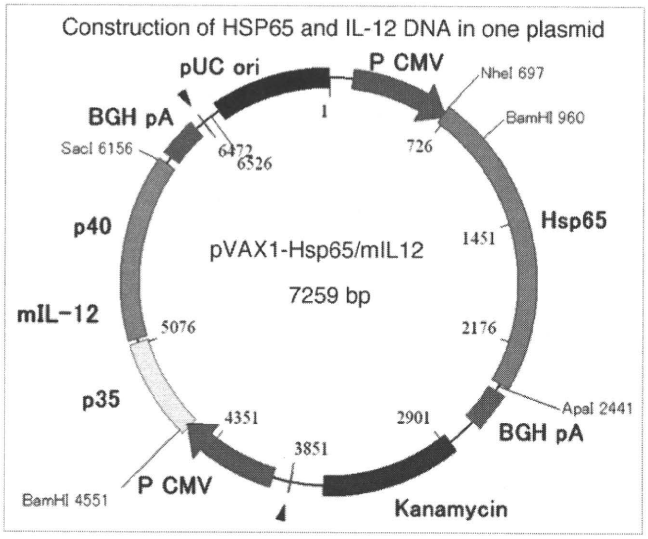

Figure 1. Construction of DNA vaccine for clinical trial. HVJ-Envelope/HSP65DNA + IL-12DNA vaccine was constructed for GMP-level-vaccine which contains two kinds of DNA in one plasmid vector (pVAX1) for clinical prophylactic trial.

vaccine was investigated using monkey models. In vivo humanized immune models of IL-2 receptor γ-chain disrupted NOD-SCID mice constructed with human PBL (SCID-PBL/hu) provide a useful tool for investigating human immune responses activated by vaccine.[4,5] Transgenic mice which contain the components of vaccine also provide a lot of information about novel TB vaccines. Therefore, using cynomolgus monkey model, SCID-PBL/hu mice model and transgenic mice model, we have developed several kinds of novel vaccines against TB.

Granulysin, a member of the saposin-like protein family, colocalizes with perforin and granzymes in the cytolytic granules of human CTL and NK cells, has cytotoxic activity against intracellular pathogens in infected cells in the presence of perforin and has a cytotoxic effect against tumor cells.[6-8] The granulysin is expressed in human CD8 positive cytotoxic T cells and NK cells. 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.[7] However, the precise role of granulysin in the in vivo defense for the tuberculosis infection has not been elucidated yet. Therefore, we have established 15 K and 9 K granulysin transgenic mice to elucidate in vivo role of granulysin and to develop novel vaccines against the infection of *M. tuberculosis*. These 15 K granulysin transgenic mice and 9 K granulysin transgenic mice showed in vivo anti TB effect. This is the first demonstration of an in vivo action of granulysin for TB using granulysin transgenic mice. We have also developed novel TB vaccine of HVJ-Envelope/HSP65 DNA + IL-12 DNA.[3,9,10] Therefore, these findings suggest that granulysin or granulysin DNA may be useful as a TB vaccine, in the combination of other DNA vaccine.

Results

Cynomolgus monkey model. The prophylactic efficacy of HVJ-Envelope/Hsp65 DNA + IL-12 DNA vaccine against TB was

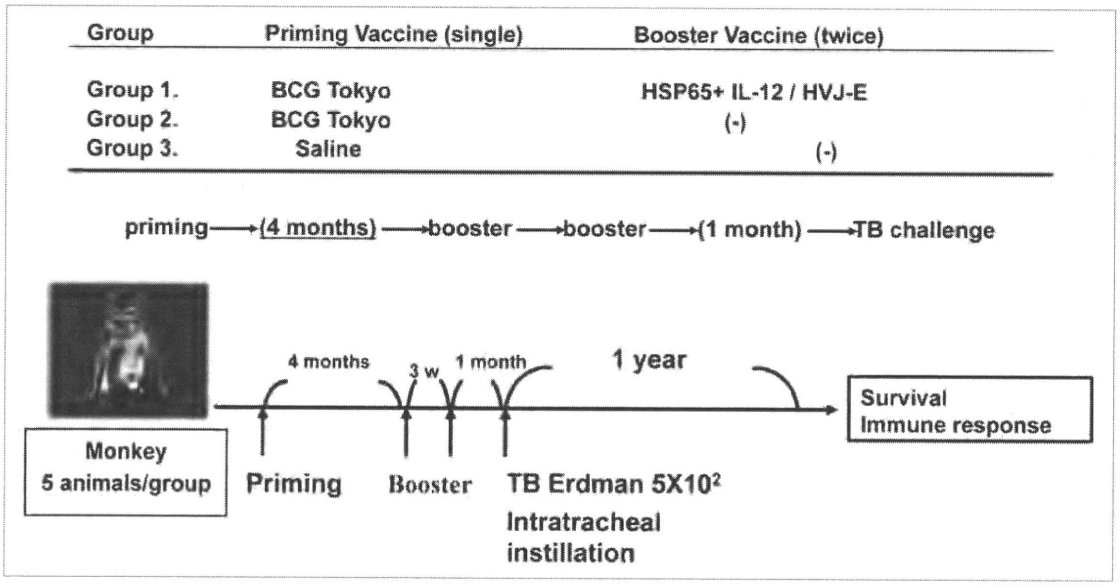

Figure 2. Evaluation of prophylactic efficacy of HVJ-Envelop/HSP65DNA + IL-12DNA vaccine on the infection of cynomolgus monkeys. Protective efficacy of HSP65 + IL-12/HVJ and BCG using prime-boost method against TB challenged cynomolgus monkeys. Group of animals were vaccinated three times (every 3 weeks) with (1°) BCG Tokyo, (2°) HSP65 + IL-12/HVJ, (3°) HSP65 + IL-12/HVJ = G_1. BCG prime-HVJ/DNA boost group; (1°) BCG, (2°) saline, (3°) saline = G_2. G_2 group animals were vaccinated with BCG once; (1°) saline, (2°) saline, (3°) saline = G_3. 4 month after the prime BCG vaccine, 2° vaccine was immunized. 3 weeks after the 2° vaccine, 3° vaccine was treated. One month after the third vaccination, monkeys were challenged with the *M. tuberculosis* (5 x 10^2 CFU) by intratracheal instillation. Prophylactic efficacy was evaluated by survival periods, erythrocyte sedimentation rate (ESR), body weight, chest X-rays, immune responses and DTH reaction against PPD for 16 months.

investigated using GMP-level-vaccine which contains two kinds of DNA in one plasmid vector for clinical trial (**Fig. 1**).

Long-term interval model (4 months) between prime and boost vaccinations was used in this study prior to intratracheal instillation of the challenge dose (**Fig. 2**).

In Group1 (BCG prime—DNA vaccine boost) monkeys, the regimen of vaccines improved ESR, compared to the regimen of Group3 (saline control group) or that of Group2 (BCG alone control group) (**Fig. 3**).

This vaccination method (BCG prime—DNA vaccine boost) also increased the body weight of 4 TB-infected monkeys out of 5 in Group1 as shown in **Figure 4**. In contrast, 2 monkeys in Group3 (saline) or 2 monkeys in Group2 (BCG alone) showed the decrease in body weight after the infection of TB.

The proliferation of PBL from monkeys in the base-line period was almost same among these G_1, G_2 and G_3 groups. However, proliferation of PBL from monkeys in G_1 group, (BCG prime-DNA vaccine boost group), was higher than those in G_2 (BCG alone group) and G_3 (saline control group) at 4 weeks after third vaccinations (G_1–G_3: $p < 0.05$) (**Fig. 5**).

Furthermore, IFNγ production from PBL in G_1 group (BCG prime-DNA vaccine boost group) was higher than those in G_2 (BCG alone group) and G_3 (saline control group) (data not shown).

By using long-term prime-boost method and vector containing two kinds of genes in one plasmid, the most reproducible and prophylactic efficacy based on the prolongation of survival was observed in Group1 monkeys (BCG prime-DNA boost, **Fig. 6**). The combination of BCG prime and DNA vaccine boost improved the survival (100% at 230 days and 80% at 360 days after TB challenge, respectively). In contrast, BCG vaccine alone in G_2 group monkeys showed 60% survival at 355 days and 40% survival at 360 days. The treatment of saline (G_3) showed 50% survival at 360 days.

Thus, even using the experimental model of long-term interval (4 months interval) between prime period and boost period, we could observe the prophylactic efficacy of this BCG prime-HVJ-Envelope/Hsp65 DNA + IL-12 DNA vaccine in monkeys.

Transgenic mice model. The granulysin expression in the $CD3^+CD8^+CD4^-$ PBL-T cells of the patients with drug sensitive TB and MDR-TB was significantly lower than that of normal volunteer (data not shown).

We also analyzed the 15 K granulysin in the culture supernatants of PBL from patients with MDR-TB and healthy volunteer. The amounts of 15 K granulysin were measured after the stimulation with PPD, Hsp65 protein and killed TB H37Ra antigen. The production of 15 K granulysin was suppressed in the culture supernatants of PBL from patients with MDR-TB, compared to that from healthy volunteer (data not shown). Thus, it was suggested that granulysin treatment might improve the tuberculosis disease in human.

Therefore, to elucidate the in vivo mechanism of granulysin, we have established granulysin transgenic mice. We established eleven distinct transgenic mice including 15 K granulysin transgenic mice and 9 K granulysin transgenic mice. We confirmed the expression of mRNAs and proteins of 15 K granulysin and

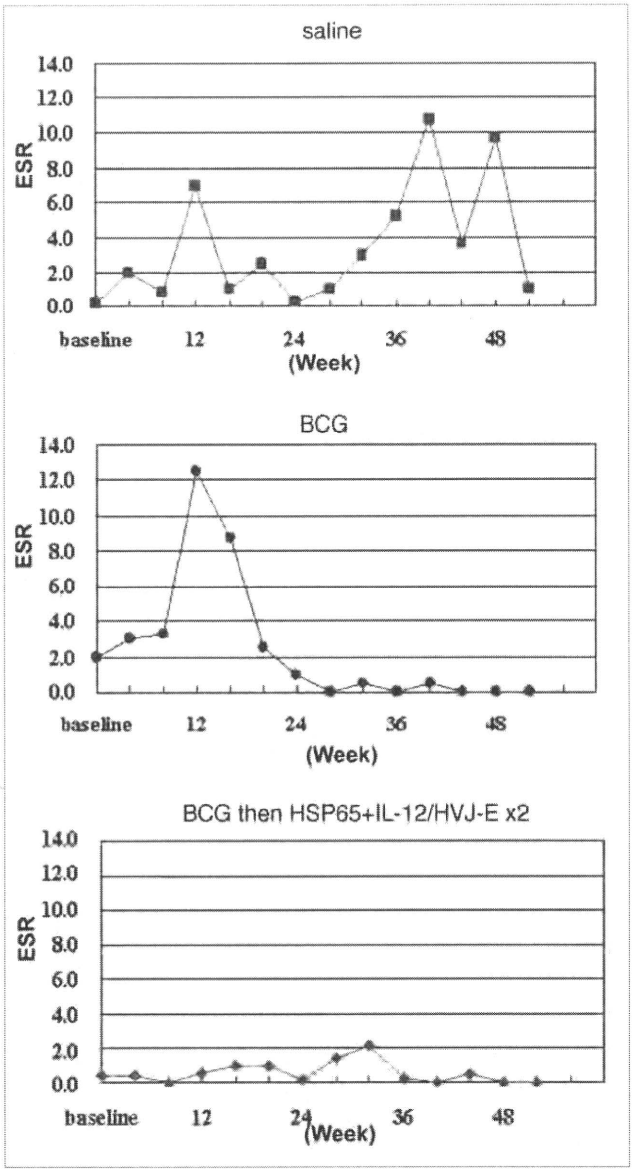

Figure 3. Improvement of erythrocyte sedimentation rate (ESR) in the cynomolgus monkeys immunized with BCG prime-HVJ-envelope/HSP65DNA + IL-12DNA boost vaccine. Cynomolgus monkeys were immunized and challenged as described in Figure 2. ESR of all monkeys was evaluated every month and mean values of ESR of 5 monkeys were shown.

