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NOTE

Comparison of rifabutin susceptibility and *rpoB* mutations in multi-drug-resistant *Mycobacterium tuberculosis* strains by DNA sequencing and the line probe assay

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Abstract We compared rifabutin susceptibility and *rpoB* mutations in 98 multi-drug-resistant strains of *Mycobacterium tuberculosis* (MDR-TB) by DNA sequencing and with a line probe assay using the commercially available INNO-LiPA Rif. TB kit (the LiPA). Our results indicated that rifabutin continues to remain active against MDR-TB strains harboring certain genetic alterations and also that the LiPA might be useful in identifying MDR-TB strains susceptible to rifabutin.

Keywords Tuberculosis · Drug resistance · Rifabutin · *rpoB* · Line probe assay

The recent global expansion of multi-drug-resistant *Mycobacterium tuberculosis* (MDR-TB) poses a serious threat to human health. Numerous previous studies have shown that the majority of rifampicin-resistant isolates of *M. tuberculosis* are also isoniazid resistant [1]. The detection of rifampicin resistance therefore has the potential benefit of simultaneously detecting MDR-TB [1, 2]. One of the commercial kits used to determine drug resistance is the INNO-LiPA Rif. TB kit (the LiPA; Innogenetics, Ghent, Belgium). This assay is an excellent tool for detecting mutations in hot-spot regions of *rpoB*, a gene that encodes a subunit of RNA polymerase. Such mutations occur in up to 95% of rifampicin-resistant strains [2].

Rifabutin is a semisynthetic spiropiperidyl derivative of rifampicin, which is more active than rifampicin itself against *M. tuberculosis* in immunocompromised patients [3]. Rifabutin is also useful as an alternative to rifampicin when serious side effects occur during tuberculosis treatment [4]. Moreover, the minimum inhibitory concentration (MIC) of rifabutin in rifampicin-resistant strains of *M. tuberculosis* carrying *rpoB* mutations varies depending on the specific site of the mutation in the *rpoB* gene [5–10]. Rifabutin might therefore be active against some MDR-TB strains. However, rifabutin susceptibility testing using the time-consuming proportional method on Middlebrook 7H10 medium or by 7H9 microdilution could postpone the effective treatment of patients infected with MDR-TB.

This study aimed to determine the MICs of rifampicin and rifabutin for MDR-TB isolates with known *rpoB* sequences and also to assess results of the LiPA, thereby helping to establish whether this test enables detection of rifabutin susceptibility in MDR-TB strains.

A total of 128 *M. tuberculosis* strains retrieved from a culture collection of the Kinki-chuo Chest Medical Center were tested by the mycobacterial growth-indicator tube–aspartate aminotransferase (MGIT-AST) method (Becton–Dickinson and Company, Fukushima, Japan), and WelPack method (Nihon BCG Inc, Tokyo, Japan) that was established by the egg-based Ogawa medium in commercial susceptibility test systems. Ninety-eight of these strains

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were considered to be resistant to rifampicin as determined by these media. Thirty pan-drug-sensitive (DS) strains were collected between 1 and 31 August 2008, and 98 MDR strains were collected between 1 January 2001 and 31 December 2008. All patients from whom the strains were derived were negative for both human immunodeficiency virus (HIV)-1 and HIV-2. With the exception of one MDR patient, these patients represent all of the DS- and MDR-TB patients treated in this hospital during the strain collection periods.

The MICs for these strains were determined by the validation protocol, performing the commercial and the in-house-prepared microdilution method in parallel for a series of these strains. We elected to use the BrothMIC MTB-1 (Kyokuto Pharmaceutical Industrial Co. Ltd., Tokyo, Japan) and a similar system for slowly growing mycobacteria by using 7H9 broth [11]. BrothMIC MTB-1 susceptibility test system with a shorter incubation period has been previously demonstrated to determine MICs that correlate with those obtained from the standardized agar proportion method. According to the manufacturer's instructions, the proposed breakpoints for rifampicin are $\leq$0.06 μg/ml (susceptible), 0.125–2 μg/ml (intermediate), and $\geq$4 μg/ml (resistant). For the microdilution method using 7H9 broth, 100 μl of serial twofold dilutions of rifampicin or rifabutin were dispensed into each well. The final concentrations of the test drugs ranged from 0.015 to 256 μg/ml. All microdilution plates were incubated at 37°C in plastic bags to increase carbon dioxide (CO_2) and were read after 7, 14, and 21 days by looking for macroscopic growth with an indirect light source. MICs were the lowest dilutions exhibiting no growth. Quality control testing using *M. tuberculosis* H37Rv was performed once each testing. Each microdilution plate included basal medium without antimicrobial agents to assess viability of the test organisms. Each microdilution testing was performed in duplicate on different days.

The MDR-TB strains were analyzed for the presence of mutations in the rifampicin-resistance-determining region (RRDR). A set of primers described by Kim et al. [12], MF (5′-CGACCACTTCGGCAACCG) and MR (5′-TCGATC GGGCACATCCGG), were used to amplify a 342-bp fragment of the *rpoB* gene containing the 81-bp RRDR. The polymerase chain reaction (PCR) product was sequenced using an automated DNA sequencer (ABI Genetic Analyzer 310, Applied Biosystems, Foster City, CA, USA) with MF and MR primers. The LiPA we employed was used in accordance with the manufacturer's instructions. This kit comprises the *M. tuberculosis* complex-specific probe, five overlapping sensitive probes (wild-type S: 19–23 bases long), and four resistance probes (R-type) from a region of the *rpoB* gene encoding amino acids 509–534. The lack of reactivities of an amplified fragment with the wild-type S probes (probes S1 through S5) was used to detect mutations that lead to rifampicin resistance. Furthermore, R-type probes were specifically designed to hybridize to the sequences of the four most frequently observed mutations: R2 (Asp-516-Val), R4a (His-526-Tyr), R4b (His-526-Asp), and R5 (Ser-531-Leu). When all the wild-type S probes gave a positive signal and all the R-type probes reacted negatively (wild-type profile), the *M. tuberculosis* isolate was considered susceptible to rifampicin. When at least one negative signal was obtained with the wild-type S probes, the isolate was considered rifampicin resistant (ΔS profiles). When the resistance to rifampicin was due to one of the four most frequently observed mutations described above, a positive reaction was obtained with one of the four R-type probes and was always accompanied by a negative reaction with the corresponding wild-type S probe (R profiles). We used *M. tuberculosis* strain H37RV as a positive control.

The ranges of the MICs in DS-TB strains were $\leq$0.03 μg/ml for rifampicin and $\leq$0.015 μg/ml for rifabutin. The corresponding ranges of the MICs in MDR-TB strains were 0.5 to $\geq$256 μg/ml and $\leq$0.015 to $\geq$256 μg/ml, respectively. Whereas rifabutin MICs for 78 of the 98 MDR-TB strains ranged between 0.5 and $\geq$256 μg/ml, which were threefold lower than or equal to those of rifampicin, the other 20 MDR-TB strains had rifabutin MICs ranging between $\leq$0.015 and $\leq$0.25 μg/ml, which were 4- to 15-fold lower than those of rifampicin. As shown in Table 1, our study revealed 20 mutations in the *rpoB* gene. Single-point mutation at codon 513, 525, 526, 531, 533, or 572, which was detected in 72 MDR-TB strains, influenced susceptibility to rifabutin. We also demonstrated that novel mutations such as two strains with double-point mutations (Asp516Ala and Leu533Pro, or Ser512Ile and His526Pro), one strain with an insertion (at codon 525), and one strain with an His526Ser mutation showed rifabutin resistance. In contrast, 20 (20.4%) of the MDR-TB strains that had single-point mutation at codon 511, 516, or 522 and double-point mutation (Asp516Gln and Ser522Leu) were susceptible to rifabutin (MIC, <0.5 μg/ml). The observations that some rifampicin-resistant strains remained susceptible to rifabutin suggest that *rpoB* mutation position and type of amino acid change influence rifabutin susceptibility.

In this study, four MDR-TB strains with a wild-type profile by the LiPA exhibited rifabutin resistance as well. Moreover, 72 strains exhibiting R4a, R4b, R5, ΔS4, ΔS5, ΔS1 + ΔS4, or ΔS2 + ΔS4 + R5 profiles were also resistant to rifabutin. Conversely, 19 strains that exhibited R2 (one of the four most frequently observed mutations), ΔS3, or ΔS2 + ΔS3 profiles were characterized by low rifabutin MICs. The susceptibility of rifabutin conflicted among the remaining three strains that exhibited ΔS1 profile. In detail, one strain had a mutation at codon 511

Table 1 Comparison of *rpoB* genotype, susceptibility of rifampicin and rifabutin, and the LiPA profiles

Isolate phenotype and mutation position[a]	Isolates (*n*)	MIC (μg/ml)		LiPA
		Rifampicin	Rifabutin	
DS-TB				
Wild type	30	≤0.03 to 0.03	≤0.015	WT
MDR-TB[b]				
511Leu → Pro	1	0.5	0.03	ΔS1
513Gln → Lys	2	8, 16	4, 16	ΔS1
516Asp → Val	17	4 to ≥256	0.015 to 0.25	R2
522Ser → Leu	1	2	0.06	ΔS3
525ACG insertion	1	32	32	WT[c]
526His → Tyr	2	32, 64	8, 64	R4a
526His → Asp	3	64, 128, 128	16, 64, 128	R4b
526His → Ser	3	2, 4, 64	2, 4, 32	ΔS4
526His → Arg	1	32	32	ΔS4
526His → Pro	2	8, 64	4,32	ΔS4
526His → Leu	1	256	64	ΔS4
526His → Cys	1	4	1	ΔS4
526His → Arg, 529Arg → Gln	1	64	64	ΔS4
531Ser → Leu	54	0.5 to ≥256	0.5 to ≥256	R5
533Leu → Pro	1	32	32	ΔS5
512Ser → Ile, 526His → Pro	1	≥256	≥256	ΔS1 + ΔS4
516Asp → Glu, 522Ser → Leu	1	128	0.25	ΔS2 + ΔS3
516Asp → Ala, 533Leu → Pro	1	128	64	ΔS5[c]
Mixed peak in 516 (GAC (Asp) → GTC (Val)), 526 (CAC (His) → CAA (His), 530 (CTG (Leu) → ATG (Met)), and 531 (TCG (Ser) →, TTC (Leu))	1	256	256	ΔS2 + ΔS4 + R5[c]
572Ile → Phe	1	1	1	WT
Non-RRDR	2	16, 128	2, 128	WT

