It is well established that protective immunity to *M. tuberculosis* depends on both $CD4^+$ and $CD8^+$ T cells [1–6]. Because DNA vaccination results in the generation of cellular immune responses, including those of a Th-1-type response, and protection in animal models of infectious diseases [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 [10,11] and HIV infections [12]. DNA vaccination has also shown potential for the development of tuberculosis vaccines in the mouse model [13–16]. However, in a guinea pig model, which is arguably one of the most biologically relevant systems available for studying human pulmonary tuberculosis, DNA vaccines has not proven more efficacious than BCG [17]. The efficacy of any experimental tuberculosis vaccine remains to be evaluated in human clinical trials and, thus, a vaccine against tuberculosis is still anxiously awaited.

Mycobacterial heat shock protein 65 (Hsp65) is a potential target for protective immunity and has been extensively studied [18]. Several groups have reported that *hsp65* DNA vaccines can induce strong protective immune responses in mice against virulent *M. tuberculosis* infections [19–21]. Protection is attributed to the establishment of a cellular immune response dominated by Hsp65-specific T cells that both produce IFN-γ and are cytotoxic towards infected cells. Furthermore, Lowrie et al. have reported that this vaccine reduces bacterial loads in mice infected with *M. tuberculosis* when given therapeutically after infection [22]. Interleukin-12 (IL-12) is a cytokine with a major role in the induction of IFN-γ-dominated immune responses to microbial pathogens. Orme and colleagues have demonstrated the importance of IL-12 in generation of the protective response to tuberculosis [23]. Co-administration of the IL-12 gene, which participates in the induction of IFN-γ dominated immune responses to microbial pathogens, with various tuberculosis DNA vaccines including the *hsp65* DNA [20,24] and 35 K MW DNA [25] may boost the efficacy of these DNA vaccines to levels achieved with BCG in the mouse model, although inhibitory effect rather than synergistic effect on immunotherapy was observed in mice co-administrated with *hsp65* DNA vaccine plus the *IL-12* gene.

In order to explore the preclinical use of tuberculosis DNA vaccine combinations of the *IL-12* DNA with the *hsp65* DNA, we chose the viral-based hybrid antigen delivery system hemagglutinating virus of Japan (HVJ)-liposome because this delivery system results in a high transfection efficacy, repeated gene transfection without reduction of gene transfer efficiency in vivo, and no apparent toxicity. These characteristics 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 transfection of 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 into mice [26]. 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 [27]. HVJ-liposomes were also very effective as a mucosal vaccine against HIV infection [28]. Thus, it is likely that HVJ proteins may be responsible for inducing a robust immune response. No side effects from repetitive injections of HVJ-liposomes into mice, rats or monkeys were observed.

We designed this study to clarify the clinical feasibility of HVJ-liposome-mediated DNA vaccines for tuberculosis. First, we clarify that co-administration of IL-12 DNA with Hsp65 DNA via gene gun delivery enhanced protection in mice compared with Hsp65 DNA alone. Second, we show that vaccination with HVJ-liposome encapsulated Hsp65 DNA resulted in better protection than did gene gun vaccination. Third, we demonstrate that HVJ-liposome encapsulated Hsp65 DNA and IL-12 DNA induce enhanced protective immunity in the mouse model compared to that seen with BCG. This protective efficacy was associated with the emergence of IFN-γ-secreting T cells upon stimulation with Hsp65 and purified protein derivative. These results suggest that Hsp65 + IL-12/HVJ could be a promising candidate for a new tuberculosis DNA vaccine, which is superior to BCG vaccine. We also discuss in this paper the prospects of this HVJ-liposome-based DNA vaccine for testing in primate models [29] and, ultimately, in a clinical setting.

2. Materials and methods

2.1. Bacteria

M. tuberculosis strain 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 medium (Wako Chemicals, Osaka, Japan). For the mouse infection studies, a single colony of *M. tuberculosis* H37Rv was grown in Middlebrook 7H9 (DIFCO Laboratories, Detroit, MI: lot 137971 XA MD) medium supplemented with albumin–dextrose complex and grown at 37 °C until approximately mid-log phase. Aliquots were stored at −80 °C and thawed at 10 days before use, grown to mid-log phase in 7H9 medium.

2.2. Reagents and antibodies

Purified protein derivative (PPD: lot T-3-4) was obtained from JAPAN BCG Co., Ltd. (Tokyo, Japan). Killed *M. tuberculosis* H37Ra (lot 13971XA) was obtained from DIFCO Laboratories. Fetal calf serum (FCS: lot AGC6341) was obtained from Hyclone (Logan, UT). Anti-L3T4, anti-Lyt2.2 monoclonal antibodies and anti-Thy1.2 antibody were kindly provided by Dr. K. Kuribayashi (Mie University, Tsu, Japan) and Dr. E. Nakayama (Okayama University, Okayama, Japan) [30].

2.3. Animals

Inbred and specific pathogen-free female BALB/c mice were purchased from Clea Japan Inc. (Tokyo, Japan). Mice were maintained in isolator cages, manipulated in laminar flow hoods and used between 8 and 10 weeks of age. 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.

2.4. Cell lines

COS-7 cells were kindly provided by Dr. H. Endoh (Jichi Medical School, Tochigi, Japan). COS-7 cells were maintained at 37 °C in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% FBS, 2 mM L-glutamine, and antibiotics. A mouse mastocytoma cell line (P815: DBA/2 origin) was kindly provided by Dr. C.S. Henney (Fred Hutchinson Cancer Research Center, Seattle) [31]. A mouse macrophage cell line (J774.1: BALB/c origin) was kindly provided by Dr. P. Ralph (Sloan Kettering Cancer Inst., New York, NY) [32]. The P815 and J774.1 cells were maintained in RPMI 1640 medium (Flow Laboratories, Inc., Mclean, VA) supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml) and 5×10^{-5} M 2-mercaptoethanol [33,34].

2.5. Plasmid construction

The *hsp65* gene was amplified from *M. tuberculosis* H37Rv genomic DNA by PCR using a set of primers, phsp65-F1 and phsp65-R1, and cloned into the *Bam*HI/*Not*I sites of pcDNA3.1 (+) (Invitrogen, San Diego, CA) to generate pcDNA-hsp65. pcDNA-hsp65 was designated as Hsp65 DNA in this text. For the construction of the *hsp65* gene fused with the mouse Igκ secretion signal sequence, the PCR product was cloned into the *Bam*HI/*Not*I sites of pcDNA-CS87 [35] to generate pcDNA-Ighsp65. pcDNA-Ighsp65 was designated as IgHsp65 DNA in this text. For the construction of the mouse IL-12 (mIL-12) *p40* and *p35* single-chain gene, the *mIL12p35*and *mIL12p40* genes were cloned from pcDNA-p40p35 [35] by PCR using sets of primers, pmIL12p35-F1 and pmIL12p35-R1, and pmIL12p40-F1 and pmIL12p40-R1, respectively, and cloned into pcDNA3.1 (+) to generate pcDNA-mIL12p40p35-F. pcDNA-mIL12p40p35-F was designated as mIL-12 DNA in this text. As a control, pcDNA-EGFP vector expressing the *EGFP* gene was used. Sequences of oligonucleotide primers used are available as request.

2.6. Protein production and antibody preparation

Recombinant Hsp65 (rHsp65) protein was expressed in *E. coli* BL21 (λDE3) and purified by affinity chromatography on Ni-NTA columns (Qiagen).

2.7. Transfection

DNA transfection of COS-7 cells was performed with the PolyFect Transfection Reagent (Qiagen) according to the manufacturer's instructions. After 24 h, supernatant and cells were harvested separately. Immunoprecipitation of cell lysates and supernatants with antibodies were performed as described previously [36]. Rat anti-mouse IL-12p70 (BD Biosciences Pharmingen, San Diego, CA) and mouse anti-rHsp65 polyclonal antibody were used for immunoprecipitation. For IL-12 bioassay, COS-7 cells (1×10^6 cells/plate) were plated into 60-mm cell culture plates and transfected with 2.5 μg of pcDNA 3.1, pcDNA-mIL12p40 + p35, or pcDNA-mIL12p40p35-F using the PolyFect Transfection Reagent. At 48 h after transfection, culture supernatants were collected and stored at −70 °C until use. Various volumes of the supernatants were added to the mouse spleen cells (2×10^6 cells/ml). Murine culture supernatants after 60 h incubation were collected and the level of mouse IFN-γ measured using sandwich ELISA kits (BD Opt EIA™ Set. BD Biosciences Pharmingen), according to manufacturer's instructions.

2.8. Vaccination

2.8.1. Gene gun vaccination

Gold particles coated with plasmid DNAs and their cartridges were prepared as described previously [35]. The abdomen was shaved and gold particles coated with plasmid DNA (1 μg plasmid DNA per shot) was delivered once into the abdomen using a Helios Gene gun (Nippon Bio-Rad Laboratory, Tokyo, Japan) at a helium discharge pressure of 300 psi. A separate group was vaccinated once subcutaneously with 1×10^6 colony-forming units (CFU) of *M. bovis* BCG Tokyo strain.

