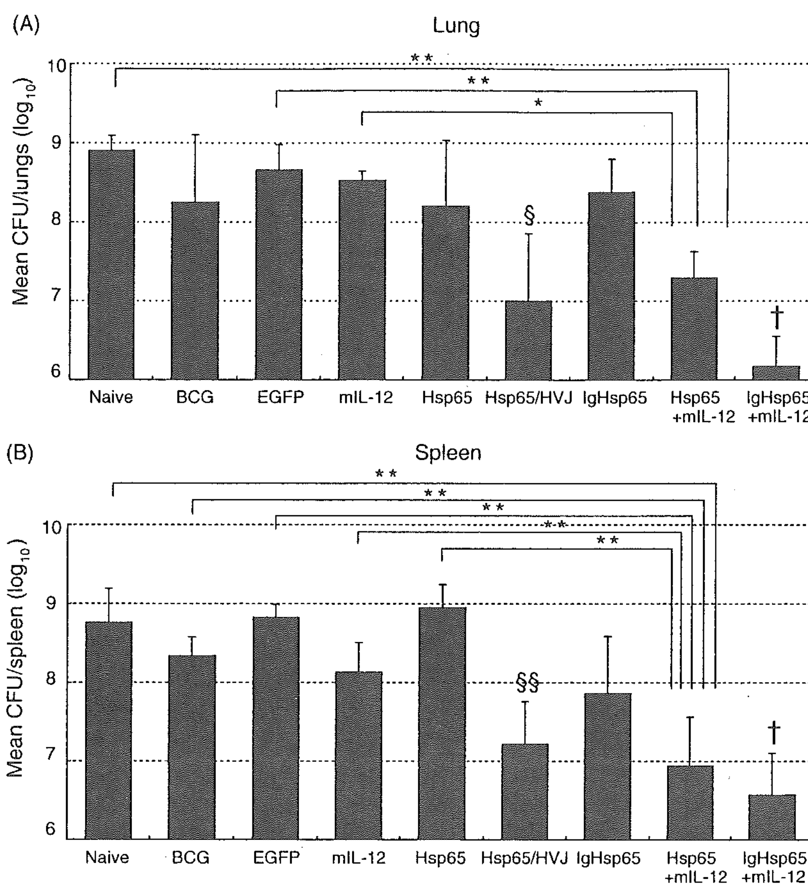

Fig. 2. The effect of a combination of mIL-12 expression vector and Hsp65-based DNA vaccines and comparison of different vaccines on the protective efficacy against challenge with *M. tuberculosis*. Groups of mice were vaccinated once with Hsp65 DNA, IgHsp65 DNA and a combination of mIL12 DNA via gene gun or three times with Hsp65/HVJ via intramuscular route and challenged intravenously with *M. tuberculosis* H37Rv as described in Section 2. Ten weeks after challenge, protection was measured by enumerating bacterial loads (CFU) in the lungs and spleen from vaccinated mice. Reduction of bacterial load was expressed as the mean $\log_{10}$ difference in CFU in the organs of the naive and vaccinated mice. The statistical significance of differences between individual groups in the number of CFU was determined by Tukey–Kramer's HSD test ($n = 4$–5). * and **, the statistical significance of differences ($P < 0.05$ and $P < 0.01$) compared to Hsp65 DNA + mIL-12 DNA group, respectively; †, the statistical significance of differences ($P < 0.01$) of IgHsp65 DNA + mIL-12 DNA group compared to the naive, BCG, EGFP DNA, mIL12 DNA, Hsp65 DNA and IgHsp65 DNA groups; §, the statistical significance of differences of Hsp65/HVJ group compared to BCG group ($P < 0.05$) in the lungs; §§, the statistical significance of differences of Hsp65/HVJ group compared to Hsp65 DNA ($P < 0.01$) and BCG ($P < 0.05$) groups in the spleen.

mIL-12 DNA via gene gun were challenged intravenously with *M. tuberculosis* H37Rv. The bacterial loads of the naive and vaccinated mice were compared 10 weeks after challenge (Fig. 2). Consistent with the previous report by Lima et al. [43], gene gun vaccination with Hsp65 DNA alone did not result in significant protective immunity as assessed by the bacterial load in the lungs or spleen. Vaccination with IgHsp65 DNA, which encodes the additional mouse Igκ signal sequence upstream of the *hsp*65 gene, did not significantly improve the protective efficacy in the bacterial load in the lungs, although there was a modest decrease in the bacterial load in the spleen. In contrast, the combination with mIL-12 DNA markedly improved the protective efficacy both in the lungs and spleen ($P < 0.01$). In particular, vaccination of IgHsp65 DNA plus mIL-12 DNA conferred the greatest reduction of the bacterial load both in the lungs and spleen. Similar to IgHsp65 DNA plus mIL-12 DNA, the increased protection in the lungs and spleen was also observed in mice vaccinated with Hsp65 DNA plus mIL-12 DNA compared to IgHsp65 DNA alone and mIL-12 DNA alone. Thus, a strong synergistic effect on protection was achieved when Hsp65 DNA was co-administrated with IL-12 DNA. It is notable that the prophylactic effect of IgHsp65 DNA plus mIL-12 DNA in the lungs was more than 100-fold greater than that of BCG. These vaccinations of IgHsp65 DNA plus mIL-12 DNA and Hsp65 DNA plus mIL-12 DNA also exerted the significant reduction in the liver compared to the naive ($P < 0.05$) and control EGFP DNA groups ($P < 0.01$), whereas there was no significant difference of the naive group compared with Hsp65 or mIL-12 group (data not shown). In mice vaccinated with IgHsp65 DNA plus mIL-12 DNA, increased protection in the lungs were also observed at 5 weeks after challenge, which was equivalent to that obtained by vaccination with BCG (data not shown).

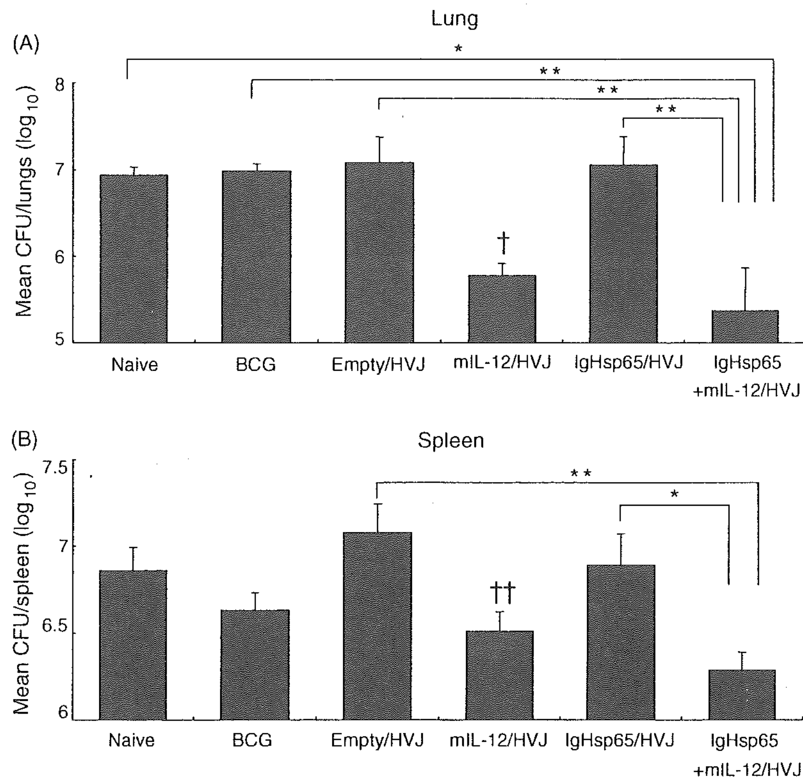

Fig. 3. Mouse protection studies using HVJ-liposome vaccines. Groups of mice vaccinated with HVJ-liposome DNA or BCG were challenged by intravenous injection with *M. tuberculosis* H37Rv. Five weeks after challenge, protection was measured by enumerating the bacterial loads (CFU) in the lungs (A) and spleen (B) from vaccinated mice. Results are expressed as the mean $\log_{10} \pm$ S.D. of CFU. The statistical significance of differences between individual groups in the number of CFU was determined by Tukey–Kramer's HSD test ($n = 4$–5). $^{*}P < 0.05$; $^{**}P < 0.01$; †, the statistical significance of differences ($P < 0.05$) of mIL-12/HVJ group compared to BCG, Empty/HVJ, and Hsp65/HVJ groups in the lungs; ††, the statistical significance of differences ($P < 0.05$) of mIL-12 DNA group compared to Empty/HVJ group in the spleens.

3.3. Comparison of the protective efficacy of gene gun versus HVJ-liposome delivery of Hsp65 DNA vaccines

We next compared methods of DNA vaccine delivery on vaccine efficacy at 10 weeks after challenge. Hsp65/HVJ vaccination and challenge experiments were conducted simultaneously with gene gun experiments. As shown in Fig. 2, Hsp65/HVJ vaccination significantly reduced the bacterial loads as compared to Hsp65 gene gun immunization in the spleen ($P < 0.01$). IgHsp65 gene gun immunization significantly reduced the bacterial loads as compared to Hsp65 gene gun immunization in the spleen ($P < 0.05$, data not shown). Therefore, we used IgHsp65/HVJ for further experiments.