WT wild-type S profile, *DS-TB* drug-sensitive tuberculosis, *MDR-TB* multi-drug-resistant tuberculosis

[a] Numbers correspond to *Escherichia coli* RNA polymerase amino acid positions

[b] Resistant to rifampicin at 1.0 μg/ml by the Clinical and Laboratory Standards Institute method of proportion in 7H10 agar and mycobacterial growth-indicator tube–aspartate aminotransferase (MGIT-AST) method or 40 μg/ml by WelPack method

[c] The LiPA also did not reveal the correct type of mutation

and appeared to have a low rifabutin MIC, but the remaining two strains, at codon 513, were characterized by high rifabutin MICs. Thus, except for ΔS1, profiles of the LiPA could predict rifabutin susceptibility rather faithfully (Table 1).

According to previous studies, rifabutin MICs against rifampicin-susceptible strains were ≤0.06 μg/ml [13], and all strains susceptible to 1 μg/ml of rifampicin and 12% of the strains resistant to 10 μg/ml of rifampicin were susceptible to 0.5 μg/ml of rifabutin [14]. In the study by Uzun et al. [15], all rifampicin-susceptible strains and 12% of rifampicin-resistant strains were also susceptible to rifabutin (MIC, ≤1 μg/ml). All 30 DS-TB strains and 20 of 98 MDR-TB strains were susceptible to rifabutin (MIC, <0.5 μg/ml) in our study. Clinical outcome regarding the efficacy of rifabutin therapy for isolates of MDR-TB with the MICs of ≤0.5 μg/ml has not yet been obtained, but the proposed critical concentration for rifabutin (≤ 0.5 μg/ml) in this study was the same as that recommended by The Clinical and Laboratory Standards Institute (CLSI) using agar-plate testing. However, the relevant critical concentration of rifabutin should be determined by future clinical outcome study.

Our data indicated that all MDR-TB strains with an R2 profile, which was associated with a specific point mutation (Asp516Val), were almost always identified as rifabutin susceptible. The LiPA may offer improvement in the management of MDR-TB, as these vulnerable patients can commence treatment with rifabutin before the strain's isolation. This study further confirmed that rifabutin remains active against MDR-TB strains harboring certain genetic alterations. We also indicate that the LiPA is useful for rapid detection of strains susceptible to rifabutin in MDR-TB before examining susceptibility testing.

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Research Article

Novel Prophylactic Vaccine Using a Prime-Boost Method and Hemagglutinating Virus of Japan-Envelope against Tuberculosis

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Objective. *Mycobacterium tuberculosis* infection is a major global threat to human health. The only tuberculosis (TB) vaccine currently available is bacillus Calmette-Guérin (BCG), although it has no efficacy in adults. Therefore, the development of a novel vaccine against TB for adults is desired. *Method.* A novel TB vaccine expressing mycobacterial heat shock protein 65 (HSP65) and interleukin-12 (IL-12) delivered by the hemagglutinating virus of Japan- (HVJ)- envelope was evaluated against TB infection in mice. Bacterial load reductions and histopathological assessments were used to determine efficacy. *Results.* Vaccination by BCG prime with IgHSP65+murine IL-12/HVJ-envelope boost resulted in significant protective efficacy (>10,000-fold versus BCG alone) against TB infection in the lungs of mice. In addition to bacterial loads, significant protective efficacy was demonstrated by histopathological analysis of the lungs. Furthermore, the vaccine increased the number of T cells secreting IFN-γ. *Conclusion.* This vaccine showed extremely significant protection against TB in a mouse model, consistent with results from a similar paper on cynomolgus monkeys. The results suggest that further development of the vaccine for eventual testing in clinical trials may be warranted.

1. Introduction

Tuberculosis (TB) is a major global threat to human health, with about 2 million people dying every year from *Mycobacterium tuberculosis* infection. The only TB vaccine currently available is an attenuated strain of *Mycobacterium bovis*, bacillus Calmette-Guerin (BCG), although its efficacy against adult TB disease is unclear. Furthermore, multidrug-resistant TB (MDR-TB) and extremely drug-resistant TB (XDR-TB) are becoming big problems worldwide. For these reasons, a prophylactic and therapeutic vaccine against TB is sought. TB vaccines are classified into 4 main groups: (1) DNA vaccines, (2) recombinant BCG vaccines, (3) subunit vaccines, and (4) attenuated vaccines.

It is well established that protective immunity to *M. tuberculosis* depends on both $CD4^+$ and $CD8^+$ T cells [1–6]. DNA vaccines induce cellular immune responses, including the Th-1-type cellular immune response, and they prevent infections in animal models [7, 8]. In fact, several human clinical trials have recently been initiated to test the efficacy of DNA vaccines against emerging and re-emerging infectious diseases including hepatitis B [9], malaria [10–12], and HIV infections [13]. DNA vaccines have also shown their potential as TB vaccines in mouse

models [14–17]. However, in a guinea pig model, which is one of the most biologically relevant systems available for studying human pulmonary TB, DNA vaccines have not been proven more efficacious than BCG [18]. The efficacy of any experimental TB vaccine must be evaluated in human clinical trials, and a vaccine against TB is still anxiously awaited.

We have been developing a novel TB vaccine that is a DNA vaccine expressing mycobacterial heat shock protein 65 (HSP65) and interleukin-12 (IL-12), delivered by the hemagglutinating virus of Japan- (HVJ)- liposome or -envelope (HVJ-E) (HSP65 + IL-12/HVJ) [19–22]. The former vaccine was 100-fold more efficacious than BCG in a murine model on the basis of the elimination of *M. tuberculosis* [19]. In the present study, we demonstrated that the combination of BCG prime with HSP65 + IL-12/HVJ-E vaccine-boost was 10,000-fold more efficacious than BCG alone in a murine TB prophylactic model.

2. Materials and Methods

2.1. Bacteria. *M. tuberculosis* strains H37Rv and *M. bovis* BCG Tokyo, were kindly provided by Dr. I. Sugawara (JATA, Tokyo, Japan). *M. bovis* BCG Tokyo was maintained in synthetic Sauton's medium (Wako Chemicals, Osaka, Japan). For the mouse infection studies, a single colony of *M. tuberculosis* H37Rv was grown in Middlebrook 7H9 medium (DIFCO Laboratories, Detroit, MI; lot 137971 XA MD) supplemented with albumin-dextrose complex and grown at 37°C until approximately midlog phase. Aliquots were stored at −80°C and thawed 10 days before use. Each bacterium was grown to midlog phase in 7H9 medium.

2.2. Animals. Inbred and specific pathogen-free female BALB/c mice were purchased from Japan SLC (Shizuoka, Japan). Mice were maintained in isolator cages, manipulated in laminar flow hoods, and used between 8 and 10 weeks of age. All animal experiments were approved by the National Hospital Organization Kinki-chuo Chest Medical Center Animal Care and Use Committee. All vaccinations and experiments on isolated tissues were performed on anesthetized animals with sevoflurane. Infected animals were housed in individual microisolator cages in Biosafety Level (BL) 3 animal facility of the NHO Kinki-chuo Chest Medical Center.

2.3. Plasmid Construction. The *HSP65* gene was amplified from *M. tuberculosis* H37Rv genomic DNA, and cloned into pcDNA3.1 (+) (Invitrogen, San Diego, CA) to generate pcDNA-hsp65 (designated as HSP65 DNA) as described previously [19]. The *hsp65* gene was fused with mouse Igκ secretion signal sequence, and pcDNA-Ighsp65 (designated as IgHSP65 DNA) was generated. For construction of the mouse IL-12 (mIL-12) *p40* and *p35* single-chain genes, *mIL12p35* and *mIL12p40* genes were cloned from pcDNA-p40p35 [21], fused and cloned into pcDNA3.1 (+) to generate pcDNA-mIL12p40p35-F (designated as mIL-12 DNA).

2.4. HVJ-E Vaccination. HVJ-E was prepared as described previously (**Figure 1**) [19–23]. The HVJ-E complex was aliquoted and stored at −70°C until use. Groups of BALB/c mice were vaccinated 3 times at 3-week intervals with 100 μL of HVJ-E solution containing 50 μg of pcDNA-IgHSP65 and 50 μg of mIL12 DNA. These DNA vaccines were injected into both anterior muscles in the tibia. Mice were vaccinated using 1×10^6 colony-forming units (CFU) *M. bovis* BCG Tokyo by subcutaneous injection at 4 different sites (left-upper, right-upper, left-lower, and right-lower back). HVJ-E DNA vaccine containing pcDNA-IgHSP65 and -mIL12 DNA was designated as IgHSP65 + mIL-12/HVJ-E in this text.

