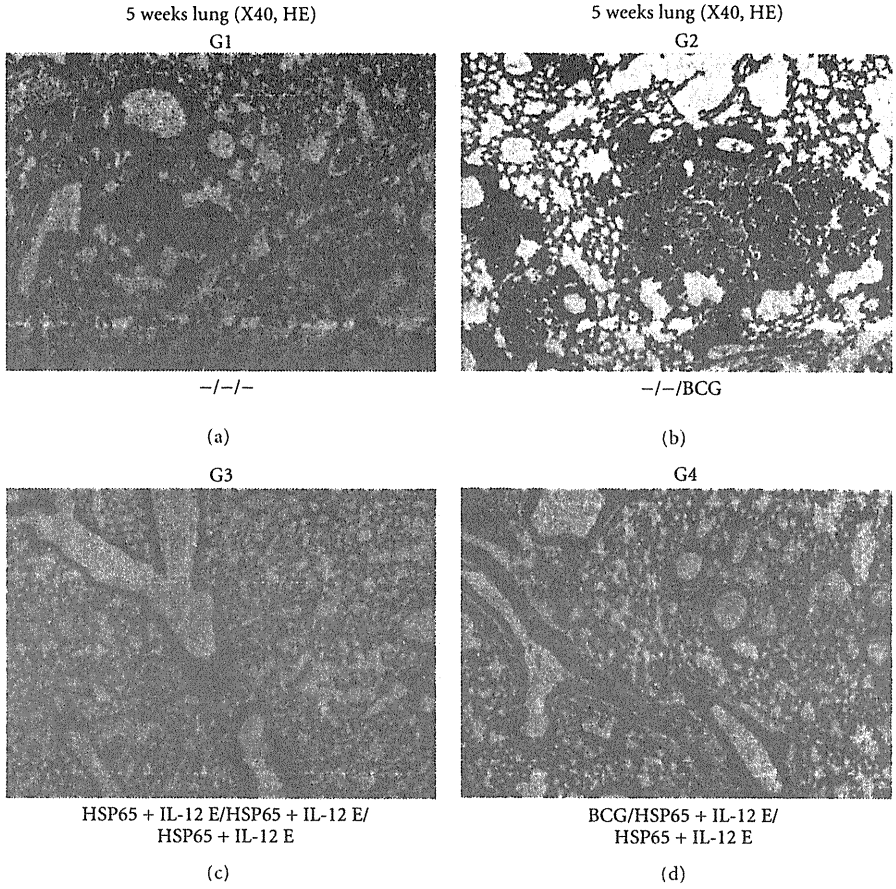

FIGURE 6: Histopathological analysis of vaccinated mice 5 weeks after *M. tuberculosis* challenge. Representative photomicrographs of lung tissue sections harvested from the G1 naive control group, G2 (BCG alone) group, G3 group (DNA/DNA/DNA), and G4 group (BCG/DNA/DNA) are shown (5 weeks after *M. tuberculosis* challenge, hematoxylin and eosin staining, × 4 objective). There was much infiltration of mononuclear cells and extensive parenchymal destruction by large, poorly demarcated granuloma in the lungs from the G1 (naive control) group and G2 (BCG alone) group. In the G3 (DNA/DNA/DNA) group and G4 (BCG/DNA/DNA) group, there was less inflammation, and only a few granulomas were observed.

Boosting BCG vaccination with MVA85A downregulates the immunoregulatory cytokine TGF-β1 [29]. 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 [30]. Several vaccines have been used with a prime-boost strategy to complement immune responses [31].

DNA vaccines are a relatively new approach to induce immunities for the protection of infectious diseases [14, 19, 22, 32–34]. Prophylactic and therapeutic DNA vaccines were established by using several kinds of vectors such as HVJ-liposome, HVJ-E, adenovirus vector, adenoassociated virus vector, and lentivirus vector [19–22, 35, 36]. In order to explore the preclinical use of a tuberculosis DNA vaccine combination of *IL-12* DNA with *hsp65* DNA, we chose the HVJ-based delivery system (HVJ-liposome and HVJ-E). These systems have high transfection efficiency and are available for repeated *in vivo* gene transfection without reduction of gene transfer efficiency or apparent toxicity. These characters of HVJ-liposomes support the feasibility of its clinical application not only for cancer gene therapy but also for DNA vaccinations. In a recent study, highly efficient gene expression in muscle cells was observed for several weeks when pcDNA3 plasmid containing the human tumor antigen genes, *MAGE-1* and *MAGE-3*, were encapsulated in HVJ-liposomes and injected intramuscularly in mice [37]. Effective induction of $CD4^+$ T-cell responses by a hepatitis B core particle-based HIV vaccine was achieved by subcutaneous administration of HVJ-liposomes in mice [38]. HVJ-liposomes were also very effective as a mucosal vaccine against HIV infection [39]. Thus, it is likely that HVJ proteins may be responsible for the induction of a robust immune response. No side effects were observed when repetitive injections of HVJ-liposomes were performed in mice, rats, or monkeys. We have previously developed an HVJ-E using inactivated Sendai virus, as a nonviral vector for drug delivery [40–42]. It can be used for efficient delivery of DNAs, siRNAs, proteins, and anticancer drugs into cells

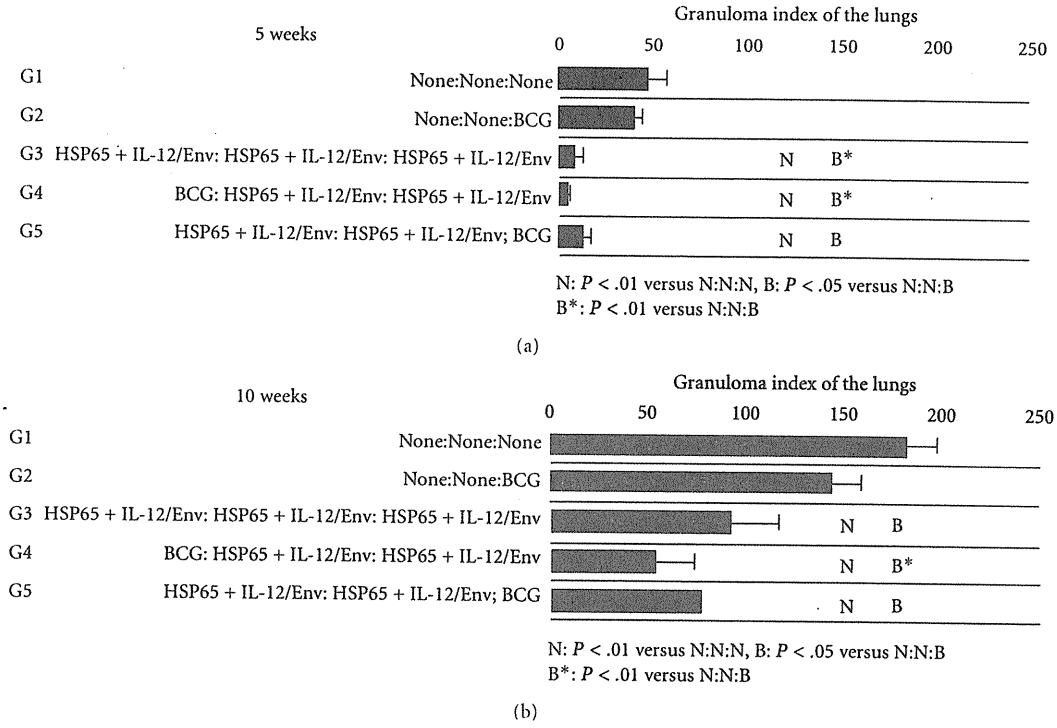

FIGURE 7: Granuloma index of the G1, G2, G3, G4, and G5 (DNA/DNA/BCG) groups in the lungs 5 weeks and 10 weeks after *M. tuberculosis* challenge. Results are expressed as the mean ± S.D. of triplicates of 5 mice per group. The statistical significance of differences between the groups was determined by Dunnett test, $P < .01$ as compared with the naive (N) group or the BCG alone (B) group. $P < .05$ as compared with the BCG alone (B*) group. The statistical significance of differences ($P < .05$) of granuloma index of 5 weeks G3 group compared to the G4 group.

both *in vitro* and *in vivo* [40, 43, 44]. Therefore, HVJ-E was used as an efficient and safe vector for DNA vaccine against TB in the present study.

Mycobacterial heat shock protein 65 (HSP65) is a potential target for protective immunity and has been studied extensively [19]. Several studies have reported that *hsp65* DNA vaccines can strongly induce protective immune responses in mice against virulent *M. tuberculosis* infections [20–22]. Protection is attributed to the establishment of a cellular immune response dominated by HSP65-specific T cells which produce IFN-γ and are cytotoxic towards infected cells. Furthermore, Lowrie and colleagues have reported that this vaccine reduces bacterial loads in mice infected with *M. tuberculosis* when given therapeutically after infection [32].

