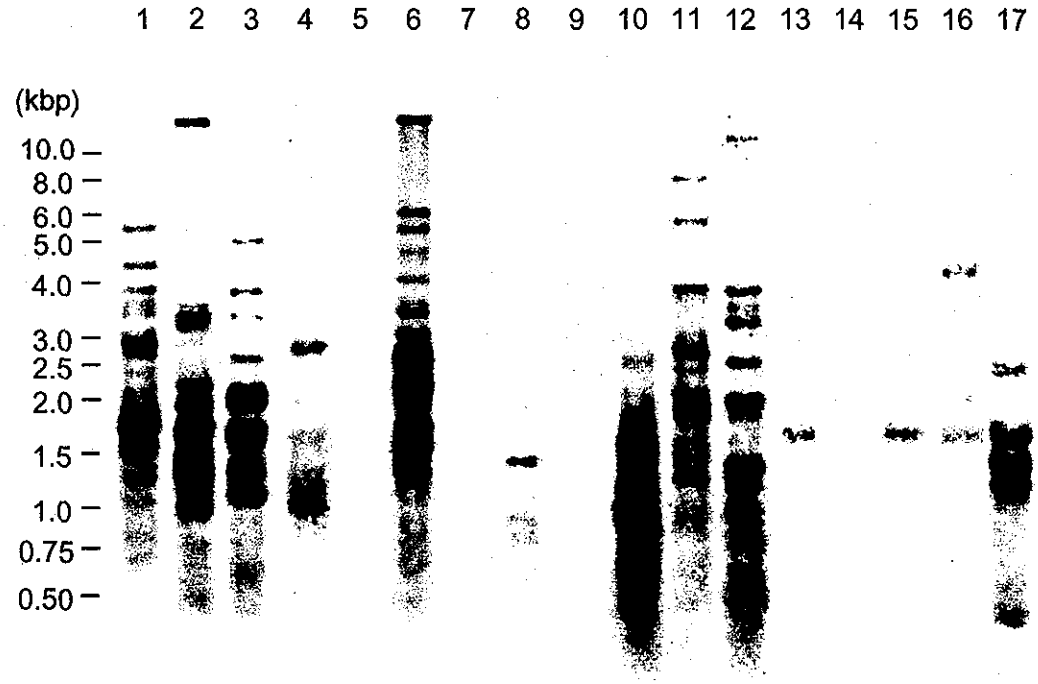

FIG. 5. $(CGG)_5$-probed fingerprinting of AluI-digested DNA from various mycobacterial species. Lane 1, *M. bovis* BCG; lane 2, *M. marinum*; lane 3, *M. kansasii*; lane 4, *M. simiae*; lane 5, *M. scrofulaceum*; lane 6, *M. szulgai*; lane 7, *M. avium*; lane 8, *M. intracellulare*; lane 9, *M. xenopi*; lane 10, *M. gastri*; lane 11, *M. terrae*; lane 12, *M. nonchromogenicum*; lane 13, *M. fortuitum*; lane 14, *M. abscessus*; lane 15, *M. chelonae*; lane 16, *M. peregrinum*; lane 17, *M. smegmatis*.

PPE, PE, and PE_PGRS genes translated to neutral-charged amino acids of poly(*Ala*) and poly(*Gly*), respectively, with no special substitution, indicating that these regions do not participate in the formation of unique structures within these proteins. Thus, the $(CGG)_5$ sequences in these genes will likely not have characteristic properties regarding function.

It is unclear whether TRS in bacteria, particularly $(CGG)_5$ in *M. tuberculosis* and *M. bovis*, participate in their pathogenesis. There was no difference between virulent strain H37Rv and the derived avirulent strain H37Ra in $(CGG)_5$-probed fingerprinting (Fig. 2). No correlation was found between the virulency of mycobacterial species and the numbers of bands in $(CGG)_5$-probed fingerprinting or copies of $(CGG)_5$ (Table 2 and Fig. 5). For example, *M. leprae* had no $(CGG)_5$ repeats (Table 2). Some rare etiologic agents of nontuberculous mycobacteria, such as *M. smegmatis* and *M. szulgai* (20), did possess several copies of $(CGG)_5$ in their genomes (Fig. 5), whereas some common etiologic agents, such as *M. avium*, *M. xenopi*, and *M. abscessus* (20), possessed no $(CGG)_5$ repeats (Fig. 5). These results indicate that $(CGG)_5$ repeats do not participate directly in the virulency of mycobacterial species.

Whereas fingerprinting analysis showed that both $(CGG)_5$ and IS*6110* were sufficiently stable epidemiologic markers, $(CGG)_5$ appeared to be more stable than IS*6110* (Fig. 1). We were unable to find any differences between strains H37Rv and H37Ra in $(CGG)_5$-probed fingerprinting by extensive studies with various restriction enzymes. However, four different bands were detected between these strains with PvuII-IS*6110* fingerprinting (Fig. 2B). Lari et al. (11) compared H37Rv and H37Ra strains maintained at their institution by IS*6110* fingerprinting with EcoNI, PstI, and PvuII and found different patterns between these strains. Bifani et al. (3) compared the PvuII-IS*6110* fingerprints of 15 and 3 different catalogued variants of H37Rv and H37Ra, respectively. Ten distinct fingerprint patterns, making up nine H37Rv variants and one H37Ra variant, were identified. A discrepancy between IS*6110*- and $(CGG)_5$-probed fingerprints of laboratory strains was observed in three pairs of clinical isolates (Fig. 4). In these cases, each isolate was identical in $(CGG)_5$ fingerprinting pattern but differed in its IS*6110* fingerprinting pattern. Our recent epidemiological case report of intrafamilial tuberculosis transmission showed that two clinical isolates from a father and son were identical in $(CGG)_5$-probed fingerprinting patterns, whereas one different band was detected between them by IS*6110*-probed fingerprinting (25). Collectively, IS*6110*-probed fingerprint patterns changed more rapidly than did $(CGG)_5$-probed patterns, suggesting that there are different mechanisms by which these patterns change. In other terms, although $(CGG)_5$-probed fingerprinting will hardly detect a few mutations in a clone of *M. tuberculosis*, it will easily detect an origin among the clones. The $(CGG)_5$-probed fingerprinting combined with IS*6110*-probed fingerprinting will provide more powerful information about tuberculosis epidemiology.