2.5. Challenge Infection of Vaccinated Animals and Bacterial Load Determination. Mice were challenged by the intravenous route with 5×10^5 CFU of *M. tuberculosis* H37Rv 4 weeks after the third vaccination as described previously (**Figure 2**) [19, 24]. 0.2 mL of saline containing 5×10^5 CFU of H37Rv *Mycobacterium tuberculosis* were injected into tail vein of mice. At 5 and 10 weeks after *M. tuberculosis* H37Rv challenge, lungs, spleens, and livers were aseptically homogenized by using a homogenizer in saline, and serial dilutions of the organ homogenates were plated on 7H11 Middlebrook agar (Kyokuto. Tokyo, Japan). Plates were sealed and incubated at 37°C, and the number of colonies was counted 2 weeks later. Results were converted to $\log_{10}$ values. The $\log_{10}$ [mean ± standard deviation (S.D.)] values for CFU/organs/animals were calculated for each experimental group. Weight of lungs, liver, or spleen was measured by a balance (Sartorius Co. LP620S).

2.6. Histological Analysis. The lungs obtained from the mice were fixed with 10% buffered formalin and embedded in paraffin. Each block was sliced into 4-μm-thick sections and stained using hematoxylin and eosin. Semiquantitative morphometric analysis of pathological slides was performed by a method modified over that of Dascher et al. (2003) using a micrometer-attached microscope (Microphot-FXA, Nikon, Japan) [19, 25, 26]. The longer axis and minor axis of each granuloma in the field (×40 magnification) were measured and then multiplied and summed. Three random fields from each tissue section of mice were evaluated. The average score of the fields was designated as the granuloma index ($\times 10^{-2}$ mm^2). This method for the evaluation of granuloma area was significantly correlated with the granuloma area determined by a hematoxylin and eosin section scanning method.

2.7. ELISPOT Assay. The spleens were removed aseptically from vaccinated mice 3 weeks after the third vaccination. Antigen-specific IFN-γ-producing cells were determined by enzyme-linked immunosorbent spot (ELISPOT) as described previously [19]. Briefly, ELISPOT plates (MultiScreen IP Filtration plate MAIPS45; Millipore, Bedford, MA) were coated with antimouse IFN-γ MAb R4-6A2 (BD Biosciences Pharmingen. San Diego, CA). Spleen cells from vaccinated mice were suspended at 1×10^7 cells/mL (1×10^6 cells/well). The cells were placed into 6 antibody-coated wells, and rHSP65 protein (10 μg/mL) or PPD

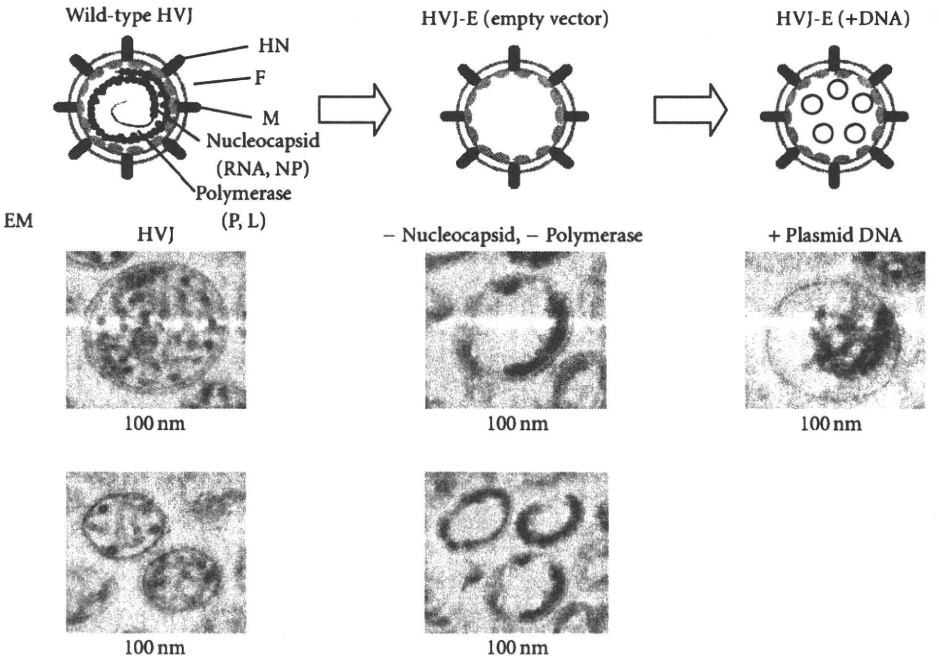

FIGURE 1: Hemagglutinating virus of Japan- (HVJ)- envelope vaccination: pcDNA3.1/HSP65DNA + IL-12DNA were incorporated into an HVJ-envelope empty vector (nonviral vector). Graphical representations of the HVJ-envelope empty vector in the presence or absence of DNA are shown. Electronic microscopy (EM) photographs of the HVJ-envelope empty vector are also shown.

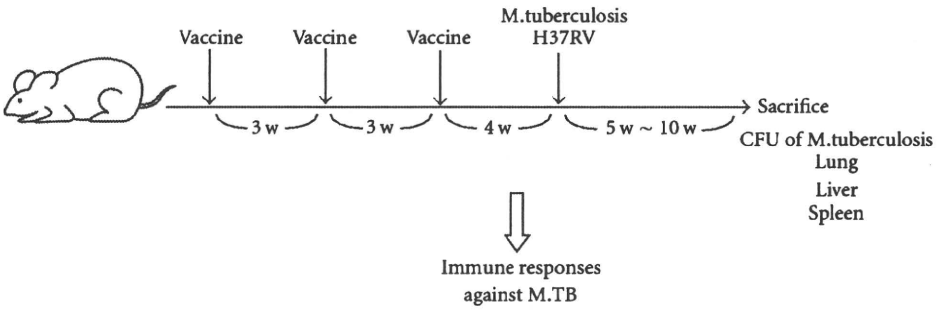

FIGURE 2: Groups of mice were vaccinated 3 times every 3 weeks using the prime-boost method and challenged intravenously with *M. tuberculosis* H37Rv as described in the Materials and Methods section. Five or 10 weeks after challenge with TB, protection was measured by enumerating bacterial loads (CFU) in the lungs, liver, and spleen of the vaccinated mice.

(10 μg/mL) was added to each well. After 20 h of incubation at 37°C, cells were removed by washing the plates, and the site of cytokine secretions was detected using biotinylated antimouse IFN-γ MAb XMG1.2 (BD Biosciences Pharmingen) and streptavidin-alkaline phosphatase conjugate (BD Biosciences Pharmingen). The enzyme reaction was developed with BCIP-NBT substrate (Vector Laboratories Inc., Burlingame, CA). Spot-forming cells (SFCs) were enumerated using the KS ELISPOT system (Carl Zeiss, Hallbergmoos, Germany).

2.8. Statistical Analysis. Dunnett's tests (multiple comparisons) were used to compare $\log_{10}$ values of CFUs between groups following challenge and used to compare T-cell responses between groups in the ELISPOT assay. A P-value of $< .05$ was considered significant.

3. Results and Discussion

3.1. Results

3.1.1. Prophylactic Efficacy Using Prime-Boost Method. The IgHSP65 + mIL-12/HVJ-E and BCG vaccines were administered using the prime-boost method as shown in **Table 1**.

At 5 and 10 weeks after intravenous challenge of *M. tuberculosis* H37Rv, the number of CFUs in the lungs, spleen, and liver were determined. **Figure 3(a)** shows the result of bacterial loads 5 weeks after challenge.

Table 1: BCG-HVJ-E/HSP65 DNA + IL-12 DNA Prime/Boost Experiment. Groups of mice were vaccinated 2 or 3 times with IgHSP65 + mIL-12/HVJ-E vaccine and/or BCG by using the prime-boost method. IgHSP65 + mIL-12/HVJ-E vaccine was injected intramuscularly, and BCG was injected subcutaneously. 4 weeks after the last immunization, *M. tuberculosis* H37Rv was challenged intravenously. 5 weeks and 10 weeks after TB challenge, protection was measured by enumerating bacterial loads (CFU) in the lungs, liver, and spleen from vaccinated mice. One week before the TB challenge, the immune responses of cytotoxic T cells, proliferation of T cells, and cytokines (IFN-γ, IL-2, IL-6) production were assayed.

Group	First immunization	Second immunization	Third immunization
1	—	—	—
2	—	—	BCG
3	HSP65 + IL-12/HVJ-E	HSP65 + IL-12/HVJ-E	HSP65 + IL-12/HVJ-E
4	BCG	HSP65 + IL-12/HVJ-E	HSP65 + IL-12/HVJ-E
5	HSP65 + IL-12/HVJ-E	HSP65 + IL-12/HVJ-E	BCG

13 mice per group.
3 mice for the *in vitro* assay prior to challenge (IFN-γ ELISPOT, etc.).
10 mice for the protection study (half of the mice were used for necropsy at 5 weeks after challenge and half at 10 weeks).

Vaccination by BCG prime + IgHSP65 + mIL-12/HVJ-E boost showed significant protective effects on the bacterial loads in the lungs as compared to BCG alone ($P < .01$). The prime-boost method using DNA and BCG vaccines showed extremely strong protective efficacy (>10,000-fold versus BCG alone) regardless of the order of administration (**Figure 3(a)**). Vaccination with BCG vaccine alone decreased TB CFUs in the lungs by 1 log unit as compared to nonvaccinated mice.

Vaccination with IgHSP65 + mIL-12/HVJ-E and BCG by the prime-boost method also showed significant protective efficacy on the bacterial loads in the liver as compared to BCG (>100-fold, $P < .05$; **Figure 3(b)**). The combination of 2 vaccines and administration by the prime-boost method also exerted a significant protective effect on the bacterial load in the spleen as compared to naive control group (10-fold higher, $P < .05$; **Figure 3(c)**).

