Bacterial culture and freeze stock

Bacterial culture and freeze stocking were performed as reported by Hayashi *et al.* (2009).

Biochemical tests

Tests for nitrate reduction, catalase, Tween 80 hydrolysis, urease, pyrazinamidase and resistance to thiophene 2-carboxylic acid hydrazide (TCH) were performed by standard procedures except as described below (Gangadharam & Jenkins, 1998). Nitrate reduction was performed by the classical procedure with liquid reagent. Pyrazinamidase activity was tested on Middlebrook 7H11 broth (BD, Franklin Lakes, NJ) instead of Dubos broth. Resistance to TCH was determined on solid Ogawa medium containing 1 or 10 $\mu g\,mL^{-1}$ TCH. Niacin accumulation was detected using the Kyokuto Niacin Test (Kyokuto Pharmaceutical Industries, Tokyo, Japan) in accordance with the manufacturer's instruction. Degradation of *p*-amino salicylate (PAS) was determined according to Tsukamura (1961). *Mycobacterium tuberculosis*, *M. bovis*, *M. avium* and *M. smegmatis* were used as controls. In the urease test, urease-deficient recombinant BCG (Mukai *et al.*, 2008) was used as a negative control.

Culture and differentiation of THP-1 cells

The human monocytic cell line THP-1 (ATCC TIB202) was purchased from ATCC and maintained in RPMI 1640 medium containing 100 $U\,mL^{-1}$ penicillin G and 5% heat-inactivated fetal bovine serum (FBS). THP-1 cells were stimulated with 10 nM phorbol 12-myristate 13-acetate (PMA; Wako Pure Chemical Industries, Osaka, Japan) for 24 h to be differentiated to macrophages. Cells were washed three times with culture medium and used for the assays.

Isolation and culture of bone marrow-derived macrophages (BMMs)

Bone marrow was isolated from the tibias and femurs of C57BL/6J female mice at 4–8 weeks of age. Bone marrow cells haemolysed in 0.83% NH_4Cl–Tris buffer were cultured in RPMI 1640 supplemented with 10% FBS, 100 $U\,mL^{-1}$ penicillin G, 50 μM 2-mercaptoethanol and 10 $ng\,mL^{-1}$ granulocyte-macrophage colony-stimulating factor (Wako) in 24-well culture plates; the culture medium was refreshed every 2 days. On day 7, adherent cells were collected and used for the assays.

Macrophage infection

Macrophages infected with bacilli at a multiplicity of infection (MOI) of 20 were incubated at 37 °C for 6 h. Extracellular bacilli were washed out three times and killed by 100 $\mu g\,mL^{-1}$ amikacin treatment for 6 h. Interferon (IFN)-γ (final concentration of 100 $U\,mL^{-1}$) was added to some of the wells as a stimulator. Following incubation, cells were washed three times and ruptured with 100 μL of sterile distilled water. To determine the number of intracellular live bacteria, the lysates were diluted and plated on 7H11 agar in triplicate. Colonies were counted after 3 weeks' incubation.

Tolerance test for hydrogen peroxide and nitric oxide

Bacilli (2×10^6 CFU) were incubated in 7H9 broth containing albumin, dextrose (without catalase) and 0–10 mM H_2O_2 for 6 h. In the same manner, bacilli were incubated in 7H9 broth supplemented with ADC (albumin, dextrose, catarase) and containing 0–10 mM $NaNO_2$, as an NO donor, at pH 6.6, 6.0 or 5.5 for 3 days. Following incubation, bacilli were washed with 7H9 medium three times, diluted and plated on 7H11 agar. Plates were incubated for 3 weeks and the percentage of live bacilli relative to control (0 mM H_2O_2 or $NaNO_2$) was calculated.

Determination of permissive pH range for growth of bacilli

Bacterial log-phase cultures in Middlebrook 7H9 (BD) supplemented with 10% ADC (BD) were adjusted to an OD of 0.1 at 530 nm and mixed with 100-fold volume of various pH-adjusted broths (pH 3, 4, 5, 5.4, 5.7, 6.2, 6.6, 7, 8, 9, 10, 11 and 12, adjusted with HCl or NaOH). Following incubation at 37 °C for 21 days, bacterial growth was evaluated by measuring OD at 530 nm.

Statistical analysis

Each experiment was repeated three times. Statistically significant differences between two series were assessed by Student's *t*-test or Aspin–Welch's *t*-test following an *F*-test assessment of variance.

Results and discussion

Eight different biochemical tests, nitrate reduction, niacin, catalase, Tween 80 hydrolysis, urease, pyrazinamidase, PAS degradation and resistance to TCH, were applied to 14 substrains of BCG, BCG-Russia, -Moreau, -Japan, -Sweden, -Birkhaug, -Danish, -Glaxo, -Mexico, -Tice, -Connaught, -Montreal, -Phipps, -Australia and -Pasteur (Table 1). BCG-Birkhaug was positive for nitrate reduction whereas BCG-Mexico, -Australia and -Pasteur were negative; the other BCG strains were weakly positive, although *M. bovis*, the parental strain of BCG, was negative. The nitrate respiration system may be responsible for the survival of *M. tuberculosis* under anaerobic conditions (Sohaskey, 2008), and the nitrate reductase gene *narGHJI* contributes to the virulence of BCG in immunodeficient mice (Weber *et al.*, 2000). BCG-Russia and -Japan survived better both in THP-1 and in mouse BMMs than other substrains (Fig. 1 and Table 1). Although host *M. bovis* was negative for nitrate reduction,

Table 1. Summary of characteristics of BCG substrains *in vitro**

Organism	Nitrate reduction	Niacin accumulation	Tween 80 hydrolysis	Urease	Pyrazinamidase Day 4	Pyrazinamidase Day 7	PAS degradation	Resistance to TCH (μg mL^{-1}) 1	Resistance to TCH (μg mL^{-1}) 10	Catalase (room temperature) Bubble column (mm)	Activity	68 °C catalase	H_2O_2 tolerance	NO tolerance	Optimal pH	Viability in THP-1 −IFN-γ	Viability in THP-1 +IFN-γ	Viability in BMM −IFN-γ	Viability in BMM +IFN-γ
BCG†																			
Russia	±	+	–	+	–	–	–	+	–	9.3 ± 2.4	Low	–	+	–	6.6	+	+	+	+
Moreau	±	+	±	+	–	–	–	+	–	7.1 ± 1.8	Low	–	ND	ND	ND	ND	ND	ND	ND
Japan	±	+	–	+	–	–	–	+	–	14.8 ± 2.3	Low	–	+	+	6.6	+	+	+	+
Sweden	±	+	±	+	–	–	–	+	–	6.7 ± 1.7	Low	–	–	–	8–9	–	–	+	+
Birkhaug	+	+	–	+	–	–	–	+	–	11.8 ± 2.3	Low	–	+	–	8–9	+	+	–	–
Danish	±	–	±	+	–	–	–	+	–	9.4 ± 2.4	Low	–	–	+	7–8	+	+	–	+
Glaxo	±	–	–	+	–	–	–	+	–	7.4 ± 1.1	Low	–	–	+	7–8	–	–	–	–
Mexico	–	–	–	+	–	–	–	+	–	6.4 ± 1.8	Low	–	ND	ND	ND	ND	ND	ND	ND
Tice	±	–	–	+	–	–	–	+	–	6.3 ± 1.6	Low	–	ND	ND	ND	ND	ND	ND	ND
Connaught	±	–	±	+	–	–	–	+	–	7.9 ± 1.9	Low	–	+	–	7–8	–	–	–	+
Montreal	±	+	±	+	–	–	–	+	–	6.0 ± 2.3	Low	–	ND	ND	ND	ND	ND	ND	ND
Phipps	±	+	±	+	–	–	–	+	–	6.0 ± 2.2	Low	–	–	–	6.6	–	–	–	–
Australia	–	–	±	+	–	–	–	+	–	6.1 ± 2.1	Low	–	ND	ND	ND	ND	ND	ND	ND
Pasteur	–	–	±	+	–	–	–	+	–	7.3 ± 2.6	Low	–	–	+	6.6	+	+	–	+
M. bovis	–	–	–	+	–	–	–	+	–	5.4 ± 0.7	Low	–	+	+	6.6	+	+	+	+
M. tuberclosis																			
H37Rv	+	+	±	+	+	+	–	+	+	8.4 ± 1.1	Low	–	ND	ND	ND	ND	ND	ND	ND
H37Ra	+	+	±	+	+	+	–	+	+	10.0 ± 1.6	Low	–	ND	ND	ND	ND	ND	ND	ND
M. avium																			
724S	–	–	–	–	+	+	–	+	+	35.8 ± 13.0	Low	–	ND	ND	ND	ND	ND	ND	ND
2151SmO	–	–	–	–	+	+	–	+	+	27.6 ± 3.5	Low	+	ND	ND	ND	ND	ND	ND	ND
M. smegmatis	+	–	+	–	+	+	–	+	+	14.0 ± 1.3	Low	–	ND	ND	ND	ND	ND	ND	ND

*Summarizing the data from biochemical tests, tolerance to oxidative stress (Fig. 1) and survival activities in host cells (Fig. 2).

†Scores indicate the numbers that are positive (+) and slightly positive (±).

‡BCG substrains, which were historically distributed from the Pasteur Institute, are given in chronological order.

Methods for conventional biochemical tests for mycobacteria are described in Materials and methods. Experiments were conducted more than three times. Representative results are indicated.

ND, no data

the viability in host cells was higher than BCG (Table 1 and Fig. 1). According to the standard method for the nitrate reductase test, the assay period was 2 h. Under different conditions, for example longer incubation times and anaerobic conditions, nitrite production has been found in some BCG strains (Weber *et al.*, 2000; Sohaskey & Wayne, 2003; Stermann *et al.*, 2003; Sohaskey & Modesti, 2009). Therefore, different incubation times could explain the discrepancy observed between nitrate reductase test results and intercellular survival. Nitrate reductase activity is not the sole explanation, but we believe it is partly responsible for the survival in host cells, as shown in previous reports (Weber *et al.*, 2000; Sohaskey, 2008) and the present study.

Heterogeneity of niacin accumulation was also observed among BCG substrains (Table 1). Recycling of NAD favours the latent infection of *M. tuberculosis* (Boshoff *et al.*, 2008), and NAD-quinoline reductase is responsible for resistance to oxidative stress (Akhtar *et al.*, 2006). These reports suggest that the activity of NAD metabolism is associated with the survival of BCG in macrophages or host cells. Whether the long or short survival of BCG in host cells favours the effectiveness of BCG has not been determined. However, the different characteristics of BCG substrains as reported here provide the basic information for further investigation of immunological characteristics and evaluation.

Parker *et al.* (2007) purified and characterized MPLA. MPLA is associated with cutinase, a serine esterase and catalyses the hydrolysis of lipids including Tween 80. MPLA activity was observed not only in pathogenic *M. tuberculosis*, but also in BCG-Pasteur. BCG-Pasteur was weakly positive for Tween 80 hydrolysis (Table 1). In fact, eight of the 14 substrains, namely BCG-Moreau, -Sweden, -Danish, -Connaught, -Montreal, -Phipps, -Australia and -Pasteur, were weakly positive. Mycobacteria are known to use this fatty acid as carbon source at the dormant stage. Therefore, this activity could contribute to survival under starvation conditions during dormancy (Jackson *et al.*, 1989; Deb *et al.*, 2009).