We collected and analyzed the isolates in this study in Japan and Poland. If isolates could be collected worldwide, it would provide more exact epidemiological data. In conclusion, the $(CGG)_5$ repeat is a useful probe for DNA fingerprinting of *M. tuberculosis*, because all strains tested here possessed more than eight copies. In addition, $(CGG)_5$-probed fingerprinting will be a useful tool for the investigation of *M. bovis*, *M. marinum*, *M. kansasii*, and *M. szulgai*.

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Laboratory and Epidemiology Communications

Further Acquisition of Drug-Resistance in Multidrug-Resistant Tuberculosis during Chemotherapy

Emiko Toyota*, Jun-ichiro Sekiguchi, Hisahiro Shimizu, Tomoko Fujino, Yayoi Otsuka, Hiroshi Yoshikura[1], Tadatoshi Kuratsuji, Teruo Kirikae and Koichiro Kudo

International Medical Center of Japan, Tokyo 162-8655 and
[1]National Institute of Infectious Diseases, Tokyo 162-8640

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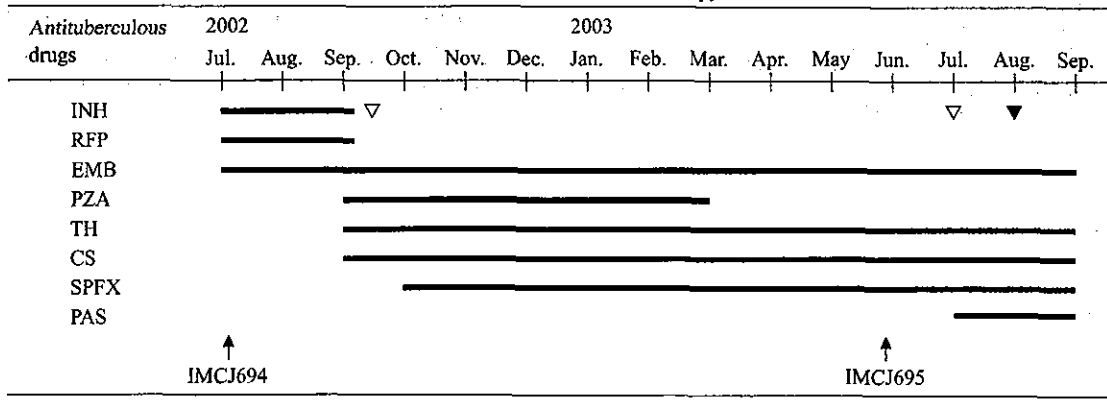
Multidrug-resistant tuberculosis (MDR-TB) resulting from failure to control primary tuberculosis (1) poses a serious clinical problem. Understanding how an organism can acquire resistance to multiple drugs is essential to prevent the emergence of a multidrug-resistant organism in an individual receiving antituberculous chemotherapy.

A 41-year-old man visited a doctor complaining of a left chest pain. A chest radiograph revealed pneumothorax and his sputum was positive for acid-fast bacilli (AFB). He was referred to a hospital in Tokyo in July 2002. The patient had a history of antituberculous chemotherapy from 1992 to 1997 in South Korea with isoniazid (INH), rifamipicin (RFP), ethambutol (EMB), and streptomycin (SM) for 1 month, and then with INH, RFP, EMB, and cycloserine (CS) for 5 years. After the chemotherapy, he still continued to have a productive cough.

Four-drug chemotherapy consisting of INH, RFP, pyrazinamide (PZA), and EMB was started in July 2002 (Table 1). Table 2 shows the drug sensitivity to various drugs (Vit Spectrum-SR™; Kyokuto Pharmaceutical Industrial Co. Ltd., Tokyo, Japan). The drug sensitivity to PZA was not tested at that time. The isolate was found to be already resistant to INH, RFP, SM, and levofloxacin (LVFX). Therefore, in September 2002, the drugs were immediately changed to PZA, EMB, CS, and ethionamide (TH) and then to five drugs (PZA, EMB, CS, TH, and sparfloxacin [SPFX]) in October 2002. Because the sputa smears remained AFB positive and the symptoms did not improved, the patient received right thoracoplasty in December 2002. The drugs were changed to the four drugs EMB, CS, TH, and SPFX in March 2003. Drug sensitivity testing was conducted in June 2003 (Tables 1 and 2). The isolates were resistant to EMB and TH. The PZA sensitivity test that measures *M. tuberculosis* pyrazinamidase (PZase), which converts PZA to an active form, was conducted in August 2003, and was negative for both July 2002 and June 2003 isolates; i.e., they were resistant to PZA. The drugs were changed to EMB, CS, *p*-aminosalicylic acid (PAS), and SPFX in July 2003. The sputum smear was still AFB-positive. He again received pulmonary resection at the left S6 segment in August 2003. After the resection, the smear turned AFB-negative, and he was discharged from the hospital in September 2003.

To determine whether isolates in July 2002 and in June 2003 came from a single clone, chromosomal DNA was analyzed by restriction fragment length polymorphism (RFLP) (2) using a *IS6110* probe (3) and a trinucleotide

Table 1. Timetable of chemotherapy



INH, Isoniazid; RFP, Rifampicin; EMB, Ethambutol; PZA, Pyrazinamide; TH, Ethionamide; CS, Cycloserine; SPFX, sparfloxacin; PAS, *p*-aminosalicylic acid. ▽: The results of susceptibility testing of antituberculous drugs except for PZA were reported. ▼: The PZase activity was determined. ↑: Isolation date.

*Corresponding author: Mailing address: International Medical Center of Japan, Toyama 1-21-1, Shinjuku-ku, Tokyo 162-8655, Japan. Fax: +81-3-3202-7364, E-mail: etoyota@imcj.hosp.go.jp

Table 2. Patterns of antimicrobial susceptibility of MDR-TB isolates

Month of isolation	Isolate No.	Antibiotics [μg/ml]										
		INH [0.2, 1.0]	RFP [40]	EMB [2.5]	SM [10]	KM [20]	TH [20]	PAS [0.5]	CS [30]	EVM [20]	LVFX [1.0]	PZA[1)]
July 2002	IMCJ694	R, S	R	S	R	S	S	S	S	S	R	ND
June 2003	IMCJ695	R, S	R	R	R	S	R	S	S	S	R	ND

Abbreviations are in Table 1. R, Resistance; S, Sensitive; RFP, Rifampicin; SM, Streptomycin; KM, Kanamycin; EVM, Enviomycin; LVFX, Levofloxacin; ND, not determined.
[1)]: Isolates IMCJ694 and IMCJ695 were negative for PZase activity, which was measured in August 2003.