Body weight of vaccinated mice was similar in all vaccinated groups. Tissue weights of spleens and livers in the prime-boost groups were lower than those of naive group (Figures **4** and **5**).

We also confirmed the greater enhancement of protective effects in the BCG-DNA vaccine combination groups than those in the naive control group or BCG-alone group 10 weeks after challenge (data not shown). These results indicate that treatment using 2 vaccines by the prime-boost method was more effective than BCG alone.

3.1.2. Histological Analysis. In addition to the reduction of bacterial loads, the efficacies of each vaccine were assessed by histological analysis. The number and size of granulomatous lesions in the lungs were significantly lower and smaller, respectively, in the mice vaccinated by the BCG prime-DNA boost group than in the naive control mice and BCG control mice groups (**Figure 6**). Quantitative evaluation of the granulomatous lesions clearly showed that the BCG prime with IgHSP65 + mIL-12/HVJ-E boost significantly reduced the granuloma index in the lungs as compared to naive and BCG groups ($P < .05$; **Figure 7**). Thus, vaccination by the prime-boost method has the capability to reduce pulmonary lesions caused by *M. tuberculosis* infection.

3.1.3. Immunological Analysis. Furthermore, BCG prime with IgHSP65 + mIL-12/HVJ-E boost augmented the proliferation and IFN-γ production of HSP65 antigen-specific T cells in the K-S ELISPOT Assay. The efficacy of BCG prime with IgHSP65 + mIL-12/HVJ-E boost was higher compared with BCG Tokyo alone or IgHSP65 + mIL-12/HVJ-E prime with BCG boost (**Figure 8**).

These data indicate that the protective efficacies of BCG prime with IgHSP65 + mIL-12/HVJ-E boost are strongly associated with the number and activity of IFN-γ-secreting and HSP65-specific T cells. Taken together, combinational vaccination with BCG and IgHSP65 + mIL-12/HVJ-E by the prime-boost method is capable of augmenting T-cell activation. In addition, increase of IFN-γ-secreting cells is involved in the reduction of bacterial burden and lesions in the lungs. The efficacies of the prime-boost method are greater than those achieved by vaccination with BCG alone.

3.2. Discussion. In this study, we evaluated the protective efficacy of IgHSP65 + mIL-12/HVJ-E vaccine, using the prime-boost method. One of the significant findings was that the combination of IgHSP65 + mIL-12/HVJ-E and BCG led to a remarkably high degree of protection against intravenous challenge infection with virulent *M. tuberculosis*; bacterial numbers exponentially declined in 3 organs, especially in the lungs (10,000-fold lower than that of mice vaccinated with BCG alone; **Figure 3(a)**).

The pathological parameters of protection included reductions in the mean lung granulomatous lesion score in our study. The protective efficacies of BCG with IgHSP65 + mIL-12/HVJ-E administered by the prime-boost method were indicated on the basis of histopathological methods as well as bacterial loads. Histopathological analysis showed that mice vaccinated with BCG prime with IgHSP65 + mIL-12/HVJ-E boost had fewer and smaller lesions in the lungs and significantly less lung granuloma than naive mice and mice treated with BCG alone. These results suggest that severe toxicities (Koch phenomenon) were suppressed by the combination of two kinds of vaccines.

The data in the present study also show that the protective efficacy of BCG prime with IgHSP65 + mIL-12/HVJ-E boost is strongly associated with the emergence of IFN-γ-secreting T cells upon stimulation with HSP65. In the previous study, we demonstrated that *in vivo* function of CD8-positive T cells as well as CD4-positive T cells is involved in prophylactic efficacy of the IgHSP65 + mIL-12/HVJ-E in mice [22].

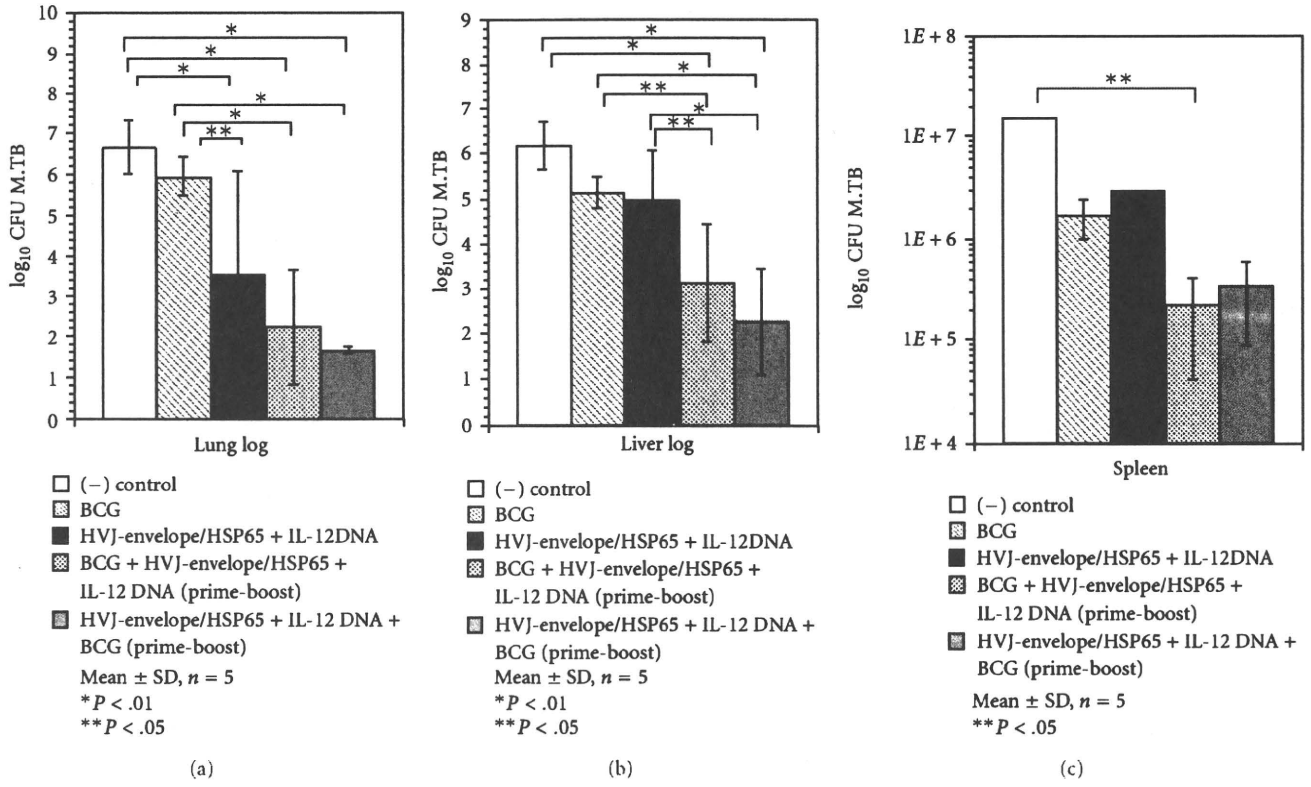

FIGURE 3: (a) Mouse protection studies using the prime-boost method. Groups of mice vaccinated with HVJ-envelope (HVJ-E) DNA and/or BCG were challenged by intravenous injection with *M. tuberculosis* H37Rv. Five weeks after challenge, protection was measured by enumerating the bacterial loads (CFU) in the lungs. Results are expressed as the mean $\log_{10}$ ± S.D. of CFU. The statistical significance of differences between individual groups in the CFU number was determined by Dunnett test ($n = 5$); $^*P < .01$ and $^{**}P < .05$; the statistical significance of differences ($P < .01$) of the G1 (naive) group compared to the G3 group (DNA/DNA/DNA), G4 group (BCG/DNA/DNA), or the G5 group (DNA/DNA/BCG). The statistical significance of differences ($^{**}P < .05$) of the G2 group (BCG-alone group) compared to the G3 group (DNA/DNA/DNA), that of differences ($P < .01$) of the G2 group compared to the G4 group (BCG/DNA/DNA), or the G5 group (DNA/DNA/BCG). (b) Mouse protection studies using the prime-boost method. Groups of mice vaccinated with HVJ-E DNA and/or BCG were challenged by intravenous injection with *M. tuberculosis* H37Rv. Five weeks after challenge, protection was measured by enumerating the bacterial loads (CFU) in the liver. Results are expressed as the mean $\log_{10}$ ± S.D. of CFU. The statistical significance of differences between individual groups in the CFU number was determined by Dunnett test ($n = 5$), $^*P < .01$; the statistical significance of differences ($P < .01$) of the G1 (naive) group compared to the G4 group (BCG/DNA/DNA), or the G5 group (DNA/DNA/BCG). The statistical significance of differences ($P < .05$) of the G2 group (BCG-alone group) compared to the G4 group (BCG/DNA/DNA). The statistical significance of differences($P < .01$) of the G2 group compared to the G5 group (DNA/DNA/BCG). The statistical significance of differences ($P < .05$) of the G3 group (DNA/DNA/DNA) compared to G4 (BCG/DNA/DNA). That of differences ($P < .01$) of the G3 group compared to the G5 group. (c) Mouse protection studies using the prime-boost method. Groups of mice vaccinated with HVJ-E DNA and/or BCG were challenged by intravenous injection with *M. tuberculosis* H37Rv. Five weeks after challenge, protection was measured by enumerating the bacterial loads (CFU) in the spleen. Results are expressed as the mean $\log_{10}$ ± S.D. of CFU. The statistical significance of differences between individual groups in the number of CFU was determined by Dunnett test ($n = 5$); $^{**}P < .05$; the statistical significance of differences ($P < .05$) of the G1 (naive) group compared to the G4 group (BCG/DNA/DNA).

