#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.

References

1. Walsh GP, Tan EV, dela Cruz EC, Abalos RM, Villahermosa LG, Young LJ, et al. The *Philippine cynomolgus* monkey provides a new nonhuman primate model of tuberculosis that resembles human disease. Nat Med 1996; 2:430-6.
2. Kita Y, Tanaka T, Yoshida S, Ohara N, Kaneda Y, Kuwayama S, et al. Novel recombinant BCG and DNA-vaccination against tuberculosis in a cynomolgus monkey model. Vaccine 2005; 23:2132-5.
3. Okada M, Kita Y, Nakajima T, Kanamaru N, Hashimoto S, Nagasawa T, et al. Evaluation of a novel vaccine (HVJ-liposome/HSP65 DNA + IL-12 DNA) against tuberculosis using the cynomologus monkey model of TB. Vaccine 2007; 25:2990-3.
4. Tanaka F, Abe M, Akiyoshi T, Nomura T, Sugimachi K, Kishimoto T, et al. The anti-human tumor effect and generation of human cytotoxic T cells in SCID mice given human peripheral blood lymphocytes by the in vivo transfer of the Interleukin-6 gene using adenovirus vector. Cancer Res 1997; 57:1335-43.
5. Oda M, Okuno Y, Hashimoto S, Kita Y, Kanamaru N, Nishida Y, et al. Development of vaccines and passive immunotherapy against SARS corona virus using SCID-PBL/hu mouse models. Vaccine 2007; 25:3038-40.
6. Pena SV, Hanson DA, Carr BA, Goralski TJ, Krensky M. Processing, subcellular localization and function of 519 (granulysin), a human late T cell activation molecule with homology to small, lytic, granule proteins. J Immunol 1997; 158:2680-8.
7. Strenger S, Hanson DA, Teitelbaum R, Dewan P, Niazi KR, Froelich CJ, et al. An antimicrobial activity of cytolytic T cells mediated by granulysin. Science 1998; 282:121-5.
8. Huang LP, Lyu SC, Clayberger C, Krensky AM. Granulysin-mediated tumor rejection in transgenic mice. J Immunol 2007; 178:77-84.
9. Yoshida S, Tanaka T, Kita Y, Kuwayama S, Kanamaru N, Muraki Y, et al. DNA vaccine using hemagglutinating virus of Japan-liposome encapsulating combination encoding mycobacterial heat shock protein 65 and interleukin-12 confers protection against *Mycobacterium tuberculosis* by T cell activation. Vaccine 2006; 24:1191-204.
10. Kita Y, Tanaka T, Yoshida S, Ohara N, Kaneda Y, Kuwayama S, et al. Novel recombinant BCG and DNA-vaccination against tuberculosis in a cynomolgus monkey model. Vaccine 2005; 23:2132-5.
11. Okada M, Kitahara M, Kishimoto S, Matsuda T, Hirano T, Kishimoto T. IL-6/BSF-2 functions as a killer helper factor in the in vitro induction of cytotoxic T cells. J Immunol 1988; 141:1543-9.
12. Okada M, Yoshimura N, Kaieda T, Yamamura Y, Kishimoto T. Establishment and characterization of human T hybrid cells secreting immunoregulatory molecules. Proc Natl Acad Sci USA 1981; 78:7717-21.
13. Okada M, Sakaguchi N, Yoshimura N, Hara H, Shimizu K, Yoshida N, et al. B cell growth factor and B cell differentiation factor from human T hybridomas. Two distinct kinds of B cell growth factor and their synergism in B cell proliferation. J Exp Med 1983; 157:583-90.
14. McShane H, Pathan AA, Sander CR, Keating SM, Gilbert SC, Huygen K, et al. Recombinant modified vaccinia virus Ankara expressing antigen 85A boosts BCG-primed and naturally acquired antimycobacterial immunity in humans. Nature Med 2008; 10:1240-4.
15. Fletcher HA, Pathan AA, Berthoud TK, Dunachie SJ, Whela KT, Alder NC, et al. Boosting BCG vaccination with MVA85A domn-regulates the immunoregulatory cytokine TGFβ1. Vaccine 2008; 26:5264-75.
16. Okada M, Kita Y. Tuberculosis vaccine development: The development of novel (preclinical) DNA vaccine. Human Vaccine 2010; 6:297-308.
17. Kaufmann SHE. New vaccine for tuberculosis. The Lancet 2010; 375:85-94.

REVIEW

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

Novel therapeutic vaccine
Granulysin and new DNA vaccine against tuberculosis

Masaji Okada,[1,*] Yoko Kita,[1] Toshihiro Nakajima,[2] Noriko Kanamaru,[1] Satomi Hashimoto,[1] Tetsuji Nagasawa,[2] Yasufumi Kaneda,[3] Shigeto Yoshida,[4] Yasuko Nishida,[1] Hitoshi Nakatani,[1] Kyoko Takao,[1] Chie Kishigami,[1] Shiho Nishimatsu,[1] Yuki Sekine,[1] Yoshikazu Inoue,[1] Makoto Matsumoto,[5] David N. McMurray,[6] E.C. Dela Cruz,[7] E.V. Tan,[7] R.M. Abalos,[7] J.A. Burgos,[7] Paul Saunderson[7] and Mitsunori Sakatani [1]

[1]Clinical Research Center; National Hospital Organization Kinki-chuo Chest Medical Center; Kitaku, Sakai; [2]Ikeda Laboratory; GenomIdea Inc.; Ikeda, Osaka; [3]Division of Gene Therapy Science; Graduate School of Medicine; Osaka University; Suita, Osaka; [4]Department of Medical Zoology; Jichi-Med. Sch; Minamikawachi-machi, Tochigi; [5]Otsuka Pharmaceutical Co., Ltd.; Tokushima Japan; [6]Texas A & M University; System Health Science Center; College of Medicine; College Station, TX USA; [7]Leonard Wood Memorial; Mandaue City, Cebu Philippines

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.

