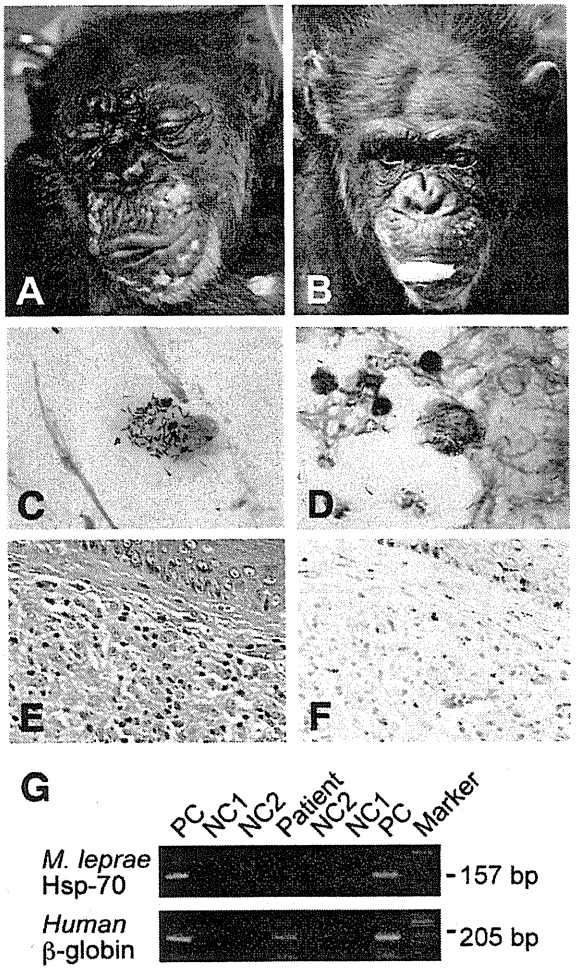

FIG. 1. (A) Facial leproma-like lesions of the chimpanzee Haruna (13 May 2009). (B) The same chimpanzee without lesions 1 year earlier (16 May 2008). (C) Ziehl-Neelsen staining of a nasal swab specimen showing globus-filled acid-fast bacilli (×1,000 magnification). (D) A skin smear from a left forearm nodule also showing multiple acid-fast bacilli (×1,000 magnification). Tissue staining was performed as previously described (18). (E) Hematoxylin and eosin staining of a skin biopsy sample from a right forearm nodule, showing accumulation of foamy histiocytes in the upper dermis (×400 magnification). (F) Fite staining demonstrating numerous acid-fast bacilli within the histiocytes (×400 magnification). (G) PCR analysis demonstrating *M. leprae* Hsp70 DNA. Tissue DNA was prepared using a QIAamp DNA Micro kit (Qiagen Inc., Valencia, CA) according to the manufacturer's protocol. PCR was performed as previously described (18) using specific primers (10, 17). PCR products were sequenced using an ABI Prism 310 genetic analyzer and GeneScan Collection software (Applied Biosystems). PC, positive control; NC1, negative control 1 (nuclease-free water was used as a template for PCR); NC2, negative control 2 (nuclease-free water was used instead of skin tissue to purify DNA). Human β-globin served as a positive control for DNA extraction from the skin biopsy sample.

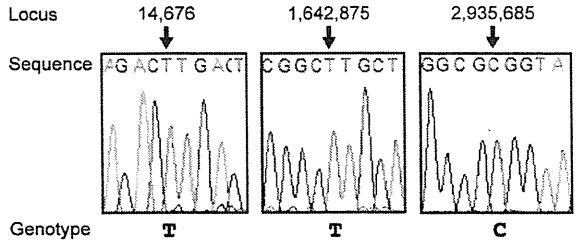

FIG. 2. Sequence analysis of the three reported SNPs of *M. leprae* DNA. PCR was performed using previously described primer sets (13), and the PCR products were sequenced using an ABI Prism 310 genetic analyzer and GeneScan Collection software (Applied Biosystems).

is primarily transmitted by repeated airborne exposure to *M. leprae* through the nasorespiratory passage via close contact with multibacillary leprosy patients during infancy or early childhood and that the clinical disease becomes apparent only after a long incubation period (3). Although it is thought that *M. leprae* parasitizes histiocytes in the dermis and Schwann cells of the peripheral nerves (15), there is no available serologic or biologic method to demonstrate the presence of subclinical infection during latency. Therefore, it has not yet been possible to determine the length of incubation following infection.

M. leprae infection in this chimpanzee was highly unlikely to have occurred in Japan, particularly given the strict biosafety standards of primate housing facilities in experimental laboratories and the very low prevalence of leprosy in Japan. Therefore, the evidence strongly suggests that Haruna contracted a *M. leprae* infection when she was in West Africa before the age of 2 and that she developed lepromatous leprosy after a 30-year incubation period. Our results also suggest that the levels of serum anti-PGL-I antibody reflect the bacterial load in the patient but may not represent a marker for subclinical *M. leprae* infection.

To date, only three cases of leprosy in chimpanzees have been reported in the literature. All three chimpanzees were born in Africa and brought to the United States at a young age (4–6). One male chimpanzee captured in Sierra Leone developed leprosy some time between 5 and 6 years of age (4). Gormus et al. suggested the possibility that *M. leprae* might be transmitted among chimpanzees in Africa (5). Others have suggested that contact with an infected human had potentially occurred during the 2-to-3-month period when the chimpanzees were housed in outdoor cages while awaiting shipment after capture (4).

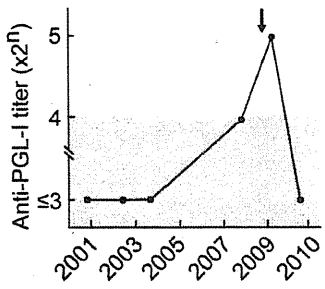

FIG. 3. Changes in the serum anti-PGL-I antibody titer before and after disease onset. The arrow indicates the approximate date when skin lesions appeared. The shaded area indicates the negative range. The serum anti-PGL-I antibody titer was measured using a gelatin particle agglutination test kit, Serodia-Leprae (Fujirebio, Tokyo, Japan), according to the manufacturer's instructions. Antibody titers > 2^5 were judged to represent positive results.