Table 3. Analysis of MDR-TB isolates for mutations conferring resistance to isoniazid, rifampicin, ethambutol, pyrazinamide, streptomycin, kanamycin, and levofloxacin

Isolate No. (Month of isolation)	INH		RFP	EMB	PZA	SM	SM, KM	LVFX
	katG mutation	*inhA* promoter mutation	*rpoB* mutation	*embB* mutation	*pncA* mutation	*rpsL* mutation	*rrs* mutation	*gyrA* mutation
IMCJ694 (July 2002)	L48Q (CTG→CAG), R463L[1)] (CGG→CTG)	−15C→T	S531L (TCG→TTG)	WT	P54L (CCG→CTG)	K43R (AAG→AGG)	WT	D94G (GAC→GGC) S95T[1)] (AGC→ACC)
IMCJ695 (June 2003)	L48Q (CTG→CAG), R463L[1)] (CGG→CTG)	−15C→T	S531L (TCG→TTG)	M306V (ATG→GTG)	P54L (CCG→CTG)	K43R (AAG→AGG)	WT	D94G (GAC→GGC) S95T[1)] (AGC→ACC)

Abbreviations are in Tables 1 and 2. L48Q, Leu48Gln; R463L, Arg463Leu; S531L, Ser531Leu; M306V, Met306Val; P54L, Pro54Leu; K43R, Lys43Arg; D94G, Asp94Gly; S95T, Ser95Thr.
WT: wild type. Corresponding with nucleotide sequences of a laboratory strain of *M. tuberculosis* H37Rv.
[1)]: Natural polymorphism with no association with drug-resistance (5).
Amino acid numbering based on the GenBank database under accession no. NC_000962.

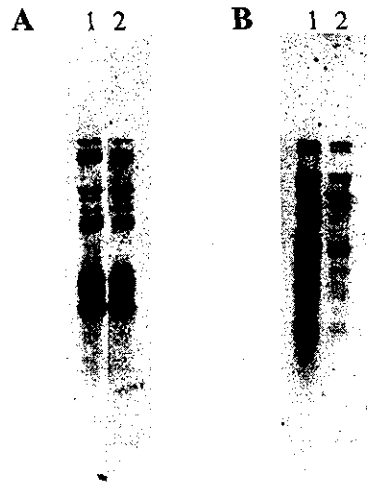


Fig. 1. IS*6110* and $(CGG)_5$ restriction fragment length polymorphism (RFLP) typing.
*Pvu*II- or *Alu*I-digested genomic DNA was analyzed by Southern blot hybridization with a peroxidase-labeled DNA probe for IS*6110* (A) and $(CGG)_5$ (B), respectively. Lane 1, *M. tuberculosis* isolate obtained in July 2002; Lane 2, *M. tuberculosis* isolate obtained in June 2003.

repeat sequence $(CGG)_5$ probe (4). The IS*6110* patterns (Fig. 1A) and $(CGG)_5$ patterns (Fig. 1B) were identical between July 2002 and June 2003 isolates. The results indicate that these isolates were of the same origin.

Drug resistance in *M. tuberculosis* is caused by mutations in restricted regions of its genome (5). We PCR-amplified eight drug resistance-associated regions, including *rpoB* for RFP, *katG* and the *inhA* promoter for INH, *emb*B for EMB, *pncA* for PZA, *rpsL* and *rrs* for SM and KM, and *gyrA* for LVFX, and sequenced them (J. Sekiguchi et al., unpublished data). As shown in Table 3, the July 2002 isolate had eight mutations among these regions, including Ser531Leu in *rpoB*, Leu48Gln and Arg463Leu in *katG*, nucleotide substitution C to T in the *inhA*-promoter region, Pro54Leu in *pncA*, Lys43Arg in *rpsL*, and Asp94Gly and Ser95Thr in *gyrA*. Of these mutations, Arg463Leu in *katG* and Ser95Thr in *gyrA* are known to be natural polymorphisms with no association with drug resistance (5). The other six mutations are known to be associated with resistance to RFP, INH, PZA, SM, and LVFX, respectively. The profile of the mutations was well correlated with that of the drug susceptibility (Tables 2 and 3). The June 2003 isolates showed nine mutations. Among them, eight mutations were identical to those of the July 2002 isolates. In addition to these mutations, the June 2003 isolates had the mutation Met306Val in *embB*. This explains the EMB-resistance of the isolate. The isolate was also resistant to TH. However, mutations that may be associated with TH-resistance were not found in the present study. In general, mutations that play a role in resistance to EMB were not identified.

The patient had been treated with ineffective drugs against MDR-TB organisms, such as INH and RFP from July to September 2002, PZA from September 2002 to March 2003, TH at least in March 2003, and EMB from March to July 2003. SPFX that was given from October 2002 to July 2003 may also have been ineffective, given that SPFX, like LVFX, is a fluoroquinolone and the organisms were resistant to LVFX. Insufficient information about the drug resistance of the bacteria resulted in the use of ineffective drugs. PZA was given from September 2002 to March 2003 without monitor-

ing PZA susceptibility or PZase activity. PZA susceptibility testing or a PZase assay should be added to routine mycobacterial examination. The genetic diagnosis system is useful for rapidly diagnosing drug-resistant *M. tuberculosis*.

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Detection of *Mycobacterium bovis* Bacillus Calmette-Guerin Using Quantum Dot Immuno-Conjugates

Yayoi Otsuka, Ken-ichi Hanaki, Jizi Zhao, Ryuji Ohtsuki, Kiminori Toyooka, Hiroshi Yoshikura[1], Tadatoshi Kuratsuji, Kenji Yamamoto and Teruo Kirikae*

International Medical Center of Japan, Tokyo 162-8655 and
[1]National Institute of Infectious Diseases, Tokyo 162-8640

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Luminescent quantum dots (QDs) are a novel and promising class of fluorophores for cellular imaging (1,2). The benefits of QDs include their photostability, high brightness, multi-target labeling with several colors, and single-source excitation for QDs of all colors. We have developed procedures for using QDs to detect mycobacteria in a species-specific manner.

