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Original Article

TRENDS IN TUBERCULOSIS INFECTION AMONG FOREIGNERS IN JAPAN ACCORDING TO WORK STATUS

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Abstract [Purpose] TB among foreigners is presently a serious issue in some developed countries and could become so in Japan. The purpose of this report is to assess the epidemiological situation of TB among foreigners in Japan.

[Materials and Methods] The trend of TB reporting among foreigners in Japan was examined with regard to work status.

[Results] The number of reported TB cases among employees and students in Japan increased between 1998 and 2008, but that among housekeepers was level throughout the same period. The increase among employees and students might be due to the increased numbers of foreign employees and students. In the case of housekeepers, the increase in the number of permanent residents did not lead to an increase in TB among these housekeepers. Estimates of TB reporting rates decreased during the study period, so the changes in reporting rates would not have caused the increase in TB cases. This downward trend may have been caused by an increase in longer-term residents and a decrease in TB incidence in home countries. Even though the TB reporting rate is decreasing, the rates in those countries are much higher than in Japan in the same work categories.

[Discussion] To control the spread of TB, it is important to identify high-risk individuals. The Japanese TB control program should further strengthen mass health examination programs for foreign housekeepers and employees (especially temporary and daily employees), case-finding based on individuals' access to hospitals or clinics when suffering from TB symptoms, and flexible and periodic adjustment of TB control activities for foreigners according to future changes in the number and distribution of foreigners in Japan. Furthermore, improving the TB epidemiological situation in home countries might contribute to the downward trend of TB reporting rates among foreigners in Japan. Therefore, Japanese assistance in TB control activities in surrounding countries such as China, South Korea, and the Philippines might contribute to TB control activities for foreigners in Japan.

Key words: Tuberculosis, Foreigners, Labor status, Incidence

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Tuberculosis vaccine development

The development of novel (preclinical) DNA vaccine

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Keywords: MDR-TB therapy, monkey, CTL, recombinant DNA

A third of the world's population is infected with *Mycobacterium tuberculosis* and 2 million people die from tuberculosis every year. The only tuberculosis vaccine currently available is an attenuated strain of *Mycobacterium bovis* BCG, although its efficacy against adult tuberculosis disease remains controversial. Furthermore multi-drug resistant tuberculosis is becoming big problems in the world. Therefore, the development of novel therapeutic vaccine as well as novel prophylactic vaccine against tuberculosis is required.

This review provides a summary of novel vaccines (especially DNA vaccines) in preclinical stage using mouse, guinea pig and monkey models. In several promising novel vaccines, the studies were extended to a cynomolgus monkey model, which is currently the best animal model of human tuberculosis. The review also provides recent advances of the precise studies of induction of immunity including CD8 positive cytotoxic T cells and effector molecules such as granulysin by these vaccines, against multi-drug resistant tuberculosis and extremely drug resistant tuberculosis.

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 was not effective. 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 as well as prophylactic vaccine against TB is required. TB vaccines were classified into four main vaccines (1) DNA vaccines, (2) recombinant BCG vaccines (3) subunit vaccines and (4) attenuated TB vaccines.

It is well established that protective immunity to *M. tuberculosis* depends on both $CD4^+$ and $CD8^+$ T cells.[1-8] 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.[9,10] 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,[11] malaria[12,13] and HIV infections.[14] DNA vaccination has also shown potential for the development of tuberculosis vaccines in the mouse model.[15-18] 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 been proven more efficacious than BCG.[19] 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.

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Vaccines for TB (DNA Vaccines)

DNA vaccines are a relatively new approach to immunization for infectious diseases.[1,2,15,16,20-23] Many gene therapies, including DNA vaccines, have been used for the treatment of several kinds of cancers for more than twenty years via the activation of $CD8^+$ cytotoxic T cells.[24-26] Plasmids containing genes have also been used to induce protection against a variety of bacterial, viral (such as SARS corona virus), protozoal and helminth infection in animal models.[27,28] DNA vaccines by the use of several kinds of vectors including HVJ-envelope, HVJ-liposome, adenovirus vector and AAV vector were established.

DNA vaccines can induce strong cellular immunity against TB and can invoke both CD4 and CD8 T-cell responses.[2,29] The first data showing significant protection against *M. tuberculosis* came from naked DNA immunization in mice. Several mycobacterial antigens delivered as naked DNA have elicited protection against *M. tuberculosis*. Hsp65 antigens (derived from H37Rv *M. tuberculosis*)-, Ag 85B-, Ag 85A-, M.tb8.4-, M.tb41-, PPT39-, MPT51-, MPT63-, MPT64-, MPT83-, ESAT-6-, Pst-3- and the 38 kDA lipoprotein-DNA vaccines were studied (**Table 1**).[1,20,30-44]

Ag85A antigen has been used to prime immune responses followed by homologous or heterologous boosting vaccines in mice. Protection as defined by a reduction in the numbers of bacteria recovered from the lungs was observed. Antigen Ag85B, an abundant 30 kDa secreted protein of *M. tuberculosis*, has shown protection in mice as well as in guinea pigs against TB.[34] Similarly DNA vaccines encoding secreted proteins Ag85B and MPT64 have also been reported to protect mice from *M. tuberculosis* H37Rv challenge by prompting a Th1 response. The effectiveness of DNA vaccines can be increased by codelivery of multiple DNA plasmids or chimeric DNA vaccines.[45-47] Derrick et al. used a DNA vaccine cocktail consisting of Ag85B, ESAT.

Table 1. Mycobacterial antigens and cytokines delivered as DNA vaccine

DNA Vaccines
HSP65
IL-12
Ag 85A
Ag 85B
MPT51
HSP70
ESAT-6
IL-6 + IL-6 Receptor + gp130
γIFN
Mtb8.4
Mtb41
Mtb39
MPT63
MPT64
Psts-3
38 kDa lipoprotein
MPT12
IL-15
IL-23
IL-27

Table 2. Vectors for DNA vaccines against tuberculosis

Types	Characteristics
(1) HVJ-Envelope	Very Good Expression (GMP Level)
(2) HVJ-liposome	Good Expression
(3) Adenovirus	Good Expression. Transient
(4) Adeno Assosiated Virus (AAV) Vector	AAV 2/5 Good Expression. Long Term
(5) Lentivirus	Non-proliferating cell
(6) Liposome	safety
(7) Sendai virus Vector	Good Expression
(8) Gene Gun	safety
(9) Vaccinia Virus (Attenuated: MVA)	
(10) BCG (recombinant BCG)	
(11) Attenuated Listeria	

KatG, MPT8.4, MPT12, MPT63, MPT64 and MPT83 to reduce the bacterial burden in the lungs of normal mice as well as mice lacking CD4 T cells after aerosol challenge.

DNA vaccines are safe, cheap, stable and effective for inducing cellular immunity (helper T cell, cytotoxic T cell, macrophages) and humoral immunity in preclinical models of infectious diseases.[48-50] These vaccines are also able to activate innate immune responses. They interact with Toll-like receptor 9 (TLR 9: the pattern recognition receptor) through unmethylated CpG oligodeoxynucleotides (CpG ODNs9),[51,52] resulting in the upregulation of cytokines gene expression through MyD88 molecule.[53-56]

DNA vaccines have been in general proved to be safe and well tolerated in preclinical and clinical studies.[57,58] The naked plasmid DNA molecules have not caused any adverse effects on the biochemical and hematological blood values and have caused neither detectable organ pathology nor systemic toxicity. Furthermore, there has been no evidence of autoimmunity, development of anti-nuclear or anti-DNA antibodies, or plasmid DNA integration into chromosomes.

TB Vaccine Strategies

Pre-infection vaccination strategy. From a public health perspective, delivering a vaccine prior to mycobacterial infection and soon after birth makes most sense.

Booster vaccination strategy (prophylactic). A second option would be to use a new TB vaccine as a booster sometime after neonatal BCG vaccination.