*Correspondence to: Masaji Okada; Email: okm@kch.hosp.go.jp
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

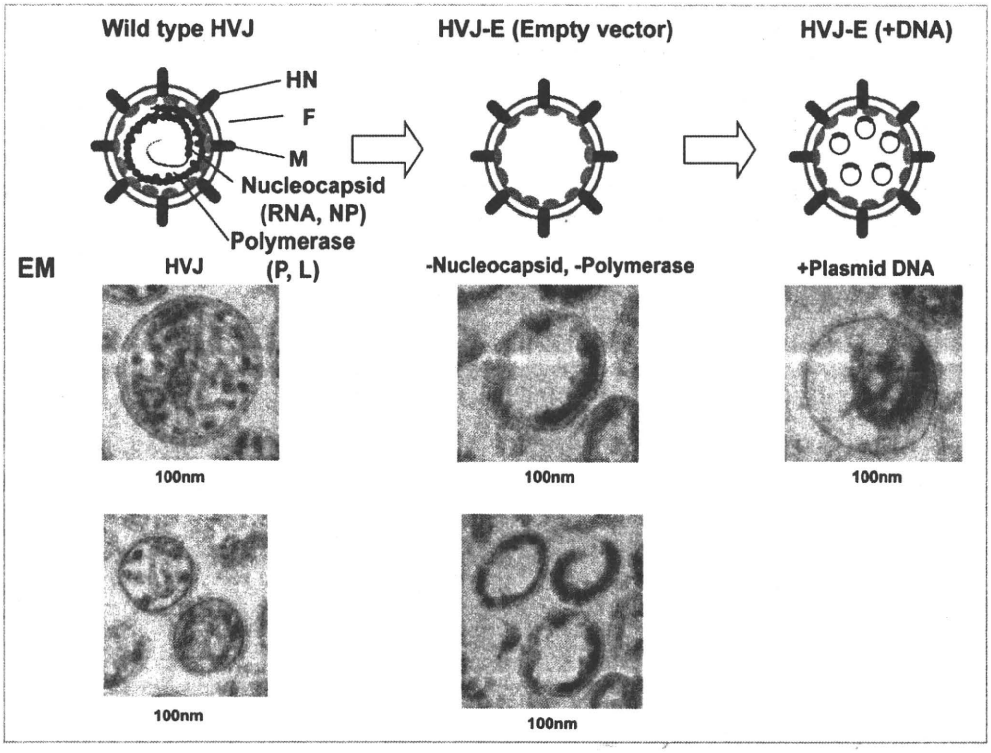


Figure 1. HVJ-envelope vaccination. pcDNA3-1/HSP65DNA+IL-12DNA were incorporated into HVJ-Envelope Empty Vector (Non-Viral Vector). Cartoons of HVJ-Envelope Empty Vector in the presence or absence of DNA were shown. Photographs of an electronic microscope (EM) of HVJ-Envelope Empty Vector were also shown.

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

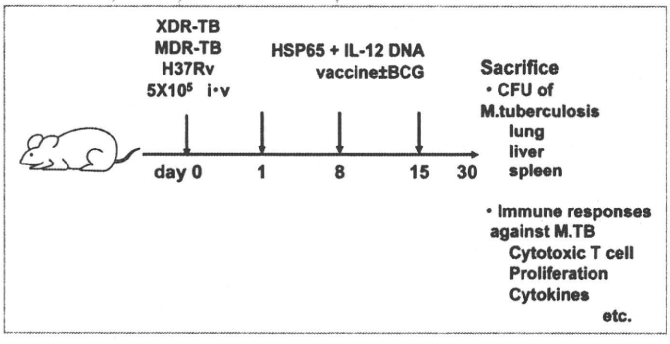


Figure 2. TNFR gene disrupted DBA/1 mice and IL-6 gene disrupted DBA/1 mice were treated with HVJ-Envelope/HSP65 DNA+IL-12 DNA vaccine three times i.m at 1, 8 and 15 days after the challenge of 5 x 10^5 CFU MDR-TB i.v. At 30 days after MDR-TB challenge, the lungs, spleens and livers were aseptically 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

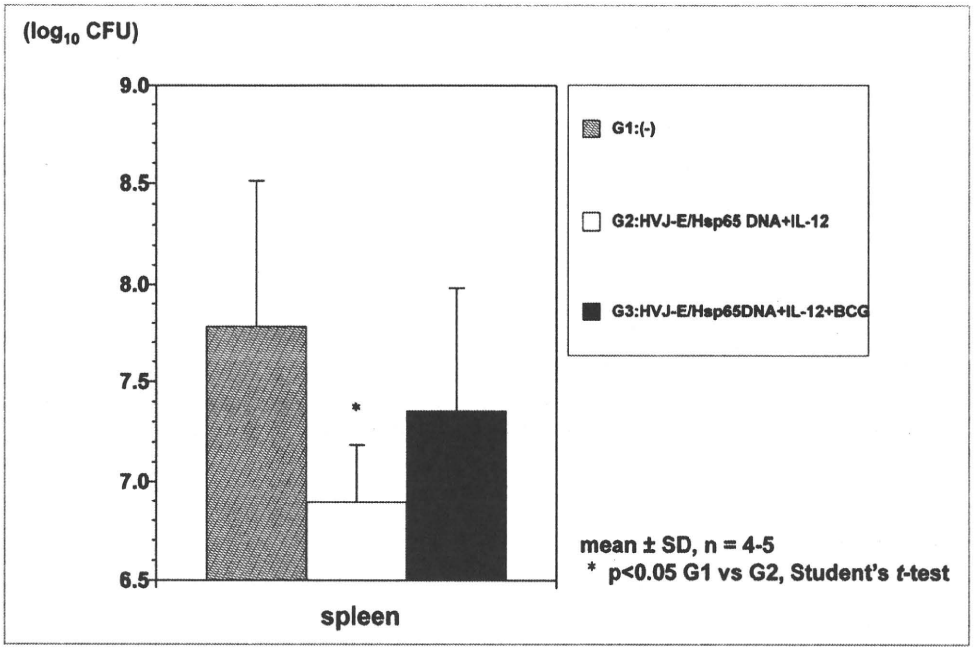


Figure 3. ~~Therapeutic efficacy of HVJ-Envelope/HSP65 DNA+IL-12 DNA vaccine on the MDR-TB infection in the TNFR gene disrupted DBA/1 mice. Groups of mice were challenged by intravenous injection with 5 x 10^5 CFU MDR-TB, and then treated three times with HVJ-Envelope/HSP65 DNA+IL-12 DNA vaccine, as 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.