One of the major roles of IL-12 is the induction of IFN-γ-mediated immune responses to microbial pathogens. Cooper and colleagues have demonstrated the importance of IL-12 in generation of the protective response to tuberculosis [45]. Coadministration of the *IL-12* gene, which induces an IFN-γ-mediated immune response to microbial pathogens, with various tuberculosis DNA vaccines including *hsp65* DNA [46], and 35 K MW DNA [47], may boost the efficacy of these DNA vaccines to the levels achieved with BCG in the mouse model, although an inhibitory effect rather than a synergistic effect on immunotherapy was observed in mice coadministered *hsp65* DNA vaccine plus the *IL-12* gene [32].

In conclusion, we have shown efficacy of a novel HVJ-E DNA vaccine encapsulating HSP65 DNA with IL-12 DNA in the mouse model of TB. These results suggest that HSP65 + IL-12/HVJ could be a promising candidate for a new tuberculosis vaccine superior to BCG. To this aim, protective efficacy and immune responses were further studied in nonhuman primates before proceeding to human clinical trials.

In Japan and other countries, BCG is inoculated into human infants up to 6 months after birth. Therefore, BCG prime in infants and HSP65 + hIL-12/HVJ boost in adults (including junior high school students, high school students, and the elderly) may be required for significant improvement of clinical protective efficacy against TB. Thus, our results with the HSP65 + hIL-12/HVJ vaccine in a murine prophylactic model and cynomolgus monkey prophylactic model provide a significant rationale for moving this vaccine into clinical trials. Indeed, multiple animal models are available to accumulate essential data on the HVJ-E DNA vaccine in anticipation of a phase I clinical trial.

4. Conclusions

Vaccination by BCG prime with a novel vaccine (IgHSP65 + mIL-12/HVJ-E) boost resulted in significant protective efficacy (10,000-fold greater than BCG alone) against TB

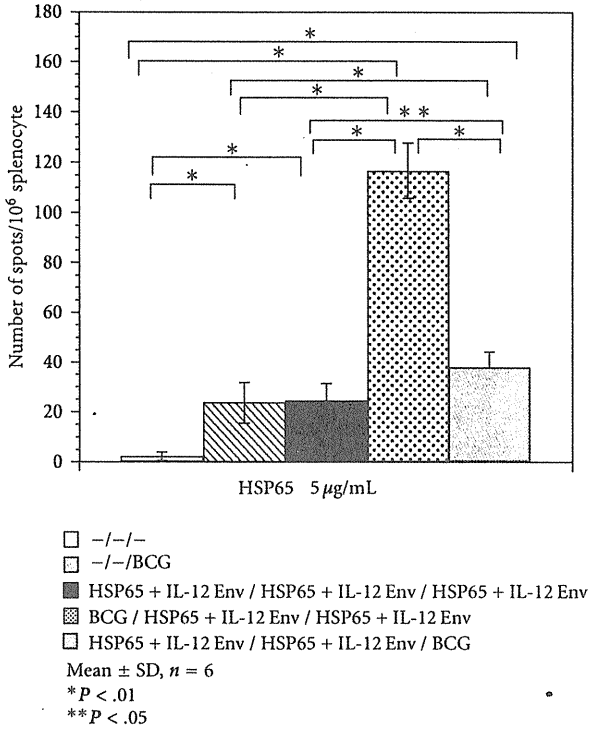

FIGURE 8: ELISPOT assay for IFN-γ antigen-specific responses in the spleens of vaccinated mice following stimulation with rHSP65 protein. Spleen cell cultures were stimulated with rHSP65 protein for 20 h. The numbers of IFN-γ-secreting cells specific for rHSP65 protein per million cells were determined individually by ELISPOT assay. Results are expressed as the mean ± S.D. of 6 wells of 3 mice per group. The statistical significance of differences between individual groups in the number of IFN-γ-secreting cells was determined by Dunnett test. The statistical significance of differences ($P < .01$) of the G1 (naive) group compared to the G2 (BCG alone group), G3 (DNA/DNA/DNA), G4 (BCG/DNA/DNA), or G5 (DNA/DNA/BCG). The statistical significance of the G2 group difference ($P < .01$) compared to the G4 or the G5. The statistical significance of the G3 group differences ($P < .01$) compared to the G4. $P < .01$; the G4 group compared to the G5. The statistical significance of the G3 group differences ($P < .05$) compared to the G5.

infection in the lungs of mice. In addition to bacterial loads, significant protective immunity was demonstrated by histopathological analysis of the lungs. This vaccine showed extremely significant protection against TB, suggesting that further development for eventual testing in clinical trials may be warranted.

Acknowledgments

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

References

[1] J. L. Flynn and J. Chan, "Immunology of tuberculosis," *Annual Review of Immunology*, vol. 19, pp. 93–129, 2001.

[2] J. Hess, U. Schaible, B. Raupach, and S. H. E. Kaufmann, "Exploiting the immune system: toward new vaccines against intracellular bacteria," *Advances in Immunology*, vol. 75, pp. 1–88, 2000.

[3] A. Geluk, K. E. Van Meijgaarden, K. L. M. C. Franken et al., "Identification of major epitopes of *Mycobacterium tuberculosis* AG85B that are recognized by HLA-A*0201-restricted $CD8^+$ T cells in HLA-transgenic mice and humans," *Journal of Immunology*, vol. 165, no. 11, pp. 6463–6471, 2000.

[4] A. Lalvani, R. Brookes, R. J. Wilkinson et al., "Human cytolytic and interferon γ-secreting CD8+ T lymphocytes specific for *Mycobacterium tuberculosis*," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 1, pp. 270–275, 1998.

[5] P. Wong and E. G. Pamer, "CD8 T cell responses to infectious pathogens," *Annual Review of Immunology*, vol. 21, pp. 29–70, 2003.

[6] H. McShane, S. Behboudi, N. Goonetilleke, R. Brookes, and A. V. S. Hill, "Protective immunity against *Mycobacterium tuberculosis* induced by dendritic cells pulsed with both CD8(+)- and CD4(+)-T-cell epitopes from antigen 85A," *Infection and Immunity*, vol. 70, no. 3, pp. 1623–1626, 2002.

[7] I. K. Srivastava and M. A. Liu, "Gene vaccines," *Annals of Internal Medicine*, vol. 138, no. 7, pp. 550–148, 2003.

[8] M. Okada and T. Kishimoto, "The potential application and limitation of cytokine/growth factor manipulation in cancer therapy," in *Cell proliferation in Cancer: Regulatory Mechanisms of Neoplastic Cell Growth*, L. Pusztai, C. Lewis, and E. Yap, Eds., pp. 218–244, Oxford University Press, New York, NY, USA, 1996.

[9] M. J. Roy, M. S. Wu, L. J. Barr et al., "Induction of antigen-specific $CD8^+$ T cells, T helper cells, and protective levels of antibody in humans by particle-mediated administration of a hepatitis B virus DNA vaccine," *Vaccine*, vol. 19, no. 7-8, pp. 764–778, 2000.

[10] T. P. Le, K. M. Coonan, R. C. Hedstrom et al., "Safety, tolerability and humoral immune responses after intramuscular administration of a malaria DNA vaccine to healthy adult volunteers," *Vaccine*, vol. 18, no. 18, pp. 1893–1901, 2000.

[11] A. C. Moore and A. V. S. Hill, "Progress in DNA-based heterologous prime-boost immunization strategies for malaria," *Immunological Reviews*, vol. 199, pp. 126–143, 2004.

[12] S. Yoshida, S. I. Kashiwamura, Y. Hosoya et al., "Direct immunization of malaria DNA vaccine into the liver by gene gun protects against lethal challenge of Plasmodium berghei sporozoite," *Biochemical and Biophysical Research Communications*, vol. 271, no. 1, pp. 107–115, 2000.

[13] J. D. Boyer, M. A. Chattergoon, K. E. Ugen et al., "Enhancement of cellular immune response in HIV-1 seropositive individuals: a DNA-based trial," *Clinical Immunology*, vol. 90, no. 1, pp. 100–107, 1999.

[14] K. Huygen, "DNA vaccines: application to tuberculosis," *International Journal of Tuberculosis and Lung Disease*, vol. 2, no. 12, pp. 971–978, 1998.

[15] D. B. Lowrie, "DNA vaccines against tuberculosis," *Current Opinion in Molecular Therapeutics*, vol. 1, no. 1, pp. 30–33, 1999.

[16] I. M. Orme, "The search for new vaccines against tuberculosis," *Journal of Leukocyte Biology*, vol. 70, no. 1, pp. 1–10, 2001.

[17] T. M. Doherty and P. Andersen, "Tuberculosis vaccine development," *Current Opinion in Pulmonary Medicine*, vol. 8, no. 3, pp. 183–187, 2002.

[18] D. M. McMurray, "Guinea pig model of tuberculosis," in *Tuberculosis: Pathogenesis, Protection, and Control*, pp. 113–134, ASM Press, Washington, DC, USA, 1994.

[19] S. Yoshida, T. Tanaka, Y. Kita 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*, vol. 24, no. 8, pp. 1191–1204, 2006.

[20] Y. Kita, T. Tanaka, S. Yoshida et al., "Novel recombinant BCG and DNA-vaccination against tuberculosis in a cynomolgus monkey model," *Vaccine*, vol. 23, no. 17-18, pp. 2132–2135, 2005.