9 K granulysin in established transgenic mice, respectively (data not shown). 15 K granulysin transgenic mice as well as 9 K granulysin transgenic mice exerted in vivo anti-TB effects, in vivo induction of cytotoxic T cells specific for TB, proliferation of T cells after the stimulation with TB antigens and augmentation of cytokine production.

As shown in **Figure 7**, in vivo anti-TB efficacy of 15 K granulysin transgenic mouse was observed.

CFU of *M. tuberculosis* was decreased at 4 weeks after the intravenous injection of 5×10^5 TB in the lungs of 15 K granulysin transgenic mice compared to those of wild type mice and

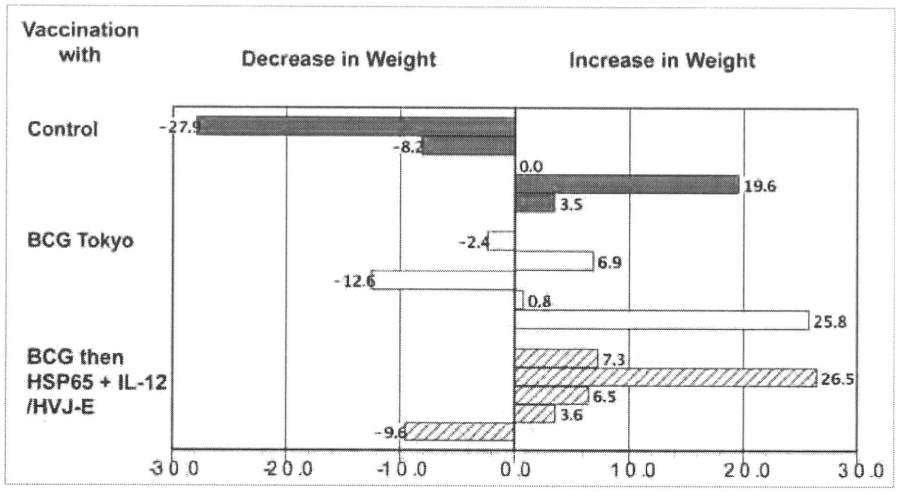

Figure 4. The increase in the body weight of monkeys vaccinated with HVJ-Envelope/Hsp65 DNA + IL-12 DNA. Monkeys vaccinated with BCG prime-HVJ-Envelope/Hsp65 DNA + IL-12 DNA vaccine boost were challenged as described in Figure 2. Body weight of all monkeys was evaluated every month and values of body weight of monkeys at 16 weeks after TB challenge were shown.

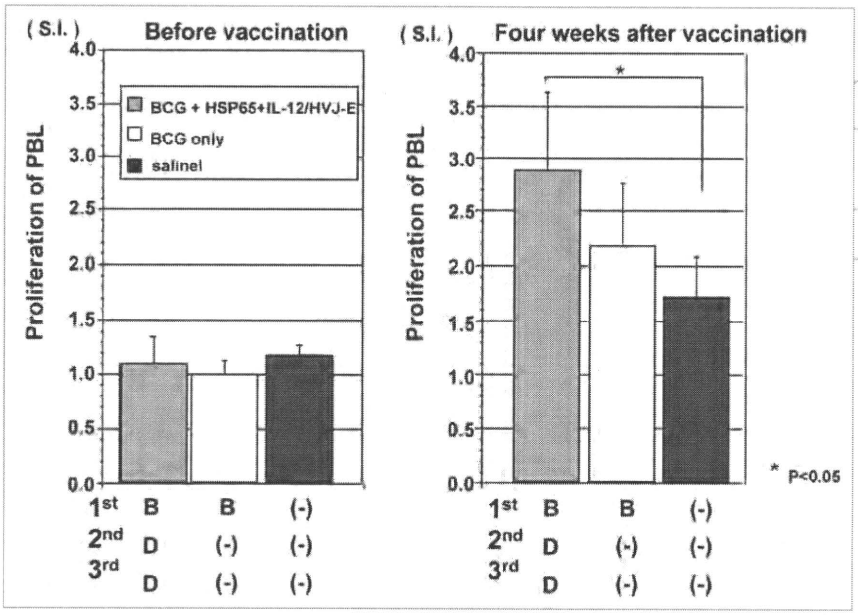

Figure 5. Augmentation of the proliferation of PBL in the monkeys immunized with BCG prime—HVJ-Envelope/Hsp65 DNA + IL-12 DNA boost vaccine. The proliferation of PBL (base line: before vaccination) from monkeys and PBL from monkeys vaccinated and challenged as described in Figure 2 were shown. Stimulation Index (S.I) of the ^{3}H-TdR uptake of monkey PBL at 11 weeks after TB challenge were shown. Student's t test were used to compare T cell proliferation between groups (p-value: G_1–G_3 $p < 0.05$).

normal C57BL/6 mice ($p < 0.05$). Furthermore, CFU of TB in the lungs of 9 K granulysin transgenic #1 mice and 9 K granulysin transgenic #17 mice were also decreased at 4 weeks after TB injection compared to that of wild type mice ($p < 0.05$) (**Fig. 8**). Thus, 15 K granulysin transgenic mice as well as 9 K granulysin transgenic mice exerted in vivo anti-TB efficacy and decreased the number of TB in the lungs.

SCID-PBL/hu model. We have very important and interesting SCID-PBL/hu models capable of analyzing in vivo human T cell immune responses and evaluating the efficacy of novel vaccines against TB, as reported first in Cancer Research 1997.

We used IL-2 receptor γ-chain gene knock out SCID-PBL/hu mice to analyze human immune responses.

Now, the therapeutic effects of HSP65 + IL-12 DNA vaccine in G_3 group (50 μg i.m.) on TB infection is observed in this IL-2 receptor γ-chain gene disrupted SCID-PBL/hu-model ($p < 0.05$) (**Table 1**). Human CTL activity against TB was associated with the efficacy of TB vaccine (data not shown).

Taken together, using cynomolgus monkey model, SCID-PBL/hu mice model and granulysin transgenic mice model, we have developed two kinds of novel vaccines, HVJ-Envelope/Hsp65 DNA + IL-12 DNA vaccine and granulysin vaccine, against TB.

Discussion

In the present study, using cynomolgus monkey model, SCID-PBL/hu mice model and granulysin transgenic mice model, we have developed two kinds of novel vaccines, HVJ-Envelope/Hsp65 DNA + IL-12 DNA vaccine and granulysin vaccine, against TB.

Most importantly, we demonstrated that even when the boost was performed after long-term period (4 month) from prime, the prolongation of monkey survival was observed by the combination of BCG and this HVJ-Envelope/Hsp65 DNA + IL-12 DNA vaccine in the present study. This combination improved the erythrocyte sedimentation rate (ESR), increased the body weight and augmented the proliferation of PBL and IFNγ production, more than BCG alone or saline.

In the mouse system, by using BCG prime-HVJ-Envelope/Hsp65 DNA + IL-12 DNA vaccine boost method, the number of *M. Tuberculosis* in the lungs of DNA-vaccinated mice were 10,000 (ten thousand) times lower compared to BCG alone vaccinated mice in our study (data not shown).

In parallel with the protective effect of HVJ-Envelope/DNA vaccines + BCG vaccine using prime-boost method on bacterial loads, histopathological analysis shows that mice vaccinated with

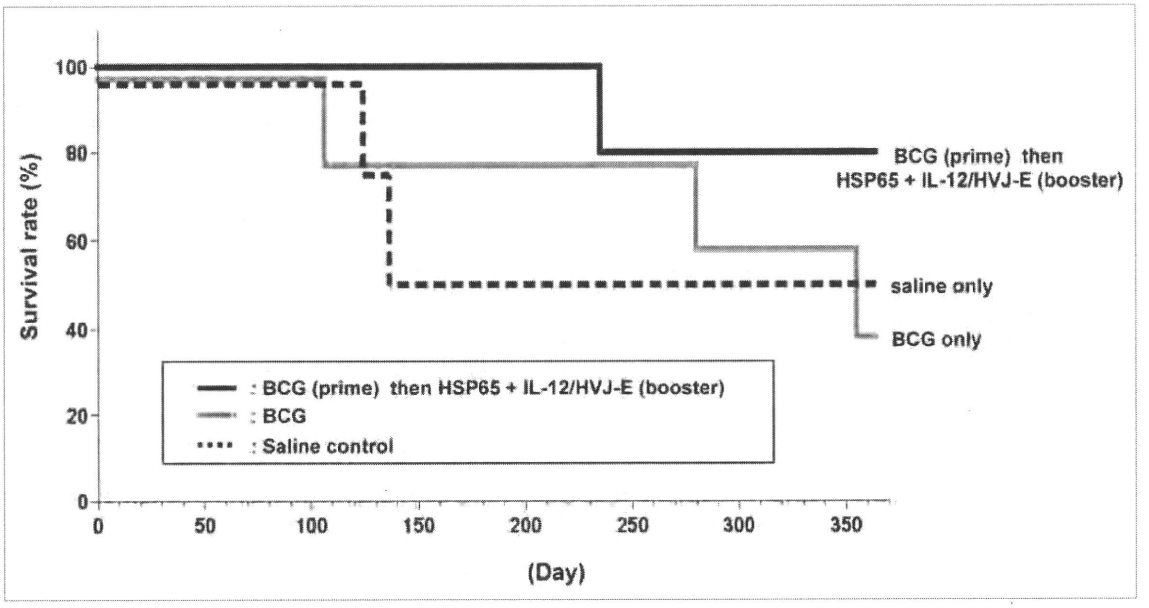

Figure 6. Protective efficacy of Hsp65 + IL-12/HVJ and BCG using prime—boost method against TB challenged cynomolgus monkeys. Group of animals were vaccinated three times as described in Figure 2. Group of animals were vaccinated with (1°) BCG Tokyo, (2°) Hsp65 + IL-12/HVJ, (3°) Hsp65 + IL-12/HVJ = G_1(—). BCG prime-HVJ/DNA boost group; (1°) BCG, (2°) saline, (3°) = G_2(—). G_2 group animals were vaccinated with BCG once; (1°) saline, (2°) saline, (3°) saline = G_3(----). One month after the third vaccination, monkeys were challenged with the *M. tuberculosis*.

this BCG prime-HVJ-Envelope/HSP65 DNA + IL-12 DNA boost had fewer and smaller lesions in the lungs and significantly less lung granuloma than the naïve mice and mice vaccinated with BCG alone (data not shown).