3.4. Protective efficacy of HVJ-liposome DNA vaccines

At 5 and 10 weeks after intravenous challenge of *M. tuberculosis* H37Rv, the number of CFU in the lungs, spleen, and liver were determined. Fig. 3 shows the results of bacterial loads 5 weeks after challenge. Vaccination with mIL-12/HVJ group resulted in significant protective immunity in the bacterial as compared to BCG, Empty/HVJ and Hsp65/HVJ groups in the lung ($P < 0.05$) and as compared to Empty/HVJ group in the spleen ($P < 0.05$). Vaccination with IgHsp65 + mIL-12/HVJ induced better protective immunity in the bacterial load both in the lungs and spleens than IgHsp65/HVJ alone and mIL-12/HVJ alone. Thus, the synergistic effect of IgHsp65 DNA and mIL-12 DNA resulted in improving the protective efficacy. At 10 weeks after challenge, the same reduction was also observed in these organs from mice vaccinated with IgHsp65 + mIL-12/HVJ (data not shown). Body weights of vaccinated mice were similar in all vaccinated groups. Tissue weight of lungs, liver, and spleen in the IgHsp65 + mIL-12/HVJ group were slightly lower than that from the naive mice (data not shown). In this experiment, BCG vaccination did not provide significant reduction of the bacterial load compared to the naive group. This may be due to the single-dose of vaccination used usually, the use of BCG Tokyo strain requires a three-dose vaccination to achieve 10 to 30-fold reduction of the bacterial loads compared to a non-vaccinated group. Although, 5 weeks after challenge, no reduction of bacterial loads was observed in IgHsp65/HVJ group compared with the naive control group, we confirmed the increased protection 10 weeks after challenge compared with the naive control group

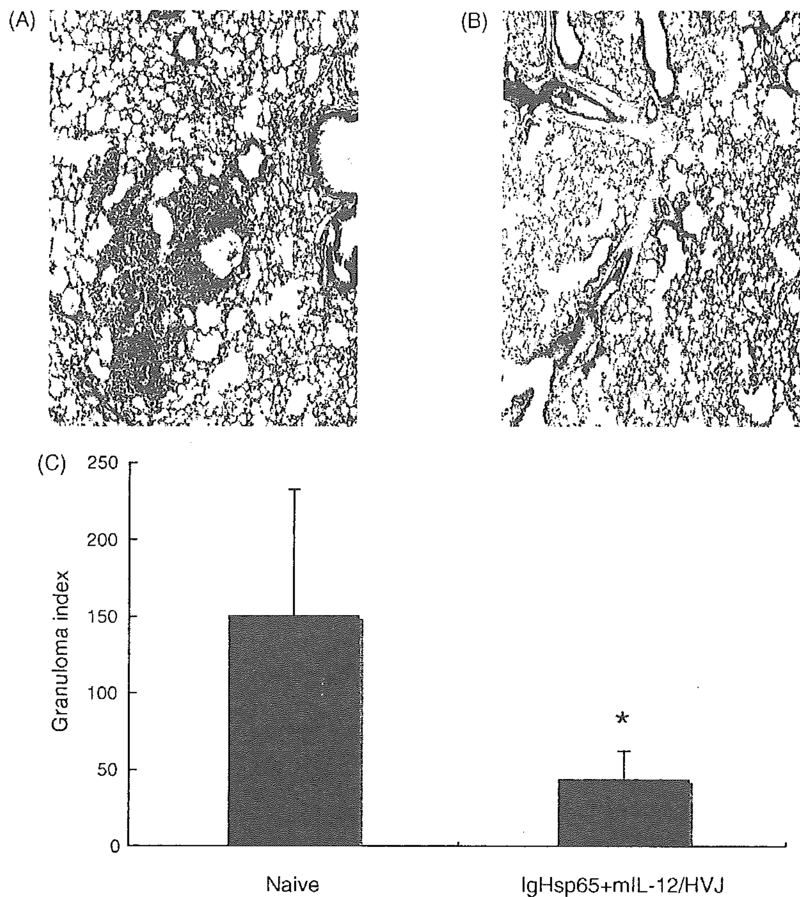

Fig. 4. Histopathological analysis of vaccinated mice 10 weeks after *M. tuberculosis* challenge. Representative photomicrographs of lung tissue sections harvested from the naive control group (A) and from the IgHsp65 + mIL-12/HVJ group (B) are shown (10 weeks after *M. tuberculosis* challenge, hematoxylin and eosin staining, ×10 objective). There were much infiltration of mononuclear cells and extensive parenchymal destruction by large, poorly demarcated granuloma in the lung from the naive control group. In the IgHsp65 + mIL-12/HVJ group, the lungs were less inflamed and only a few granuloma was observed. (C) Granuloma index of the naive control group and the IgHsp65+mIL-12/HVJ group in the lungs. Results are expressed as the mean ± S.D. of triplicates of five mice per group. The statistical significance of differences between the groups was determined by Student's *t*-test. $^{*}P < 0.05$ as compared with the naive control group.

at the same experiments (data not shown). These results indicate that co-vaccination with IL-12 DNA was effective for inducing protective immunity at as early as 5 weeks after challenge.

3.5. IgHsp65 + mIL-12/HVJ vaccination markedly reduced granuloma formation in the lung

In addition to the reduction of bacterial loads, the effects of vaccination on the mice were assessed by histological analysis. The granulomatous lesions in the lungs from IgHsp65 + mIL-12/HVJ mice were significantly less in number and size than from the naive control group (Fig. 4A and B). Quantitative evaluation of the granulomatous lesions clearly shows that IgHsp65 + mIL-12/HVJ vaccinated mice group exhibited significant reduction in granuloma index in the lungs, compared to the naive group ($P < 0.05$) (Fig. 4C). Thus IgHsp65 + mIL-12/HVJ vaccine provided significant protection against the pulmonary pathology caused by *M. tuberculosis* infection.

3.6. HVJ-liposome DNA vaccines generated T-helper response and cytokine production

To investigate lymphocyte proliferative and cytokine responses induced by HVJ-liposome DNA vaccines, spleen cells from vaccinated mice were re-stimulated with antigen in vitro. As shown in Fig. 5, substantial lymphocyte proliferation was observed in response to rHsp65 protein in spleen cells from mice vaccinated with IgHsp65/HVJ or IgHsp65 + mIL-12/HVJ but not with the naïve control. IgHsp65 + mIL-12/HVJ vaccination induced significantly better proliferative response to rHsp65 protein than did IgHsp65/HVJ vaccination ($P < 0.01$). In addition to lymphocyte proliferative responses, vaccination with IgHsp65 + mIL-12/HVJ induced elevated levels of IFN-γ and

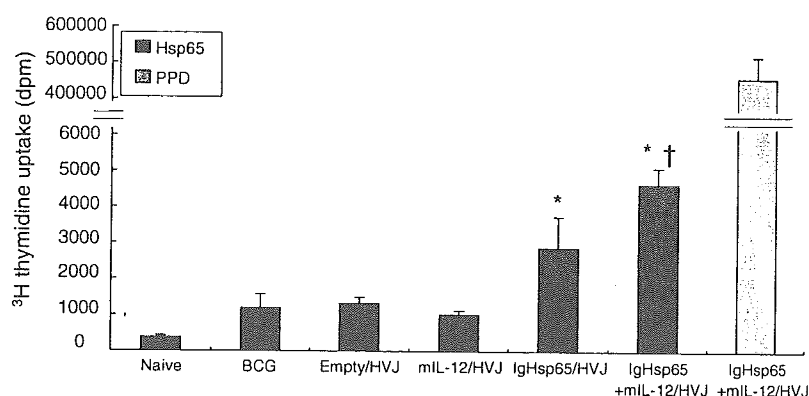

Fig. 5. The effect of vaccination with HVJ-liposome DNA on T cell proliferation. Proliferative responses of splenic lymphocytes from mice vaccinated with IgHsp65/HVJ, mIL-12/HVJ, IgHsp65 + mIL-12/HVJ, BCG, or Empty/HVJ. Incorporation of [^{3}H]thymidine in response to rHsp65 protein (black bars) or PPD (gray bar) was measured as described in Section 2. Results are expressed as the mean ± S.D. of triplicates of three mice per group. The statistical significance of differences between individual groups in T cell proliferation was determined by Tukey–Kramer's HSD test. The statistical significance of differences ($P < 0.01$) compared to the naive and BCG groups are indicated as (*) and (†), respectively.

IL-2 in response to rHsp65 protein, but not with the naive control or BCG group (Fig. 6). In response to PPD, vaccination with IgHsp65 + mIL-12/HVJ markedly increased both IFN-γ and IL-2 production as compared to the BCG group. Moderate but significant levels of IFN-γ and IL-2 were also induced in Hsp65/HVJ vaccination in response to Hsp65 protein and PPD. Thus, the synergistic effect of IgHsp65 DNA and mIL-12 DNA resulted in the strongest response not only to T cell proliferation but also to cytokine production.

