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DNA vaccine using hemagglutinating virus of Japan-liposome encapsulating combination encoding mycobacterial heat shock protein 65 and interleukin-12 confers protection against *Mycobacterium tuberculosis* by T cell activation

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Abstract

We investigated the immunogenicity and protective efficacy of DNA vaccine combinations expressing mycobacterial heat shock protein 65 (Hsp65) and interleukin-12 (IL-12) using gene gun bombardment and the hemagglutinating virus of Japan (HVJ)-liposome method. A mouse IL-12 expression vector (mIL-12 DNA) encoding single-chain IL-12 proteins comprised of p40 and p35 subunits were constructed. In a mouse model, a single gene gun vaccination with the combination of Hsp65 DNA and mIL-12 DNA provided a remarkably high degree of protection against challenge with virulent *Mycobacterium tuberculosis*; bacterial numbers were 100-fold lower in the lungs compared to BCG-vaccinated mice. To explore the clinical use of the DNA vaccines, we evaluated HVJ-liposome encapsulated Hsp65 DNA and mIL-12DNA (Hsp65 + mIL-12/HVJ). The HVJ-liposome method improved the protective efficacy of the Hsp65 DNA vaccine compared to gene gun vaccination. Hsp65 + mIL-12/HVJ induced $CD8^+$ cytotoxic T lymphocyte activity against Hsp65 antigen. Most importantly, Hsp65 + mIL-12/HVJ vaccination 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 and activation of proliferative T cells and cytokines (IFN-γ and IL-2) production upon stimulation with Hsp65 and antigens from *M. tuberculosis*. 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.
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Keywords: Tuberculosis; DNA vaccine; HVJ-liposome

1. Introduction

Tuberculosis is a major global threat to human health, with about 2 million people dying each year from *Mycobacterium tuberculosis* infections. The only tuberculosis vaccine currently available is an attenuated strain of *Mycobacterium bovis* BCG. BCG continues to be widely administered to children in developing countries, yet its efficacy remains controversial, particularly against the pulmonary form of the disease in adults. In recent years, the increasing frequency of drug-resistant *M. tuberculosis* isolates has further complicated the clinical management of this disease. Clearly, a more effective vaccine for the control of tuberculosis is urgently needed.

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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™), 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

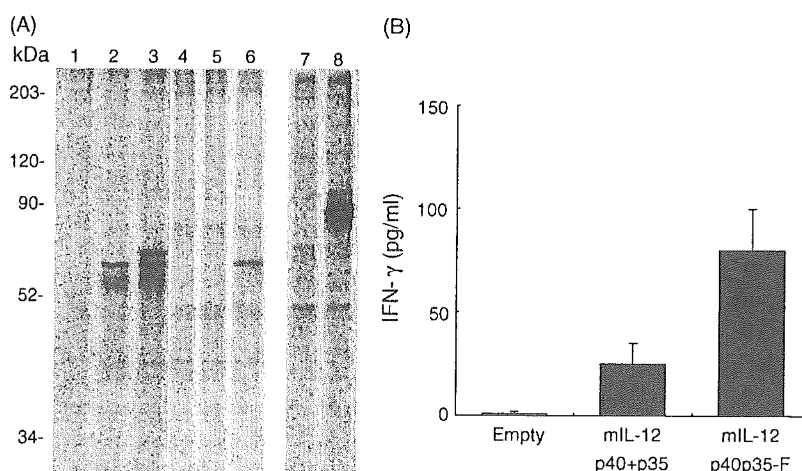

Fig. 1. Expression and biological analysis of Hsp65 and mIL-12. (A) In vitro expression analysis of Hsp65 and mIL-12 from cells transiently transfected with DNA vaccines. COS-7 cells were transfected with EGFP DNA (lanes 1, 4 and 7), Hsp65 DNA (lanes 2 and 5), IgHsp65 DNA (lanes 3 and 6), and mILp40p35-F 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.