[21] M. Okada, Y. Kita, T. Nakajima et al., "Evaluation of a novel vaccine (HVJ-liposome/HSP65 DNA + IL-12 DNA) against tuberculosis using the cynomolgus monkey model of TB," *Vaccine*, vol. 25, no. 16, pp. 2990–2993, 2007.

[22] M. Okada, Y. Kita, T. Nakajima et al., "Novel prophylactic and therapeutic vaccine against tuberculosis," *Vaccine*, vol. 27, no. 25-26, pp. 3267–3270, 2009.

[23] Y. Saeki, N. Matsumoto, Y. Nakano, M. Mori, K. Awai, and Y. Kaneda, "Development and characterization of cationic liposomes conjugated with HVJ (Sendai virus): reciprocal effect of cationic lipid for in vitro and in vivo gene transfer," *Human Gene Therapy*, vol. 8, no. 17, pp. 2133–2141, 1997.

[24] K. Miki, T. Nagata, T. Tanaka et al., "Induction of protective cellular immunity against *Mycobacterium tuberculosis* by recombinant attenuated self-destructing *Listeria monocytogenes* strains harboring eukaryotic expression plasmids for antigen 85 complex and MPB/MPT51," *Infection and Immunity*, vol. 72, no. 4, pp. 2014–2021, 2004.

[25] C. C. Dascher, K. Hiromatsu, X. Xiong et al., "Immunization with a mycobacterial lipid vaccine improves pulmonary pathology in the guinea pig model of tuberculosis," *International Immunology*, vol. 15, no. 8, pp. 915–925, 2003.

[26] I. Sugawara, T. Udagawa, S. C. Hua et al., "Pulmonary granulomas of guinea pigs induced by inhalation exposure of heat-treated BCG Pasteur, purified trehalose dimycolate and methyl ketomycolate," *Journal of Medical Microbiology*, vol. 51, no. 2, pp. 131–137, 2002.

[27] Y. Kita, M. Okada, T. Nakajima et al., "Development of therapeutic and prophylactic vaccine against Tuberculosis using monkey and transgenic mice models," *Human Vaccines*, vol. 7, pp. 108–114, 2011.

[28] H. McShane, A. A. Pathan, C. R. Sander et al., "Recombinant modified vaccinia virus Ankara expressing antigen 85A boosts BCG-primed and naturally acquired antimycobacterial immunity in humans," *Nature Medicine*, vol. 10, no. 11, pp. 1240–1244, 2004.

[29] H. A. Fletcher, A. A. Pathan, T. K. Berthoud et al., "Boosting BCG vaccination with MVA85A down-regulates the immunoregulatory cytokine TGF-β1," *Vaccine*, vol. 26, no. 41, pp. 5269–5275, 2008.

[30] M. Okada and Y. Kita, "Tuberculosis vaccine development: the development of novel (preclinical) DNA vaccine," *Human Vaccines*, vol. 6, no. 4, pp. 297–308, 2010.

[31] S. H. Kaufmann, G. Hussey, and P. H. Lambert, "New vaccines for tuberculosis," *The Lancet*, vol. 375, no. 9731, pp. 2110–2119, 2010.

[32] D. B. Lowrie, R. E. Tascon, V. L. D. Bonato et al., "Therapy of tuberculosis in mice by DNA vaccination," *Nature*, vol. 400, no. 6741, pp. 269–271, 1999.

[33] D. F. Hoft, "Tuberculosis vaccine development: goals, immunological design, and evaluation," *The Lancet*, vol. 372, no. 9633, pp. 164–175, 2008.

[34] U. D. Gupta, V. M. Katoch, and D. N. McMurray, "Current status of TB vaccines," *Vaccine*, vol. 25, no. 19, pp. 3742–3751, 2007.

[35] F. Tanaka, M. Abe, T. Akiyoshi 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 Research*, vol. 57, no. 7, pp. 1335–1343, 1997.

[36] H. McShane, A. A. Pathan, and C. R. Sander, "Recombinant modified vaccinia virus Ankara expressing antigen 85A boosters BCG-primed and naturally acquired antimycobacterial immunity in humans," *Nature Medicine*, vol. 10, pp. 1240–1244, 2008.

[37] M. Tanaka, Y. Kaneda, S. Fujii et al., "Induction of a systemic immune response by a polyvalent melanoma-associated antigen DNA vaccine for prevention and treatment of malignant melanoma," *Molecular Therapy*, vol. 5, no. 3, pp. 291–299, 2002.

[38] S. Takeda, K. Shiosaki, Y. Kaneda et al., "Hemagglutinating virus of Japan protein is efficient for induction of CD4(+) T-cell response by a hepatitis B core particle-based HIV vaccine," *Clinical Immunology*, vol. 112, no. 1, pp. 92–105, 2004.

[39] G. Sakaue, T. Hiroi, Y. Nakagawa et al., "HIV mucosal vaccine: nasal immunization with gp160-encapsulated hemagglutinating virus of Japan-liposome induces antigen-specific CTLs and neutralizing antibody responses," *Journal of Immunology*, vol. 170, no. 1, pp. 495–502, 2003.

[40] Y. Kaneda, T. Nakajima, T. Nishikawa et al., "Hemagglutinating virus of Japan (HVJ) envelope vector as a versatile gene delivery system," *Molecular Therapy*, vol. 6, no. 2, pp. 219–226, 2002.

[41] Y. Kaneda, "New vector innovation for drug delivery: development of fusigenic non-viral particles," *Current Drug Targets*, vol. 4, no. 8, pp. 599–602, 2003.

[42] Y. Kaneda, S. Yamamoto, and T. Nakajima, "Development of HVJ Envelope Vector and Its Application to Gene Therapy," *Advances in Genetics*, vol. 53, pp. 307–332, 2005.

[43] M. Ito, S. Yamamoto, K. Nimura, K. Hiraoka, K. Tamai, and Y. Kaneda, "Rad51 siRNA delivered by HVJ envelope vector enhances the anti-cancer effect of cisplatin," *Journal of Gene Medicine*, vol. 7, no. 8, pp. 1044–1052, 2005.

[44] H. Mima, S. Yamamoto, M. Ito et al., "Targeted chemotherapy against intraperitoneally disseminated colon carcinoma using a cationized gelatin-conjugated HVJ envelope vector," *Molecular Cancer Therapeutics*, vol. 5, no. 4, pp. 1021–1028, 2006.

[45] A. M. Cooper, J. Magram, J. Ferrante, and I. M. Orme, "Interleukin 12 (IL-12) is crucial to the development of protective immunity in mice intravenously infected with *Mycobacterium tuberculosis*," *Journal of Experimental Medicine*, vol. 186, no. 1, pp. 39–45, 1997.

[46] K. M. Baek, S. Y. Ko, M. Lee et al., "Comparative analysis of effects of cytokine gene adjuvants on DNA vaccination against *Mycobacterium tuberculosis* heat shock protein 65," *Vaccine*, vol. 21, no. 25-26, pp. 3684–3689, 2003.

[47] E. Martin, A. T. Kamath, H. Briscoe, and W. J. Britton, "The combination of plasmid interleukin-12 with a single DNA vaccine is more effective than Mycobacterium bovis (bacille Calmette-Guèrin) in protecting against systemic *Mycobacterim avium* infection," *Immunology*, vol. 109, no. 2, pp. 308–314, 2003.

Human Vaccines 7: Supplement, 60-67; January/February 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 Japan; [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 Japan; [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: 9/24/10; Accepted 10/7/10
DOI: 10.4161.hv.7.0.14563

Introduction

Tuberculosis is a major global threat to human health, with about two 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

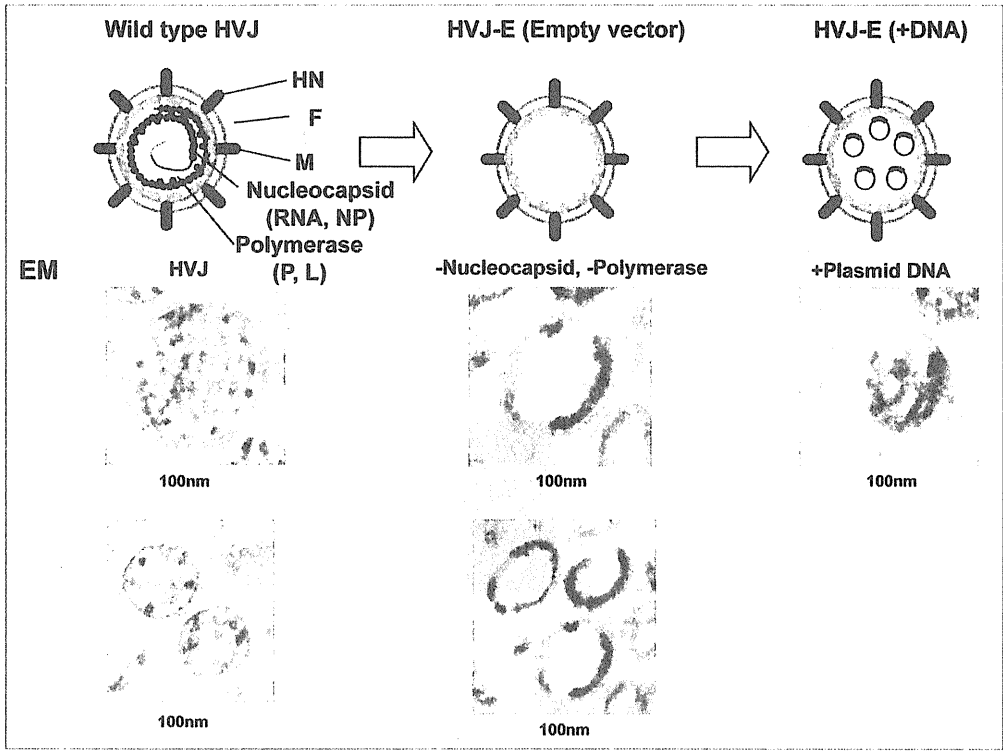

Figure 1. HVJ-envelope vaccination. pcDNA3-1/HSP65DNA+IL-12DNA were incorporated into HVJ-Envelope Empty Vector (Non-Viral Vector). Cartoons of HVJ-Envelope Empty Vector in the presence or absence of DNA were shown. Photographs of an electronic microscope (EM) of HVJ-Envelope Empty Vector were also shown.