3.7. HVJ-liposome DNA vaccines generated cytotoxic CD8$^+$ T cells

Because CD8$^+$ CTLs have been considered critical effectors of protective immunity to *M. tuberculosis*, it was of interest to determine whether a tuberculosis specific response could be induced in the vaccinated mice. We characterized CD8$^+$ T cells specific for Hsp65, PPD or killed *M. tuberculosis* by using a conventional ^{51}Cr release assay in the

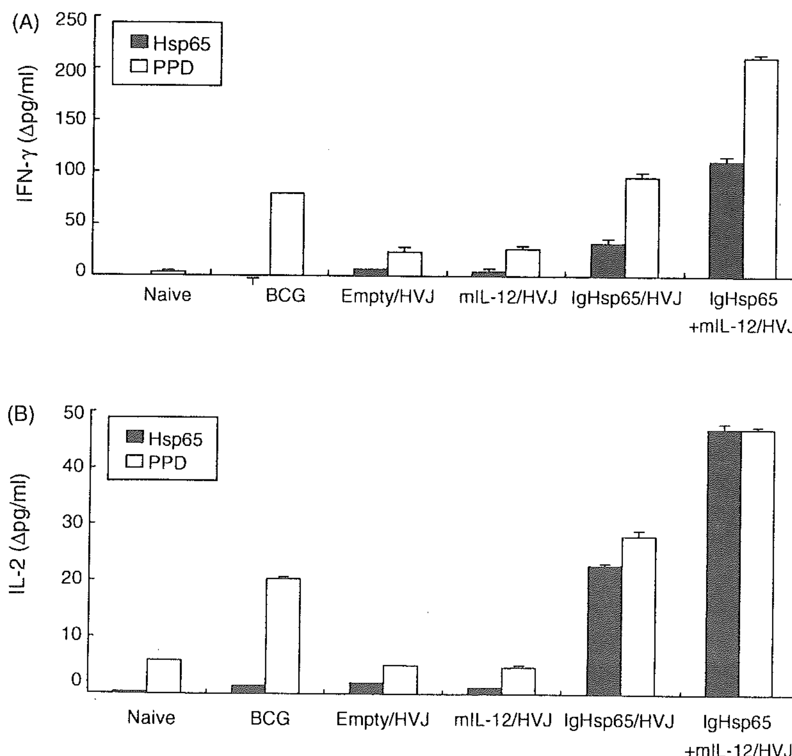

Fig. 6. IFN-γ (A) and IL-2 (B) production in spleen cell culture supernatants from vaccinated mice following stimulation with rHsp65 protein and PPD. Spleen cell cultures were stimulated with rHsp65 protein (black bars) or PPD (white bars) for 48 h, and the levels of IFN-γ and IL-2 production were determined by ELISA. Results are expressed as the mean ± S.D. of duplicates of three mice per group with antigens minus the mean ± S.D. of triplicates of three mice per group with medium alone.

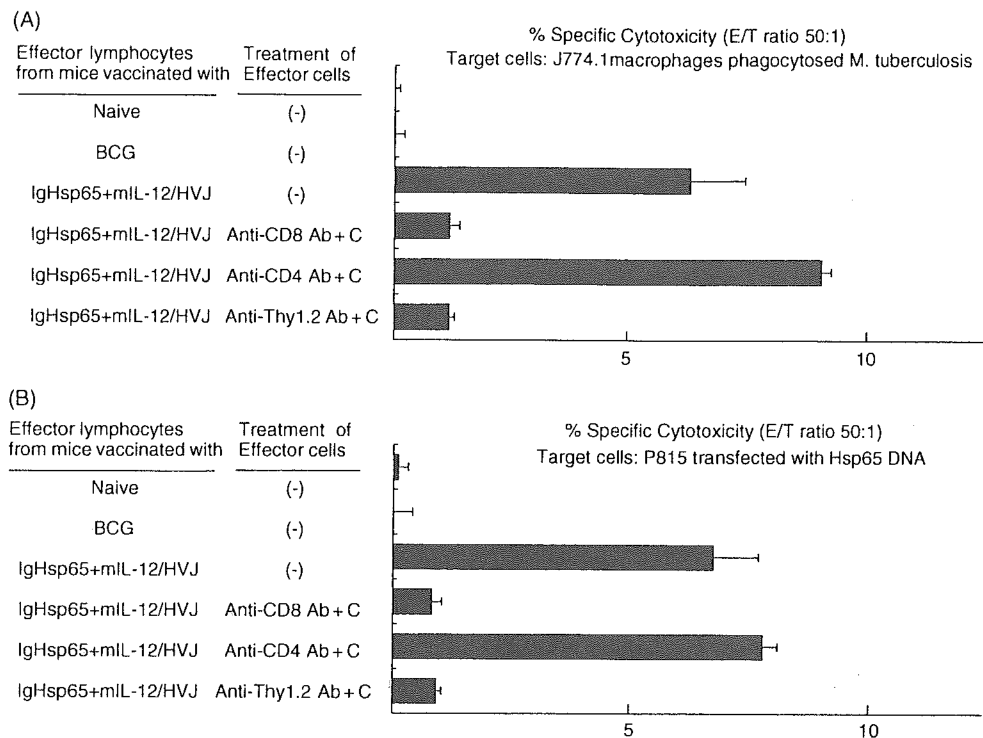

Fig. 7. Induction of CD8$^+$ CTL specific for Hsp65 protein and *M. tuberculosis* by vaccination with IgHsp65 + mIL-12/HVJ. Spleen cells from the naive, BCG-, and IgHsp65 + mIL-12/HVJ-vaccinated mice were obtained 8 weeks after the final vaccination. Cytotoxicity was assayed as release of radioactivity from ^{51}Cr-labeled J774.1 macrophages that had phagocytosed *M. tuberculosis* (killed H37Ra) (A) or from ^{51}Cr-labeled P815 target that had been transfected with Hsp65 DNA (B) using a conventional ^{51}Cr release assay at E:T ratio of 50:1. The effector cells were pre-incubated with anti-CD8, anti-CD4 or anti-Thy1.2 antibody, followed by treatment with complement. Percent specific lysis was determined as: [(experimental release–medium control release)/(maximum release–medium control release)] × 100. Ab: antibody; C: complement; (−), non-treatment.

absence of re-stimulation. As shown in Fig. 7, high levels of Hsp65- and *M. tuberculosis*-CTL specific lysis against J774.1 macrophages phagocytosed *M. tuberculosis* and P815 mastocytomas transfected with Hsp65 DNA were detected in mice vaccinated with IgHsp65 + mIL-12/HVJ, whereas little CTL response was detectable in either the naive or BCG-vaccinated mice. In vitro depletion of CD8$^+$ T cells eliminated the specific lysis. Depletion of CD4$^+$ T cells had no effect. Stronger (more than twenty percent) cytotoxicity against Hsp65 was detected in the spleen cells from mice 2 weeks after the last vaccination with IgHsp65 + mIL-12/HVJ (data not shown). These results indicate that IgHsp65 + mIL-

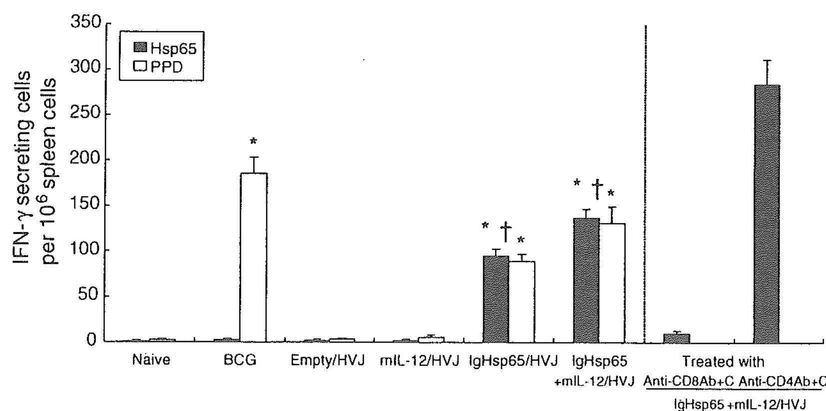

Fig. 8. ELISPOT assay for IFN-γ antigen-specific responses in the spleens of vaccinated mice following stimulation with rHsp65 protein and PPD. Spleen cell cultures were stimulated with rHsp65 protein or PPD for 20 h or pre-incubated with anti-CD8 antibody or anti-CD4 antibody followed by treatment with complement and then stimulated with rHsp65 protein for 20 h. The number of IFN-γ-secreting cells specific for rHsp65 protein (black bars) or PPD (white bars) per million cells were determined individually by ELISPOT assay. Results are expressed as the mean ± S.D. of five-wells of three mice per group. The statistical significance of differences between individual groups in the number of IFN-γ-secreting cells was determined by Tukey–Kramer's HSD test. The statistical significance of differences ($P < 0.01$) compared to the naive and BCG groups are indicated as (*) and (†), respectively.

12/HVJ vaccine induced long-term immune response with strong CD8+ CTL activity.

3.8. ELISPOT assay

In order to determine whether enhanced protection was associated with increased IFN-γ production, the frequency of IFN-γ-secreting cells was enumerated by ELISPOT. Vaccination with IgHsp65/HVJ and IgHsp65 + mIL-12/HVJ resulted in a marked increase of IFN-γ secreting cells following stimulation with rHsp65 protein (Fig. 8). Moreover, the increase of IFN-γ secreting cells was also seen in IgHsp65/HVJ and IgHsp65 + mIL-12/HVJ groups following stimulation with PPD. These results indicate that vaccination with IgHsp65/HVJ and IgHsp65 + mIL-12/HVJ activated antigen-specific T cells producing IFN-γ. Depletion of CD8+ cells from responder cells by treatment with anti-CD8 antibody and complement almost abrogated the IFN-γ producing cells. In contrast, an increase in the number of IFN-γ producing cells was observed in the responder cells when treated with anti-CD4 antibody and complement. BCG vaccination resulted in significant increase of IFN-γ secreting cells following stimulation with PPD but not rHsp65 protein. These data indicate that the protective efficacy of IgHsp65 + mIL-12/HVJ is strongly associated with the emergence of IFN-γ-secreting cells upon stimulation with Hsp65. Taken together, vaccination with IgHsp65 + mIL-12/HVJ capable of augmenting T cell activation and frequency of IFN-γ-secreting cells proves to reduce bacterial burden and pathology in the lungs—all to an extent greater than those achieved by vaccination with BCG.