Mycobacterium bovis BCG strain 172 was obtained from Japan BCG Laboratory, Tokyo, Japan. A green fluorescent protein (GFP) expressing *M. bovis* BCG, containing plasmid pGFM-11, was supplied by C. Locht, Institut Pasteur de Lille, France. The BCG strains were grown in liquid Middlebrook 7H9 medium (Difco Laboratories, Detroit, Mich., USA) supplemented with 10% oleic acid-albumin-dextrose-catalase enrichment (OADC, Difco) and incubated at 37°C. Ten microliters of liquid medium was mounted on a glass coverslip beneath a hole in a plastic petri dish bottom (Matsunami Glass Industry., Ltd., Tokyo, Japan; code. D110100) and were subsequently air dried. Two percent glutaraldehyde in PBS was applied for 1 h at room temperature. After several rinses with PBS, the 1% bovine serum albumin (BSA) in PBS (BSA/PBS) was applied for 20 min at room temperature to block nonspecific binding. Antiserum obtained from rabbits immunized with heat-killed BCG was applied at a dilution of 1:4000 with BSA/PBS, and the dishes were incubated for 1 h at room temperature. After several rinses with 0.02% Tween 20 in PBS (PBS/Tween 20), Qdot™ 655 goat $F(ab')_2$ anti-rabbit IgG conjugate (H+L) highly cross-absorbed (antibodies QD-conjugate: Quantum Dot Corp., Hayward, Calf., USA) was applied at a dilution of 1:1000 with 1% BSA for 1 h at room temperature. The dishes were then rinsed three times with PBS/Tween 20, and microscopic examinations were conducted with a confocal laser scanning microscope (LSM 510, Carl Zeiss, Oberkochen, Germany) equipped with a × 100/1.40 oil immersion objective, an HBO 50 illuminator, and an FITC/Rhodamine dual-band filter set.

The results of immunofluorescent staining (A, B), conventional mycobacterial staining (C, D), and Ziehl-Neelsen staining (E, F) are shown in Fig. 1. BCG strains were labeled in red when treated with anti-BCG antibodies (Fig. 1A), whereas *Mycobacterium smegmatis* (Fig. 1B) was not labeled when treated with anti-BCG antibodies, indicating that these antibodies was specific to *M. bovis* BCG.

As shown by the confocal image in Fig. 2A, the surface of

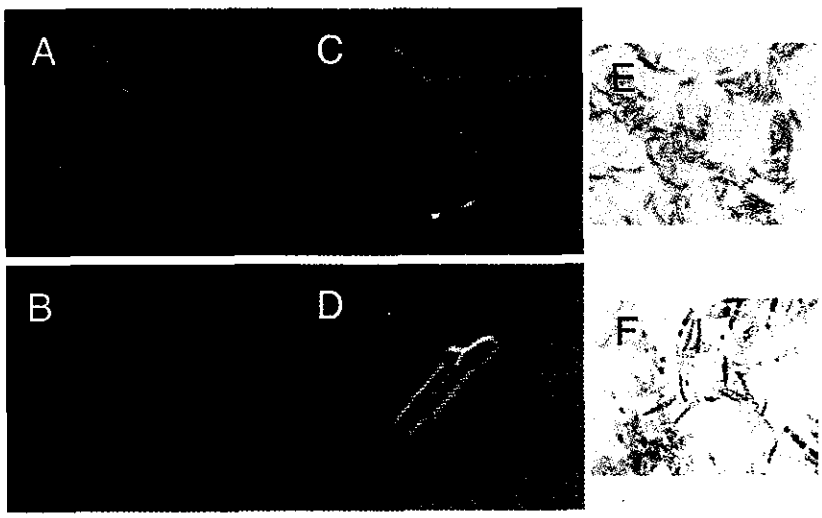

Fig. 1. Immunofluorescence staining of BCG (A, C, E) and *M. smegmatis* (B, D, F) strains (×1000).

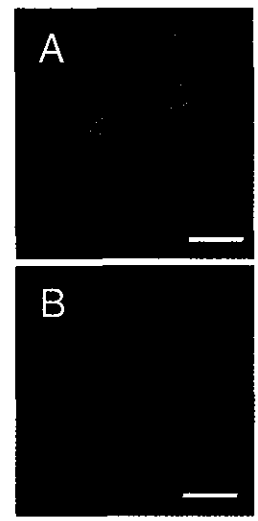

Fig. 2. Labeling of BCG (A) and GFP-expressed BCG (B) with anti-BCG antiserum and QD-conjugated anti-rabbit IgG. Scale bar, 1 μm.

*Corresponding author: Mailing address: International Medical Center of Japan, Toyama 1-21-1, Shinjuku-ku, Tokyo 162-8655, Japan. Fax: +81-3-3202-7364, E-mail: tkirikae@ri.imcj.go.jp

BCG strain 172 was labeled with red-colored QD-conjugated anti-rabbit IgG when treated with antiserum against BCG. The size of the labeled BCG was 3.5 (SD: 0.4) × 0.5 (SD: 0.1) μm ($n = 4$). The microorganisms were not labeled when treated with pre-immune serum. GFP-expressing BCG was stained using the same procedure (Fig. 2B). GFP was detected in the bacteria's intracellular region and was labeled only negligibly by QD-conjugate. The anti-BCG antibodies in combination with the QD-conjugated anti-IgG antibodies labeled the surface of BCG in a specific manner.

Acid-fast staining, such as Ziehl-Neelsen stain and auramine-rhodamine stain, are well-established procedures for detecting *Mycobacterium tuberculosis* and other mycobacterial spp. The immunostaining using QD-conjugates may be useful for identification of mycobacterial-specific antigen.