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.[1,2] Vaccination with BCG prime-HSP65 + IL-12/HVJ boost provided better protective efficacy as assessed by the Erythrocyte Sedimentation Rate, chest X-ray findings and immune responses than BCG alone. Importantly, HSP65 + IL-12/HVJ resulted in an increased survival for over a year. This was the first report of successful DNA vaccination against *M. Tuberculosis* in the monkey model which closely mimics human TB disease.[3]

Furthermore, the protective efficacy of the HSP65 + IL-12/HVJ and BCG using the prime-boost method in the TB-infected cynomolgus monkeys was very strong. All four monkeys from the group of BCG-prime and the DNA vaccine (HVJ-liposome/HSP65 + IL-12 DNA vaccine) boost were alive more than 12 months post-infection.[3] In contrast, only 2 monkeys out of 6 from the BCG Tokyo alone group were alive (33% survival).

Prime-boost method was reported in the study of MVA85A vaccine, which is a modified vaccine virus Ankara (MVA) strain expressing antigen 85A. In phase I studies in humans, this vaccine has induced high immune responses in previously BCG-vaccinated individuals.[14] Boosting of BCG vaccination with MVA85A downregulates the immunoregulatory cytokine TGFβ.[15] Aeras-402 DNA (DNA that expressed 85A, 85B and TB10.4) vaccine using adenovirus vector is intended for use as a boosting vaccine in BCG-primed individuals.[16] Several vaccines use a prime-boost strategy to enhance the immune responses.[17]

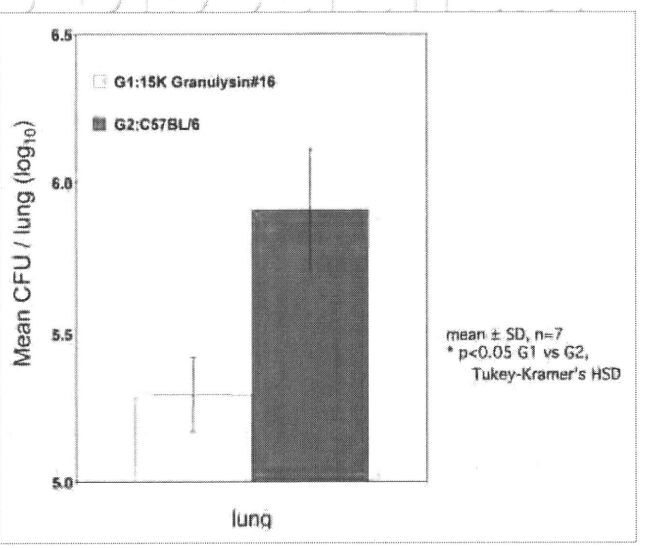

Figure 7. In vivo inhibition of the growth of *M. tuberculosis* in the 15 K granulysin transgenic mice. (In vivo anti-TB effect of 15 K granulysin transgenic mouse). Seven 15 K granulysin #16 transgenic mice and seven wild type C57BL/6 mice were injected with 5 x 10^5 H37RV *M. tuberculosis* i.v. 4 weeks after the challenge of *M. tuberculosis*, mice were sacrificed. CFU of *M. tuberculosis* in the lungs of these mice were assessed described in Material and Methods. CFU of 15 K granulysin #16 transgenic mice (□). CFU of control wild C57BL/6 mice (■). Student's t-test was used ($p < 0.05$).

In Japan and other countries, the BCG vaccine is inoculated into human infants (0–6 months after birth). Therefore, BCG prime in infants and HSP65 + hIL-12/HVJ boosts for adults

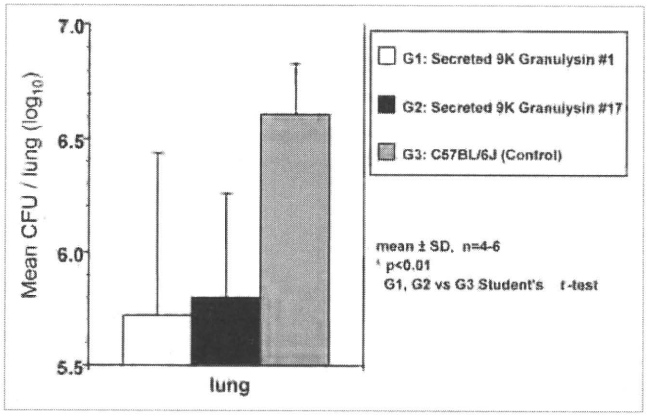

Figure 8. In vivo inhibition of the growth of *M. tuberculosis* in the 9 K granulysin transgenic mice. (In vivo anti-TB effect of 9 K granulysin transgenic mouse). Five 9 K granulysin #1 transgenic mice, five 9 K granulysin #7 mice and five wild type C57BL/6 mice were injected with 5 x 10^5 H37RV *M. tuberculosis* i.v. and 4 weeks after the challenge of *M. tuberculosis*, mice were sacrificed. CFU of *M. tuberculosis* in the lungs of these mice were assessed described in Material and Methods. CFU of 9 K granulysin #1 transgenic mice G_1 (□). CFU of 9 K granulysin #17 transgenic mice G_2 (■). CFU of control wild type C57BL/6 mice G_3 (■). Student's t-test was used to compare the CFU of each group(G_1–G_3: $p < 0.01$ G_2–G_3: $p < 0.01$).

Table 1. Efficacy of HVJ-Envelope/HSP65 DNA + IL-12 DNA vaccine against tuberculosis infection using IL-2 Receptor (-/-) NOD-SCID mice (SCID-PBL/hu)

Group	Treated	CFU of TB (log)
G1	(-)	6.03 ± 0.06
G2	HSP65DNA + IL-12 DNA vaccine (10 μg)	5.96 ± 0.15
G3	HSP65DNA + IL-12 DNA Vaccine (50 μg)	5.40 ± 0.97

Therapeutic efficacy of HVJ-envelope/HSP65DNA + IL-12DNA, using in vivo humanized immune models of IL-2 receptor γ-chain disrupted NOD-SCID mice (SCID-PBL/hu). Groups of animals were treated with 3 times with HVJ-envelope/HSP65DNA + IL-12DNA (50 ug i.m. or 10 ug i.m.). 10 days after the third vaccination, mice were sacrificed and CFU of TB in the liver of mice were assessed as described in Materials and Methods. 1 x 10^7 PBL from a healthy human volunteer were injected i.p. into IL-2 receptor γ-chain disrupted NOD-SCID mice. 21 days after injection of PBL, mice were challenged with 5 x 10^5 H37Rv i.v. and then treated with vaccine. G1, (-) control; G2, treated with HVJ-envelope/HSP-65DNA + IL-12DNA 10 μg; G3, treated with HVJ-envelope/HSP65DNA + IL-12DNA 50 μg. Student's t-test was used to compare the CFU of TB of each group (G1–G3: $p < 0.05$).

(including junior high school students, high school students and old persons) may be required for the significant improvement of clinical protective efficacy against TB.

In the present study, using very long-term period (4 month interval between prime and boost), protective efficacy of the combination of vaccines was evaluated. In human, long-term interval (5~15 years) between prime vaccine and boost vaccine might be used in the clinical application of a novel TB prophylactic vaccine.

Thus, our results with the HVJ-Envelope/HSP65 DNA + IL-12 DNA vaccine in the murine prophylactic model and cynomolgus monkey prophylactic model should provide a significant rationale for moving this vaccine into clinical trial.

On the other hand, we established transgenic mice and a vaccine expressing granulysin. The granulysin expression in the $CD3^+CD8^+$ PBL-T cells of the patients with drug sensitive TB was significantly lower than that of normal volunteer (data not shown). The granulysin expression in $CD3^+CD8^+$ T cells from MDR-TB patients was lower than that in $CD8^+$ T cells from drug sensitive TB patients.

The production of 15 K granulysin was also suppressed in the culture supernatants of PBL from 10 patients with MDR-TB, compared to that from healthy volunteer (data not shown). Thus, these data suggest that granulysin vaccine treatment provides a useful tool to regulate the human TB infection disease.

Two major protein products, 15 K granulysin and 9 K 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.

We found that 15 K granulysin was secreted from CD8 positive CTL, and it could enter human macrophages and kill *M. tuberculosis* in the cytoplasm (data not shown). Therefore, we established 15 K granulysin transgenic mice and 9 K granulysin transgenic mice.

It was demonstrated that 15 K granulysin transgenic mice as well as 9 K granulysin transgenic mice exerted in vivo anti-TB effect and decrease in the number of TB. Thus, granulysin DNA vaccine therapy and recombinant granulysin therapy might provide a weapon against MDR-TB and XDR-TB.

In conclusion, we have the advantage of the availability of multiple animal models to accumulate essential data on the HVJ-Envelope DNA vaccine and granulysin vaccine in anticipation of a phase I clinical trial.

Materials and Methods

Methods for the evaluation of the prophylactic efficacy of the vaccine on the TB infection of the monkeys. Cynomolgus monkeys were housed in a BSL 3 animal facility of the Leonard Wood Memorial Research Center. All animal experiments were approved by the Leonard Wood Memorial Animal Care and Use Committee and the National Hospital Organization Kinki-chuo Chest Medical Center Animal Care and Use Committee. The animals were vaccinated three times with the HVJ-envelope with expression plasmid of both HSP65 and human IL-12 (HSP65 + hIL-12/HVJ: 400 ug i.m.), and then challenged with the *M. tuberculosis* Erdman strain (5 x 10^2) by intratracheal instillation. Survival, immune responses (proliferation of PBL and cytokines production), body weight, ESR, PPD skin test and chest X-P findings were examined as described in our previous studies.[3,9,10]

Methods for the establishment of granulysin transgenic mouse. 15 K granulysin gene, 9 K granulysin gene or secreted 9 K granulysin DNA (15 K granulysin secretory signal DNA was fused into N terminal of 9 K granulysin DNA) were transferred to expressing plasmid DNA (pCAGGS) having CAG promoter. DNA fragment was injected to pronuclei embryo and grafted to 200 foster parents. 2 types of 15 K granulysin Tg mice (#3, #16), 3 types of 9 K granulysin Tg mice (#15,

#17, #18) and 6 types of secreted 9 K granulysin Tg mice(#1, #3, #11, #14, #17, #25) were made. Granulysin activity was assessed by monoclonal antibody targeting 15 K granulysin and 9 K granulysin. *Mycobacterium tuberculosis* H37Rv 5 x 10^5 CFU was intravenously injected to 15 K granulysin Tg mice, 9 K granulysin Tg mice, wild type (control) mice and normal C57BL/6 mice (8~12 weeks).[3,10] From 2 to 12 weeks after injection, these mice were sacrificed. The lungs, the liver and the spleen of these mice were removed, homogenized and cultivated for 14 days on 7H11 agar medium. Then, the number of colony of *Mycobacterium tuberculosis* was measured.[3,10] Mice were maintained in isolator cages, manipulated in laminar flow hoods and used between 8–10 weeks of age. All animal experiments were approved by the National Hospital Organization Kinki-chuo Chest Medical Center Animal Care and Use Committee. All vaccinations and experiments on isolate tissue of animal were done under anaesthetic state with sevoflurane. Infected animals were housed in individual micro-isolator cages in a Biosafety Level (BSL) 3 animal facility of the NHO Kinki-chuo Chest Medical Center.