Nonetheless, leprosy is clearly a common disease among humans and apes. Even after the worldwide efforts to reduce the disease burden of leprosy spearheaded by WHO (http://www.searo.who.int/LinkFiles/GLP_SEA-GLP-2009_3.pdf) were successfully completed (8, 16), isolated leprosy cases in chimpanzees or other animals (e.g., armadillos) may still exist in the wild (11, 12, 19) and serve as potential sources of human infection. Therefore, it might be of particular importance to perform a survey of chimpanzee leprosy in West Africa (11).

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Invited Review

Whole-Genome Expression Analysis of *Mycobacterium leprae* and Its Clinical Application

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3. Characteristics of *M. leprae* genome
4. Detection of pseudogene expression by membrane array
5. Detection of RNA expression by whole-genome tiling array
6. Clinical application of *M. leprae* RNA analysis
7. Conclusion

SUMMARY: The whole-genome sequence analysis of *Mycobacterium leprae*, which was completed in 2001, revealed the characteristics of this microbe's genomic structure. Half of the *M. leprae* genome consists of a limited number of protein-coding genes and the rest comprises non-coding regions and pseudogenes. We performed membrane array and tiling array analyses to analyze the gene-expression profile of the *M. leprae* genome and found that pseudogenes and non-coding regions were expressed similarly to coding regions at the RNA level. The RNA expressions were confirmed by real-time PCR analysis. Expression of these RNAs in clinical samples showed varying patterns among patients, thus indicating that the analysis of RNA expression patterns, including non-coding regions and pseudogenes, may be useful for understanding the pathological state, prognosis, and assessment of therapeutic progress in leprosy.

1. Introduction

More than 240,000 new cases of leprosy, a chronic infectious disease caused by *Mycobacterium leprae* that involves the skin and nerves (1), were detected worldwide in 2009 (2). The discovery of its causative agent, *M. leprae*, was made earlier than that of *Mycobacterium tuberculosis*, but it is still impossible to culture this bacillus or to transfer the disease to experimental animals. Although it is known that *M. leprae* preferentially parasitizes macrophages in a foamy or enlarged phagosome filled with lipids (3) and the mechanism by which such a large amount of lipids is recruited and accumulated in phagosomes, and how the expression of host proteins is modified following infection, have been elucidated (4,5), the biological characteristics of *M. leprae* in the phagosome are still largely unknown. Previous studies on the nature of *M. leprae* were limited to methods other than gene manipulation, such as evaluation of respiration or adenosine triphosphate production (6), detection of RNA, and the use of other mycobacteria to study the function of specific *M. leprae* genes (7). The exact nature of *M. leprae* therefore remains unclear.

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2. Advances in the DNA microarray technique

The microarray technique permits the analysis of hundreds of thousands of probes mounted on a glass slide in a single experiment. Recent advances in genome science and the more precise annotation of published genomes for many organisms has enabled probe design for all of the organism's genes and comprehensive analysis of whole-genome RNA expression. Conventional DNA microarray analysis has been applied to various species such as *Escherichia coli* (8,9), *Helicobacter pylori* (10), *Yersinia pestis* (11), and *Salmonella enterica* serovar Typhi (12), as well as to *M. tuberculosis* (13) and *M. leprae* transcriptomes (14,15). However, only known or predicted genes were examined in such arrays, therefore, it was difficult to analyze the RNA expression of non-coding regions and potential pseudogenes that did not have the appropriate annotation and array probes.

The use of tiling arrays makes it possible to design and arrange overlapping probes spanning the entire genome of an organism (16,17). A genome tiling array enables the comprehensive detection of short and long transcripts, including products from non-coding regions, over the entire genome without bias. With typical eukaryote genomes, the tiling array is performed for each chromosome or smaller limited region because of the large genome size (18–20). However, whole-genome tiling array probes for the smaller genomes of prokary-

Table 1. The genome structure of *Mycobacterium* spp.

Mycobacterium spp.	Pseudogene	Gene	% of genome	Genome size (bp)	Reference
M. tuberculosis	6	3,959	90.8	4,411,532	41
M. bovis	27	3,953	90.6	4,345,492	56
M. marinum	65	5,424	90.0	6,636,827	57
M. smegmatis	168	6,897	92.4	6,988,209	58
M. ulcerans	771	4,160	72.3	5,631,606	28
M. leprae	1,116	1,605	49.5	3,268,203	26

otes and fungi have been designed (21) and applied in various species such as *E. coli* (22), *Bacillus subtilis* (23), *Neisseria meningitidis* (24), and *Saccharomyces cerevisiae* (25).

3. Characteristics of the *M. leprae* genome

It is thought that the high degree of host dependency of *M. leprae* is related to its genetic characteristics. The whole-genome sequence of *M. leprae*, which was published in 2001, revealed that only half of the small genome contains protein-coding genes, while the remainder consists of pseudogenes and non-coding regions (26). The number of pseudogenes in the *M. leprae* genome is much larger than in other mycobacteria (Table 1) (27). *Mycobacterium ulcerans* has the second-highest number of pseudogenes, 771, although the proportion of pseudogenes based on genome size is less than half that of *M. leprae* (28). The number and proportion of pseudogenes in *M. leprae* are also exceptionally large in comparison with other pathogenic and non-pathogenic bacteria and archaea (29,30). It has been shown that many *M. leprae* pseudogenes are caused by insertions of stop codons (31), and this is speculated to be caused by the dysfunction of sigma factors (32) or the insertion of repetitive sequences derived from transposons, which comprise up to 2% of the *M. leprae* genome (33). Despite such genetic damage, specialized intracellular environments that are free from evolutionary competition allow its survival (26,34,35). Moreover, the proportion of non-coding regions in the *M. leprae* genome (24%) is also greater than that of *M. tuberculosis* (9%). It has been speculated that *M. leprae* has lost over 1,500 genes from its complete genome set and that non-coding regions are functionally silent and useless "junk DNA" (36). However the expression patterns of non-coding regions have never been analyzed, therefore the functional roles of these unique pseudogenes and non-coding regions are unknown.