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INTERNATIONAL PROCEEDINGS

Novel (Recombinant BCG- and DNA-) Vaccination Against Tuberculosis using cynomolgus monkey

[1]Y. Kita, [1]T. Tanaka, [2]S. Yoshida, [3]N. Ohara, [4]Y. Kaneda, [1]S. Kuwayama, [1]Y. Muraki, [1]N. Kanamaru, [1]S. Hashimoto, [1]H. Takai, [1]C. Okada, [1]Y. Fukunaga, [1]Y. Sakaguchi, [1]I. Furukawa, [1]K. Yamada, [1]Y. Inoue, [1]Y. Takemoto, [3]M. Naito, [3]T. Yamada, [5]M. Matsumoto, [6]E.C. Cruz, [6]E.V. Tan, [6]R.M. Abalos, [6]L.J. Young, [6]J.A. Burgos, [6]R. Gelber, [7]Y. Skeiky, [7]S. Reed, [1]M. Sakatani and [1]M. Okada

[1] Clinical Research Center, National Kinki-Chuo Hospital for Chest Disease., Sakai, Osaka, JAPAN [2] Dep. Med. Zoology, Jichi-Med. Sch, Tochigi, JAPAN [3] Nagasaki Univ. Grad. Sch. Biomed. Sci. Div. of Microbiol. Oral Infect., Nagasaki, JAPAN [4] Div. of Gene Ther. Sci. Grad. Sch. of Med. Osaka, Osaka, JAPAN [5] Otsuka Pharma. Co., Ltd., Tokushima, JAPAN [6] Leonard Wood Memorial, Mandaue, Philippines [7] Corixa Corp. Seattle, USA

Summary

HVJ-liposome / HSP65 DNA + IL-12 DNA vaccination were 100 fold more efficient than BCG on the elimination of Mycobacterium tuberculosis (M.TB) in lungs, liver, and spleen in the BALB/c mice. Cytotoxic T cells activity against M.TB in the mice was augmented. The recombinant(r) 72f BCG vaccine as well as HSP65 DNA + IL-12 DNA vaccine showed stronger anti-TB immunity than BCG in the mice, and guinea pigs. By using these new vaccines (HSP65 DNA + IL-12 DNA, r72f BCG and 72f fusion protein + BCG) and the cynomolgus monkey models which are very similar to human tuberculosis, the prophylactic effect (survival, Erythrocyte Sedimentation Rate, chest X-P finding, immune responses) of vaccines was observed. Thus, these novel vaccines should provide a useful tool for the prevention of human TB infection.

Introduction

The development of new vaccine against tuberculosis (TB) is essential to protect many people from the infection of M.tuberculosis in the world. New vaccine is also necessary to cure the patients with MDR-TB. Therefore, the development of new vaccine was studied using three distinct methods; 1. DNA 2. rBCG and 3. subunit vaccination. In our previous report, IFN-γ gene and IL-6 related gene (IL-6 gene + IL-6R gene + gp130 gene) using adenovirus vector exerted significant efficacy against TB(1). Cytotoxic T lymphocyte (CTL) is suggested to play an important role in the anti-tuberculosis immunity in mice vaccinated related genes as well as tumor immunity (1-4). In the present paper, HVJ-liposome / HSP65 DNA+ IL-12 DNA or r72f BCG showed the prophylactic effect in the cynomolgus monkey as well as mice challenged with M.TB.

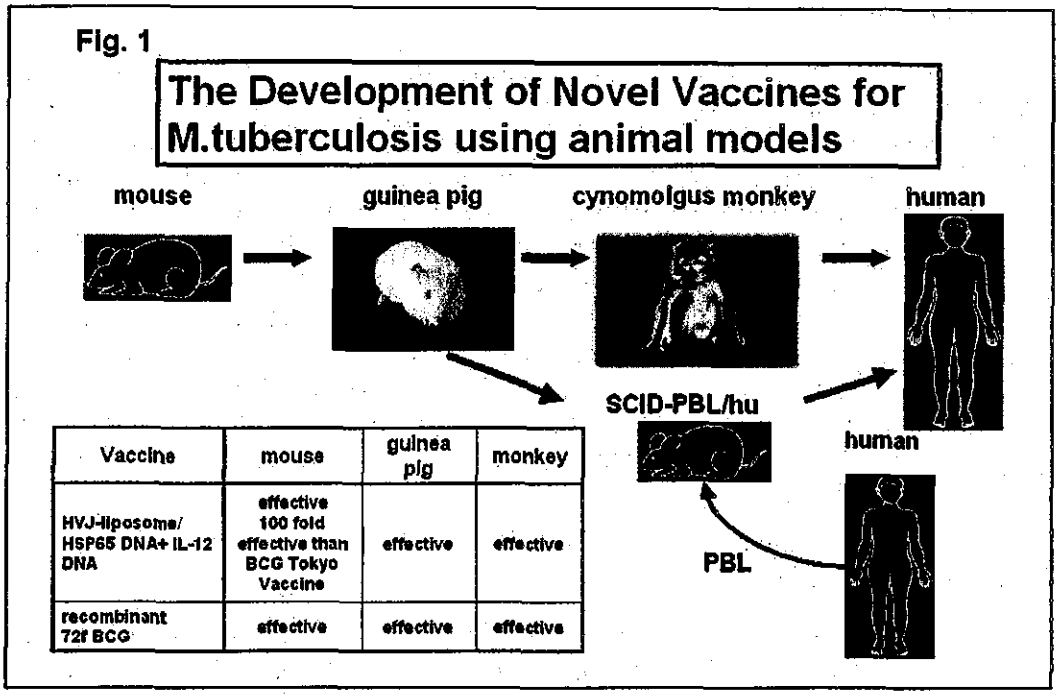
Materials and Methods

IL-12 gene or heat shock protein (HSP65) gene derived from M.TB was constructed as DNA vaccine into plasmid using CMV promoter. DNA vaccination into female BALB/c (8 weeks, CEA Japan Inc., Japan) was performed using HVJ-liposome CTL activity was assessed by the detection of IFN-γ activity in the culture supernatant of 20 hrs. culture consisting effector spleen cells from vaccine(s)-treated mice in the presence of J774.1 macrophage cells pulsed with M.TB. Cynomologus monkeys were immunized three times with (1)r72f BCG vaccine (2)HVJ-liposome / HSP65 DNA + IL-12 DNA vaccine (3)72f fusion protein + BCG Tokyo (4)BCG Tokyo (5)Saline. One month after last immunization, M.TB (Erdman strain $5X10^2$) were challenged by intratracheally instillation. BSR, body weight, chest X-P, immune responses, DTH reaction against PPD, and survival were studied.