In this study, we used the murine model of TB, which may not reflect the pathologic status of human TB. As to the difference of the infection route, our previous results in a guinea pig model used in a collaborative study with Dr. D. McMurray (Texas A&M University) showed that vaccination with HSP65 + guinea pig IL-12/HVJ resulted in better protection against pulmonary pathology caused by aerosol challenge with *M. tuberculosis* than BCG vaccination (data not shown).

In addition, we have recently confirmed that the prime-boost method was also effective in a cynomolgus monkey model [20–22]. We evaluated our HSP65 + human IL-12/HVJ (HSP65 + hIL-12/HVJ) in the monkey model infected by an intratracheal instillation (aerogenic route), which is currently the best animal model of human TB. Vaccination with HSP65 + hIL-12/HVJ resulted in better protective efficacy than that with BCG alone on the basis of the erythrocyte sedimentation rate test, chest X-ray findings, and immune responses. In addition, vaccination with HSP65 + hIL-12/HVJ resulted in increased survival for over a year. This was the first report of successful DNA vaccination against M. tuberculosis in a monkey model [21].

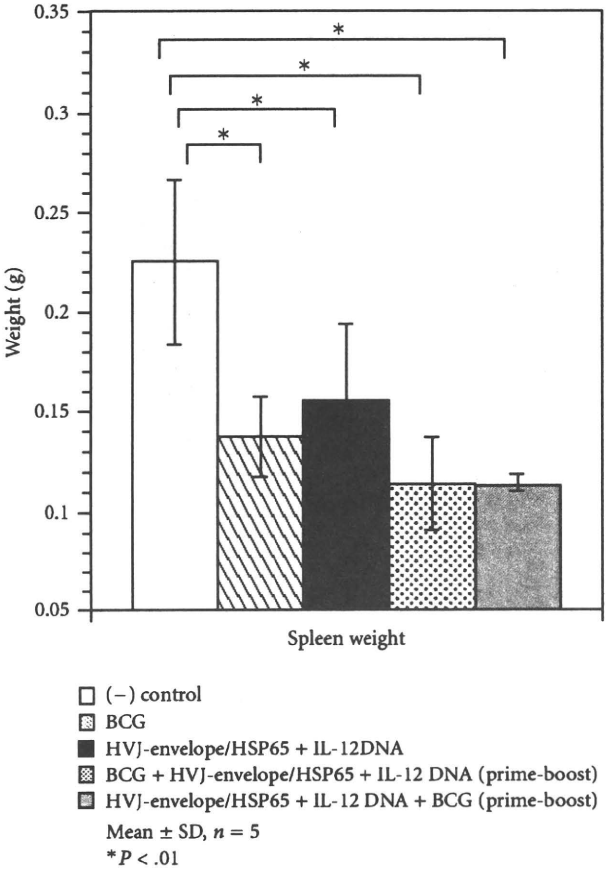

FIGURE 4: Tissue weight in mouse protection studies using the prime-boost method. Groups of mice vaccinated with HVJ-E DNA and/or BCG were challenged by intravenous injection with *M. tuberculosis* H37Rv. Five weeks after challenge, spleen weight was measured. Results are expressed as the mean ± S.D. in grams (g). The statistical significance of differences between individual groups in the weight was determined by Dunnett test ($n = 5$), $^*P \leq .01$; the statistical significance of differences ($P < .01$) of the G1 (naive) group compared to the G2 group (BCG-alone group), G3 group (DNA/DNA/DNA), G4 group (BCG/DNA/DNA), or G5 group (DNA/DNA/BCG).

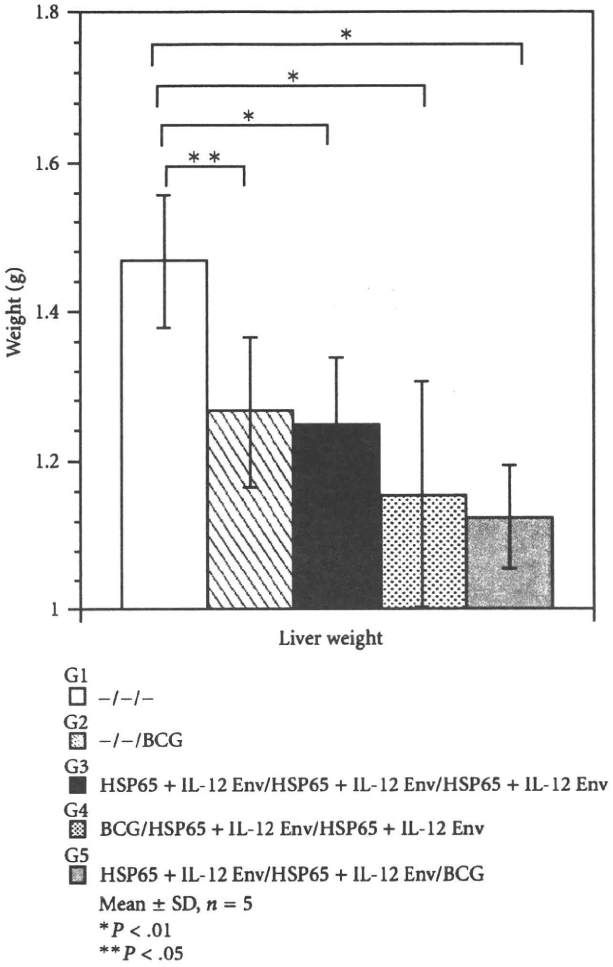

FIGURE 5: Tissue weight in mouse protection studies using the prime-boost method. Groups of mice vaccinated with HVJ-E DNA and/or BCG were challenged by intravenous injection with *M. tuberculosis* H37Rv. Five weeks after challenge, liver weight was measured. Results are expressed as the mean ± S.D. in grams (g). The statistical significance of differences between individual groups in the weight was determined by Dunnett test ($n = 5$), $^*P < .01$; the statistical significance of differences ($P \leq .01$) of the G1 (naive) group compared to the G3 group (DNA/DNA/DNA), G4 group (BCG/DNA/DNA), or G5 group (DNA/DNA/BCG). $^{**}P < .05$; that of differences ($P < .05$) of the G1 group compared to the G2 group (BCG alone group).

Most importantly, protective efficacy was augmented when BCG and HSP65 + hIL-12/HVJ were administered by the prime-boost method. Survival rates of BCG alone, saline control, HSP65 + hIL-12/HVJ-prime with BCG-boost, and BCG-prime with HSP65 + hIL-12/HVJ-boost groups were 33%(2/6), 50%(3/6), 50%(2/4), and 100%(4/4) at 12 months after the infection (aerogenic route), respectively [21]. We also evaluated immune responses in the monkey model of TB. Antigen-specific IFN-γ-production and proliferation of peripheral blood lymphocyte (PBL) were enhanced by the vaccination using the prime-boost method.

We also demonstrated efficacies in the monkey model when the boost was performed after a long-term period (4 months) from the prime. The prolongation of the survival was observed in the BCG-prime and HSP65 + IL-12/HVJ-booster group [27]. Improvement of ESR, increase of the body weight and augmentation of IFN-γ production, and proliferation of PBL were also observed in the BCG-prime and HSP65 + IL-12/HVJ-booster group.

Taken together, these results clearly demonstrated that BCG-prime with HSP65 + hIL-12/HVJ-boost could provide extremely strong protective efficacy against M. tuberculosis in a cynomolgus monkey model (intratracheal infection route), which is currently the best animal model of human TB [21].

The prime-boost method was reported in a study of the MVA85A vaccine, which is a modified vaccinia virus Ankara (MVA) strain expressing antigen 85A. In phase I studies in humans, this vaccine has induced high immune responses in previously BCG-vaccinated individuals [28].