All BCG strains belong to the low-catalase group, although there were variations in the height of bubble column among them (Table 1). It was over 10 mm in BCG-Japan (14.8 mm) and -Birkhaug (11.8 mm) (Table 1). No mutation in the coding region of the *ahpC* gene among was observed among the substrains (data not shown). The

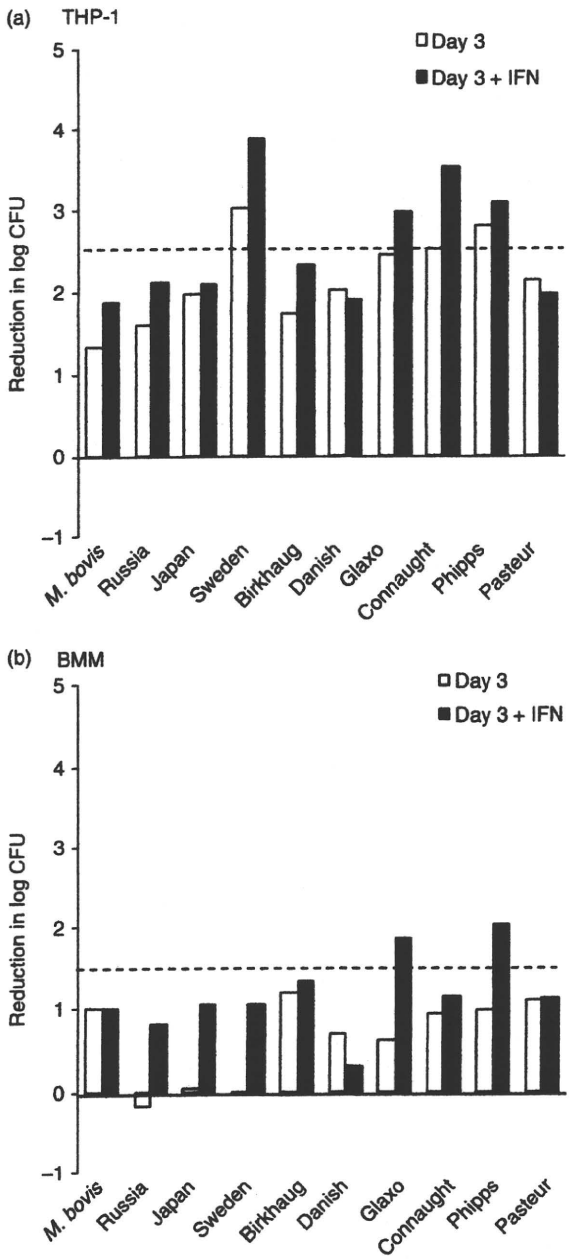


Fig. 1. Viability of BCG strains in THP-1 and mouse BMMs. PMA-differentiated THP-1 (a) or mouse BMMs (b) were infected with BCG at an MOI of 20 with (solid) or without (open) 100 U mL^{-1} of IFN-γ as a stimulator. After 6 h of infection, BCG CFU counts were determined from infected cell lysates and were monitored on days 0, 3 and 7. The data are expressed as the reduction in $\log_{10}$ CFU compared with control at day 0. Error bars represent means+SD for triplicate results from one of two similar experiments. Statistically significant differences between BCG group Russia, Japan, Birkhaug, Danish and Pasteur and BCG group Sweden, Glaxo, Connaught and Phipps were observed in (a) (Student's *t*-test, $P < 0.05$). In (b) there were statistically significant differences between BCG group Russia, Japan and Sweden and BCG group Birkhaug, Danish, Glaxo, Connaught, Phipps and Pasteur in the absence of IFN-γ (open column) (Aspin–Welch's *t*-test, $P < 0.05$). In the presence of IFN-γ (solid column) there were statistically significant differences between BCG group Russia, Japan, Sweden, Birkhaug, Danish, Connaught and Pasteur and BCG group Glaxo and Phipps (b) (Aspin–Welch's *t*-test, $P < 0.05$).

differences between transcription of the genes and the activities have not yet been analysed. Catalase (*katG*) and peroxidase (*ahpC*) activities of *M. tuberculosis* are related to resistance to oxidative killing in human monocytes *in vitro* (Manca *et al.*, 1999). The expression of *katG* is partially regulated by ferric uptake regulators (*fur*), and contributes to the virulence of *M. tuberculosis* (Lucarelli *et al.*, 2008). Resistance to hydrogen peroxide of *M. bovis*, BCG-Russia and -Japan was higher than that of other BCG substrains (Fig. 1). This resistance relates well to survival in host cells, THP-1 and BMMs (Fig. 1). These findings suggest that resistance to H_2O_2 contributes to survival of BCG substrains in host cells and that enzyme activities other than of catalase could be relevant to the resistance to oxidative stress from host cells.

We next investigated the susceptibility of BCG substrains to nitrosative stress by exposing them to sodium nitrite for 3 days (Fig. 2b). BCG-Pasteur was tolerant to nitric oxide, and moderate susceptibility was observed in BCG-Japan, -Danish and -Glaxo. BCG-Russia, -Sweden, -Birkhaug, -Connaught and -Phipps were sensitive to NO. The parental strain of BCG, *M. bovis*, was able to tolerate NO. To assess NO production from the bacilli, reduction of pH of the media is required to generate NO from sodium nitrate (Darwin *et al.*, 2003; MacMicking *et al.*, 2003). Intriguingly, optimal pH levels were found to be different among the BCG substrains (Table 2). The optimal pH of BCG-Russia, -Moreau, -Japan, -Phipps, -Pasteur and *M. bovis* was 6.6. Optimal pH of BCG-Sweden and -Birkhaug was 8–9, and that of BCG-Danish, -Glaxo and -Connaught was 7–8. According to maturation state, pH in phagosomes decreases from about 6 to 4. All BCG strains were positive for urease (Table 1). The changes in pH of the culture broths for each BCG strain were not significantly different (data not shown). Therefore, these data indicate that the increasing pH of the culture broth, such as by generating ammonium, is not responsible for the tolerance of BCG strains to a reduction of pH. The precise mechanisms of adaptability to pH changes have not been elucidated.

In summary, we have evaluated the usefulness of various biochemical tests currently used for identifying mycobacterial species. Surprisingly, there were differences in the results of these tests among BCG substrains. These differences could be generated during the long time of passage of BCG vaccine strains. Their characteristics are quality controlled by lyophilizing techniques. A good correlation between oxidative and nitrosative stress and survival in host cells were observed among BCG substrains. The relationship between antigen presentation and viability in host cells is not clear. The longer persistence of the bacilli in the host cells may favour antigen presentation by continuous supply of the antigens, while short persistent bacilli may stimulate antigen presentation through a different pathway (Grode L

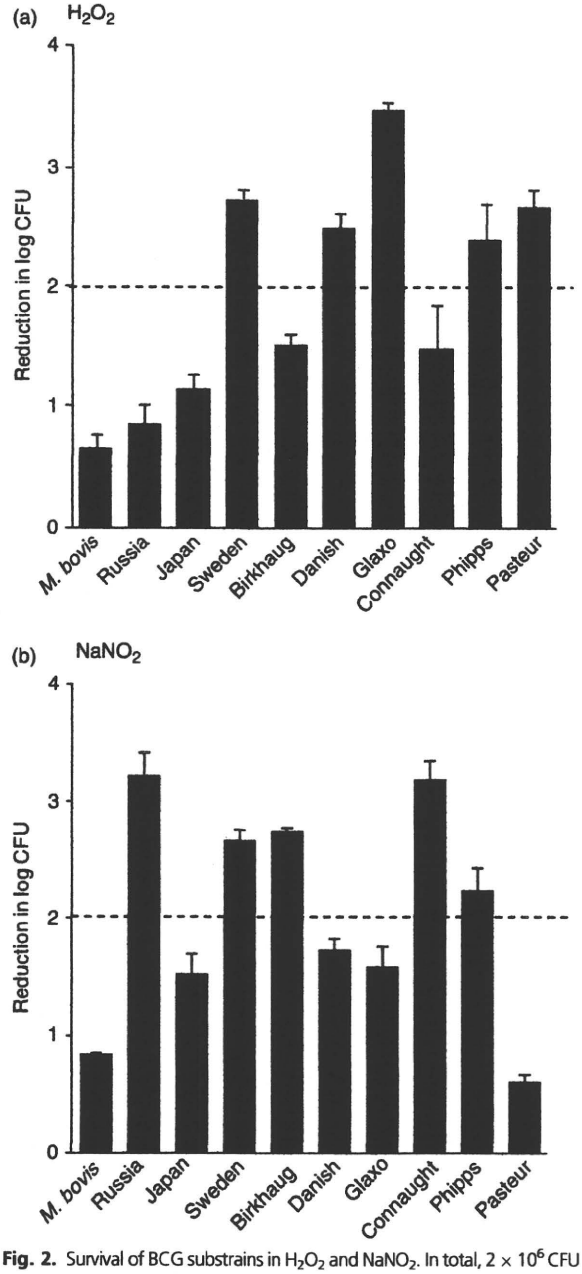
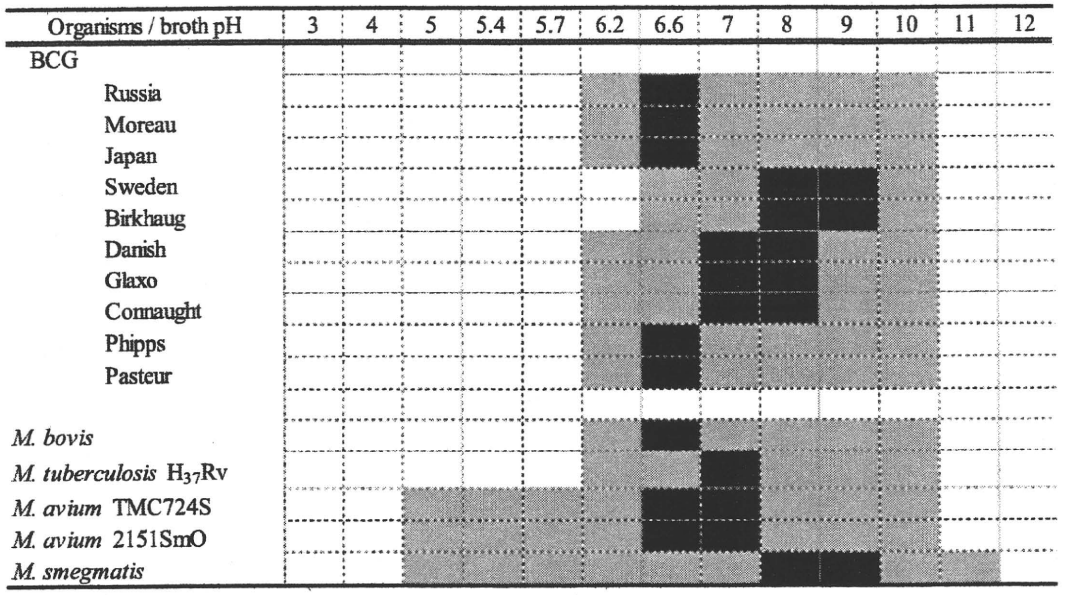


Fig. 2. Survival of BCG substrains in H_2O_2 and $NaNO_2$. In total, 2×10^6 CFU of *Mycobacterium bovis* or BCG substrains were treated with (a) 10 mM H_2O_2 for 6 h or (b) 10 mM $NaNO_2$ for 3 days. Treated and washed cells were serially diluted, and aliquots from four serial dilutions were plated in duplicate on 7H11 agar. The results are expressed as the reduction in $\log_{10}$ CFU compared with control at day 0. Error bars show means+SD of triplicate results from one of three similar experiments. BCG substrains, which were historically distributed from the Pasteur Institute, are aligned in chronicle order. In (a), statistically significant differences were found between BCG group Russia, Japan, Birkhaug and Connaught and BCG group Sweden, Danish, Glaxo, Phipps and Pasteur (Student's *t*-test, $P < 0.05$). In (b), statistically significant differences were found between BCG group Japan, Danish, Glaxo and Pasteur and BCG group Russia, Sweden, Birkhaug, Connaught and Phipps (Student's *t*-test, $P < 0.05$).

Table 2. The range of pH permissive for growth of BCG and other mycobacteria



BCG substrains, *Mycobacterium bovis*, *Mycobacterium tuberculosis*, *Mycobacterium avium* and *Mycobacterium smegmatis* were cultured in 7H9 broth at the indicated pH for 21 days and OD at 530 nm was monitored every 3 days.
Grey, pH ranges that the broth OD was above 0.1; black, maximal pH.

et al., 2005). Comparative analysis of BCG substrains on acquired immunity should be undertaken. This and our previous studies provide basic information on the biological characteristics and the effect on the innate immunological characteristics of BCG substrains, and these studies could contribute to the re-evaluation of BCG vaccine.