4. Discussion

In the first stage of this study, we evaluated the protective efficacy of Hsp65 DNA vaccines via gene gun vaccination. One of the significant findings of the present study is that a single gene gun vaccination with the combination of IgHsp65 DNA and mIL-12 DNA led to a remarkably high degree of protection against intravenous challenge infection with virulent *M. tuberculosis*; bacterial numbers declined exponentially in internal organs and were 100-fold lower in the lungs than in BCG-vaccinated mice. Consistent with previous studies [43], gene gun vaccination with Hsp65 DNA alone did not promote reduction in bacterial burden compared to the naive mice. However, co-vaccination of Hsp65 DNA or IgHsp65 DNA plus mIL-12 DNA significantly improved the protective efficacy compared to either Hsp65 DNA alone or IgHsp65 DNA alone. Since the importance of IL-12 in the control of mycobacterial infections has been well documented, these results are consistent with other studies describing an adjuvant effect of IL-12 gene when administrated in combination with various tuberculosis DNA vaccines [20,24,25]. The mIL-12 DNA, which express both p40 and p35 chains as a single molecule, is able to induce four-fold higher levels of IFN-γ from mouse T lymphocytes than mIL12p40 + p35, which has previously been constructed as a murine expression vector with IL-12 p40 and p35 expression cassettes in tandem array [35]. Culture supernatants from the mIL-12 DNA-transfected COS-7 cells were effectively induced IFN-γ from mouse spleen cells. Thus, the improved expression levels of IL-12 DNA and the biologically active IL-12 explain the enhanced protection observed.

The second stage of this study demonstrated the protective efficacy of HVJ-liposome DNA vaccines in mouse and guinea pig models. We originally developed HVJ-liposomes, a viral/nonviral hybrid vector, as a gene transfer vector for cancer gene therapy. HVJ-liposome gene transfer method can deliver DNA directly and efficiently into host cells in vivo by means of the HVJ virus cell fusion machinery. We found that HVJ-liposome-mediated gene transfer was 30–100 times more efficient in gene expression in skeletal muscle than naked DNA transfer (unpublished data) and over three times more efficient in delivering intact oligodeoxyribonucleotide within the nuclei of transfected cells than Lipofectin®, a different cation liposome [44]. In addition to its high transfection efficiency, there are numerous safety advantages of HVJ-liposomes including: (i) no apparent toxicity or inflammation and (ii) repeated gene transfection without reduction of transfection efficiency. In fact, no significant adverse effects were induced in monkeys by intravenous injection of HVJ-liposomes [45]. Using this novel vector, we observed the enhancement of protection conferred by Hsp65 DNA compared to gene gun vaccination. This result is encouraging for the development of a novel tuberculosis DNA vaccine that is applicable both for prophylactic and therapeutic uses with no side-effects after repeated injections.

The most significant finding of this study is that vaccination with IgHsp65 + IL-12/HVJ provided greater protective efficacy than vaccination with BCG. In the mouse model, IgHsp65 + mIL-12/HVJ preferentially triggered a Th1 type T helper response, characterized by elevated levels of IFN-γ and IL-2, and augmentation of lymphocyte proliferation. After challenge, vaccination with IgHsp65 + mIL-12/HVJ resulted in a greater degree of protection than that evoked by BCG. This protective efficacy was associated with the emergence of IFN-γ-secreting T cells directed against Hsp65 and PPD. CD8+ CTL activity against macrophage target cells, which had previously phagocytosed *M. tuberculosis* or expressed Hsp65 protein, was still observed in the spleen cells from mice vaccinated with IgHsp65 + mIL-12/HVJ at 8 weeks after the final vaccination, IgHsp65 + mIL-12/HVJ vaccine capable of augmenting long-term immune response with anti-tuberculosis CTL activity proves IgHsp65 + mIL12/HVJ to be a promising tuberculosis vaccine candidate.

Although the *hsp65* DNA vaccines have been shown to have significant promise as a new prophylactic vaccine against tuberculosis [19,21,46], negative outcomes have also been reported [47,48]. In the case of vaccination with *hsp65* DNA alone, our results are consistent with the previous report

that vaccination with *hsp65* DNA alone did not provide significant protective effect in the bacterial load in the lung either in the mouse model or in the guinea pig model [43,47]. However, as described above, the combination with mIL-12 DNA expressing biologically active IL-12 and the use of HVJ-liposome as a DNA vaccine delivery system remarkably improved the protective efficacy. In addition, our preliminary results of a guinea pig model in the collaborative study with Dr. D. McMurray (Texas A&M University) show that vaccination with IgHsp65 + guinea pig IL-12 (gpIL-12)/HVJ provided better protection against the pulmonary pathology caused by aerosol challenge with *M. tuberculosis* than did BCG vaccination (data not shown). For immunotherapeutic use, *hsp60*/lep DNA vaccine (*hsp65* DNA derived from *Mycobacterium leprae*) has been shown to be effective in a Cornel-type model [22], although others have argued that this vaccine induced progressively severe pulmonary necrosis in the model [48]. In support of the effectiveness, when administered to mice or SCID-PBL/hu mice [49] already infected with *M. tuberculosis*, neither IgHsp65 + mIL-12/HVJ vaccine nor IgHsp65 + human IL-12 (hIL-12)/HVJ vaccine, respectively, resulted in exacerbation of the granulomatous response in the lungs (unpublished data). Moreover, therapeutic administration of IgHsp65 + mIL-12/HVJ resulted in significant reduction of bacterial loads (paper in submission). The pathological parameter of protection included reductions in the mean lung granulomatous lesion score in our study. In parallel with the protective efficacy of HVJ-liposome vaccines on bacterial loads, histopathological analysis shows that mice vaccinated with IgHsp65 + mIL-12/HVJ had fewer and smaller lesions in the lung and significantly less lung granuloma than the naive mice. These results suggest that severe toxicities (Koch phenomenon) could not be induced by this vaccine. One possible explanation for these diverging results may be different *hsp65* DNA construct (secreted form versus cytoplasmic form; derived from *M. tuberculosis* versus *M. leprae*), different mIL-12 DNA construct (p40p35 fusion form versus p40-p35 tandem form), and different vaccine delivery (HVJ-liposome versus gene gun or naked DNA).

In conclusion, we demonstrate the development of a novel HVJ-liposome DNA vaccine encapsulating Hsp65 DNA plus IL-12 DNA. These results suggest that Hsp65 + IL-12/HVJ could be a promising candidate for a new tuberculosis DNA vaccine, which is superior to the currently available BCG vaccine. The goal of our study is to develop a new tuberculosis vaccine superior to BCG. To this aim, we believe that the protective efficacy and protective immune responses for vaccine candidates should be addressed in larger animals, such as non-human primates, before proceeding to human clinical trials. Although other DNA vaccine candidates that appear to protect against virulent *M. tuberculosis* in mice better than BCG have failed to provide better protection than BCG in guinea pigs against aerosol challenge of a low dose of virulent *M. tuberculosis* [47,50,51], some of them are being prepared to enter early human clinical trials [52]. More recently, we evaluated the IgHsp65 + hIL-12/HVJ vaccine in the cynomolgus monkey model [29], which is currently the best non-human primate animal model of human tuberculosis. Monkeys were subsequently challenged with virulent *M. tuberculosis* by the intra-tracheal route after the third vaccination. This challenge dose normally causes death from acute respiratory infection within 4–6 months. In this particular experiment, monkeys vaccinated with IgHsp65 + hIL-12/HVJ induced Hsp65-specific T cell proliferation and improvement of chest X-P findings, resulting in an increased survival for over a year, superior to BCG group [29]. Thus, we are taking advantage of the availability of multiple animal models (mouse, guinea pig, and monkey) to accumulate essential data of the HVJ-liposome DNA vaccine, including the vaccine efficacy and safety, for up-coming Phase I clinical trials.

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Novel recombinant BCG and DNA-vaccination against tuberculosis in a cynomolgus monkey model

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Abstract

We have developed two novel tuberculosis (TB) vaccines: a DNA vaccine combination expressing mycobacterial heat shock protein 65 (Hsp65) and interleukin-12 (IL-12) by using the hemagglutinating virus of Japan (HVJ)-liposome (HSP65 + IL-12/HVJ) and a recombinant BCG harboring the 72f fusion gene (72f rBCG). These vaccines provide remarkable protective efficacy in mouse and guinea pig models, as compared to the current by available BCG vaccine. In the present study, 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 and 72f rBCG vaccines. Vaccination with HSP65 + IL-12/HVJ as well as 72f rBCG vaccines provided better protective efficacy as assessed by the Erythrocyte Sedimentation Rate, chest X-ray findings and immune responses than BCG. Most importantly, HSP65 + IL-12/HVJ resulted in an increased survival for over a year. This is the first report of successful DNA vaccination and recombinant BCG vaccination against *M. tuberculosis* in the monkey model.
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Keywords: HSP65 DNA + IL-12 DNA vaccine; Tuberculosis; Monkey

1. Introduction

Tuberculosis (TB) is a major global threat to human health, with more than 3 million people dying each year from *M. tuberculosis* (TB) infections. The only tuberculosis vaccine currently available is an attenuated strain of *M. bovis* BCG (BCG), although its efficacy against adult TB disease remains controversial. Therefore, we have recently developed two novel TB vaccines: a DNA vaccine combination expressing mycobacterial heat shock protein 65 (Hsp65) and interleukin-12 (IL-12) by using the hemagglutinating virus of Japan (HVJ)-liposome (HSP65 + IL-12/HVJ) and a recombinant BCG harboring the 72f fusion gene (r72f BCG). The former vaccine was 100-fold more efficient than BCG in the elimination of *M. tuberculosis* in mice by the induction of CTL [9].

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Researchers have recognized that a nonhuman primate model of TB will be able to provide critical information for vaccine development. However, several TB vaccine candidates who appear to protect better than BCG against virulent *M. tuberculosis* in mice, have rarely been tested in the nonhuman primate model because of cost and limited facilities.

In the present study, we evaluated the protective efficacy of HSP65 + IL-12/HVJ and r72f BCG in the cynomolgus monkey model, which is an excellent model of human tuberculosis [1]. These vaccines provided a strong prophylactic effect in monkeys challenged with *M. tuberculosis* as we have seen previously in mice.