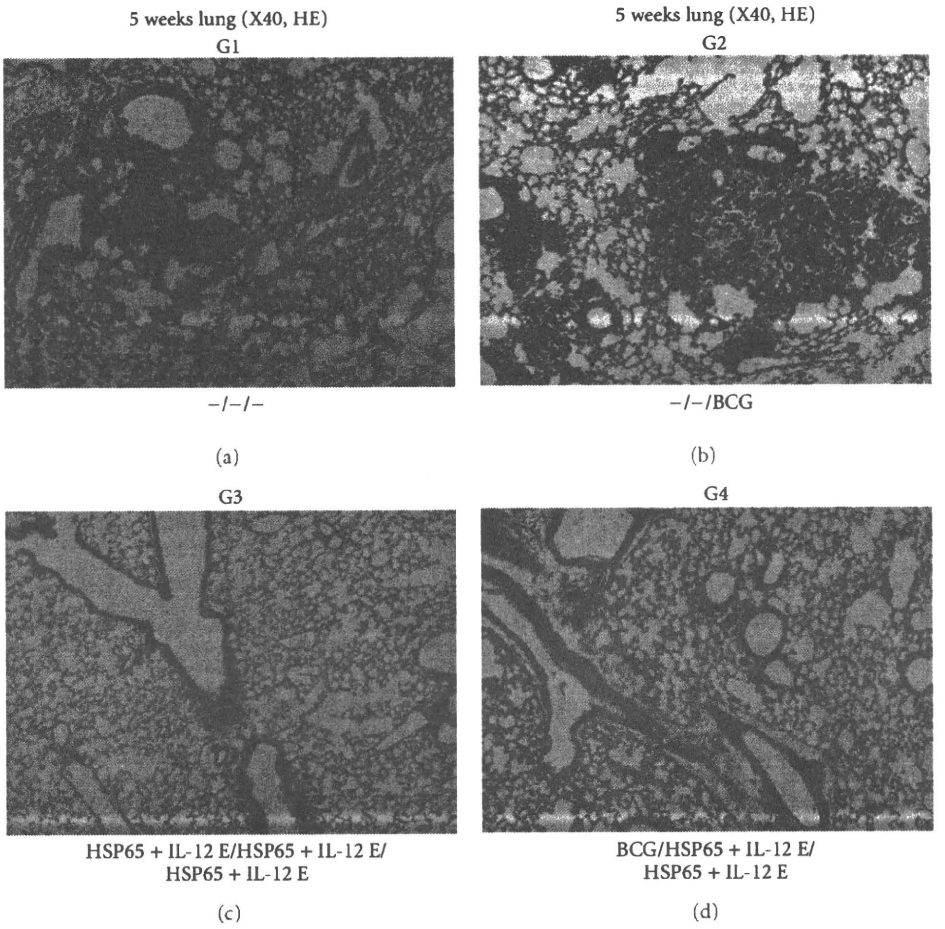

FIGURE 6: Histopathological analysis of vaccinated mice 5 weeks after *M. tuberculosis* challenge. Representative photomicrographs of lung tissue sections harvested from the G1 naive control group, G2 (BCG alone) group, G3 group (DNA/DNA/DNA), and G4 group (BCG/DNA/DNA) are shown (5 weeks after *M. tuberculosis* challenge, hematoxylin and eosin staining, × 4 objective). There was much infiltration of mononuclear cells and extensive parenchymal destruction by large, poorly demarcated granuloma in the lungs from the G1 (naive control) group and G2 (BCG alone) group. In the G3 (DNA/DNA/DNA) group and G4 (BCG/DNA/DNA) group, there was less inflammation, and only a few granulomas were observed.

Boosting BCG vaccination with MVA85A downregulates the immunoregulatory cytokine TGF-β1 [29]. Aeras-402 DNA (DNA that expressed 85A, 85B, and TB10.4) vaccine using adenovirus vector is intended for use as a boosting vaccine in BCG-primed individuals [30]. Several vaccines have been used with a prime-boost strategy to complement immune responses [31].

DNA vaccines are a relatively new approach to induce immunities for the protection of infectious diseases [14, 19, 22, 32–34]. Prophylactic and therapeutic DNA vaccines were established by using several kinds of vectors such as HVJ-liposome, HVJ-E, adenovirus vector, adenoassociated virus vector, and lentivirus vector [19–22, 35, 36]. In order to explore the preclinical use of a tuberculosis DNA vaccine combination of *IL-12* DNA with *hsp65* DNA, we chose the HVJ-based delivery system (HVJ-liposome and HVJ-E). These systems have high transfection efficiency and are available for repeated *in vivo* gene transfection without reduction of gene transfer efficiency or apparent toxicity. These characters of HVJ-liposomes support the feasibility of its clinical application not only for cancer gene therapy but also for DNA vaccinations. In a recent study, highly efficient gene expression in muscle cells was observed for several weeks when pcDNA3 plasmid containing the human tumor antigen genes, *MAGE-1* and *MAGE-3*, were encapsulated in HVJ-liposomes and injected intramuscularly in mice [37]. Effective induction of $CD4^+$ T-cell responses by a hepatitis B core particle-based HIV vaccine was achieved by subcutaneous administration of HVJ-liposomes in mice [38]. HVJ-liposomes were also very effective as a mucosal vaccine against HIV infection [39]. Thus, it is likely that HVJ proteins may be responsible for the induction of a robust immune response. No side effects were observed when repetitive injections of HVJ-liposomes were performed in mice, rats, or monkeys. We have previously developed an HVJ-E using inactivated Sendai virus, as a nonviral vector for drug delivery [40–42]. It can be used for efficient delivery of DNAs, siRNAs, proteins, and anticancer drugs into cells

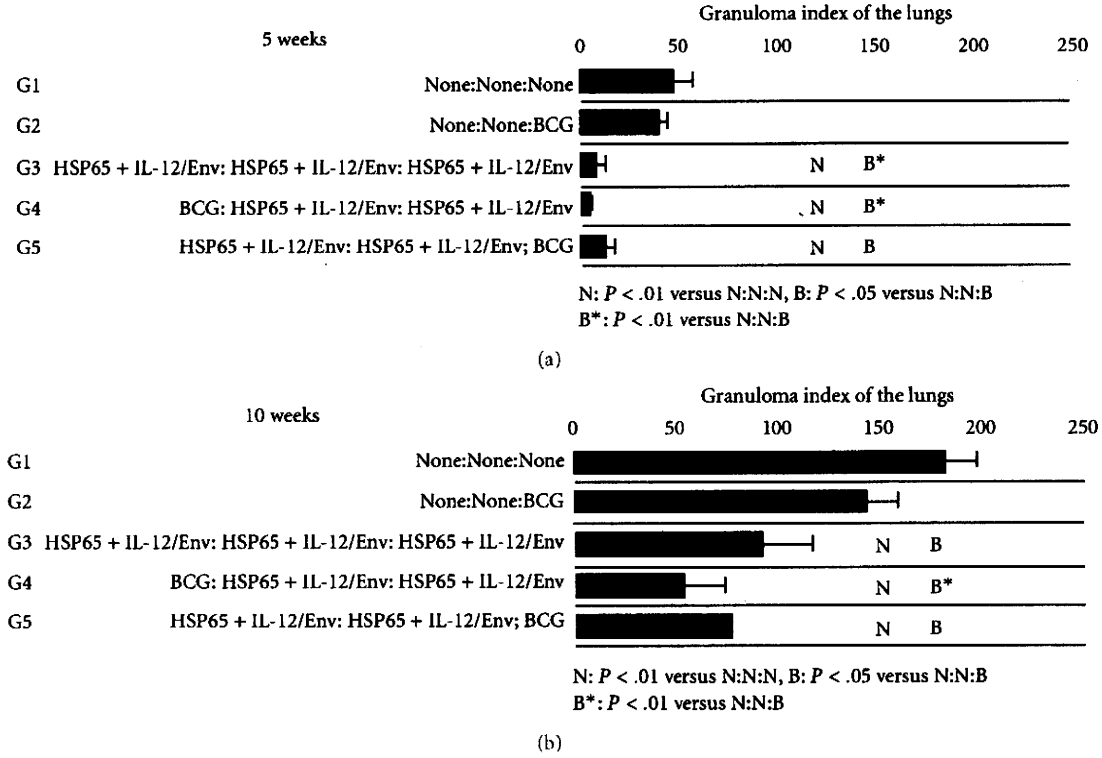

FIGURE 7: Granuloma index of the G1, G2, G3, G4, and G5 (DNA/DNA/BCG) groups in the lungs 5 weeks and 10 weeks after *M. tuberculosis* challenge. Results are expressed as the mean ± S.D. of triplicates of 5 mice per group. The statistical significance of differences between the groups was determined by Dunnett test, $P < .01$ as compared with the naive (N) group or the BCG alone (B) group. $P < .05$ as compared with the BCG alone (B*) group. The statistical significance of differences ($P < .05$) of granuloma index of 5 weeks G3 group compared to the G4 group.

both *in vitro* and *in vivo* [40, 43, 44]. Therefore, HVJ-E was used as an efficient and safe vector for DNA vaccine against TB in the present study.

Mycobacterial heat shock protein 65 (HSP65) is a potential target for protective immunity and has been studied extensively [19]. Several studies have reported that *hsp65* DNA vaccines can strongly induce protective immune responses in mice against virulent *M. tuberculosis* infections [20–22]. Protection is attributed to the establishment of a cellular immune response dominated by HSP65-specific T cells which produce IFN-γ and are cytotoxic towards infected cells. Furthermore, Lowrie and colleagues have reported that this vaccine reduces bacterial loads in mice infected with *M. tuberculosis* when given therapeutically after infection [32].

One of the major roles of IL-12 is the induction of IFN-γ-mediated immune responses to microbial pathogens. Cooper and colleagues have demonstrated the importance of IL-12 in generation of the protective response to tuberculosis [45]. Coadministration of the *IL-12* gene, which induces an IFN-γ-mediated immune response to microbial pathogens, with various tuberculosis DNA vaccines including *hsp65* DNA [46], and 35 K MW DNA [47], may boost the efficacy of these DNA vaccines to the levels achieved with BCG in the mouse model, although an inhibitory effect rather than a synergistic effect on immunotherapy was observed in mice coadministered *hsp65* DNA vaccine plus the *IL-12* gene [32].

In conclusion, we have shown efficacy of a novel HVJ-E DNA vaccine encapsulating HSP65 DNA with IL-12 DNA in the mouse model of TB. These results suggest that HSP65 + IL-12/HVJ could be a promising candidate for a new tuberculosis vaccine superior to BCG. To this aim, protective efficacy and immune responses were further studied in nonhuman primates before proceeding to human clinical trials.

In Japan and other countries, BCG is inoculated into human infants up to 6 months after birth. Therefore, BCG prime in infants and HSP65 + hIL-12/HVJ boost in adults (including junior high school students, high school students, and the elderly) may be required for significant improvement of clinical protective efficacy against TB. Thus, our results with the HSP65 + hIL-12/HVJ vaccine in a murine prophylactic model and cynomolgus monkey prophylactic model provide a significant rationale for moving this vaccine into clinical trials. Indeed, multiple animal models are available to accumulate essential data on the HVJ-E DNA vaccine in anticipation of a phase I clinical trial.