2.8.2. HVJ-liposome vaccination

HVJ-liposomes were prepared as described previously [37]. The HVJ–liposome complex was aliquoted with 10% DMSO and stored at −70 °C until use. HVJ-liposomes without plasmid DNA was used and designated as Empty/HVJ in this text. Groups of BALB/c mice were vaccinated three times at 3-week intervals with 100 μl of HVJ-liposome solution containing 50 μg of pcDNA-IgHsp65 and/or 50 μg of pcDNA-mIL12p40p35-F in the tibia both anterior muscles. A separate group was vaccinated once with 1×10^6 CFU *M. bovis* BCG Tokyo by subcutaneous injection at four different sites (left upper, right upper, left lower, right lower back) at the same time. HVJ-liposome DNA vaccines encapsulating pcDNA-IgHsp65, pcDNA-mIL12p40p35-F, or combination of pcDNA-IgHsp65 and pcDNA-mIL12p40p35-F was designated as IgHsp65/HVJ, mIL-12/HVJ, and IgHsp65 + mIL-12/HVJ, respectively, in this text.

2.9. 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 3 weeks after the third vaccination as described previously [38]. At 5 and 10 weeks after *M. tuberculosis* H37Rv challenge, the lungs, spleens, and livers were aseptically homogenized by using homogenizer in saline, and serial dilutions of the organ homogenates were plated on Ogawa agar (Kyokuto, Tokyo, Japan) or 7H11 Middlebrook agar (Kyokuto). Plates were sealed up and incubated at 37 °C and the number of CFU was counted 2 or 4 weeks later. Results are converted to $\log_{10}$ values and $\log_{10}$ [mean ± standard deviation (S.D.)] for CFU/organ/animal were calculated for each experimental group.

2.10. Histological analysis

The lungs were obtained from the mice, fixed with 10% buffered formalin, and embedded in paraffin. Each block was cut into 4 μm-thick sections and stained using hematoxylin and eosin. Semi-quantitative morphometric analysis of pathological slides was performed by our modified method of Dascher et al. [39] using a micrometer-attached microscope (Microphot-FXA, Nikon, Japan) [39,40]. The longer axis and minor axis of each granuloma in the field (×4 magnification) were measured. Longer axis to minor axis of each granuloma were multiplied and added up. Three random fields from each tissue section of mice and six random fields of guinea pigs were evaluated, and 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 is significantly correlated with the granuloma area by other scanning method of hematoxylin and eosin section.

2.11. Tuberculosis-specific cytotoxic test using ^{51}Cr release

Eight weeks after the final vaccination, CTL activity of spleen cells and mesenteric lymph node cells from vaccinated mice was assessed by using the ^{51}Cr-release assay. P815 mastocytoma cells, which have the same major histocompatibility complex (MHC) (H-2^d) as BALB/c mice, were transfected with pcDNA-hsp65 and used as Hsp65 protein-expressing target cells. J774.1 macrophage cells were pulsed with *M. tuberculosis* (killed H37Ra) for 24 h and used as target cells. A total of 2×10^6 cells/ml effector splenic cells were treated with anti-CD8 antibody, anti-CD4 antibody or anti-Thy1.2 antibody followed by complement as described above. ^{51}Cr release was assessed using the ^{51}Cr-release assay [31,33] at the effector:target (E:T) ratio of 50:1. Spontaneous lysis (with medium alone) and maximum lysis (^{51}Cr release after three cycles of freeze-thaw) were set up for background and targets. Percent specific lysis was determined as:

$$\left[\frac{(\text{experimental release} - \text{spontaneous release})}{(\text{maximum release} - \text{spontaneous release})}\right] \times 100.$$

2.12. Proliferative responses of lymphocytes

Vaccinated mice were sacrificed immediately prior to challenge, and 1×10^5 single spleen cells were cultured in a 96-well flat bottom plate (Linbro) with rHsp65 protein (10 μg/ml) or PPD (20 μg/ml) for 60 h at 37 °C, and then pulsed with 1 μCi of [^{3}H]thymidine per well for the final 12 h of incubation [30]. Cells were harvested onto glass wool fiber filters, and [^{3}H]thymidine incorporation was measured using a Liquid Scintillation Counter LSC-6100 (ALOKA Co. Ltd., Tokyo, Japan).

2.13. Production of cytokines (IL-2 and IFN-γ)

Mouse cytokines were measured in quantitative ELISAs for IL-2 and IFN-γ as described previously [38]. Briefly, spleen cells from vaccinated mice were cultured at a concentration of 5×10^6 cells/ml in 200 μl of medium at various antigen concentrations. Culture supernatants were collected 48 h later and the levels of IFN-γ and IL-2 measured using sandwich ELISA kits (BD Opt EIA^{TM}), according to manufacturer's instructions.

2.14. ELISPOT assay

The spleens were removed aseptically from vaccinated mice three weeks after the third vaccination. Antigen-specific IFN-γ-producing cells were determined by ELISPOT as described previously [41]. Briefly, ELISPOT plates (MultiScreen IP Filtration plate MAIPS45; Millipore, Bedford, MA) were coated with anti-mouse IFN-γ MAb R4-6A2 (BD Biosciences Pharmingen). Spleen cells from vaccinated mice were suspended to 1×10^7 cells/ml (1×10^6 cells/well). In some experiments, the spleen cells from mice vaccinated with IgHsp65 + mIL-12/HVJ were pre-incubated with anti-CD8 antibody or anti-CD4 antibody (1:50 dilution) for 15 min at 4 °C and then incubated with rabbit complement (1:10 dilution) (Cedarlane, Hornby, Ont., Canada) for 45 min at 37 °C as described previously [30,33]. The cells were placed in five wells into antibody-coated wells, and rHsp65 protein (10 μg/ml) or PPD (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 biotynylated anti-mouse 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 KS ELISPOT system (Carl Zeiss, Hallbergmoos, Germany).

2.15. Statistical analysis

Tukey–Kramer's HSD tests were used to compare $\log_{10}$ value of CFU between groups following challenge and T cell responses between groups in ELISPOT assay. Student's *t* tests were performed to compare T cell responses between groups in T cell proliferation assay and granuloma formation between groups following challenge. A *P*-value of <0.05 was considered significant.

3. Results

3.1. In vitro expression of Hsp65 and IL-12

The DNA vaccines encoding mature and secreted forms of Hsp65 were constructed as Hsp65 DNA and IgHsp65 DNA, respectively. Hsp65 DNA contains the full-length *M. tuberculosis hsp65* gene. IgHsp65 DNA contains the full-length *M. tuberculosis hsp65* gene fused to the mouse Igκ signal sequence. Each construct is driven by CMV promoter and terminated at a bovine growth hormone polyadenylation sequence. Hsp65 DNA or IgHsp65 DNA was transfected into COS-7 cells and cell lysates and supernatants were analyzed for the *hsp65* gene expression. As shown in Fig. 1A, the mature form was detected as a single band in cell lysates (lane 2), whereas the secreted form was detected as a doublet band in cell lysates (lane 3). The doublet of slightly higher molecular weight than the mature form is most likely due to incomplete cleavage of the Igκ signal peptide in COS-7 cells because only a single band corresponding to the mature form was seen when HeLa cells or HepG2 cells were transfected with pcDNA-IgHsp65 (data not shown).

Based on the results of study reporting with high levels of IL-12 expression [42], we constructed a mouse IL-12 expression vector, mIL-12 DNA. The vector encodes mouse single-chain IL-12 protein comprised of p40 and p35 subunits linked by Gly_6Ser polypeptide linkers. As shown Fig. 1A, COS-7 cells transfected with mIL-12 DNA transiently expressed the mouse single-chain IL-12 protein with molecular weight of 80 kDa (lane 8). Quantitative analysis using ELISA showed that the COS-7 cells transfected with the mIL-12 DNA secreted four-fold higher levels of mIL-12p70 (125 ng/ml) than those transfected with from pcDNAmIL-12p40 + p35 (30 ng/ml), which previously constructed as a murine expression vector with IL-12 p40 and p35 expression cassettes in tandem array [35] (data not shown). Consistent with the mIL-12p70 expression level, the supernatant form the mIL-12 DNA transfectant cells induced 3.2-fold higher levels of IFN-γ from murine T lymphocytes than that from pcDNAmIL-12p40 + p35 transfectant cells (Fig. 1B). Thus, the mIL-12 DNA construct expresses biologically active IL-12, indicating that the single-chain IL-12 DNA is an effective DNA vaccine adjuvant capable of inducing primary Th-1 responses.