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Enhanced Activation of T Lymphocytes by Urease-Deficient Recombinant Bacillus Calmette-Guérin Producing Heat Shock Protein 70-Major Membrane Protein-II Fusion Protein

Tetsu Mukai, Yumi Maeda, Toshiki Tamura, Masanori Matsuoka, Yumiko Tsukamoto, and Masahiko Makino

To activate naive T cells convincingly using *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), recombinant BCG (BCG-D70M) that was deficient in urease, expressed with gene encoding the fusion of BCG-derived heat shock protein (HSP) 70 and *Mycobacterium leprae*-derived major membrane protein (MMP)-II, one of the immunodominant Ags of *M. leprae*, was newly constructed. BCG-D70M was more potent in activation of both $CD4^+$ and $CD8^+$ subsets of naive T cells than recombinant BCGs including urease-deficient BCG and BCG-70M secreting HSP70–MMP-II fusion protein. BCG-D70M efficiently activated dendritic cells (DCs) to induce cytokine production and phenotypic changes and activated $CD4^+$ T cells even when macrophages were used as APCs. The activation of both subsets of T cells was MHC and CD86 dependent. Pretreatment of DCs with chloroquine inhibited both surface expression of MMP-II on DCs and the activation of T cells by BCG-D70M–infected APCs. The naive $CD8^+$ T cell activation was inhibited by treatment of DCs with brefeldin A and lactacystin so that the T cell was activated by TAP- and proteosome-dependent cytosolic cross-priming pathway. From naive $CD8^+$ T cells, effector T cells producing perforin and memory T cells having migration markers were produced by BCG-D70M stimulation. BCG-D70M primary infection in C57BL/6 mice produced T cells responsive to in vitro secondary stimulation with MMP-II and HSP70 and more efficiently inhibited the multiplication of subsequently challenged *M. leprae* than vector control BCG. These results indicate that the triple combination of HSP70, MMP-II, and urease depletion may provide a useful tool for inducing better activation of naive T cells. *The Journal of Immunology*, 2010, 185: 6234–6243.

Mycobacterium leprae is a causative bacterium of leprosy (1, 2). Leprosy is clinically divided into two major categories, paucibacillary and multibacillary leprosy (2). In the lesion of paucibacillary leprosy, $CD1a^+$ dendritic cells (DCs) are involved, and the substantially activated T cells are observed (3, 4). These observations indicate that host defense activity against *M. leprae* is chiefly conducted by adaptive immunities, and both IFN-γ–producing type 1 $CD4^+$ T cells and $CD8^+$ T cells act to inhibit the active multiplication of *M. leprae*. Thus, few numbers of bacilli are observed in the lesion of paucibacillary leprosy. The activation of T cells is induced by DCs loaded with bacilli or its component, which display various antigenic molecules on their surface, including the immunodominant Ags (5, 6). Previously, we identified major membrane protein (MMP)-II (gene name bfrA or ML 2058) as one of the immunodominant Ags of *M. leprae* (7). MMP-II ligates TLR2 and consequently activates the NF-κB pathway (7). DCs pulsed with MMP-II protein activate both naive and memory type $CD4^+$ and $CD8^+$ T cells to produce IFN-γ in an Ag-specific fashion (7, 8). Further, the MMP-II is supposed to be recognized by T cells in vivo of *M. leprae*-infected individuals, including paucibacillary leprosy patients (8).

Multidrug therapy introduced by the World Health Organization has been effective to reduce the number of leprosy patients registered. However, the drug therapy seems ineffective to reduce the number of newly developed leprosy patients; thus, the useful vaccine is essential to control the number of new patients. So far, *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) is used as a vaccine against leprosy, although not broadly (9–11). However, nowadays, BCG is not recognized as a reliable vaccine, because an overall efficacy of BCG against leprosy is reported to be 26%, which is calculated by meta-analyses enrolling several studies and observations (12). However, BCG intrinsically possesses the ability to activate type 1 $CD4^+$ T cells, although not convincingly, and may share some antigenic molecules with *M. leprae* (9, 10). These observations suggest that the improvement of BCG may be one of the critical ways to develop new effective vaccines against leprosy. However, BCG also has its intrinsic defect, an activity to inhibit the fusion of BCG-infected phagosomes with lysosomes (13–15). This defect seems to be a major factor associated with unconvincing activation of naive T cells. Therefore, we tried to upregulate the T cell-stimulating activity of BCG by overcoming the intrinsic defect of the bacteria. First of all, we produced recombinant BCG (rBCG) (BCG-SM) that secretes MMP-II in the infected cells (16). As expected, BCG-SM substantially activated both naive $CD4^+$ and $CD8^+$ T cells and consequently inhibited the growth of *M. leprae* to some extent, but not completely, in the

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Abbreviations used in this paper: BCG, bacillus Calmette-Guérin; DC, dendritic cell; HSP, heat shock protein; MMP, major membrane protein; MOI, multiplicity of infection; rBCG, recombinant BCG.

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footpad of C57BL/6 mice (17). It is known that parent BCG partially activates naive $CD4^+$ T cells but is not efficient in stimulating naive $CD8^+$ T cells quickly to produce IFN-γ (14, 16). In this respect, the fact that BCG-SM can activate DCs to produce IL-12p70 and both subsets of naive T cells to produce IFN-γ indicates that secretion of MMP-II of *M. leprae* presumably in phagosomes of APCs of host cells is a useful strategy to activate both APCs and T cells (16). Usefulness of the enhancement of secretion of vaccinated BCG-derived Ags is revealed in the other intracellular infection system such as *Mycobacterium tuberculosis*, in which the active secretion of Ag85 complex was effective in inhibiting the replication of subsequently challenged *M. tuberculosis* (18).

Then, we undertook two other strategies to further enhance the T cell-stimulating activity of BCG. One of them was aimed at potentiating the activation of naive $CD4^+$ T cells. BCG possesses urease, which produces ammonia and inhibited the acidication of BCG-infected phagosomes to avoid the fusion with lysosomes (19, 20). To inhibit the ammonia production, we produced urease-deficient BCG (BCG-ΔUT-11-3) (15). BCG-ΔUT-11-3 was feasibly translocated into lysosomes and activated both DCs and naive $CD4^+$ T cells of human (15). Further, BCG-ΔUT-11-3 efficiently produces memory type $CD4^+$ T cells in mice that can recognize *M. leprae*-derived proteins (15). Thus, the disruption of the *UreC* gene of BCG is useful tool to enhance the $CD4^+$ T cell-activating activity of BCG. The second strategy for potentiation of BCG activity is aimed to provide BCG the ability of activating IFN-γ–producing $CD8^+$ T cells quickly and strongly. To this end, we used heat shock protein 70 (HSP70) (21–24). The gene encoding HSP70 was directly connected with that of MMP-II and was extrachromosomally transformed into BCG (production of BCG-70M). BCG-70M secreted HSP70–MMP-II fusion protein and activated not only Ag-specific naive $CD8^+$ T cells polyclonally, but also naive $CD4^+$ T cells and DCs (25). Thus, the production and secretion of HSP70 in phagosomes accompanied by MMP-II seems an effective strategy to activate human naive $CD8^+$ T cells using BCG.

Because we employed two independent strategies to overcome the intrinsic defect of BCG (inhibition of phagosome-lysosome fusion), in this study, we combined the two strategies and produced another rBCG (BCG-D70M) that is deficient in urease activity but is introduced with the gene encoding HSP70–MMP-II fusion protein and evaluated its immunostimulatory activities. The BCG-D70M showed the strongest activities in terms of activation of naive $CD4^+$ and $CD8^+$ T cells among the rBCGs produced by us so far.

Materials and Methods

Preparation of cells and Ags

Peripheral blood was obtained from healthy purified protein derivative-positive individuals under informed consent. In Japan, BCG vaccination is compulsory for children (0–4 y old). PBMCs were isolated using Ficoll-Paque Plus (Pharmacia, Uppsala, Sweden) and cryopreserved in liquid nitrogen until use, as previously described (26). The viability of T cells obtained from cryopreserved PBMCs was >90%, and no selection in terms of functionality was induced in both monocytes and 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 mAb (Dynabeads 450, Dynal Biotech, Oslo, Norway). 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 (27). Monocyte-derived DCs were differentiated as described previously (26, 28). Briefly, monocytes were cultured in the presence of 50 ng rGM-CSF (PeproTech EC, London, U.K.) and 10 ng/ml rIL-4 (PeproTech) (28). On day 4 of culture, immature DCs were infected with rBCG at an indicated multiplicity of infection (MOI), and, on day 6 of culture, DCs were used for further analyses of surface Ag and mixed lymphocyte assays. Macrophages were differentiated as described previously (29, 30). In brief, monocytes were cultured in the presence of 10 ng/ml rM-CSF (R&D Systems, Minneapolis, MN). On day 5 of culture, macrophages were infected with rBCG at an indicated MOI, and, on day 7 of culture, they were used for further analyses of mixed lymphocyte assay. The rMMP-II protein was produced as described previously (7, 31), and the rHSP70 Ag was purchased (HyTest, Turku, Finland).

Vector construction and preparation of rBCG

The genomic DNAs from BCG substrain Tokyo and from *M. leprae* Thai-53 strain were extracted by using phenol-chloroform. The oligonucleotide primers for the *hsp70* gene were FMb70BaI (5′-aaaTGGCCAtggctcgtgcggtcggg-3′; capital letters indicate a BalI site) and RMb70Eco (5′-aaaGAATTCcttggcctcccggccg-3′; capital letters indicate an EcoRI site). The primers for MMP-II sequence from *M. leprae* genomic DNA was amplified with FMMP Eco4 (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 BalI-SalI–digested parental pMV261 plasmid. For replacing kanamycin resistance gene to hygromycin resistance cassette, the XbaI-NheI fragment from pYUB854 (32) was cloned into SpeI-NheI fragment of each plasmid (32). The rBCG that lacks *ure*C gene (BCG-ΔUT-11) was produced as described previously (15). The hygromycin cassette was removed by using a plasmid encoding γδ-resolvase (γδ-*tnp*R) encoded in pTYUB870 (32). The unmarked BCG having the hygromycin gene was named BCG-ΔUT-11-3. The HSP70–MMP-II fusion protein expression vector was introduced into BCG-ΔUT-11-3 by electroporation method. BCG-70M was produced as described previously (25). BCG-ΔUT-11-3 containing pMV-HSP70-MMP-II as an extrachromosomal plasmid is referred to as BCG-D70M and that containing pMV-261-hygromycin is referred to as BCG-261H (BCG vector control). rBCGs were grown to log phase and stored at 10^8 CFU/ml at −80°C. Preinfection to DCs and macrophages, BCGs were counted by colony assay method. There is no significant difference in the in vitro culture growth between BCG-261H and BCG-D70M.

Western blot analyses of the fusion protein HSP70–MMP-II extracted from rBCGs

To verify the expression of MMP-II and HSP70 in rBCGs, we prepared cell lysates from BCG-70M and BCG-D70M as described previously (16). Briefly, the protein fraction of the rBCGs was prepared as follows: harvested cells were washed with PBS and sonicated. Disrupted cells were centrifuged at 10,000 × *g* at 4°C, and the supernatant was taken as the cell lysate. SDS-PAGE and electroblotting were carried out using standard methods. Western blotting was performed as follows: a polyvinylidene difluoride 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 homologs. Anti-Ag85B rabbit polyclonal Ab was used as an internal control. An alkaline phosphatase-conjugated anti-mouse IgG Ab (Biosource International, Camarillo, CA) or alkaline phosphatase-conjugated anti-rabbit IgG Ab (Tago, Burlingame, CA) was used as the secondary Ab. Color development was performed using NBT/BCIP detection reagent (Calbiochem, San Diego, CA).

Analysis of cell surface Ag

The expression of cell surface Ag on DCs and lymphocytes was analyzed using FACSCalibur (BD Biosciences). Dead cells were eliminated from the analysis by staining with propidium iodide (Sigma-Aldrich, St. Louis, MO), and 1×10^4 live cells were analyzed. For the analysis of the cell surface Ag, the following mAbs were used: FITC-conjugated mAb against HLA-ABC (G46-2.6, BD Biosciences, San Jose, CA), HLA-DR (L243, BD Biosciences), CD86 (FUN-1, BD Biosciences), CD83 (HB15a, Immunotech, Marseille, France), CD1a (NA1/34, DakoCytomation, Glostrup, Denmark), CD62L (Dreg 56, BD Biosciences), CCR7 (clone 150503, R&D Systems), CD27 (M-T271, BD Bioscience), and PE-conjugated mAb to CD162 (TB5, Exbio, Praha, Czech), CD8 (RPA-T8, BD Biosciences), and CD4 (RPA-T4, BD Biosciences).