CTL activity in the spleen cells of mice were assessed using ^{51}Cr release assay.[11-13]

Methods for the establishment of SCID-PBL/hu model. IL-2 receptor γ-chain disrupted NOD-SCID-PBL/hu was constructed as described in our previous study.[4,5] CTL activity was assessed using the method as described previously in reference 12 and 13.

Statistical analysis. Student's t tests were used to compare log 10 value of CFU between groups following challenge of TB. Student's t test were also performed to compare immune responses between groups in T cell proliferation assay. A p-value of <0.05 was considered significant.

Acknowledgements

This study was supported by a Health and Labour Science Research Grant from MHLW (H11-shinko-2, H14-shinko-1, H17-shinko-5, H20-Shinko-14), grants from Osaka Tuberculosis research foundation and Grant-in-Aid for Scientific Research (B) from the Ministry of Education, Culture, Sports, Science and Technology Japan.

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Human Vaccines 7:1 Supplement, 1-2; January 2011; © 2011 Landes Bioscience

Novel therapeutic vaccine

Granulysin and new DNA vaccine against tuberculosis

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

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

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

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

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

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

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Submitted: xx/xx/xx; Accepted: xx/xx/xx
DOI:

Introduction

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

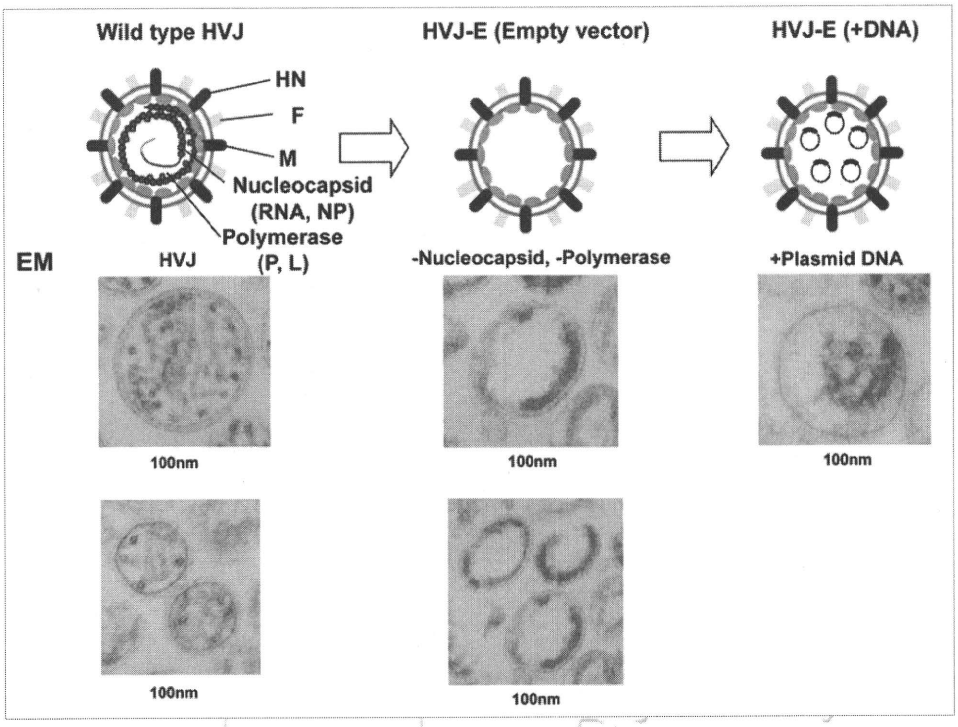


Figure 1. HVJ-envelope vaccination. pcDNA3-1/HSP65DNA+IL-12DNA were incorporated into HVJ-Envelope Empty Vector (Non-Viral Vector). Cartoons of HVJ-Envelope Empty Vector in the presence or absence of DNA were shown. Photographs of an electronic microscope (EM) of HVJ-Envelope Empty Vector were also shown.

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

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

DNA vaccines encoding *M. tuberculosis* HSP65 and human IL-12 were encapsulated into HVJ-Envelope or HVJ-liposomes.[6] HVJ-liposomes and HVJ-Envelope were prepared as described previously in reference 7–11 (**Fig. 1**). The HVJ-Envelope complex was aliquoted and stored at -70°C until use. Groups of mice were vaccinated three times with 100 μl of HVJ-Envelope solution containing 50 μg of pcDNA-IgHsp65 and 50 μg of pcDNA-mIL12p40p35-F in the tibia both anterior muscles. Mice were vaccinated with 1 x 10^6 CFU *M. bovis* BCG Tokyo by subcutaneous injection at 4 different sites (left upper, right upper, left lower, right lower back). HVJ-Envelope DNA vaccines encapsulating combination of pcDNA-IgHsp65 and pcDNA-mIL12p40p35-F was designated as IgHsp65+mIL-12/HVJ in this text. CTL activity was assessed by $^{51}C_r$-release assay.[1,12] At 30 days after intravenous challenge of *M. tuberculosis* H37RV, the number of CFU in the lungs, spleen and liver were counted and therapeutic efficacy of HVJ-Envelope DNA vaccines was evaluated.[3,13] TNFR gene disrupted DBA/1 mice and IL-6 gene disrupted DBA/1 mice were treated with HVJ-Envelope/HSP65 DNA + IL-12 DNA vaccine three times i.m at 1, 8 and 15 days after the challenge of 5 x 10^5 CFU MDR-TB i.v. (**Fig. 2**). Therapeutic efficacy was

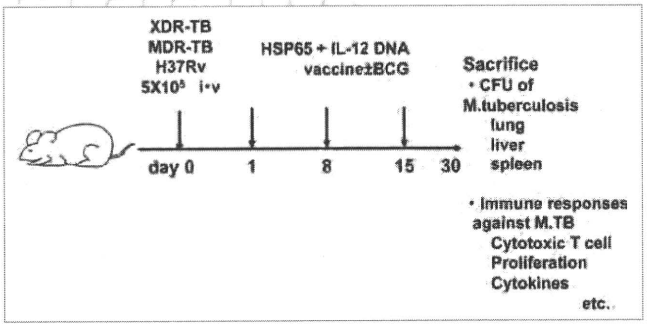


Figure 2. TNFR gene disrupted DBA/1 mice and IL-6 gene disrupted DBA/1 mice were treated with HVJ-Envelope/HSP65 DNA+IL-12 DNA vaccine three times i.m at 1, 8 and 15 days after the challenge of 5 x 10^5 CFU MDR-TB i.v. At 30 days after MDR-TB challenge, the lungs, spleens and livers were aseptically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

also evaluated by chronic TB infection model of mice using aerosol challenge of TB (15 CFU/mouse: Madison aerosol exposure chamber, University of Wisconsin). Mice were maintained in isolator cages, manipulated in laminar flow hoods and used between 8–10 weeks of age. All animal experiments were approved by the National Hospital Organization Kinki-chuo Chest Medical Center Animal Care and Use Committee. All vaccinations and

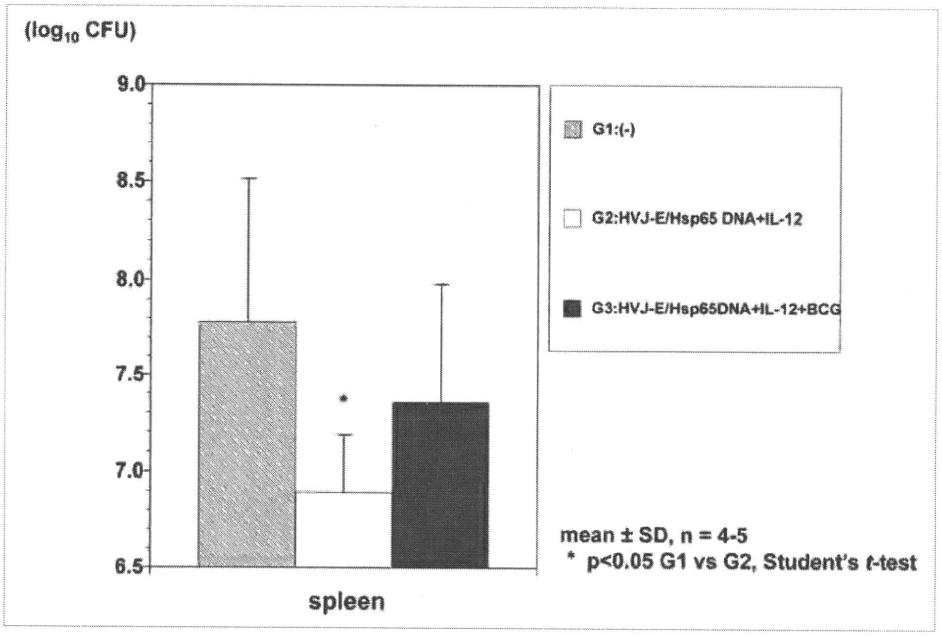


Figure 3. Therapeutic efficacy of HVJ-Envelope/HSP65 DNA+IL-12 DNA vaccine on the MDR-TB infection in the TNFR gene disrupted DBA/1 mice. Groups of mice were challenged by intravenous injection with 5 x 10^5 CFU MDR-TB, and then treated three times with HVJ-Envelope/HSP65 DNA+IL-12 DNA vaccine, as described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

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

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

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

Results

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

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

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

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

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

Table 1. The development of Novel vaccines for *M. tuberculosis* using animal model

Vaccine	Mouse	Guinea pig	Monkey	SCID-PBL/hu	Human
	Prophylactic Effect 10,000 fold than BCG	effective	effective		plan (phase I, II)
HVJ-Envelope/HSP65 DNA+IL-12 DNA	Therapeutic Effect	plan	effective	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)		
recombinant 15 K granulysin	Therapeutic Effect		plan		
15 K granulysin DNA	Therapeutic Effect		plan		

HVJ-Envelope/HSP65 DNA+IL-12 DNA vaccine was evaluated by using mouse, guinea pig, monkey and SCID-PBL/hu model. Therapeutic efficacy as well as prophylactic efficacy was shown in this vaccine. HVJ-liposome/HSP65 DNA+IL-12 DNA vaccine and granulysin vaccine were also evaluated by using these models.