Results

An infection initiated by intravenous injection of virulent M.TB H37RV ($5X10^5$) was allowed to develop for 10 weeks, the duration time of which the number of bacteria in the internal organs such as lung, liver, spleen increased. 10 weeks after challenge, mice coimmunized with HSP65 DNA vaccines and IL-12 expression vector had significantly reduced numbers of CFU in the all three organs (lungs, liver, spleen) as compared with mice immunized with Mycobacterium bovis BCG vaccine. (Fig. 1) Spleen cells from the mice vaccinated with HSP65 plus IL-12 DNA produced more IFN-γ than those from the mice vaccinated with BCG Tokyo in the assay for CTL activity. Thus CTL activity correlated with the efficacy of vaccination.

Fig. 1



Vaccine	mouse	guinea pig	monkey
HVJ-liposome/ HSP65 DNA+ IL-12 DNA	effective 100 fold effective than BCG Tokyo Vaccine	effective	effective
recombinant 72f BCG	effective	effective	effective

Fusion protein Mtb72f (Mtb39 + Mtb32) vaccine was developed by Dr. Steven Reed et al, Corixa. To develop more strong vaccination, rBCG secreting 72f was constructed using 72f fusion gene. R72f BCG exerted stronger effect on the increase in the number of IFN-γ producing T cells than BCG Tokyo in mice by ELISPOT. In guinea pig model, r72f BCG as well as HSP65 + IL-12 DNA vaccine improved the histopathological observations of the lungs.

Survival of monkeys vaccinated with HSP65 + IL-12 DNA vaccine was better than that of control (saline) monkeys. (Table 1) All 4 monkeys of control saline groups died of TB infection. In contrast, 2 monkeys out of 4 in the HSP65 + IL-12 DNA group were alive more than 14 months. Survival of monkeys vaccinated with r72f BCG was better than that of BCG vaccine group. All 4 monkeys in the 72f fusion protein + BCG Tokyo vaccine group were alive. HSP65 + IL-12 DNA vaccine and r72f BCG vaccine improved ESR and chest X-P finding and induced the increase in weight of TB- infected cynomolgus monkeys. The augmentation of IFN-γ production was significantly augmented in the monkeys immunized with these three kinds of vaccines. IL-2 production and proliferation of PBL were strongly enhanced in the monkeys vaccinated with HSP65 + IL-12 DNA vaccine.

Conclusions

(1)Novel HVJ-liposome / HSP65 DNA + IL-12 DNA vaccine showed

Table 1

The Development of Novel vaccines using cynomolgus monkey
Anti-tuberculosis effect of ① HSP65DNA+IL-12 DNA vaccine,
② recombinant 72f BCG vaccine

Three distinct kind of TB vaccines	Prophylactic effect against M.tb	survival	Improvement of BSR	Increase in Body weight	Improvement of Chest X-ray finding	Immune Responses		
						Proliferative responses of lymphocyte	IFN-γ production	IL-2 production
① HVJ-liposome /HSP65 DNA + IL-12 DNA vaccine	++	++	++	+	+	+++	+	+
② recombinant 72f BCG vaccine	++	++	+	+	+	+	+	+

prophylactic effect on TB infection using murine, guinea pig and cynomolgus monkey models. (2)HSP65 + IL-12 DNA vaccination was more efficient (100 fold) than parental BCG Tokyo vaccination in mice. CTL activity against M.TB was augmented. (3)By using new vaccines (HSP65 DNA + IL-12 DNA and r72f BCG) and the cynomolgus monkey models which are very similar to human tuberculosis, the significant prophylactic effect of vaccines are observed. (4)HSP65 + IL-12 DNA vaccine, r72f BCG vaccine and (72f fusion protein + BCG Tokyo) vaccine (a)Prolonged the survival time (b)Improved the BSR, chest X-P findings, and immune responses.

The SCID-PBL/hu mouse model, which in capable of analyzing in vivo human immune responses, was also used for theses studies because it is a highly relevant translational for human cases. (4) (Fig. 1) We plan to do clinical trial using, HVJ-Envelope / HSP65 + IL-12DNA vaccine.

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Novel recombinant BCG and DNA-vaccination against tuberculosis in a cynomolgus monkey model

Yoko Kita[a], Takao Tanaka[a], Shigeto Yoshida[b], Naoya Ohara[c], Yasufumi Kaneda[d], Sachiko Kuwayama[a], Yumiko Muraki[a], Noriko Kanamaru[a], Satomi Hashimoto[a], Hiroko Takai[a], Chika Okada[a], Yukari Fukunaga[a], Yayoi Sakaguchi[a], Izumi Furukawa[a], Kyoko Yamada[a], Yoshikazu Inoue[a], Yuji Takemoto[a], Mariko Naito[c], Takeshi Yamada[c], Makoto Matsumoto[e], David N. McMurray[f], E.C. Dela Cruz[g], E.V. Tan[g], R.M. Abalos[g], J.A. Burgos[g], Robert Gelber[g], Yasir Skeiky[h], Steven Reed[h], Mitsunori Sakatani[a], Masaji Okada[a,*]

[a] *Clinical Research Center, National Hospital Organization Kinki-chuo Chest Medical Center, 1180 Nagasone, Sakai, Osaka 591-8555, Japan*
[b] *Department of Medical Zoology, Jichi-Med. Sch, 3311-1 Yakushiji, Minamikawachi-machi, Tochigi 329-0498, Japan*
[c] *Nagasaki University Graduate School of Biomedical Sciences, Division of Microbiology of Oral Infection, 1-7-1 Sakamoto, Nagasaki 852-8588, Japan*
[d] *Division of Gene Therapy Science Graduate School of Medicine, Osaka University, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan*
[e] *Otsuka Pharmaceutical Co. Ltd., 463-10, Kagasuno, Kawauchi-cho, Tokushima 771-019, Japan*
[f] *System Health Science Center, Texas A & M University, Reynolds Medical Building, Mail Stop 1114 College Station, TX 77843-1114, USA*
[g] *Leonard Wood Memorial, Jagobino, Mandaue City, Cebu 6000, Philippines*
[h] *Corixa Corp., 1124 Columbia Street, Suite 200, Seattle, WA 98104, USA*