The expression of MMP-II on rBCG-infected DCs was determined using the mAb against MMP-II (M270-13, IgM, κ), which probably detects MMP-II complexed with MHC molecules on the surface of DC (8), followed by FITC-conjugated anti-mouse Igs Ab (Tago-immunologicals,

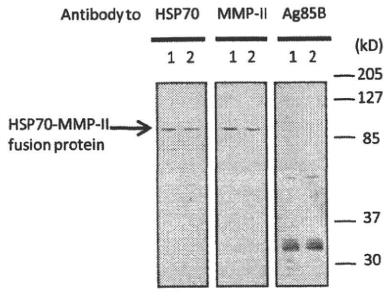

FIGURE 1. Western blotting analysis of protein fraction extracted from BCG-70M and BCG-D70M. An mAb to either MMP-II or HSP70 was used to detect HSP70–MMP-II fusion protein. An anti-Ag85B rabbit polyclonal Ab was also used to detect Ag85B (an internal control). *Lane 1*, Cell lysate of BCG-70M; *lane 2*, cell lysate of BCG-D70M.

Camarillo, CA). For the inhibition of the intracellular processing of phagocytosed bacteria, DCs were treated with 50 μM chloroquine (Sigma-Aldrich) for 2 h, washed, and subsequently infected with rBCG and subjected to analyses of MMP-II surface expression. The intracellular production of perforin was assessed as follows: naive $CD8^+$ T cells were stimulated with rBCG-infected DCs for 5 d in the presence of naive $CD4^+$ T cells, and $CD8^+$ T cells were surface stained with PE-labeled mAb to CD8 and were fixed in 2% formaldehyde. Subsequently, the cells were permeabilized using Permeabilizing solution (BD Biosciences) and stained with FITC-conjugated mAb to perforin (δG9, BD Biosciences) or FITC-labeled isotype control.

APC functions of DCs

The ability of BCG-infected DCs and macrophages to stimulate T cells was assessed using an autologous APC–T cell coculture as previously described (6, 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 >95% when assessed using an FACS-Calibur (BD Biosciences). Naive $CD4^+$ and $CD8^+$ T cells were produced by further treatment of these T cells with mAb to CD45RO, which were followed by beads coated with mAb to goat anti-mouse IgGs (Dynal Biotech). The purity of both subsets of naive T cells was >97%. However, there was no contamination of memory type T cells in the naive T cell preparations. More than 98% of $CD45RA^+$ T cells were positive in the expression of CCR7 molecule. Memory type T cells were similarly produced by the treatment of cells with mAb to CD45RA Ag. The purified responder cells (1×10^5/well) were plated in 96-well round-bottom tissue-culture plates, and DCs or macrophages infected with rBCG 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, rBCG-infected DCs and macrophages were treated with mAb to HLA-ABC (W6/32, mouse IgG2a, κ), HLA-DR (L243, mouse IgG2a, κ), CD86 (IT2.2, mouse IgG2b, κ, BD Biosciences), MMP-II (M270-13), or normal mouse IgG. The optimal concentration was determined in advance. Also, in some cases, immature DCs and macrophages were treated with the indicated dose of chloroquine, brefeldin A (Sigma-Aldrich), or lactacystin (Sigma-Aldrich) and subsequently infected with BCG-D70M. The optimal dose of these reagents was determined in advance.

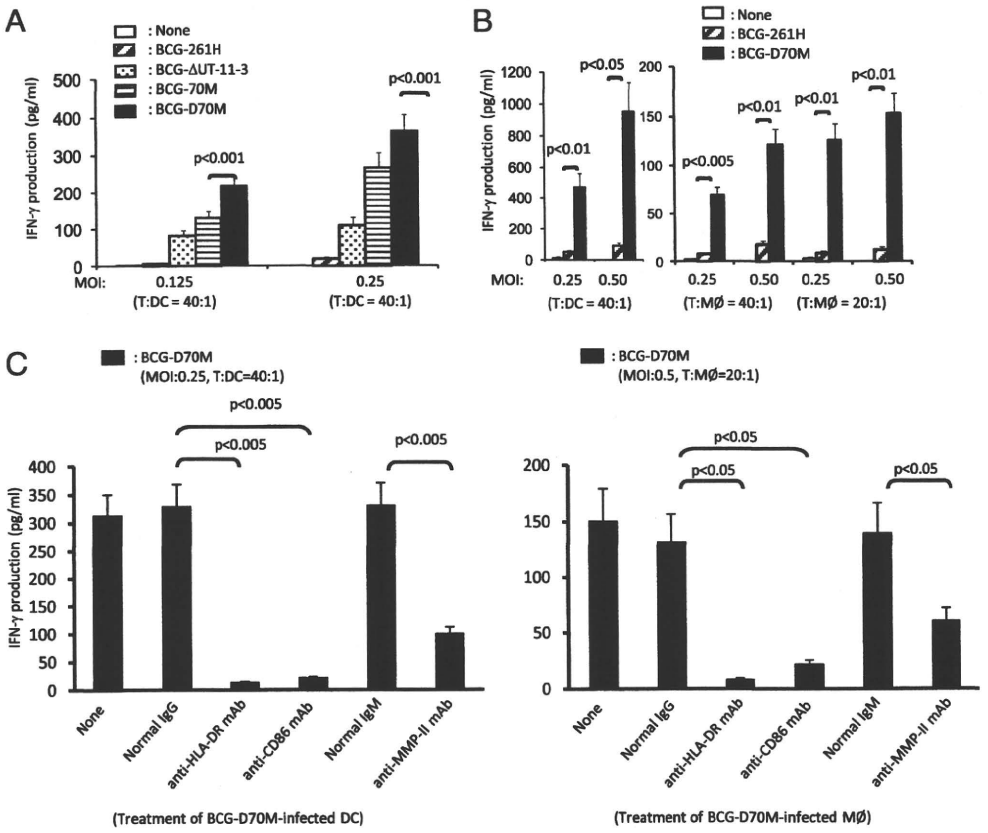

FIGURE 2. *A*, IFN-γ production from naive $CD4^+$ T cells by stimulation with rBCG. Monocyte-derived DCs were infected with BCG-261H, BCG-ΔUT-11-3, BCG-70M, or BCG-D70M at the indicated MOI and were used as a stimulator of naive $CD4^+$ T cells in a 4-d culture. Total of 10^5 responder T cells were stimulated with the BCG-infected DCs at T/DC ratio of 40:1. *B*, IFN-γ production from unseparated $CD4^+$ T cells by stimulation with rBCG. Monocyte-derived DCs or macrophages were infected with either BCG-261H or BCG-D70M at the indicated MOI and were used as a stimulator of unseparated $CD4^+$ T cells in a 4-d culture. Total of 10^5 responder T cells were stimulated with the indicated dose of BCG-infected DCs or macrophages. *C*, Inhibition of naive or unseparated $CD4^+$ T cell activation by the treatment of BCG-D70M–infected DCs or BCG-D70M–infected macrophages, respectively, with mAb. Monocyte-derived DCs or macrophages were infected with BCG-D70M at the indicated MOI and subsequently treated with 10 μg/ml mAb to HLA-DR, CD86, or MMP-II Ags or normal murine IgG or IgM. These APCs were used as the stimulator of responder $CD4^+$ T cells (1×10^5/well) at the indicated T/APC ratio. IFN-γ produced by T cells was measured. A representative of four separate experiments is shown. Assays were performed in triplicate, and the results are expressed as the mean ± SD. Titers were statistically compared using Student *t* test.

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 DCs or macrophages stimulated for 24 or 48 h with rBCGs. The concentrations of these cytokines were quantified using the enzyme assay kit Opt EIA Human ELISA Set (BD Biosciences).

Animal studies

For inoculation into mice, rBCGs were cultured in Middlebrook 7H9 medium to log phase 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 Middlebrook 7H10 agar plate. Three 5-wk-old C57BL/6J mice (Clea Japan, Tokyo, Japan) per group were inoculated s.c. with 0.1 ml PBS or PBS containing 1×10^3 rBCGs. The animals were kept in specific pathogen-free conditions and supplied with sterilized food and water. Four or 12 wk postinoculation, 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 or rHSP70 (HyTest) in triplicate in 96-well round-bottom microplates (15, 16). The individual culture supernatants were collected 3 to 4 d poststimulation, and IFN-γ was measured using the Opt EIA Mouse ELISA Set (BD Biosciences). Five C57BL/6 mice per group were vaccinated with 1×10^3 CFU/mouse either BCG-261H or BCG-D70M for 4 wk and were challenged with 5×10^3/mouse of *M. leprae* in footpad. Thirty-two wk later, the footpad was removed. The number of *M. leprae* grown in the footpad was enumerated by Shepard method (33). 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 *t* test was applied to determine the statistical differences.

Results

Activation of naive T lymphocytes by BCG-D70M

Because BCG-D70M possess two modified characteristics, 1) a lack of urease activity and 2) an expression of HSP70 and MMP-II fusion protein, we assessed the expression level of HSP70 and MMP-II proteins in BCG strains: BCG-70M and BCG-D70M. Both BCG-70M and BCG-D70M equivalently expressed both HSP70 and MMP-II molecules as examined by Western blot analyses using Ag85B as an internal control (Fig. 1). Further, the T cell activation activity of BCG-D70M was evaluated by using not only vector control BCG (BCG-261H), but also rBCGs that lacks urease activity (urease-deficient BCG-ΔUT-11-3 and BCG-70M that secretes HSP70–MMP-II fusion protein) as control BCG (Fig. 2*A*). When these rBCGs were infected to DCs to use as a stimulator of naive $CD4^+$ T cells, both BCG-ΔUT-11-3 and BCG-70M showed higher T cell-stimulating activity than vector control BCG. However, BCG-D70M showed the highest T cell-stimulating activity among these rBCGs at both MOIs: 0.125 and 0.25. More than 350 pg/ml IFN-γ was produced by stimulation with BCG-D70M (MOI: 0.25; T/DC ratio: 40:1). Also, at different T/DC ratios, BCG-D70M exhibited the highest activity (not shown). On addition to IFN-γ, TNF-α was also efficiently produced by BCG-D70M stimulation (not shown). So far, when macrophages were used as APCs, it was difficult to activate $CD4^+$ T cells to produce IFN-γ significantly. In contrast to the activity of rBCGs, such as BCG-261H, BCG-ΔUT-11-3, and BCG-70M (15, 25), newly constructed BCG-D70M efficiently stimulated $CD4^+$ T cells to produce the cytokine through macrophages at various conditions, although the T cell-stimulating activity of macrophages was much less efficient comparing to that of DCs (Fig. 2*B*). More than 100 pg/ml IFN-γ was produced from responder $CD4^+$ T cells; however, BCG-D70M–infected macrophages failed to induce the production of significant dose of IFN-γ from naive $CD4^+$ T cells (not shown). Also, BCG-D70M did not induce IFN-γ production from DCs or macrophages (not shown). Although normal murine IgG did not affect the T cell-stimulating activity of both BCG-D70M–infected DC and the BCG-D70M–infected macrophages, the treatment of these APCs with either anti–HLA-DR mAb, anti-CD86 mAb, or anti–MMP-II mAb significantly inhibited the activation of naive $CD4^+$ T cells and $CD4^+$ T cells, respectively (Fig. 2*C*). More than 90% of IFN-γ production was inhibited by the treatment of APCs when mAb to HLA-DR or CD86 was used. Furthermore, when naive $CD8^+$ T cells were stimulated with DC infected with various rBCGs, BCG-D70M induced the strongest activation of naive $CD8^+$ T cells (Fig. 3*A*). Both BCG-70M and BCG-D70M induced significant IFN-γ production, but BCG-D70M activated the T cells more strongly than BCG-70M. More than 400 pg/ml IFN-γ can be produced from naive $CD8^+$ T cells. These phenomena were observed consistently at various conditions including the different MOIs and T cell/DC ratios, although high doses of BCG-D70M and high doses of BCG-D70M–infected DCs were required to induce the production of abundant IFN-γ from naive $CD8^+$ T cells compared with the dose required to stimulate naive $CD4^+$ T cells. Again, when BCG-D70M–infected DCs were treated with mAb to either HLA-ABC or CD86, the IFN-γ production from naive $CD8^+$ T cells was significantly inhibited, whereas normal murine IgG did not affect the activation of the responder T cells (Fig. 3*B*).