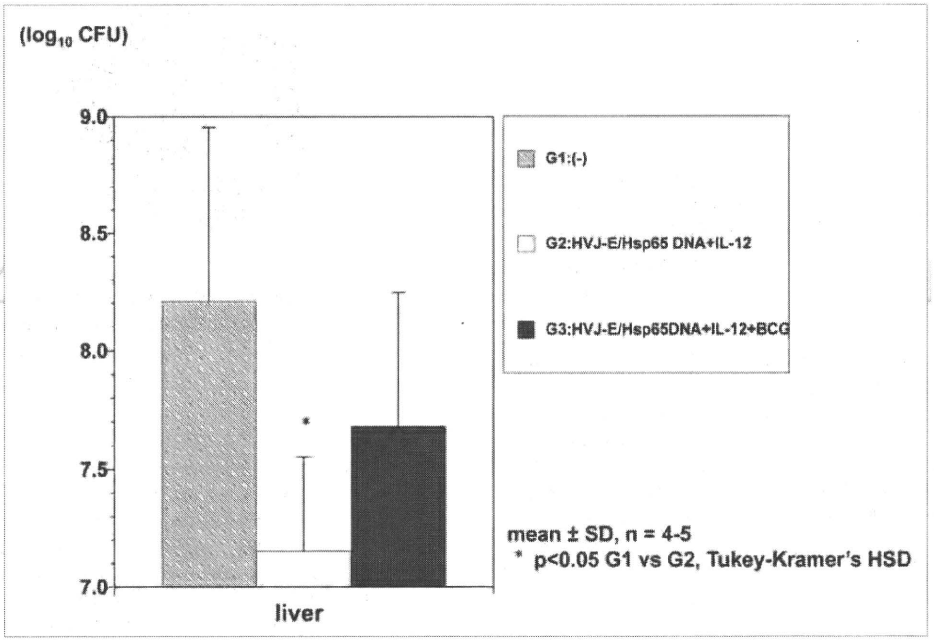


Figure 4. (A) Therapeutic efficacy of TB vaccines using chronic TB infection model by aerosol challenge of TB. Therapeutic efficacy was evaluated by chronic TB infection model of mice using aerosol challenge of TB. Mice were challenged intratracheally by aerosol of *M. tuberculosis* H37Rv in saline (15 CFU/mouse) using Madison aerosol exposure chamber. Five weeks after the challenge of TB, mice were treated with HVJ-Envelope/HSP65 DNA+IL-12 DNA six times, every 3 days i.m. Eight weeks after the challenge of TB, therapeutic efficacy was measured by enumerating bacterial loads (CFU) in the lungs, liver and spleen from vaccinated mice. (B) Therapeutic efficacy of HVJ-Envelope/HSP65 DNA+IL-12 DNA vaccine on chronic TB infection model of mice using aerosol chamber. DBA/1 mice were challenged intratracheally by aerosol of *M. tuberculosis* H37Rv (15 CFU/mouse). After the treatment of HVJ-Envelope/HSP65 DNA+IL-12 DNA vaccine 6 times i.m., therapeutic efficacy was measured by enumerating bacterial loads (CFU) in the lungs from vaccinated mice.

significantly therapeutic efficacy even in SCID-PBL/hu mice which exerted human T cell immune responses (**Table 1**).

Therapeutic efficacy of granulysin vaccine on TB infected mice. Two major protein products, 15 kDa (15 K) granulysin and 9 kDa (9 K) 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. We found that 15 K granulysin was secreted from CD8 positive CTL, could enter into human macrophages and killed *M. tuberculosis* in the cytoplasm of macrophages (**Fig. 5**). Recombinant 15 K granulysin protein enhanced the in vitro induction of human cytotoxic T cells in the 5 day MLC culture (**Fig. 6**). Synergistic effect of recombinant 15 K granulysin in the presence of IL-6-related DNA vaccine (IL-6 DNA+IL-6 receptor DNA+gp130 DNA vaccine) was shown by in vivo induction of CTL specific for HSP65 TB antigen in the mice stimulated with killed TB antigens (**Fig. 7**). Granulysin vaccines (recombinant 15 K granulysin and 15 K granulysin DNA vaccine) exerted strong

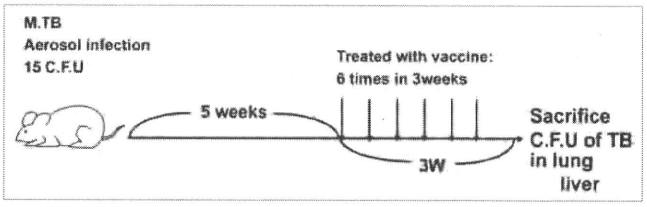


Figure 5. The hypothesis models of anti-tuberculosis immunity by granulysin produced from human cytotoxic T cell.

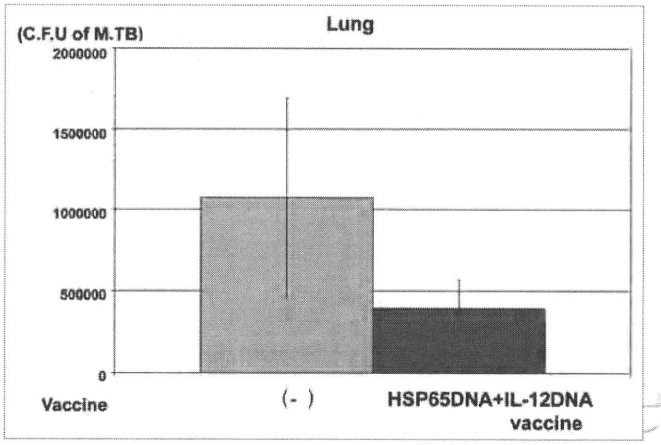


Figure 6. In vitro induction of human cytotoxic T cell by the stimulation with recombinant 15 K granulysin protein. T cells from human PBL were obtained by nylon wool column method. 1 x 10^6 T cells were cultured with human $CESS_{MMC}$ cells (Mitomycic C treated CESS tumor cells) in the presence of 15 K granulysin for 5 days. CTL activity of effector cells was assayed using ^{51}Cr labbelled CESS cells. Results are expressed as % Specific cytotoxicity ± SD. % Specific cytotoxicity was calculated as

$$\frac{\text{Experimental } ^{51}\text{Cr release-Spontaneous } ^{51}\text{Cr release}}{\text{Maximum } ^{51}\text{Cr release-Spontaneous } ^{51}\text{Cr release}} \times 100.$$

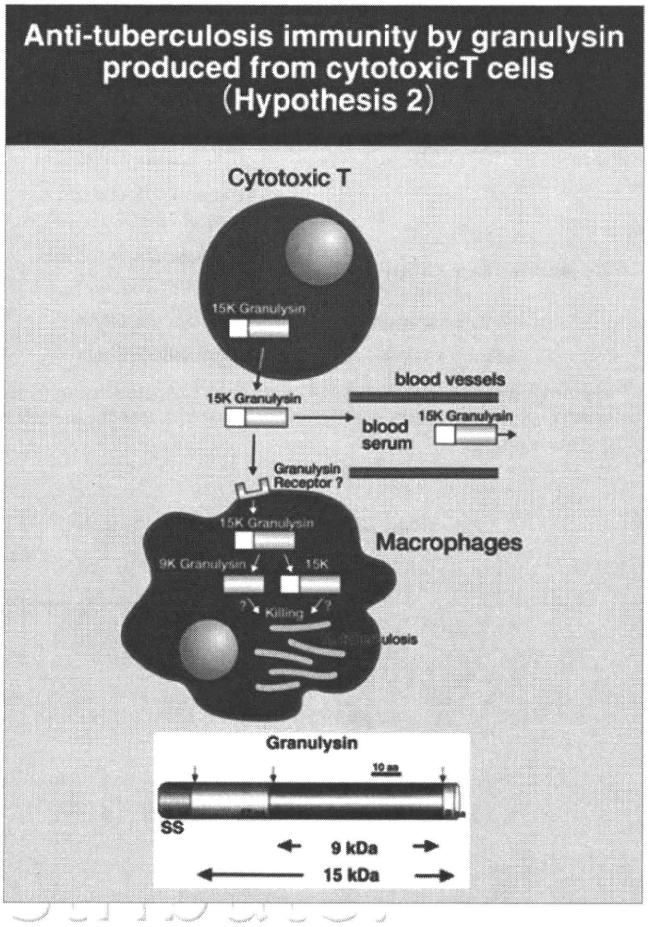


Figure 7. Synergistic effect of recombinant 15 K granulysin + IL-6 related DNA on the in vivo induction of CTL specific for HSP65 antigen. C57BL/6 mice were injected with killed TB H37Ra and then treated with recombinant 15 K granulysin protein i.p 6 times and/or IL-6 DNA + IL-6 Receptor DNA + gp130 DNA using adenovirus vector i.m. 3 weeks after killed TB challenge, CTL activity against HSP65 antigens of TB in the spleen cells was assessed by ^{51}Cr release assay. HSP65 DNA (derived from TB) was transfected into EL-4 tumor cells syngenic to C57BL/6 mice, and used for target cells. Results are expressed as % specific cytotoxicity ± SD.

therapeutic efficacy (decrease in the number of TB in the lungs, liver and spleen) in the mice infected with TB by aerosol challenge (**Table 1**).

Monkey models. Furthermore, the therapeutic activity of this vaccine was evaluated in a nonhuman primate model infected with *M. tuberculosis*. We studied therapeutic efficacy of HVJ-Envelope/HSP65 DNA+IL-12 DNA vaccine on TB-infected monkeys using GMP-level-vaccine which contains two kinds of DNA in one plasmid vector for clinical therapeutic trial (**Fig. 8A**).

Therapeutic efficacy was evaluated by survival, ESR, body weight, immune responses, chest X-ray findings and PPD skin test (**Fig. 8B**).

Immune responses of cynomolgus monkey were augmented at 11 weeks after the challenge of *M. tuberculosis* Erdman strain by intratracheal instillation. The proliferation of PBL was also augmented by therapeutic vaccination of monkeys with HVJ-Envelope/HSP65 DNA+IL-12 DNA (data not shown). This vaccine also improved the survival of monkeys, compared to the saline (control) group, after TB challenge (**Fig. 9**). All 5 monkeys were alive in the group of HVJ-Envelope/HSP65DNA+IL-12DNA vaccine (100% survival) at 16 weeks after challenge. In contrast, only 3 monkeys out of 5 were alive in the saline control group (60% survival) (**Fig. 9 and Table 2**). The number of monkeys which showed an increase in body weight was larger in the group treated with this DNA vaccine than in control group (**Table 2**). This vaccine improved ESR (Erythrocyte Sedimentation Rate) of TB-infected monkeys as shown in **Figure 10**. The proliferation of PBL by the stimulation with HSP65 antigens, H37Ra-killed TB antigens and PPD antigens was examined, and it was more augmented by the treatment with this DNA vaccine than the treatment with saline (data not shown). Furthermore, IL-2 production from PBL by the stimulation with killed TB H37Ra antigens was also examined and it was more augmented by the treatment with this vaccine than that with saline (**Fig. 11**). The induction of IL-2

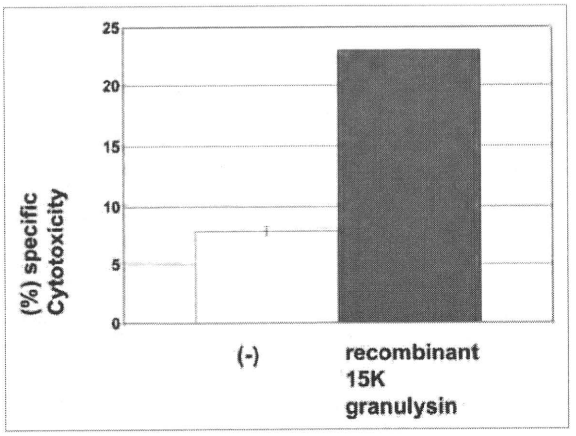


Figure 8. (A) Construction of DNA vaccine for clinical trial. HVJ-Envelope/HSP65 DNA+IL-12DNA vaccine was constructed for GMP-level vaccine which contains two kinds of DNA in one plasmid vector (pVAX1) for clinical therapeutic trial. (B) Evaluation of therapeutic efficacy of HVJ-Envelope/HSP65 DNA+IL-12DNA vaccine on TB-infected cynomolgus monkeys. Cynomolgus monkeys were vaccinated nine times with HVJ-Envelope with expression plasmid of both HSP65 and human IL-12 (HSP65+hIL-12/HVJ: 400 ug i.m.), one week after the challenge with the *M. tuberculosis* Erdman strain (5×10^2) by intratracheal instillation. Therapeutic efficacy was evaluated by survival, chest X-P findings, immune responses, body weight and erythrocyte sedimentation rate (ESR) for one year.