4. Detection of pseudogene expression by membrane array

In the course of studying the gene-expression profile of *M. leprae* upon infection, we found that some *M. leprae* pseudogenes are highly expressed as RNA (37). In this experiment, genomic DNA fragments prepared from the *Thai*-53 strain of *M. leprae* were cloned into shuttle cosmid vectors for *E. coli* and mycobacteria to construct a genomic DNA library. Clones overlapping in sequence and covering 98% of the *M. leprae* genome (137 clones) were selected and each cosmid was spotted onto a nylon filter. Messenger RNA was purified from *M. leprae* with or without infection into macrophages, then each mRNA was enriched by cDNA subtraction. Radiolabeled cDNA was hybridized using the membrane array technique (Figs. 1A and B) and the signal intensities determined (Fig. 1C). Eight cosmid clones whose expression was significantly modified by infection were digested with eight different restriction enzymes; Southern blot analysis was then performed using the same probe used for the membrane array (Fig. 1D). The location of each hybridized fragment was analyzed in silico by comparison with the restriction map and gene annotations of the *M. leprae* genome. As a result, 12 genes, including six pseudogenes, were identified as genes whose expression level was high and modulated by infection. The high expression level of the pseudogenes suggests that they may have some functional role, such as antisense activity (38), and that they might be involved in infection and/or intracellular parasitization.

5. Detection of RNA expression by whole-genome tiling array

We then designed a whole-genome tiling array to analyze the comprehensive RNA expression of genes, pseudogenes, and non-coding regions in *M. leprae* (39). Since shorter probes achieve higher resolution but result in low signal specificity, the probe length was set to 60-mer and the adjacent probe was shifted by 18 nucleotides (42-nt overlaps). A total of 363,116 probes were designed for the sense and antisense strands based on sequences obtained from the GenBank database (accession no. NC_002677) (26). For this genomic tiling array, *M. leprae* RNA from SHR/NCrj-*rnu* hypertensive nude rats (40) was labeled and hybridized, and the fluorescent signal intensity was normalized. The signal-intensity pattern for each probe was found to be similar to that obtained from an *M. leprae* ORF array, which was hybridized using the same RNA sample, thereby confirming the specificity of the tiling array. The strongest signal was identified in the rRNA, with most probes in this region showing significantly higher intensity (Fig. 2A). Other highly expressed areas were also detected in the gene (Fig. 2B), pseudogene (Fig. 2C), and non-coding regions (Fig. 2D).

For this array, highly expressed regions were defined as areas where the intensities of four probes within 500 bp were higher than 60% of the maximum intensity. As a result, a total of 209 highly expressed regions were determined from gene, pseudogene, and non-coding regions based on annotation (Table 2). In this study, non-coding regions were defined as regions that are not

annotated. Surprisingly, the RNA expression from non-coding regions was found to be the highest in terms of number, mean length, and mean peak signal intensity, whereas that from genes (ORFs) was statistically the lowest. RNA expression from 18 of the 209 highly expressed regions was detected by RT-PCR and real-time PCR.

Gene and pseudogene expression profiles were obtained from the tiling array of the *M. leprae* genome. Since *M. leprae* genes and pseudogenes are functionally classified into six groups (26,41), the highly expressed genes were classified according to these same criteria (Table 3). *M. leprae* grown in hypertensive nude rats was found to have relatively high expression levels in the "cell processes" class, which is related to the folding of synthesized proteins like chaperones and transporters (9.8%) (42), whereas a low expression ratio was found for the pseudogenes (3.0%). This pattern is consistent with the proposal that genes in this class play very essential roles. Furthermore, a high degree of expression was detected for lipoproteins and the PE and PPE families (3 out of 11) in the "other functions" class. The PE and PPE families are specific to *Mycobacterium* spp. (41) and are associated with the early secreted antigenic target 6-kDa (ESAT6) antigen (43), therefore they may play an important role in virulence, as exemplified in *M. tuberculosis* (44). Because *M. leprae* has fewer PE, PPE, and ESAT6-like genes than *M. tuberculosis*, information on these expressed genes will facilitate further functional analysis of a PE, PPE and ESAT6-like protein complex.

