 4 wk, and were challenged with 5×10^3/mouse *M. leprae* in footpad. Thirty-two weeks later, footpad was removed. The number of *M. leprae* grown in footpad was enumerated by Shepard method (29). Animal studies were reviewed and approved by the Animal Research Committee of Experimental Animals of the National Institute of Infectious Diseases, and were conducted according to their guidelines.

Statistical analysis

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

Results

Secretion of HSP70-MMP-II fusion protein from the rBCG (BCG-70M)

To verify the secretion of MMP-II protein from BCG-70M, culture filtrates of BCG transformants including BCG-261H (vector control) and BCG-70M were concentrated and examined by Western blotting analysis using mAbs to MMP-II and HSP70 (Fig. 1*A*). When probed by the MMP-II mAb, BCG-70M showed distinct band at 90-kDa equivalent to the molecular mass of the fusion protein comprising HSP70 and MMP-II, and control rMMP-II showed a 22-kDa band. Because BCG-Tokyo, a parental strain of BCG-70M and BCG-261H, has the gene encoding BCG-derived MMP-II, a faint 22-kDa band was observed in the culture filtrate of BCG-261H. In addition, when the culture filtrates were examined using the mAb to HSP70, the BCG-70M-derived filtrates expressed the 90-kDa protein, whereas the filtrates obtained from BCG-261H and rMMP-II protein did not express any obvious band. These results indicate that BCG-70M efficiently secreted the fusion protein comprising HSP70 and MMP-II. Furthermore, the HSP70-MMP-II fusion protein stimulated DC and induced a significant level of IL-12p70 production (Fig. 1*B*). To address the contribution of TLR2 expressed on DC to the IL-12p70 production, DC were pretreated with an antagonistic Ab to TLR2 and subsequently stimulated with the fusion protein. More than 80% of IL-12p70 production was inhibited by the anti-TLR2 antagonistic Ab, whereas pretreatment of DC with normal murine IgG did not affect the level of production. Although BCG-261H induced IL-12p70 production from DC, production was only partially inhibited by the antagonistic Ab to TLR2 (data not shown).

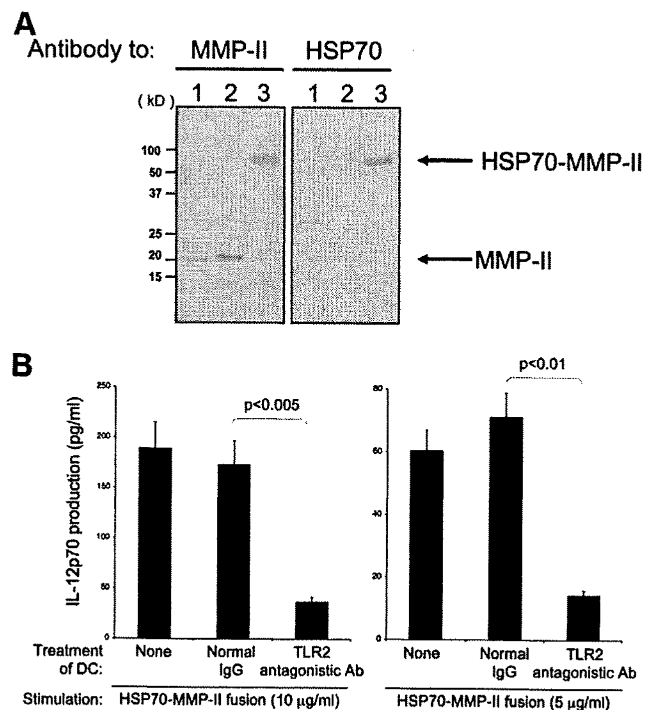

FIGURE 1. *A*, Western blotting analysis of protein secreted from BCG-70M. A mAb to either MMP-II or HSP70 was used to detect HSP70-MMP-II fusion protein. *Lane 1*, Culture filtrates of BCG-261H. *Lane 2*, rMMP-II protein. *Lane 3*, Culture filtrates of BCG-70M. *B*, Contribution of TLR2 to IL-12p70 production by DC by stimulation with HSP70-MMP-II fusion protein. PBMCs were obtained from one donor. Monocyte-derived DC were pretreated with either normal murine IgG or TLR2 antagonistic Ab (10 μg/ml) and subsequently stimulated with BCG-70M-derived HSP70-MMP-II fusion protein (10 or 5 μg/ml) for 24 h. The concentration of IL-12p70 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 ± SD. Titers were statistically compared using Student's *t* test.

Characteristics of BCG-70M

To define infectivity and survival in APCs, we examined the recovery rate of BCG-261H and BCG-70M. There was no significant difference between the two strains, and similar amounts of BCG were recovered as that of infected number (data not shown). Both HSP70 and MMP-II are known to be immunostimulators (6, 30). To see the effect of the secretion of HSP70-MMP-II fusion protein from BCG on the activation of DC, we analyzed the expression of surface Ags of BCG-infected DC (Fig. 2*A*). Both BCG-261H and BCG-70M enhanced the expression of HLA-ABC, HLA-DR, CD86, and CD83 Ags, but BCG-70M was significantly more efficient in up-regulating the expression of these molecules than BCG-261H. Furthermore, when various MOIs of BCG were used, a similar difference between BCG-261H and BCG-70M was observed (data not shown). Thus, BCG-70M phenotypically activated DC. Furthermroe, BCG-70M-infected DC significantly,

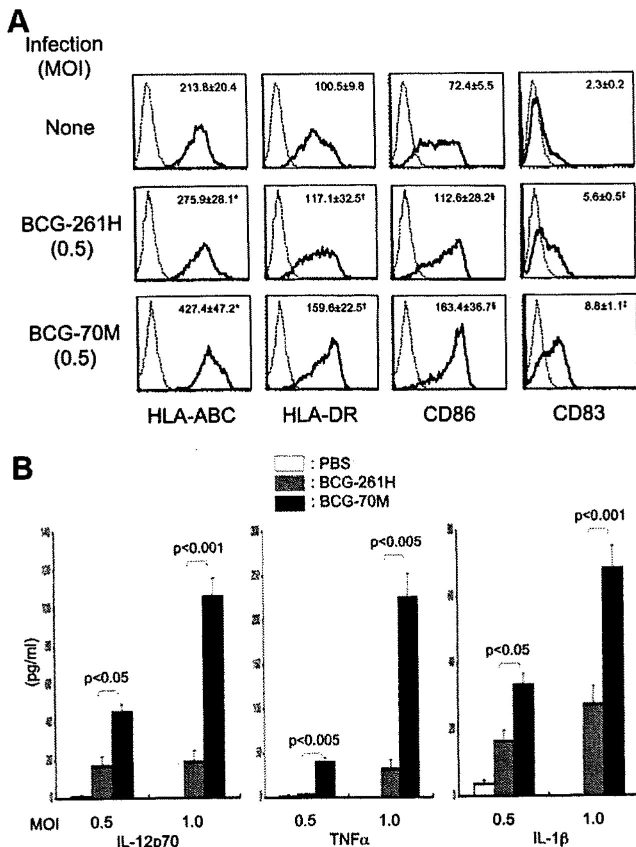

FIGURE 2. *A*, Up-regulated expression of APC-associated molecules on DC by infection with BCG-70M. PBMCs were obtained from one donor. Monocyte-derived immature DC were infected with either BCG-261H or BCG-70M at a MOI of 0.5 and cultured for another 2 days in the presence of rGM-CSF and rIL-4. The DC from day 5 were gated and analyzed. Dotted lines, isotype-matched control IgG; solid lines, the indicated test mAb. Representative results of three separate experiments are shown. The number in the *top right-hand corner* of each panel represents the mean ± SD for three independent experiments of the difference in mean fluorescence intensity between the control IgG and the test mAb. Titers were statistically compared using Student's *t* test. *, $p < 0.01$; †, $p < 0.05$; §, $p < 0.01$; ‡, $p < 0.05$. *B*, Cytokine production from DC stimulated with BCG. PBMCs were obtained from one donor. Monocyte-derived DC from 4 days of culture in the presence of rGM-CSF and rIL-4 were stimulated with the indicated dose of either BCG-261H or BCG-70M for 24 h. The concentration of the indicated cytokine 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 ± SD. Titers were statistically compared using Student's *t* test.

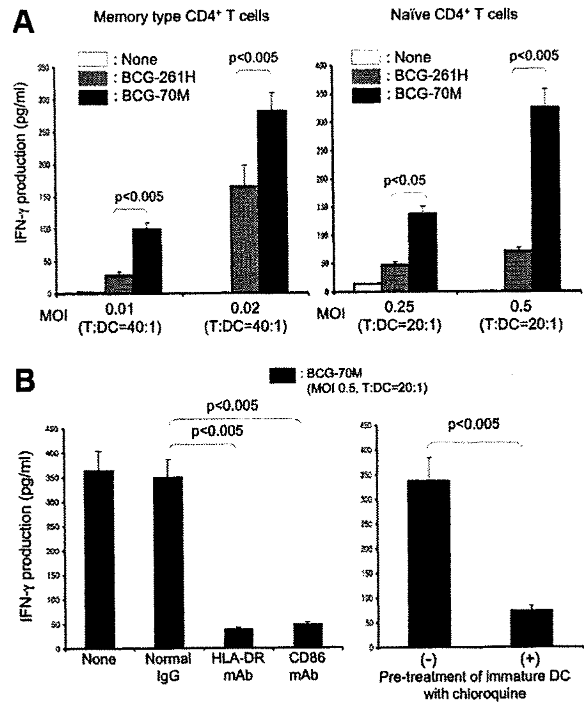

FIGURE 3. *A*, IFN-γ production from CD4+ T cells by stimulation with BCG. PBMCs were obtained from one donor. Monocyte-derived DC were infected with either BCG-261H or BCG-70M at the indicated MOI, and used as a stimulator of memory-type or naive CD4+ T cells in a 4-day culture. Responder CD4+ T cells (1×10^5) were stimulated with the indicated dose of BCG-infected DC. A representative of three separate experiments is shown. Assays were performed in triplicate, and the results are expressed as the mean ± SD. Titers were statistically compared using Student's *t* test. *B*, Inhibition of naive CD4+ T cell activation by the treatment of BCG-70M-infected DC with mAb or the treatment of immature DC with chloroquine. PBMCs were obtained from one donor. Monocyte-derived DC were infected with BCG-70M at a MOI of 0.5, and subsequently treated with 10 μg/ml mAb to HLA-DR, CD86 Ags, or normal murine IgG. Immature DC were treated with 50 μM chloroquine for 2 h and subsequently infected with BCG-70M at a MOI of 0.5. These DC were used as the stimulator of naive CD4+ T cells (1×10^5/well) at T:DC = 20:1. IFN-γ produced from T cells was measured. A representative of three separate experiments is shown. Assays were performed in triplicate, and the results are expressed as the mean ± SD. Titers were statistically compared using Student's *t* test.

though faintly, expressed MMP-II on their surface, and the MMP-II expression was inhibited by the pretreatment of DC with chloroquine, an inhibitor of phagosomal acidification, before BCG-70M infection (data not shown). Moreover, when we examined the influence of BCG-70M infection in DC in terms of the production of proinflammatory cytokines, BCG-70M was superior to BCG-261H in the production of IL-12p70, TNF-α, and IL-1β (Fig. 2*B*). These results indicate again that BCG-70M more efficiently activated DC than did the parental BCG.