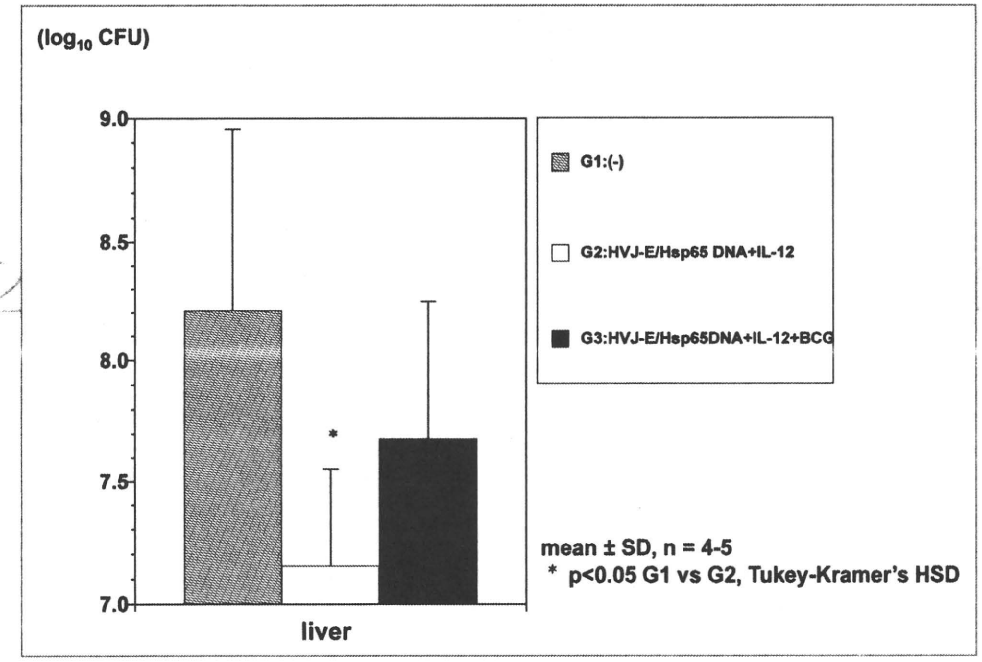


Figure 4. ~~(A) Therapeutic efficacy of TB vaccines using chronic TB infection model by aerosol challenge of TB. Therapeutic efficacy was evaluated by chronic TB infection model of mice using aerosol challenge of TB. Mice were challenged intratracheally by aerosol of *M. tuberculosis* H37Rv in saline (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

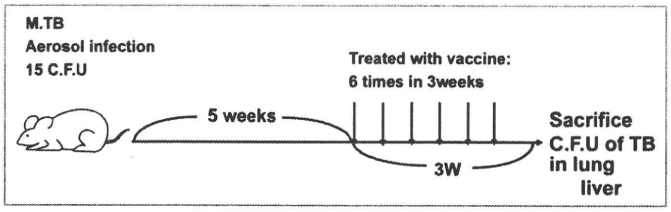


Figure 5. The hypothesis models of anti-tuberculosis immunity by granulysin produced from human cytotoxic T cell.

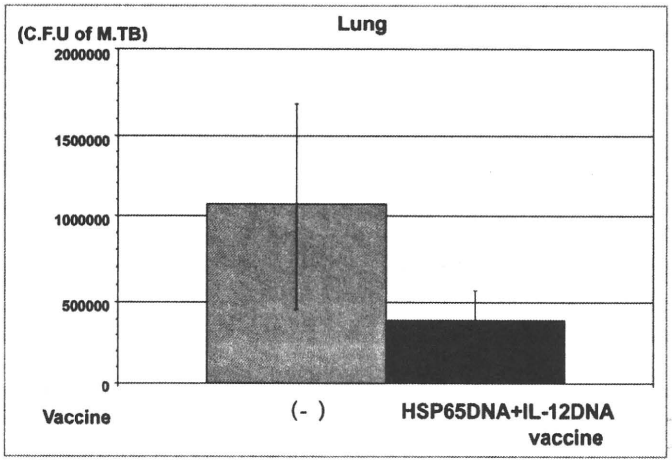


Figure 6. In vitro induction of human cytotoxic T cell by the stimulation with recombinant 15 K granulysin protein. T cells from human PBL were obtained by nylon wool column method. 1×10^6 T cells were cultured with human $CESS_{MMC}$ cells (Mitomycic C treated CESS tumor cells) in the presence of 15 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

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

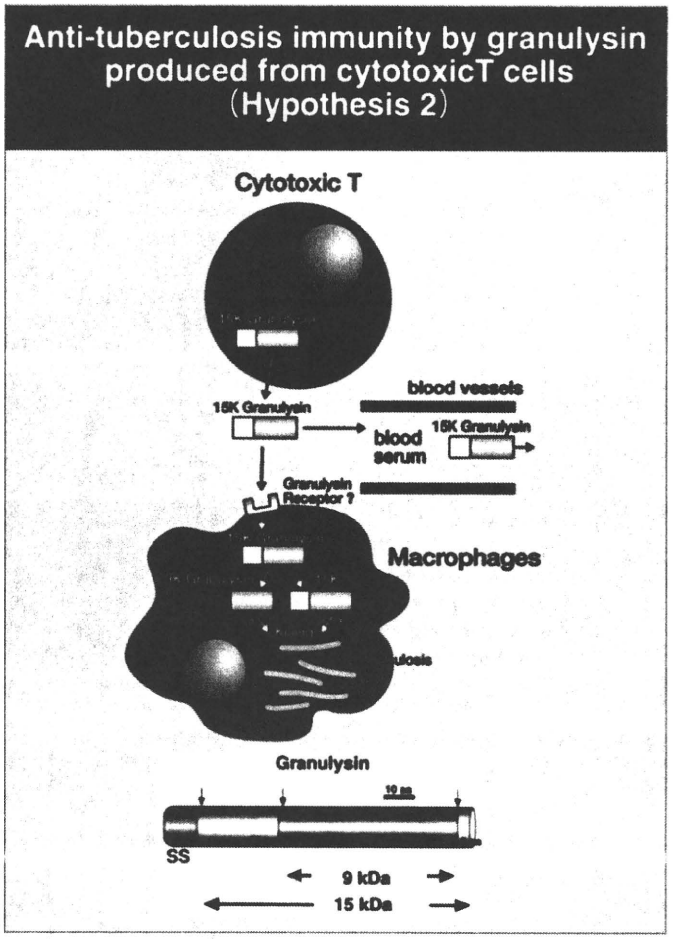


Figure 7. Synergistic effect of recombinant 15 K granulysin + IL-6 related DNA on the in vivo induction of CTL specific for HSP65 antigen. C57BL/6 mice were injected with killed TB H37Ra and then treated with recombinant 15 K granulysin protein i.p 6 times and/or IL-6 DNA + IL-6 Receptor DNA + gp130 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