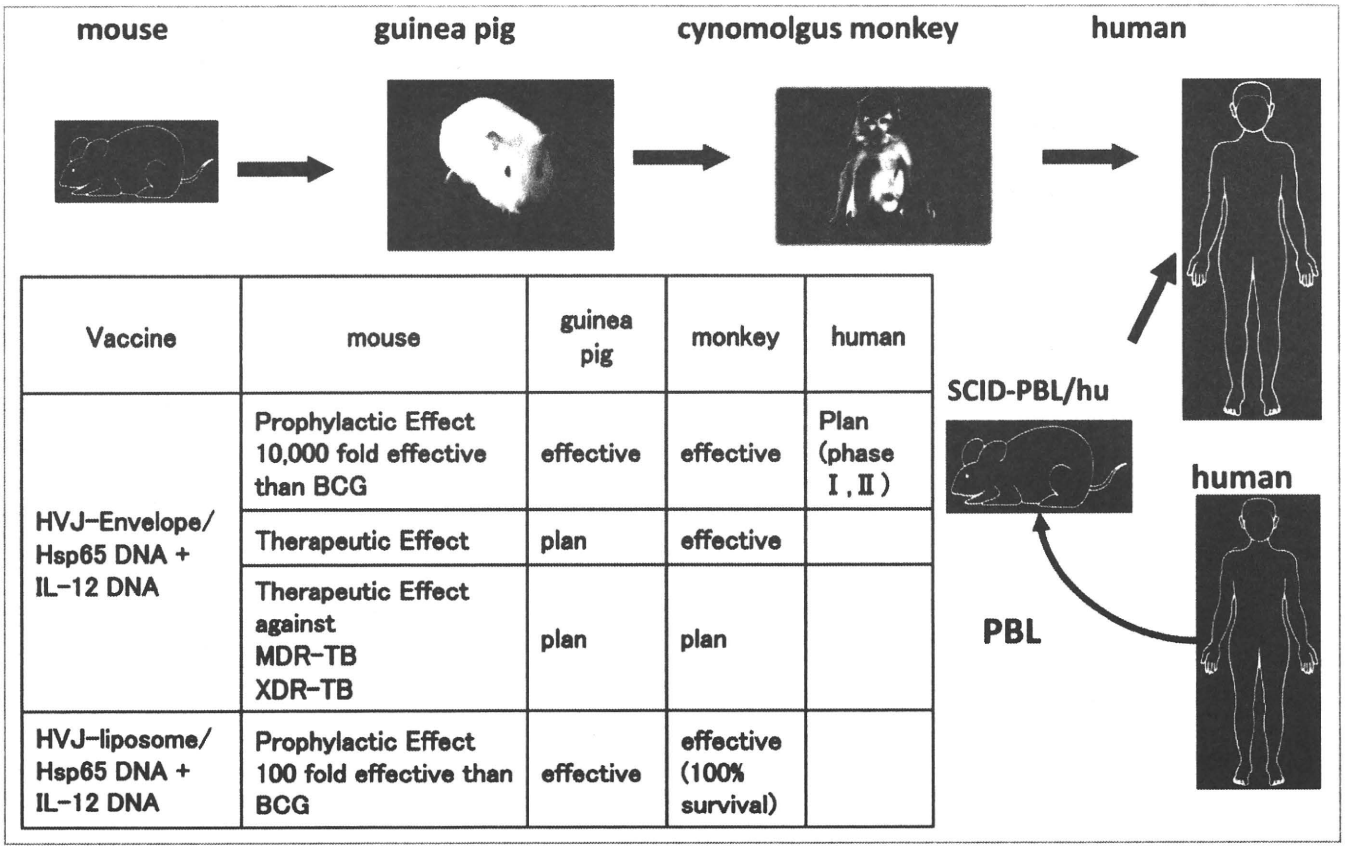
Post-infection vaccine strategy. A third option is to prevent disease by enhancing or boosting immunity in persons already infected a post-infection vaccine strategy. This approach is attractive because more than 2 billion persons worldwide are already infected and therefore at risk of progression to disease.

Therapeutic vaccine. A fourth option would be to use a vaccine as an adjunct to anti-TB treatment, to shorten therapy or reduce the risk of relapse, a therapeutic vaccine. This may be particularly relevant in situations where multi-drug resistant TB cases are common.

Vector and Adjuvant

Prophylactic and therapeutic DNA vaccines were established by using several kinds of vectors such as HVJ-liposome, HVJ-envelope, adenovirus vector, adeno-associated virus vector (AAV), lenti-virus vector, vaccinia-virus vector, poliovirus vector, BCG, attenuate Listeria[1,2,20,24,59-61] (**Table 2**).

MPL + QS21 + Squallene (AS101), or cationic liposome dimethyl dioctadecyl ammonium bromide (DDA) is promising adjuvant for TB vaccines.[62] Lipopolysaccharide (LPS), a component of bacterial cell walls, is driven by the adaptor proteins myeloid differentiation factor 88 (MyD88) and Toll-interleukin 1 receptor domain-containing adapter inducing interferon-β (TRIF), which together mediate signaling by the endotoxin receptor Toll-like receptor 4 (TLR4). Monophosphoryl lipid A (MPLA) is a low-toxicity derivative of LPS with useful immunostimulatory properties, which is nearing regulatory approval for use as a human vaccine adjuvant. Mata-Havo et al. reported that, in mice, the low toxicity of MPLA's adjuvant function is associated with a bias toward TRIF signaling which is likely caused by the active suppression, rather than passive loss, of proinflammatory activity of this LPS derivative. This finding



Vaccine	mouse	guinea pig	monkey	human
HVJ-Envelope/ Hsp65 DNA + IL-12 DNA	Prophylactic Effect 10,000 fold effective than BCG	effective	effective	Plan (phase I, II)
	Therapeutic Effect	plan	effective	
	Therapeutic Effect against MDR-TB XDR-TB	plan	plan	
HVJ-liposome/ Hsp65 DNA + IL-12 DNA	Prophylactic Effect 100 fold effective than BCG	effective	effective (100% survival)	

Figure 1. The development of novel vaccines for *M. tuberculosis* using animal models.

may have important implication for the development of future vaccine adjuvants.[63]

Animal Model

Murine models are usually used for the first screening of novel TB vaccines, because (1) genetic background of mice is homogeneous, (2) only small spaces are required for the experiments (3) we can use TB-resistant C57BL/6 mice and TB sensitive BALB/C mice.[1,2]

Guinea pig models are also used for the evaluation of efficacy of novel TB vaccines. Guinea pig is sensitive to TB and makes granulomatous TB lesions in the lungs.[19]

Cynomolgus monkey model is the best animal TB model as reported by Walsh and E.V. Tan in Leonard Wood Memorial Institute. TB infection in the cynomolgus monkeys is very similar to human TB disease[1,20,21,64] (Fig. 1). Monkeys make caseous necrosis in TB granulomas in the lungs as human make caseous necrosis.

HVJ-Envelope/HSP65 DNA + IL-12 DNA Vaccine

DNA vaccines against TB using murine models. *Prophylactic DNA vaccines.* 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.[65] 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 *M. 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.[66-70] 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 IFNγ-secreting T cells and activation of proliferative T cells and cytokines (IFNγ and IL-2) production upon stimulation with Hsp65 and antigens from *M. tuberculosis* (Fig. 2). 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.[2]

The in vivo necessity of CD8 positive T cells as well as CD4 positive T cells to exert the prophylactic efficacy of the HVJ-envelope/HSP65 DNA + IL-12 DNA vaccine was demonstrated in mice.

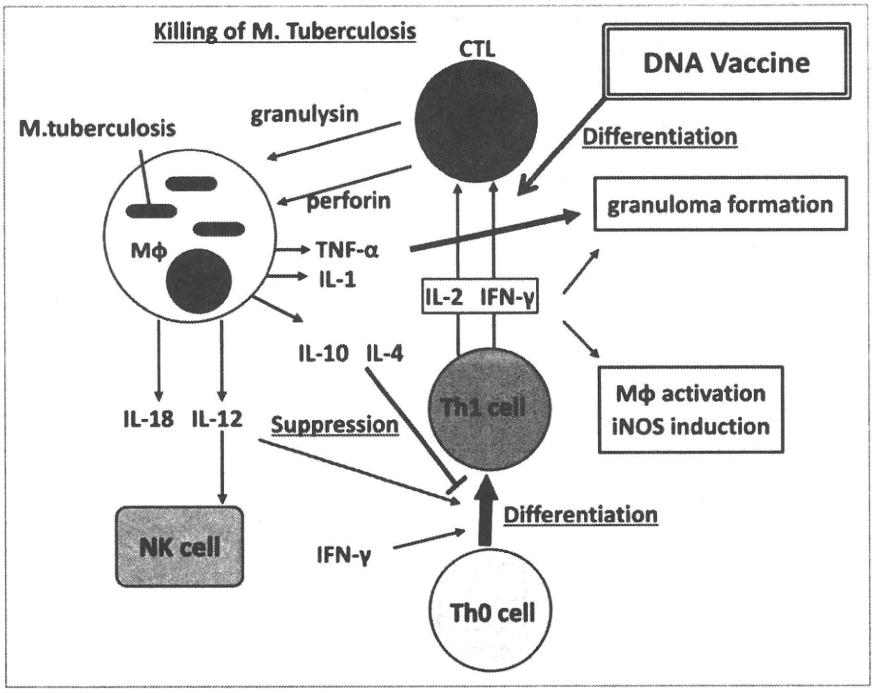


Figure 2. Induction of CTL and prophylactic effect by DNA vaccines against *Mycobacterium tuberculosis*.