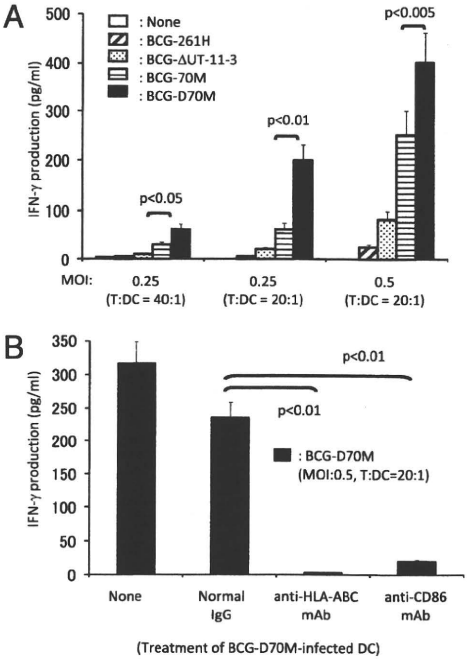

FIGURE 3. *A*, IFN-γ production from naive $CD8^+$ T cells by stimulation with BCG. Monocyte-derived DCs were infected with BCG-261H, BCG-ΔUT-11-3, BCG-70M, or BCG-D70M at the indicated MOI and were used as a stimulator of responder naive $CD8^+$ T cells in a 4-d culture. Total of 10^5 responder T cells were stimulated with the BCG-infected DCs at the indicated T/DC ratio. *B*, Inhibition of naive $CD8^+$ T cell activation by the treatment of BCG-D70M–infected DCs with mAb. Monocyte-derived DCs were infected with BCG-D70M at an MOI of 0.5 and subsequently treated with 10 μg/ml mAb to HLA-ABC or CD86 Ags or normal murine IgG. These DCs were used as the stimulator of naive $CD8^+$ T cells (1×10^5/well) at T/DC ratio of 20:1. IFN-γ produced by 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 *t* test.

Effect of BCG-D70M on DCs

To stimulate responder T cells efficiently, APCs susceptible to BCG infection should be adequately activated. We assessed the activation of DCs from the aspects of cytokine production and phenotypic changes (Fig. 4). When DCs were stimulated with various rBCGs including BCG-261H, BCG-ΔUT-11-3, BCG-70M, and BCG-D70M, BCG-D70M stimulated DCs to produce IL-12p70 most efficiently at both MOIs: 0.25 and 0.5 (Fig. 4*A*). Further, BCG-D70M induced significantly higher dose of IL-1β and TNF-α production from DCs and also induced a higher dose of TNF-α from macrophages compared with BCG-261H (Fig. 4*B*). To assess the phenotypic changes induced by BCG-D70M infection, we assessed the expression of MHC, CD86, CD83, and CD1a molecules on DCs (Fig. 4*C*). Infection with BCG-D70M induced significantly higher level of expression of HLA-ABC, HLA-DR, CD86, and CD83 Ags compared with BCG-261H infection. The expression of CD1a was significantly downregulated. We used various dose of rBCGs for the assessment, and the similar changes were observed (not shown). These results indicated that BCG-D70M more efficiently activated DCs than BCG-261H.

Characteristics of BCG-D70M

Previously, we reported that BCG-70M, which was genetically manipulated to produce HSP70–MMP-II fusion protein, induced expression of MMP-II on the surface of BCG-70M–infected DCs (25). We then analyzed the BCG-D70M–infected DCs in terms of MMP-II expression (Fig. 5*A*). Whereas DCs uninfected or infected with BCG-261H did not express MMP-II significantly, BCG-D70M induced significant expression of MMP-II. Further, when immature DCs were treated with chloroquine, an inhibitor of phagosomal acidification, in advance to the infection with BCG-D70M, the MMP-II expression was significantly inhibited. In addition to the MMP-II expression, the chloroquine treatment on DCs affects the activation of responder T cell by BCG-D70M (Fig. 5*B*). IFN-γ production from naive $CD4^+$ T cells by stimulation with BCG-D70M (MOI: 0.125 and 0.25) was significantly

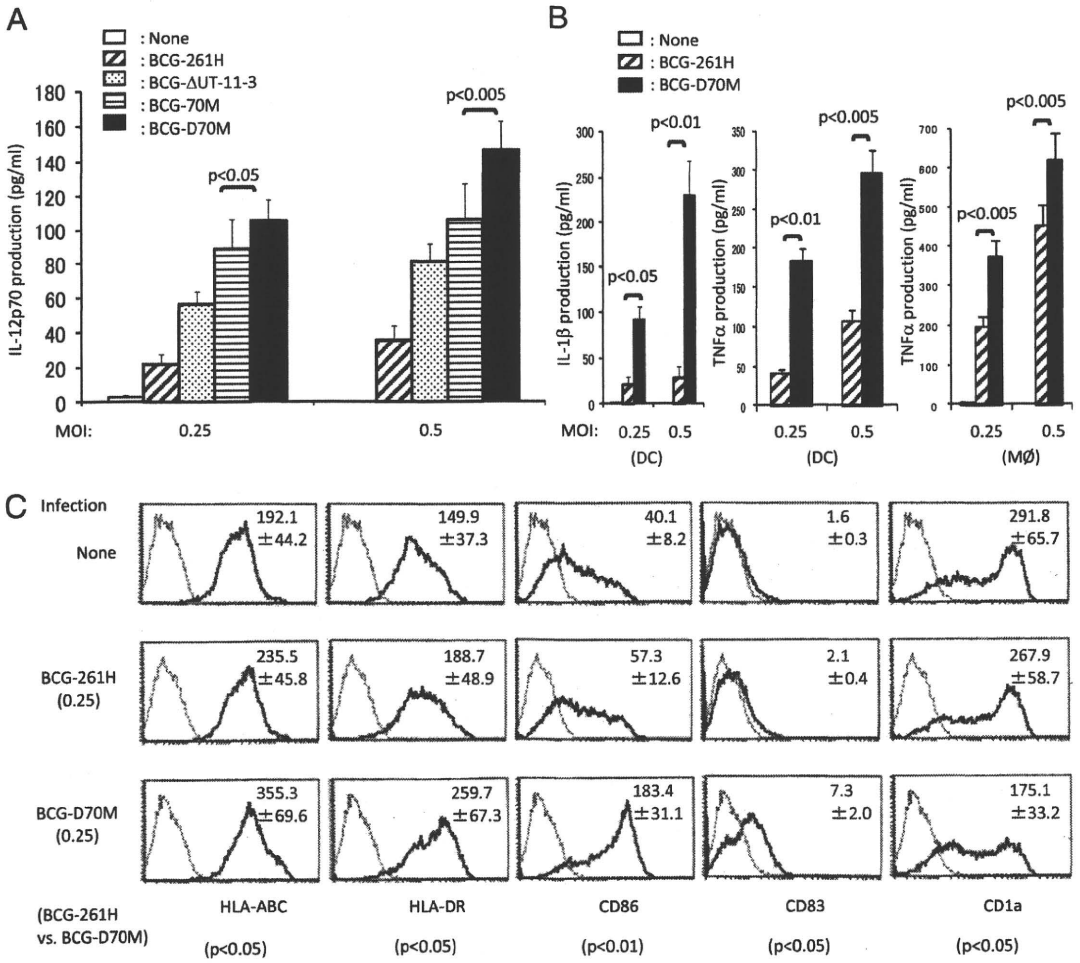

FIGURE 4. *A*, IL-12p70 production from DCs stimulated with rBCG. Monocyte-derived DCs from 5 d of culture in the presence of rGM-CSF and rIL-4 were stimulated with the indicated dose of BCG-261H, BCG-ΔUT-11-3, BCG-70M, or BCG-D70M for 48 h. The concentration of IL-12p70 was determined by the ELISA method. *B*, Cytokine production from DCs or macrophages stimulated with rBCG. DCs produced using rGM-CSF and rIL-4 or macrophages from 5 d of culture in the presence of rM-CSF were stimulated with either BCG-261H or BCG-D70M 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 *t* test. *C*, Upregulation of APC-associated molecules and activation marker on DCs by BCG-D70M infection. Monocyte-derived immature DCs were infected with either BCG-261H or BCG-D70M at an MOI of 0.25 and cultured for another 2 d in the presence of rGM-CSF and rIL-4. The DCs from day 5 of culture 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 corner of each panel represents the mean ± SD for three independent experiments of the difference in the fluorescence intensity between the control IgG and test mAb. Titers of BCG-261H–infected DCs and BCG-D70M–infected DCs were statistically compared using Student *t* test.

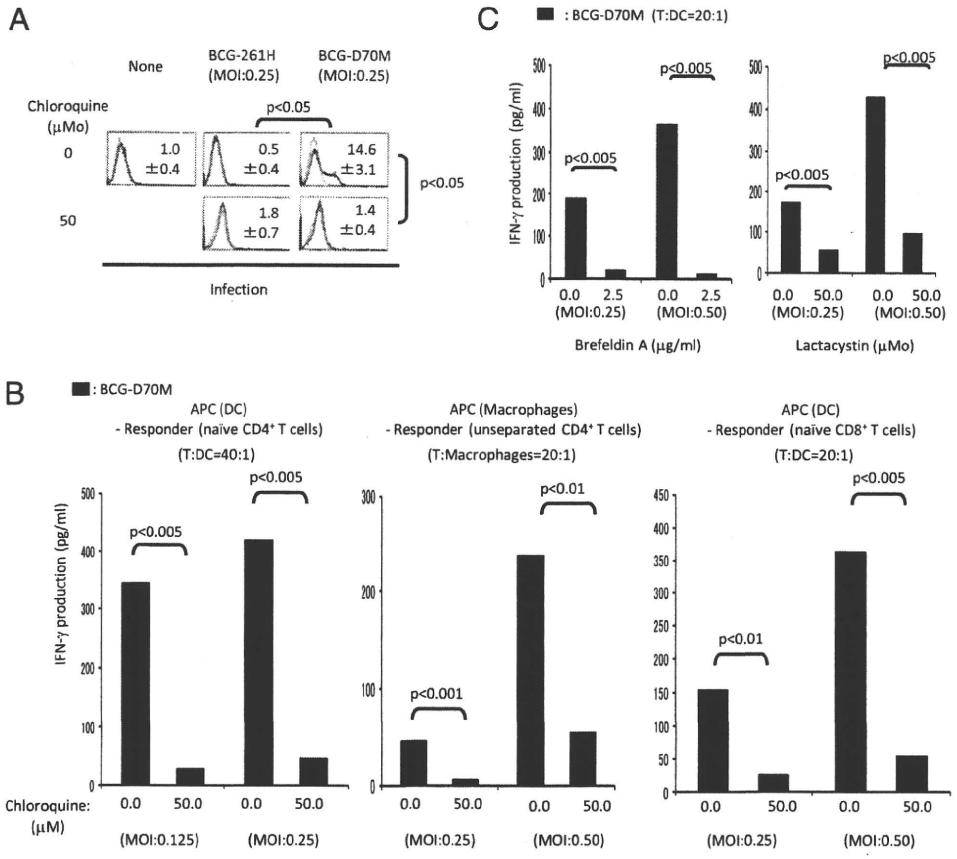

FIGURE 5. *A*, Expression of MMP-II on DCs. Immature DCs were either treated with 50 μM chloroquine for 2 h or untreated and subsequently infected with either BCG-261H or BCG-D70M at an MOI of 0.25. After 2 d culture in the presence of rGM-CSF and rIL-4, DCs were gated and analyzed. Dotted lines, control normal IgM; solid lines, anti–MMP-II mAb (IgM). The number in the top right corner of each panel represents the mean ± SD for six independent experiments of the difference in mean fluorescence intensity between the control IgM and the test mAb. Representative results of six separate experiments are shown. *B*, Effect of chloroquine treatment of DCs and macrophages on the activation of T cells. Immature DCs from 4 d of culture were treated with chloroquine (50 μM, 2 h) or untreated and subsequently infected with BCG-D70M at the indicated MOI. These DCs were used as the stimulator of responder autologous naive $CD4^+$ T cells or naive $CD8^+$ T cells (1×10^5/well) at T/DC ratio of 40:1 or 20:1, as indicated. Macrophages from 5 d of culture in the presence of M-CSF were treated with chloroquine as above and subsequently infected with BCG-D70M at the indicated MOI. These macrophages were used as the stimulator of responder autologous unseparated $CD4^+$ T cells (1×10^5/well) at T/DC ratio of 20:1. IFN-γ produced by T cells was measured. *C*, Effect of treatment of immature DCs with brefeldin A or lactacystin on the activation of naive $CD8^+$ T cells. Immature DCs from 4 d of culture were treated with the indicated dose of brefeldin A or lactacystin or untreated and subsequently infected with BCG-D70M at the indicated MOI. These DCs were used as the stimulator of responder autologous naive $CD8^+$ T cells (1×10^5/well) at T/DC ratio of 20:1. IFN-γ produced by 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 *t* test.

inhibited by chloroquine. Also, on treatment of macrophages with chloroquine, ~90% of IFN-γ produced from $CD4^+$ T cells by BCG-D70M stimulation (MOI: 0.25 and 0.50) was inhibited. Similarly, the production of IFN-γ from naive $CD8^+$ T cells was significantly inhibited by the chloroquine treatment of DCs, which were subsequently infected with BCG-D70M (MOI: 0.25 and 0.50). We further confirmed that BCG-D70M secreted 92 kDa protein (molecular mass: MMP-II 22 kDa and HSP70 70 kDa) in vitro (not shown). These results suggest the possibility that the secreted HSP70–MMP-II fusion protein is one of the responsible elements for the activation of both $CD4^+$ T cells and $CD8^+$ T cells as observed in BCG-70M. Further, we previously reported that BCG-70M stimulated naive $CD8^+$ T cells through TAP- and proteosome-dependent cytosolic cross-presentation pathway, because the T cell activation was inhibited by the pretreatment of DCs with brefeldin A and lactacystin (25). In this study, we assessed whether BCG-D70M activates naive $CD8^+$ T cells by using the similar cross-presentation pathway (Fig. 5*C*). When immature DCs were treated with either brefeldin A or lactacystin and were subsequently infected with BCG-D70M at MOI 0.25 or 0.50, the IFN-γ production from naive $CD8^+$ T cells was significantly inhibited.