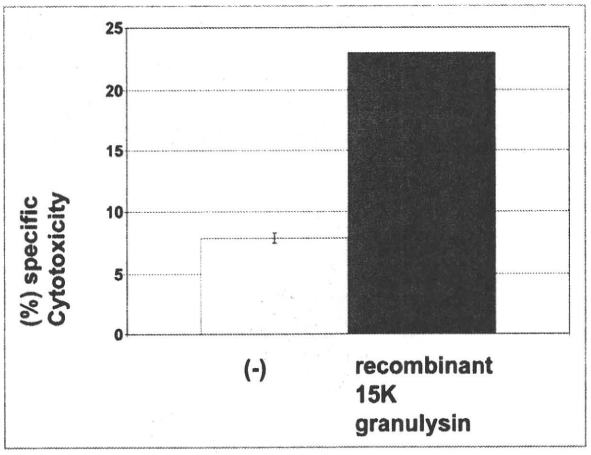


Figure 8. (A) Construction of DNA vaccine for clinical trial. HVJ-Envelope/HSP65 DNA+IL-12DNA vaccine was constructed for GMP-level vaccine which contains two kinds of DNA in one plasmid vector (pVAX1) for clinical therapeutic trial. (B) Evaluation of therapeutic efficacy of HVJ-Envelope/HSP65 DNA+IL-12DNA 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.

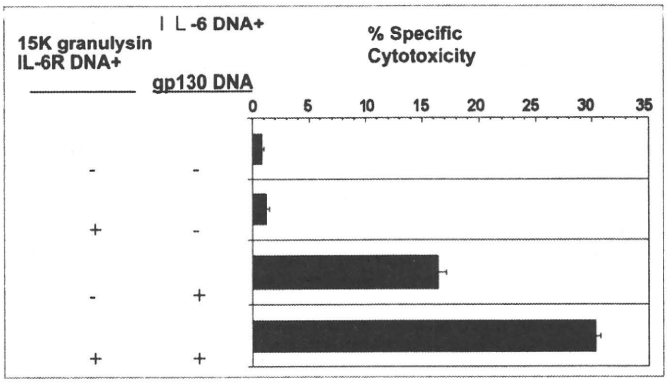


Figure 9. Survival of monkeys treated with HVJ-Envelope/HSP65 DNA+IL-12DNA vaccine after the infection of TB. Therapeutic efficacy was evaluated by survival of monkeys. Survival of monkeys treated with HVJ-Envelope/HSP65 DNA+IL-12DNA at 19 weeks after the challenge of TB by intratracheal instillation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

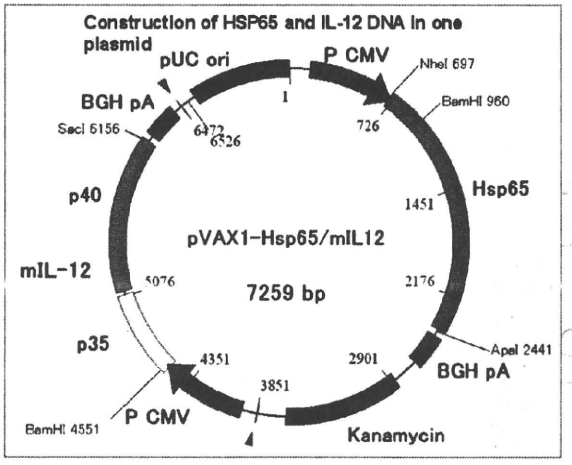


Figure 10. ~~Improvement of ESR by the treatment of HVJ-Envelope/HSP65 DNA+IL-12DNA vaccine. Therapeutic efficacy was evaluated by ESR of the monkeys at 11 weeks after *M. tuberculosis* infection.~~

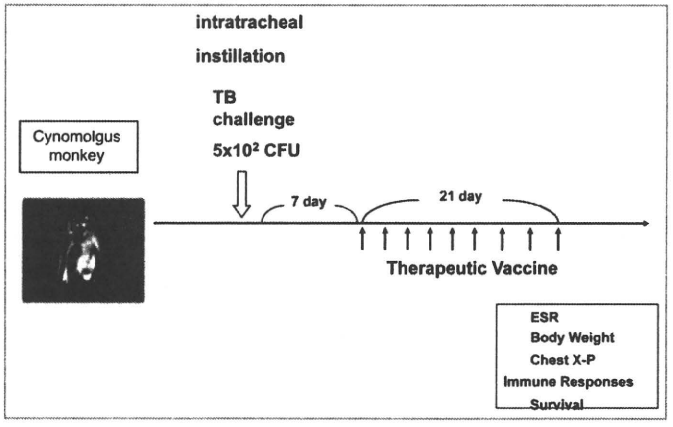


Figure 11. Augmentation ~~of IL-2 production from PBL in the monkeys treated with HVJ-Envelope/HSP65 DNA+IL-12 DNA vaccine. Peripheral blood lymphocytes (PBL) were cultured with killed TB, H37Ra for 3 days. Supernatants were harvested after 3 day culture. IL-2 activity in the culture supernatants was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

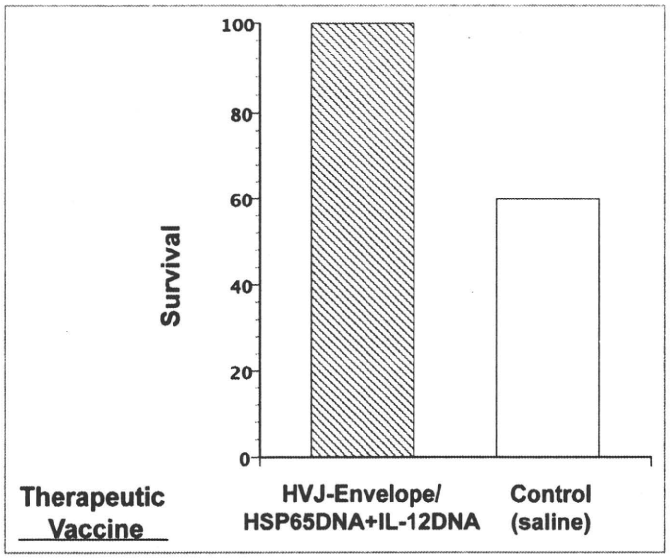


Figure 12. ~~AUTHOR: Please provide figure legend.~~

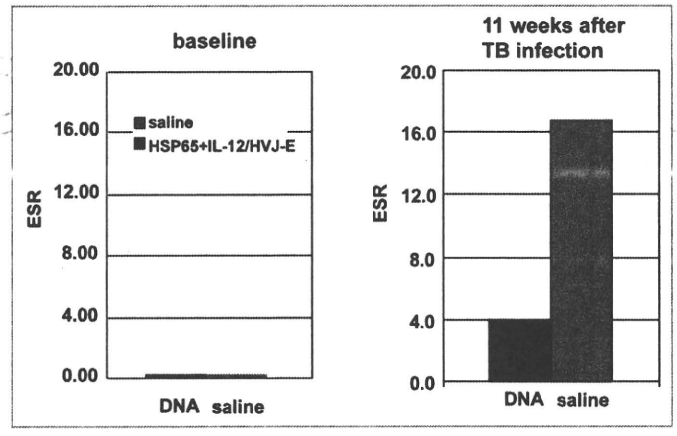


Figure 13. AUTHOR: ~~Please provide figure legend~~.