therapeutic effect of this 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 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

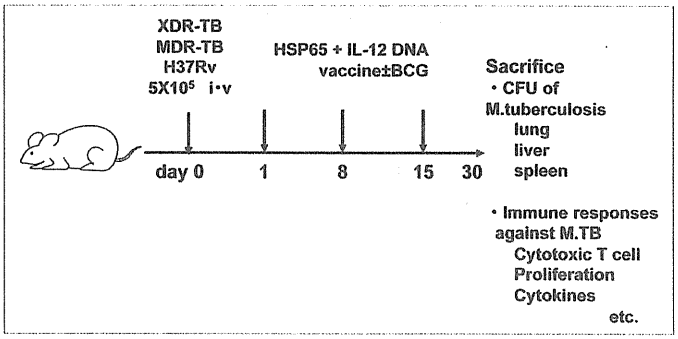

Figure 2. TNFR gene disrupted DBA/1 mice and IL-6 gene disrupted DBA/1 mice were treated with HVJ-Envelope/HSP65 DNA+IL-12 DNA vaccine three times i.m at 1, 8 and 15 days after the challenge of 5 x 10^5 CFU MDR-TB i.v. At 30 days after MDR-TB challenge, the lungs, spleens and livers were aseptically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

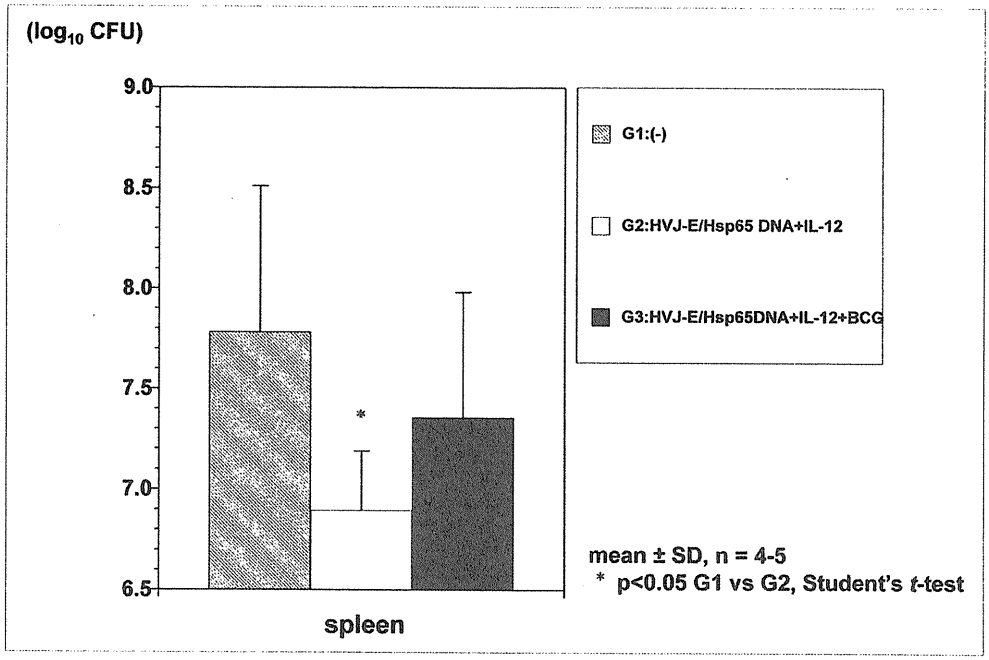

Figure 3. Therapeutic efficacy of HVJ-Envelope/HSP65 DNA+IL-12 DNA vaccine on the MDR-TB infection in the TNFR gene disrupted DBA/1 mice. Groups of mice were challenged by intravenous injection with 5×10^5 CFU MDR-TB, and then treated three times with HVJ-Envelope/HSP65 DNA+IL-12 DNA vaccine, as described in Materials and Methods. Thirty days after challenge, therapeutic efficacy was measured by enumerating the bacterial loads (CFU) in the spleen. Results are expressed as the mean log10 ± S.D.of CFU. The statistical significance of differences between individual groups in the number of CFU was determined studen't test (n=4-5). *P<0.05, the statistical significance of differences (P<0.05) of G1 (naive) group compared to G2 (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 (5×10^2) by intratracheal instillation. Immune responses and survival were examined as described in our previous studies in references 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 3 and 4**, 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. 5**). By using this model, therapeutic efficacy of this vaccine was also observed (**Fig. 6**). 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 significantly therapeutic efficacy even in SCID-PBL/hu mice which exerted human T-cell immune responses (**Table 1**).

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.

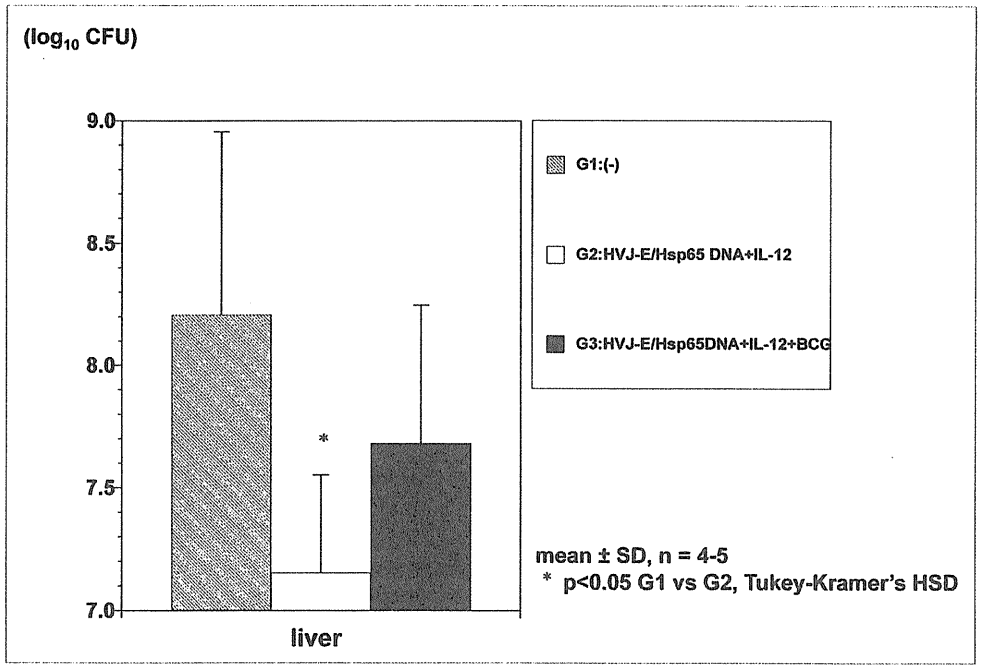

Figure 4. Therapeutic efficacy of HVJ-Envelope/HSP65 DNA+IL-12 DNA vaccine on the MDR-TB infection in the TNFR gene disrupted DBA/1 mice. Groups of mice were challenged by intravenous injection with 5×10^5 CFU MDR-TB, and then treated three times with HVJ-Envelope/HSP65 DNA + IL-12 DNA vaccine, as described in Materials and Methods. Thirty days after challenge, therapeutic efficacy was measured by enumerating the bacterial loads (CFU) in the liver. Results are expressed as the mean log10 ± S.D.of CFU. The statistical significance of differences between individual groups in the number of CFU was determined studen't test (n=4-5). *P<0.05, the statistical significance of differences (P<0.05) of G1 (naïve) group compared to G2 (HVJ-Envelope/HSP65 DNA+IL-12 DNA).

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. 7**). Recombinant 15 K granulysin protein enhanced the in vitro induction of human cytotoxic T cells in the 5 day MLC culture (**Fig. 8**). 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. 9**). Granulysin vaccines (recombinant 15 K granulysin and 15 K granulysin DNA vaccine) exerted strong 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

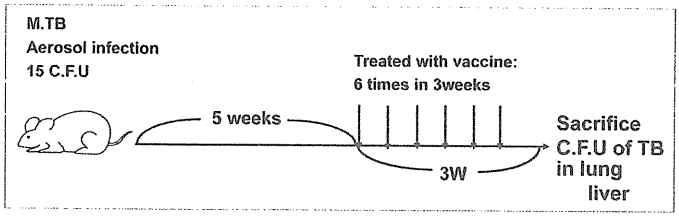

Figure 5. Therapeutic efficacy of TB vaccines using chronic TB infection model by aerosol challenge of TB. Therapeutic efficacy was evaluated by chronic TB infection model of mice using aerosol challenge of TB. Mice were challenged intratracheally by aerosol of *M.tuberculosis* H37Rv in saline (15 CFU/mouse) 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.