Effect of $CD4^+$ T cells on the activation of naive $CD8^+$ T cells

Because BCG-D70M activated both naive $CD4^+$ T cells and naive $CD8^+$ T cells, we assessed the influence of the presence of $CD4^+$ T cells on the activation of naive $CD8^+$ T cells (Fig. 6*A*). The naive unseparated T cell population was stimulated with DCs infected with BCG-261H or BCG-D70M, and $CD8^+$ T cells were gated and analyzed by FACS. Compared to $CD8^+$ T cells stimulated with BCG-261H, the BCG-D70M–stimulated naive $CD8^+$ T cells produced significantly higher number of perforin-producing $CD8^+$ T cells and $CD62L^{low}$ $CD8^+$ T cells. Further, $CCR7^{low}$ $CD8^+$ T cells and $CD27^{low}$ $CD8^+$ T cells were more efficiently produced by BCG-D70M stimulation (Fig. 6*A*). The efficient production of these $CD8^+$ T cells was observed with different doses of BCG; however, in the absence of naive $CD4^+$ T cells, these changes were not induced (not shown). Also, $CD4^+$ T cells producing intracellular perforin was not produced from naive $CD4^+$ T cells by the stimulation with BCG-D70M–infected

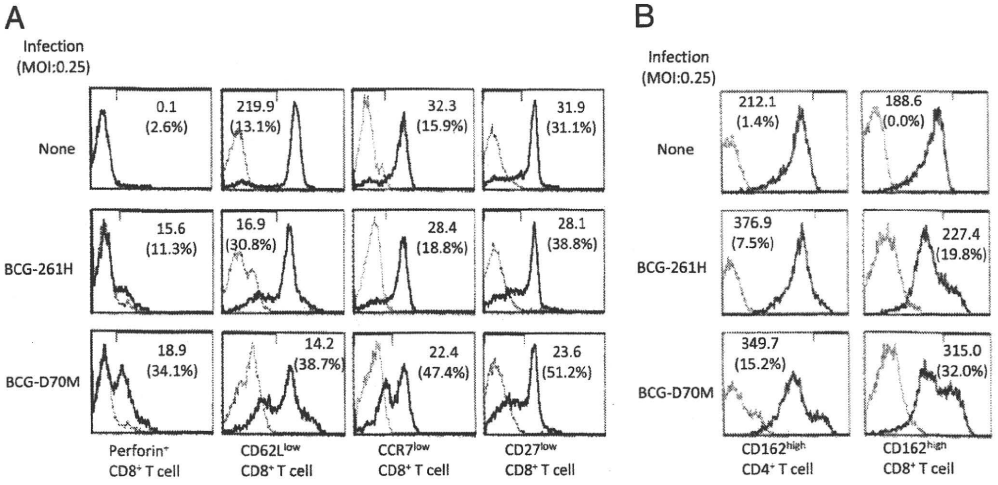

FIGURE 6. *A*, Perforin production and expression of activation or memory marker on naive $CD8^+$ T cells stimulated with DCs infected with BCG-D70M. Monocyte-derived DCs were infected with either BCG-261H or BCG-D70M at an MOI of 0.25 and were cocultured with naive T cells (T/DC 20:1) for 5 d. The stimulated $CD8^+$ T cells were gated and analyzed for perforin production and expression of the indicated molecules. The number in the top right corner of each panel represents the difference in the fluorescence intensity between the control IgG and the test mAb. The number in the parenthesis indicated either percent perforin-positive $CD8^+$ T cells or $CD8^+$ T cells expressing the test Ags among $CD8^+$ T cell population. *B*, Expression of migration marker on naive T cells. Monocyte-derived DCs were infected with either BCG-261H or BCG-D70M at an MOI of 0.25 and were cocultured with naive T cells (T/DC 20:1) for 5 d. The stimulated T cells of either $CD4^+$ or $CD8^+$ subsets were gated and analyzed for expression of CD162 molecules. The number in the top right or left corner of each panel represents the difference in the fluorescence intensity between the control IgG and the test mAb. The number in the parentheses indicated percent $CD162^{high}$ T cells among $CD4^+$ or $CD8^+$ T cell population. 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 *t* test.

DCs. These results indicate that BCG-D70M may produce effector $CD8^+$ T cells having killing activity and memory type $CD8^+$ T cells efficiently from naive population. When BCG is used as a vaccine, it is required to produce memory T cells having a high migratory function (34). To examine this point, we assessed the expression of CD162 molecules on both $CD4^+$ T cells and $CD8^+$ T cells, which were stimulated with DCs infected with BCG-D70M (Fig. 6*B*). On BCG-D70M stimulation, both $CD4^+$ T cells and $CD8^+$ T cells that express high levels of CD162 Ag were produced more efficiently than by the stimulation with BCG-261H. A similar difference between BCG-261H and BCG-D70M was induced when different doses of BCG were used (not shown).

Production of T cells responsive to the secondary stimulation by BCG-D70M in vivo

The ability of BCG-D70M to produce T cells highly responsive to the secondary in vitro stimulation was examined by in vivo functional studies (Fig. 7). C57BL/6 mice were s.c. inoculated with 1×10^3/mouse of rBCGs 4 wk prerestimulation in vitro. Both MMP-II and HSP70 proteins were used as a restimulator. These proteins induced IFN-γ production from T cells in all infected or uninfected mice because they have high immunogenicity, and BCG-Tokyo, a parental strain of all rBCGs, has the gene encoding BCG-derived MMP-II. However, splenic T cells from BCG-D70M–infected mice respond most vigorously to the stimulators and produced higher doses of IFN-γ (Fig. 7*A*) and IL-2 (not shown) than those from mice uninfected or infected with control rBCGs including BCG-261H, BCG-ΔUT-11-3, and BCG-70M. To examine the long-term effect of the inoculation of rBCGs on the production of such responsive T cells, C57BL/6 mice were s.c. inoculated with 1×10^3/mouse of rBCGs 12 wk before the restimulation. Again, a significantly higher dose of IFN-γ (Fig. 7*B*) was produced from splenic T cells obtained from mice inoculated with BCG-D70M by the stimulation with MMP-II and HSP70 than those from mice uninfected or infected with control rBCGs.

Effect of BCG-D70M vaccination on the multiplication of M. leprae *in vivo*

C57BL/6 mice vaccinated with either BCG-261H or BCG-D70M (1×10^3 CFU/mouse) for 4 wk were challenged with 5×10^3 *M. leprae* in the footpad. Thirty-two weeks later, the footpad was removed, and the *M. leprae* recovered from the footpad was enumerated (Fig. 8). A total of 2×10^5 *M. leprae* were recovered from mice inoculated with PBS and challenged with *M. leprae*. Although the mice vaccinated with BCG-261H inhibited the multiplication of *M. leprae* significantly, the BCG-D70M vaccination significantly and more efficiently inhibited the *M. leprae* multiplication than BCG-261H. A similar difference was observed when 1×10^2 CFU/mouse rBCG was inoculated for the inhibition of *M. leprae*.

Discussion

M. leprae is well-known as a representative slow-growing *Mycobacterium*. Usually, *M. leprae* needs 12–14 d for one division and at least 2–5 y for the manifestation of the disease. In vivo studies using the immunodeficient nude mouse indicate that adaptive immunities play an important role in inhibiting the multiplication of *M. leprae*, and the activation of both $CD4^+$ T cells and $CD8^+$ T cells is an essential element for controlling *M. leprae* infection (5, 6, 35). Although $CD4^+$ T cells chiefly act at the initial phase of infection, the contribution of $CD8^+$ T cells in terms of IFN-γ production and killing of mycobacteria-infected host cells is necessary in the chronic phase of the infection (36). BCG was used so far as vaccine against leprosy; however, its efficacy is nowadays considered not as convincing as expected (12). The reason for why BCG cannot prevent the leprosy manifestation convincingly may be due to its inadequate ability to stimulate T cells. The poor T cell-stimulating activity seems to be based on the intrinsic defect of BCG not being able to enter the lysosome feasibly. Also, poor stimulation of T cells would result in the meager production of competent memory T cells, including both $CD4^+$ and $CD8^+$ subsets, capable of convincingly responding

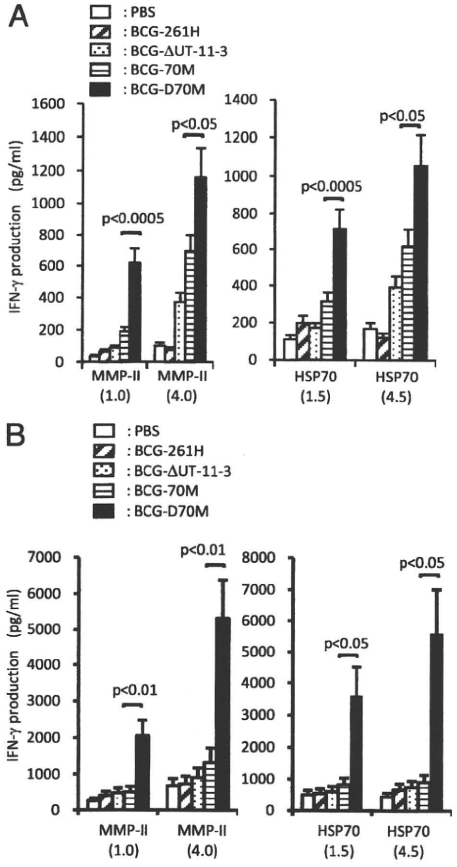

FIGURE 7. *A*, Production of T cells responsive to the secondary in vitro stimulation in C57BL/6 mice by infection with rBCG. Three 5-wk-old C57BL/6 mice per group were infected with 1×10^3 CFU/mouse BCG-261H, BCG-ΔUT-11-3, BCG-70M, or BCG-D70M s.c. Four weeks postinoculation, splenocytes (2×10^5 cells/well) were stimulated with the indicated dose of either MMP-II or HSP70 for 4 d in vitro, and IFN-γ produced in the cell supernatant was measured. *B*, Production of T cells capable of responding to the secondary stimulation in C57BL/6 mice a long time postinfection with rBCG. Three 5-wk-old C57BL/6 mice per group were infected with 1×10^3 CFU/mouse rBCGs s.c. Twelve weeks postinoculation, splenocytes (2×10^5 cells/well) were stimulated with the indicated dose of either MMP-II or HSP70 for 3 d in vitro, 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 three separate experiments are shown. Titers were statistically compared using Student *t* test.