2. Materials and methods

DNA vaccines encoding *M. tuberculosis* HSP65, mouse IL-12 and guinea pig IL-12 were encapsulated with HVJ-liposomes [2]. Groups of animals (mice and guinea pigs) were vaccinated intramuscularly with HVJ-liposome DNA vaccines. CTL activity was assessed by ^{51}Cr-release and IFN-γ activity [3,4]. A total of 16 cynomolgus monkeys were housed in a BL 3 animal facility of the Leonard Wood Memorial. Groups of animals were vaccinated three times with either the HVJ-liposome combination with HSP65 DNA plus human IL-12 DNA (HSP65 + hIL-12/HVJ: 400 μg i.m.), r72f BCG (1×10^6 CFU i.d.), BCG Tokyo (1×10^6 CFU i.d.) or saline. One month after the third vaccination, monkeys were challenged with the *M. tuberculosis* Erdman strain (5×10^2) by intratracheally instillation, Erythrocyte Sedimentation Rate (ESR), body weight, chest X-ray, immune responses, DTH reaction against PPD and survival periods were examined during 14 months [1].

3. Results

Mice vaccinated with HSP65 + mIL-12/HVJ had significantly reduced numbers of CFU [5] in the lungs, liver and spleen as compared with mice vaccinated with BCG [9]. CTL activity correlated with the protective efficacy of vaccination. The fusion protein Mtb72f (Mtb39 + Mtb32) vaccine was developed by Skeiky et al. [6]. To improve its vaccine efficacy, a recombinant BCG harboring the 72f fusion gene (r72f BCG) was generated [7]. The ELISPOT assay showed that r72f BCG induced a greater number of IFN-γ producing T-cells than BCG in the mouse model. In the guinea pig model, r72f BCG as well as HSP65 + gpIL-12/HVJ provided better protection against the pulmonary pathology caused by pulmonary challenge with TB than BCG vaccination (data not shown).

The purpose of this study was to evaluate two TB vaccines we have developed in a nonhuman primate model of *M. tuberculosis* infection. To this end, a total of 16 monkeys were vaccinated either with HSP65 + hIL-12/HVJ, r72f BCG, BCG or saline, followed by TB challenge by intratracheally instillation. Table 1 shows survival periods of vaccinated monkeys after TB challenge. All four monkeys in the control (saline) group died of TB infection within 8 months. In contrast, three and two monkeys from the 72f rBCG and HSP65 + hIL-12/HVJ groups, respectively, were alive more than 14 months post-infection (the termination period of the experiment). Survival periods of the remaining monkeys in the both groups were much longer than those of saline control group. In addition, both HSP65 + hIL-12/HVJ and r72f BCG significantly improved ESR and chest X-ray findings (Table 2). Body weights of the HSP65 + hIL-

Table 1
Survival of cynomolgus monkeys immunized with HVJ-liposome/HSP65 DNA + IL-12 DNA vaccine and recombinant 72f BCG vaccine

Vaccination	Total monkeys	Survival	Dead	% Survival
HVJ-liposome/HSP65 DNA + IL-12 DNA	4	2	2	50
Recombinant 72f BCG	4	3	1	75
BCG Tokyo	4	2	2	50
Saline	4	0	4	0

Cynomolgus monkey (4 monkeys/group) were immunized three times (every 3 weeks) with (1) HVJ-liposome/ HSP65 DNA + IL-12 DNA vaccine, (2) r72f BCG vaccine, (3) BCG Tokyo and (4) saline as control group as described in Section 2. One month after last immunization, M.TB (Erdman strain 5×10^2) was challenged by intratracheally instillation. Survival was studied more than 14 months.

Table 2
Improvement of Erythrocyte Sedimentation Rate (ESR) in the cynomolgus monkeys immunized with HVJ-liposome/HSP65 DNA + IL-12 DNA vaccine and recombinant 72f vaccine

Vaccination	ESR (nm/h)	Mean ± S.D.	Statistical significance *P*-value compared to saline group (Student *t*-test)
HVJ-liposome/HSP65 DNA + IL-12 DNA	2	3.5 ± 1.9	<0.01
	6		
	4		
	2		
Recombinant 72f BCG	3	6.75 ± 8.9	Not significant
	1		
	20		
	3		
BCG Tokyo	22	11.25 ± 11.3	Not significant
	2		
	20		
	1		
Saline	50	29.75 ± 18.1	
	14		
	15		
	40		

Cynomolgus monkey (4 monkeys/group) were immunized and challenged as described in Table 1. Elevation of Erythrocyte Sedimentation Ratio (ESR) of all monkeys was evaluated every month and maximum values of ESR in each monkey were shown.

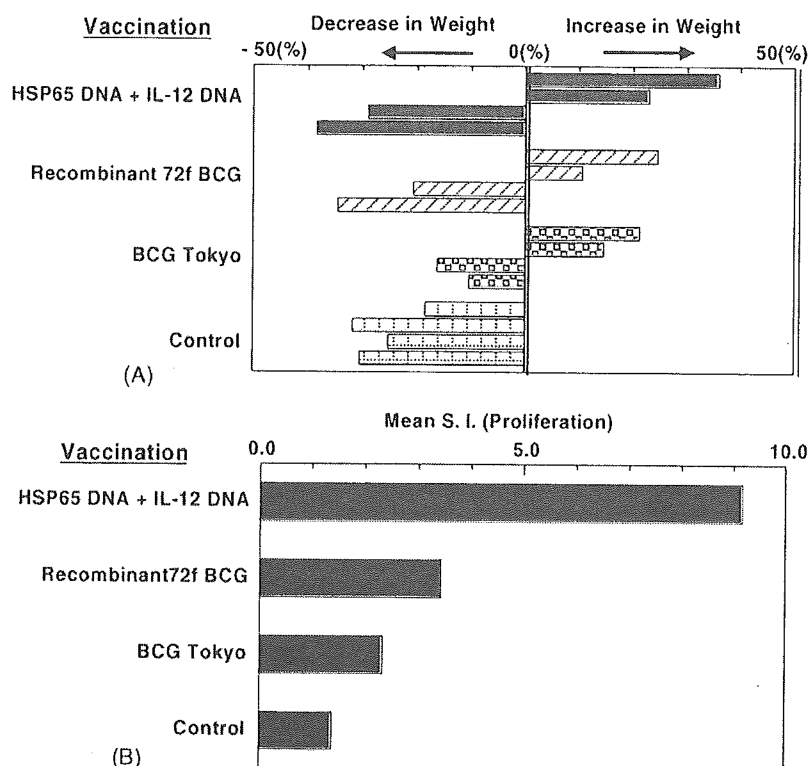

Fig. 1. (A) Increase in body weight: the prophylactic effect of novel vaccines (HSP65 DNA + IL-12 DNA, recombinant 72f BCG) on *M. tuberculosis* infection of cynomolgus monkeys. Percent of increase or decrease in body weight of monkeys immunized with (1) HSP65 DNA + IL-12 DNA (■), (2) recombinant 72f BCG (▨), (3) BCG Tokyo vaccines (▩) and (4) saline (control) (⊟) and challenged with *M. tuberculosis*, compared to the weight of pre-immunized monkeys. (B) Lymphocyte proliferation activity (LPA) against recombinant HSP65 protein in the peripheral blood (whole blood) from the cynomolgus monkeys immunized with novel vaccines and challenged with *M. tuberculosis*. Peripheral blood lymphocytes (whole blood) 4 weeks after TB challenge were cultured with 10 μg/ml of recombinant HSP65 antigen in a 96-microwell plate for 5 days at 37 °C and then pulsed with 1 μCi of [3H] thymidine per well for the final 16–18 h of incubation. Results are expressed as a stimulation index (S.I.) and compared to the pre-immune LPA from the same monkey.

12/HVJ group also increased significantly, as compared to saline control group (Fig. 1A). IL-2 and IFN-γ production were augmented in the two groups vaccinated with HSP65 + hIL-12/HVJ and r72f BCG (data not shown). Furthermore, proliferation of PBL was strongly enhanced in the group vaccinated with HSP65 + hIL-12/HVJ in response to HSP65 protein 4 weeks after TB challenge (Fig. 1B). Taken together, these results clearly demonstrate that both HSP65 + hIL-12/HVJ and r72f BCG could provide protective efficacy against *M. tuberculosis* in the cynomolgus monkey model.

4. Discussion

HSP65 + hIL-12/HVJ vaccine as well as r72f BCG vaccine exerted the significant prophylactic effect against TB, as indicated by: (1) prolongation of survival for over a year, (2) improvement of ESR and chest X-ray findings, (3) increase in the body weight and (4) augmentation of immune responses, in a cynomolgus monkey model which closely mimics human TB disease. It is very important to evaluate the long survival period in a monkey model, as human TB is a chronic infection disease. Furthermore, the decrease in the body weight of TB patients with TB is usually accompanied by progress of TB disease. Suppression of IFN-γ production, CTL activity and T-cell proliferation has also been observed in patients with TB [8].

Our results with the HSP65 + hIL-12/HVJ vaccine in the cynomolgus monkey model should provided a significant rational for moving this vaccine into clinical trials. In fact, the 72f fusion protein vaccine entered Phase I testing after its evaluation in cynomolgus monkeys in Leonard Wood Memorial [4] by Reed and Skeiky. Thus, we are taking advantage of the availability of multiple animal models (mouse, guinea pig, and monkey) to accumulate essential data on the HVJ-liposome DNA vaccine in anticipation of a Phase I clinical trial.