3.2. Evaluation of the best combination of Hsp65-based DNA vaccines with mIL-12 DNA for vaccine efficacy via gene gun

We compared the protective abilities of two versions of Hsp65-based DNA vaccine (Hsp65 DNA versus IgHsp65 DNA), and combinations with mIL-12 DNA (Hsp65 DNA versus Hsp65 DNA plus mIL-12 DNA, or IgHsp65 DNA versus IgHsp65 DNA plus mIL-12 DNA). Mice vaccinated with Hsp65 DNA, IgHsp65 DNA, and the combination with

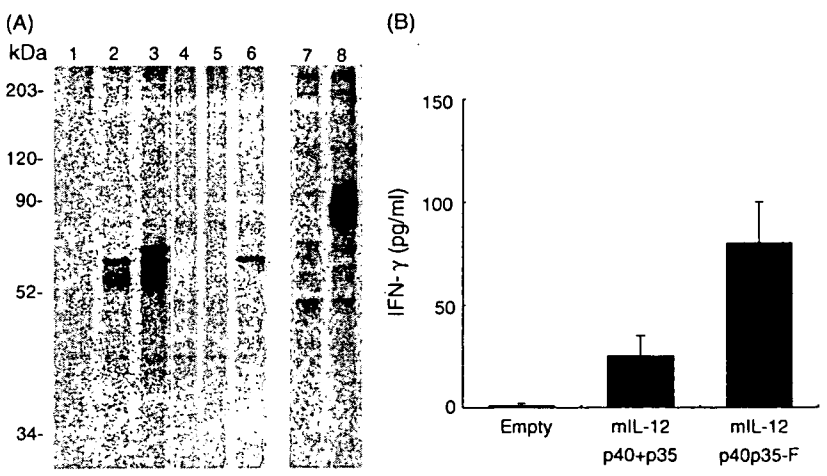

Fig. 1. Expression and biological analysis of Hsp65 and mIL-12. (A) In vitro expression analysis of Hsp65 and mIL-12 from cells transiently transfected with DNA vaccines. COS-7 cells were transfected with EGFP DNA (lanes 1, 4 and 7), Hsp65 DNA (lanes 2 and 5), IgHsp65 DNA (lanes 3 and 6), and mILp40p35-F DNA (lane 8). Following metabolically labeling with [^{35}S]methionine, cell lysates (lanes 1, 2, 3, 7 and 8) and supernatants (lanes 4–6) were immunoprecipitated with mouse anti-Hsp65 polyclonal antibody (lanes 1–6) or rabbit anti-murine IL-12p70 antibody (lanes 7 and 8). (B) The biological activities of IL-12 expressed in transfected cell supernatants in vitro. Culture supernatants from COS-7 cells transfected with pcDNA3.1 (no insert empty vector), pcDNA-mIL12p40 + p35 or pcDNA-mIL12p40p35-F were added to the mouse spleen cells (2×10^6 cells/ml) at the final concentration of 4% (v/v) and incubated for 60 h. The levels of mouse IFN-γ were measured using sandwich ELISA kits as described in Section 2.

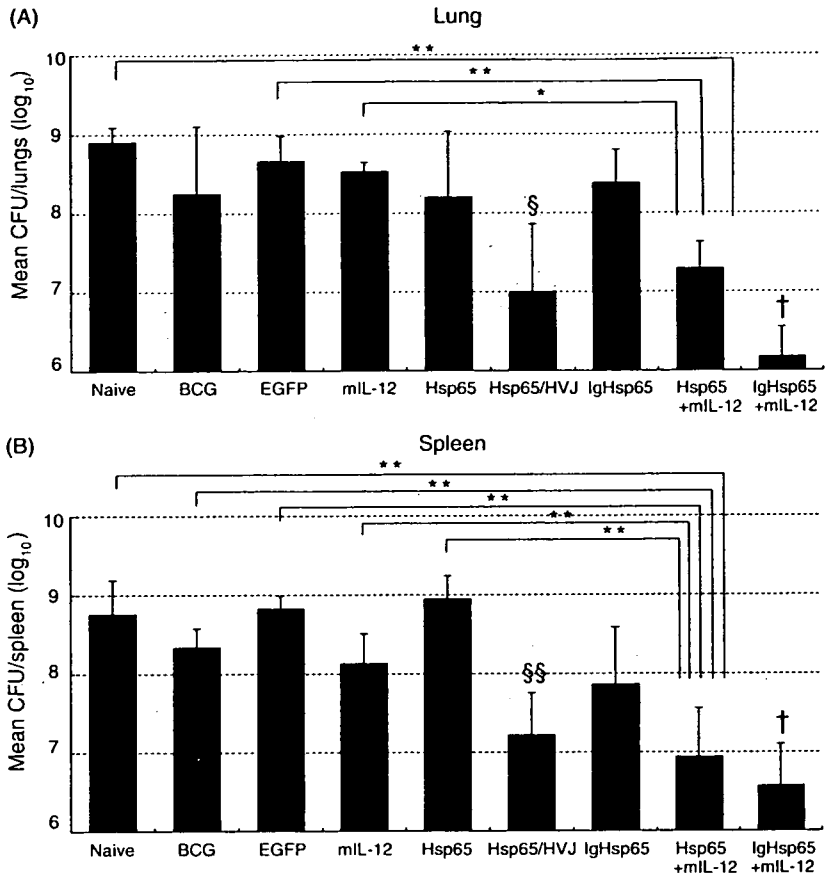

Fig. 2. The effect of a combination of mIL-12 expression vector and Hsp65-based DNA vaccines and comparison of different vaccines on the protective efficacy against challenge with *M. tuberculosis*. Groups of mice were vaccinated once with Hsp65 DNA, IgHsp65 DNA and a combination of mIL12 DNA via gene gun or three times with Hsp65/HVJ via intramuscular route and challenged intravenously with *M. tuberculosis* H37Rv as described in Section 2. Ten weeks after challenge, protection was measured by enumerating bacterial loads (CFU) in the lungs and spleen from vaccinated mice. Reduction of bacterial load was expressed as the mean $\log_{10}$ difference in CFU in the organs of the naive and vaccinated mice. The statistical significance of differences between individual groups in the number of CFU was determined by Tukey–Kramer's HSD test ($n = 4$–5). * and **, the statistical significance of differences ($P < 0.05$ and $P < 0.01$) compared to Hsp65 DNA + mIL-12 DNA group, respectively; †, the statistical significance of differences ($P < 0.01$) of IgHsp65 DNA + mIL-12 DNA group compared to the naive, BCG, EGFP DNA, mIL12 DNA, Hsp65 DNA and IgHsp65 DNA groups; §, the statistical significance of differences of Hsp65/HVJ group compared to BCG group ($P < 0.05$) in the lungs; §§, the statistical significance of differences of Hsp65/HVJ group compared to Hsp65 DNA ($P < 0.01$) and BCG ($P < 0.05$) groups in the spleen.

mIL-12 DNA via gene gun were challenged intravenously with *M. tuberculosis* H37Rv. The bacterial loads of the naive and vaccinated mice were compared 10 weeks after challenge (Fig. 2). Consistent with the previous report by Lima et al. [43], gene gun vaccination with Hsp65 DNA alone did not result in significant protective immunity as assessed by the bacterial load in the lungs or spleen. Vaccination with IgHsp65 DNA, which encodes the additional mouse Igκ signal sequence upstream of the *hsp*65 gene, did not significantly improve the protective efficacy in the bacterial load in the lungs, although there was a modest decrease in the bacterial load in the spleen. In contrast, the combination with mIL-12 DNA markedly improved the protective efficacy both in the lungs and spleen ($P < 0.01$). In particular, vaccination of IgHsp65 DNA plus mIL-12 DNA conferred the greatest reduction of the bacterial load both in the lungs and spleen. Similar to IgHsp65 DNA plus mIL-12 DNA, the increased protection in the lungs and spleen was also observed in mice vaccinated with Hsp65 DNA plus mIL-12 DNA compared to IgHsp65 DNA alone and mIL-12 DNA alone. Thus, a strong synergistic effect on protection was achieved when Hsp65 DNA was co-administrated with IL-12 DNA. It is notable that the prophylactic effect of IgHsp65 DNA plus mIL-12 DNA in the lungs was more than 100-fold greater than that of BCG. These vaccinations of IgHsp65 DNA plus mIL-12 DNA and Hsp65 DNA plus mIL-12 DNA also exerted the significant reduction in the liver compared to the naive ($P < 0.05$) and control EGFP DNA groups ($P < 0.01$), whereas there was no significant difference of the naive group compared with Hsp65 or mIL-12 group (data not shown). In mice vaccinated with IgHsp65 DNA plus mIL-12 DNA, increased protection in the lungs were also observed at 5 weeks after challenge, which was equivalent to that obtained by vaccination with BCG (data not shown).