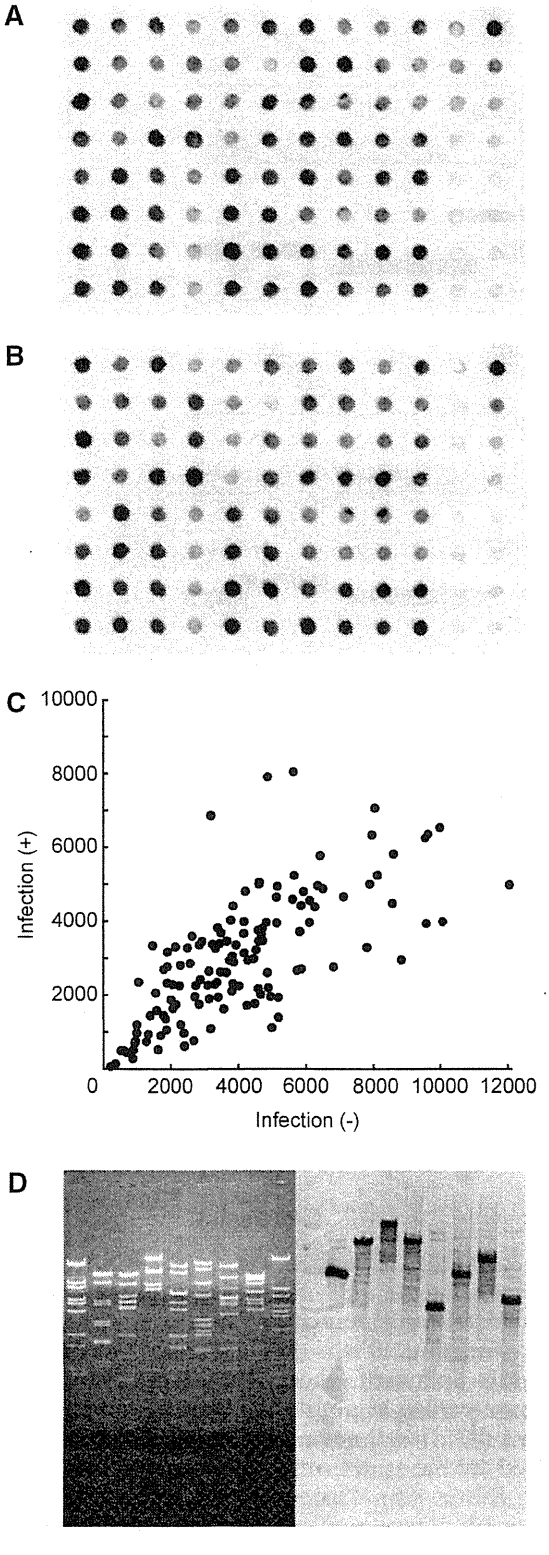

Fig. 1. Hybridization of *Mycobacterium leprae* genome arrays with ^{33}P-dCTP-labeled cDNA probes. *M. leprae* DNA covering over 98% of the genome was purified from a cosmid library, spotted on membranes and probed with cDNA from bacilli-infected macrophages (A) or cDNA from control bacilli (B) after subtractive enrichment. (C) Correlation of signal intensity from arrays hybridized with subtracted cDNA. Densitometric analysis of signal intensity was performed on the array hybridizations and results from the same clones were compared. The *y* axis denotes the results from bacilli-infected macrophages, as shown in Fig. 1A. The *x* axis denotes results from the control bacilli shown in Fig. 1B. (D) Identification of fragments containing differentially expressed cDNAs. The cosmids demonstrating significant differences in signal with or without infection were digested with eight restriction enzymes and separated on a 1% agarose gel (left panel) with marker DNA (leftmost lane). Gels were subsequently transferred to nylon filters and hybridized with the same probes that were used for dot blot hybridization (right panel).

6. Clinical application of *M. leprae* RNA analysis

There is currently no sensitive marker to reflect the disease type or clinical course of leprosy, therefore we decided to analyze clinical specimens to see how the mRNAs were expressed in *M. leprae* from infected patients. PCR primer sets were designed against expressed regions in the *M. leprae* genome and RT-PCR was performed on *M. leprae* RNA extracted from skin smear samples from lepromatous leprosy patients (45). The results showed that the RNA expression patterns were quite different among the patients, although *M. leprae* Hsp70 was detected in all specimens examined (Fig. 3). The differences in pseudogene and non-coding region expression suggest that these may have some functional role and that their expression may be related to disease type, clinical course, or treatment (46). Although further studies are needed, it should be possible to establish an RT-PCR-based diagnostic panel to rapidly diagnose the effects of drug therapy, disease type, and lepra reaction.

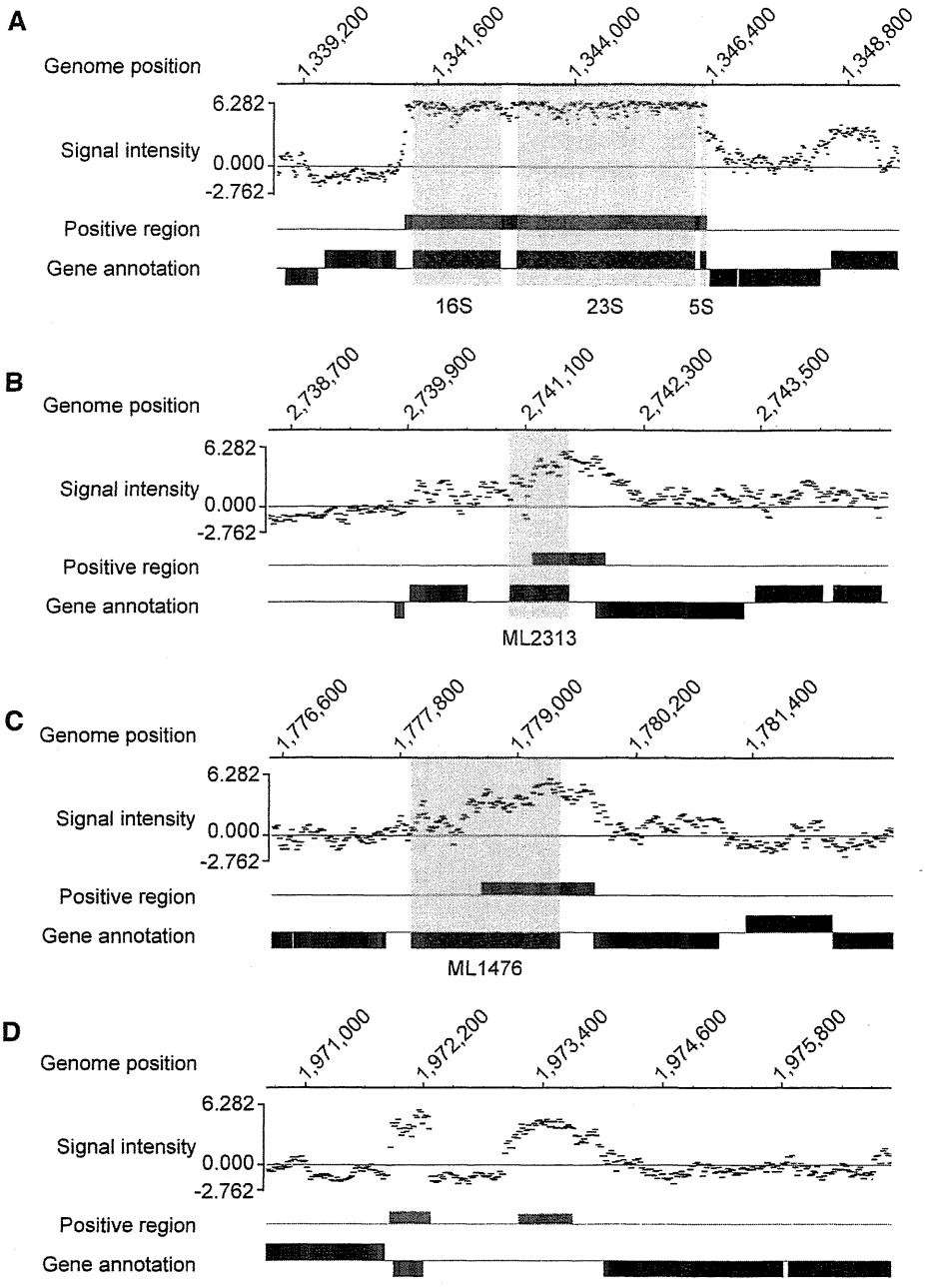

Fig. 2. Signal intensity patterns detected as highly expressed areas in the tiling array. Each probe was arrayed against the corresponding *M. leprae* genome sequence. Positive areas were extracted and are depicted under the signal pattern of probes with gene and pseudogene annotations. (A) Genomic region of rRNA showing nearly saturated signal intensity. (B) Highly expressed region of the gene for the ML2313 hypothetical protein (shaded area). (C) Highly expressed region of the pseudogene ML1476, pseudo-probable oxidoreductase alpha subunit (shaded area). (D) Highly expressed non-coding region in genomic position 1,973,155 to 1,973,700 bp, which showed no homology to genes or other functional sequences by BLASTN search.