4. Conclusions

Vaccination by BCG prime with a novel vaccine (IgHSP65 + mIL-12/HVJ-E) boost resulted in significant protective efficacy (10,000-fold greater than BCG alone) against TB

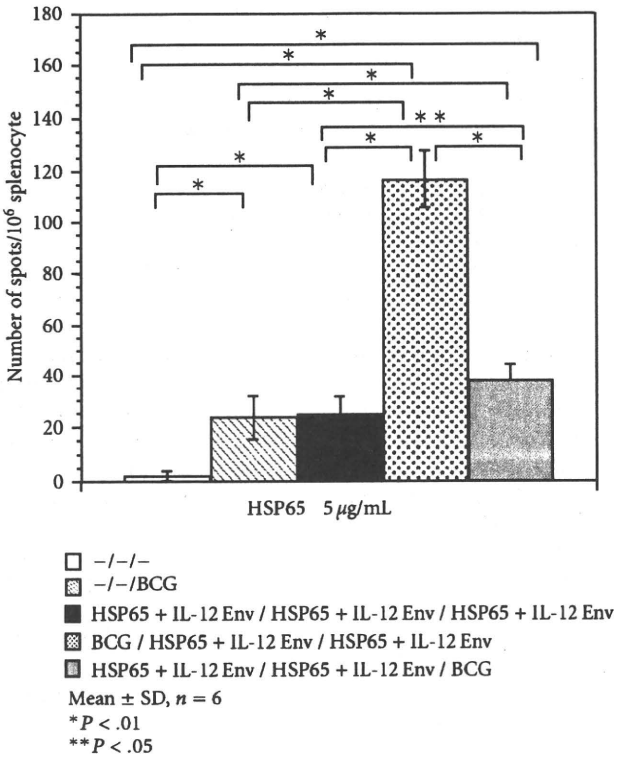

FIGURE 8: ELISPOT assay for IFN-γ antigen-specific responses in the spleens of vaccinated mice following stimulation with rHSP65 protein. Spleen cell cultures were stimulated with rHSP65 protein for 20 h. The numbers of IFN-γ-secreting cells specific for rHSP65 protein per million cells were determined individually by ELISPOT assay. Results are expressed as the mean ± S.D. of 6 wells of 3 mice per group. The statistical significance of differences between individual groups in the number of IFN-γ-secreting cells was determined by Dunnett test. The statistical significance of differences ($P < .01$) of the G1 (naive) group compared to the G2 (BCG alone group), G3 (DNA/DNA/DNA), G4 (BCG/DNA/DNA), or G5 (DNA/DNA/BCG). The statistical significance of the G2 group difference ($P < .01$) compared to the G4 or the G5. The statistical significance of the G3 group differences ($P < .01$) compared to the G4. $P < .01$; the G4 group compared to the G5. The statistical significance of the G3 group differences ($P < .05$) compared to the G5.

infection in the lungs of mice. In addition to bacterial loads, significant protective immunity was demonstrated by histopathological analysis of the lungs. This vaccine showed extremely significant protection against TB, suggesting that further development for eventual testing in clinical trials may be warranted.

Acknowledgments

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Novel therapeutic vaccine

Granulysin and new DNA vaccine against tuberculosis

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Key words: *Mycobacterium tuberculosis*, therapeutic vaccine, HVJ-envelope, monkey

granulysin, multi-drug resistant tuberculosis, cytotoxic T cell, IL-2, mouse, XDR-TB

Abbreviations: HVJ, hemagglutinating virus of japan; CTL, cytotoxic T cell; MDR-TB, multi-drug resistant tuberculosis; XDR-TB, extremely drug resistant tuberculosis

Purpose: Multi-drug resistant (MDR) *Mycobacterium Tuberculosis* (M.TB) is a big problem in the world. We have developed novel TB therapeutic vaccines.

Results and Methods: DNA vaccine expressing mycobacterial heat shock protein 65 and IL-12 was delivered by the hemagglutinating virus of Japan (HVJ)-envelope. M. TB, MDR-TB or extremely drug resistant (XDR-TB) was injected i.v. into DBA/1 mice, and treated with the vaccine three times. This HVJ-E/Hsp65DNA+IL-12DNA vaccine provided strong therapeutic efficacy against MDR-TB and XDR-TB (prolongation of survival time and the decrease in the number of TB) in mice. Therapeutic effect of this vaccine on TB infection was also demonstrated in chronic TB infection murine model using aerosol infection intratracheally. On the other hand, granulysin protein produced from CTL has lethal activity against TB. Granulysin protein vaccine also exerted strong therapeutic effect. Furthermore, we extended our studies to monkey model, which is currently the best animal model of human TB. Hsp65DNA+IL-12 DNA vaccine exerted strong therapeutic efficacy (100% survival and augmentation of immune responses) in the TB-infected monkeys. In contrast, the survival of the saline control group was 60% at 16 week post-challenge. HVJ-Envelope/HSP65 DNA+IL-12 DNA vaccine increased the body weight of TB-infected monkeys, improved the erythrocyte sedimentation rate and augmentated the immune responses (proliferation of PBL and IL-2 production). The enhancement of IL-2 production from monkeys treated with this vaccine was correlated with the therapeutic efficacy of the vaccine.

Conclusion: These data indicate that novel vaccines might be useful against TB including XDR-TB and MDR-TB for human therapeutic clinical trials.

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Introduction

Tuberculosis is a major global threat to human health, with about 2 million people dying every year from *Mycobacterium tuberculosis* (TB) infection. The only tuberculosis vaccine currently available is an attenuated strain of *Mycobacterium bovis* BCG (BCG), although its efficacy against adult TB disease remains controversial. Furthermore, multi-drug resistant tuberculosis (MDR-TB) and extremely drug resistant TB (XDR-TB) are becoming big problems in the world. In such circumstances, the development of therapeutic vaccine against TB as well as prophylactic vaccine against TB is required. Therefore, we have recently developed a novel TB vaccine, a DNA vaccine expressing mycobacterial heat shock protein 65 (HSP65) and interleukin-12 (IL-12) delivered by the hemagglutinating virus of Japan (HVJ)-liposome (HSP65+IL-12/HVJ). This vaccine was 100 fold more efficient than BCG in the murine model on the basis of the elimination of *M. tuberculosis* mediated by the induction of CTL.[1,3] Furthermore the HSP65+IL-12/HVJ vaccine using HVJ-envelope was 10,000 fold more efficient than BCG in the murine TB-prophylactic model. A nonhuman primate model of TB will provide information for vaccine development. In fact, in the previous study we evaluated the protective efficacy of HSP65+IL-12/HVJ in the cynomolgus monkey model, which is an excellent model of human tuberculosis.[1,3,4] We observed the synergistic effect of the HSP65+IL-12/HVJ and BCG using a priming-booster method in the TB-infected cynomolgus monkeys. The combination of the two vaccines showed a very strong prophylactic efficacy against *M. tuberculosis* (100% survival) as we have seen previously in the murine model of TB.[2,5] Furthermore, the granulysin produced from T cells and NK cells exerted therapeutic efficacy against TB. In the present study, we evaluated therapeutic effect of the HSP65+IL-12/HVJ vaccine on the MDR-TB infection and XDR-TB infection in murine and therapeutic effect of this

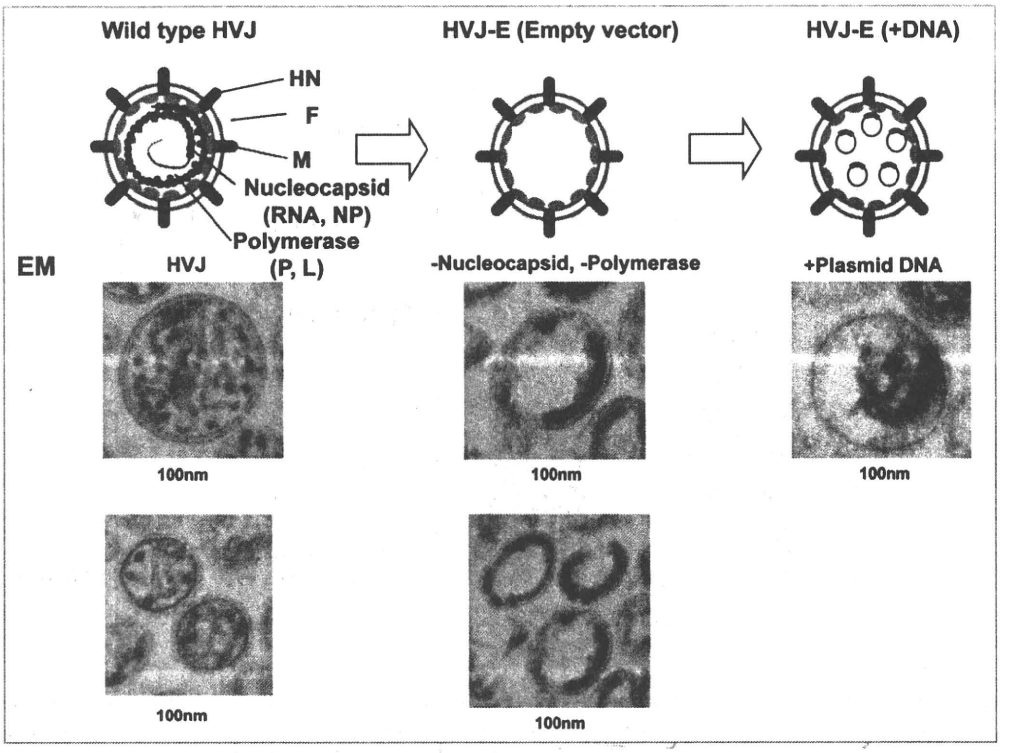

Figure 1. HVJ-envelope vaccination. pcDNA3-1/HSP65DNA+IL-12DNA were incorporated into HVJ-Envelope Empty Vector (Non-Viral Vector). Cartoons of HVJ-Envelope Empty Vector in the presence or absence of DNA were shown. Photographs of an electronic microscope (EM) of HVJ-Envelope Empty Vector were also shown.

vaccine on TB infection monkey models, and obtained the results indicating that the vaccine exerts therapeutic efficacy against TB, MDR-TB and XDR-TB.