Furthermore, by using BCG priming-DNA vaccine booster method, *M. tuberculosis* numbers in the lungs of DNA vaccinated mice were 10,000 (ten thousand) lower compared to BCG alone vaccinated mice (**Fig. 1**).

Therapeutic DNA vaccines. MDR-TB and XDR-TB are becoming big problems in the world. About 500,000 new patients with MDR-TB are shown every year. However, the effective drugs against MDR-TB are few.

Figure 1 shows the therapeutic efficacy of HVJ-Envelope/Hsp65DNA + IL-12DNA vaccine against XDR-TB (extremely drug resistant TB).[1] Mice treated with this DNA vaccine prolonged the survival periods significantly by statistical analysis. The vaccine exerted the therapeutic activity even against XDR-TB, which is resistant to RFP, INH, SM, EB, KM, EVM, TH, PAS, LVFX, PZA and only sensitive to CS.

This DNA vaccine exerted a significant therapeutic effect against TB, as indicated by: (1) extension of survival of mice infected with XDR-TB, (2) decrease in the CFU of TB in lungs, liver and spleen of mice infected with MDR-TB as well as drug-sensitive TB (H37Rv), (3) decrease in the CFU of TB in these organs of mice challenged with TB in the in vivo humanized immune model of SCID-PBL/hu (**Fig. 1**).

Furthermore, we have established chronic TB disease model using mouse infected with TB in the aerosol chamber (data not shown).[1] By using this model, therapeutic efficacy of this vaccine was also observed.

DNA vaccines against TB using guinea pig models. In the guinea pig model, HSP65 + gpIL-12/HVJ provided better protection against the pulmonary pathology caused by pulmonary infection with TB than BCG vaccination (data not shown) (**Fig. 1**).

DNA vaccines against TB using cynomolgus monkey models. *Prophylactic DNA vaccines.* We have developed two novel TB vaccines: HSP65 + IL-12/HVJ and a recombinant BCG harboring the 72f fusion gene (72f rBCG).[21,71] We extended our studies to a cynomolgus monkey model,[64] 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 which closely mimics human TB disease[21] (**Fig. 3**).

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.

Furthermore, the protective efficacy of the HSP65 + IL-12/HVJ and BCG using the priming-booster method in the TB infected cynomolgus monkeys was very strong. All four monkeys from the group of BCG-priming and the DNA vaccine (HVJ-liposome/HSP65 + IL-12 DNA vaccine) booster were alive more than 12 months post-infection (**Fig. 3**).[20] In contrast, only 2 monkeys out of 6 from the BCG Tokyo alone group were alive (33% survival).[20] 50% of the monkeys from the saline control group and DNA vaccine-priming and the BCG Tokyo vaccine booster group, respectively, were alive more than 12 months in the study. Furthermore, IFNγ production and proliferation of PBL from monkeys vaccinated with these vaccines were strongly enhanced. Taken together, these results clearly demonstrated that

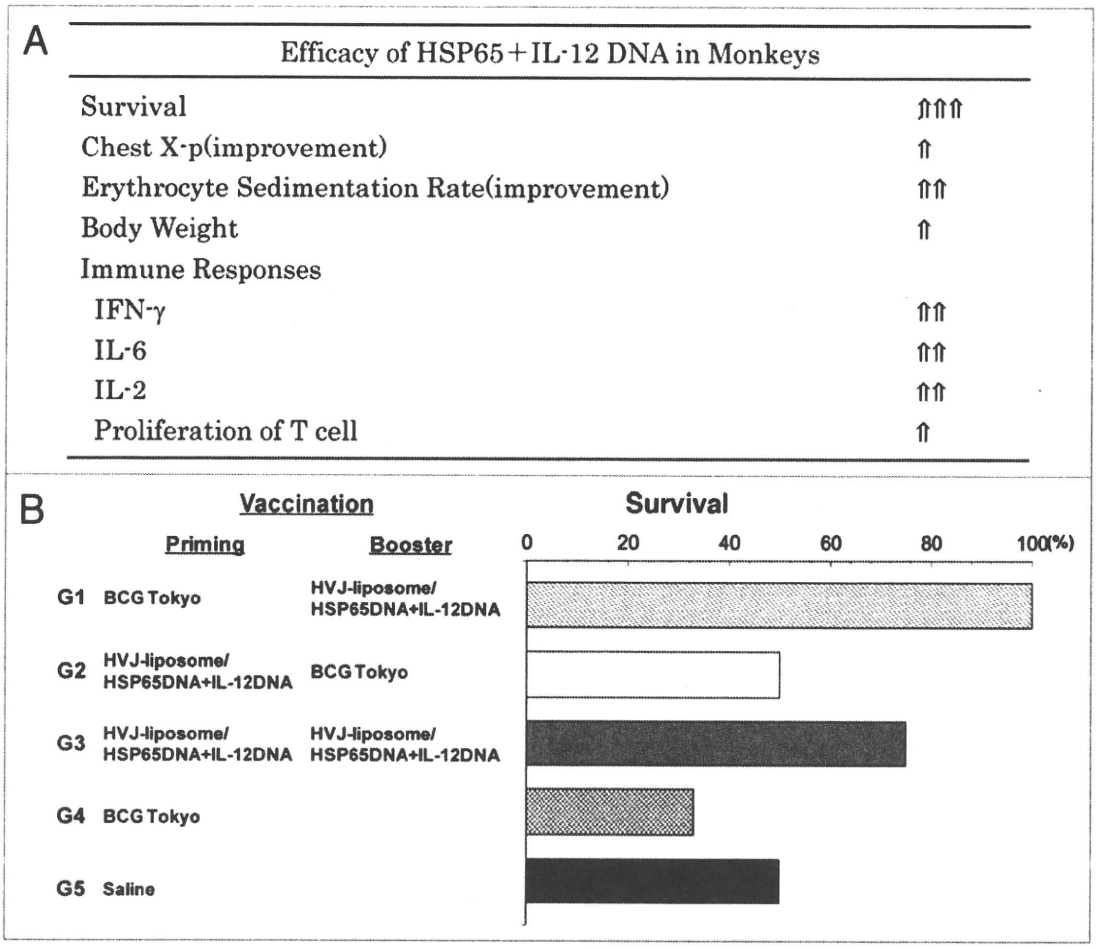


Figure 3. (A) Efficacy of HSP65 + IL-12 DNA in monkeys. (B) Protective efficacy (survival) of HSP65 + IL-12/HVJ and BCG using priming-booster method against TB challenged cynomolgus monkey 350 days after TB infection.

BCG priming and the HSP65 + hIL-12/HVJ booster could provide extremely strong protective efficacy against *M. tuberculosis* in the cynomolgus monkey model.[20]

In Japan and other countries, the BCG vaccine is inoculated into human infants (0–6 months after birth). Therefore, BCG priming in infants and HSP65 + hIL-12/HVJ boosters for adults (including junior high school students, high school students and old persons) may be required for the significant improvement of clinical protective efficacy against TB.

Our results with the HSP65 + hIL-12/HVJ vaccine in the cynomolgus monkey model should provide a significant rationale 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[64] by Reed and Skeiky.

Therapeutic DNA vaccines. Furthermore, the therapeutic activity of this vaccine was evaluated in a nonhuman primate model infected with *M. tuberculosis*.[1]

Figure 4 shows the results of immune responses of cynomolgus monkey at 11 weeks after challenge of *M. tuberculosis* Erdman strain (5×10^2) by intratracheal instillation. The proliferation of PBL in therapeutic vaccination of monkeys in the group with HVJ-Env/HSP65 DNA + IL-12 DNA was augmented (**Fig. 4B**). This vaccine also improved the survival of monkeys, compared to the saline (control) group after TB challenge (**Fig. 4C**).[1]

This vaccine exerted a significant therapeutic effect against TB, as indicated by augmentation of survival and immune responses, in a cynomolgus monkey model. It is important to evaluate the survival of monkey.[20,21]

Thus, our results with this DNA vaccine in the murine therapeutic model and cynomolgus monkey therapeutic model should provide a significant rationale for moving this vaccine into clinical trial.