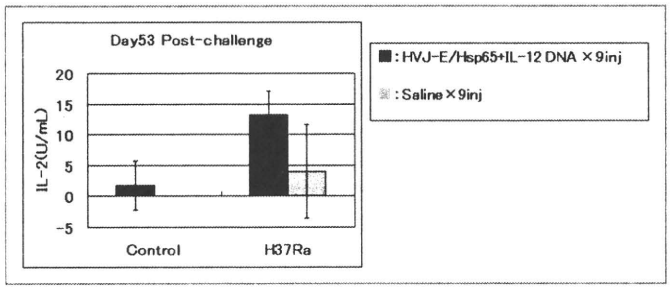


Figure 14. ~~AUTHOR: Please provide figure legend~~.

References

1. Yoshida S, Tanaka T, Kita Y, Kuwayama S, Kaneda Y, Okada M, et al. DNA vaccine using hemagglutinating virus of Japan-liposome encapsulating combination encoding mycobacterial heat shock protein 65 and interleukin-12 confers protection against *Mycobacterium tuberculosis* by T cell activation. Vaccine 2006; 24:1191-204.
2. Okada M, Kita Y, Nakajima T, Kanamaru N, Hashimoto S, Nagasawa T, et al. Evaluation of a novel vaccine (HVJ-liposome/HSP65 DNA+IL-12 DNA) against tuberculosis using the cynomologus monkey model of TB. Vaccine 2007; 25:2990-3.
3. Okada M, Kita Y. Tuberculosis vaccine development: The development of novel (preclinical) DNA vaccine. Human Vaccines 2010; 6:297-308.
4. Walsh GP, Tan EV, dela Cruz EC, Abalos RM, Villahermosa LG, Young LJ, et al. The *Philippine cynomolgus* monkey provides a new nonhuman primate model of tuberculosis that resembles human disease. Nat Med 1996; 2:430-6.
5. Kita Y, Tanaka T, Yoshida S, Ohara N, Kaneda Y, Kuwayama S, et al. Novel recombinant BCG and DNA-vaccination against tuberculosis in a cynomolgus monkey model. Vaccine 2005; 23:2132-5.
6. Saeki Y, Matsumoto N, Nakano Y, Mori M, Awai K, Kaneda Y. Development and characterization of cationic liposomes conjugated with HVJ (Sendai virus). Hum Gene Ther 1997; 8:2133-41.
7. Kaneda Y, Nakajima T, Nishikawa T, Yamamoto S, Ikegami H, Suzuki N, et al. Hemagglutinating virus of Japan (HVJ) envelope vector as a versatile gene delivery system. Mol Ther 2002; 6:219-26.
8. Kaneda Y. New vector innovation for drug delivery: development of fusigenic non-viral particles. Curr Drug Targets 2003; 4:599-602.
9. Kaneda Y, Yamamoto S, Nakajima T. Development of HVJ envelope vector and its application to gene therapy. Adv Genet 2005; 53:307-32.
10. Ito M, Yamamoto S, Nimura K, Hiraoka K, Tamai K, Kaneda Y. Rad51 siRNA delivered by HVJ envelope vector enhances the anti-cancer effect of cisplatin. J Gene Med 2005; 7:1044-52.
11. Mima H, Yamamoto S, Ito M, Tomoshige R, Tabata Y, Tamai K, et al. Targeted chemotherapy against intraperitoneally disseminated colon carcinoma using a cationized gelatin-conjugated HVJ envelope vector. Mol Cancer Ther 2006; 5:1021-8.
12. Okada M, Yoshimura N, Kaieda T, Yamamura Y, Kishimoto T. Establishment and characterization of human T hybrid cells secreting immunoregulatory molecules. Proc Natl Acad Sci USA 1981; 78:7717-21.
13. Okada M, Kita Y, Nakajima T, Kanamaru N, Hashimoto S, Nagasawa T, et al. Novel prophylactic and therapeutic vaccine against tuberculosis. Vaccine 2009; 27:3267-70.
14. Okada M, Okuno Y, Hashimoto S, Kita Y, Kanamaru N, Nishida Y, et al. Development of vaccines and passive immunotherapy against SARS corona virus using SCID-PBL/hu mouse models. Vaccine 2007; 25:3038-40.
15. Tanaka F, Abe M, Akiyoshi T, Nomura T, Sugimachi K, Kishimoto T, et al. The anti-human tumor effect and generation of human cytotoxic T cells in SCID mice given human peripheral blood lymphocytes by the in vivo transfer of the Interleukin-6 gene using adenovirus vector. Cancer Res 1997; 57:1335-43.
16. Huygen K. DNA vaccines: application to tuberculosis. Int J Tuberc Lung Dis 1998; 2:971-8.
17. Lowrie DB. DNA vaccines against tuberculosis. Curr Opin Mol Ther 1999; 1:30-3.
18. Hoft D. Tuberculosis vaccine development: goals, immunological design and evaluation. Lancet 2008; 372:164-75.
19. Gupta UD, Katoch VM, McMurray DN. Current status of TB vaccines. Vaccine 2007; 25:3742-51.
20. Flynn JL, Chan J. Immunology of tuberculosis. Annu Rev Immunol 2001; 19:93-129.
21. Rook GAW, Lowrie DB, Hernandez-Pando R. Immunotherapeutics for Tuberculosis on experimental animals: Is there a common pathway activated by effective protocols? J Inf Dis 2007; 196:191-8.
22. Kaufmann SHE. New vaccine for tuberculosis. The Lancet 2010; 375:85-94.