Table 2. Number of highly expressed genes, pseudogenes, and non-coding regions identified by tiled microarray analysis

	Gene	Pseudogene	Non-coding region	Total
No. identified	63	78	68	209
% of total	30.1	37.3	32.5	100
Mean length (bp)	637	611	634	—
Mean peak intensity[1)]	4.88	5.11*	5.38**	—

[1)]: Mean peak intensity of pseudogenes and non-coding regions were statistically compared with the intensity of coding genes (*, $P < 0.05$; **, $P < 0.00001$ by Student's t test).

7. Conclusion

We have been able to detect strong RNA expression from pseudogenes and other non-coding regions by using an *M. leprae* genome tiling array. Although the biological function of the RNA transcribed from these regions is unknown, short RNA fragments generated from non-coding regions have been found in many organisms (47). It is known that most of the microRNAs detected in the transcriptome of eukaryotes are non-coding RNAs (48). Moreover, it is reported that pseudogenes also have some biological functions in proc-

Table 3. Number and percentage of expressed genes and pseudogenes based on functional classification (26,41)

Classification	Gene[1)]	Pseudogene[1)]
Small-molecule metabolism (Synthesis and degradation of amino acid, polyamine, nucleotide, cofactor and lipid, energy metabolism enzymes)	19/467 (4.1%)	19/334 (5.7%)
Macromolecule metabolism (Synthesis and degradation of protein, RNA, DNA, and cell envelope)	16/458 (3.5%)	10/163 (6.1%)
Cell processes (Transporter and chaperon)	10/102 (9.8%)	2/67 (3.0%)
Other functions (Virulence, repeated sequence, PE and PPE families)	6/77 (7.8%)	29/133 (21.8%)
Conserved hypotheticals	6/360 (1.7%)	18/416 (4.3%)
Unknown	6/141 (4.3%)	0/2 (0%)
Total	63/1605 (3.9%)	78/1115 (7.0%)

[1)]: The ratio is the number of expressed genes or pseudogenes/ total number of genes or pseudogenes.

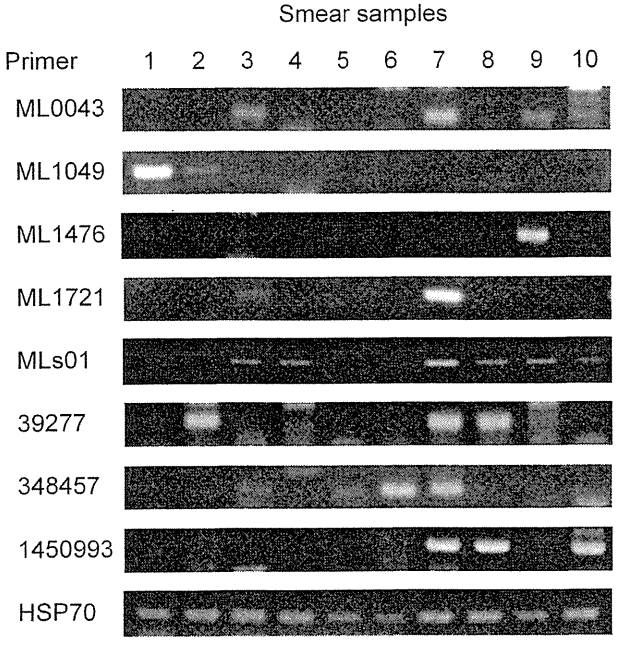

Fig. 3. RT-PCR analysis of *M. leprae* RNA derived from skin smear samples from 10 lepromatous leprosy patients. Total RNA was isolated from skin smear samples. After treatment with DNase I, RT-PCR was performed for pseudogenes ML0043, ML1049, ML1476, ML1721, non-coding genomic region positions 39277, 348457, 1450993 with known non-coding RNA MLs01, and Hsp70.

esses such as cell growth and organogenesis (49), or cancer and the central nervous system (50), through the regulation of gene expression (51,52). Although the proportion of non-coding regions in microbes, as well as their genome sizes, is much smaller than for eukaryotes, RNA expression from non-coding regions was also been detected from the whole-genome analysis of *E. coli* (53) and *Prochlorococcus* and *Synechococcus* (54). Tiling array analysis also identified novel non-coding RNAs in yeast (55). Since exceptionally large numbers of pseudogenes and non-coding regions were shown to be expressed as RNAs in *M. leprae*, this may prove to be a useful model organism for the study of the molecular mechanisms underlying the creation and the role of these transcripts.

The diagnosis of leprosy primarily relies on clinical and microscopic examination. It is difficult to predict the efficacy of treatment or the occurrence of lepra reactions. Sensitive and feasible molecular analyses should therefore provide important information for clinical diagnosis. In particular, it is possible that the analysis of *M. leprae* RNA expression will provide a method to assess the efficacy of multidrug therapy. Further analysis of large numbers of clinical specimens will hopefully enable the construction of an RT-PCR-based diagnostic panel.

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Conflict of interest None to declare.

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RESEARCH LETTER

Biochemical characteristics among *Mycobacterium bovis* BCG substrains

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Abstract

In order to evaluate the biochemical characteristics of 14 substrains of *Mycobacterium bovis* bacillus Calmette Guérin (BCG) – Russia, Moreau, Japan, Sweden, Birkhaug, Danish, Glaxo, Mexico, Tice, Connaught, Montreal, Phipps, Australia and Pasteur – we performed eight different biochemical tests, including those for nitrate reduction, catalase, niacin accumulation, urease, Tween 80 hydrolysis, pyrazinamidase, *p*-amino salicylate degradation and resistance to thiophene 2-carboxylic acid hydrazide. Catalase activities of the substrains were all low. Data for nitrate reduction, niacin accumulation, Tween 80 hydrolysis, susceptibility to hydrogen peroxide and nitrate, and optimal pH for growth were all variable among these substrains. These findings suggest that the heterogeneities of biochemical characteristics are relevant to the differences in resistance of BCG substrains to environmental stress. The study also contributes to the re-evaluation of BCG substrains for use as vaccines.