Thus, we are taking advantage of the availability of multiple animal models (mouse, guinea pig and monkey) (**Fig. 1**) to accumulate essential data on the HVJ-envelope DNA vaccine in anticipation of a phase I clinical trial.

DNA vaccines against TB using SCID-PBL/hu model. Therapeutic efficacy of HVJ-Envelope/HSP65 DNA + IL-12 DNA was also observed, using in vivo humanized immune models of IL-2 receptor γ-chain disrupted NOD-SCID mice constructed with human PBL (SCID-PBL/hu).[21,24,59] This DNA vaccine resulted in significantly therapeutic activity even in SCID-PBL/hu mice which exerted human T-cell immune responses (**Fig. 1**).

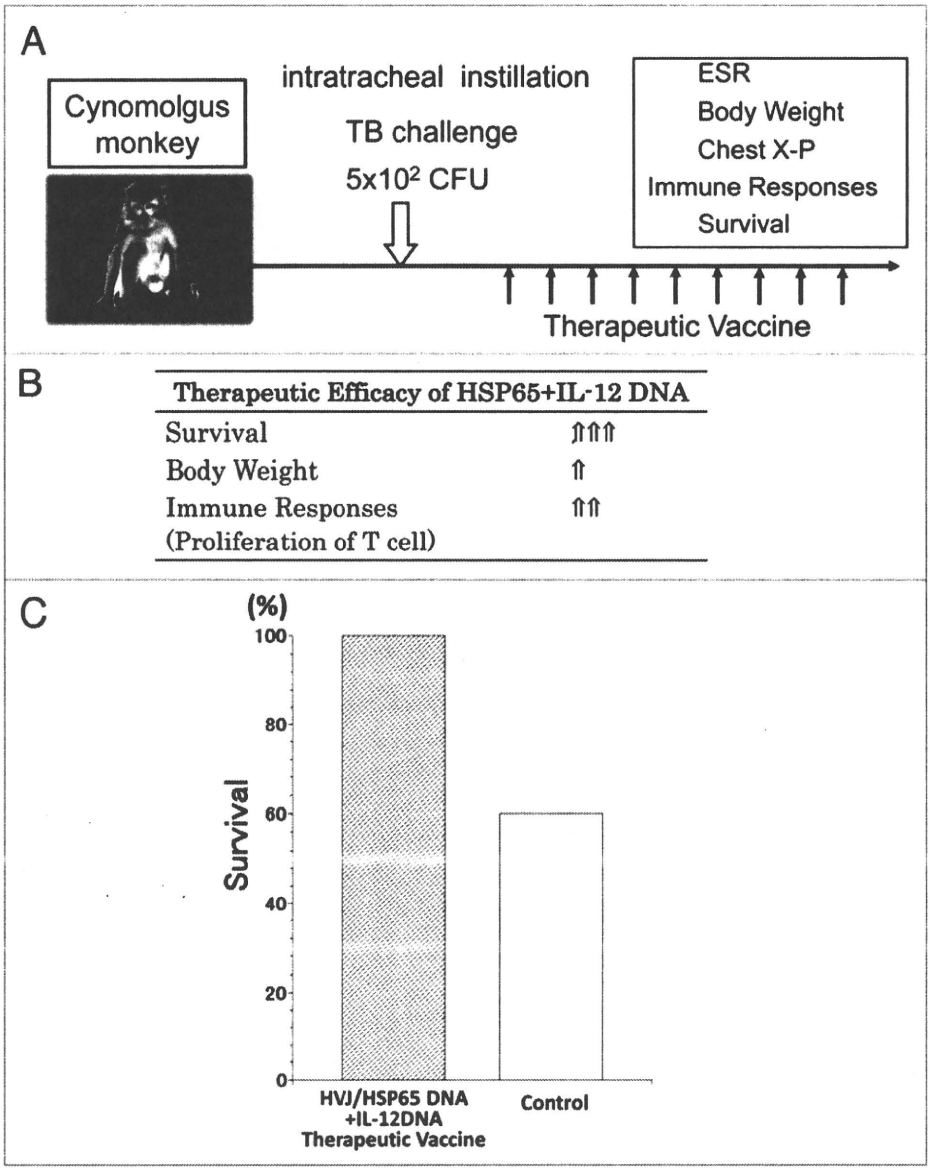


Figure 4. (A) Therapeutic effect of HVJ-Envelope/HSP65DNA + IL-12DNA vaccine on TB-infected cynomolgus monkeys. (B) Therapeutic efficacy of HSP65 + IL-12DNA. (C) Therapeutic efficacy (survival) of HSP65 + IL-12 DNA vaccine 130 days after TB infection.

CTL and Granulysin Vaccine

The increasing threat posed by drug-resistant strain of *M. tuberculosis* is to a reappraisal of the possibility of treating TB by immunotherapy (Fig. 2). Rook et al. analyze six strategies that have been shown to be therapeutic in animal models of TB and identify a common pathway underlying the activity of the superficially different immunotherapeutic protocols.[29] Six strategies are (1) DNA vaccine encoding hsp65 from *M. leprae*, (2) heat-killed *M. vaccae*, (3) fragmented, lipid-depleted *M. tuberculosis* delivered in liposomes (RUTI), (4) HE2000, (5) inhibition of IL-4, (6) inhibition of TGFβ. This pathway involves enhanced induction of $CD8^+$ CTLs and downregulation of interleukin-4 and pathways. This unifying analysis strengthens the rationale for future trials of immunotherapy in humans and points to surrogate markers that could be studied in such trials.[29]

There is increasing evidence for the importance of cytotoxic cells.[72] Their role in immunity to TB in mice has been revealed by knocking out genes involved in presentation via major histocompatibility complex (MHC) class I (e.g., transporter associated with antigen processing-1, CD8, β2m and MHC class I heavy chain), by cell-transfer experiments, and by depletion of $CD8^+$ CTLs with antibodies.[73] $CD8^+$ CTLs play a major role in the control of the latent TB.[74] N-acetyl muramyl dipeptide (MDP: N-acetylmuramyl-L-alanyl-D-isoglutamine mycobacterial peptidoglycan(PGN)) presented adjuvant activity, augmented the CTL differentiation against TB as well as cancer cells, and activated innate immunity via the host sensor NOD2.[75,76]

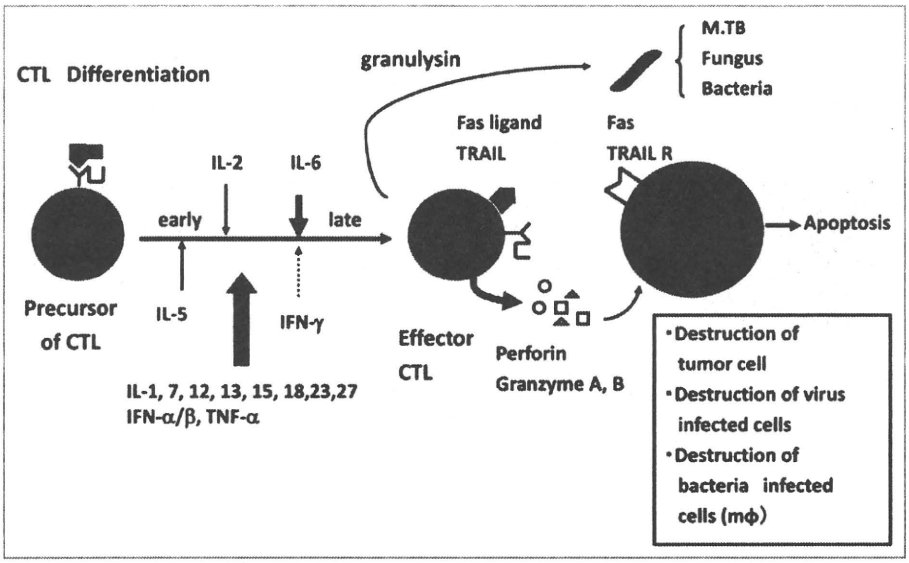


Figure 5. Induction of cytotoxic T cells and killing mechanism.