Abstract

We have developed two novel tuberculosis (TB) vaccines: a DNA vaccine combination expressing mycobacterial heat shock protein 65 (Hsp65) and interleukin-12 (IL-12) by using the hemagglutinating virus of Japan (HVJ)-liposome (HSP65 + IL-12/HVJ) and a recombinant BCG harboring the 72f fusion gene (72f rBCG). These vaccines provide remarkable protective efficacy in mouse and guinea pig models, as compared to the current by available BCG vaccine. In the present study, we extended our studies to a cynomolgus monkey model, which is currently the best animal model of human *tuberculosis*, to evaluate the HSP65 + IL-12/HVJ and 72f rBCG vaccines. Vaccination with HSP65 + IL-12/HVJ as well as 72f rBCG vaccines provided better protective efficacy as assessed by the Erythrocyte Sedimentation Rate, chest X-ray findings and immune responses than BCG. Most importantly, HSP65 + IL-12/HVJ resulted in an increased survival for over a year. This is the first report of successful DNA vaccination and recombinant BCG vaccination against *M. tuberculosis* in the monkey model.

Keywords: HSP65 DNA + IL-12 DNA vaccine; Tuberculosis; Monkey

1. Introduction

Tuberculosis (TB) is a major global threat to human health, with more than 3 million people dying each year from *M. tuberculosis* (TB) infections. The only tuberculosis vaccine currently available is an attenuated strain of *M. bovis* BCG (BCG), although its efficacy against adult TB disease remains controversial. Therefore, we have recently developed two novel TB vaccines: a DNA vaccine combination expressing mycobacterial heat shock protein 65 (Hsp65) and interleukin-12 (IL-12) by using the hemagglutinating virus of Japan (HVJ)-liposome (HSP65 + IL-12/HVJ) and a recombinant BCG harboring the 72f fusion gene (r72f BCG). The former vaccine was 100-fold more efficient than BCG in the elimination of *M. tuberculosis* in mice by the induction of CTL (Yoshida et al., submitted for publication).

* Corresponding author. Tel.: +81 72 252 3021; fax: +81 72 21 2153.
E-mail address: okm@kinchu.hosp.go.jp (M. Okada).

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Researchers have recognized that a nonhuman primate model of TB will be able to provide critical information for vaccine development. However, several TB vaccine candidates who appear to protect better than BCG against virulent *M. tuberculosis* in mice, have rarely been tested in the nonhuman primate model because of cost and limited facilities.

In the present study, we evaluated the protective efficacy of HSP65 + IL-12/HVJ and r72f BCG in the cynomolgus monkey model, which is an excellent model of human tuberculosis [1]. These vaccines provided a strong prophylactic effect in monkeys challenged with *M. tuberculosis* as we have seen previously in mice.

2. Materials and methods

DNA vaccines encoding *M. tuberculosis* HSP65, mouse IL-12 and guinea pig IL-12 were encapsulated with HVJ-liposomes [2]. Groups of animals (mice and guinea pigs) were vaccinated intramuscularly with HVJ-liposome DNA vaccines. CTL activity was assessed by ^{51}Cr-release and IFN-γ activity [3,4]. A total of 16 cynomolgus monkeys were housed in a BL 3 animal facility of the Leonard Wood Memorial. Groups of animals were vaccinated three times with either the HVJ-liposome combination with HSP65 DNA plus human IL-12 DNA (HSP65 + hIL-12/HVJ: 400 μg i.m.), r72f BCG (1×10^6 CFU i.d.), BCG Tokyo (1×10^6 CFU i.d.) or saline. One month after the third vaccination, monkeys were challenged with the *M. tuberculosis* Erdman strain (5×10^2) by intratracheally instillation, Erythrocyte Sedimentation Rate (ESR), body weight, chest X-ray, immune responses, DTH reaction against PPD and survival periods were examined during 14 months [1].

3. Results

Mice vaccinated with HSP65 + mIL-12/HVJ had significantly reduced numbers of CFU [5] in the lungs, liver and spleen as compared with mice vaccinated with BCG (Yoshida et al., submitted for publication). CTL activity correlated with the protective efficacy of vaccination. The fusion protein Mtb72f (Mtb39 + Mtb32) vaccine was developed by Skeiky et al. [6]. To improve its vaccine efficacy, a recombinant BCG harboring the 72f fusion gene (r72f BCG) was generated [7]. The ELISPOT assay showed that r72f BCG induced a greater number of IFN-γ producing T-cells than BCG in the mouse model. In the guinea pig model, r72f BCG as well as HSP65 + gpIL-12/HVJ provided better protection against the pulmonary pathology caused by pulmonary challenge with TB than BCG vaccination (data not shown).

The purpose of this study was to evaluate two TB vaccines we have developed in a nonhuman primate model of *M. tuberculosis* infection. To this end, a total of 16 monkeys were vaccinated either with HSP65 + hIL-12/HVJ, r72f BCG, BCG or saline, followed by TB challenge by intratracheally instillation. Table 1 shows survival periods of vaccinated monkeys after TB challenge. All four monkeys in the control (saline) group died of TB infection within 8 months. In contrast, three and two monkeys from the 72f rBCG and HSP65 + hIL-12/HVJ groups, respectively, were alive more than 14 months post-infection (the termination period of the experiment). Survival periods of the remaining monkeys in the both groups were much longer than those of saline control group. In addition, both HSP65 + hIL-12/HVJ and r72f BCG significantly improved ESR and chest X-ray findings (Table 2). Body weights of the HSP65 + hIL-

Table 1
Survival of cynomolgus monkeys immunized with HVJ-liposome/HSP65 DNA + IL-12 DNA vaccine and recombinant 72f BCG vaccine

Vaccination	Total monkeys	Survival	Dead	% Survival
HVJ-liposome/HSP65 DNA + IL-12 DNA	4	2	2	50
Recombinant 72f BCG	4	3	1	75
BCG Tokyo	4	2	2	50
Saline	4	0	4	0

Cynomolgus monkey (4 monkeys/group) were immunized three times (every 3 weeks) with (1) HVJ-liposome/ HSP65 DNA + IL-12 DNA vaccine, (2) r72f BCG vaccine, (3) BCG Tokyo and (4) saline as control group as described in Section 2. One month after last immunization, M.TB (Erdman strain 5×10^2) was challenged by intratracheally instillation. Survival was studied more than 14 months.