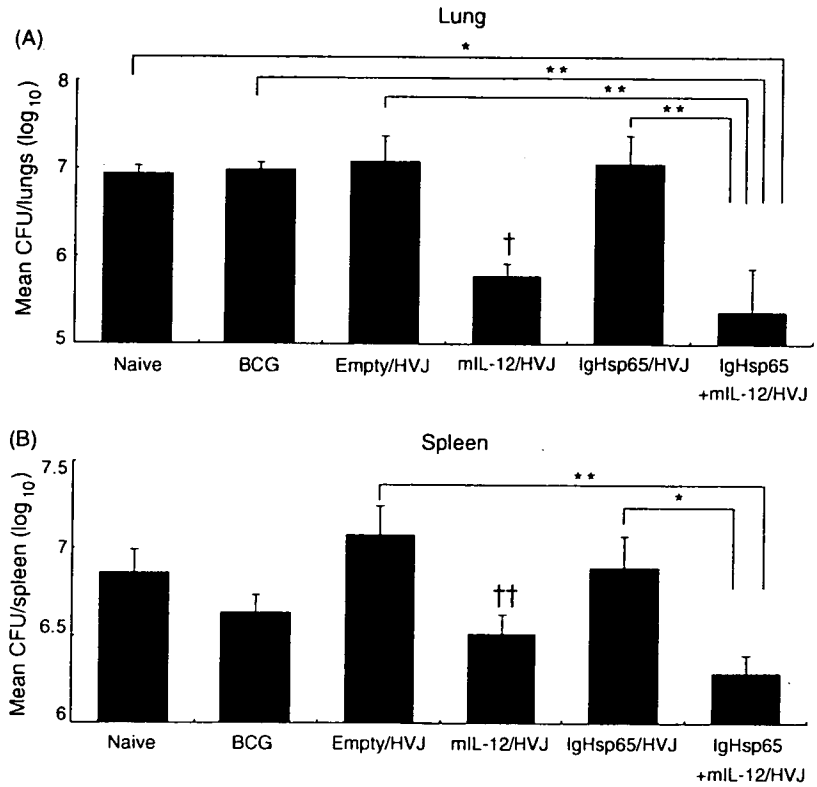

Fig. 3. Mouse protection studies using HVJ-liposome vaccines. Groups of mice vaccinated with HVJ-liposome DNA or BCG were challenged by intravenous injection with *M. tuberculosis* H37Rv. Five weeks after challenge, protection was measured by enumerating the bacterial loads (CFU) in the lungs (A) and spleen (B) from vaccinated mice. Results are expressed as the mean $\log_{10} \pm$ S.D. of CFU. The statistical significance of differences between individual groups in the number of CFU was determined by Tukey–Kramer's HSD test ($n = 4$–5). $^{*}P < 0.05$; $^{**}P < 0.01$; †, the statistical significance of differences ($P < 0.05$) of mIL-12/HVJ group compared to BCG, Empty/HVJ, and Hsp65/HVJ groups in the lungs; ††, the statistical significance of differences ($P < 0.05$) of mIL-12 DNA group compared to Empty/HVJ group in the spleens.

3.3. Comparison of the protective efficacy of gene gun versus HVJ-liposome delivery of Hsp65 DNA vaccines

We next compared methods of DNA vaccine delivery on vaccine efficacy at 10 weeks after challenge. Hsp65/HVJ vaccination and challenge experiments were conducted simultaneously with gene gun experiments. As shown in Fig. 2, Hsp65/HVJ vaccination significantly reduced the bacterial loads as compared to Hsp65 gene gun immunization in the spleen ($P < 0.01$). IgHsp65 gene gun immunization significantly reduced the bacterial loads as compared to Hsp65 gene gun immunization in the spleen ($P < 0.05$, data not shown). Therefore, we used IgHsp65/HVJ for further experiments.

3.4. Protective efficacy of HVJ-liposome DNA vaccines

At 5 and 10 weeks after intravenous challenge of *M. tuberculosis* H37Rv, the number of CFU in the lungs, spleen, and liver were determined. Fig. 3 shows the results of bacterial loads 5 weeks after challenge. Vaccination with mIL-12/HVJ group resulted in significant protective immunity in the bacterial as compared to BCG, Empty/HVJ and Hsp65/HVJ groups in the lung ($P < 0.05$) and as compared to Empty/HVJ group in the spleen ($P < 0.05$). Vaccination with IgHsp65 + mIL-12/HVJ induced better protective immunity in the bacterial load both in the lungs and spleens than IgHsp65/HVJ alone and mIL-12/HVJ alone. Thus, the synergistic effect of IgHsp65 DNA and mIL-12 DNA resulted in improving the protective efficacy. At 10 weeks after challenge, the same reduction was also observed in these organs from mice vaccinated with IgHsp65 + mIL-12/HVJ (data not shown). Body weights of vaccinated mice were similar in all vaccinated groups. Tissue weight of lungs, liver, and spleen in the IgHsp65 + mIL-12/HVJ group were slightly lower than that from the naive mice (data not shown). In this experiment, BCG vaccination did not provide significant reduction of the bacterial load compared to the naive group. This may be due to the single-dose of vaccination used usually, the use of BCG Tokyo strain requires a three-dose vaccination to achieve 10 to 30-fold reduction of the bacterial loads compared to a non-vaccinated group. Although, 5 weeks after challenge, no reduction of bacterial loads was observed in IgHsp65/HVJ group compared with the naive control group, we confirmed the increased protection 10 weeks after challenge compared with the naive control group

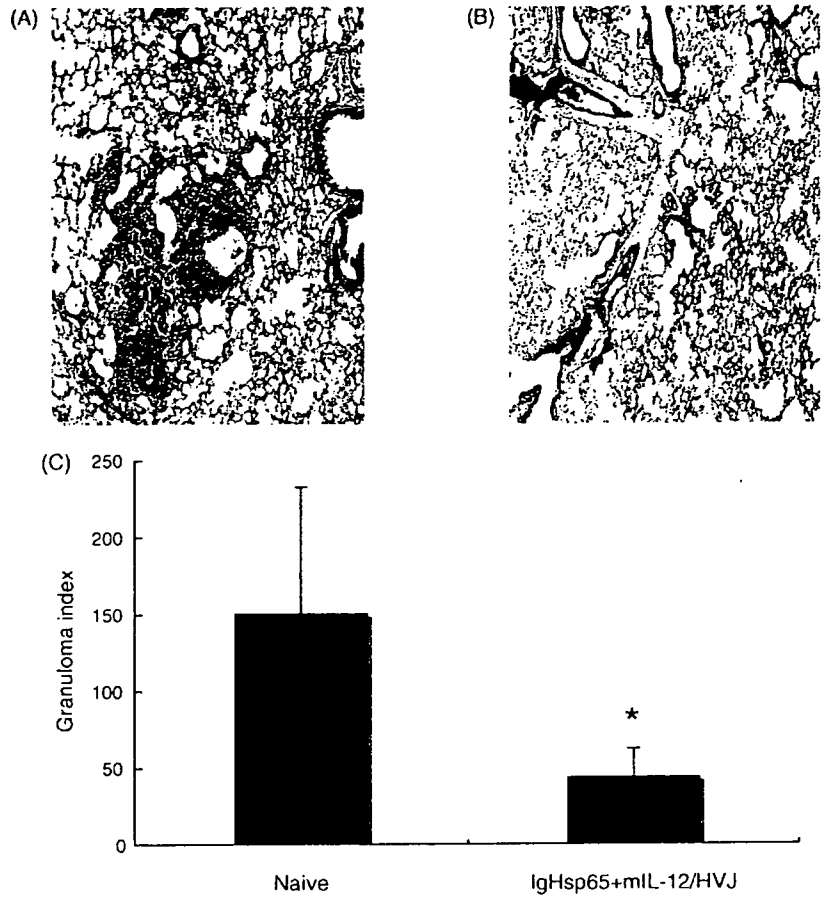

Fig. 4. Histopathological analysis of vaccinated mice 10 weeks after *M. tuberculosis* challenge. Representative photomicrographs of lung tissue sections harvested from the naive control group (A) and from the IgHsp65 + mIL-12/HVJ group (B) are shown (10 weeks after *M. tuberculosis* challenge, hematoxylin and eosin staining, ×10 objective). There were much infiltration of mononuclear cells and extensive parenchymal destruction by large, poorly demarcated granuloma in the lung from the naive control group. In the IgHsp65 + mIL-12/HVJ group, the lungs were less inflamed and only a few granuloma was observed. (C) Granuloma index of the naive control group and the IgHsp65+mIL-12/HVJ group in the lungs. Results are expressed as the mean ± S.D. of triplicates of five mice per group. The statistical significance of differences between the groups was determined by Student's *t*-test. $^{*}P < 0.05$ as compared with the naive control group.

at the same experiments (data not shown). These results indicate that co-vaccination with IL-12 DNA was effective for inducing protective immunity at as early as 5 weeks after challenge.

3.5. IgHsp65 + mIL-12/HVJ vaccination markedly reduced granuloma formation in the lung

In addition to the reduction of bacterial loads, the effects of vaccination on the mice were assessed by histological analysis. The granulomatous lesions in the lungs from IgHsp65 + mIL-12/HVJ mice were significantly less in number and size than from the naive control group (Fig. 4A and B). Quantitative evaluation of the granulomatous lesions clearly shows that IgHsp65 + mIL-12/HVJ vaccinated mice group exhibited significant reduction in granuloma index in the lungs, compared to the naive group ($P < 0.05$) (Fig. 4C). Thus IgHsp65 + mIL-12/HVJ vaccine provided significant protection against the pulmonary pathology caused by *M. tuberculosis* infection.