T cell activation by BCG-70M

Enhanced activation of DC may be closely associated with the efficient activation of both CD4+ and CD8+ T cell subsets. Because BCG-infected mitomycin C-treated DC were confirmed not to produce IFN-γ (data not shown), the T cell-activating ability of BCG-70M was assessed by using DC as APCs. Memory-type and naive CD4+ T cells were purified from healthy BCG-vaccinated individuals, and were stimulated with DC infected with rBCG (Fig. 3*A*). As expected, memory T cells responded more vigorously to DC infected with smaller dose of BCG than naive CD4+ T cells. Although both BCG-261H and BCG-70M stimulated memory and naive CD4+ T cells, BCG-70M induced a significantly higher level of IFN-γ production in both types of CD4+ T cells than BCG-261H. Note that high levels of IFN-γ could be produced from naive CD4+ T cells by BCG-70M. In addition, various MOIs and T:DC ratios were assessed, and a similar difference between BCG-261H and BCG-70M was observed (data not shown). To address the mechanism leading to the activation of naive CD4+ T cells, BCG-70M-infected DC were treated with mAbs to HLA-DR and CD86 molecules and subsequently used as a stimulator of the T cells. The IFN-γ production from naive CD4+ T cells was significantly inhibited by the surface treatment of BCG-70M-infected DC with the mAb to HLA-DR or CD86 (Fig. 3*B*), and similarly, IL-2 production from naive CD4+ T cells

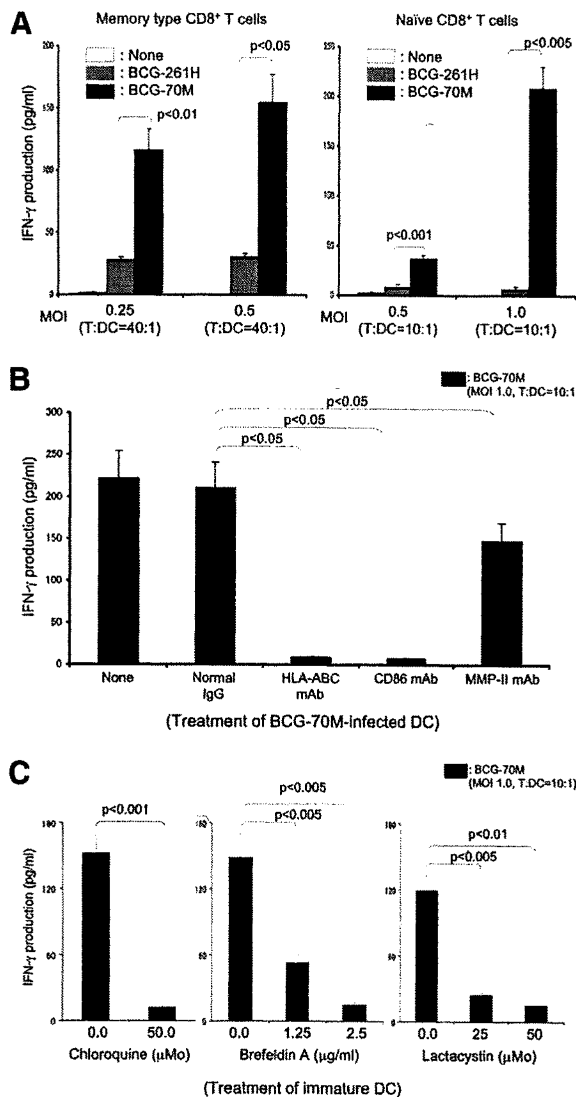

FIGURE 4. *A*, IFN-γ production from $CD8^+$ T cells by stimulation with BCG. PBMCs were obtained from one donor. Monocyte-derived DC were infected with either BCG-261H or BCG-70M at the indicated MOI, and used as a stimulator of memory-type or naive $CD8^+$ T cells in a 4-day culture. Responder $CD8^+$ T cells (1×10^5) were stimulated with the indicated dose of BCG-infected DC. A representative of three separate experiments is shown. Assays were performed in triplicate, and the results are expressed as the mean ± SD. Titers were statistically compared using Student's *t* test. *B*, Inhibition of naive $CD8^+$ T cell activation by the treatment of BCG-70M-infected DC with mAb. PBMCs were obtained from one donor. Monocyte-derived DC were infected with BCG-70M at a MOI of 1.0, and subsequently treated with 10 μg/ml mAb to HLA-ABC, CD86, or MMP-II (M270-13). The DC were used as the stimulator of naive $CD8^+$ T cells (1×10^5/well) at T:DC = 10:1. IFN-γ produced from the 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 the mean ± SD. Titers were statistically compared using Student's *t* test. *C*, Effect of treatment of immature DC with reagents on the activation of naive $CD8^+$ T cells. PBMCs were obtained from one donor. Monocyte-derived immature DC were treated with the indicated dose of either chloroquine, brefeldin A, or lactacystin, and subsequently infected with BCG-70M at a MOI of 1.0. These DC were used as the stimulator of naive $CD8^+$ T cells (1×10^5/well) at T:DC = 10:1. IFN-γ produced from the T cells was measured. A representative of three separate experiments is shown. Assays were performed in triplicate, and the results are expressed as the mean ± SD. Titers were statistically compared using Student's *t* test.

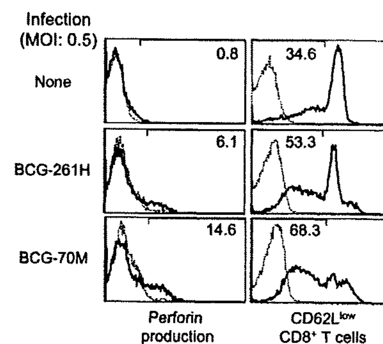

FIGURE 5. Influence of naive $CD4^+$ T cells on the activation of naive $CD8^+$ T cells. PBMCs were obtained from one donor. Monocyte-derived DC were infected with BCG (MOI 0.5) and cocultured with unseparated naive T cells (T:DC = 10:1) for 7 days. The stimulated $CD8^+$ T cells were gated and analyzed for perforin production and for expression of CD62L Ag. Numbers indicate either percentage of perforin-positive $CD8^+$ T cells or $CD62L^{low}$ $CD8^+$ T cells among $CD8^+$ T cell population. A representative of three separate experiments is shown.

was inhibited (data not shown). Furthermore, pretreatment of immature DC with chloroquine before infection with BCG-70M significantly inhibited the IFN-γ production from naive $CD4^+$ T cells (Fig. 3*B*). These results indicated that the secreted HSP70-MMP-II protein or BCG-70M itself may be processed in the DC, and some of the antigenic peptides were used for the stimulation of autologous Ag-specific naive $CD4^+$ T cells. Similarly, BCG-70M-infected DC stimulated memory $CD8^+$ T cells more efficiently than BCG-261H-infected DC, although a higher dose of BCG-70M was necessary to induce a similar level of IFN-γ production from $CD8^+$ T cells than the dose of BCG-70M required to produce the cytokine from memory $CD4^+$ T cells. As reported, BCG-261H did not activate naive $CD8^+$ T cells efficiently (15); however, BCG-70M-infected DC induced a significant level of IFN-γ production from naive $CD8^+$ T cells (Fig. 4*A*). Using a higher dose of BCG-70M (MOI 1.0) and a larger number of BCG-70M-infected DC (T:DC = 10:1), a high concentration (~200 pg/ml) of IFN-γ could be produced from naive $CD8^+$ T cells. In addition, various MOIs and T:DC ratios were assessed, and a similar difference between BCG-261H and BCG-70M was observed (data not shown). To clarify the mechanism leading to the activation of naive $CD8^+$ T cells by BCG-70M, BCG-70M-infected DC were treated with mAbs. Again, the activation of naive $CD8^+$ T cells by BCG-70M-infected DC was significantly inhibited by the treatment of the DC with the mAb to HLA-ABC or CD86. However, surface treatment of the DC with the mAb to MMP-II significantly, but only partially, inhibited the T cell activation (Fig. 4*B*). These results may indicate that BCG-70M-infected DC cross-primed naive $CD8^+$ T cells in an Ag-specific manner. To elucidate the mechanisms leading to the cross-presentation by BCG-70M-infected DC, we treated immature DC with various reagents in advance of the BCG-70M infection (Fig. 4*C*). On the pretreatment of DC with chloroquine, IFN-γ production from naive $CD8^+$ T cells was significantly inhibited, indicating that protein derived from BCG-70M was degraded in presumably the phagolysosome. Furthermore, on the pretreatment of DC with brefeldin A, an inhibitor of antegrade Golgi transportation, and lactacystin, an inhibitor of proteosomal protein degradation, IFN-γ production from naive $CD8^+$ T cells was inhibited significantly in a manner dependent on the concentration of the reagents. Because BCG-70M activated both naive $CD4^+$ T cells and naive $CD8^+$ T cells, we stimulated naive $CD8^+$ T cells with BCG-70M in the presence of the $CD4^+$ T cells (Fig. 5). The expression level of CD62L on some $CD8^+$ T cells was