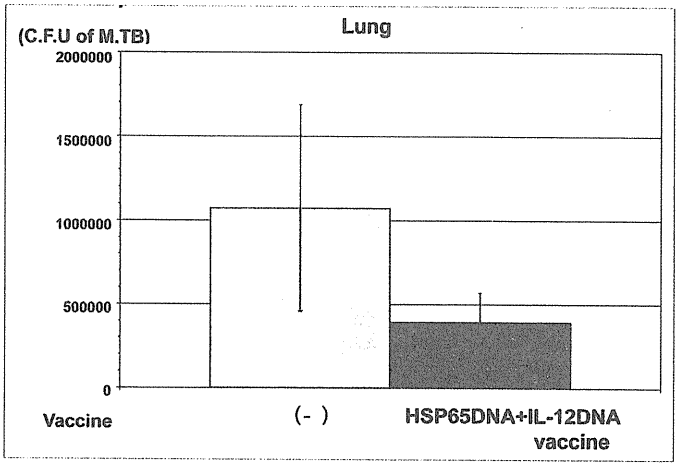

Figure 6. Therapeutic efficacy of HVJ-Envelope / HSP65 DNA + IL-12 DNA vaccine on chronic TB infection model of mice using aerosol chamber. DBA/1 mice were challenged intratracheally by aerosol of *M.tuberculosis* H37Rv (15 CFU/mouse). After the treatment of HVJ-Envelope / HSP65 DNA + IL-12 DNA vaccine 6 times i.m., therapeutic efficacy was measured by enumerating bacterial loads (CFU) in the lungs from vaccinated mice.

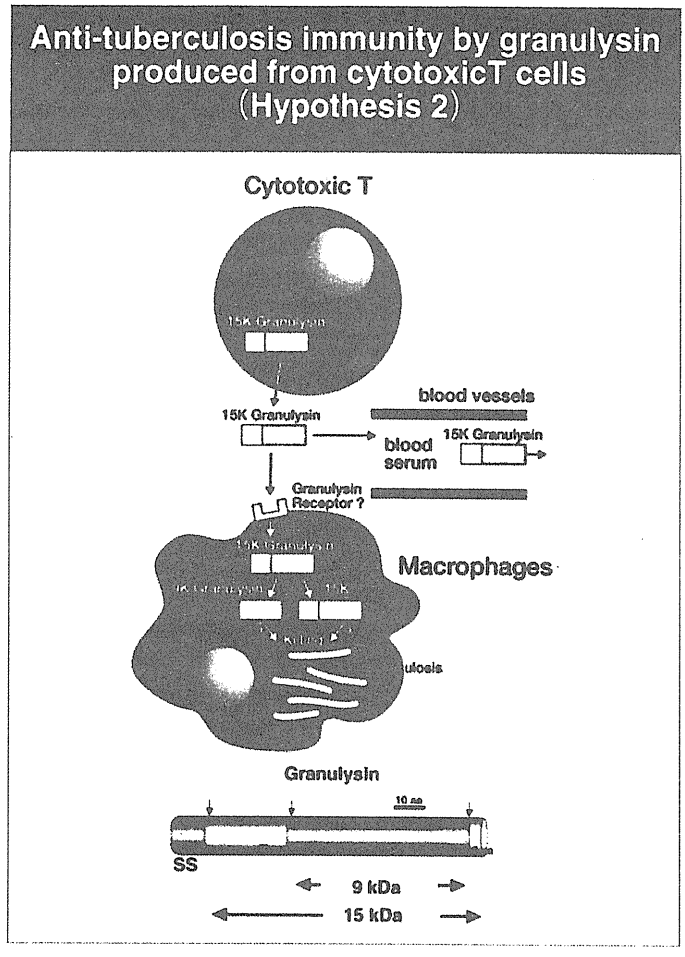

Figure 7. The hypothesis models of anti-tuberculosis immunity by granulysin produced from human cytotoxic T cell.

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. 10).

Therapeutic efficacy was evaluated by survival, ESR, body weight, immune responses, chest X-ray findings and PPD skin test (Fig. 11).

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. 12). All five monkeys were alive in the group of HVJ-Envelope/HSP65DNA+IL-12DNA vaccine (100% survival) at 16 weeks after challenge. In contrast, only three monkeys out of five were alive in the saline control group (60% survival) (Fig. 12 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 13. 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. 14). The induction of IL-2 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

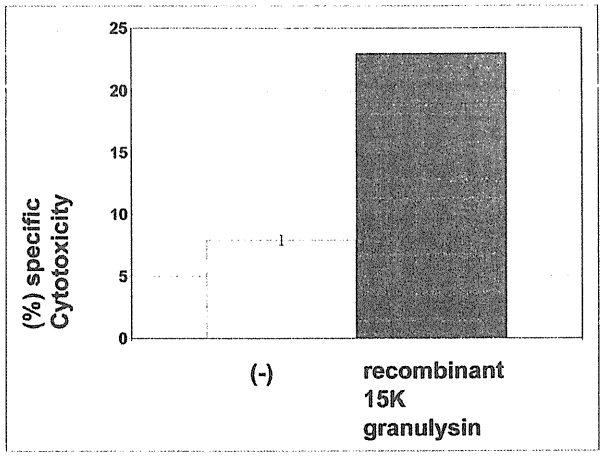

Figure 8. In vitro induction of human cytotoxic T cell by the stimulation with recombinant 15K granulysin protein. T cells from human PBL were obtained by nylon-wool column method. 1×10^6 T cells were cultured with human $CESS^{MMC}$ cells (Mitomycic C treated CESS tumor cells) in the presence of 15K granulysin for 5 days. CTL activity of effector cells was assayed using ^{51}Cr-labbelled CESS cells. Results are expressed as % Specific cytotoxicity ± S.D.. % Specific cytotoxicity was calculated as

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

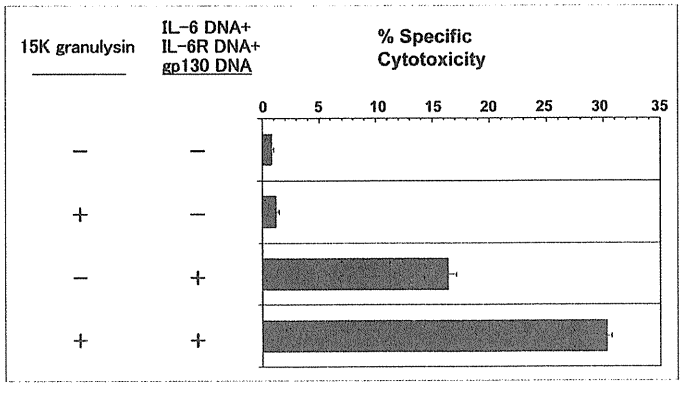

Figure 9. Synergistic effect of recombinant 15K granulysin + IL-6 related DNA on the in vivo induction of CTL specific for HSP65 antigen. C57BL/6mice were injected with killed TB H37Ra and then treated with recombinant 15K granulysin protein i.p 6 times and / or IL-6 DNA + IL-6 Receptor DNA + gp130 DNA using adenovirus vector i.m. 3weeks 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 ± S.D.

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 references 21 and 22.

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%.

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.

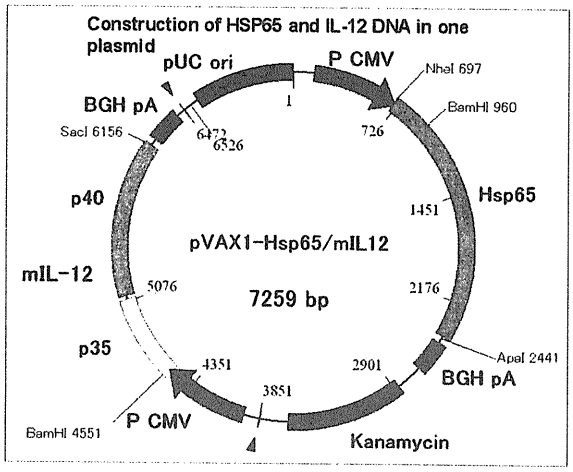

Figure 10. Construction of DNA vaccine for clinical trial. HVJ-Envelope/HSP65 DNA+IL-12DNA vaccine was constructed for GMP-level-vaccine which contains two kinds of DNA in one plasmid vector (pVAX1) for clinical therapeutic trial.