3.6. HVJ-liposome DNA vaccines generated T-helper response and cytokine production

To investigate lymphocyte proliferative and cytokine responses induced by HVJ-liposome DNA vaccines, spleen cells from vaccinated mice were re-stimulated with antigen in vitro. As shown in Fig. 5, substantial lymphocyte proliferation was observed in response to rHsp65 protein in spleen cells from mice vaccinated with IgHsp65/HVJ or IgHsp65 + mIL-12/HVJ but not with the naïve control. IgHsp65 + mIL-12/HVJ vaccination induced significantly better proliferative response to rHsp65 protein than did IgHsp65/HVJ vaccination ($P < 0.01$). In addition to lymphocyte proliferative responses, vaccination with IgHsp65 + mIL-12/HVJ induced elevated levels of IFN-γ and

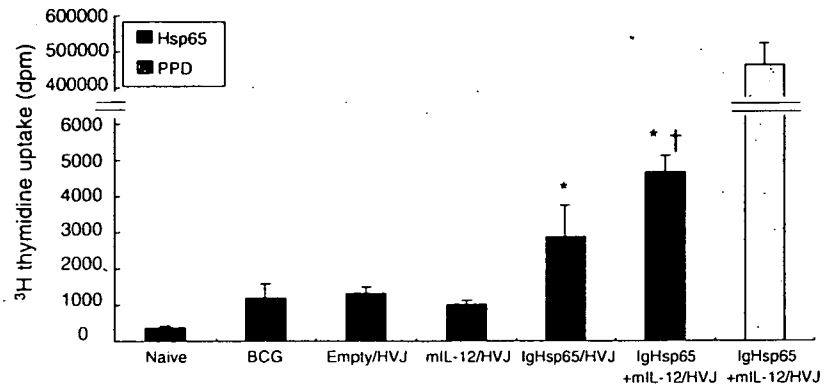

Fig. 5. The effect of vaccination with HVJ-liposome DNA on T cell proliferation. Proliferative responses of splenic lymphocytes from mice vaccinated with IgHsp65/HVJ, mIL-12/HVJ, IgHsp65 + mIL-12/HVJ, BCG, or Empty/HVJ. Incorporation of [^{3}H]thymidine in response to rHsp65 protein (black bars) or PPD (gray bar) was measured as described in Section 2. Results are expressed as the mean ± S.D. of triplicates of three mice per group. The statistical significance of differences between individual groups in T cell proliferation was determined by Tukey–Kramer's HSD test. The statistical significance of differences ($P < 0.01$) compared to the naive and BCG groups are indicated as (*) and (†), respectively.

IL-2 in response to rHsp65 protein, but not with the naive control or BCG group (Fig. 6). In response to PPD, vaccination with IgHsp65 + mIL-12/HVJ markedly increased both IFN-γ and IL-2 production as compared to the BCG group. Moderate but significant levels of IFN-γ and IL-2 were also induced in Hsp65/HVJ vaccination in response to Hsp65 protein and PPD. Thus, the synergistic effect of IgHsp65 DNA and mIL-12 DNA resulted in the strongest response not only to T cell proliferation but also to cytokine production.

3.7. HVJ-liposome DNA vaccines generated cytotoxic CD8$^+$ T cells

Because CD8$^+$ CTLs have been considered critical effectors of protective immunity to *M. tuberculosis*, it was of interest to determine whether a tuberculosis specific response could be induced in the vaccinated mice. We characterized CD8$^+$ T cells specific for Hsp65, PPD or killed *M. tuberculosis* by using a conventional ^{51}Cr release assay in the

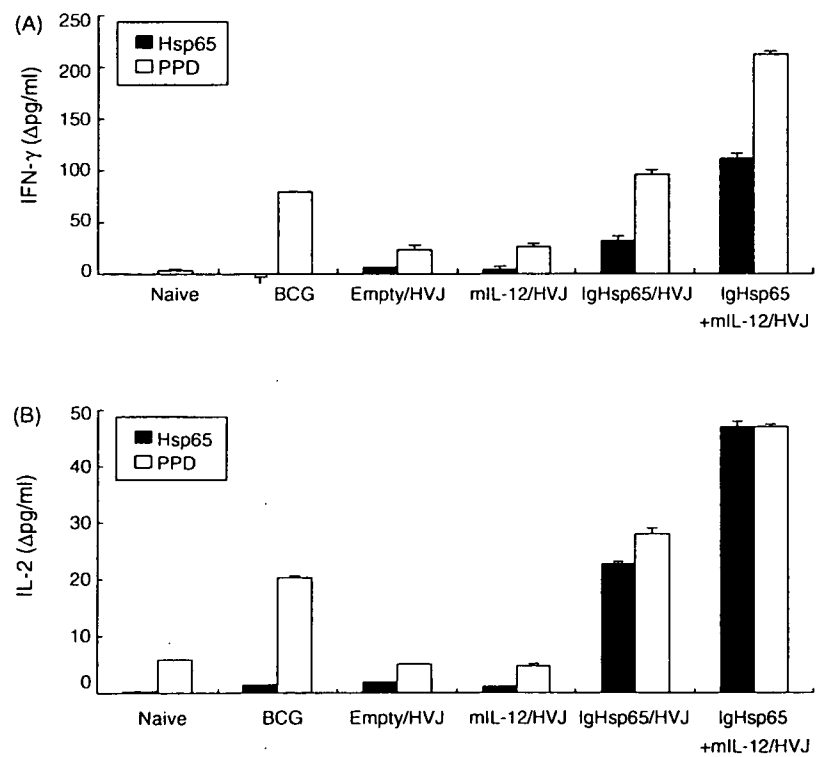

Fig. 6. IFN-γ (A) and IL-2 (B) production in spleen cell culture supernatants from vaccinated mice following stimulation with rHsp65 protein and PPD. Spleen cell cultures were stimulated with rHsp65 protein (black bars) or PPD (white bars) for 48 h, and the levels of IFN-γ and IL-2 production were determined by ELISA. Results are expressed as the mean ± S.D. of duplicates of three mice per group with antigens minus the mean ± S.D. of triplicates of three mice per group with medium alone.

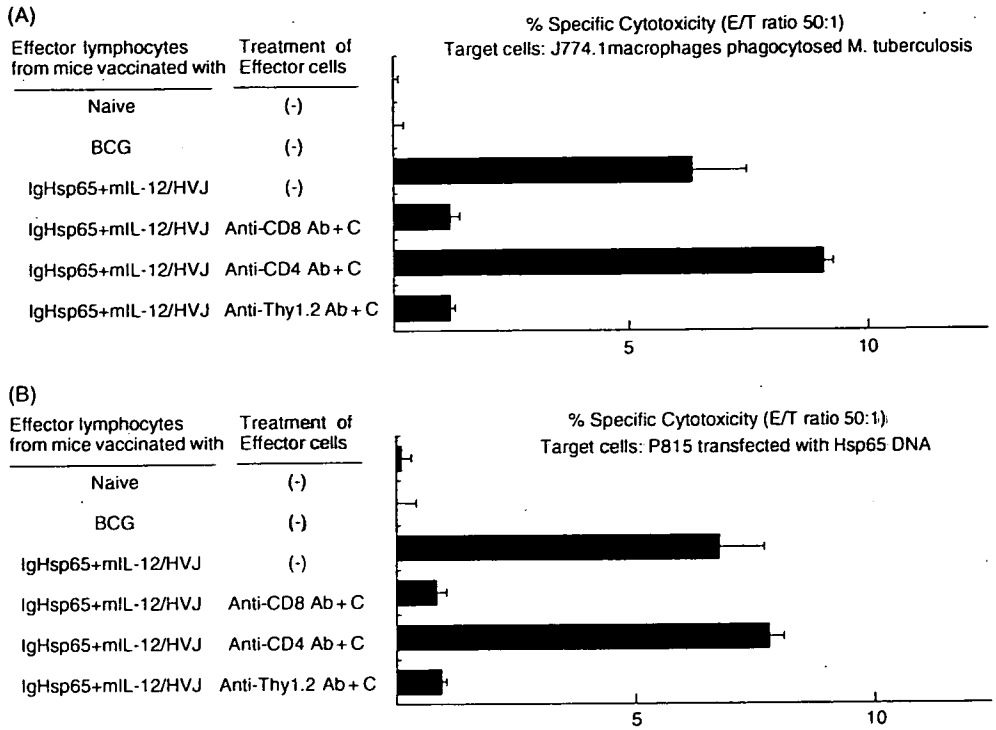

Fig. 7. Induction of $CD8^+$ CTL specific for Hsp65 protein and *M. tuberculosis* by vaccination with IgHsp65 + mIL-12/HVJ. Spleen cells from the naive, BCG-, and IgHsp65 + mIL-12/HVJ-vaccinated mice were obtained 8 weeks after the final vaccination. Cytotoxicity was assayed as release of radioactivity from ^{51}Cr-labeled J774.1 macrophages that had phagocytosed *M. tuberculosis* (killed H37Ra) (A) or from ^{51}Cr-labeled P815 target that had been transfected with Hsp65 DNA (B) using a conventional ^{51}Cr release assay at E:T ratio of 50:1. The effector cells were pre-incubated with anti-CD8, anti-CD4 or anti-Thy1.2 antibody, followed by treatment with complement. Percent specific lysis was determined as: [(experimental release–medium control release)/(maximum release–medium control release)] × 100. Ab: antibody; C: complement; (−), non-treatment.