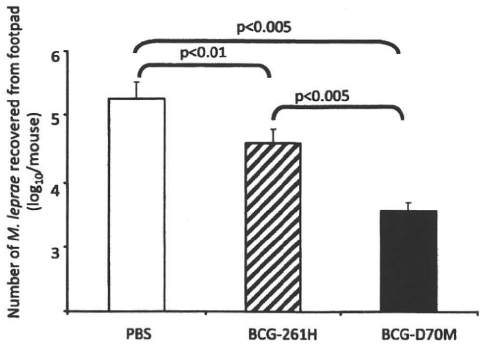

FIGURE 8. Inhibition of *M. leprae* multiplication by s.c. vaccination with BCG-D70M. Five-week-old C57BL/6 mice (5 mice/group) were vaccinated with 1×10^3 CFU/mouse either BCG-261H or BCG-D70M s.c. and were challenged with 5×10^3 bacilli/mouse *M. leprae* in footpad 4 wk postvaccination. The number of *M. leprae* recovered from the footpad at 32 wk postchallenge was enumerated by Shepard's method (33). Representative results of three separate experiments are shown. Titers were statistically compared using Student *t* test.

to mycobacterial Ags. Especially, BCG cannot activate naive $CD8^+$ T cells adequately in the absence of $CD4^+$ T cell-derived help (14), so that BCG may poorly control the disease in the chronic phase or in the inhibition of disease manifestation for a long time postinfection (14). This fact is important when the growth rate of *M. leprae* is taken into account.

However, BCG has also intrinsic benefit, because it activates human naive $CD4^+$ T cells to produce IFN-γ to some extent. Therefore, we tried to improve the potency of BCG, especially with regard to immunostimulatory activities. We chiefly focused on overcoming the defect of BCG—that is, the ability to avoid the fusion of BCG-infected phagosomes with lysosomes. One of the approaches we carried out previously is the production of *UreC* gene-deficient rBCG (BCG-ΔUT-11-3), which successfully produces acidic phagosomes and facilitates them to fuse with lysosomes (15). In fact, BCG-ΔUT-11-3 efficiently colocalizes with lysosomes and preferentially and effectively stimulates human naive $CD4^+$ T cells (15). Therefore, the disruption of the *UreC* gene of BCG seems to be a useful strategy to translocate BCG to lysosomes. However, unfortunately, BCG-ΔUT-11-3 did not activate naive $CD8^+$ T cells effectively. Then, the second approach for overcoming the lack of phagosome–lysosome fusion was carried out—that is, to induce the secretion of immunodominant Ag into phagosome. In this study, we used MMP-II as the immunodominant Ag of *M. leprae* (7). In one case, sole MMP-II protein (production of BCG-SM) and in the other case HSP70–MMP-II fusion protein (production of BCG-70M) secreting BCG was constructed (16, 25). Both BCGs were quite efficient in the induction of activation of not only naive $CD8^+$ T cells, but also naive $CD4^+$ T cells. However, BCG-70M was superior to BCG-SM in activating both subsets of T cells, especially naive $CD8^+$ T cells (not shown). The activation of naive $CD8^+$ T cells by BCG-70M is highly dependent on the secretion of HSP70–MMP-II fusion protein, because the activation seems to be induced by TAP- and proteosome-dependent cross-presentation of the secreted protein (24, 25, 37). Therefore, the secretion of MMP-II in the combination with HSP70 seems to be an efficient strategy to overcome the intrinsic defect of BCG.

Because the two independent strategies for overcoming the intrinsic defect of BCG were useful, we tried to combine both strategies and produced new rBCG (BCG-D70M), in which BCG-ΔUT-11-3 was integrated with gene encoding HSP70–MMP-II fusion protein. As previously reported (25), BCG-70M secreted 92 kDa HSP70–MMP-II fusion protein after being phagocytozed by APCs, and the secreted protein was transported to functional lysosomes. In the phagolysosomes, some portions of HSP70–MMP-II fusion protein could be degraded, but rest of the protein may be sequestrated into the cytosol, where they could be degraded and used for cross-priming $CD8^+$ T cells. In this respect, when immature DCs were pretreated with chloroquine and subsequently infected with newly produced BCG-D70M, both the expression of MMP-II and the activation of naive $CD4^+$ and $CD8^+$ T cells by the rBCG were inhibited. Thus, protein secreted from BCG-D70M seems to be responsible for the activation of naive T cells. Further, the activation of naive $CD8^+$ T cells by BCG-D70M was also abolished by pretreatment of immature DCs with lactacystin, a proteosomal protein degradation blocker and brefeldin A that is an inhibitor of antegrade Golgi transportation and of TAP-dependent transportation. Therefore, it is highly likely that the 92-kDa fusion protein secreted from BCG-D70M could be sequestrated into cytosol from lysosome, degraded in proteosome, and used for loading on MHC class I molecules through the TAP-

dependent pathway. Thus, similar to BCG-70M, BCG-D70M also used the cytosolic pathway, which is known as the most effective cross-presenting pathway (38), to cross-prime $CD8^+$ T cells. In this respect, it is known that HSP plays an important role in the induction of the cytosolic cross-presentation pathway (39, 40). HSP70 secreted as a part of the fusion protein seems to be closely associated with the cross-priming $CD8^+$ T cells. The activation of both naive $CD8^+$ T cells and naive $CD4^+$ T cells by BCG-D70M was induced in an Ag-specific fashion, because treatment of BCG-D70M–infected DCs with mAb to MHC molecules or CD86 Ag inhibited the IFN-γ production from naive T cells. However, the naive $CD4^+$ T cells seemed to be polyclonally activated by the stimulation, because the treatment of DCs with mAb to MMP-II partially, but significantly, inhibited the activation (Fig. 2*C*). In C57BL/6 mice, a single injection of BCG-D70M produced T cells capable of responding to both MMP-II and HSP70 several weeks postinoculation. Therefore, the HSP70–MMP-II fusion protein activated both APCs and T cells by the similar mechanisms as observed in in vitro experiments and was probably used as antigenic molecules in vivo. Because *M. leprae*-infected DCs expressed MMP-II–derived antigenic determinants on their surface (7, 16), the production of T cells responsive to MMP-II in vivo may be useful to prevent the disease manifestation. This speculation seems to be supported by the present observation that the vaccination with BCG-D70M more efficiently inhibited the multiplication of *M. leprae* in vivo than that with vector control BCG.

The activities stimulating both subsets of naive T cells of BCG-D70M were strongest among the all rBCGs produced so far including BCG-70M. Although all of the rBCGs showed the dose-dependent effect in the T cell activation, BCG-D70M showed the strongest activity in terms of the T cell activation, even if an MOI 1.0 of BCG was used. Further, BCG-D70M most strongly activated DCs as revealed by IL-12p70 production from DCs. Because BCG-70M activated DCs through the binding of HSP70–MMP-II fusion protein with TLR2 (25), BCG-D70M seems to activate DCs with a similar mechanism, at least partially. However, it did not induce an apoptotic cell death of target APCs including DCs and macrophages, the in vitro growth rate of BCG-D70M was almost identical with that of BCG-261H, and further, the infectivity of these rBCGs to host cells in both in vitro and in vivo was identical (not shown). It is likely that the stronger DC- and T cell-activating ability of BCG-D70M than BCG-70M might be due to the absence of ammonia, products of *UreC* gene-encoding urease, in the phagosome. The urease depletion may facilitate the translocation of HSP70–MMP-II fusion protein secreted in phagosomes from BCG-D70M into lysosomes. However, another explanation could be that the absence of ammonia may facilitate the translocation of BCG-D70M itself to lysosomes, because it has previously reported that BCG-ΔUT-11-3 more efficiently entered lysosomes than parent BCGs, which possess the *UreC* gene (15, 19). BCG-D70M translocated into lysosomes or phagolysosomes secreted HSP70–MMP-II fusion protein. Therefore, it can be speculated that a larger dose of secreted protein that could be efficiently processed would be available in lysosomes, so that much or many types of antigenic peptides could be loaded on the MHC molecules. This speculation is important because it has recently been reported that quick activation of $CD8^+$ T cells by BCG requires the high antigenic load on MHC class I molecules (41). These results indicate that the deletion of urease from BCG and integration of gene encoding fusion protein into BCG may act synergistically, although further detailed analyses is required.

The strong ability of BCG-D70M to stimulate T cells enables macrophages to activate $CD4^+$ T cells. The $CD4^+$ T cells stimulated by BCG-D70M through macrophages seemed to be activated in an Ag-specific manner, because the IFN-γ production from the T cells was largely blocked by the treatment of BCG-D70M–infected macrophages with mAbs to MHC class II and CD86 Ags. So far, rBCG including BCG-ΔUT-11-3 and BCG-70M did not activate $CD4^+$ T cells efficiently through macrophages in the absence of costimulators such as CD40L and IFN-γ (15, 25). The definite reason for why BCG-D70M, but not BCG-70M, could activate $CD4^+$ T cells through macrophages remains unanswered. However, the secreted fusion protein either in the phagosome or phagolysosome could be associated with $CD4^+$ T cell activation through macrophages, because pretreatment of macrophages with chloroquine abolished their T cell-stimulating activities. BCG infects not only DCs, but also macrophages, which are highly active in phagocytosis of bacteria; thus, the successful activation of $CD4^+$ T cells by macrophages upon an infection with BCG-D70M would provide many chances to heterogeneous $CD4^+$ T cells to receive antigenic stimuli. The $CD4^+$ T cell activation by macrophages should contribute to the efficient production of high doses of IFN-γ and to the production and maintenance of abundant memory T cells. In addition, in the presence of the help of $CD4^+$ T cells, naive $CD8^+$ T cells were differentiated into $CCR7^{low}CD8^+$ and $CD27^{low}CD8^+$ memory type T cells by the stimulation with BCG-D70M. Also, they produced phenotypically activated $CD62L^{low}CD8^+$ T cells as well as perforin-producing effector $CD8^+$ T cells. Therefore, the efficient activation of naive and memory type $CD4^+$ T cells may contribute to the efficient production of effector and memory $CD8^+$ T cells. In our hands, we could not confirm the possibility that the functional perforin-producing $CD8^+$ T cells, which are produced from naive T cells, can be further differentiated into memory subsets. If this were the case, effector $CD8^+$ T cells having killing activity can be immediately and efficiently produced from such memory T cells upon an infection with *M. leprae* in vivo.

It has been reported that to prevent the disease manifestation induced by infection with mycobacteria, such as *M. tuberculosis*, by vaccination, the vaccinating agents should be able to produce memory T cells that have a high potency to migrate into the infection site (34). Thus, we evaluated whether BCG-D70M can produce T cells with a migration activity by monitoring the surface expression of CD162 molecules. The stimulation of naive T cells with BCG-D70M–infected DCs induced the expression of CD162 on both $CD4^+$ T cells and $CD8^+$ T cells. Therefore, it could be assumed that BCG-D70M may be a convincing stimulator of naive T cells.

Taken together, in this study, we newly constructed an rBCG that is deficient in production of urease, but instead produced HSP70–MMP-II fusion protein and is capable of effectively and strongly activating both naive $CD4^+$ and $CD8^+$ T cells, thus overcoming the intrinsic defect of BCG. Using the triple combination of expressing HSP70 and MMP-II protein in BCG and depletion of urease may result in sufficient production of memory T cells by activating both subsets of naive T cells in human.

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Disclosures

The authors have no financial conflicts of interest.