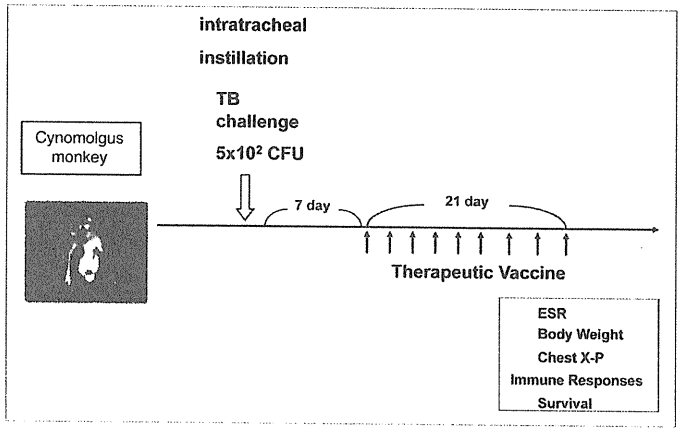

Figure 11. Evaluation of therapeutic efficacy of HVJ-Envelope/HSP65 DNA+IL-12DNA vaccine on TB-infected cynomolgus monkeys. Cynomolgus monkeys were vaccinated nine times with HVJ-Envelope with expression plasmid of both HSP65 and human IL-12 (HSP65+hIL-12/HVJ:400ug i.m.), one week after the challenge 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.

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.

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.

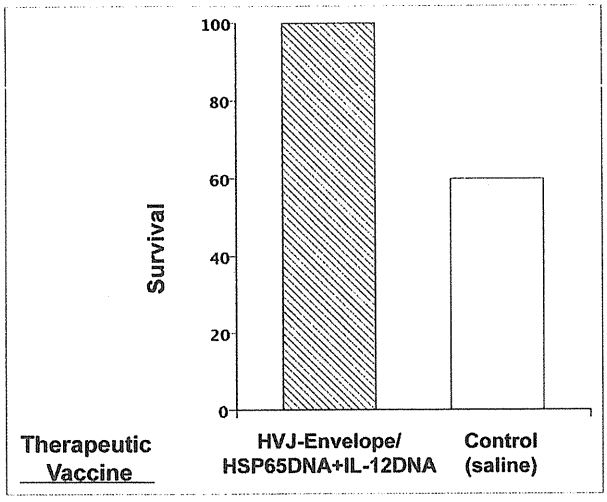

Figure 12. Survival of monkeys treated with HVJ-Envelope/HSP65 DNA+IL-12DNA vaccine after the infection of TB. Therapeutic efficacy was evaluated by survival of monkeys. Survival of monkeys treated with HVJ-Envelope/HSP65 DNA+IL-12DNA at 19 weeks after the challenge of TB by intratracheal instillation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

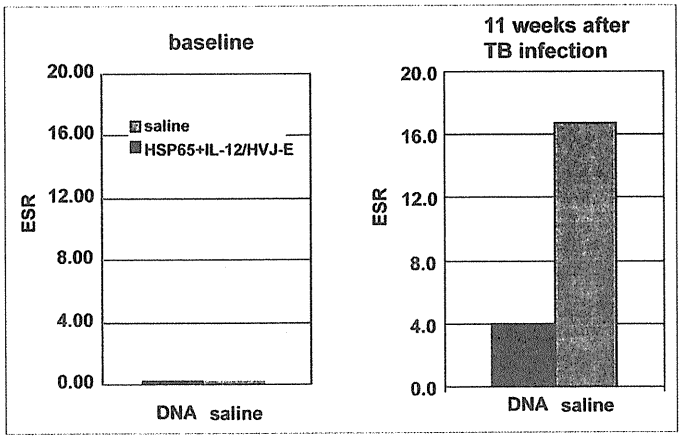

Figure 13. Impovement of ESR by the treatment of HVJ-Envelope/HSP65 DNA+IL-12DNA vaccine. Therapeutic efficacy was evaluated by ESR of the monkeys at 11 weeks after M.tuberculosis infection.

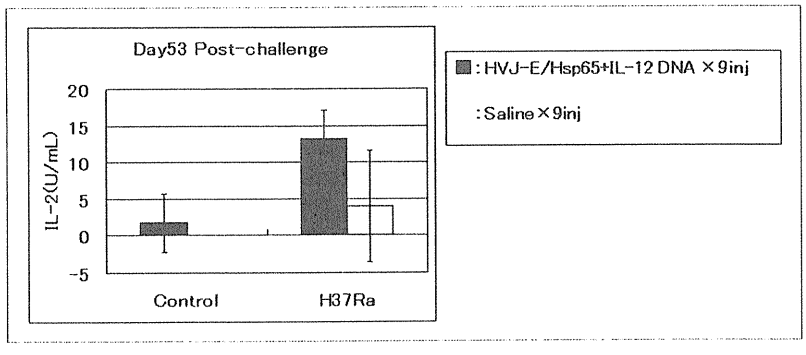

Figure 14. Augmentation of IL-2 production from PBL in the monkeys treated with HVJ-Envelope/HSP65 DNA+IL-12 DNA vaccine. Peripheral blood lymphocytes (PBL) were cultured with killed TB, H37Ra for 3 days. Supernatants were harvested after 3 day culture. IL-2 activity in the culture supernatants was assessed by ELISA. IL-2 activity (U/ml) was shown.

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.

Human Vaccines 7: Supplement, 108-114; January/February 2011; © 2011 Landes Bioscience

Development of therapeutic and prophylactic vaccine against tuberculosis using monkey and transgenic mice models

Yoko Kita,[1] Masaji Okada,[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] Yasushi Takamori,[3] David N. McMurray,[6] E.C. de la 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 Japan; [2]Ikeda Laboratory; GenomIdea Inc.; Midorigaoka, Ikeda; [3]Division of Gene Therapy Science; Graduate School of Medicine; Osaka University; Suita, Osaka Japan; [4]Department of Medical Zoology; Jichi-Med.Sch; Minamikawachi-machi; Tochigi Japan; [5]Cell Therapy Research Division; Stem Cell Institute; Minatoku, 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: monkey, prime-boost method, HVJ-envelope/HSP65DNA + IL-12DNA, *Mycobacterium tuberculosis*, vaccine, 15 KDa granulyin, granulysin transgenic mouse, patients with tuberculosis, prophylactic efficacy, SCID-PBL/hu

Abbreviations: HVJ, hemagglutinating virus of Japan; Tg, transgenic; MDR-TB, multi-drug resistant tuberculosis; 15 K granulysin, 15 kilodalton granulysin; 9 K granulysin, 9 kilodalton granulysin; PBL, peripheral blood lymphocyte; ESR, erythrocyte sedimentation rate

Purpose: BCG is not efficacious against *M. tuberculosis* (TB) in adult. Therefore, novel TB vaccines were established by using three kinds of animal models (cynomolgus monkey model which is the best animal model of human TB, IL-2R knock out SCID mice as a human immune model and granulysin transgenic mouse).

Methods and Results: DNA vaccine expressing TB Hsp65 and IL-12 was delivered by the hemagglutinating virus of Japan (HVJ)-envelope. The BCG prime followed by Hsp65 + IL-12/HVJ vaccine boost showed a synergistic effect in the TB-infected cynomolgus monkey (100% survival). In contrast, 33% of monkeys were alive in BCG alone group. Furthermore, the prolongation of survival period of the monkey was observed by the combination of BCG and DNA vaccine even when the boost was performed after long-term period (4 mounths) from prime. This combination also improved the erythrocyte sedimentation rate (ESR), increased the body weight and augmented the proliferation of PBL and IL-12 production at higher levels than BCG alone or saline. Furthermore, this vaccine exerted therapeutic efficacy in IL-2R knock out SCID-PBL/hu mice, which were transplanted with human T cells. Granulysin is an important defensive molecule expressed by human T cells and NK cells and has a cytolytic activity against microbes including *Mycobacterium tuberculosis* (TB) and tumors. Expression of 15 kD (15 K) granulysin protein and mRNA in CD8 positive T cells in the patients infected with drug sensitive (TB) or multi-drug resistant M. tuber*culosis (MDR-TB)* were lower than that in the healthy volunteers, suggesting that granulysin treatment might improve the tuberculous disease in human. Therefore, we established two kinds of granulysin transgenic mice (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, including the decrease of the number of TB and augmentation of the CTL activity. These are the first findings which demonstrate in vivo effects of 15 K granulysin and 9 K granulysin against TB infection. Moreover, DNA vaccine expressing 15 K granulysin showed a therapeutic activity against TB in mice.

Conclusion: These data indicate that monkey, IL-2R gene-knock out SCID-PBL/hu and granulysin transgenic mice models provide useful tools for the development of novel vaccines (HVJ-Envelope/Hsp65 DNA + IL-12 DNA vaccine and granulysin vaccine) against TB.

*Correspondence to: Masaji Okada; Email: okm@kch.hosp.go.jp
Submitted: 09/24/10; Accepted: 10/10/10
DOI: 10.4161/hv.7.0.14571

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 as well as prophylactic vaccines against TB is required.