absence of re-stimulation. As shown in Fig. 7, high levels of Hsp65- and *M. tuberculosis*-CTL specific lysis against J774.1 macrophages phagocytosed *M. tuberculosis* and P815 mastocytomas transfected with Hsp65 DNA were detected in mice vaccinated with IgHsp65 + mIL-12/HVJ, whereas little CTL response was detectable in either the naive or BCG-vaccinated mice. In vitro depletion of $CD8^+$ T cells eliminated the specific lysis. Depletion of $CD4^+$ T cells had no effect. Stronger (more than twenty percent) cytotoxicity against Hsp65 was detected in the spleen cells from mice 2 weeks after the last vaccination with IgHsp65 + mIL-12/HVJ (data not shown). These results indicate that IgHsp65 + mIL-

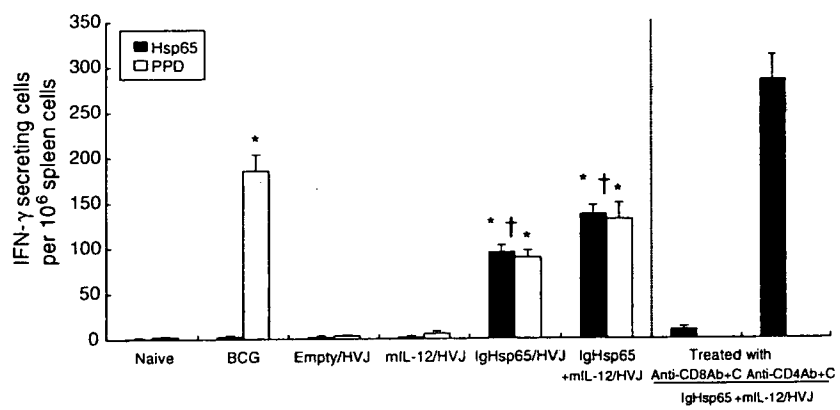

Fig. 8. ELISPOT assay for IFN-γ antigen-specific responses in the spleens of vaccinated mice following stimulation with rHsp65 protein and PPD. Spleen cell cultures were stimulated with rHsp65 protein or PPD for 20 h or pre-incubated with anti-CD8 antibody or anti-CD4 antibody followed by treatment with complement and then stimulated with rHsp65 protein for 20 h. The number of IFN-γ-secreting cells specific for rHsp65 protein (black bars) or PPD (white bars) per million cells were determined individually by ELISPOT assay. Results are expressed as the mean ± S.D. of five-wells of three mice per group. The statistical significance of differences between individual groups in the number of IFN-γ-secreting cells was determined by Tukey–Kramer's HSD test. The statistical significance of differences ($P < 0.01$) compared to the naive and BCG groups are indicated as (*) and (†), respectively.

12/HVJ vaccine induced long-term immune response with strong $CD8^+$ CTL activity.

3.8. ELISPOT assay

In order to determine whether enhanced protection was associated with increased IFN-γ production, the frequency of IFN-γ-secreting cells was enumerated by ELISPOT. Vaccination with IgHsp65/HVJ and IgHsp65 + mIL-12/HVJ resulted in a marked increase of IFN-γ secreting cells following stimulation with rHsp65 protein (Fig. 8). Moreover, the increase of IFN-γ secreting cells was also seen in IgHsp65/HVJ and IgHsp65 + mIL-12/HVJ groups following stimulation with PPD. These results indicate that vaccination with IgHsp65/HVJ and IgHsp65 + mIL-12/HVJ activated antigen-specific T cells producing IFN-γ. Depletion of $CD8^+$ cells from responder cells by treatment with anti-CD8 antibody and complement almost abrogated the IFN-γ producing cells. In contrast, an increase in the number of IFN-γ producing cells was observed in the responder cells when treated with anti-CD4 antibody and complement. BCG vaccination resulted in significant increase of IFN-γ secreting cells following stimulation with PPD but not rHsp65 protein. These data indicate that the protective efficacy of IgHsp65 + mIL-12/HVJ is strongly associated with the emergence of IFN-γ-secreting cells upon stimulation with Hsp65. Taken together, vaccination with IgHsp65 + mIL-12/HVJ capable of augmenting T cell activation and frequency of IFN-γ-secreting cells proves to reduce bacterial burden and pathology in the lungs—all to an extent greater than those achieved by vaccination with BCG.

4. Discussion

In the first stage of this study, we evaluated the protective efficacy of Hsp65 DNA vaccines via gene gun vaccination. One of the significant findings of the present study is that a single gene gun vaccination with the combination of IgHsp65 DNA and mIL-12 DNA led to a remarkably high degree of protection against intravenous challenge infection with virulent *M. tuberculosis*; bacterial numbers declined exponentially in internal organs and were 100-fold lower in the lungs than in BCG-vaccinated mice. Consistent with previous studies [43], gene gun vaccination with Hsp65 DNA alone did not promote reduction in bacterial burden compared to the naive mice. However, co-vaccination of Hsp65 DNA or IgHsp65 DNA plus mIL-12 DNA significantly improved the protective efficacy compared to either Hsp65 DNA alone or IgHsp65 DNA alone. Since the importance of IL-12 in the control of mycobacterial infections has been well documented, these results are consistent with other studies describing an adjuvant effect of IL-12 gene when administrated in combination with various tuberculosis DNA vaccines [20,24,25]. The mIL-12 DNA, which express both p40 and p35 chains as a single molecule, is able to induce four-fold higher levels of IFN-γ from mouse T lymphocytes than mIL12p40 + p35, which has previously been constructed as a murine expression vector with IL-12 p40 and p35 expression cassettes in tandem array [35]. Culture supernatants from the mIL-12 DNA-transfected COS-7 cells were effectively induced IFN-γ from mouse spleen cells. Thus, the improved expression levels of IL-12 DNA and the biologically active IL-12 explain the enhanced protection observed.

The second stage of this study demonstrated the protective efficacy of HVJ-liposome DNA vaccines in mouse and guinea pig models. We originally developed HVJ-liposomes, a viral/nonviral hybrid vector, as a gene transfer vector for cancer gene therapy. HVJ-liposome gene transfer method can deliver DNA directly and efficiently into host cells in vivo by means of the HVJ virus cell fusion machinery. We found that HVJ-liposome-mediated gene transfer was 30–100 times more efficient in gene expression in skeletal muscle than naked DNA transfer (unpublished data) and over three times more efficient in delivering intact oligodeoxyribonucleotide within the nuclei of transfected cells than Lipofectin®, a different cation liposome [44]. In addition to its high transfection efficiency, there are numerous safety advantages of HVJ-liposomes including: (i) no apparent toxicity or inflammation and (ii) repeated gene transfection without reduction of transfection efficiency. In fact, no significant adverse effects were induced in monkeys by intravenous injection of HVJ-liposomes [45]. Using this novel vector, we observed the enhancement of protection conferred by Hsp65 DNA compared to gene gun vaccination. This result is encouraging for the development of a novel tuberculosis DNA vaccine that is applicable both for prophylactic and therapeutic uses with no side-effects after repeated injections.

The most significant finding of this study is that vaccination with IgHsp65 + IL-12/HVJ provided greater protective efficacy than vaccination with BCG. In the mouse model, IgHsp65 + mIL-12/HVJ preferentially triggered a Th1 type T helper response, characterized by elevated levels of IFN-γ and IL-2, and augmentation of lymphocyte proliferation. After challenge, vaccination with IgHsp65 + mIL-12/HVJ resulted in a greater degree of protection than that evoked by BCG. This protective efficacy was associated with the emergence of IFN-γ-secreting T cells directed against Hsp65 and PPD. $CD8^+$ CTL activity against macrophage target cells, which had previously phagocytosed *M. tuberculosis* or expressed Hsp65 protein, was still observed in the spleen cells from mice vaccinated with IgHsp65 + mIL-12/HVJ at 8 weeks after the final vaccination, IgHsp65 + mIL-12/HVJ vaccine capable of augmenting long-term immune response with anti-tuberculosis CTL activity proves IgHsp65 + mIL12/HVJ to be a promising tuberculosis vaccine candidate.