Table 3. Induction of decrease in TB number in vivo and CTL differentiation by 15K granulysin and 9K granulysin

Function					Granulysin expression in $CD8^+T$	
Two Kinds of Granulysin	**Decrease in TB number**	**Induction of CTL against TB**	**Proliferation of T cells against TB**	**IFNγ production**	**Patients with MDR-TB**	**Patients with Drag-sensitive TB**
15K Granulysin	++ (strong augmentation)	++	++	++	⇓⇓	⇓
9K Granulysin	++	+ (augmentation)	+	++	N.D	N.D

(++, strong augmentation; +, augmentation; ⇓⇓, strong suppression; ⇓, suppression

$CD8^+$ cells and other CTLs might be even more important in humans, in whom they have additional effector modalities not present in the mouse, such as granulysin, which can kill *M. tuberculosis*,[77] and lymphocyte subsets that recognize antigens presented by HLA-E or by group1 CD1 molecules (CD1a, CD1b, etc.,) in addition to MHC class 1.[73,78-81]

It has been suggested that the granulysin has the function of in vitro cytotoxic activity against *M. tuberculosis* outside the macrophage cells, and contributes the in vitro reduction of *M. tuberculosis* in the macrophage in the presence of perforin.[71] However, the precise role of granulysin in the in vivo defense for the tuberculosis infection has not been elucidated yet.

CTL play an important role for the protection against TB[1,2] (Fig. 5). The granulysin protein expression as well as IL-6, IFNγ and IL-2 activities in the culture supernatants of PBL from patients with MDR-TB and patients with Diabetes Mellitus (DM)-TB were evaluated since IL-6, IFNγ and IL-2 act as cytotoxic T-cell differentiation factor.[59,82] All these activities were very low in MDR-TB and DM-TB in comparison with healthy volunteers.

Two major protein products, 15 kDa (15K) granulysin and 9 kDa (9K) granulysin, are detected in CTL and NK cells. Granulysin exhibits potent cytotoxic activity against a broad panel of microbial targets, including bacteria, fungi and parasites. Granulysin is present in human $CD8^+$ (and some $CD4^+$) CTLs, NK cells, NK T cells and γ/δ T cells. It is a member of the saposin-like protein family, colocalizes with perforin and granzymes in the cytolytic granules of human CTL and NK cells[82-85] (**Table 3**).

We found that 15K granulysin was secreted from CD8 positive CTL, and 15K granulysin could enter human macrophages and killed *M. tuberculosis* in the cytoplasm of macrophages.[82]

Table 4. Therapeutic efficacy against tuberculosis by 15K granulysin transgenic mice and 9K granulysin transgenic mice

Tg mouse	CFU of TB (log) (*p < 0.05)
15K Granulysin Tg mouse	5.3 ± 0.1*
wild type C57BL/6 mouse	5.9 ± 0.2
9K Granulysin Tg mouse	5.8 ± 0.4*
wild type C57BL/6 mouse	6.7 ± 0.2
Secreted 9K Granulysin Tg mouse	5.7 ± 0.6*
wild type C57BL/6 mouse	6.7 ± 0.2

CFU, colony forming unit; *,significant ($p < 0.05$) by Student's test

Table 5. Preclinical and/or phase I, II clinical trial vaccines for tuberculosis

A) Priming, Pre-Exposure	
(1) Phase I: 2008	Vaccine Type
(a) **rBCG30**	**recombinant 85B BCG**
(b) **rBCG30ΔureC:Hly (VPM1002)**	**recombinant listeriolysin BCG**
(c) AERAS-407	recombinant perfringiolysin
(d) rBCG30ARMF, rBCG Mtb B30, rBCG h IFNγ	recombinant 85B BCG
(e) Nas L3/Htk BCG	nasal vaccine/heat killed whole BCG
(f) $mc^2$6220, $mc^2$6221, $mc^2$6222, $mc^2$6231	non-replicating, *M. Tuberculosis* strain (Δlys A Δpan CD)
(g) $mc^2$5059	replicating pro-apoptotic *M. bovis* BCG (ΔnuoG)
(2) Phase I 2009 or Later	
(a) HBHA (heparin-binding haemagglutinin)	methylated 21-KDA protein
(b) Attenuated Live Vaccine based on Phop	attenuated TB (virulence gene phop inactivation)
(c) paBCG (pro-apoptotic BCG)	decrease in anti-apoptotic enzyme activity

Expression of 15K granulysin protein and mRNA in CD8 positive T cells in the patients with drug sensitive TB were lower than that in the healthy volunteers.[86] Expression of 15K granulysin protein in CD8 positive T cells in the patients with multi-drug resistant tuberculosis (MDR-TB) was significantly lower than that in the patients with drug-sensitive TB (**Table 3**). 15K granulysin production stimulated with PHA-P, ConA, alloantigens and PPD antigens was suppressed significantly in the supernatants of PBL from MDR-TB patients.[82] Furthermore, we established 15K granulysin transgenic mice and 9K granulysin transgenic mice (**Table 4**). It was demonstrated first that 15K granulysin transgenic mice as well as 9K granulysin transgenic mice exerted in vivo anti-TB effect, decrease in the number of TB and in vivo induction of cytotoxic T cells against TB, proliferation of T cells against TB, and augmentation of cytokine production. As shown in **Table 4**, in vivo anti-TB effect of 15K granulysin transgenic mouse was observed. CFUs of *M. tuberculosis* in the lungs, 4 weeks after TB injection, were decreased in 15K granulysin transgenic mice, compared to wild type mice. Furthermore, CFUs of TB in the lungs of 9K granulysin transgenic mice, were also decreased compared to wild type mice (**Table 4**). These findings demonstrate for the first time an in vivo effect of 15K granulysin and 9K granulysin against TB infection. Thus, granulysin vaccine therapy might provide a weapon against MDR-TB and XDR-TB (extremely drug resistant TB).[78]

Anti-TNF therapy reduced the expression in lymphocytes of perforin and granulysin, two components of the T-cell-mediated antimicrobial response to intercellular pathogens. Specifically, *M. tuberculosis*-reactive $CD8^+CCR7\text{-}CD45RA^+$ effector memory T cell (T_{EMRA} cells) expressed the highest levels of granulysin, lysed *M. tuberculosis*, and infected macrophages and mediated an antimicrobial activity against intracellular *M. tuberculosis*. Furthermore, T_{EMRA} cells expressed cell surface TNF and bound the anti-TNF therapeutic infliximad in vitro, making them susceptible to complement-mediated lysis. Immune therapy with anti-TNF was associated with reduced numbers of $CD8^+T_{EMRA}$ cells and decreased antimicrobial activity against *M. tuberculosis*.[53,87]

Long-term memory T-cell immunity for tuberculosis vaccines. Standard BCG vaccinations (intradermal) protect infants from severe TB meningitis and milliary TB, but provide highly variable protection against pulmonary TB later in life (adult). Therefore, an important issue for new TB vaccination strategies attempting to do better than BCG is the ability to induce long-term memory immunity that is protective against primary-disease progression and reactivation of latent TB. Memory T cells express CD44, CD45RO and IL-7 receptor etc. Two subpopulations (central memory T cells and effector memory T cells) of memory T cells are identified.[88,89] Some cytokine, such as IL-7 and IL-15 enhance memory T cell responses and their induction by vaccines or their exogenous addition to vaccines might be useful in optimizing long-term memory responses.[90]

HSP65 DNA + IL-12 vaccine showed significant prophylactic efficacy on TB infection even when the interval between BCG priming and HSP65 + IL-12 DNA vaccine booster was very long in a monkey model, suggesting that this vaccine may augment the memory T cell differentiation and survival against TB.

Other Vaccines

Recombinant BCG vaccine. Recombinant BCG strains overexpressing specific mycobacterial antigens or engineered to escape from the phagosome, live, attenuated vectors expressing mycobacterial antigens have been tested. Tuberculosis is the leading cause of death in AIDS patient, yet the current tuberculosis vaccine, BCG is contraindicated for immunocompromised individuals, including human immunodeficiency virus (HIV)-positive person, although Tullius et al. reported that a replication-limited recombinant BCG (30 kDa) vaccine designed for HIV-positive person is safer and more efficacious than BCG using SCID mice[91] (**Table 5**).