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第80回総会シンポジウム

Ⅲ. 抗酸菌症研究の最前線

座長 [1]岡田 全司 [2]白川 太郎

キーワーズ：結核，抗酸菌症，基礎研究

シンポジスト：

1. 結核感染症例のSNP解析
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 慶長直人（国立国際医療センター研究所呼吸器疾患研究部）
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 岡田全司（独立行政法人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）
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5. 結核菌の薬剤耐性に関与する遺伝子
 阿部千代治（日本ベクトン・ディッキンソン株式会社）
6. Toll-like receptorと結核感染
 竹田 潔（九州大学生体防御医学研究所発生工学分野）

結核菌を含む抗酸菌に対する宿主側の抵抗性は主としてT細胞免疫によって担われている。事実，HIV感染症やT細胞免疫不全（生体の抗酸菌感受性）に伴う結核感染合併が大きな問題となっている。したがって，T細胞免疫を増強するワクチン療法や免疫療法が理論的のみでなく，実際的にも開発されつつある。

一方，自然免疫系の作動メカニズムはほとんど理解されていなかった。最近，Toll-like receptor（TLR）ファミリーが病原体の構成成分の認識に関与していることが，明らかになってきた。結核菌に対する生体防御においても，TLRファミリーによる結核菌の認識が重要な役割を果たす可能性が考えられる。本シンポジウムでは，竹田潔が自然免疫系による結核などの病原体の生体内への侵入を察知するメカニズムをTLRを中心とした受容体の解析から明らかにし，結核感染における免疫系作動の分子機構を包括的に理解することを目的とし，特に，TLRを介したシグナル伝達機構を中心に解析した。

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。岡田全司はBCGよりも100倍以上強力な結核予防ワクチン効果を示す新しいDNAワクチン（Hsp 65 DNA＋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。したがって，さらに岡田全司は結核患者のT細胞免疫低下解析とT細胞免疫増強ワクチンの研究成果を中心に，新しい抗結核ワクチンの臨床応用への動きと課題について検討した。

さらに，宿主側の多因子疾患の感受性遺伝子を同定する方法として，多型マーカーとの連鎖不平衡を利用し，大規模な領域を関連解析でスクリーニングする手法が広く検討されている。本研究では，多型性に富むマイクロサテライトをマーカーとして利用し，非結核性抗酸菌症と関連する一塩基多型（SNP）を同定する研究を慶長直人が発表し，結核菌症と関連するSNP同定の研究は白川太郎が報告した。本解析により，未だ確実な方法論のない疾患感受性候補領域の絞り込みから感受性遺伝子の同定に至る戦略を確立し，それをシステム化することで，今後，抗酸菌症の疾患感受性遺伝子の同定が可能となると期待される。

一方，抗酸菌側から見た薬剤抵抗性や宿主の免疫力に対する抵抗性について，阿部千代治は抗酸菌における

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種々の薬剤感受性遺伝子の長年にわたる解析をクリアーカットに講演した。また最近，谷口初美は結核菌のmIHFおよびその近傍遺伝子の放線菌の遺伝子との類似性に着目し，これらの遺伝子が抗酸菌の増殖やphase variationに与える影響を明らかにしつつある。これにより休眠状態への変換機序を明らかにする手掛かりを得たことより，今まで不明であった，結核菌が宿主の抵抗性をエスケープするメカニズム解明につながる研究が紹介された。

1. 結核感染症例のSNP解析

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1. はじめに

Common diseaseあるいは多因性疾患と呼ばれる糖尿病，虚血性心疾患，高血圧といった疾患の病態形成においては遺伝要因が重要であることが広く認識されている。結核などの感染症も例外ではなく，その発症には宿主側の環境衛生・生活栄養状態といった要因に加え，免疫反応に影響をもたらす遺伝要因が深く関わることがこれまでに強く示されてきた。特筆すべきは，近年，非定型抗酸菌やBCGによる致死性の重症感染をきたした家系や小児患者群から，インターロイキン-12（IL-12）およびインターフェロン-γ（IFN-γ）といったTH1免疫反応の中心を担うサイトカイン関連遺伝子の欠損・変異が相次いで見出された点である。これら一連の報告により，著しいTH1活性の低下が抗酸菌症をはじめとした細胞内寄生性病原体への選択的易感染性をきたすことが明らかとなり，"Mendelian susceptibility to mycobacterial disease"（MSMD; MIM）という新たな疾患概念が確立されるに至った。

2. 結　果

これらの知見をふまえ，われわれは独自の多型解析の結果，IL-12受容体β1鎖（IL12RB1）に3カ所のミスセンス多型があることを見出し，そのリスクアリルがIL-12（およびIL-23）の受容体に対する反応性を低下させ，最終的にIFN-γを介したTH1免疫反応が減弱することで結核感染への感受性に寄与していることを報告した。また，その後のわれわれの行った関連解析では他のTH1反応に関連した候補遺伝子については結核感染との相関は認めなかった。

一方で，ヒトやマウスのゲノム情報の整備に伴い，これまで様々な遺伝解析手法を駆使して抗酸菌感染症の感受性遺伝子（座）の同定が試みられてきた。代表的に用いられてきたのは，3つの手法，すなわち①動物モデルを使った解析，②候補遺伝子アプローチ，③全ゲノム連鎖解析，である。マウスの解析から同定された例としては，*Nramp1*がよく知られており，その後の患者対照研究でも，ヒトにおける*NRAMP1*の遺伝子多型と結核との強い相関が確認されている。ノックアウトマウスの解析からも宿主の感染防御に関わる遺伝要因の研究が進められており，例えばIL-12欠損マウスはBCGや結核感染により感受性が高いことが知られている。関連解析を用いた研究からは*NRAMP1*以外の有力な候補遺伝子として，MHC class II，vitamin D receptor（VDR），mannose binding lectin（MBL），IL1RA/IL1B，IL12RB1などが報告されている（Table 1）。また全ゲノム連鎖解析によって，主として2q35（*NRAMP1*領域），15q11-13，Xq27の3領域が結核と連鎖のある遺伝子座として同定されている。しかし，これまでに見出されたこれらの候補遺伝子は結核感染における宿主側の全遺伝要因の一部を説明しているにすぎず，今後さらなる結核感受性遺伝子同定へ向けての幅広い研究が必要である。

このような状況を踏まえてわれわれは，すでに発表された結核の感受性遺伝子11個における18 SNPについてわれわれのサンプルを用いて検討を行うこととした。サンプルは和歌山のサンプル，東京のサンプル，九州のサンプルの，3地域のサンプルを用い合計764名，各々の地域での正常対照を用いて解析を行った。その結果，*NRAMP1*遺伝子（GTとSNP）とIL12RB1遺伝子に関連を認めたが，すべてのサンプルにおいて関連を認めたのは，*NRAMP1*遺伝子内のSNPのみであった。これらの結果は，すでに発表された論文の結果と一致するもので，現在世界で人種を超えて関連を認めている遺伝子は*NRAMP1*のみである。しかしながら，その機能的な意味は現在のところ不明である。

結核感染の特殊な例として，薬剤耐性の問題が次にあげられるが，本来この問題は，結核菌側の問題として処理されてきた。しかし，集団発生例において，一部に薬剤耐性が生じることから，一部には宿主側の問題もあると考えられる。そこで，薬剤耐性を獲得した症例においてどのような遺伝子が関与するかの検討が必要と考えられた。

Table Relationship between tuberculosis and candidate genes in many populations

Candidate gene	Chromosome	Allelic gene/Polymorphism	Population	Sample (patients/normal)	Odds ratio /p value	Reference
IL10	1q31-q32	−1082A→C	Gambian	358/106	1.8 (1.2–2.9)	1)
IL1RA/IL1B	2q14-14.2	IL1RA A2-/IL1B (+3953) A1+	Gujarati Asian	54/65	0.028	2)
IL1RA	2q14-14.2	allele2	Gambian	>400/400	0.03	3)
NRAMP1	2q35	INT4 C+3'UTR del	Gambian	410/417	4.1 (1.9–9.1)	4)
NRAMP1	2q35	3'UTR	Korean	192/192	1.8 (1.1–3)	5)
NRAMP1	2q35	5'(GT) n, Asn543Asp	Japanese	267/202	1.9 (1.3–2.6)	6)
NRAMP1	2q35	INT4 C	Guinea-Conaky	44 pedigree	<0.04	7)
IL8	4q13-q21	−251T→A	White/African American	167/180	3.5 (1.5–8.1)	8)
IL12B	5q31.1-q33.1	$(ATT)_8$	Hong Kong Chinese	516/514	2.1 (1.5–3.2)	9)
HLA class I	6p21.3	A1, Cw6, Cw7	Indian	235/289	<0.001	10)
HLA-DRB1	6p21.3	DRB1*1501	North Indian	20/46	4.8 (1–23.3)	11)
HLA-DRB1	6p21.3	DRB1*1501	South Indian	126/87	2.7 (1.3–5.9)	12)
HLA-DRB1	6p21.3	DRB1*1501	Mexican	50/95	7.9 (2.7–23.1)	13)
HLA-DQB1	6p21.3	DQB1*0503	Cambodian	78/49	0.005	14)
MBL	10q11.2-21	R52C, G54D, G57Q	Indian	202/109	0.008	15)
MBL	10q11.2-21	G54D	South African	91 (64) /79	<0.017 (<0.002)	16)
SP	10q22-q23	$1A^3$	Mexican	107/101	9.3 (1.6–53.4)	17)
VDR	12q12-14	codon352 (tt)	Gambian	408/414	0.01	18)
VDR	12q12-14	*Fok* I ff or undetectable VD	Gujarati Asian	71/42	5.1 (1.4–18.4)	19)
IFNG	12q14	874A→T	South African	313/235	0.0055	20)
P2X7	12q24	−762T→C	Gambian	323/347	0.55 (0.32–0.93)	21)
IL12RB1	19p13.1	R214-T365-R378	Japanese	98/197	2.5 (1.2–5.0)	22)
IL12RB1	19p13.1	−2C→T	Moroccan	101 pedigree	2.7 (1.2–6.1)	23)