Introduction

Biochemical tests are currently used as a technique for the identification of bacterial species. Recently, several studies have investigated the physiological meaning of the biochemical characters in the genus *Mycobacterium*. Sohaskey and colleagues reported variable nitrate production among *Mycobacterium bovis* bacillus Calmette Guérin (BCG) substrains in relation to survival in host cells (Sohaskey, 2008; Sohaskey & Modesti, 2009). Recycling of NAD and NAD-quinoline reductase relevant to the latent infection of *Mycobacterium tuberculosis* and resistance to oxidative stress, respectively, have also been reported (Boshoff *et al.*, 2008). Mycobacterial phospholipase A (MPLA) catalyses the hydrolysis of lipids including Tween 80 (Parker *et al.*, 2007), and this activity appears to contribute to survival under starvation at the dormant stage of growth (Jackson *et al.*, 1989; Deb *et al.*, 2009). Here, we analysed the biochemical characteristics and their relationship to susceptibility to environmental stress, such as oxidative stress, nitrosative stresses and pH changes, among BCG substrains.

Materials and methods

Bacterial strains

Mycobacterium bovis BCG strains Australia (ATCC 35739), Birkhaug (ATCC 35731), Connaught (ATCC 35745), Danish (ATCC 35733), Glaxo (ATCC 35741), Mexico (ATCC 35738), Montreal (ATCC 35735), Pasteur (ATCC 35734), Phipps (ATCC 35744), Tice (ATCC 35743), Russia (ATCC 35740) and *M. tuberculosis* strain $H_{37}Rv$ (ATCC 25618) were purchased from American Type Culture Collection (ATCC, Manassas, VA). BCG-Moreau, *M. bovis* (JATA) and *Mycobacterium smegmatis* were provided by Dr M. Takahashi (The Research Institute of Tuberculosis Japan Anti-tuberculosis Association, Kiyose, Tokyo, Japan). BCG-Japan (Tokyo 172) was purchased from Japan BCG Laboratory (Kiyose, Tokyo, Japan). BCG-Sweden (vaccine seed) was provided by Dr S. Yamamoto (Japan BCG Laboratory). *Mycobacterium avium* strains 724S and 2151SmO were kindly provided by Drs J. Inamine and E. Torsten (Colorado State University, Fort Collins, CO).

FEMS MICROBIOLOGY LETTERS

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

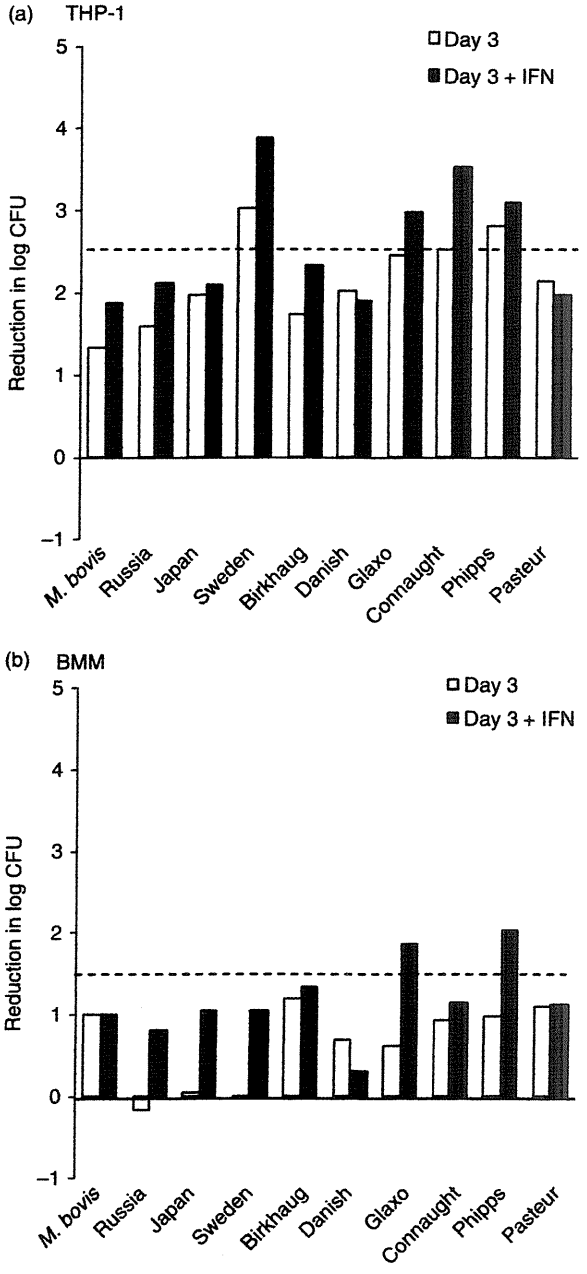


Fig. 1. Viability of BCG strains in THP-1 and mouse BMMs. PMA-differentiated THP-1 (a) or mouse BMMs (b) were infected with BCG at an MOI of 20 with (solid) or without (open) 100 U mL^{-1} of IFN-γ as a stimulator. After 6 h of infection, BCG CFU counts were determined from infected cell lysates and 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