Table 2
Improvement of Erythrocyte Sedimentation Rate (ESR) in the cynomolgus monkeys immunized with HVJ-liposome/HSP65 DNA + IL-12 DNA vaccine and recombinant 72f vaccine

Vaccination	ESR (nm/h)	Mean ± S.D.	Statistical significance *P*-value compared to saline group (Student *t*-test)
HVJ-liposome/HSP65 DNA + IL-12 DNA	2	3.5 ± 1.9	<0.01
	6		
	4		
	2		
Recombinant 72f BCG	3	6.75 ± 8.9	Not significant
	1		
	20		
	3		
BCG Tokyo	22	11.25 ± 11.3	Not significant
	2		
	20		
	1		
Saline	50	29.75 ± 18.1	
	14		
	15		
	40		

Cynomolgus monkey (4 monkeys/group) were immunized and challenged as described in Table 1. Elevation of Blood Sedimentation Ratio (BSR) of all monkeys was evaluated every month and maximum values of BSR in each monkey were shown.

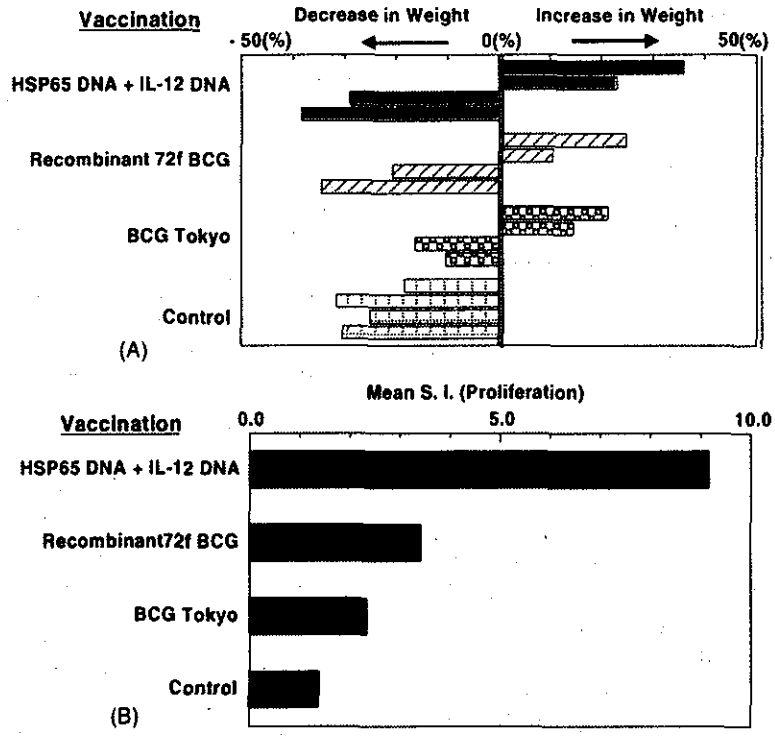

Fig. 1. (A) Increase in body weight: the prophylactic effect of novel vaccines (HSP65 DNA + IL-12 DNA, recombinant 72f BCG) on *M. tuberculosis* infection of cynomolgus monkeys. Percent of increase or decrease in body weight of monkeys immunized with (1) HSP65 DNA + IL-12 DNA (■), (2) recombinant 72f BCG (▨), (3) BCG Tokyo vaccines (▩) and (4) saline (control) (▤) and challenged with *M. tuberculosis*, compared to the weight of pre-immunized monkeys. (B) Lymphocyte proliferation activity (LPA) against recombinant HSP65 protein in the peripheral blood (whole blood) from the cynomolgus monkeys immunized with novel vaccines and challenged with *M. tuberculosis*. Peripheral blood lymphocytes (whole blood) 4 weeks after TB challenge were cultured with 10 μg/ml of recombinant HSP65 antigen in a 96-microwell plate for 5 days at 37 °C and then pulsed with 1 μCi of [3H] thymidine per well for the final 16–18 h of incubation. Results are expressed as a stimulation index (S.I.) and compared to the pre-immune LPA from the same monkey.

12/HVJ group also increased significantly, as compared to saline control group (Fig. 1A). IL-2 and IFN-γ production were augmented in the two groups vaccinated with HSP65 + hIL-12/HVJ and r72f BCG (data not shown). Furthermore, proliferation of PBL was strongly enhanced in the group vaccinated with HSP65 + hIL-12/HVJ in response to HSP65 protein 4 weeks after TB challenge (Fig. 1B). Taken together, these results clearly demonstrate that both HSP65 + hIL-12/HVJ and r72f BCG could provide protective efficacy against *M. tuberculosis* in the cynomolgus monkey model.

4. Discussion

HSP65 + hIL-12/HVJ vaccine as well as r72f BCG vaccine exerted the significant prophylactic effect against TB, as indicated by: (1) prolongation of survival for over a year, (2) improvement of ESR and chest X-ray findings, (3) increase in the body weight and (4) augmentation of immune responses, in a cynomolgus monkey model which closely mimics human TB disease. It is very important to evaluate the long survival period in a monkey model, as human TB is a chronic infection disease. Furthermore, the decrease in the body weight of TB patients with TB is usually accompanied by progress of TB disease. Suppression of IFN-γ production, CTL activity and T-cell proliferation has also been observed in patients with TB [8].

Our results with the HSP65 + hIL-12/HVJ vaccine in the cynomolgus monkey model should provided a significant rational for moving this vaccine into clinical trials. In fact, the 72f fusion protein vaccine entered Phase I testing after its evaluation in cynomolgus monkeys in Leonard Wood Memorial [4] by Reed and Skeiky. Thus, we are taking advantage of the availability of multiple animal models (mouse, guinea pig, and monkey) to accumulate essential data on the HVJ-liposome DNA vaccine in anticipation of a Phase I clinical trial.

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