IL1RA, IL-1 receptor antagonist; NRAMP1, natural resistance-associated macrophage protein 1 (SLC11A1); MBL, mannose binding lectin; SP, surfactant protein; VDR, vitamin D receptor; IFNG, IFN-γ; P2X7, P2X7 purinergic receptor; IL12RB1, IL-12 receptor β1

1) Delgado et al. J Infec Dis, 2002. 2) Wilkinson et al. J Exp Med, 1999. 3) Bellamy et al. Tuber Lung Dis, 1998. 4) Bellamy et al. N Eng J Med, 1998. 5) Ryu et al. Int J Tuberc Lung Dis, 2000. 6) Gao et al. Clin Genet, 2000. 7) Cervino et al. Ann Hun Genet, 2000. 8) Ma et al. J Infect Dis, 2003. 9) Tso et al. J Infect Dis, 2004. 10) Balamurugan et al. J Infect Dis, 2004. 11) Mehra et al. Int J Lepr Other Mycobsct Dis, 1995. 12) Ravikumar et al. Tuber Lung Dis, 1999. 13) Teran-Escandon et al. Chest, 1999. 14) Goldfeld et al. JAMA, 1998. 15) Selvaraj et al. Tuber Lung Dis, 1999. 16) Hoal-Van Helden et al. Pediatr Res, 1999. 17) Floros J et al. J Infect Dis, 2000. 18) Bellamy et al. J Infect Dis, 1999. 19) Wilkinson et al. Lancet, 2000. 20) Rossouw M et al. Lancet, 2003. 21) Li et al. J Infect Dis, 2002. 22) Akahoshi et al. Hum Genet, 2003. 23) Remus et al. J Infect Dis, 2004.

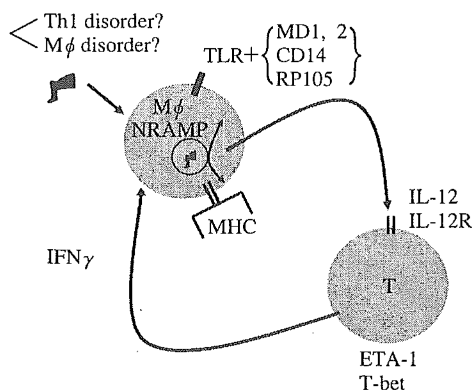

Fig. 1 Cells and genes in the process of degradation of *M. tuberculosis*

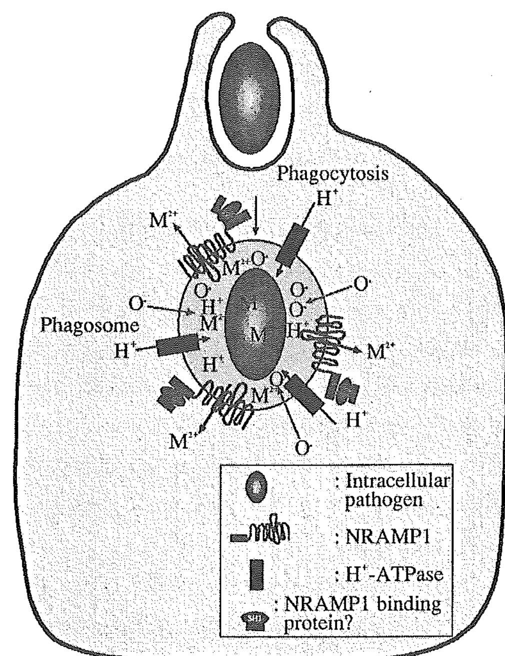

Fig. 2 Role of *NRAMP1* in the phagosome (hypothesis): *NRAMP1* play a role of proton influx-efflux, and regulate *M. tuberculosis* requiring O_2.

以上のような状況を踏まえて，結核患者において薬剤耐性現象が生じる理由として，

(i) 結核菌に対する感受性あるいは耐性が異なると考える

(ii) 結核菌への感受性は同じであるが，単に薬剤への感受性が異なる

以上の２つが考えられる。この2つの仮説のどちらが正しいかを解決するには，

(i) これまでの感受性遺伝子群について解析を行う

(ii) 薬剤感受性については，全ゲノムで解析を行う

以上２つの作業を行うことで解決が可能であると考えられる。

3. まとめ

上記の討議を踏まえて解析を行っており，結核感受性遺伝子群では，*NRAMP1*のSNPパターンに違いが見られる可能性があり，また耐性結核患者との比較でもこの遺伝子のみが関連すると考えられる。この理由として細胞内処理の違いが関係する可能性があることが認められた。

2. MAC症の疾患感受性遺伝子研究

国立国際医療センター研究所呼吸器疾患研究部　慶長　直人

はじめに

免疫関連遺伝子変異研究によって，感染の成立，発症，病型にどれだけの遺伝子多型がどの程度関与するかが明らかになれば，難治であるヒトMAC症など抗酸菌感染症の新たな予防，治療戦略へ道が開けるものと期待される。動物モデルや細胞生物学的な研究により，抗酸菌症研究は，大きな進歩を遂げたが，複雑な人における感染症の病態を解析する有効な手段は未だに限られており，遺伝子多型を用いたアプローチは，まさに人を対象とするその直截性と解析方法の客観性の点から，抗酸菌症研究においても，重要な位置を占めつつある。

今日，感染症の免疫遺伝学研究が盛んに行われる背景には，候補遺伝子アプローチの基礎となる多型情報が十分，公共データベース上に蓄積されてきたこと，ゲノムワイドアプローチによる新規疾患関連遺伝子の局在の推定が技術的にも比較的手軽に行われるようになったことがあげられる。いずれも，ヒトゲノムプロジェクトの発展がもたらした産物である。抗酸菌感染症の候補遺伝子は，サイトカイン，ケモカインやそれらのレセプターなどオーソドックスな免疫関連分子，マウスを中心とした動物モデルから得られた疾患関連分子のヒト相同性遺伝子，ノックアウトマウスにおける感染実験の結果などから選択されている。

そもそも感染症における疾患感受性，抵抗性に関連する遺伝素因として最も医学的によく知られている事例は，マラリア抵抗性を示す遺伝性異常ヘモグロビン症であろう。遺伝的に異常のあるヒトの赤血球は，マラリア原虫にとって，感染，生育しづらい環境を与えることになる。この例は，遺伝要因が感染症の発症に対して，ときとしてきわめて顕著な働きを示すことがあること，また遺伝子変異の選択圧として感染症が大きな役割を果たしてきたことを推測させるものである。

このような特殊な例だけでなく，おそらく一般集団においても，宿主側の遺伝要因が，感染症の疾患感受性に重要な役割を果たすものと推測されている。多くの感染性疾患では家族内集積の傾向が見られる。遺伝要因が存在すれば，一般人口における発症危険率に対して，発症者の同胞に同一疾患が発症する危険率（λs）が高くなる。たとえば，I型糖尿病や，多発性硬化症などではこの値は15～20くらいとされている。感染症では，同一家族内では，同一環境で，同一病原体へ曝露されやすいことから，λsは過大評価されやすいものの，多くの場合，1近くから10程度と考えられている[1]。

感染症発症に関する双生児研究や養子研究においては，環境要因の関与をできるだけ一定にした状態でも，遺伝要因の関与が十分認められることが報告されてきた。一卵性と二卵性双生児の研究では，結核症の一卵性双生児における発症頻度の高さが知られている。人種集団間の有病率の差異は，しばしば遺伝素因が発症に関与する根拠とされる。ただし，多くの環境要因，病原微生物の株にも，地理的分布の違いが認められるため，この解釈は慎重である必要がある。家系研究の結果からは，これまで感染症は，多因子疾患ではあるものの，主要な少数の感受性遺伝子と病態を修飾するそれ以外の多くの遺伝子の存在が示唆されている。

MAC症の候補遺伝子アプローチ

感染症の疾患感受性遺伝子研究として，急速に発展し，現在でも，最も頻繁に行われている手法は，候補遺伝子の遺伝子多型頻度を症例対照群間で比較して，関連解析により，疾患感受性，抵抗性を明らかにしようとす

Table Search for predisposing factors in pulmonary MAC disease

A. Candidate gene
Sporadic pulmonary MAC disease
(1) HLA-DR6 (Takahashi M, et al.; Kubo K, et al.; AJRCCM. 2000)
(2) *NRAMP1* (our study)

Familial mycobacterial disease
(1) IFNγ-receptor 1 (Newport MJ, et al.; NEJM. 1996)
(2) IFNγ-receptor 2 (Dorman SE, et al.; JCI. 1998)
(3) STAT1 (Dupuis S, et al.; Science. 2001)
(4) IL-12 p40 subunit (Picard C, et al.; AJHG. 2002)
(5) IL-12 receptor β 1 (Altare F, et al.; de Jong R, et al.; Science. 1998)