Methods for the Evaluation of the Efficacy of Vaccines on the *M. tuberculosis*-infected Mice

DNA vaccines encoding *M. tuberculosis* HSP65 and human IL-12 were encapsulated into HVJ-Envelope or HVJ-liposomes.[6] HVJ-liposomes and HVJ-Envelope were prepared as described previously in reference 7–11 (**Fig. 1**). The HVJ-Envelope complex was aliquoted and stored at -70°C until use. Groups of mice were vaccinated three times with 100 μl of HVJ-Envelope solution containing 50 μg of pcDNA-IgHsp65 and 50 μg of pcDNA-mIL12p40p35-F in the tibia both anterior muscles. Mice were vaccinated with 1 x 10^6 CFU *M. bovis* BCG Tokyo by subcutaneous injection at 4 different sites (left upper, right upper, left lower, right lower back). HVJ-Envelope DNA vaccines encapsulating combination of pcDNA-IgHsp65 and pcDNA-mIL12p40p35-F was designated as IgHsp65+mIL-12/HVJ in this text. CTL activity was assessed by $^{51}C_r$-release assay.[1,12] At 30 days after intravenous challenge of *M. tuberculosis* H37RV, the number of CFU in the lungs, spleen and liver were counted and therapeutic efficacy of HVJ-Envelope DNA vaccines was evaluated.[3,13] TNFR gene disrupted DBA/1 mice and IL-6 gene disrupted DBA/1 mice were treated with HVJ-Envelope/HSP65 DNA + IL-12 DNA vaccine three times i.m at 1, 8 and 15 days after the challenge of 5 x 10^5 CFU MDR-TB i.v. (**Fig. 2**). Therapeutic efficacy was also evaluated by chronic TB infection model of mice using aerosol challenge of TB (15 CFU/mouse: Madison aerosol exposure chamber, University of Wisconsin). Mice were maintained in isolator cages, manipulated in laminar flow hoods and used between 8–10 weeks of age. All animal experiments were approved by the National Hospital Organization Kinki-chuo Chest Medical Center Animal Care and Use Committee. All vaccinations and

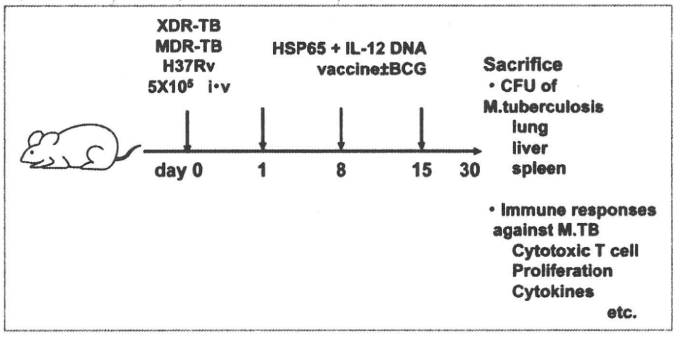

Figure 2. TNFR gene disrupted DBA/1 mice and IL-6 gene disrupted DBA/1 mice were treated with HVJ-Envelope/HSP65 DNA+IL-12 DNA vaccine three times i.m at 1, 8 and 15 days after the challenge of 5 x 10^5 CFU MDR-TB i.v. At 30 days after MDR-TB challenge, the lungs, spleens and livers were aseptically homogenized by using homogenizer in saline and serial dilutions of the organ homogenates were plated on 7H11 Middlebrook agar. Plates were sealed up and incubated at 37°C and the number of CFU was counted 2 weeks later. Results are converted to $\log_{10}$ values and $\log_{10}$ [mean ± standard deviation (SD)] for CFU/organ/animal were calculated.

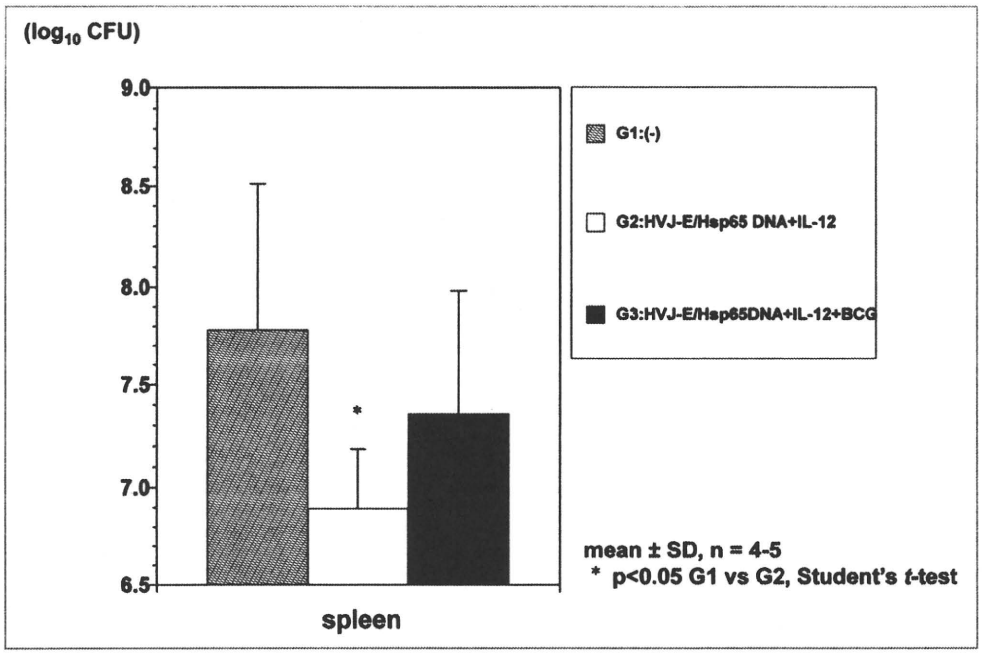

Figure 3. Therapeutic efficacy of HVJ-Envelope/HSP65 DNA+IL-12 DNA vaccine on the MDR-TB infection in the TNFR gene disrupted DBA/1 mice. Groups of mice were challenged by intravenous injection with 5 x 10^5 CFU MDR-TB, and then treated three times with HVJ-Envelope/HSP65 DNA+IL-12 DNA vaccine, as described in Materials and Methods. Thirty days after challenge, therapeutic efficacy was measured by enumerating the bacterial loads (CFU) in the spleen (A) and in the liver (B). Results are expressed as the mean $\log_{10}$ ± SD of CFU. The statistical significance of differences between individual groups in the number of CFU was determined student's t test (n = 4–5). *p < 0.05, the statistical significance of differences (p < 0.05) of G_1 (naïve) group compared to G_2 (HVJ-Envelope/HSP65 DNA+IL-12 DNA).

experiments on isolate tissue of animal were done under anaesthetic state with sevoflurane. Infected animals were housed in individual micro-isolator cages in a Biosafety Level (BL) 3 animal facility of the NHO Kinki-chuo Chest Medical Center.

Methods for the Evaluation of the Efficacy of the Vaccine on the *M. tuberculosis*-infected Monkeys

Cynomolgus monkeys were housed in a BL 3 animal facility of the Leonard Wood Memorial Research Center. All animal experiments were approved by the Leonard Wood Memorial Animal Care and Use Committee and the National Hospital Organization Kinki-chuo Chest Medical Center Animal Care and Use Committee. The animals were vaccinated nine times with the HVJ-envelope with expression plasmid of both HSP65 and human IL-12 (HSP65+hIL-12/HVJ: 400 ug i.m.), one week after the challenge with the *M. tuberculosis* Erdman strain (5 x 10^2) by intratracheal instillation. Immune responses and survival were examined as described in our previous studies in reference 2 and 5.

Results

Murine models. *Therapeutic efficacy of HSP65 DNA+IL-12 DNA vaccine using murine models.* At 30 days after intravenous challenge of MDR-TB, the CFU of TB in the lungs, spleen and liver were counted and therapeutic efficacy of HVJ-Envelope DNA vaccine was evaluated.

As shown in Figure ~~3A and B,~~ HVJ-Envelope/HSP65 DNA+IL-12 DNA vaccine treatment significantly reduced the bacterial loads of MDR-TB in the liver of mice as well as spleen as compared to saline control group ($p < 0.05$).

The survival of vaccinated mice after XDR-TB (extremely drug resistant TB) was investigated. All mice in the control group died of TB within 160 days after XDR-TB infection. In contrast, mice treated with HVJ-Envelope/HSP65 DNA+IL-12 DNA prolonged the survival periods significantly by statistical analysis($p < 0.05$) (data not shown). It was demonstrated that this vaccine had a therapeutic activity against XDR-TB as well as MDR-TB and drug-sensitive TB (**Table 1**).

Therapeutic efficacy using chronic TB disease models. Furthermore, we have established chronic TB disease models using a mouse infected with TB in the aerosol chamber (Fig. ~~4A~~). By using this model, therapeutic efficacy of this vaccine was also observed (Fig. ~~4B~~). At 8 weeks after intratracheal aerosol infection of TB, the number of CFU in the lung was determined. Vaccination with HVJ-Envelope/HSP65 DNA+IL-12 DNA exerted therapeutic efficacy in the bacterial loads as compared to saline control.

Therapeutic efficacy using SCID-PBL/hu mice. Therapeutic efficacy of HVJ-Envelope/HSP65 DNA+IL-12 DNA was also observed, when we used in vivo humanized immune models of IL-2 receptor γ-chain disrupted NOD-SCID mice constructed with human PBL (SCID-PBL/hu).[14,15] Therapeutic vaccination with HVJ-Envelope/HSP65 DNA+IL-12 DNA showed