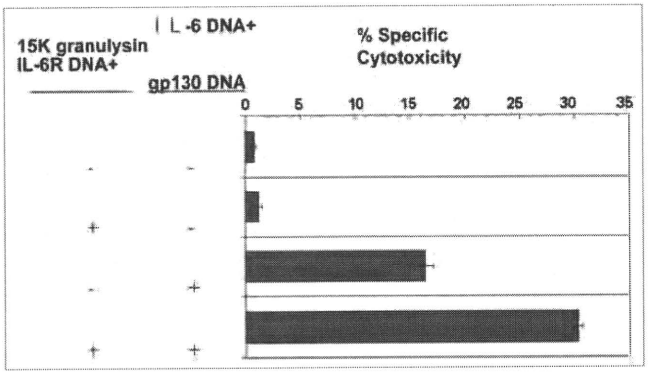


Figure 9. Survival of monkeys treated with HVJ-Envelope/HSP65 DNA+IL-12DNA vaccine after the infection of TB. Therapeutic efficacy was evaluated by survival of monkeys. Survival of monkeys treated with HVJ-Envelope/HSP65 DNA+IL-12DNA at 19 weeks after the challenge of TB by intratracheal instillation was shown.

from PBL by the stimulated with PPD was significantly lower in control monkeys died of TB within 19 weeks after TB challenge than that in survived monkeys in the same group, (data not shown). IL-2 production by the stimulation with HSP65 protein was also extremely low in the control monkeys died of TB (data not shown). Thus, this GMP-level of DNA vaccine which contains two kinds of genes in one plasmid vector exerted therapeutic efficacy in TB-infected monkeys. These results demonstrate that HVJ-Envelope/HSP65DNA+IL-12DNA vaccine could provide strong therapeutic efficacy against TB in the cynomolgus monkey models as well as murine models.

Discussion

In the present study, 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 and (4) prolongation of survival and augmentation of immune responses, in a cynomolgus monkey model which closely mimics human TB disease. It is important to evaluate the survival of monkey.[2,5,13] Increases in the survival rate of the monkeys treated with this vaccine were observed, compared to the control monkeys treated with saline. In the recent study, it is demonstrated that granulysin vaccine shows therapeutic efficacy against TB in mice (**Table 1**). Therefore, the combination of these therapeutic vaccines might be useful in the future.

MDR-TB and XDR-TB are becoming big problems around 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 a mouse infected with TB in the aerosol chamber. Therapeutic efficacy of this vaccine was also observed in this model.

DNA vaccine is a relatively new approach to immunization for infectious diseases.[1,2,5,16-19]

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) and (5) lenti-virus vector.[1,2]

We have developed a hemagglutinating virus of Japan envelope (HVJ-Envelope) using inactivated Sendai virus, as a non-viral vector for drug delivery.[7-9] It can efficiently deliver DNAs, siRNAs, proteins and anti-cancer drugs into cells both in vitro and in vivo.[9-11] Therefore, HVJ-Envelope was used as an efficient and safe vector for DNA vaccine against TB in the present study.

It will 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.

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.[20]

Most importantly, this is the leading report of novel therapeutic vaccine using monkey models as well as murine models.

According to our knowledge, only a few therapeutic vaccine against TB has been reported in reference 21 and 22.

Table 2. Body weight and survival of cynomolgus monkeys treated with HSP65 DNA+IL-12 DNA vaccine

	Increase in body weight at 16 weeks		Survival
G_1 (DNA 9 times)	+	2/5 (40%)	5/5
	+		
	-		
	-		
	0		
	-		
G_2 (control saline)	+	1/5 (20%)	3/5
	0		
	-		
	-		

Increase in body weight and survival of monkeys treated this DNA vaccine at 16 weeks after TB challenge.

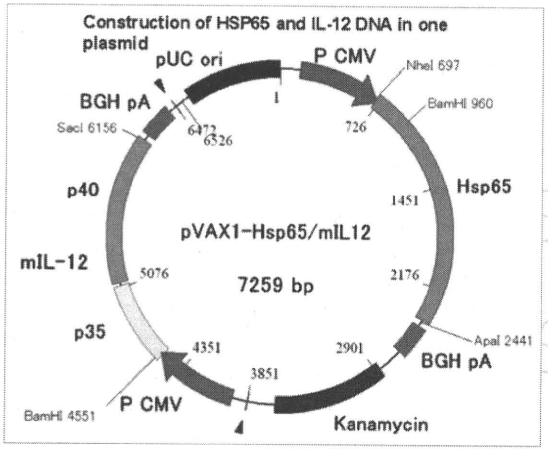


Figure 10. ~~Impovement of ESR by the treatment of HVJ-Envelope/HSP65 DNA+IL-12DNA vaccine. Therapeutic efficacy was evaluated by ESR of the monkeys at 11 weeks after *M. tuberculosis* infection.~~

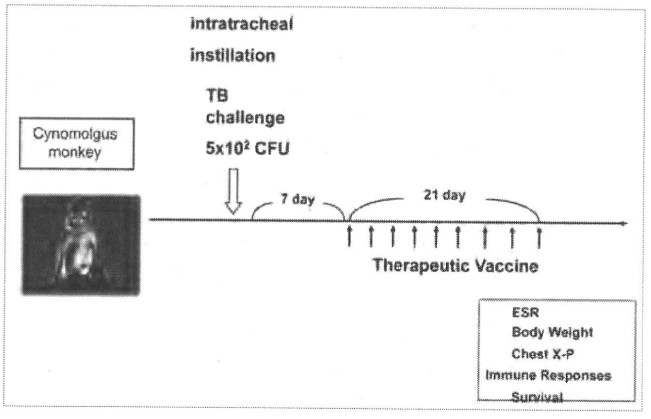


Figure 11. Augmentation ~~of IL-2 production from PBL in the monkeys treated with HVJ-Envelope/HSP65 DNA+IL-12 DNA vaccine. Peripheral blood lymphocytes (PBL) were cultured with killed TB, H37Ra for 3 days. Supernatants were harvested after 3 day culture. IL-2 activity in the culture supernatants was assessed by ELISA. IL-2 activity (U/ml) was shown.~~

Hsp65DNA+IL-12 DNA vaccine exerted strong therapeutic efficacy (100% survival at 16 weeks after challenge and augmentation of immune responses) in the TB-infected monkeys. In contrast, the survival rate of the saline control group was 60%. HVJ-Envelope/HSP65 DNA+IL-12 DNA vaccine increased the body weight of TB-infected monkeys, improved the erythrocyte sedimentation rate and augmentated the immune responses (proliferation of PBL, IL-2 production). The enhancement of IL-2 production from monkeys treated with this vaccine was correlated with the therapeutic efficacy of the vaccine.

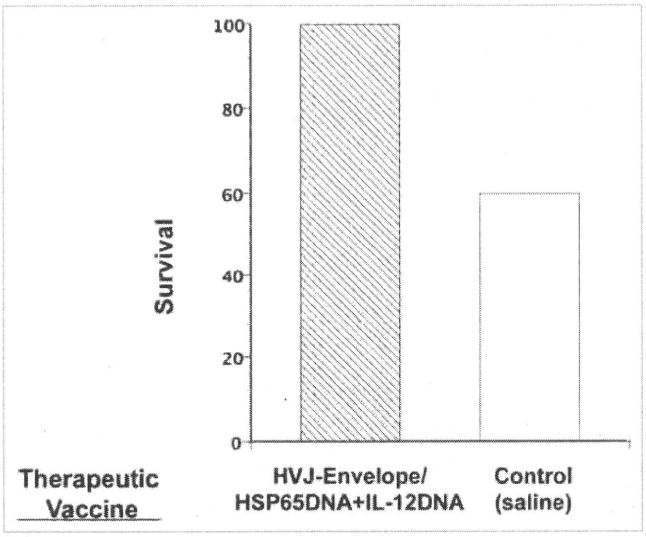
Thus, we are taking advantage of the availability of multiple animal models to accumulate essential data on the HVJ-envelope DNA vaccine in anticipation of a phase I clinical trial.

In conclusion, these data indicate that HVJ-Envelope/HSP65DNA+IL-12DNA vaccine and granulysin vaccine might be useful against TB including XDR-TB and MDR-TB for human therapeutic clinical trials.

Acknowledgements

This study was supported by Health and Labour Science Research Grants from MHLW, Research on Publicly Essential Drugs and Medical Devices, Japan Health Science Foundation and Grant-in-Aid for Scientific Research (B) from the Ministry of Education, Culture, Sports, Science and Technology Japan and Grant of Osaka Tuberculosis Foundation.

AUTHOR: Please refer for Figures 12–14 within the body text.



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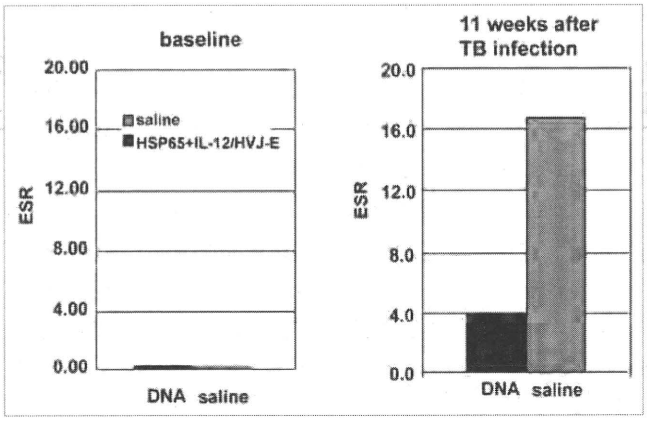

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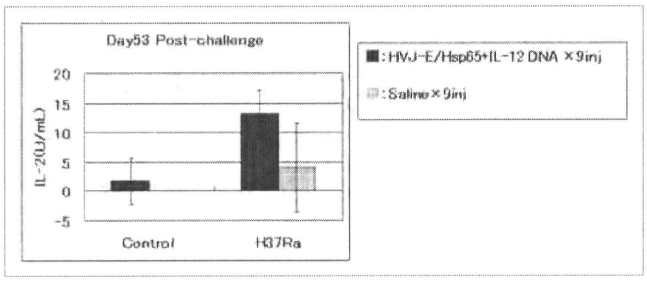

Figure 14. AUTHOR: Please provide figure legend.