B. Genome-wide search for predisposing genes

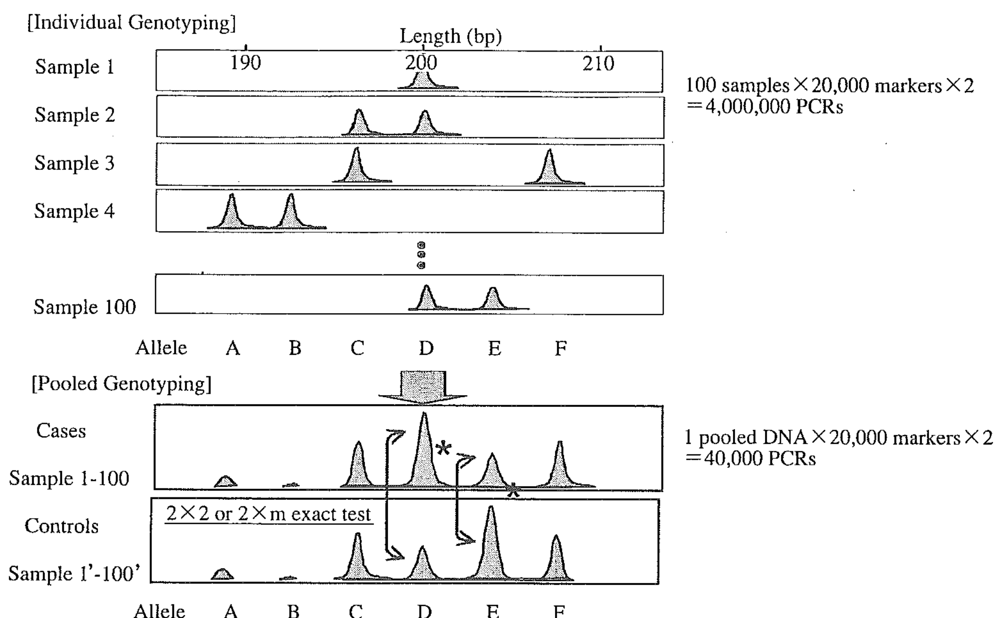

Fig. Principle of pool typing of microsatellite markers

るアプローチである。感染症の候補遺伝子を選択する際には，感染症の免疫機構の解明に伴い，重要と認識された分子，すなわち，HLAおよび抗原提示機構に関連する分子群，サイトカイン，ケモカインなどを対象にすることが一般的である。次に動物（主にマウス）における病原体感受性に関わる遺伝子がポジショナルクローニングなどにより同定された場合，そのヒト相同遺伝子が候補になっている。細胞内寄生細菌抵抗性に関わるヒト*NRAMP1*遺伝子と結核との関連がよい例である[2]。

MAC症に関しては，単一遺伝子による，免疫不全を伴う全身性播種性のMAC感染症の家系が国外から報告されている（Table）。Th1サイトカインに関連した遺伝子の異常によるものがほとんどである。このことは，MAC症においても，結核その他の肉芽腫性疾患同様，Th1系免疫応答が感染防御に重要な役割を果たしていることを示している。

一方，明らかな全身免疫異常を伴わない，中高年女性に多く見られるMAC症孤発例について，症例を集積して，症例対照研究の形で，候補遺伝子の遺伝子多型の頻度が，症例群に有意に高い（疾患感受性），有意に低い（疾患抵抗性）ことを検討している報告は，限られている。オッズ比2程度までの弱い関連を示す遺伝子多型の有意差を再現性よく検出するには，それに相応したサンプル数が必要である[3)~6)]。MAC症においては，HLA-DR6との関連が報告されているが，検討は必ずしも十分ではなく，多重比較の補正の問題をクリアしたとしても，DR6自体の機能が，MAC症発症と関連するのか，HLA領域にあるHLA遺伝子以外の免疫関連遺伝子が，MAC症の疾患関連遺伝子となっているか，不明な点は多い。

MAC症と候補遺伝子との関連性

われわれは，国立病院機構東京病院との共同研究によ

り，2001年より，MAC症の候補遺伝子関連解析を開始して，これまで，自然抵抗性関連マクロファージ蛋白(*NRAMP1*)，ビタミンDレセプター (VDR)，マンノース結合レクチン (MBL) など，結核症ですでに関連が報告されたことのある主な遺伝子多型について，症例111例，対照177例で検討を行い，*NRAMP1*遺伝子多型とMAC症との間に有意な関連を認めた(田中：投稿準備中)。

MAC症とゲノムワイド関連解析

われわれは，さらに国立病院機構近畿中央胸部疾患センターを中心とした多施設研究によって得られた300症例のMAC症検体を用いて，東海大学猪子英俊教授との共同研究により，同教授の開発されたpooled samplesによる，約2万マーカーのゲノムワイドマイクロサテライト関連解析を実施した (Fig.)。3段階のスクリーニングにより，抽出された43マーカーについて，現在，individual typingを行っている。有意なマーカーが得られれば，その周辺にMAC症感受性遺伝子の存在が期待される。

おわりに

抗酸菌感染症の疾患感受性，抵抗性に影響を与える遺伝因子の詳細は，まだ十分に解明されたとはいえず，現在，他の多因子疾患同様，罹患同胞対解析やゲノムワイド関連解析などの方法が試行されている。感染症関連遺伝子多型の解明は，現存の生物が病原微生物に対する防御機構を進化させてきた道のりをたどる研究でもあり，その長期にわたる攻防を垣間見ることにより，これまでに予想されなかった，新たな予防，治療法が開ける可能性もあり，今後の進展が期待される。

謝　　辞

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[1]J. Shojima, [1]G. Tanaka, [1]I. Matsushita, [2]Y. Inoue, [2]K. Suzuki, [2]M. Sakatani, [2]M. Okada, [2]K. Kimura, [3]E. Toyoda, [3]N. Kobayashi, [3]K. Kudo, [4]H. Nagai, [4]A. Kurashima, [5]A. Kajiki, [6]N. Oketani, [7]H. Hayakawa, [8]T. Shirakawa, [9]M. Tamari, [10]K. Nakata, [11]A. Oka, [11]S. Ando, [11]G. Tamiya, [12]T. Sasazuki, [11]H. Inoko

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3. 生体の抗酸菌症感受性と結核ワクチン研究

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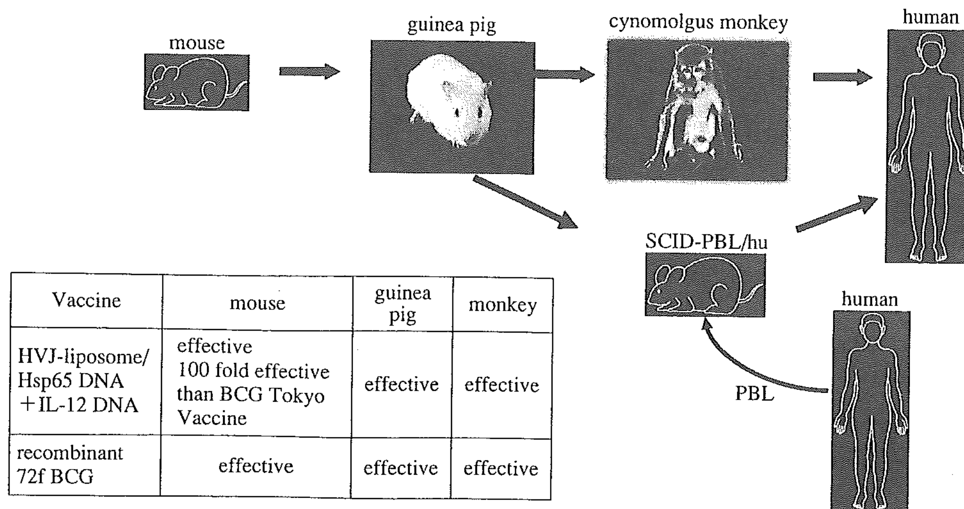
はじめに

いまだに世界の人口の3分の1が結核菌の感染を受け，その中から毎年800万人の結核患者が発生し，200万人が死亡している，最大の感染症の1つである。

結核菌に対する宿主側の抵抗性は主としてT細胞免疫によって担われている。事実，HIV感染症やT細胞免疫不全 (生体の抗酸菌感受性) に伴う結核感染合併が大き



Vaccine	mouse	guinea pig	monkey
HVJ-liposome/ Hsp65 DNA +IL-12 DNA	effective 100 fold effective than BCG Tokyo Vaccine	effective	effective
recombinant 72f BCG	effective	effective	effective

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

Table Survival of cynomolgus monkeys immunized with HVJ-liposome/ Hsp65 DNA＋IL-12 DNA vaccine and recombinant 72f BCG vaccine

Vaccination	Total monkeys	Survival	% Survival
HVJ-liposome/Hsp65 DNA ＋IL-12 DNA	4	2	50
Recombinant 72f BCG	4	3	75
BCG Tokyo	4	2	50
Saline	4	0	0

Cynomolgus monkeys (4 monkeys/group) were immunized three times (every 3 weeks) with (1) HVJ-liposome/ Hsp65 DNA＋IL-12 DNA vaccine, (2) r72f BCG vaccine, (3) BCG Tokyo and (4) saline as control group as described in Section 2. One month after last immunization, M.TB (Erdman strain 5×10^{2}) was challenged by intratracheally instillation. Survival was studied more than 14 months.

な問題となっている。したがって，T細胞免疫を増強するワクチン療法や免疫療法が理論的のみでなく，実際的にも開発されつつある[1)~4)]。

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よりも100倍以上強力な結核予防ワクチン効果を示す新しいDNAワクチン（Hsp65 DNA＋IL-12 DNAワクチン）やリコンビナント72f BCGワクチンを開発した（Fig.）。したがって，結核患者のT細胞免疫低下解析とT細胞免疫増強ワクチンの研究成果を中心に，新しい抗結核ワクチンの臨床応用への動きと課題について検討する。

方法と結果

〔A〕生体の結核菌抵抗性

CD8^{+}T細胞が結核菌で感染したMϕ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。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。

多剤耐性結核患者末梢血リンパ球をPPDで4日刺激して，リンパ球のgranulysin mRNAをRT-PCRにて定量した。その結果多剤耐性結核患者では健常人に比し著明な低下が認められた。また，糖尿病合併の難治性結核ではキラーT細胞低下例が認められた。

〔B〕結核ワクチン

［I］HVJ-liposome/Hsp 65 DNA＋IL-12 DNAワクチン

マウスでは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（Table）。われわれは① Hsp 65 DNA＋IL-12 DNA（HVJ-liposomeベクター）のワクチンはBCGよりも100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。このワクチンはキラーTの分化を増強しIFN-γ，IL-2，IL-6の産生を増強した。

〔治療ワクチン〕さらに，Hsp 65 DNA＋IL-12 DNAワクチンは治療結核ワクチン効果も示した。欧米では治療ワクチンは未開発。

［II］リコンビナント72f BCGワクチン