Cynomolgus monkey model is the best animal TB model as reported by Walsh and Tan.[1] TB infection in the cynomolgus monkey is very similar to human TB disease.[1-3] In the present

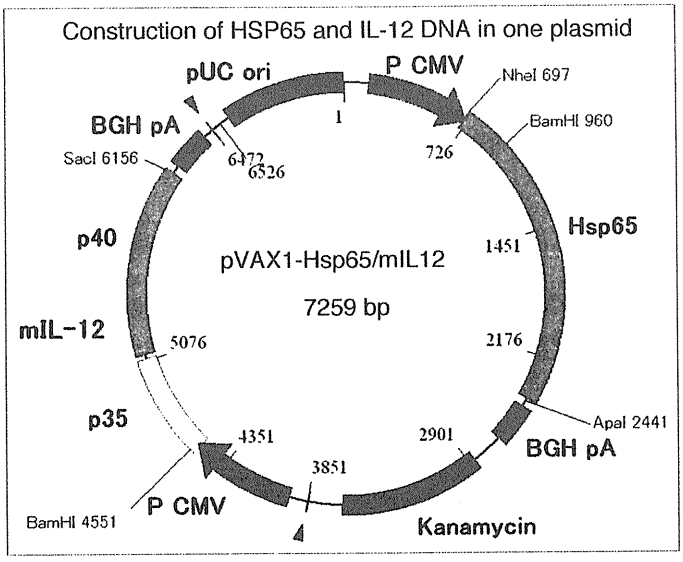

Figure 1. Construction of DNA vaccine for clinical trial. HVJ-Envelope/HSP65DNA + IL-12DNA vaccine was constructed for GMP-level-vaccine which contains two kinds of DNA in one plasmid vector (pVAX1) for clinical prophylactic trial.

study, the long-term prime-boost period prophylactic efficacy of 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 investigated using GMP-level-vaccine which contains

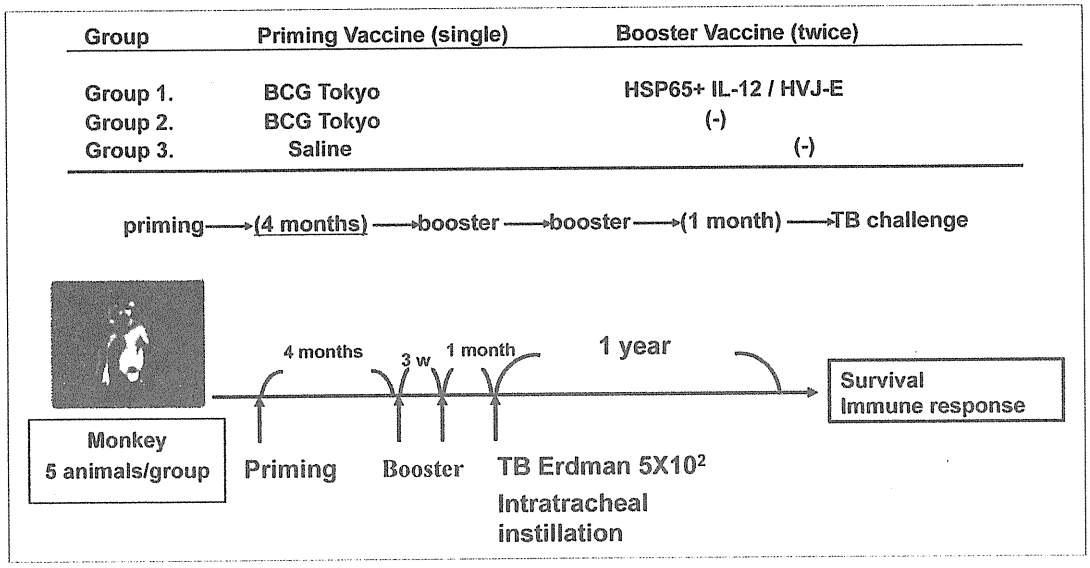

Figure 2. Evaluation of prophylactic efficacy of HVJ-Envelop/HSP65DNA + IL-12DNA vaccine on the infection of cynomolgus monkeys. Protective efficacy of HSP65 + IL-12/HVJ and BCG using prime-boost method against TB challenged cynomolgus monkeys. Group of animals were vaccinated three times (every three 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. Four months after the prime BCG vaccine, 2° vaccine was immunized. Three 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.

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 Group 1 (BCG prime—DNA vaccine boost) monkeys, the regimen of vaccines improved ESR, compared to the regimen of Group 3 (saline control group) or that of Group 2 (BCG alone control group) (Fig. 3).

This vaccination method (BCG prime—DNA vaccine boost) also increased the body weight of four TB-infected monkeys out of five in Group 1 as shown in Figure 4. In contrast, two monkeys in Group 3 (saline) or two monkeys in Group 2 (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 four 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 Group 1 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

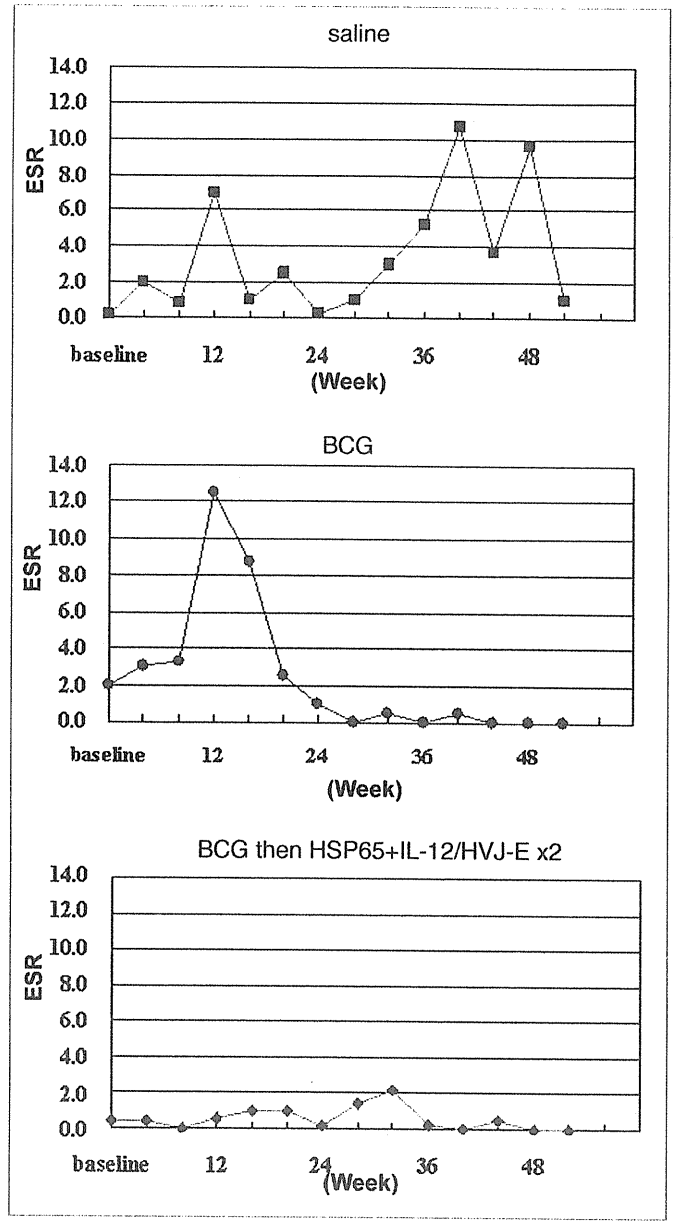

Figure 3. Improvement of erythrocyte sedimentation rate (ESR) in the cynomolgus monkeys immunized with BCG prime-HVJ-envelope/HSP65DNA + IL-12DNA boost vaccine. Cynomolgus monkeys were immunized and challenged as described in Figure 2. ESR of all monkeys was evaluated every month and mean values of ESR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

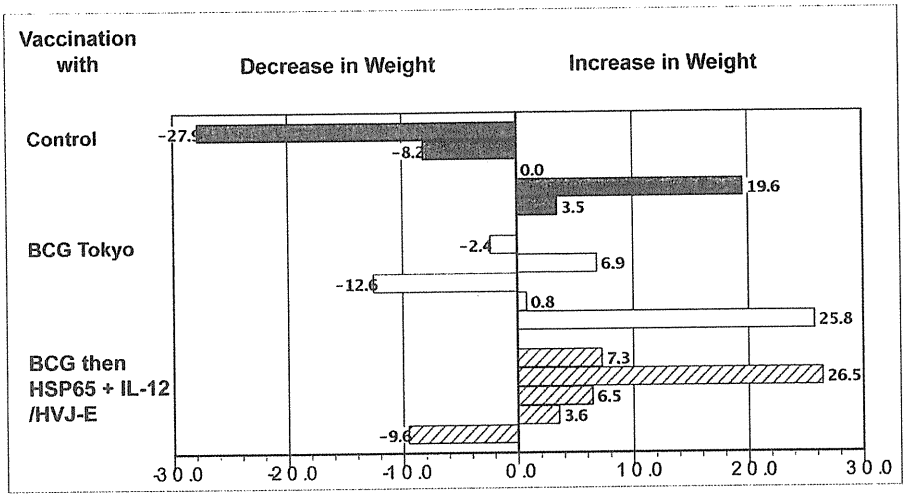

Figure 4. The increase in the body weight of monkeys vaccinated with HVJ-Envelope/Hsp65 DNA + IL-12 DNA. Monkeys vaccinated with BCG prime-HVJ-Envelope/Hsp65 DNA + IL-12 DNA vaccine boost were challenged as described in Figure 2. Body weight of all monkeys was evaluated every month and values of body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