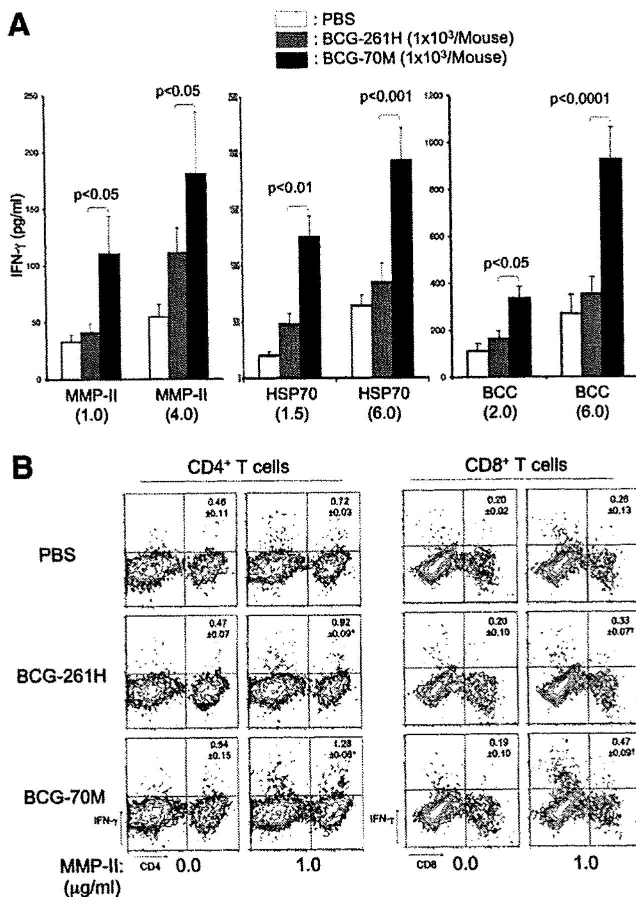

FIGURE 6. *A*, Production of memory-type T cells in C57BL/6 mice by infection with BCG. Five-week-old C57BL/6 mice were infected with 1×10^3 CFU/mouse of either BCG-261H or BCG-70M s.c. Four weeks after the inoculation, splenocytes (2×10^5 cells/well) were stimulated with the indicated dose of either MMP-II-, HSP70-, or BCG-derived cytosolic protein for 4 days, and IFN-γ produced in the cell supernatant was measured. Assays were performed in triplicate for each mouse, and the results of three mice per group are shown as the mean ± SD. Representative results of four separate experiments are shown. Titers were statistically compared using Student's *t* test. *B*, Intracellular IFN-γ production from CD4$^+$ T cells and CD8$^+$ T cells in C57BL/6 mice s.c. inoculated with BCG by secondary stimulation. Groups of 5-wk-old C57BL/6 mice were infected with 1×10^3/mouse BCG-261H or BCG-70M intradermally. Four weeks after the inoculation, splenocytes (2×10^5 cells/well) were stimulated with 1.0 µg/ml rMMP-II for 3 days. The CD4$^+$ T cells and CD8$^+$ T cells were gated separately and analyzed for intracellular production of IFN-γ. The number in the *top right-hand corner* of each panel represents the mean ± SD for three mice in the percentage of IFN-γ-producing cells among the gated T cell population. Representative results of four separate experiments are shown. Titers were statistically compared using Student's *t* test. *, $p < 0.05$; †, $p < 0.01$.

significantly reduced by stimulation with BCG-70M-infected DC, and a significant amount of intracellular perforin was produced in naive CD8$^+$ T cells by the stimulation. These changes were more efficiently induced by BCG-70M-infected DC than by BCG-261H-infected DC (Fig. 5). The CD62L^{low}CD8$^+$ T cells and perforin-producing CD8$^+$ T cells were not produced when naive CD8$^+$ T cells were stimulated in the absence of naive CD4$^+$ T cells (data not shown).

Memory T cell production by BCG-70M in vivo

Another important aspect to be studied is the production of Ag-specific memory T cells in vivo. C57BL/6 mice were infected with

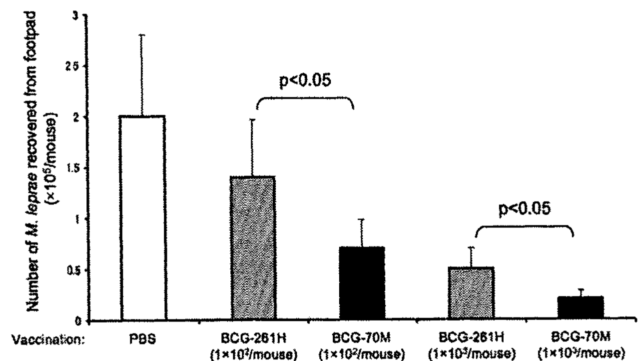

FIGURE 7. Inhibition of *M. leprae* multiplication by s.c. vaccination with BCG-70M. Five-week-old C57BL/6 mice (8 mice per group) were vaccinated with 1×10^2 or 1×10^3 CFU/mouse either BCG-261H or BCG-70M s.c., and were challenged with 5×10^3 bacilli/mouse *M. leprae* in footpad 4 wk after the vaccination. The number of *M. leprae* recovered from the footpad at 32 wk after the challenge was enumerated by Shepard's methods (29). Representative results of two separate experiments are shown. Titers were statistically compared using Student's *t* test.

1000 CFU/mouse rBCG for 4 wk, and their splenocytes were secondarily stimulated in vitro with rMMP-II protein, or recall Ags, like BCC (Fig. 6*A*). When a lower dose of MMP-II was used as a stimulator, only T cells obtained from BCG-70M-infected mice responded to the stimulator. Because BCG-Tokyo, the parental strain of the rBCGs, has the gene encoding MMP-II, a higher dose of *M. leprae*-derived MMP-II induced IFN-γ production from both T cells obtained from BCG-261H- and BCG-70M-inoculated mice; however, T cells from BCG-70M-infected mice more efficiently produced the cytokine than those from BCG-261H-infected mice. Also, T cells from BCG-70M-inoculated mice produced significantly higher level of IFN-γ than T cells from mice inoculated with BCG-261H on stimulation with HSP70 in vitro. The efficient generation of memory T cells responding to mycobacteria-derived Ags in mice infected with BCG-70M was confirmed, because only T cells from mice infected with BCG-70M significantly responded to BCC (Fig. 6*A*). To clarify the T cell subsets responsible for the IFN-γ production on secondary MMP-II stimulation, T cells producing the cytokine intracellulary were analyzed (Fig. 6*B*). Both CD4$^+$ T cells and CD8$^+$ T cells derived from not only non-BCG-inoculated mice, but also BCG-infected mice, produced intracellular IFN-γ by MMP-II stimulation. Both noninoculated and PBS-inoculated mice showed the similar responses (data not shown). However, significantly larger populations of both CD4$^+$ T cells (~1.3%) and CD8$^+$ T cells (~0.5%) obtained from BCG-70M-infected mice produced the cytokine. There were no CD4$^+$ T cells or CD8$^+$ T cells that were positively stained with the isotype control IgG (data not shown).

Effect of BCG-70M vaccination on the multiplication of M. leprae *in vivo*

C57BL/6 mice vaccinated with either BCG-261H or BCG-70M (1×10^2 or 1×10^3 CFU/mouse) for 4 wk were challenged with 5×10^3 of *M. leprae* in footpad. Thirty-two weeks later, footpad was removed and *M. leprae* recovered from the footpad was enumerated (Fig. 7). A total of 2×10^5 *M. leprae* was recovered from mice inoculated with PBS and challenged with *M. leprae*. Both mice inoculated with BCG-261H or BCG-70M inhibited the multiplication of *M. leprae* in the manner dependent on the dose of rBCG vaccinated; however, BCG-70M vaccination was significantly more efficient than BCG-261H vaccination in inhibiting the

multiplication, and only 2×10^4 *M. leprae* was recovered from mice vaccinated with 1×10^3 CFU/mouse BCG-70M.

Discussion

M. leprae is a representative mycobacterium among slow-growing prokaryotes, which needs 12–14 days for one division and 10–70 years for the manifestation of the disease, depending on the dose of bacilli entering the hosts. Host defense against *M. leprae* is chiefly conducted by adaptive immunity involving both $CD4^+$ and $CD8^+$ T cell subsets (3–5). Although $CD4^+$ T cells usually act at the initial phase of infection, $CD8^+$ T cells inhibit the multiplication of *M. leprae* in the chronic phase or when it is reactivated from a dormant state (31). Therefore, the vaccine should have an ability to competently activate not only $CD4^+$ T cells, but also $CD8^+$ T cells to produce memory T cells. To date, BCG is used as sole, but safe, vaccine against leprosy; however, nowadays, its efficacy is considered not so convincing (13). We have made several attempts to improve the potency of BCG, especially its immunostimulatory activities. We chiefly focused on achieving the fusion of BCG-infected phagosomes with lysosomes, without which the full and polyclonal activation of Ag-specific T cells cannot be expected. One approach we tried was the production of an *ure C*-deficient rBCG that successfully produces acidic phagosomes and facilitates their fusion with lysosomes (15, 28, 32, 33). Actually, the rBCG efficiently colocalized with lysosomes and effectively stimulated $CD4^+$ T cells when DC were targeted (28). However, it did not activate naive $CD8^+$ T cells. Then, we produced a second rBCG that secretes MMP-II (BCG-SM) in the phagosome (14). BCG-SM was useful in the activation of not only naive $CD4^+$ T cells, but also naive $CD8^+$ T cells to some extent (14). The T cell activation presumably occurs as a consequence of the translocation of the antigenic determinants of the secreted MMP-II to the cell surface, although the precise mechanism has not been clarified. Therefore, the intracellular secretion of immunodominant Ag by BCG is thought necessary to enhance the T cell-stimulating activity of BCG. However, BCG-SM vaccinations only partially inhibited the multiplication of *M. leprae* in the footpads of mice (our unpublished observation). These observations indicate the need for another rBCG capable of activating both naive $CD4^+$ and $CD8^+$ T cells more strongly.

Because the strong activation of naive $CD8^+$ T cells by mycobacteria required the cross-presentation of mycobacteria-derived Ags to $CD8^+$ T cells, in this study, we used BCG-derived HSP70 as a mediator facilitating the cross-presentation by DC, because HSPs of both mammalian host cells and bacterial origin are reported to have chaperone activity (34) and can effectively prime a cytolytic response (35). The usefulness of HSP65 as a convincing stimulator of $CD8^+$ T cells was revealed in animal studies to develop vaccine against *M. tuberculosis* (36). A DNA vaccine containing the *hsp65* gene activated naive $CD8^+$ T cells, and consequently inhibited the development of tuberculosis, although precisely how was not clarified. Our newly constructed rBCG (BCG-70M) secreted a fusion protein comprising HSP70 and MMP-II in vitro in the absence of any exogenous secretion signal. The secretion of HSP70-MMP-II fusion was confirmed by Western blotting analyses (Fig. 1*A*) and by the surface expression of MMP-II on DC (data not shown). The exact mechanism leading to the secretion of the fusion protein from BCG-70M is not known, but the secretion could be due to the inherent characteristics of HSP70 to be secreted (16, 37). Although we tried to enhance the secreting activity of BCG-70M by additionally inserting *M. tuberculosis*-derived secretion signal to BCG-70M, the secretion efficacy was rather inhibited and the construct stimulated naive T cells less efficiently than BCG-70M (data not shown).