Although the *hsp65* DNA vaccines have been shown to have significant promise as a new prophylactic vaccine against tuberculosis [19,21,46], negative outcomes have also been reported [47,48]. In the case of vaccination with *hsp65* DNA alone, our results are consistent with the previous report

that vaccination with *hsp65* DNA alone did not provide significant protective effect in the bacterial load in the lung either in the mouse model or in the guinea pig model [43,47]. However, as described above, the combination with mIL-12 DNA expressing biologically active IL-12 and the use of HVJ-liposome as a DNA vaccine delivery system remarkably improved the protective efficacy. In addition, our preliminary results of a guinea pig model in the collaborative study with Dr. D. McMurray (Texas A&M University) show that vaccination with IgHsp65 + guinea pig IL-12 (gpIL-12)/HVJ provided better protection against the pulmonary pathology caused by aerosol challenge with *M. tuberculosis* than did BCG vaccination (data not shown). For immunotherapeutic use, *hsp60*/lep DNA vaccine (*hsp65* DNA derived from *Mycobacterium leprae*) has been shown to be effective in a Cornel-type model [22], although others have argued that this vaccine induced progressively severe pulmonary necrosis in the model [48]. In support of the effectiveness, when administered to mice or SCID-PBL/hu mice [49] already infected with *M. tuberculosis*, neither IgHsp65 + mIL-12/HVJ vaccine nor IgHsp65 + human IL-12 (hIL-12)/HVJ vaccine, respectively, resulted in exacerbation of the granulomatous response in the lungs (unpublished data). Moreover, therapeutic administration of IgHsp65 + mIL-12/HVJ resulted in significant reduction of bacterial loads (paper in submission). The pathological parameter of protection included reductions in the mean lung granulomatous lesion score in our study. In parallel with the protective efficacy of HVJ-liposome vaccines on bacterial loads, histopathological analysis shows that mice vaccinated with IgHsp65 + mIL-12/HVJ had fewer and smaller lesions in the lung and significantly less lung granuloma than the naive mice. These results suggest that severe toxicities (Koch phenomenon) could not be induced by this vaccine. One possible explanation for these diverging results may be different *hsp65* DNA construct (secreted form versus cytoplasmic form; derived from *M. tuberculosis* versus *M. leprae*), different mIL-12 DNA construct (p40p35 fusion form versus p40-p35 tandem form), and different vaccine delivery (HVJ-liposome versus gene gun or naked DNA).

In conclusion, we demonstrate the development of a novel HVJ-liposome DNA vaccine encapsulating Hsp65 DNA plus IL-12 DNA. These results suggest that Hsp65 + IL-12/HVJ could be a promising candidate for a new tuberculosis DNA vaccine, which is superior to the currently available BCG vaccine. The goal of our study is to develop a new tuberculosis vaccine superior to BCG. To this aim, we believe that the protective efficacy and protective immune responses for vaccine candidates should be addressed in larger animals, such as non-human primates, before proceeding to human clinical trials. Although other DNA vaccine candidates that appear to protect against virulent *M. tuberculosis* in mice better than BCG have failed to provide better protection than BCG in guinea pigs against aerosol challenge of a low dose of virulent *M. tuberculosis* [47,50,51], some of them are being prepared to enter early human clinical trials [52]. More recently, we evaluated the IgHsp65 + hIL-12/HVJ vaccine in the cynomolgus monkey model [29], which is currently the best non-human primate animal model of human tuberculosis. Monkeys were subsequently challenged with virulent *M. tuberculosis* by the intra-tracheal route after the third vaccination. This challenge dose normally causes death from acute respiratory infection within 4–6 months. In this particular experiment, monkeys vaccinated with IgHsp65 + hIL-12/HVJ induced Hsp65-specific T cell proliferation and improvement of chest X-P findings, resulting in an increased survival for over a year, superior to BCG group [29]. Thus, we are taking advantage of the availability of multiple animal models (mouse, guinea pig, and monkey) to accumulate essential data of the HVJ-liposome DNA vaccine, including the vaccine efficacy and safety, for up-coming Phase I clinical trials.

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

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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 [9].

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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 [9]. 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 Erythrocyte Sedimentation Ratio (ESR) of all monkeys was evaluated every month and maximum values of ESR in each monkey were shown.

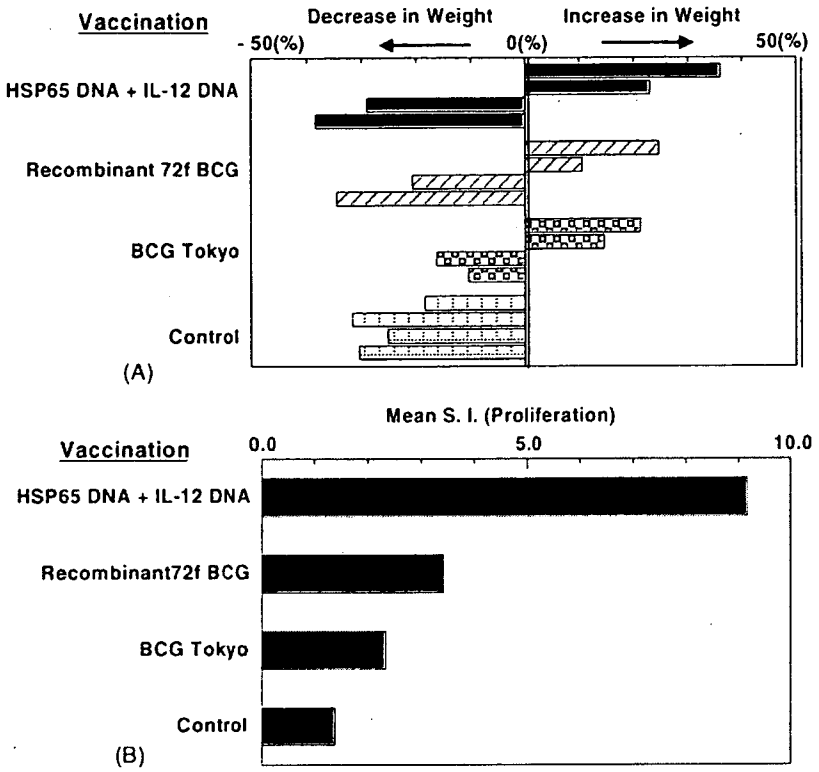

Fig. 1. (A) Increase in body weight: the prophylactic effect of novel vaccines (HSP65 DNA + IL-12 DNA, recombinant 72f BCG) on *M. tuberculosis* infection of cynomolgus monkeys. Percent of increase or decrease in body weight of monkeys immunized with (1) HSP65 DNA + IL-12 DNA (■), (2) recombinant 72f 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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ゆえに結核病棟内での再感染が判明した事実をもとに，国内外の文献の考察も含め，宿主側の因子，菌側の因子および結核菌の再曝露程度により，外来性再感染が普遍的に起こり得ることを，分子疫学的解析により実証した。低蔓延国に近づいている本邦では，外来性再感染は結核入院病棟を中心に起こるので，これを意識した入院患者・職員への感染防止対策が必要であることを各演者は警告した。

1. 多剤耐性結核の再感染

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はじめに

わが国の結核医療は，長年にわたってそのほとんどが隔離入院治療という形で行われてきた。しかし，結核病棟が特に他の一般病棟と比べて特別な感染対策が施されていたわけではなく，空気感染防止のための空調管理設備を備えた病室が整備され始めたのもごく近年のことである。結核病室の多くは大部屋であり，感受性結核患者と耐性結核患者が同室となることも多かった。これは，①結核患者が新たに他の結核菌の感染を受ける（再感染）ことは稀である，②耐性結核菌は変異菌であるので毒力は弱い，という漠然とした認識があったからと考えられる。すなわち，感受性結核患者が耐性結核菌の外来性再感染を受けることはまずあり得ないと想定されていたのである。

しかし近年分子疫学の進歩により結核の再感染発病を確実に証明することが可能となり，耐性結核菌による再感染発病が起こり得ることも報告されている。ここでは，われわれが経験した多剤耐性結核菌による再感染発病と考えられる2事例について概説し，今後の結核感染対策のあり方について考えてみたい。

事　例　1

本事例は多剤耐性結核の院内集団感染事例である。初発患者Aは56歳の男性で，平成12年3月発症の初回多剤耐性結核患者である。発症時の分離結核菌の薬剤感受性検査でisoniazid（INH），rifampicin（RFP）を含む多剤に既に耐性を示しており，近医入院にて化学療法を施行されるも大量排菌持続していた。平成14年6月に他患者とのトラブルのため当院転院となる。

当院転院までの約2年間における患者Aの接触者から後に5名の多剤耐性結核患者が発生し，5名の分離菌株はRFLP分析により患者Aの菌株と同一であると考えられた。うち3名は特に基礎疾患のない若年女性であった。他の2名は63歳男性と53歳男性であり，基礎疾患として肺気腫，糖尿病を有していた。2名とも全剤感受性肺結核にて入院加療を受けており，入院中にのみ患者Aと接触歴があった。2名とも感受性肺結核治癒後に多剤耐性肺結核を発症している。従って感受性結核罹患中に多剤耐性結核菌の再感染を受けたと考えられる。なお，2名とも感受性肺結核罹患時の分離菌は保存されておらず，RFLP分析は行えなかった。本事例の患者は6名全員HIV陰性であった。