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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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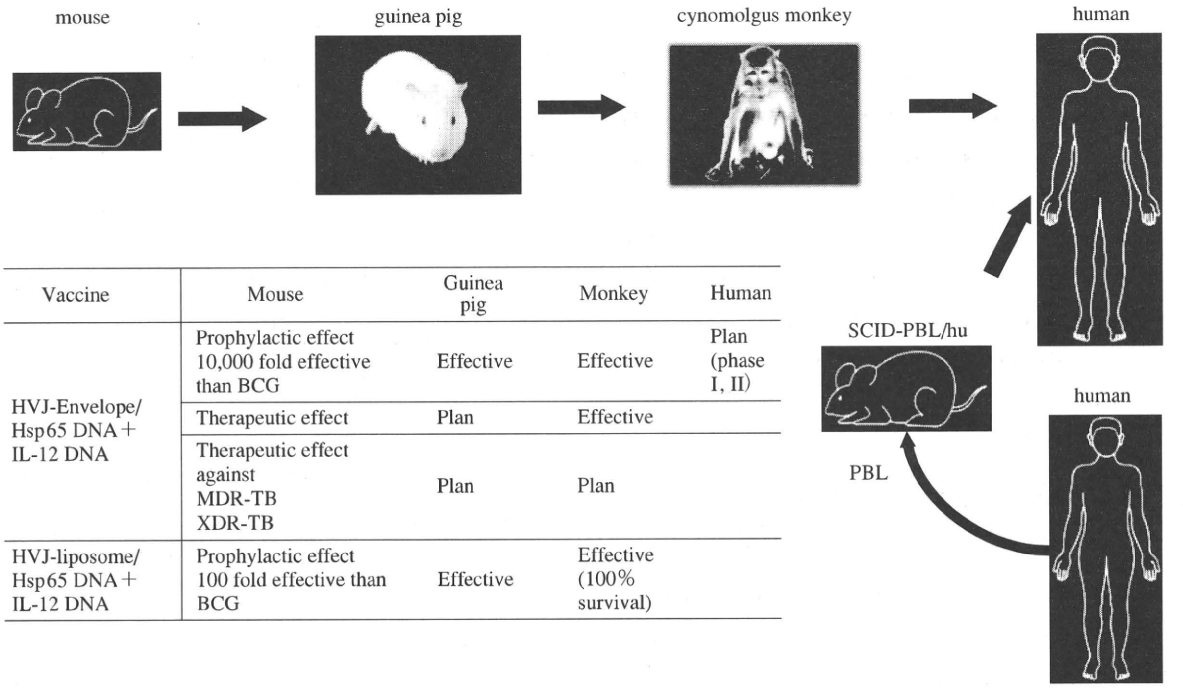
(Received 14 Mar. 2010)

を Fas-independent, granule-dependent の機構で溶かし，最終的には結核菌を殺すことが報告されている[9]。このT細胞は CD1-restricted でミコール酸，LAM，phosphatidyl inositol mannoside, glucose monomycolate, isoprenoid glycolipid（Cd1cと結合）等の結核菌 lipid と lipoglycan を認識する。このキラーTの顆粒内の蛋白である granulysin は直接細胞外の結核菌を殺す。

一方，キラーTの TRAIL とパーフォリンが抗結核免疫に重要である興味深い結果を得た（Fig. 2）。

Ⅲ. granulysin と結核

キラーTの顆粒内の蛋白である granulysin は直接細胞外の結核菌を殺す。この機序は結核菌細胞膜を不完全な状態にすることによる。granulysin は病原細菌，真菌，寄生虫の生存を減少させる。さらにパーフォリンとの共存下でMφ内の結核菌も殺すと考えられている。これ



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)	

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

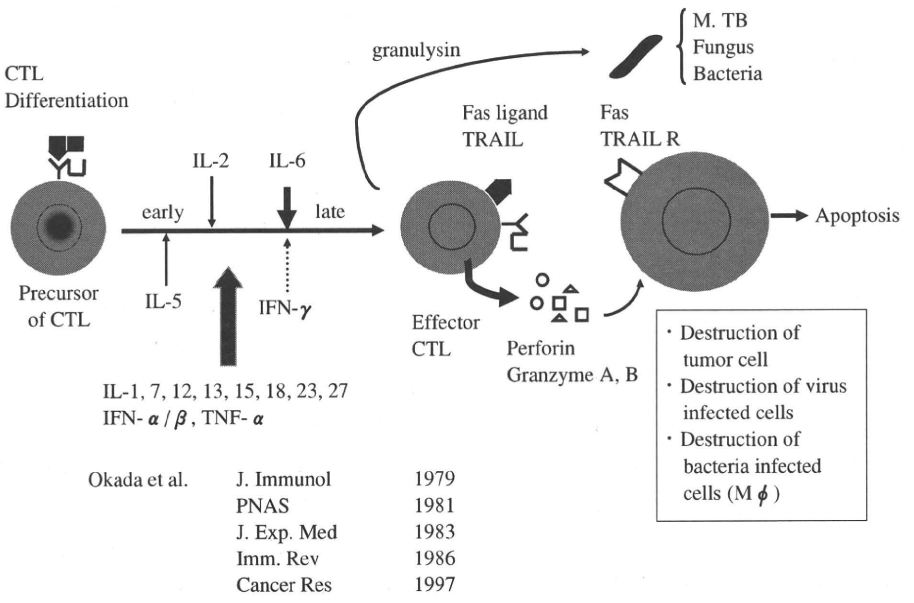


Fig. 2 Induction of cytotoxic T cells and killing mechanism

Table 1 Induction of decrease in TB number *in vivo* and CTL differentiation by 15K Granulysin and 9K Granulysin

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

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

はパーフォリンよりMφに穴が開き，Mφ内の結核菌に直接granulysinが作用するためと思われる。われわれは結核患者，特に多剤耐性結核患者ではキラーTリンパ球のmRNAの発現および蛋白の発現が低下していることを明らかにした[14)16)]。すなわち，われわれはキラーT細胞のgranulysin（分子量9000）産生低下が多剤耐性結核発症と大きな関連があるのではないかと考えている。

一方，granulysinがキラーT分化因子の一つであることを発見し，マウスで結核治療効果を示した（特許取得）。granulysin遺伝子導入マウスを作製した。

Ⅳ. 新しい結核ワクチン（HSP 65 DNA＋IL-12 DNA ワクチン，granulysinワクチン等）開発

結核ワクチンは，①サブユニットワクチン，②DNAワクチン，③リコンビナントBCGワクチン（弱毒化結核菌を含む），その他に大別される。

（1）DNAワクチン：BCGワクチンより1万倍強力な結核予防ワクチン

マウスの結核感染系ではBCGワクチンをはるかに凌駕する新しい結核ワクチンはきわめて少ない。われわれはHsp65 DNA＋IL-12 DNA（HVJ-エンベロープベクター）のワクチンはBCGワクチンよりも1万倍強力な結核予防ワクチンであることを世界に先駆けて明らかにした。

このHVJ-エンベロープ/HSP65 DNA＋IL-12 DNAワクチンでマウスを免疫して結核菌を投与すると，マウス肺の結核菌数がBCGワクチン投与の1万分の1以下となった。これを1万倍強力という。

さらに，結核菌に対するCD8陽性キラーT細胞の分化誘導を増強した[4)]。この強力なワクチン効果とキラーT活性が相関した。またTh1細胞の分化誘導，IFN-γ産生の増強をこのワクチンが発揮することも明らかにした。

この新しい結核ワクチンの開発研究が高く評価され，WHO STOP TB PartnershipおよびWHO STOP TB WGND（Working Group on New Drugs）に選出された。

（2）リコンビナントBCGワクチン

Table 2 Therapeutic efficacy against tuberculosis by 15K granulysin Transgenic mice and 9K granulysin Transgenic mice

Tg mouse	CFU of TB (log)
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

BCG東京菌に，種々の遺伝子を導入しリコンビナントBCGを作製した。われわれはAg85A＋Ag85B＋MPB51リコンビナントBCGはBCGよりも強力なワクチンであることを明らかにした[5)]。

さらに，サブユニットワクチンでサルのレベルで強力な予防効果が得られたMtb72f融合タンパク質のDNAを導入した72fリコンビナントBCGの作製に成功した。この72f rBCGワクチンはサルでも結核予防効果を示した（Fig. 1）[6)]。

（3）granulysinワクチン（Table 1，Table 2）

キラーT細胞は結核感染防御に重要な働きをする[3)4)]（Fig. 2）。granulysin蛋白発現を多剤耐性結核や糖尿病患者の難治性結核のPBLの培養上清中の活性で検討した。その結果IL-6，IFN-γ，IL-2のキラーT細胞分化因子のみでなくgranulysin（15K Granulysin）の産生低下を認めた[14)15)]。

さらにわれわれは15K Granulysinが$CD8^+$キラーT細胞から直接分泌され，ヒトのMφに直接入り，Mφ内の結核菌を殺傷することを明らかにした[14)15)]。薬剤感受性結核患者PBL中のCD8陽性T細胞の15K Granulysin蛋白発現とmRNAの発現は健常人よりも有意に低下していた[14)15)]（Table 1）。さらに，多剤耐性結核患者PBL中のCD8陽性T細胞の15K Granulysin蛋白発現とmRNAの発現は，有意差をもって，薬剤感受性結核患者のそれらよりも低下していた（Table 1）。また，多剤耐性結核患者のPBLをPHA-P，ConA，アロ抗原（CESS），PPD抗

原で刺激すると，15K Granulysinの培養上清中への分泌が低下していることを明らかにした（Table 1）。

15K Granulysinの遺伝子導入マウスと9K granulysin遺伝子導入マウスをそれぞれ作製し，*in vivo*の抗結核作用を解析した。Table 2に示したごとく，15K Granulysin transgenic（Tg）マウスの結核菌感染4週間後の肺結核菌数（CFU）はwild typeマウスに比較して低下が認められた。また9K Granulysin Tgマウスの肺内結核菌数もwild typeマウスに比較して低下していた（Table 2）。さらに，これらの2つのTgマウス（15K Granulysin Tgと9K Granulysin Tg）は*in vivo*のキラーT誘導（結核に対する）の増強，結核に対するT細胞増殖反応の増強やIFN-γ産生の増強等を示した。これらの生体内における15K Granulysinと9K Granulysinの結核感染に対する効果は世

Table 3

A. Priming, Pre-Exposure	
1. Phase I：現在—2008年	特徴
a. **rBCG30**	リコンビナント85B BCG
b. **rBCG30ΔureC：Hly** (VPM1002)	リコンビナント listeriolysin BCG
c. AERAS-407	リコンビナント perfringiolysin
d. rBCG30ARMF, rBCG Mtb B30, rBCG h IFNy	リコンビナント85B BCG
e. Nas L3/Htk BCG	鼻粘膜ワクチン/heat killed whole BCG コペンハーゲン株
f. $mc^2$6220, $mc^2$6221, $mc^2$6222, $mc^2$6231	nor-replicating, *M. tuberculosis* strain (Δ lys A Δ pan CD)
g. $mc^2$5059	replicating pro-apoptotic *M. bovis* BCG株（Δ nuoG）
2. Phase I 2009 or Later	メチル化21-K Da蛋白
a. HBHA (heparin-binding haemagglutinin)	弱毒化ヒト結核菌（virulence geneの pho Pの不活性）
b. Attenuated Live Vaccine based on Phop	anti-apoptotic酵素活性を減弱
c. paBCG (pro-apoptotic BCG)	