BCG-70M secreted a 90-kDa fusion protein composed of HSP70 and MMP-II (14). The activation of naive $CD8^+$ T cells by BCG-70M was only partially inhibited by the treatment of DC with the mAb to MMP-II. Therefore, it can be speculated that BCG-70M activated $CD8^+$ T cells polyclonally by using various epitopes, originating from MMP-II, HSP70, or other Ags of BCG, because T cells from BCG-70M-infected mice vigorously responded to MMP-II, HSP70, and BCG-derived cytosolic protein (Fig. 6*A*). Therefore, HSP70 may alter the clonality of responding $CD8^+$ T cells, and the production of such polyclonal Ag-specific $CD8^+$ T cells might be beneficial for the broad coverage of a heterogenous MHC population.

BCG-70M induced higher level of cytokine production, including IL-12p70, TNF-α, and IL-1β, than the vector control BCG. The enhanced cytokine production by BCG-70M could be due to the intracellular secretion of HSP70 as a part of the fusion protein by the BCG. MMP-II can ligate TLR2 (6), but HSP70 is also known to bind TLRs (38); thus, the secreted HSP70-MMP-II protein seems to activate DC strongly. The contribution of TLR2 to cytokine production was confirmed by the inhibition of IL-12p70 production by antagonistic Ab to TLR2. The cytokines released from DC by BCG-70M stimulation could facilitate skewing of the direction of T cell activation to type 1 and induce the efficient and strong production of IFN-γ from naive $CD8^+$ T cells.

HSPs play a varied role in enhancing the ability of APCs to stimulate T cells (39). For the activation of Ag-specific $CD4^+$ and $CD8^+$ T cells, peptides should be loaded onto the corresponding MHC pathways. For the loading of BCG-derived Ags on these pathways, the proteins secreted from phagocytozed BCG should be transported to functional lysosomes. In the phagolysosome, some portions of HSP70-MMP-II fusion protein could be degraded, but the rest would be sequestrated into the cytosol, where they are degraded and used for cross-priming $CD8^+$ T cells. BCG-70M-infected DC expressed derivatives of MMP-II and the other proteins on their surface, and they activated both naive $CD4^+$ T cells and naive $CD8^+$ T cells. However, both MMP-II expression on DC and the activation of the T cells by DC were inhibited by the pretreatment of DC with chloroquine (24, 40). These results indicate that secreted HSP70-MMP-II fusion protein was efficiently processed in lysosomes and its derivatives are used for the activation of both subsets of naive T cells. When naive $CD8^+$ T cells were stimulated by BCG-70M in the presence of naive $CD4^+$ T cells, $CD62L^{low}CD8^+$ T cells and perforin-producing $CD8^+$ T cells were efficiently produced. The activation of naive T cells was confirmed by the production of memory-type T cells by BCG-70M infection to unprimed mice, because both $CD4^+$ and $CD8^+$ T cell subsets from BCG-70M-infected mice responded to the restimulation with MMP-II in vitro. Furthermore, BCG-70M significantly and more efficiently inhibited the multiplication of *M. leprae*, which were challenged in footpad of mice, than BCG-261H.

There are two pathways of cross-presentation, as follows: cytosolic (ER-Golgi-dependent) and vacuolar pathways (20). It is known that HSP can enhance both pathways (20). In the present study, IFN-γ production from naive $CD8^+$ T cells was largely blocked by the treatment of DC with brefeldin A, an inhibitior of antegrade Golgi transportation and of TAP-dependent transportation, and also with lactacystin, a proteosomal protein degradation blocker (20, 40). Therefore, it can be presumed that the fusion protein was sequestered into the cytosol from the lysosome, degraded in the proteosome, and used for loading on MHC class I molecules through the TAP-dependent pathway. Furthermore, it has been reported that proteins that are intracellularly secreted are usually processed by a cytosolic (ER-Golgi-dependent) pathway, and DC prefer this pathway for cross-priming $CD8^+$ T cells with

protein Ag (20). Our present observations seem to fit well with these previous findings. Therefore, we concluded that BCG-70M activates naive CD8$^+$ T cells through the ER-Golgi-dependent cytosolic cross-presentation pathway. However, *M. tuberculosis*-derived HSP70 activated T cells through a post-Golgi, proteosomal-independent mechanism, and both brefeldin A and lactacystin may inhibit vacuolar pathway in some cases (20). Also, the vacuolar pathway is used more frequently by macrophages (20). Therefore, the possibility that BCG-70M may also use the post-Golgi pathway in vivo cannot be ruled out.

Taken together, in this study, we constructed a rBCG that secretes HSP70-MMP-II fusion protein, which effectively activates not only DC, but also naive T cells. Therefore, the combination of HSP70 and MMP-II may be useful for stimulating both subsets of naive T cells.

Acknowledgments

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Disclosures

The authors have no financial conflict of interest.

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Temperature dependency for survival of *Mycobacterium leprae* in macrophages

Yasuo FUKUTOMI *, Yumi MAEDA, Masanori MATSUOKA, and Masahiko MAKINO
Department of Microbiology, Leprosy Research Center, National Institute of Infectious Diseases, Tokyo, Japan

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Hansen's disease is caused by an infection with an intracellular pathogen, *Mycobacterium leprae*, which mainly inhabits macrophages and Schwann cells. However, little is known about the survival or growth mechanisms of the bacilli in mouse and human macrophages. In the present study, by using radiorespirometry analysis for the evaluation of the viability of *M.leprae*, we observed that *in vitro* incubation of *M.leprae*-infected macrophages at 35°C was more growth permissive than at 37°C, and supplementation with the immunosuppressive cytokine IL-10 supported the survival of the bacilli in the macrophages for 3 weeks, whereas viability of the bacilli was gradually lost if cultured without IL-10. In human macrophages, *M.leprae* retained its viability when cultured at 35°C for at least 4 weeks without IL-10. However, the viability of *M.leprae* was almost lost within 2 weeks if cultured at 37°C. These data suggest that temperature is a crucial factor for the survival of *M.leprae* in host cells.

Introduction

Hansen's disease is caused by an infection with *Mycobacterium leprae*. *M.leprae* is an intracellular pathogen, mainly residing in macrophages and Schwann cells. In patients, *M.leprae* is predominantly observed in the skin, nasal mucosa and peripheral nerves, particularly the more superficial ones. This clinical observation suggests that the optimal temperature of *M.leprae* for survival in human cells is less than 37°C [1]. In animal models, *M.leprae* multiplies in the mouse footpad where the temperature is lower than the core temperature, and the optimal temperature for the growth of *M.leprae* is reported to be in the range of several degrees above and below 30°C [2].

From another aspect, the growth of *M.leprae* seems to be largely affected by the host immune

*Corresponding author :
Department of Microbiology, Leprosy Research Center,
National Institute of Infectious Diseases
4-2-1, Aoba-cho, Higashimurayama-shi, Tokyo 189-0002, Japan
TEL : 81-42-391-8211 FAX: 81-42-394-9092
E-mail : fukutomi@nih.go.jp

response. Hansen's disease is characterized by a broad spectrum of the host immune response, such as lepromatous type (towards the increased load of bacteria) and tuberculoid type (towards the decreased bacterial load). In lepromatous type leprosy, Th-2 cytokines (IL-4, IL-5 and IL-10) are predominantly expressed in local lesions. In contrast, in tuberculoid type, Th-1 cytokines (IFN-γ, IL-2) are predominantly expressed [3]. Among cytokines, IFN-γ has been demonstrated to play a central role in activating macrophages to kill intracellular pathogens including *M.leprae*, whereas IL-10 is reported to inhibit the microbicidal activity of macrophages, resulting in the survival of the intracellular pathogen [4]. However, little is known about the survival and growth mechanisms of *M.leprae* in human macrophages since the viability of these uncultivable bacilli cannot be easily measured by in vitro study.

Previously we reported that metabolically active *M.leprae* were maintained in monolayer cultures of mouse peritoneal macrophages and supplemental IL-10 bolstered *M.leprae* metabolism in the macrophages for as long as 8 weeks. In the cell culture system temperature is extremely important and 31-33°C incubation temperature is more growth permissive than 37°C [5]. In the present study, we observed that incubation of mouse macrophages at 35°C was also more permissive than at 37°C and supplemental IL-10, but not TGF-β, supported the metabolic activity of *M.leprae* in the macrophages for several weeks. Moreover, *M.leprae* from infected human macrophages cultured in vitro sustained metabolic activity for at least 4 weeks if cultured at 35°C but not at 37°C. Collectively, these data demonstrate that temperature is one of the crucial factors for *M.leprae* survival in human host cells.

Materials and Methods

M.leprae inoculum: The Thai-53 strain of *M.leprae* [6] was maintained in continuous passage in athymic *nu/nu* mice (Clea Co, Tokyo, Japan) by inoculation of bacilli into both hind foot pads. Experiments with mice were performed in compliance with the guidelines of the Experimental Animal Committee of the National Institute of Infectious Diseases. At approximately one year post inoculation, the foot pads were processed to recover *M.leprae* by Nakamura's method with a slight modification [7]. Briefly, tissue was minced and homogenized with Hanks' balanced salt solution (HBSS) containing 0.05% Tween 80. The homogenate was centrifuged at 150×g for 10 min and supernatant of the sample homogenate was treated with 0.05% trypsin at 37°C for 60min. The suspension was centrifuged at 4,000×g for 20min and sediment was re-suspended in HBSS followed by treatment with 1% sodium hydroxide at 37°C for 15min. The treated material was washed and re-suspended in HBSS at the desired bacillary concentration. Bacillary number in each foot pad was enumerated individually according to standard techniques [8].

Cytokines: Murine recombinant IL-10 was obtained from Genzyme Corp. TGF-β was obtained from Kurashiki Bouseki (Kurashiki, Japan). Both cytokines were stored at -80°C until use.

Mouse macrophage culture: Mouse peritoneal resident cells (approximately 50% macrophages) were harvested from retired ICR mice and suspended as previously described [9] at a concentration of 2×10^6/ml in RPMI 1640 (Gibco BRL, Invitrogen Corp., Carlsbad, CA) supplemented with 15% fetal bovine serum (FBS, HyClone Laboratories, Logan UT), 25 mM N-2-

hydroxyethylpiperazine -N'- 2-ethanesulfonic acid (HEPES), 2 mM glutamine and 100μg/ml ampicillin (Sigma Chemical Co., St. Louis, MO). One half ml was seeded into 24 well tissue culture plates (Corning) containing 13 mm LUX coverslips (Nunc Thermanox coverslips, NalgeNunc, Thermo Scientific, Rochester, NY). After 20 hr adherence of the cells, macrophage monolayers were obtained after washing non-adherant cells from the coverslip with Hanks Balanced Salt Solution (HBSS, Sigma) leaving approximately 1×10^6 macrophages adhered per coverslip.