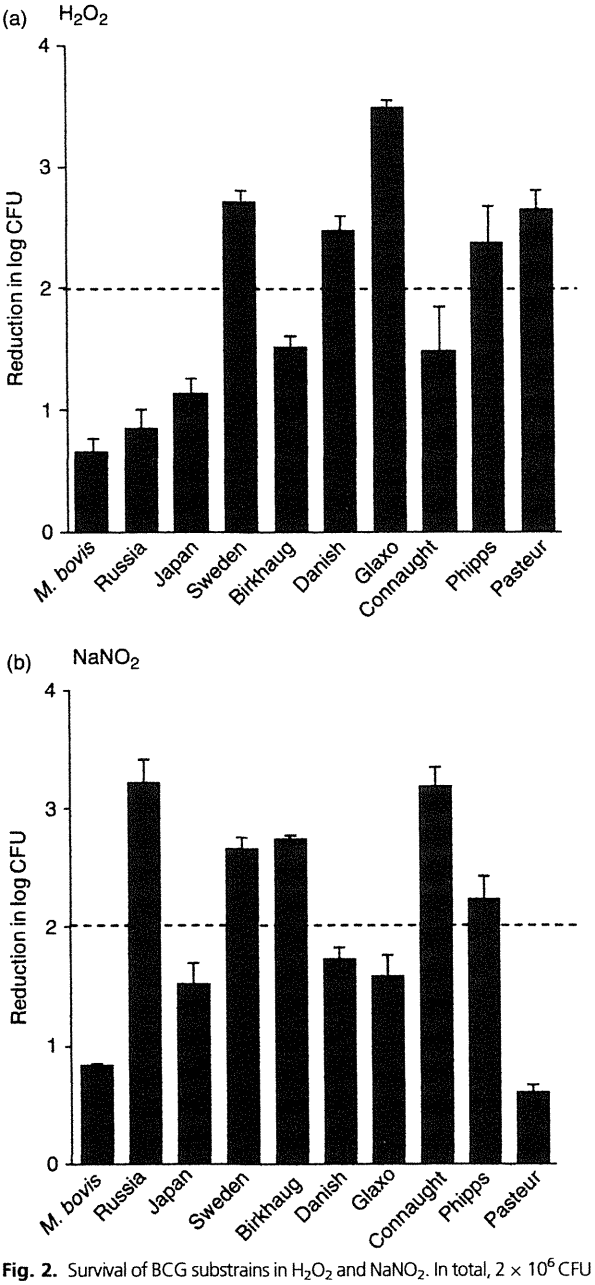
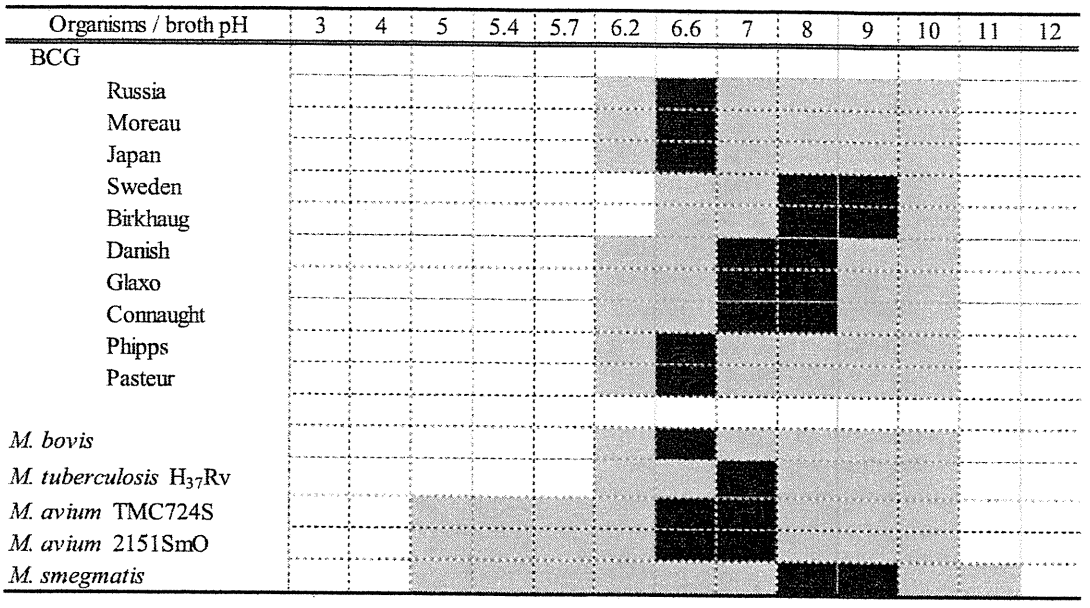


Fig. 2. Survival of BCG substrains in H_2O_2 and $NaNO_2$. In total, 2×10^6 CFU of *Mycobacterium bovis* or BCG substrains were treated with (a) 10 mM H_2O_2 for 6 h or (b) 10 mM $NaNO_2$ for 3 days. Treated and washed cells were serially diluted, and aliquots from four serial dilutions were plated in 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.

Acknowledgements

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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 FMb70Bal (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,

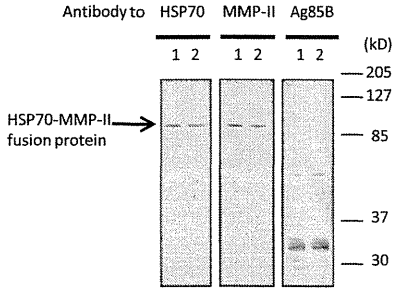

FIGURE 1. Western blotting analysis of protein fraction extracted from BCG-70M and BCG-D70M. An mAb to either MMP-II or HSP70 was used to detect HSP70–MMP-II fusion protein. An anti-Ag85B rabbit polyclonal Ab was also used to detect Ag85B (an internal control). *Lane 1*, Cell lysate of BCG-70M; *lane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