B. Boosting, Pre-Exposure	
1. Phase I：現在—2008年	特徴
a. **MVA85A**	リコンビナント MVA (Ag85Aを発現した)
b. **M72**	Mtb32＋Mtb29の fusion蛋白
c. AERAS-402	Replication-incompetent adenovirus 35 vector expressing *M. tuberculosis*
d. SSI Hybrid-1	antigens Ag85A,
e. **SSI HyVac4/AERAS-404**	Ag85B, and TB 10.4.
f. AERAS-405	fusion蛋白（Ag85B-ESAT-6）
g. r30	fusion蛋白（Ag85B-TB10.4）
h. Nas L3/Htk BCG	Shigella-delivered recombinant double-stranded RNA nucleocapsid (Ag85A, 85B, Rv3407, latency antigen)
2. Phase I：2009 or Later	リコンビナント Ag85B蛋白
a. Hsp C™ TB Vaccine	Heat shock protein antigen complexes (Hsp Cs)
b. HBHA (heparin-binding haemagglutinin)	Nasal vaccine/Man capped
c. NasL3/AM85B conjugate	Arabinomonnan oligosaccharide
d. PP1, PP2, PP3	BCG boosting
f. AC_2SGL Diacylated Sulfoglycolipids	AC_2 SGL Mycobacterial lipids
g. **HVJ-liposome/Hsp65 DNA＋IL-12 DNA**	M.Okada, 国立病院機構近畿中央胸部疾患センター

C. Post Exposure— Immunotherapy	
1. Phase I：現在—2008年	特徴
a. Mycobacterium vaccae Heat-Killed	Fragmented *M. tuberculosis* cells
b. MVA85A	naked hsp 65 DNA vaccine
c. RUTI	Chimeric ESAT6/Ag 85A DNA ワクチン
d. Nas L3/Htk BCG	Recombinant BCG overexpressing chimeric ESAT6/Ag85A fusion protein
2. Phase I：2009 or Later	Recombinant Sendai virus overexpressing chimeric ESAT6/Ag85A fusion protein
a. NasL3/AM85B conjugate	Epitope-based DNA-prime/peptide-boost vaccine.（liposomeとCpGアジュバント）
b. hspDNA vaccine	
c. HG856A	
d. HBHA (heparin-binding haemagglutinin)	
e. HG856-BCG	
f. HG856-SeV	
g. TB Vax	
h. F36, F727	
i. Mycobacterium vaccae Heat-Killed	
j. Ac_2SGL Diacylated Sulfoglycolipid	

界に先駆けての発見である。実際リコンビナントgranulysinワクチンやgranulysin DNAワクチンはマウスで結核治療効果を示した[14)15)]。したがってgranulysinワクチン治療はMDR-TBやXDR-TBに対しきわめて有用な治療法となるであろう。

V．新しい結核ワクチンの開発状況（臨床応用）

（1）Stop TB Partnership

Stop TB Partnership（WHO）は2008年に現在進行中で，しかも臨床応用に有望な新しい結核ワクチン開発のリストを発表した。

われわれのHVJ/Hsp65 DNA＋IL-12 DNAワクチンも候補の一つとしてその中に推奨されている（Table 3）。表内で太字で示したワクチンが評価されている。

2006～2015年Global Plan to Stop TBとして新しい有効な結核ワクチン開発，2050年までに結核撲滅，がWHOの目標である。

（2）結核ワクチンの応用の可能性

①新しい結核ワクチンの臨床応用

カニクイザル（cynomolgus monkey，最もヒトの肺結核に近いモデル，Nature Medicine 2, 430, 1996参照）を用いBCGよりもはるかに強力な予防ワクチン効果（生存率，血沈，体重，肺の組織）を示すワクチン2種を開発した[6)8)]。すなわち，現在最も有力なものとしてHVJリ

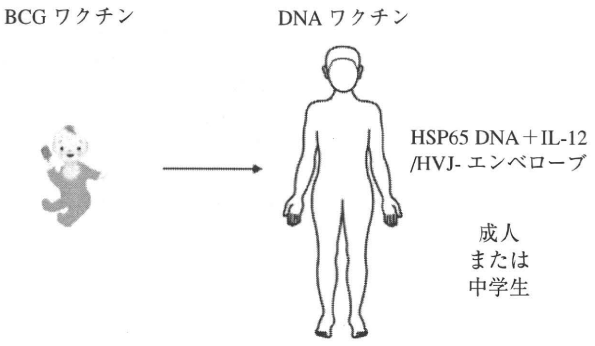


Fig. 3　新しい結核予防ワクチン（案）（DNAワクチン）

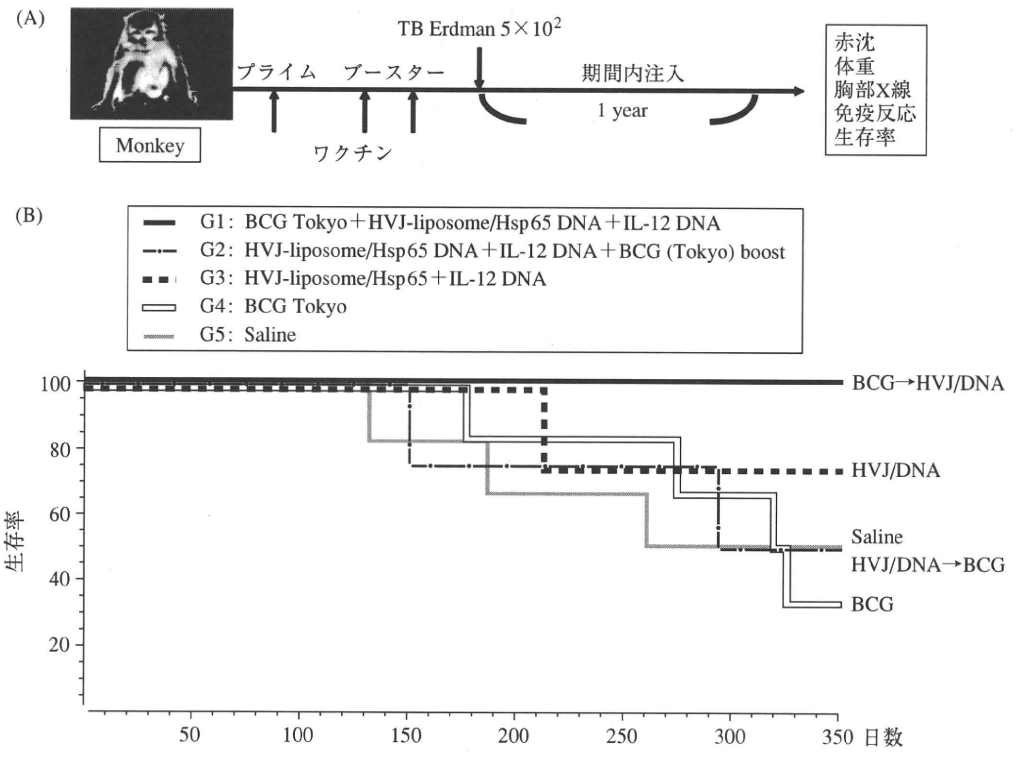


Fig. 4　ヒトの結核感染に最も近いカニクイザルを用いたHVJ-リポソーム/HSP-65 DNA＋IL12 DNAワクチンの結核予防効果

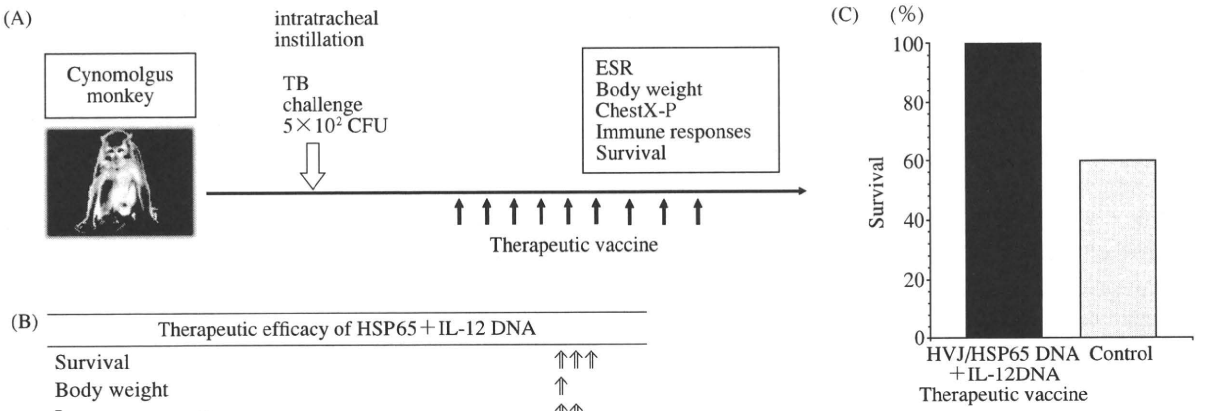


Fig. 5 Therapeutic effect of HVJ-Envelope/HSP65 DNA＋IL-12DNA vaccine on TB-infected cynomolgus monkeys

ポソーム/HSP65 DNA＋IL-12 DNAワクチンおよび，r72f BCGワクチンがあげられる。Ag85B-ESAT-6融合タンパク質（Anderson博士ら）も報告されているが，モルモット，サルでは効果は不明である。一方HuygenのAg85A DNAワクチンはマウス・モルモットで有効であったがサルの結核感染予防に対し有効でなかったという。72f融合タンパクサブユニットワクチン[16]，ワクシニアウイルスに85A DNAを導入したワクチンは第Ⅱ相，r85B BCG（Horowitzら）は第Ⅰ相clinical trialとなっている[16]。Dr.A.Hillらのワクシニアウイルス-85A DNAワクチンは，アフリカでの第Ⅰ相clinical trialでは，85A DNA蛋白に対する免疫応答増殖が認められた[17]。

②プライミング-ブースター法（乳幼児BCG—成人HVJ/HSP65 DNA＋IL-12 DNAワクチン）

さらにBCGワクチンをプライムし，新しいワクチンをブースターする方法を用いた。サルでこのプライミング-ブースター法で100%の生存を示した[3]（Fig. 2）。一方，BCGワクチン単独投与群は33%の生存率であった[3]。このように，ヒトの結核感染に最も近いカニクイザルを用いた実験系で，強力な新しい結核ワクチンをわれわれは世界に先駆けて開発した。すなわち，本邦では乳幼児にBCG接種が義務づけられていることにより，プライミングワクチンとしてBCGワクチンを用い，成人ワクチン（中学生，成人，老人）としてこのDNAワクチンをブースターワクチンとして用いる結核ワクチンの臨床応用案である（Fig. 3）。

③治療ワクチン（Fig. 4，Fig. 5）

感染したカニクイザルの系でHVJ-エンベロープ/Hsp65 DNA＋ヒトIL-12 DNAワクチンを投与した。この群では5頭中5頭100%の生存率が認められた。一方コントロール群の生食投与群では，60%の生存率であった。このDNAワクチン投与群では，体重増加が認められ，末梢血T細胞の増殖増強反応が認められた。Hsp65 DNA＋IL-12 DNAワクチンは最もヒトの結核感染症モデルに近いカニクイザルの系において予防ワクチンならびに治療ワクチン効果を示した。生存率・免疫能を増強した。したがってこのワクチンはヒトMDR-TB，XDR-TBの治療剤としてきわめて有用であることが示された。

Ⅵ．おわりに

HSP65 DNA＋IL-12 DNA/HVJエンベロープワクチンが優れていることより，このワクチンが結核の発症予防や治療に役立つ日を夢見ている。厚生科研，文部科研，大阪結核予防会研究費等により支援を受けた。

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