Human macrophage culture: Human peripheral blood was obtained under informed consent from healthy individuals. Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Paque Plus (GE Healthcare Life Sciences, Buckinghamshire, HP7 9NA, UK) gradient centrifugation[10]. The cells were suspended in AIM-V medium (Gibco BRL, Invitrogen Corp., Carlsbad, CA) and 1×10^6 PBMC were cultured in a well of a 24-well tissue culture plate (Falcon, Becton Dickinson Labware, Becton Dickinson and Company, Franklin Lakes) containing 13 mm LUX coverslips at 37°C in a 5%-CO_2 incubator for adherence of monocytes. After 1 hr incubation, the coverslips were washed with HBSS to remove non-adherent cells. The monocytes on the coverslips were cultured in a new 24-well plate containing RPMI1640 medium (Sigma) supplemented with 20% FBS (Whittaker Co., Walkersville, MD), 25mM HEPES, 2mM L-glutamine and 100μg/ml ampicillin in the presence of 10 ng/ml of human M-CSF (R&D Systems, Minneapolis, MN) or 40 ng/ml of GM-CSF (R&D Systems). After 7 days, the M-CSF-conditioned macrophages (M-macrophages) and the GM-CSF-conditioned macrophages (GM-macrophages) were used for infection with *M.leprae*.

Infection of macrophages with *M.leprae*: Purified mouse macrophage monolayers were infected with fresh *M.leprae* suspended in 0.5 ml medium per well. After 4 hr incubation for mouse macrophages and 20 hr incubation for human macrophages, non-phagocytosed bacilli were removed by washing and the cultures were incubated in 1.0 ml media supplemented with the appropriate cytokine in 5% CO_2 at the appropriate experimental temperatures[9]. Media were changed and cytokines were replenished at 5 days interval.

Radiorespirometry: The macrophages were lysed with 0.1 N NaOH to release the phagocytosed *M.leprae*, and the viability of the bacilli was determined by evaluating the oxidation of ^{14}C-palmitic acid to $^{14}CO_2$ by radiorespirometry as described previously [11]. Total isotope release was usually analyzed after one week of incubation at 31°C [9].

Staining of *M.leprae*-infected macrophages: Coverslips of *M.leprae*-infected adherent macrophages were prefixed with absolute methanol and acid-fast stained. The specimens were observed under Nikon Optiphot light microscopy.

Results

Viability of *M.leprae* in mouse macrophages cultured *in vitro*: Mouse peritoneal resident macrophages (1×10^6 cells per well) were incubated with freshly harvested *M.leprae* (multiplicity of infection (MOI), 5:1 or 10:1) for 4 hr to allow phagocytosis. Non-phagocytosed bacilli were washed off and the culture of the macrophages continued for up to 14 days. Viability (metabolic activity) of *M.leprae* in macrophages was assessed by radiorespirometry. As shown in Fig. 1, the viability of the bacilli was gradually decreased in macrophages cultured at 35°C. In contrast, the viability was significantly lost, if the macrophages were cultured at 37°C. Next, the mouse peritoneal

resident macrophages were incubated with 3 doses of *M.leprae* (MOI, 1:1, 4:1 and 10:1) for 4 hr to allow phagocytosis, and the culture continued for longer periods up to 21 days. Viability of *M.leprae* in macrophages was assessed at 7 day intervals. As shown in Fig. 2, in each dose of *M.leprae* infection, decrease in viability was significant after 21 days.

Effects of cytokines on viability of *M.leprae* in mouse macrophages: Supplementation of IL-10 to the infected macrophage culture was

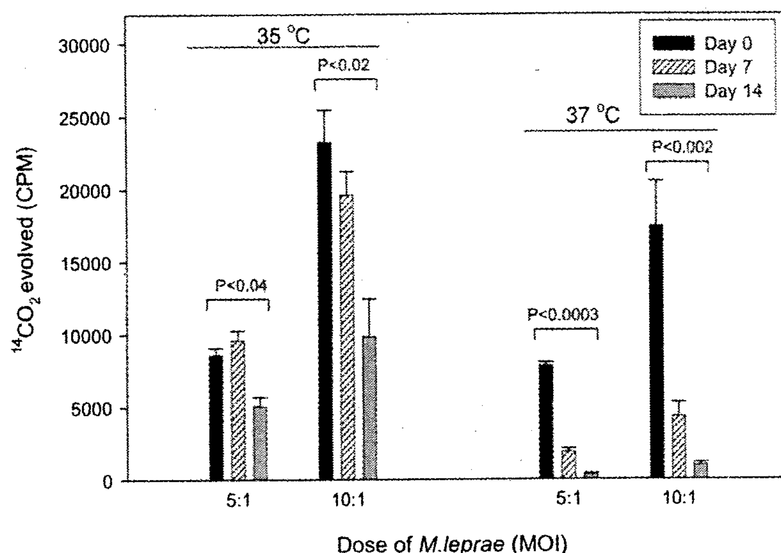

Fig.1. Viability of *M.leprae* in mouse macrophages cultured *in vitro*. Mouse peritoneal resident macrophages were incubated with 5×10^6 or 1×10^7 per well of *M.leprae* (MOI, 5:1 or 10:1), for 4 hr at 37°C to allow phagocytosis. Non-phagocytosed bacilli were washed off and the culture of the macrophages continued up to 14 days at 35°C or 37°C. The cells were lysed to obtain *M.leprae* and metabolism of the bacilli was assessed by radiorespirometry.

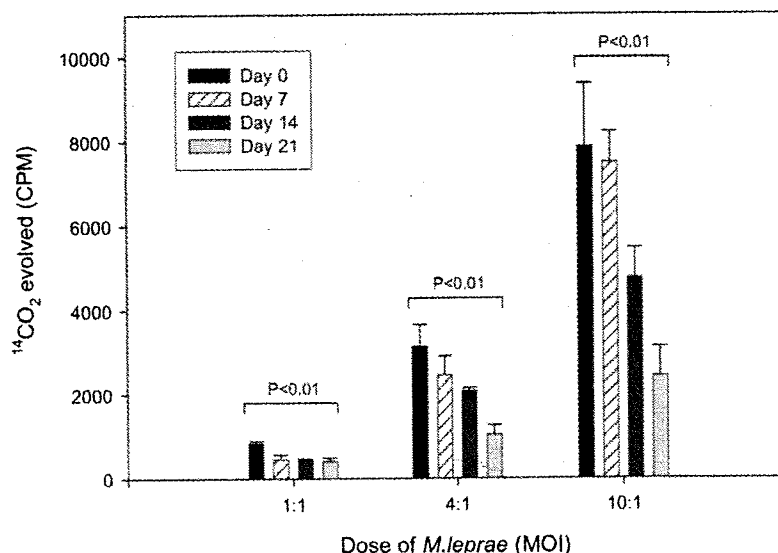

Fig.2. Viability of *M.leprae* in mouse macrophages cultured *in vitro*. Mouse peritoneal resident macrophages were incubated with 3 doses, 1×10^6, 4×10^6 and 1×10^7 per well of *M.leprae* (MOI, 1:1, 4:1 and 10:1) at 35°C for 4 hr to allow phagocytosis, and the culture continued at 35°C for longer periods up to 21 days. The cells were lysed to obtain *M.leprae* and metabolism of the bacilli was assessed by radiorespirometry.

clearly associated with sustained viability of intracellular *M.leprae* cultured at 35°C (Fig.3). In the presence of 3 U/ml of IL-10, *M.leprae* maintained their viability, whereas viability was steadily lost without IL-10. We also examined the effect of TGF- β, another suppressive cytokine for macrophage activation, on the viablility of the bacilli. To the contrary, supplementation of TGF- β

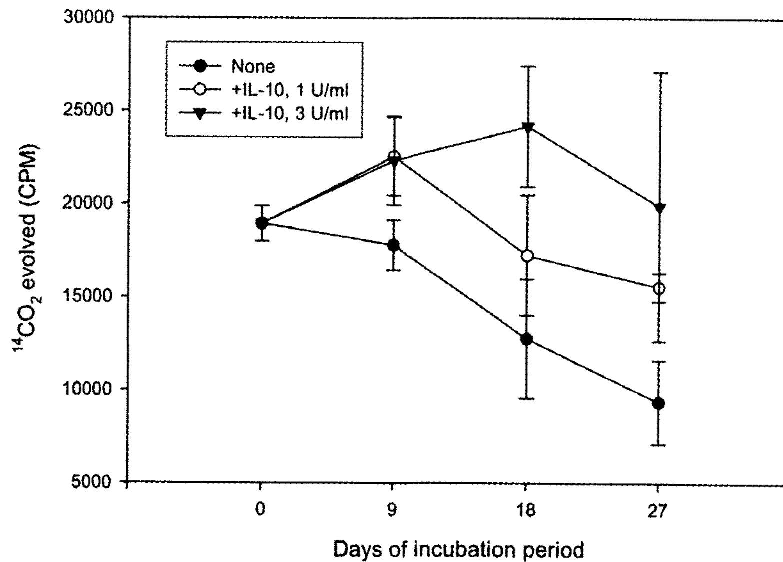

Fig.3. Effect of IL-10 on *M.leprae* survival in mouse macrophages. Mouse peritoneal resident macrophages were incubated with 1×10^7 per well of *M.leprae* (MOI, 10:1) at 35°C for 4 hr to allow phagocytosis, and the culture continued at 35°C for 9, 18 and 27 days. The cells were lysed to obtain *M.leprae* and metabolism of the bacilli was assessed by radiorespirometry.