Intranasal, intratracheal or oral vaccine. Several new TB-vaccine candidates have been evaluated for their protective efficacy in animal models using the mucosal route of immunization. The adjuvants and delivery systems are crucially important in such vaccines.[22,60,92,93]

Table 6. Preclinical and/or phase I, II clinical trial vaccines for tuberculosis

(B) BOOSTING, PRE-Exposure	
(1) Phase I: 2008	Vaccine Type
(a) MVA85A	**MVA virus expressingAg85A DNA**
(b) M72	**Mtb32 + Mtb29 fusion protein**
(c) AERAS-402	**Replication-incompetent adenovirus 35 vector expressing *M. Tuberculosis* antigens Ag85A, Ag85B, and TB 10.4.DNA**
(d) SSI Hybrid-1	**fusion protein (Ag85B-ESAT-6)**
(e) SSI HyVac4/AERAS-404	fusion protein (Ag85B-TB10.4)
(f) AERAS-405	Shigella-delivered recombinant double-stranded RNA nucleocapsid (Ag85A, 85B, Rv3407, latency antigen)
(g) r30	recombinant Ag85B protein
(h) Nas L3/Htk BCG	
(2) Phase I: 2009 or Later	
(a) Hsp C™ TB Vaccine	Heat shock protein antigen complexes (Hsp Cs)
(b) HBHA (heparin-binding haemagglutinin)	
(c) NasL3/AM85B conjugate	Nasal vaccine/Man capped Arabinomannan oligosaccharide
(d) PP1, PP2, PP3	BCG boosting
(e) AC_2SGL Diacylated Sulfoglycolipids	AC_2 SGL Mycobacterial lipids
(f) HVJ-liposome/Hsp65 DNA + IL-12 DNA	**HSP65 DNA from H37Rv TB HVJ-liposome vector**
(g) HVJ-envelope/HSP65 DNA+IL-12 DNA	**HSP65 DNA from H37Rv TB HVJ-envelope vector**

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Today, nearly 200 new "laboratory bench" vaccine candidates have been developed by different research groups.[94] They include live attenuated vaccines,[60] recombinant virus-restored vaccines, recombinant bacteria-vectored vaccines (including BCG vector),[60] DNA vaccines and subunit vaccines including fusion proteins (**Tables 5 and 6**).

Antigen specific $CD4^+$ and $CD8^+$ T cells were elicited by intranasal immunization of mice with a vaccinia virus-based vaccine or an adenoviral-based vaccine expressing Ag85A.

A vaccine of Act DNA-deleted Listeria monocytogenes including Ag85A, Ag85B or MPT51 DNA was used for oral vaccine which augmented intestinal mucosal immunity. This vaccine exerted prophylactic efficacy on TB infection in mice.[60]

However, so far no mucosal TB vaccine candidate has reached clinical trial.

MPT51DNA vaccine, Lipocalin2 DNA vaccine and SLPI (secretory leukocyte protease inhibitory protein) DNA vaccine. Aoshi et al. identified an HLA-A*0201-restricted $CD8^+$T cell epitope on MPT51 by using a strategy that included HLA-A*0201 transgenic mice and gene gun immunization with expression plasmid DNA encoding MPT51.[60,95] They found HLA-A*0201-restricted $CD8^+$CTL which may play a pivotal role in protection against *M. tuberculosis* infection. Takeda K et al. reported lipocalin 2 and SLPI produced from macrophages and lung alveolar epithelial cells stimulated by TLR have killing activity against TB in vitro.[96,97] Lipocalin 2 (-/-) mice and SLPI (-/-) mice were very sensitive to TB infection.[96,97] Therefore, we are now constructing novel stronger therapeutic vaccine containing MPT51 + Lipocalin 2 + SLPI + HSP65 + IL-12 DNA vaccine in the collaboration with Koide and Takeda.

Clinical Trial

MTB72f. The MTB72f vaccine is a fusion molecule consisting of two antigens that are strong targets for T helper 1 (TH1) cells in PPD-positive individuals. Rv1196 (MTB32) is inserted into the middle of the serine protease Rv0125 (MTB39), which is thus present as two fragments. MTB72F in the A502A adjuvant formulation has recently completed two Phase I trials in healthy PPD-negative adults in the USA and Belgium. The vaccine was well tolerated and safe, and could induce both antigen-specific humoral and cell-mediated immune responses[98-100] (**Table 6**).

MVA85A. MVA85A is a modified vaccine virus Ankara (MVA) strain expressing antigen 85A, another member of the Ag85 family of protective antigens. In phase I studies in humans, MVA85A was found to be safe and well tolerated, and this vaccine has induced strong immune responses, particularly in previously BCG-vaccinated individuals.[61]

Boosting BCG vaccination with MVA85A downregulates the immunoregulatory cytokine TGFβ1 (**Table 6**).[101] MVA85A induces cellular immune responses in UK volunteers.[102] The safety and immunogenicity of MVA85A in West Africa support its accelerated development as a booster vaccine for tuberculosis. T-cell responses were better maintained in BCG-naive Gambian than BCG-naive UK vaccine. $CD4^+$T cells responses were

predominantly stimulated. CD8+T-cell response were observed in subjects who were HLA B-35,[102] (**Table 6**).

AERAS-402 DNA. This DNA vaccine is intended for use as a boosting vaccine in BCG-primed individuals. The vaccine is a serotype 35 adenovirus which is incapable of replicating and contains DNA that expresses a fusion protein created from three *M. tuberculosis* antigens: 85A, 85B and TB 10.4.

HSP65 DNA + IL-12DNA. We reported the activities of a network of Asian investigators involved in the development of therapeutic vaccines for use in patients infected with MDR-TB, and described a network of hospitals within Japan which could be linked in the clinical testing of new TB vaccines[1,2,103] (**Table 6**).

Several new TB vaccines were reported[104-108] and some of them were currently in Phase I human testing. This requires a small study in healthy, PPD-negative individuals (usually adults) in the country in which the vaccine was developed. Additional Phase I trials may be conducted in PPD+ individual, children, infants, or other groups for which the vaccine may be indicated ultimately. The critical issues which impact upon the design of TB vaccine field trials have been reviewed recently.[109] The determination of safety and immunogenicity are prerequisites for any new TB vaccine to go forward into Phase III (efficacy) trials.

Phase I and II vaccine trials are relatively small and inexpensive, however, Phase III trials of new TB vaccines will be large, complicated, costly endeavors requiring international private/public partnership and a long planning process. The complexities of evaluating new TB vaccines during the product development phase have been analyzed recently.[110] McMurray discussed the role of the Aeras Global TB Vaccine Foundation in the movement of TB vaccines from the bench through clinical testing to the bedside.[23] True protective efficacy can only be measured in phase III trials. Because of the absence of accurate methods to measure infection rates, especially when BCG or related vaccine are given, the long latency of *M. tuberculosis* infection and delayed reactivation disease, efficacy trials need large sample sizes (at least 10,000) with long-term follow-up (5 years). New diagnostic tests capable of distinguishing between immunity induced by BCG vaccination and *M. tuberculosis* infection (i.e., Quantiferon test) might allow for an infection and point to be studied in future vaccine trials.[111]

Conclusions

Several kinds of vaccines against TB were developed by the progress of method for genes, immunity and animal models. Among the vaccine candidates shown in **Tables 5 and 6** (WHO STOP TB partnership 2008), the results of MVA (Modified vaccinia Ankara) Ag85A, HVJ-Envelope/HSP65DNA + IL-12DNA, Recombinant BCG (listeriolysin), Recombinant BCG (Ag85A), Mtb72f fusion protein, ESAT6/Ag85A fusion protein vaccines might provide a significant rationale in for moving these vaccines into clinical application. It will furthermore be a high priority for the clinical development programs to evaluate the current vaccines for post-exposure vaccine which prevents reactivation of TB in the large proportion of the global population latently infected with TB.