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WHO・薬剤耐性らい菌拠点監視事業に関する会議　報告

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平成22年11月9日、10日の両日、国立感染症研究所、戸山庁舎においてWHO, South-East Asia Region Office (SEARO), Global Leprosy Programme (GLP)による第3回Meeting on Sentinel Surveillance for Drug Resistance in Leprosyが開催された。事業の概要と今回の会議について報告する。

経　緯

ハンセン病の治療は、WHOが推進し1980年代に始まった多剤併用化学療法（MDT）により多大な効果が見られ、世界のハンセン病有病率は著しく低下した。しかしながら他の感染症において広く知られているように、ハンセン病においても抗生物質に対する耐性菌の出現が危惧された。事実、MDTが導入される前には多くのdapsoneあるいはrifampicinに対する単剤耐性菌の増加あるいは多剤耐性菌の例が報告され、MDT導入後においても主として再発例において、耐性例の報告がなされた。しかしながらそれらのほとんどは散発的なケースレポートであり、包括的なデータに基づいて耐性菌の伝搬状況を正確に把握できるものではなかった。かつて、結核はBCGと化学療法により予防と治療が可能であり、容易に制御できるものと考えられたが、近年の耐性結核菌の出現は結核対策を極めて困難なものとしている。GLPの担当官はハンセン病においても同様な轍を踏まないためにも、現行のMDT下における耐性菌の伝搬状況を把握し、その有効性を維持することを意図していた。しかしながら、周知のとおり、らい菌の薬剤感受性は、従来マウスfootpadによって実施されてきたため、多数の症例について検査し、包括的情報の収集に基づいた実態の把握は不可能であった。

2005年4月、Scotlandの Aberdeenにおいて開催された第8回WHO Technical Advisory Group on the Leprosy Controlに参加した邑久光明園の副園長であった畑野研太郎現園長は、当時GLP team leaderであったDr. Vijaykumar Pannikarから簡便な方法により、高ハンセン病有病率を示す国々で得た材料を解析する方法がないか相談を受けた。Dr. Pannikarはrifampicinがその高いbactericidal作用から、かなめの薬剤となっていることに鑑み、その耐性は現行の化学療法に基づくハンセン病対策を危うくするものであると考え、その実態把握が重要と考えた。これに対し、畑野園長は日本おいては1990年代後半より遺伝子変異の検索により、難治例、再発例を初めとしてdapsone, rifampicin, quinoloneに対する耐性の有無を検査していることを報告した。

これまで行われた会議における議題と討議の結果

以上の経緯を踏まえ、2006年4月30日および

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5月1日に、インドのAgraにおいてRifampicin Resistance in Leprosy と題する非公式協議会がSEAROにより企画された。会議には松岡も含め、11カ国16名が参加した。利用可能な薬剤耐性菌の検出技術、rifampicin耐性菌検出例に関する知見等が報告された。rifampicin耐性菌について早急に拠点監視体制を確立することが確認され、その伝播について動向を知るために、選定された場所において長期にわたり観察を継続することが合意され、「ハンセン病の薬剤耐性拠点監視事業」として当面は再発例での耐性菌の伝播状況を把握することから開始することとなった。耐性菌を検出する方法はPCR direct-sequencing法により行うこととした。検査方法のガイドラインが策定され、rifapmicin以外の薬剤の検査についても協議された。

続いて2008年10月20日から22日にベトナムのHanoiにおいて第1回の「ハンセン病の薬剤耐性拠点監視事業に関するワークショップ」が、National Institute of Dermato-Venereology の Dr. Tran Hau Khang所長の運営により開催された。12カ国より32名、WHOより8名が参加した。日本からは、松岡、甲斐と北海道大学の鈴木教授が参加した。会議においては事業遂行のための技術的諸問題、監視対象地域の選定、それらにおけるハンセン病の実態及び対策が報告され、監視拠点と検査を担当する研究機関との連携について討議された。監視対象地域には6カ国の14カ所が選定され、ハンセン病研究センターをはじめ10カ所の研究施設が遺伝子の解析を担当することになった。会議後、コロンビア、中国が加わった。ハンセン病研究センターはミャンマーの2カ所、ベトナムの1カ所について収集された検体について検査を担当することとした。拠点および検査担当機関について表1に示した。また当センターはquality controlを担当することになった。検体はslit skin法により採取され、従来我々が行ってきた方法である70%エタンールにより保存して検査施設に送付することで合意された。FTAカードの有用性が提案され、次回会議までに適応の可否を検討するデータを得ること等についても合意した。

第2回会議は2009年10月26日、27日両

表1．耐性菌監視拠点と検査担当施設

監視拠点		検査担当施設
国名	施設	
Brazil	1, Laboratorio de Hanseniase FIOCRUZ, Rio de Janeiro, RJ 2, Centro de Refréncia em Delmatorogia Snitária Dona Libánia, Fortaleza, CE 3, Fundação Alfledo da Matta, Manaus, M 4, Instituto Lauro de Souza Lima Bauru, SP 5, Centro de Referencia Estadual em Dermatogia Sanitária em Hanseniase, Uberlância	1, Laboratorio de Hanseniase Instituto Oswaldo Cruz (IOC) FIOCRUZ, Rio de Janeiro, 2, Instituto Lauro de Souza Lima Bauru, 3, Centro de Referencia Estadual em Dermatogia Sanitária em Hanseniase
Ethiopia	1, Dermatology Department, ALERT Hospital, Addis Ababa 2, Armauer Hansen Research Institute (AHRI) Addis Ababa	National Reference Center on Mycobacteria, Faculte de Madicine Pitie-Slpetriere, Paris, France
India	1, TLM Community Hospital, Shahdara, New Delhi 2, JALMA, Agra 3, Hospital of Schieffelin Institute of Health Research and Leprosy Center, Karigili	1, Stanley Brown Laboratory, New Delhi 2, JALMA, Agra 3, Laboratory of Schieffelin Institute of Health Research and Leprosy Center, Karigili
Mali	Centre for Vaccine Development-Mali (CVD-MALI), Ministry of Health, CNAM-ex-Institute Marchoux, Bamako	National Reference Center on Mycobacteria, Faculte de Madicine Pitie-Slpetriere, Paris, France
Myanmar	1, Central Special Skin Clinic, Yangon General Hospital, Yangon 2, Central Special Skin Clinic, Mandalay General Hospital, Mandalay	National Institute of Infectious Diseases, Leprosy Research Center, Tokyo, Japan
Vietnam	1.National Institute of Dermatology and Venerology, Hanoi 2. Quyhoa National Leprosy Dermatology Hospital, Quynhon	1, Department of Microbiology, Yonsei University College of Medicine, Seoul, South Korea 2, National Institute of Infectious Diseases, Leprosy Research Center, Tokyo, Japan
Philippines	Skin Clinic, Leonard Wood Memorial, Cebu,	National Hansen's Disese Programs Laboratory Research Branch, Baton Rouge ,USA
China	Institute of Dermatology and National Center for Leprosy Control, Nanjing, China	Institute of Dermatology and National Center for Leprosy Control, Nanjing, China
Colombia	Institute Colombiano de Medicina Tropical	Colorado State University, Fort Collins, Colorado, USA

日、フランスのParisにおいてProf. Emanuelle Cambauにより運営された。20カ国より51人とWHOのGLPより3名、WHOの各地域事務局担当者5名が参加した。9カ国からハンセン病の現状の報告とHanoi会議以後行った検査結果につて報告された。各国の本事業に対する取り組みにはかなりの隔たりがあり、検査体制の確立、データの収集における方法の統一等が望まれた。検査結果の詳細についてはWeekly epidemiological record 2010, 85 281-284に記載されている。quality controlの結果、FTAカードの有用性を検討した結果について報告されたが、本事業への導入は決定されなかった。検査機関からの検査結果の報告と過去1年間に検査を実施した際に問題となった技術的な件に関し改善のための提案がされた。本事業における感受性検査はPCR direct sequencingによるが、一方で新薬の有効性の検証にはマウスfootpad法による検査が必要であることから、その技術と施設の維持の重要性が認識された。新たにインドネシア、ナイジェリア、ブルキナファソが事業への参加を表明した。次回までに行われるべき課題として、より多くの再発例の検査、各拠点における再発について診断と管理をより適切に行うこと、新薬の検索を続けることが指摘された。

Parisで行われた会議において、第3回の会議は報告者らが世話人となって東京で行うことが決定された。

東京会議の概要

冒頭に記したとおり、会議は11月9日および10日に国立感染症研究所、戸山庁舎において開催された。18カ国からとWHOのGLPチームメンバー、地域事務所の担当者も含め42名が参加した。各国のハンセン病対策担当官6名、監視拠点担当者7名、検査機関から6名のほか、専門家、ハンセン病支援団体からの参加があった。(図1、2)

初めにGLP team leaderのDr. Myo Thet Htoonが過去2年間の検査状況について報告した。これまでにブラジル(135例)、中国(10例)、コロンビア(6例)、ベトナム(12例)、ミャンマー(23例)、インド(27例)において合計213例の再発について検査され、dapsoneについては92例が解析可能でそのうち12例が耐性であった。rifampicinはPCR陽性の108検体中、9例が耐性菌と判定され、国によって違いがあるものの、いずれも約10%に耐性が認められた。

続いて、ブラジル、中国、マダガスカル、イエメン、パキスタン、マリより各国の状況について報告された。

ブラジルでは患者として登録される症例の約3%が再発であることが示された。これらに対する薬剤耐性の検査は国内5検査機関においてPCRにより増幅された遺伝子を同国の中枢研究機関で

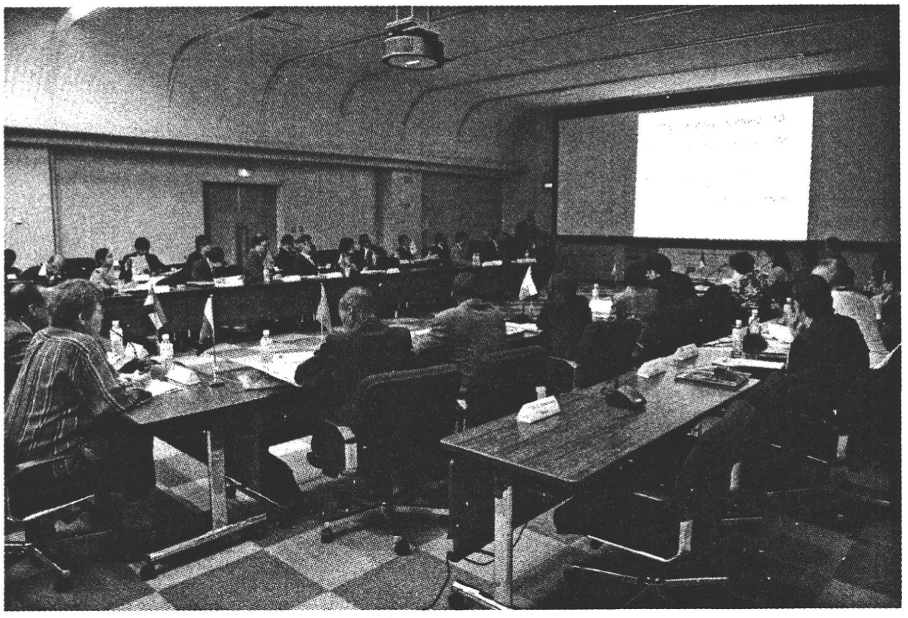

図1

ある Oswaldo Cruz Foundation（FIOCRUZ）においてまとめてシークエンスをする体制を敷いていることが示された。これに対し、PCR を担当する各施設間での能力の差について懸念が示され、技術の向上と統一の必要性が指摘された。

中国においては MDT 完了後も菌検査が行われ、陰性化するまで active case として登録され、その後は年一回パラメディカルにより、経過の観察が行われ、再発例の発見に努めていることが示された。2009 年を例にとると、新患として 1597 例が登録され、これに対し、再発の割合は MDT 以前の治療患者からは 79 例、MDT による治療を受けた者からは 69 例であることが示された。同国のハンセン病発生状況に照らし、15 省を監視拠点として選定し、監視事業が行われている。

マダガスカルからは同国のハンセン病対策の組織体制が報告され、2009 年には 1588 例の新患が登録されたことが報告された、しかしながら、2006 年までパスツール研究所と共同で行われていた再発に関する研究は打ち切りとなり、再発発見のための体制は整備されておらず、再発の実態把握はされていないことが示された。

イエメンのハンセン病は多くはなく、2009 年の有病率は 0.19/10,000、新患発見は 1.7/100,000 であった。2009 年には 6 例の再発が報告され、検体はスイスの Global Health Institute の Prof. Stewart Cole の研究室において検査された。3 例が解析可能であったが、何れにも耐性と認められる変異は検出されなかった。

パキスタンのハンセン病対策は多くの NGO の援助を受けて実施されていることが報告され、2009 年には 865 例の新患が登録され、11 例の再発があったことが示された。報告は同国のハンセン病は症例が少なく WHO の本事業の遂行については困難であるとの見解を示した。筆者の印象では同国におけるハンセン病対策が整備されていないことに対する懸念を強く感じた。

マリのハンセン病対策の概要について説明され、2009 年には 346 例が新患登録され (2.6/100,000)、有病率は 0.31/10,000 であった。再発は 2001 年から 2009 年の間で 6 例であったと報告された。同国にける再発発見のための体制が示されなかったので、はたしてこの数字が同国の再発に実態を示すものなのか否か疑問が持たれる。

続いて 9 日の午後は最初に、松岡が各 reference center に対して行った quality control について報告した。11 施設に 9 種の検体を送付し、*folP1*, *rpoB*, *gyrA* の drug resistance determining region に対する PCR の感度およびシークエンスの結果の報告を求めた。8 施設より報告があり、3 施設において PCR の感度が低くその改良が望まれた。negative control に対する false positive の例は無かったもの、2 施設のシークエンスの 4 例の結果は配布された検体とは異なるものであり、技術的

図2