J Infect Chemother (2010) 16:360–363
DOI 10.1007/s10156-010-0057-5

NOTE

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

Shiomi Yoshida · Katsuhiro Suzuki · Tomotada Iwamoto · Kazunari Tsuyuguchi · Motohisa Tomita · Masaji Okada · Mitsunori Sakatani

Received: 4 November 2009 / Accepted: 1 March 2010 / Published online: 31 March 2010

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

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

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

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

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

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

S. Yoshida (✉) · K. Suzuki · K. Tsuyuguchi · M. Okada
Clinical Research Center, National Hospital Organization, Kinki-chuo Chest Medical Center, 1180 Nagasone-cho, Sakai 591-8555, Japan
e-mail: dustin@kch.hosp.go.jp

M. Tomita
Department of Clinical Microbiology, National Hospital Organization, Kinki-chuo Chest Medical Center, Sakai, Japan

T. Iwamoto
Department of Microbiology, Kobe Institute of Health, 4-6 Minatojima-nakamachi, Chuo-ku, Kobe 650-0046, Japan

M. Sakatani
Department of Respiratory Medicine, National Hospital Organization, Kinki-chuo Chest Medical Center, 1180 Nagasone-cho, Kita-ku, Sakai 591-8555, Japan

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Acknowledgments This work was supported by a Health and Labour Science Research Grant for Research on Emerging and Re-Emerging Infectious Diseases from the Ministry of Health, Labour and Welfare, Japan, and a Grant for a National Hospital Organization respiratory network study "Study of Respiratory Diseases (Tuberculosis, Lung Cancer, Diffuse Lung Diseases and Respiratory

Insufficiency) using a Network of 54 Hospitals of National Chest Diseases in Japan".

Conflict of interest statement None of the authors has any financial interest or financial conflict with the subject matter or materials discussed in this manuscript.

References

1. Drobniewski FA, Wilson SM. The rapid diagnosis of isoniazid and rifampin resistance in *Mycobacterium tuberculosis*—a molecular story. J Med Microbiol. 1998;47:189–96.
2. Rossau R, Traore H, De Beenhouwer H, Mijs W, Jannes G, de Rijk P, et al. Evaluation of the INNO-LiPA Rif.TB assay, a reverse hybridization assay for the simultaneous detection of *Mycobacterium tuberculosis* complex and its resistance to rifampin. Antimicrob Agents Chemother. 1997;41:2093–8.
3. Davies G, Cerri S, Richel L. Rifabutin for treating pulmonary tuberculosis.Cochrane Database Syst Rev 2007; 17: (4) CD005159.
4. Luna-Herrera J, Reddy MV, Gangadharam PR. In vitro and intracellular activity of rifabutin on drug-susceptible and multiple drug-resistant (MDR) tubercle bacilli. J Antimicrob Chemother. 1995;36:355–63.
5. Bodmer T, Zűrcher G, Imboden P, Telenti A. Mutation position and type of substitution in the β-subunit of the RNA polymerase influence in vitro activity of rifamycins in rifampicin-resistant *Mycobacterium tuberculosis*. J Antimicrob Chemother. 1995;35:345–8.
6. Cavusoglu C, Hilmioglu S, Guneri S, Bilgic A. Characterization of *rpoB* mutations in rifampin-resistant clinical isolates of *Mycobacterium tuberculosis* from Turkey by DNA sequencing and line probe assay. J Clin Microbiol. 2002;40:4435–8.
7. Saribaş Z, Kocagöz T, Alp A, Günalp A. Rapid detection of rifampin resistance in *Mycobacterium tuberculosis* isolates by heteroduplex analysis and determination of rifamycin cross-resistance in rifampin-resistant isolates. J Clin Microbiol. 2003;41:816–8.
8. Williams DL, Spring L, Collins L, Miller LP, Heifets LB, Gangadharam PRJ, et al. Contribution of *rpoB* mutations to development of rifamycin cross-resistance in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 1998;42:1853–7.
9. Yang B, Koga H, Ohno H, Ogawa K, Fukuda M, Hirata Y, et al. Relationship between antimycobacterial activities of rifampicin, rifabutin and KRM-1648 and *rpoB* mutations of *Mycobacterium tuberculosis*. J Antimicrob Chemother. 1998;42:621–8.
10. Cavusoglu C, Karaca-Derici Y, Bilgic A. In vitro activity of rifabutin against rifampicin-resistant *Mycobacterium tuberculosis* isolates with known *rpoB* mutations. Clin Microbiol Infect. 2004;10:662–5.
11. Wallace RJ Jr, Nash DR, Steele LC, Steingrube V. Susceptibility testing of slowly growing mycobacteria by a microdilution MIC method with 7H9 broth. J Clin Microbiol. 1986;24:976–81.
12. Kim B-J, Lee S-H, Lyu M-A, Kim S-J, Bai G-H, Kim S-J, et al. Identification of mycobacterial species by comparative sequence analysis of the RNA polymerase gene (*rpoB*). J Clin Microbiol. 1999;37:1714–20.
13. Heifets LB, Lindholm-Levy PJ, Iseman MD. Rifabutine: minimal inhibitory and bactericidal concentrations for *Mycobacterium tuberculosis*. Am Rev Respir Dis. 1988;137:719–21.
14. Heifets LB, Iseman MD. Determination of in vitro susceptibility of mycobacteria to ansamycin. Am Rev Respir Dis. 1985;132:710–1.
15. Uzun M, Erturan Z, Anğ O. Investigation of cross-resistance between rifampin and rifabutin in *Mycobacterium tuberculosis* complex strains. Int J Tuberc Lung Dis. 2002;6:164–5.

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。プライムー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),5),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