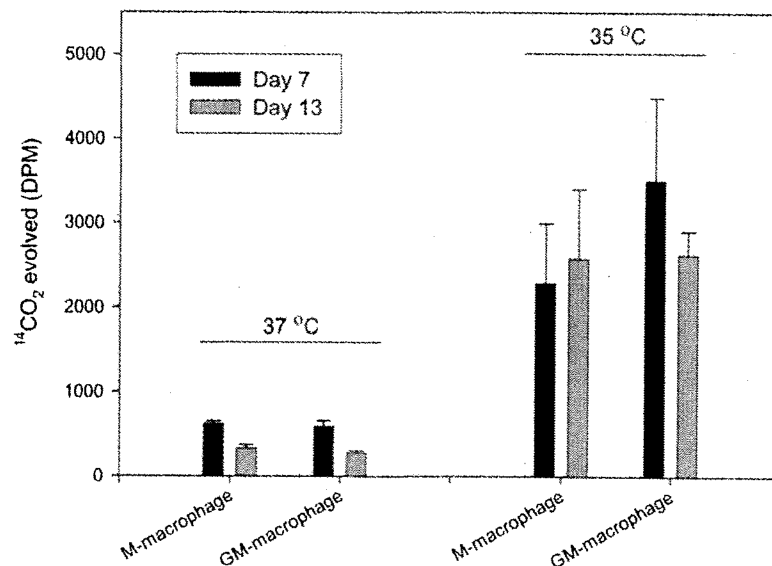

Fig.4. Viability of *M.leprae* in human macrophages cultured *in vitro*. Human M- or GM-macrophages were incubated with *M.leprae* (MOI, 50:1) for 20 hr either at 35°C or 37°C for infection and incubated at the same temperatures for indicated periods. By observation of the acid fast-stained cells under light microscopy, no difference was recognized in the number of *M.leprae* phagocytosed by macrophage cultured between at 35°C and at 37°C. So the viability at day 0 is considered equal. After 7 days and 13 days incubation period, the cells were lysed to obtain *M.leprae* and metabolism of the bacilli was assessed by radiorespirometry (dpm: disintegrations per minute).

significantly decreased the viability of *M.leprae*, when incubated for longer than 28 days post infection (Table 1).

Viability of *M.leprae* in human macrophages cultured *in vitro*: Human macrophages were obtained by culturing monocytes in the presence of either M-CSF or GM-CSF for 7 days. These macrophages (1×10^5 cells per well) were incubated with *M.leprae* (MOI, 50:1) for 20 hr either at 35°C or 37°C for infection and incubated again at the same temperatures. By observation of the acid fast-stained cells under light microscopy,

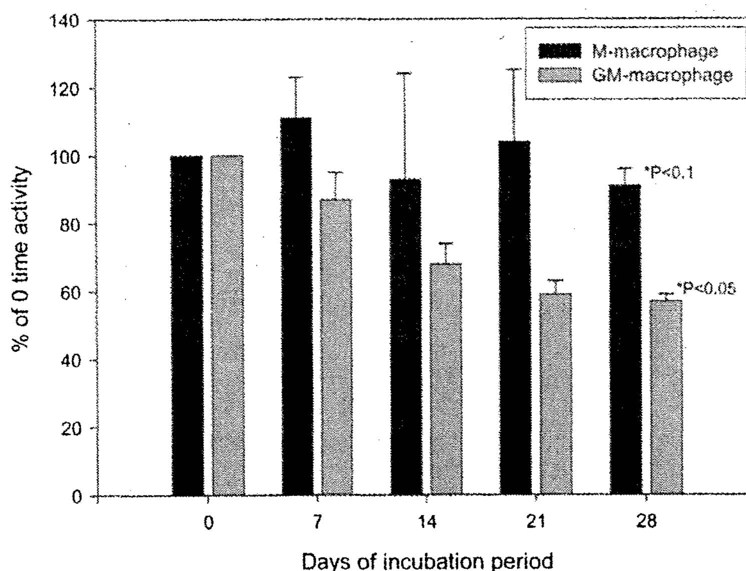

Fig.5. Viability of *M.leprae* in human macrophages cultured *in vitro*. Human M- or GM-macrophages were infected with *M.leprae* (MOI, 50:1) for 20 hr at 35°C and incubated again at 35°C for indicated periods. The cells were lysed to obtain *M.leprae* and metabolism of the bacilli was assessed by radiorespirometry. The results at day 7, 14, 21 and 28 are expressed as percentages of *M.leprae* metabolic activity at time 0. Radiorespirometry data obtained from *M.leprae* in M-macrophages at time 0 was 5,932±399 and those in GM-macrophages was 3,084±78. *P values calculated in comparison to day 0 viability.

Table 1. Effect of TGF-*β* on survival of *M.leprae* in mouse macrophages cultured at 35°C[a]

Experiment 1

Days of incubation period	At time 0	7	14	28
Medium only	5,222±936[b]	2,774±295	3,086±425	2,828±1,815
+TGF-β		2,919±535	3,119±1,339	1,973±126

Experiment 2

Days of incubation period	At time 0	14	28	49
Medium only	26,791±1,428	19,103±621	7,420±2,986	5,713±1,144
+TGF-β		14,306±2,240	3,728±410	1,594±317

[a]Mouse peritoneal resident macrophages were incubated with *M.leprae* (MOI, 1:10) for 4 hr to allow phagocytosis, and the culture continued for indicated periods. The cells were lysed to obtain *M.leprae* and metabolism of the bacilli was assessed by radiorespirometry.
[b]Radiorespirometry data, cpm.
Dose of TGF-β, 500pg/ml.
N.D., not determined.

no difference was recognized in the number of *M.leprae* phagocytosed by macrophage cultured at 35°C and at 37°C (data not shown). Viability of *M.leprae* was assessed after 7 and 13 days. The results clearly showed that the viability of *M.leprae* incubated at 35°C was maintained, whereas the viability was lost if cultured at 37°C (Fig. 4). Next, *M.leprae*-infected human M- and GM-macrophages were cultured for prolonged periods at 35°C. Viability was sustained well for 4 weeks in human macrophages, especially in M-macrophages (Fig. 5).

Discussion

In vivo M.leprae is able to enter and survive in a wide variety of tissues and cell types[12]. The preferred host cell for the leprosy bacillus appears to be the macrophages and a number of unsuccessful attempts have been made to grow *M.leprae* in macrophages *in vitro*. For example, Sharp and Banerjee[13] employed macrophages from conventional mice and rats, *nu/nu* mice or *nu/nu* rats and armadillos. The *M.leprae* inocula were derived from 3 sources (human leproma, *nu/nu* mouse footpad and frozen infected armadillo tissue). Incubation temperature was varied from 31°C to 35°C and *M.leprae*-infected cells were maintained for up to 200 days. Fieldsteel and McIntosh [14] employed a range of rat, mouse and human tissue. The conclusion of these reports is that no significant multiplication of *M.leprae* occurred in any of the cells or tissues.

Previously, we reported that metabolically active *M.leprae* could be maintained in monolayer cultures of mouse peritoneal macrophages and that supplemental IL-10 bolstered *M.leprae* metabolism in the macrophages for as long as 8 weeks. In the cell culture system, temperature is an extremely important factor for growth and 31-33°C incubation temperature is more permissive than 37°C [5]. In the present study, we further observed that incubation of mouse macrophages infected with *M.leprae* at 35°C was also more growth permissive than at 37°C. We chose 35°C as the incubation temperature, and not 31°C, because the maintenance of the integrity of the macrophage monolayer was better at 35°C than at 31-33°C. Moreover, the monolayer of *M.leprae*-infected human macrophages at 31-33°C could not be maintained for longer than one week. We observed that maintenance of the monolayer was good at 35°C, and *M.leprae* at 35°C was also more growth permissive than those at 37°C in human macrophages (Fig. 4 and 5). Our starting inoculum of *M.leprae* was freshly obtained for each experiment from infected *nu/nu* mice. We also were able to rapidly quantify the metabolic activity of *M.leprae* using the radiorespirometry technique adapted by Franzblau [11]. This assay is accurate and highly sensitive with the results available in a short duration of 1 wk (compared to 6-12 months when titrated in mouse footpads). Radiorespirometry data correlates well with other *in vitro* systems [11] but, more importantly, the data correlated well with "viability" as observed in the mouse footpad system [12].

Various clinical evidence suggests that *M.leprae* prefer a growth temperature of less than 37°C [1]. In animal models, *M.leprae* multiplies in the mouse foot pad where the temperature is lower than the body temperature [2]. In addition, *Dasypus novemcinctus*, the nine-banded armadillo has a core temperature of ~33°C, which renders it permissive as a host for the leprosy bacillus [13]. Mononuclear phagocytes in virtually every organ of the natural or experimentally infected armadillo become heavily parasitized with propagating *M. leprae* [14]. Whether intracellular or extracellular, *M.leprae* clearly prefers temperatures cooler than

normal human body temperature [12], and 37°C appeared to be highly detrimental to *M.leprae* viability. The *in vitro* results obtained in the present study confirmed the preference of lower temperature (35°C) by *M.leprae* residing in human macrophages.

In this study, supplemental IL-10, but not TGF-β supported the metabolic activity of *M.leprae* in mouse macrophages for several weeks, similar to the results obtained previously [5]. In choosing TGF-β and IL-10 as the cytokines that might bolster the intracellular survival of *M.leprae,* we were attempting to down-regulate any innate ability of the normal macrophages to cope with the organism. TGF-β is produced by activated macrophages and other inflammatory cells and has a broad array of modulatory functions on the immune response. TGF-β has been shown to interfere with macrophage antimicrobial mechanisms including the generation of reactive oxygen intermediates [15] and reactive nitrogen intermediates [16], and has been shown to enhance the intracellular growth of *M.tuberculosis* in human monocytes [17]. However, in the present studies with mouse macrophages, exogenous TGF-β had no detectable effect on sustaining intracellular *M.leprae* viability, and in fact decreased the viability (Table 1). In contrast, supplementing media with IL-10 clearly affected the long term viability of *M.leprae* in mouse macrophages (Fig. 3). IL-10 is produced by T cells, B cells and macrophages [18,19]. IL-10 has been shown to be a potent down-regulator of cell-mediated immunity to intracellular pathogens [20]. *In vivo*, endogenous IL-10 dampened the cell-mediated immune response to avirulent mycobacterial infection [4] and appeared to lead to loss of control of *M.tuberculosis* infection with widespread dissemination [21]. IL-10 functions in part at the level of the macrophage by attenuating iNOS mRNA expression, iNOS activity and, by inference, NO production [22]. In human macrophages, however, the viability of *M.leprae* was maintained for 4 weeks in the absence of IL-10 (Fig. 5), suggesting that human cells seem to be better hosts than mouse cells for *M.leprae* survival. Viability of *M.leprae* in M-macrophages seems to be maintained for a longer period (up to one month) than that in GM-macrophages (Fig. 5). One of the reasons for this may be due to the production of IL-10 by M-macrophages [23], although the mechanism by which IL-10 contributes to the maintenance and growth of *M.leprae* is unclear.

In conclusion, the present study showed that the metabolism, and presumably the viability, of *M.leprae* could be sustained under culture conditions at 35°C, which is also a moderate temperature necessary to maintain the integrity of macrophages.