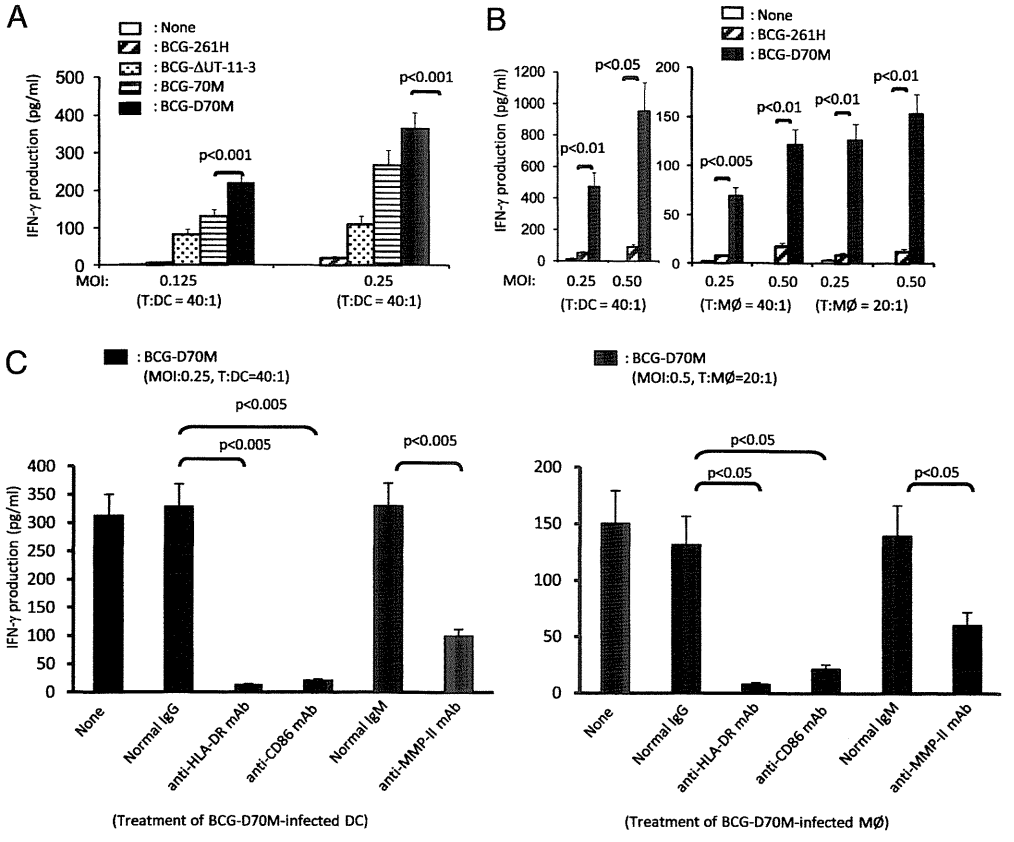

FIGURE 2. *A*, IFN-γ production from naive $CD4^+$ T cells by stimulation with rBCG. Monocyte-derived DCs were infected with BCG-261H, BCG-ΔUT-11-3, BCG-70M, or BCG-D70M at the indicated MOI and were used as a stimulator of naive $CD4^+$ T cells in a 4-d culture. Total of 10^5 responder T cells were 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*).

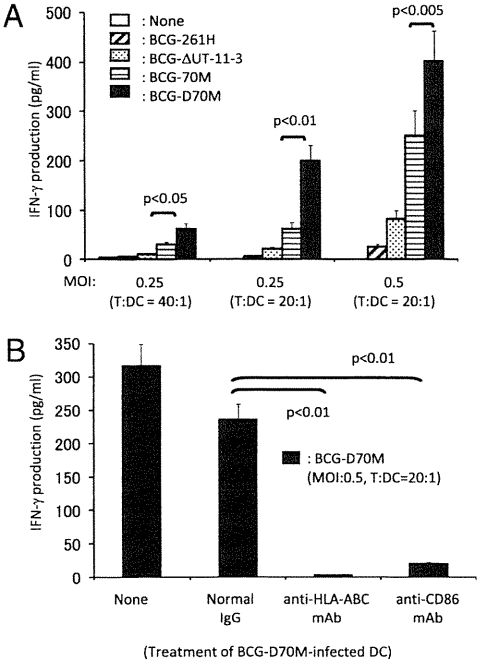

FIGURE 3. *A*, IFN-γ production from naive $CD8^+$ T cells by stimulation with BCG. Monocyte-derived DCs were infected with BCG-261H, BCG-ΔUT-11-3, BCG-70M, or BCG-D70M at the indicated MOI and were used as a stimulator of responder naive $CD8^+$ T cells in a 4-d culture. Total of 10^5 responder T cells 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

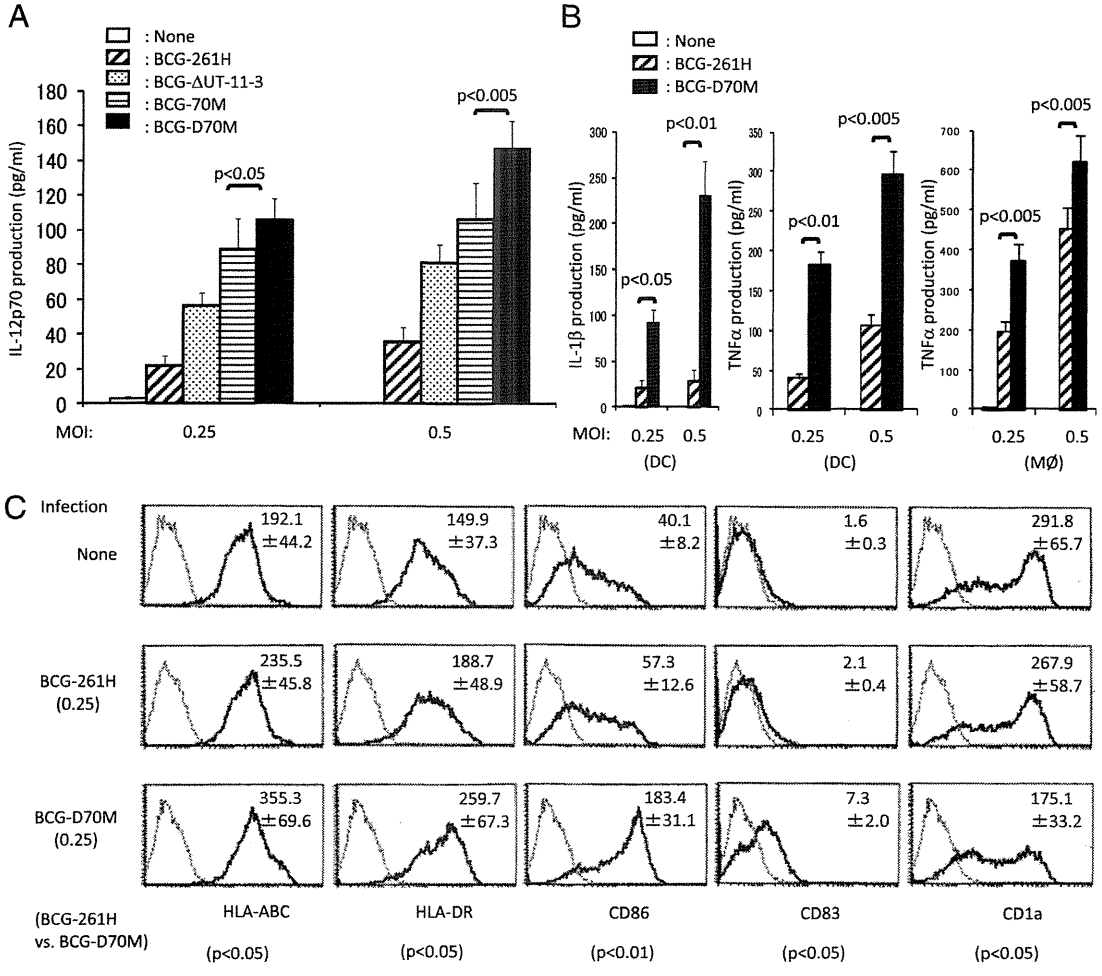

FIGURE 4. *A*, IL-12p70 production from DCs stimulated with rBCG. Monocyte-derived DCs from 5 d of culture in the presence of rGM-CSF and rIL-4 were stimulated with the indicated dose of BCG-261H, BCG-ΔUT-11-3, BCG-70M, or BCG-D70M for 48 h. The concentration of IL-12p70 was determined by the ELISA method. *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.