国立病院機構近畿中央胸部疾患センター臨床研究センター

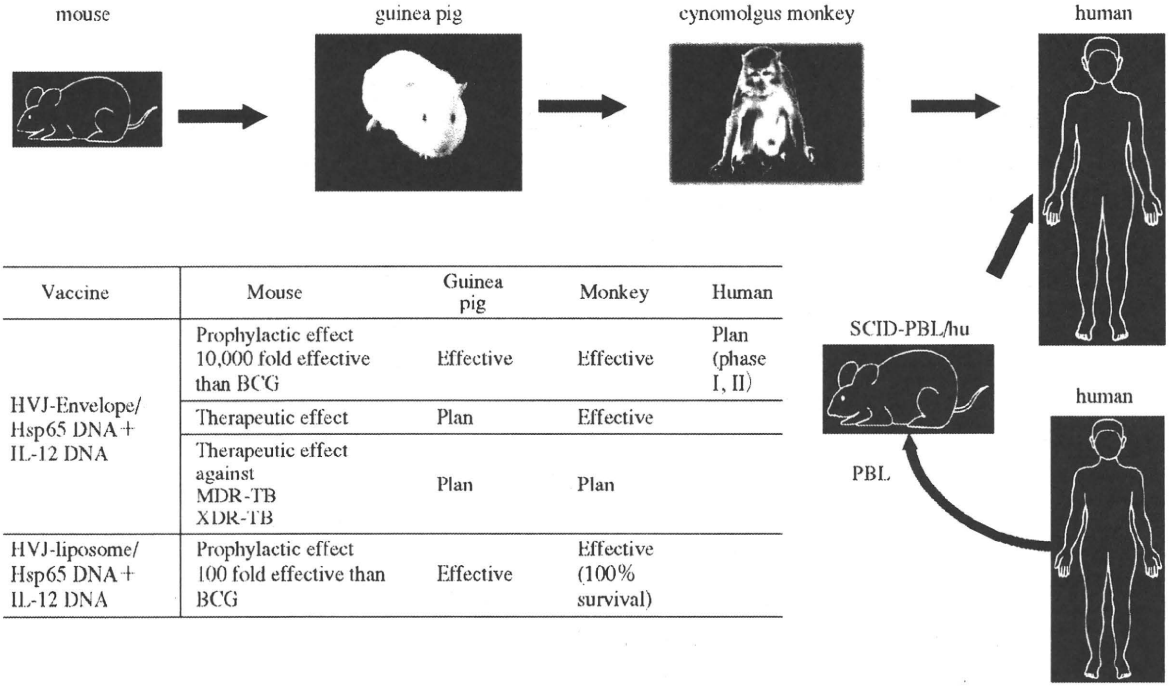
連絡先：岡田全司，国立病院機構近畿中央胸部疾患センター臨床研究センター，〒591-8555 大阪府堺市北区長曽根町1180
(E-mail: okm@kch.hosp.go.jp)
(Received 14 Mar. 2010)