Acknowledgments

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RESEARCH ARTICLE

GM-CSF-mediated T-cell activation by macrophages infected with recombinant BCG that secretes major membrane protein-II of *Mycobacterium leprae*

Masahiko Makino, Yumi Maeda, Masanori Kai, Toshiki Tamura & Tetsu Mukai

Department of Microbiology, Leprosy Research Center, National Institute of Infectious Diseases, Tokyo, Japan

Correspondence: Masahiko Makino, Department of Microbiology, Leprosy Research Center, National Institute of Infectious Diseases, 4-2-1 Aobacho, Higashimurayama, Tokyo 189-0002, Japan. Tel.: +81 42 3918059; fax: +81 42 3918212; e-mail: mmaki@nih.go.jp

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Abstract

The potential of *Mycobacterium bovis* Bacillus Calmette–Guerin (BCG) needs to be augmented to efficiently activate $CD4^+$ T cells through macrophages. *Mycobacterium leprae*-derived recombinant major membrane protein (MMP)-II induced GM-CSF production from macrophages. A recombinant BCG-SM that secretes MMP-II more efficiently produced GM-CSF and activated interferon (IFN)-γ-producing $CD4^+$ T cells than did vector control BCG when infected with macrophages. The T-cell activation by BCG-SM was dependent on the GM-CSF production by macrophages. Interleukin (IL)-10 production by macrophages stimulated with *M. leprae* was inhibited in a GM-CSF-dependent manner when the precursor monocytes were infected with BCG-SM. BCG inducing GM-CSF production was effective in macrophage-mediated T-cell activation partially through IL-10 inhibition.

Introduction

Leprosy is a chronic infectious disease induced by parasitic infection with *Mycobacterium leprae* (Stoner, 1979). Despite the marked reduction in the number of both registered leprosy cases and new cases, a significant number of new cases (254 525 for the year 2007) are still detected each year (World Health Organization, 2008). The emergence of multidrug-resistant *M. leprae* (Kai *et al.*, 2004), although still few in number, and the complexity of leprosy reactions are distressing (Moschella, 2004). These observations indicate the urgent need to develop an efficacious vaccine against leprosy. *Mycobacterium bovis* Bacillus Calmette–Guerin (BCG) has been known to provide partial protection against the development of leprosy (Ponnighaus *et al.*, 1992). However, meta-analyses conducted by Setia *et al.* (2006) demonstrated an overall protective effect of only 26% against leprosy. There seem to be several reasons why BCG is not as effective as previously predicted. One of them may be that the human immune cells most susceptible to BCG infection are macrophages (Grode *et al.*, 2005). On entry into macrophages, mycobacteria inhibit phagosome–lysosome fusion, which results in a less efficient stimulation of interferon (IFN)-γ-producing type 1 $CD4^+$ T cells (Ridley & Jopling, 1966; Frehel & Rastogi, 1987). Further, BCG as well as pathogenic mycobacteria can induce the production of an abundant amount of interleukin (IL)-10 from macrophages (Yamamura *et al.*, 1991), which inhibits activation of $CD4^+$ T cells (Jonuleit *et al.*, 2001; Granelli-Piperno *et al.*, 2004). Moreover, it has been demonstrated in a murine study that BCG primarily infects macrophages *in vivo*, and the active proliferation of T cells *in vivo* needs the enrolment of dendritic cells (DC). Further, DC are known to be the most professional antigen-presenting cells (APC) in terms of T-cell activation. Thus, the transfer to DC of antigens produced by the processing of intracellular BCG or of proteins secreted from the mycobacteria in macrophages, seems to be important (Winau *et al.*, 2006).

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We previously identified major membrane protein (MMP)-II (gene name, bfrA or ML2038), which is originally identified as bacterioferritin (Pessolani *et al.*, 1994) and localized in the cell membrane, as one of the dominant antigen of *M. leprae* (Maeda *et al.*, 2005; Makino *et al.*, 2005). Recombinant (r) MMP-II-pulsed DC activate naïve $CD4^+$ T cells to produce IFN-γ in an antigen-specific manner, and also stimulate T cells from not only paucibacillary leprosy, a representative clinical leprosy at one pole of the clinical spectrum, but also multibacillary leprosy, a representative leprosy at the opposite pole (Makino *et al.*, 2005). The activation of type 1 $CD4^+$ T cells is closely associated with the inhibition of the spread of *M. leprae in vivo* as observed in paucibacillary leprosy (Sieling *et al.*, 1999). In this respect, it was interesting to find that T cells from some paucibacillary leprosy patients seemed to be primed with MMP-II antigen *in vivo* (Makino *et al.*, 2005). Therefore, MMP-II was considered to be an immunodominant antigen of *M. leprae.* We constructed an rBCG strain (BCG-SM) that secretes MMP-II of *M. leprae* (Makino *et al.*, 2006). BCG-SM-infected DC stimulated quite efficiently both human naïve $CD4^+$ T cells and naïve $CD8^+$ T cells *in vitro*, and MMP-II-specific memory T cells were produced in mice inoculated with BCG-SM (Makino *et al.*, 2006).

Macrophages are heterogeneous in various aspects (Randolph *et al.*, 1999), and their differentiation is largely influenced by the cytokine milieu (Nakata *et al.*, 1991; Akagawa, 2002). Previously, we analysed the characteristics of two distinct macrophage subsets: rGM-CSF-mediated macrophages (GM-Mϕ) and rM-CSF-mediated macrophages (M-Mϕ) (Makino *et al.*, 2007). Both macrophages were equally susceptible to mycobacterial infection *in vitro*, but M-Mϕ infected with *M. leprae* did not activate $CD4^+$ T cells even after activation using both CD40 ligand and exogenous IFN-γ. Likewise, *Mycobacterium tuberculosis*-infected M-Mϕ failed to stimulate T cells (Verreck *et al.*, 2004). Further, a large amount of IL-10 was produced from M-Mϕ on stimulation with mycobacteria. Therefore, the fact that mycobacteria are highly susceptible to phagocytosis by M-Mϕ and poorly stimulate T cells through M-Mϕ, may be closely associated with the affinity of mycobacteria to macrophages, the induction of a latent infection and, in some cases, the development of disease. Likewise, M-Mϕ is one of the major target immune cells of BCG infection. However, to control the subsequently invading pathogenic mycobacteria, such as *M. leprae*, by producing memory T cells, modified BCG including the newly developed recombinant BCG-SM is required to be able to fully stimulate T cells even if M-Mϕ are the initial target host cells.

In this report, we examined the T-cell-stimulating ability of BCG-SM-infected M-Mϕ, and further assessed the influence of BCG-SM on the IL-10-producing activity of Mϕ upon a challenge with *M. leprae.*

Materials and methods

Preparation of cells and bacteria

Peripheral blood was obtained from healthy purified protein derivative (PPD)-positive individuals with informed consent. In Japan, most healthy individuals are PPD-positive due to a compulsory BCG vaccination for children (0–4 years old). Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Paque Plus (Pharmacia, Uppsala, Sweden) and cryopreserved in liquid nitrogen until use as previously described (Makino & Baba, 1997). For the preparation of peripheral monocytes, $CD3^+$ T cells were removed from freshly isolated heparinized blood or from cryopreserved PBMC using immunomagnetic beads coated with anti-CD3 monoclonal antiboby (mAb) (Dynabeads 450, Dynal, Oslo, Norway). The $CD3^-$ PBMC fraction was plated on collagen-coated plates and nonadherent cells were removed by extensive washing. The remaining adherent cells were used as monocytes (Makino & Baba, 1997). Macrophages were generated by culturing monocytes in the presence of 20% foetal calf serum and either rM-CSF (R and D Systems, Abingdon, UK) (M-Mϕ) or rGM-CSF (PeproTech EC Ltd, London, UK) (GM-Mϕ) (Makino *et al.*, 2007). Both GM-Mϕ and M-Mϕ were pulsed with rBCGs on day 3 or 5 of culture, and were used as a stimulator of T cells on day 5 or 7 (Makino *et al.*, 2007). A recombinant BCG that secretes *M. leprae*-derived MMP-II was constructed as described previously (Makino *et al.*, 2006). In brief, a shuttle vector, pMV-261, having a kanamycin resistance gene and origins of replication for *Escherichia coli* and mycobacteria was used to construct pMV-SM (Secreting MMP-II) having the MMP-II cDNA fragment. The BCG substrain Pasteur was cultured *in vitro* using Middlebrook 7H9 broth (BD Biosciences Pharmingen, San Jose, CA) supplemented with 0.05% Tween 80 and 10% albumin–dextrose–catalase (BD). Expression vectors were introduced into BCG by electroporation (Snapper *et al.*, 1988). Transformants were selected on Middlebrook 7H10 agar (BD) plates supplemented with 10% OADC (BD) and 25 $\mu g\,mL^{-1}$ kanamycin. Mycobacteria were subsequently grown in Middlebrook 7H9 broth containing 25 $\mu g\,mL^{-1}$ of kanamycin. BCG containing pMV-SM as an extrachromosomal plasmid is referred to as BCG-SM, and BCG containing pMV-261 is referred to as BCG-pMV (vector control BCG). *Mycobacterium leprae* (Thai-53) was isolated from the footpads of BALB/c-*nu/nu* mice (McDermott-Lancaster *et al.*, 1987). The isolated bacteria were counted using Shepard's method (McDermott-Lancaster *et al.*, 1987). The multiplicity of infection (MOI) was determined based on the assumption that macrophages were equally susceptible to infection with BCG or *M. leprae* (Hashimoto *et al.*, 2002). A recombinant MMP-II protein was produced as reported previously (Maeda *et al.*, 2005). Briefly,

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the MMP-II gene (ML2038) was inserted into the expression plasmid pET28 (Novagen, Madison, WI) and transformed into *E. coli* strain ER2566 (New England BioLabs, Ipswich, MA). The expressed protein was eluted using Whole Gel Eluter (Bio-Rad Laboratories, Hercules, CA). As a control for *M. leprae* antigen, we have purified hsp18 (ML1795) in *E. coli* using the PET expression system. The cytosolic fraction of the parental BCG was obtained as described previously (Maeda *et al.*, 2003).

Antigen-presenting function of rBCG-infected macrophages

The ability of rBCG-infected macrophages to stimulate T cells was assessed using an autologous macrophage-T cell coculture system as previously described (Wakamatsu *et al.*, 1999; Hashimoto *et al.*, 2002). The responder $CD4^+$ T cells were purified from freshly thawed PBMC using a CD4-negative isolation kit (Dynabeads 450) (Wakamatsu *et al.*, 1999). The purity of $CD4^+$ T cells was > 95% as assessed by FACS. The purified responder cells (1×10^5 per well) were plated in 96-well round-bottom tissue culture plates and macrophages were added to give the indicated macrophage : $CD4^+$ T-cell ratio. Supernatants of macrophage–T cell cocultures were collected on day 4. To identify molecules restricting T-cell activation, the following purified mAbs were used: anti-HLA-DR Ab (L243) and anti-CD86 Ab [IT2.2, Becton Dickinson (BD), San Jose, CA]. The concentration of IFN-γ produced by $CD4^+$ T cells was quantified using an enzyme assay kit, OptEIA Human enzyme-linked immunosorbent assay(ELISA) Set (BD). In some cases, M-Mϕ were pulsed with BCG-SM in the presence of $10\ \mu g\ mL^{-1}$ of either normal rat IgG or neutralizing mAb to GM-CSF (rat IgG2a) (BD).