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A Novel Therapeutic and Prophylactic Vaccine (HVJ-Envelope / Hsp65 DNA + IL-12 DNA) against Tuberculosis Using the Cynomolgus Monkey Model

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Abstract

We have developed a novel tuberculosis (TB) vaccine; a combination of the DNA vaccines expressing mycobacterial heat shock protein 65 (HSP65) and interleukin 12 (IL-12) delivered by the hemagglutinating virus of Japan (HVJ)-envelope and –liposome (HSP65 + IL-12/HVJ). An IL-12 expression vector (IL-12DNA) encoding single-chain IL-12 proteins comprised of p40 and p35 subunits were constructed. This vaccine provided remarkable protective efficacy in mouse and guinea pig models compared to the BCG vaccine on the basis of C.F.U of number of TB, survival, an induction of the CD8 positive CTL activity and improvement of the histopathological tuberculosis lesions. This vaccine also provided therapeutic efficacy against multi-drug resistant TB (MDR-TB) and extremely drug resistant TB (XDR-TB) (prolongation of survival time and the decrease in the number of TB in the lung) in murine models. Furthermore, we extended our studies to a cynomolgus monkey model, which is currently the best animal model of human tuberculosis. This novel vaccine provided a higher level of the protective efficacy than BCG based upon the assessment of mortality, the ESR, body weight, chest X-ray findings and immune responses. All monkeys in the control group (saline) died within 8 months, while 50% of monkeys in the HSP65+hIL-12/HVJ group survived more than 14 months post-infection (the termination period of the experiment). Furthermore, the BCG priming and HSP65 + IL-12/HVJ vaccine (booster) by the priming-booster method showed a synergistic effect in the TB-infected cynomolgus monkey (100% survival). In contrast, 33% of monkeys from BCG Tokyo alone group were alive (33% survival). Furthermore, this vaccine exerted therapeutic efficacy (100% survival) and augmentation of immune responses in the TB-infected monkeys. These data indicate that our novel DNA vaccine might be useful against *Mycobacterium tuberculosis* including XDR-TB and MDR-TB for human therapeutic clinical trials.

Keywords HSP65□IL-12DNA vaccine□ Tuberculosis□Monkey□Therapeutic vaccine

1. 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

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on the basis of the elimination of *M. tuberculosis* mediated by the induction of CTL [1,2]. Futhermore 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 [1,4]. In the present study, 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]. Moreover, we evaluated therapeutic effect of this vaccine on the MDR-TB infection and XDR- TB infection in murine and monkey models, indicating that the vaccine exerts therapeutic efficacy against TB, MDR-TB and XDR-TB.

2. Method 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]. CTL activity was assessed by $^{51}C_r$-release assay[1,7]. At 5 and 10 weeks 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 [1]. Therapeutic efficacy was also evaluated by chronic TB infection model of mice using aerosol challenge of TB (15CFU/mouse: Madison aerosol exposure chamber, University of Wisconsin).

3. Method 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. The animals were vaccinated nine times with the HVJ-envelope with expression plasmid of both HSP65 and human IL-12 (HSP65 + hIL-12/HVJ: 400ug i.m.), one week after the challenge with the *M.tuberculosis* Erdman strain (5×10^2) by intratracheal instillation. Immune responses and survival were examined as described in our previous studies [2,5].

4. Results and Discussion

(a) Prophylactic efficacy

All 4monkeys in the control group (saline) died within 8 months, while 50% (2monkeys out of 4) of monkeys in the HSP65+hIL-12/HVJ group survived more than 14 months post-infection (the termination period of the experiment)(data not shown).

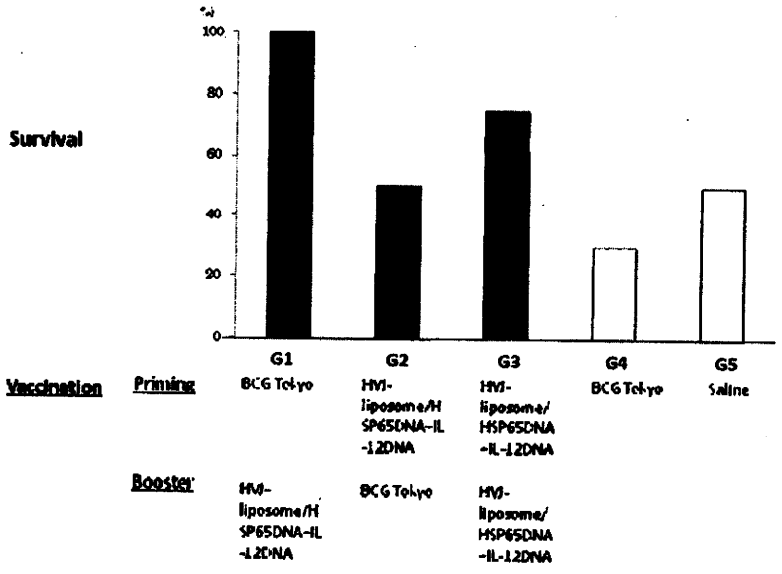

Fig 1. Protective efficacy (survival) of HSP65+IL-12/HVJ and BCG using priming- booster method against TB challenged cynomolgus monkey 350 days after TB using cynomolgus monkey models.

Table 1. Efficacy of HSP65 + IL-12 DNA Vaccine in Monkey

Efficacy of HSP65 + IL-12 DNA in Monkeys	
Survival	. . .
Chest X-p (improvement)	.
Erythrocyte Sedimentation Rate (improvement)	. .
Body Weight	.
Immune Responses	
IFN-γ	. .
IL-6	. .
IL-2	. .
Proliferation of T cell	.

Furthermore, using 32 monkeys, the protective efficacy of the HSP65+IL-12 /HVJ and BCG using the priming-booster method in the TB infected cynomolgus monkeys was very strong. All four monkeys from the group of BCG-priming and the DNA vaccine (HVJ-liposome/HSP65+IL-12 DNA vaccine) booster were alive more than 12 months post-infection (Fig.1). In contrast, only 2 monkeys out of 6 from the BCG Tokyo group were alive (33% survival). 50% of the monkeys from the saline control group and DNA vaccine-priming and the BCG Tokyo vaccine booster group, respectively, were alive more than 12 months in the study. In addition, HSP65+hIL-12/HVJ improved both ESR and chest X-ray findings. IL-2 and IL-6 production were augmented in the group vaccinated with BCG vaccine-priming and the DNA vaccine-booster (Table1). Furthermore, proliferation of PBL was strongly enhanced. Taken together, these results clearly demonstrate that BCG priming and the HSP65+hIL-12/HVJ booster could provide extremely strong protective efficacy against *M.tuberculosis* in the cynomolgus monkey model.

(b)Therapeutic efficacy

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 (Table 2A).

At 5 and 10 weeks 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.

HVJ-Envelope/HSP65 DNA +IL-12 DNA vaccine treatment significantly reduced the bacterial loads of MDR-TB as compared to saline control group($P<0.05$) (Table2).

Therapeutic efficacy of HVJ-Envelope/HSP65 DNA + IL-12 DNA was also observed, using in vivo humanized immune models of IL-2 receptor γ-chain disrupted NOD-SCID mice constructed with human PBL (SCID-PBL/hu)[8,9]. Therapeutic vaccination with HVJ-Envelope/HSP65 DNA+IL-12 DNA group resulted in significantly therapeutic activity even in SCID-PBL/hu mice which exerted human T cell immune responses(Table 2A).

Table 2A. The Development of Novel Vaccines for M.tuberculosis using animal model

Vaccine	mouse	guinea pig	monkey	SCID-PBL/hu	human
HVJ-Envelope/ Hsp65 DNA + IL-12 DNA	prophylactic Effect 10,000 fold effective than BCG	effective	effective	effective	Plan (phase. , .)
	Therapeutic Effect	plan	effective		
	Therapeutic Effect against MDR-TB XDR-TB	plan	plan		
HVJ-liposome/ Hsp65 DNA + IL-12 DNA	Prophylactic Effect 100 fold effective than BCG	effective	effective (100% survival)		

Vaccine	mouse	guinea pig	monkey	SCID-PBL/hu	human
HVJ-Envelope/ Hsp65 DNA + IL-12 DNA + Ag85B DNA + Ag85A DNA	plan	plan	Therapeutic Effect	plan	
15Kgranulysin recombinant 15K granulysin	Therapeutic Effect		plan		
15K granulysin DNA	Therapeutic Effect		plan		

Furthermore, the therapeutic activity of this vaccine was evaluated in a nonhuman primate model infected with *M.tuberculosis*.