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

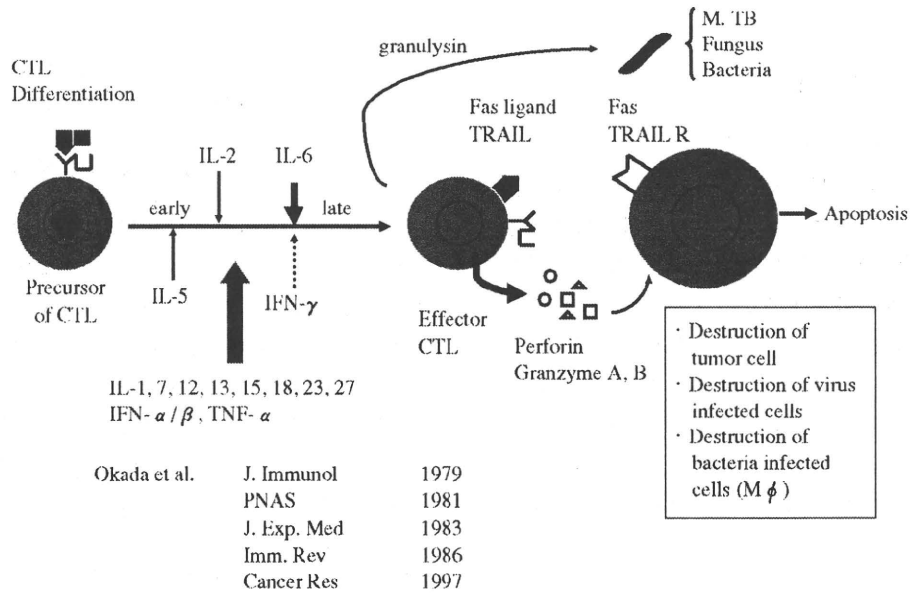


Fig. 2 Induction of cytotoxic T cells and killing mechanism

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

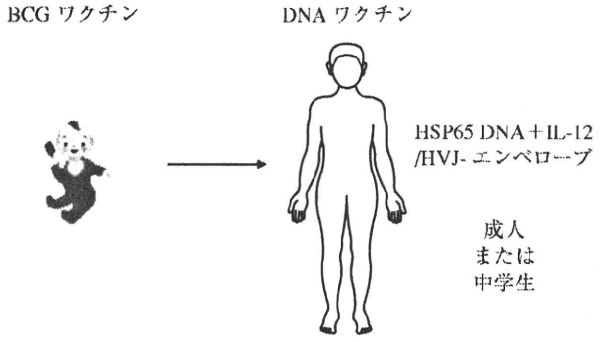


Fig. 3 新しい結核予防ワクチン（案）（DNAワクチン）

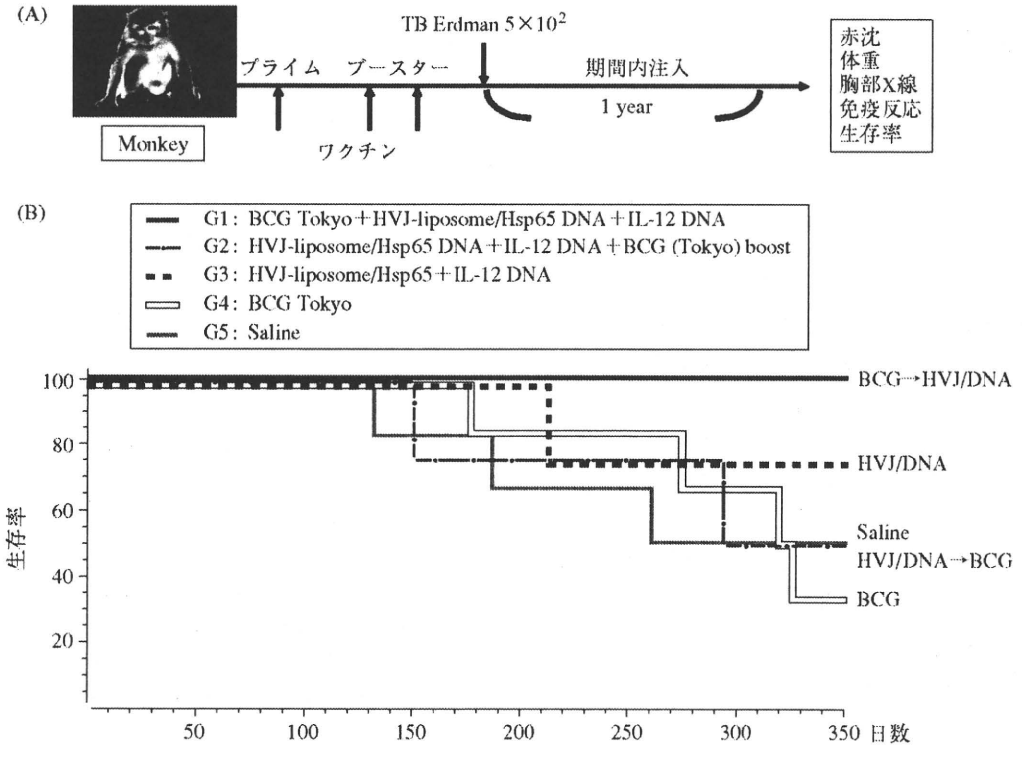


Fig. 4 ヒトの結核感染に最も近いカニクイザルを用いたHVJ-リポソーム/HSP-65 DNA＋IL12 DNAワクチンの結核予防効果

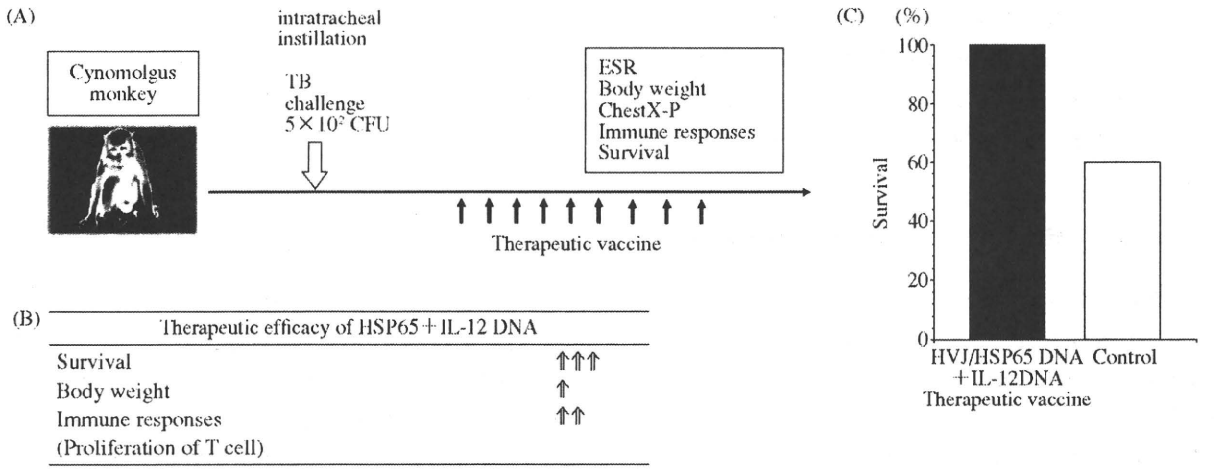


Fig. 5 Therapeutic effect of HVJ-Envelope/HSP65 DNA+IL-12DNA vaccine on TB-infected cynomolgus monkeys

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プライミングーブースター法（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ーブースター法で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。

文　献

1）岡田全司：結核“分子予防環境医学：生命科学研究の予防・環境医学への統合”（分子予防環境医学研究会編）．本の泉社，東京，2003，150-161.
2）Flynn JL, Chan J: Immunology of tuberculosis. Annu Rev Immunol. 2001 ; 19 : 93-129.
3）Okada M, Kita Y, Nakajima T, et al.: Evaluation of a novel vaccine (HVJ-liposome/HSP65 DNA+IL-12 DNA) against tuberculosis using the cynomolgus monkey model of TB. Vaccine. 2007 ; 25 : 2990-2993.
4）Yoshida S, Tanaka T, Kita Y, et al.: DNA vaccine using hemagglutinating virus of Japan-liposome encapsulating combination encoding mycobacterial heat shock protein 65 and interleukin-12 confers protection against *Mycobacterium tuberculosis* by T cell activation. Vaccine. 2006 ; 24 : 1191-1204.
5）岡田全司：The development of novel vaccines against tuberculosis. Jpn J Clin Immunol. 2008 ; 31 : 356-368.
6）Kita Y, Tanaka T, Yoshida S, et al.: Novel recombinant BCG

and DNA-vaccination against tuberculosis in a cynomolgus monkey model. Vaccine. 2005 ; 23 : 2132−2135.

7) 岡田全司：結核ワクチン.「結核」第4版, 泉　孝英, 網谷良一編, 医学書院, 東京, 2006, 50−58.

8) Okada M, Kita Y, Nakajima T, et al.: Novel prophylactic and therapeutic vaccine against tuberculosis. Vaccine. 2009 ; 27 : 3267−3270.

9) Stenger S, Hanson DA, Teitelbaum R, et al.: An antimicrobial activity of cytolytic T cells mediated by granulysin. Science. 1998 ; 282 : 121−125.

10) Tanaka F, Abe M, Akiyoshi T, et al.: The anti-human tumor effect and generation of human cytotoxic T cells in SCID mice given human peripheral blood lymphocytes by the *in vivo* transfer of the Interleukin-6 gene using adenovirus vector. Cancer Res. 1997 ; 57 : 1335−1343.

11) Okada M, Yoshimura N, Kaieda T, et al.: Establishment and characterization of human T hybrid cells secreting immunoregulatory molecules. Proc Natl Acad Sci USA. 1981 ; 78 : 7718−7721.

12) Okada M, Sakaguchi N, Yoshimura N, et al.: B cell growth factors and B cell differentiation factor from human T hybridomas. Two distinct kinds of B cell growth factor and their synergism in B cell proliferation. J Exp Med. 1983 ; 157 : 583−590.

13) Okada M, Kitahara M, Kishimoto S, et al.: IL-6/BSF-2 functions as a killer helper factor in the *in vitro* induction of cytotoxic T cells. J Immunol. 1988 ; 141 : 1543−1549.

14) Okada M, Kita Y: Tuberculosis vaccine development: The development of novel (preclinical) DNA vaccine. Human Vaccine. 2010 ; 6 : 1−12.

15) Okada M, Kita Y, Nakajima T, et al.: Procedia in Vaccinology. Vol. 2, 3rd. Vaccine Global Congress, Singapore, 2009.

16) Skeiky YA, Alderson MR, Ovendale PJ, et al.: Differential immune responses and protective efficacy induced by components of a tuberculosis polyprotein vaccine, Mtb72F, delivered as naked DNA or recombinant protein. J Immunol. 2004 ; 172 : 7618−7628.

17) McShane H, Pathan AA, Sander CR, et al.: Recombinant modified vaccinia virus Ankara expressing antigen 85A boosts BCG-primed and naturally acquired antimycobacterial immunity in humans. Nat Med. 2004 ; 10 : 1240−1244.