Production of IL-10 and GM-CSF by macrophages

The ability of M-Mϕ to produce IL-10 on stimulation with *M. leprae* was assessed. The monocytes were pretreated with the indicated dose of rBCG and subsequently made to differentiate into M-Mϕ by culturing for 5 days in the presence of rBCG and M-CSF. These macrophages were stimulated with *M. leprae* at the indicated MOI for 24 h. In some cases, monocytes were infected with BCG-SM in the presence of $10\ \mu g\ mL^{-1}$ of neutralizing mAb to GM-CSF. Also, the ability of M-Mϕ to produce GM-CSF on stimulation with rBCG for 24 h was assessed. The concentration of these cytokines was quantified using OptEIA Human ELISA Set (BD).

Statistical analysis

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

Results

Effect of rBCG-infected macrophages on T-cell-stimulating activity

We analysed the T-cell-stimulating activity of rBCG-infected GM-Mϕ and M-Mϕ (Fig. 1). GM-Mϕ infected with either BCG-SM or BCG-pMV significantly stimulated $CD4^+$

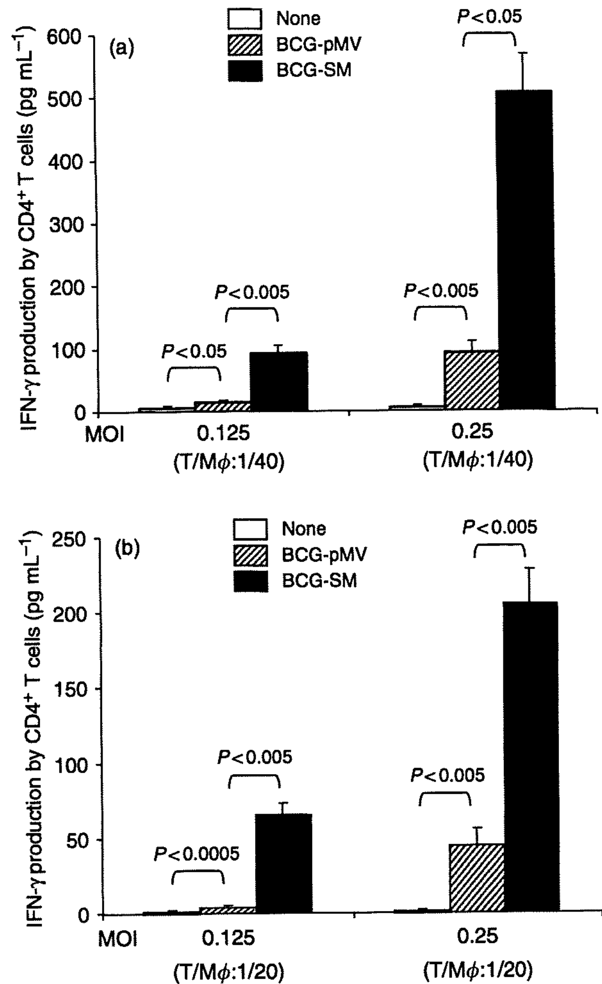

Fig. 1. Production of IFN-γ by $CD4^+$ T cells. (a) GM-Mϕ, differentiated by 3 days of culture with rGM-CSF from monocytes, were infected with BCG-pMV (vector control BCG) or BCG-SM (rBCG that secretes MMP-II) at the indicated MOI, and cultured for another 2 days. These GM-Mϕ were used as a stimulator of $CD4^+$ T cells (1×10^5 cells per well) at a T cell : GM-Mϕ ratio of 40 : 1 in a 4-day culture. (b) M-Mϕ, differentiated by 5 days of culture with rM-CSF from monocytes, were infected with BCG-pMV or BCG-SM at the indicated MOI, and cultured for another 2 days. M-Mϕ were then used as a stimulator of $CD4^+$ T cells (1×10^5 cells per well) at a T cell : M-Mϕ ratio of 20 : 1 in a 4-day culture. A representative experiment based on three separate experiments conducted using three separate PPD-positive individuals is shown. Assays were performed in triplicate and the results are expressed as the mean ± SD. Titres were statistically compared using Student's *t*-test.

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T cells. However, a larger amount of IFN-γ was produced by the T cells when GM-Mϕ were infected with BCG-SM (Fig. 1a), although BCG vaccination did not prime for MMP-II-specific T-cell response (not shown). We then analysed the T-cell-stimulating activity of BCG-infected M-Mϕ (Fig. 1b). Again, M-Mϕ infected with BCG-SM induced a higher amount of IFN-γ production by T cells than did BCG-pMV-infected M-Mϕ, although the IFN-γ production was less efficient than that induced by rBCG-infected GM-Mϕ even though higher doses of BCG-infected M-Mϕ were used as a stimulator.

Factors associated with the induction of the T-cell-stimulating activity of M-Mϕ

To define the factors associated with the CD4$^+$ T-cell activation by BCG-SM-infected M-Mϕ, we phenotypically analysed M-Mϕ infected with either BCG-pMV or BCG-SM. There was no significant difference between BCG-pMV-infected M-Mϕ and BCG-SM-infected M-Mϕ in the expression of HLA-DR, CD86 or CD40 molecules (not shown). The cytokines produced by M-Mϕ stimulated with rBCGs, including GM-CSF and IL-23, were examined. Both rBCGs induced GM-CSF production, but BCG-SM did so more efficiently than BCG-pMV (Fig. 2). However, IL-23 was not produced by M-Mϕ on stimulation with either BCG-pMV or BCG-SM. We also assessed whether rMMP-II protein can induce GM-CSF production in macrophages. Whereas *M. leprae*-derived cytosolic protein (not shown), other mycobacterial proteins such as BCG-derived cytosolic protein (5–10 μg mL^{-1}), control recombinant *M. leprae* antigen (hsp18), and lipopolysaccharide (amount present with rMMP-II protein) did not stimulate Mϕ, MMP-II induced GM-CSF production in a concentration-dependent manner (Fig. 3). rMMP-II also efficiently induced the production of other cytokines including tumour necrosis factor (TNF)α and IL-12p40 from Mϕ (not shown).

We examined the influence of surface antigens on M-Mϕ. The T-cell-stimulating activity of BCG-SM-infected M-Mϕ was significantly inhibited when the infected M-Mϕ were pretreated with the mAb to HLA-DR or CD86 antigens, whereas the control IgG did not affect IFN-γ production by T cells (Fig. 4a). However, IFN-γ production was partially inhibited when BCG-SM-infected M-Mϕ were treated with the mAb to MMP-II (not shown). Next, we examined the effect on T-cell activation of GM-CSF produced by M-Mϕ stimulated with BCG-SM (Fig. 4b). When M-Mϕ were infected with BCG-SM in the presence of the neutralizing mAb to GM-CSF, IFN-γ production by CD4$^+$ T cells was significantly inhibited. The T-cell-stimulating activity of BCG-SM-infected M-Mϕ was not affected by normal rat IgG.

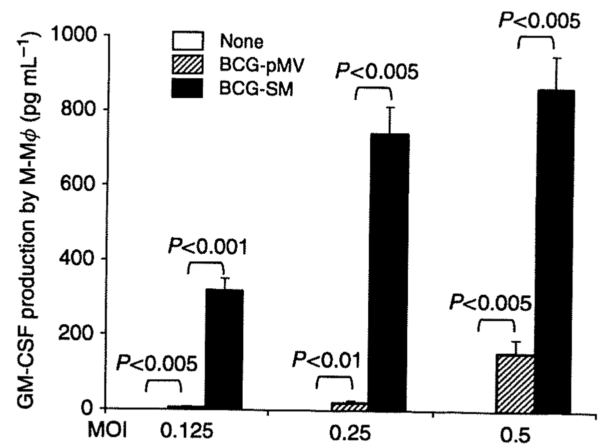

Fig. 2. Production of GM-CSF by M-Mϕ. M-Mϕ differentiated by 5 days of culture with rM-CSF from monocytes, were stimulated with BCG-pMV or BCG-SM for 24 h at the indicated MOI. A representative experiment based three separate experiments conducted using three separate PPD-positive individuals is shown. Assays were performed in triplicate and the results are expressed as the mean ± SD. Titres were statistically compared using Student's *t*-test.

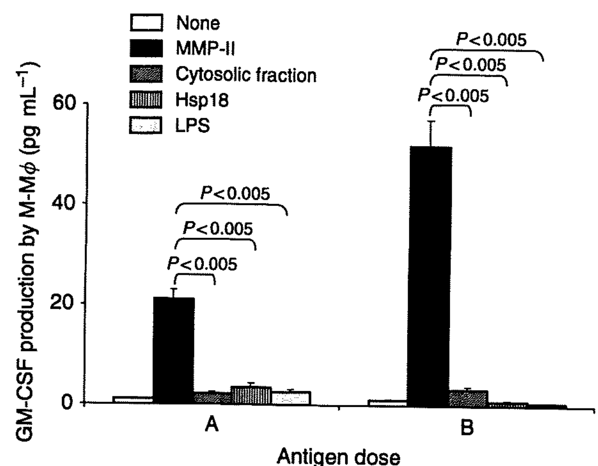

Fig. 3. GM-CSF production by M-Mϕ. M-Mϕ obtained after 5 days of culture with rM-CSF were stimulated for 24 h with rMMP-II, the BCG-derived cytosolic fraction or *Mycobacterium leprae*-derived hsp18 antigen [dose of antigen in (a) 5 μg mL^{-1} and that in (b) 10 μg mL^{-1}]. Lipopolysaccharide, assumed to be present with rMMP-II protein (660 ng mg^{-1} MMP-II protein), was used as a negative control (lipopolysaccharide in (a) 3.3 ng mL^{-1} and that in (b) 6.6 ng mL^{-1}). A representative experiment based on three separate experiments conducted using three separate PPD-positive individuals is shown. Assays were performed in triplicate and the results are expressed as the mean ± SD. Titres were statistically compared using Student's *t*-test.

Effect of infection of monocytes with BCG on IL-10 production by M-Mϕ

Macrophages are one of the cells most sensitive to *M. leprae* infection and M-Mϕ produce abundant IL-10 when infected with the bacteria (Makino *et al.*, 2007). As precursor

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