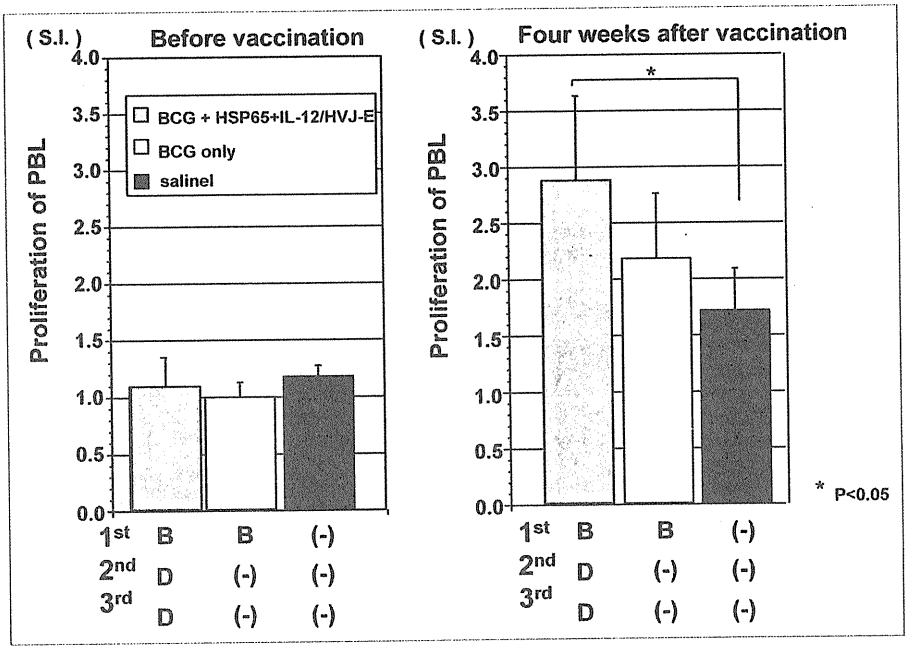

Figure 5. Augmentation of the proliferation of PBL in the monkeys immunized with BCG prime—HVJ-Envelope/Hsp65 DNA + IL-12 DNA boost vaccine. The proliferation of PBL (base line: before vaccination) from monkeys and PBL from monkeys vaccinated and challenged as described in Figure 2 were shown. Stimulation 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 (four months) 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 this BCG prime-HVJ-Envelope/HSP65 DNA + IL-12 DNA boost had fewer and smaller lesions in the lungs and significantly

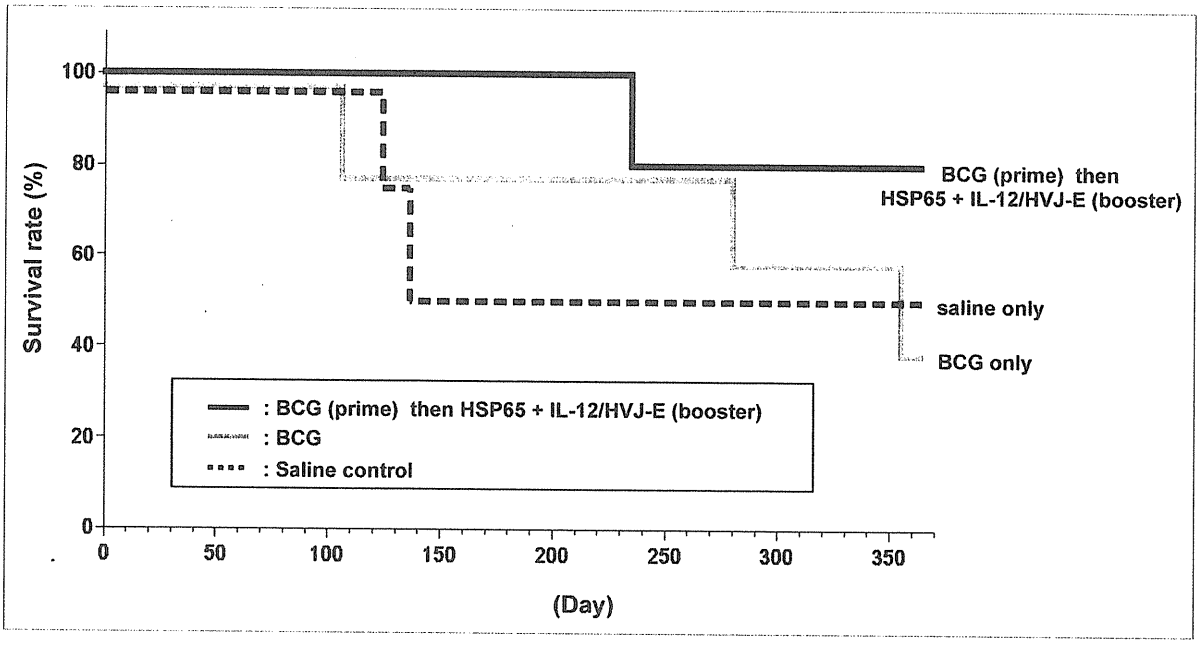

Figure 6. Protective efficacy of Hsp65 + IL-12/HVJ and BCG using prime-boost method against TB challenged cynomolgus monkeys. Group of animals were vaccinated three times as described in Figure 2. Group of animals were vaccinated with (1°) BCG Tokyo, (2°) Hsp65 + IL-12/HVJ, (3°) Hsp65 + IL-12/HVJ = G_1(—). 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*.

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 two monkeys out of six 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]

In Japan and other countries, the BCG vaccine is inoculated into human infants (0–6 months after birth). Therefore, BCG

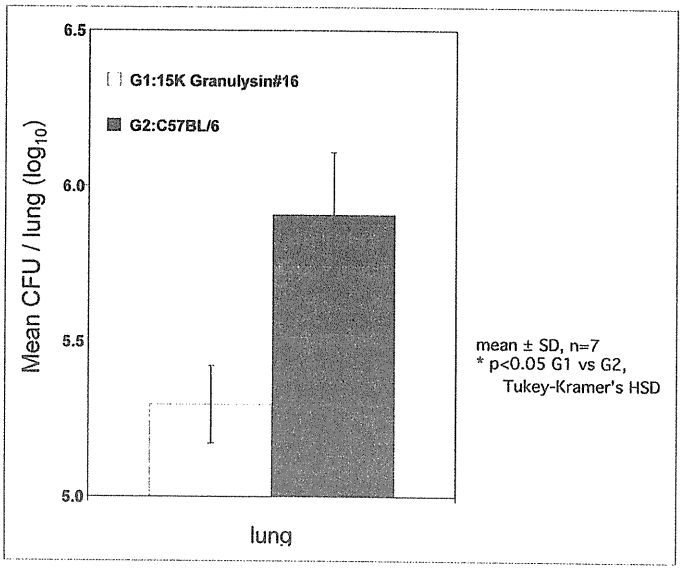

Figure 7. In vivo inhibition of the growth of *M. tuberculosis* in the 15 K granulysin transgenic mice. (In vivo anti-TB effect of 15 K granulysin transgenic mouse.) Seven 15 K granulysin #16 transgenic mice and seven wild type C57BL/6 mice were injected with 5 x 10^5 H37RV *M. tuberculosis* i.v. 4 weeks 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 ($p < 0.05$).

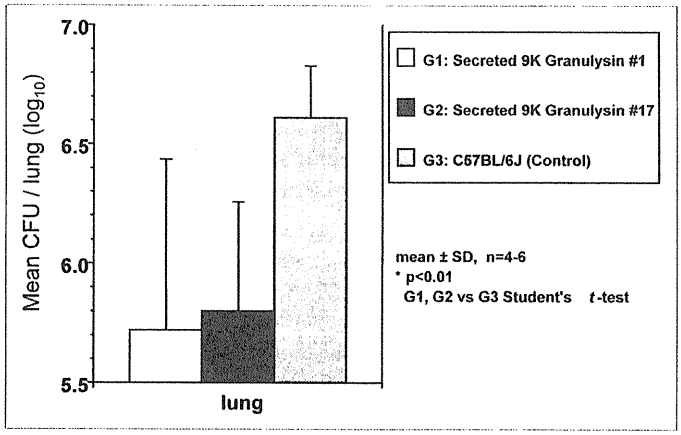

Figure 8. In vivo inhibition of the growth of *M. tuberculosis* in the 9 K granulysin transgenic mice. (In vivo anti-TB effect of 9 K granulysin transgenic mouse). Five 9 K granulysin #1 transgenic mice, five 9 K granulysin #7 mice and five wild type C57BL/6 mice were injected with 5 x 10^5 H37RV *M. 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 three 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/HSP65DNA + 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: $p < 0.05$).

prime in infants and HSP65 + hIL-12/HVJ boosts for adults (including junior high school students, high school students and old persons) may be required for the significant improvement of clinical protective efficacy against TB.

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. Two types of 15 K granulysin

Tg mice (#3, #16), three types of 9 K granulysin Tg mice (#15, #17, #18) and six 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 wks).[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. Oada 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. Stenger S, Hanson DA, Teitelbaum R, Dewan P, Niazi KR, Froelich CJ, et al. An antimicrobial activity of cytolytic T cells mediatd 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.