事　例　2

本事例は当院で経験した多剤耐性結核菌による再感染発病事例である。患者Xは特に基礎疾患を有さない28歳男性で，平成13年1月より全剤感受性結核にて当院入院し化学療法を行った。入院中の一時期，多剤耐性肺結核に罹患していた患者Yと同室であった。順調に排菌陰性化して退院し，化学療法にて治癒に至ったが，その後，平成16年6月に再発し，そのときの検出菌の薬剤感受性検査ではINH，RFP，ethambutol（EB），streptomycin（SM）を含む多剤に対して耐性を示していた。RFLP分析を行ったところ，再発時の検出菌は初回治療時の検出菌とはパターンが異なっており，患者Yの検出菌と同一パターンであった。すなわち，感受性結核治療中に多剤耐性結核菌の再感染を生じて，後に多剤耐性結核による再発を生じたと考えられた。なお，患者XもHIV陰性であった。

多剤耐性結核菌のクラスター解析

2001年から2004年までに当院で分離した多剤耐性結核菌株115株を対象に，RFLP法，spoligotyping法により解析を行った。RFLP法では48株（42%）が10群のクラスターを形成していた。5株以上からなる大きなクラスターが3群あり，クラスターa（12株），クラスターb（11株），クラスターc（7株）とした。事例1の株はクラスターc，事例2の株はクラスターaに属していた。spoligotyping法でクラスターa，クラスターbはBeijing strainと判定されたが，クラスターcはBeijing strainではなかった。

多剤耐性結核は，一般にはその多くが不十分な治療による耐性の誘導が原因と考えられているので，クラスター形成率は低くなることが予想される。しかし，今回の検討ではクラスター形成率は42%であった。また，大きなクラスターを形成するクラスターa，b，cの株は，広く蔓延する強毒株であることが示唆された。

再発時に多剤耐性を示した結核における再感染の頻度

当院において，いったん結核にて化学療法を行い治癒した後，少なくとも排菌陰性期間が6カ月以上持続した後に多剤耐性結核を発症した例につき，前後の菌株が入手できた8症例に対してRFLP分析を行った。8例中6例は前後の菌株のRFLPパターンが一致し内因性再燃であると考えられたが，残り2例（事例2を含む）はパターンが異なり再感染発病であると考えられた。この2例の再発時の耐性菌はクラスターa（事例2）とクラスターcに属する大クラスター形成株であった。

考　察

近年RFLPをはじめとする分子疫学的手法の進歩により結核の再感染発病について幅広い検討がなされている。当初はHIV感染者での報告が相次ぎ，再感染発病の宿主側の危険因子としてHIV感染が注目されたが，その後HIV陰性者を含めて様々な状況下での再感染発病事例が報告された。伊藤はこれまでの報告の分析により，かつて考えられていたほど再感染発病は稀なものではなく，宿主側の因子，菌側の因子および曝露程度により普遍的に起こり得ることを指摘している[1]。

今回の事例1では，2年間に基礎疾患をもたない若年女性3人が発病し，また，2人の中高年男性が再感染を受けて発病している。また，事例2では基礎疾患をもたないHIV陰性若年男性が再感染を受けて発病している。以上よりこの2事例の菌は強毒菌であったことがうかがわれる。いずれも大きなクラスターを形成する菌であったこともその裏付けとなる。

かつて動物実験でカタラーゼ活性を欠くINH耐性菌の増殖が感受性菌に比べて劣ることが示されたことから，変異株である耐性菌は感受性菌に比べて毒力が弱いと漠然と信じられてきた。しかし，今回われわれが経験したように，多剤耐性結核菌といえども再感染発病を引き起こす病原性の高い菌も存在する。それでは，病原性を規定するものは何であろうか？ NiemannやNarvskayaもHIV陰性者における多剤耐性結核再感染事例を報告しており[2,3]，いずれも菌はBeijing strainであった。欧米では，集団感染や再感染発病の原因となる強毒菌としてBeijing strainが関与しているとの報告が多い[4]。しかしわが国や中国ではもともと半数以上がBeijing strainである[5]。一方，事例1の菌はBeijing strainではなかった。結局，Beijing strainであることも必ずしも決め手とはならず，現時点で菌の病原性を決定するのは困難であると言わざるを得ない。あえて言えば，クラスター解析で大きなクラスターを形成する菌が強毒菌であると言えるかもしれない。

多剤耐性結核の再感染は，結核の感染対策上大きな影響を与える。多剤耐性結核菌による再感染が起こり得，しかもどの菌が再感染し得るか予測することが不可能な以上，すべての排菌陽性耐性結核患者は感受性結核患者と同室に収容すべきではない。さらに，初回耐性結核の可能性も考えると，感受性不明の排菌陽性結核患者は全員陰圧個室収容が望ましい。CDCの結核院内感染防止ガイドラインではこの点を考慮に入れ，薬剤感受性パターンが同一であると判明し有効な化学療法が行われている場合に限り患者同士を同室にしてよいとしている[6]。わが国の現状では，これを守るのはインフラの面からもコストの面からもきわめて困難である。しかし，結核患者の減少，在院日数の短縮化により結核病棟の稼働率が下がっていく中で，思い切った対策の転換を考慮する必要があるのではないだろうか。多剤耐性結核は，その医療にかかる金銭的・時間的コストの膨大さ，さらに，院内感染が生じたときの社会的なインパクトの大きさなどを考慮に入れると，その発生防止に最善の対策が講じられるべきである。

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The 80th Annual Meeting Mini-Symposium

EXOGENOUS RE-INFECTION IN TUBERCULOSIS

Chairperson: Toshiaki TSUCHIYA

Abstract Patients infected by tuberculosis (TB) had been thought to never experience exogenous re-infection. However, exogenous re-infection in HIV-positive patients is well known. Thanks to the introduction of histopathological examination, analysis of similarities in drug-resistance patterns and epidemiological surveys of genetic phage typing for TB infection, we have begun to understand that even people with a normal immune system can experience re-infection.

Recent advances in the techniques of restriction fragment length polymorphism (RFLP) and spoligotyping allow determination of similarities in tubercle bacilli, revealing a high ratio of exogenous re-infection.

In this mini-symposium, Dr. Kazunari Tsuyuguchi reported cases of nosocomial multidrug-resistant tuberculosis (MDRTB) infection, as exogenous re-infection, at 3 tuberculosis hospitals in the Osaka area. Although the virulence of MDRTB as a variant strain has generally been regarded as weaker than that of drug-sensitive strains, he reported even non-Beijing strain MDRTB, which displays strong virulence, could possess possible infectiosity with a 42% ratio of clustering formation and 2 of 8 patients with MDRTB exhibiting exogenous re-infection, as analyzed by RFLP.

Dr. Hideo Ogata reported the actual condition of exogenous re-infection, having cited a large number of reports at home and abroad. In his report he indicated that even among hosts without serious hypoimmunity, re-infection rate is high in high-prevalence countries. Conversely, endogenous TB reactivation is high in low-prevalence countries. As Japan has become a low-prevalence country, endogenous reactivation might be seen in TB wards.

Dr. Katsuhiro Kuwabara reported on his study about exogenous re-infection of *Mycobacterium avium*, which represented resident flora in the environment, using IS*1245* RFLP analysis. He demonstrated that re-infection and multiple infections were frequently observed in *M. avium* infection.

Dr. Tomoshige Matsumoto finally added that about 90% of patients with recurrence in the Osaka area exhibit endogenous reactivation, as found using molecular epidemiologic analysis of bacterial strains from initially treated and retreated patients. Compared with reports from other countries, the ratio of exogenous re-infection in Japan is lower than elsewhere. Thanks to the public health service about TB, sources of TB infection are not present, so patients with TB do not experience exogenous re-infection, he concluded. He also discussed the variable number of tandem repeats (VNTR)-typing method that has been taking the place of the IS*6110* RFLP.

In this mini-symposium referring to molecular epidemiological analyses and reports from Japan and overseas, we showed that depending on factors involving hosts, parasites and the density of TB re-exposure, the possibility of universal exogenous nosocomial re-infection exists. Each presenter alerted us to the fact that as exogenous re-infection occurs mainly in TB inpatient wards, prevention of TB infection is crucial for inpatients and medical staff in Japan as a low-prevalence country.

1. Exogenous re-infection by multidrug-resistant tuberculosis

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We describe three recurrent cases of multidrug-resistant (MDR) tuberculosis (TB) nosocomially re-infected with MDRTB strain during treatment for drug-sensitive TB. The first and the second patients, both of whom were middle-aged heavy smoker men, were associated with the outbreak caused by non-Beijing MDRTB strain. The third patient was a immunocompetent young man and the isolated strain was Beijing MDRTB strain. All the patients were HIV-seronegative. We conclude that exogenous re-infection by