Immune responses of cynomolgus monkey at 11 weeks after challenge of *M.tuberculosis* Erdman strain by intratracheal instillation were augmented. The proliferation of PBL in therapeutic vaccination of monkeys in the group with HVJ-Envelope/HSP65 DNA +IL-12 DNA was augmented (data not shown). This vaccine also improved the survival of monkeys, compared to the saline (control) group, after TB challenge(Fig.2). All five monkeys from the group of HVJ-Envelope/HSP65DNA+IL-12DNA vaccine were alive (100% survival). In contrast, 3 monkeys out of 5 from the saline control group were alive (60% survival). These results demonstrate that HVJ-Envelope/HSP65DNA+ IL-12DNA vaccine could provide strong therapeutic efficacy against TB, MDR-TB or XDR-TB in the cynomolgus monkey models as well as murine models

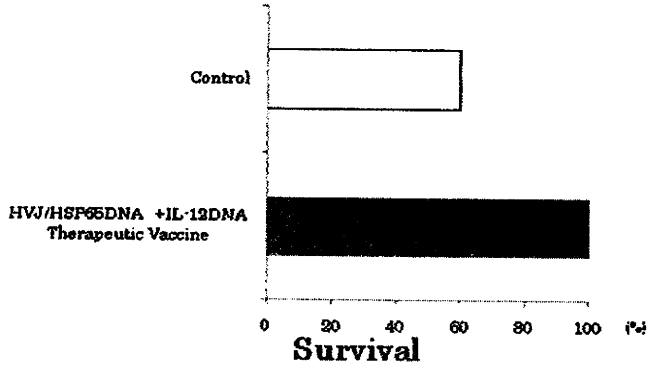

Fig 2. Therapeutic efficacy (survival) of HVJ-Envelope/HSP65DNA+IL-12DNA vaccine 130 days after TB infection using cynomolgus monkey models.

(c) Discussion

The HSP65+hIL-12/HVJ vaccine exerted a significant prophylactic effect against TB, as indicated by: 1) extension of survival for over a year; 2) improvement of ESR and chest X-ray findings; 3) increase in the body weight; 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 is usually accompanied by a progression of the disease. [10]

DNA vaccine are a relatively new approach to immunization for infectious diseases.[1,2,5,11-14]

Prophylactic and therapeutic DNA vaccines were established by using several kinds of vectors such as (1) HVJ-liposome, (2) HVJ-envelope, (3) adenovirus vector, (4) adeno-associated virus vector (AAV), (5) lenti-virus vector.[1,2,9]

We have developed a hemagglutinating virus of Japan envelope (HVJ-Envelope) using inactivated Sendai virus, as a nonviral vector for drug delivery.[15-17] It can deliver very efficiently DNA, siRNA, proteins and anti-cancer drugs into cells both in vitro and in vivo[15,18,19]. Therefore, HVJ-Envelope was used as an efficient and safe vector for DNA vaccine against TB in the present study.

In the guinea pig model, HSP65+gpIL-12/HVJ provided better protection against the pulmonary pathology caused by pulmonary infection with TB than BCG vaccination (data not shown). In the present study, it was demonstrated that BCG vaccine priming and HSP65+h IL-12/HVJ booster could provide extremely strong (100% survival) efficacy against *M.tuberculosis* compared to BCG alone (33% survival) in the cynomologus monkey model. In Japan and other countries, the BCG vaccine is inoculated into human infants (0~6months after birth). Therefore, BCG priming in infants and HSP65+h IL-12/HVJ boosters for adults (including junior high school students, high school students and old persons) may be required for the significant improvement of clinical protective efficacy against TB.

Furthermore, the HSP65+hIL-12/HVJ vaccine exerted a significant therapeutic effect against TB, as indicated by: (1) extension of survival of mice infected with XDR-TB, (2) decrease in the CFU of TB in lungs, liver and spleen of mice infected with MDR-TB as well as drug-sensitive TB(H37RV), (3) decrease in the CFU of TB in these organs of mice challenged with TB in the in vivo humanized immune model of SCID-PBL/hu, (4) augmentation of immune responses, in a cynomologus monkey model which closely mimics human TB disease. It is important to evaluate the survival of monkey [7,8]. Increases in the survival rate of the monkeys treated with this vaccine were observed, compared to the control monkeys treated with saline. Increase in the survival rate of the monkeys treated with HVJ-Envelope/HSP65DNA+IL-12DNA+Ag85B DNA+Ag85A DNA was also strongly observed in the therapeutic models of monkeys(Table 2B). In the recent study, it is demonstrated that granulysin vaccine shows therapeutic efficacy against TB in mice(Table 2B). Therefore, the combination of these therapeutic vaccines might be useful in the future.

MDR-TB and XDR-TB are becoming big problems in the world. About 500,000 new patients with MDR-TB are shown every year. However, the effective drugs against MDR-TB are few.

The HVJ-Envelope/HSP65DNA+IL-12DNA vaccine exerted the therapeutic activity even against XDR-TB, which is resistant to RFP, INH, SM, EB, KM, EVM, TH, PAS, LVFX, PZA and only sensitive to CS. Thus, our results with the HVJ-Envelope/HSP65 DNA+IL-12 DNA vaccine in the murine therapeutic model and cynomolgus monkey therapeutic model should provide a significant rationale for moving this vaccine into clinical trial. Furthermore, we have established chronic TB disease model using mouse infected with TB in the aerosol chamber (data not shown). By using this model, therapeutic efficacy of this vaccine was also observed.

Thus, we are taking advantage of the availability of multiple animal models to accumulate essential data on the HVJ-envelope DNA vaccine in anticipation of a phase I clinical trial.

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ミニ特集「免疫と結核」

キラーT細胞・granulysinによる結核免疫とワクチン（HSP65＋IL-12 DNAワクチン等）開発

岡田 全司　喜多 洋子

要旨：1998年，米国CDCおよびACETは新世代の結核ワクチン開発の必要性を発表した。しかしながら，BCGワクチンに代わる結核ワクチンは欧米でも臨床応用には至っていない。われわれはBCGワクチンをはるかに凌駕する10,000倍強力な結核予防ワクチン効果を示す新しいDNAワクチン（HVJ-エンベロープ/Hsp65＋IL-12 DNAワクチン）やリコンビナントBCGワクチンを開発した。このワクチンはマウスで長期にわたり，結核菌由来のHSP65蛋白抗原および結核菌抗原に対して特異的なCD8陽性キラーT細胞の分化を増強した。一方，BCGワクチンはキラーT細胞の分化をほとんど誘導しなかった。さらに，結核治療ワクチン効果も示した。多剤耐性結核のみならず超薬剤耐性結核に対しても治療効果（延命効果・結核菌数減少）を示した。さらに，ヒト結核感染モデルに最も近いカニクイザル（Nature Med. 1996）を用い，HSP65 DNA＋IL-12 DNAワクチンの強力な有効性を得た。カニクイザルにワクチン接種後ヒト結核菌を経気道投与し，1年以上経過観察した。リンパ球増殖反応・サイトカイン（IFN-γ，IL-2等）産生の増強および胸部X線所見・血沈，体重の改善効果が認められた。さらに生存率改善・延命効果も認められた。プライム-ブースター法を用い，このDNAワクチン投与群は100%の生存率を示した。一方，BCG投与群は33%の生存率であった。さらに，サルの系で世界に先駆けて結核治療ワクチン効果を得た。このDNAワクチン治療群では100%の生存を示したが，生食投与群では60%の生存率であった。一方，キラーT細胞から産生される結核菌殺傷タンパクgranulysinは結核治療ワクチン効果を発揮した。さらにgranulysin transgenic miceは結核菌殺傷効果を発揮した。これらについての概要を述べる。

キーワーズ：キラーT細胞，グラニュライシン，新規結核ワクチン

Ⅰ．はじめに

1998年，米国CDCは結核に対し，政府・学術機関・企業が一体となって新世代の結核ワクチン開発の必要性を強く主張する発表をした。また，ACETは国民の健康に対する大敵である結核撲滅のためには，BCGに代わる有効なワクチンが必要であることを示した。しかしながら，BCGに代わる結核ワクチンは欧米でも臨床応用には至っていない[1)~4)]。われわれはBCGよりもはるかに強力なDNAワクチンやリコンビナントBCGワクチンの開発に成功した（Fig. 1）[5)~8)]。したがって，新しい抗結核ワクチン開発と結核感染免疫におけるキラーT細胞およびgranulysin（キラーT細胞より産生される結核菌殺傷タンパク）の機能解明についても述べる。

Ⅱ．キラーT細胞と結核

CD8あるいはβ_2ミクログロブリン遺伝子やTAP遺伝子ノックアウトマウスでは抗結核免疫が十分でなく，動物は死亡する。すなわち，結核におけるCD8$^+$T細胞はマウスで抗結核免疫に重要である（Fig. 2）[1)3)4)8)~13)]。

キラーT細胞の一つの役割としてIFN-γを分泌して抗結核免疫に寄与するが，次に述べる結核感染Mφを殺して，結核菌の増殖の場をなくし結核菌を殺す役割のほうが重要である。CD8$^+$T細胞が結核菌で感染したMφ

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