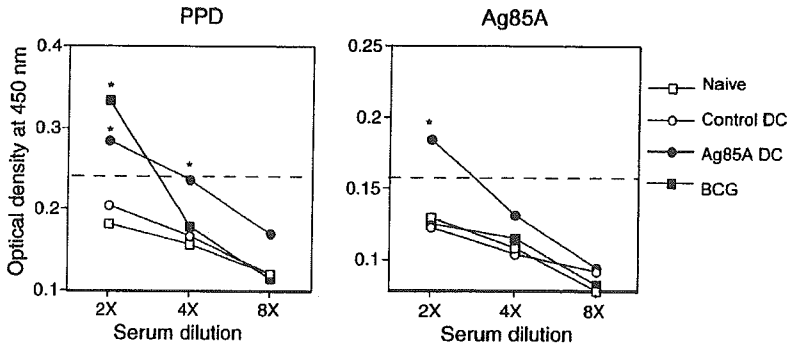

Fig. 2. Detection of PPD- and Ag85A-reactive antibodies (Abs) in the sera of Ag85A gene-transduced DC-immune mice. The sera of control untransduced DC-, Ag85A gene-transduced DC-, or BCG-immune mice or naïve mice were examined for binding to PPD (left panel) or purified Ag85A protein (right panel) by ELISA. The mean optical density at 450 nm of six mice in each group for PPD-reactive Abs and those of three mice in each group for Ag85A protein-reactive Abs are shown. Horizontal broken lines in figures indicate the two-fold greater values than the average values of 8× diluted sera of naïve mice. Asterisks indicate statistically significant ($p < 0.01$ for PPD, $p < 0.03$ for Ag85A) compared with the average value of 8× diluted sera of control untransduced DC-immune mice.

3.2. Ag85A gene-transduced DC vaccination was able to generate PPD- and Ag85A -reactive Abs in vivo

After injection of Ag85A gene-transduced DCs into BALB/c mice, we first examined the production of PPD-reactive Abs in the vaccinated mice. Ag85A molecule is one of the most abundant secreted proteins in *M. tuberculosis* and PPD contains the molecule. PPD-reactive Abs will be therefore produced if Ag85A molecule is successfully expressed in the vaccinated mice. Sera were prepared from the immunized mice 1 month after the last immunization and examined for antibodies for PPD. Sera from Ag85A gene-transduced DC-immune mice showed higher binding units to PPD than sera from control untransduced DC-immune mice and naïve mice (Fig. 2, left panel). Sera from BCG-vaccinated mice also showed PPD-binding activity. Furthermore, the sera were also examined for Abs specific for Ag85A protein (Fig. 2, right panel). Sera from Ag85A gene-transduced DC-immune mice showed binding activity to Ag85A protein. In this time, sera from BCG-immune mice did not show Ag85A protein-binding activity. These results suggest that Ag85A gene-transduced DC-vaccinated mice produced Ag85A-reactive Abs in the sera.

3.3. Ag85A gene-transduced DC vaccination induced PPD-specific spleen cell proliferation, and PPD- and Ag85A-specific IFN-γ production from the spleen cells

We then examined the proliferative response of spleen cells derived from Ag85A gene-transduced DC-immune mice in response to in vitro PPD stimulation. As shown in Fig. 3A, a significant proliferative response was observed in Ag85A gene-transduced DC-immune mice. The level of the response was comparable to that of BCG-immune mice. Only faint proliferative response was detected in control untransduced DC-immune mice.

In addition, we examined IFN-γ production from spleen cells of Ag85A gene-transduced DC-immune mice. Correlating with the proliferative response, spleen cells from mice immunized with Ag85A gene-transduced DCs produced high amounts of IFN-γ after in vitro stimulation with PPD. The IFN-γ amounts produced by the spleen cells of Ag85A gene-transduced DC-immune mice were higher than those by the spleen cells of BCG-vaccinated mice (Fig. 3B), suggesting that immunization with Ag85A gene-transduced DC efficiently generates PPD-specific IFN-γ-producing cells in vivo. Further, we also examined IFN-γ production from spleen cells of Ag85A gene-transduced DC-immune mice in response to purified Ag85A protein. As shown in Fig. 3C and D, the spleen cells of Ag85A gene-transduced DC-immune mice were capable of producing IFN-γ in response to purified Ag85A protein.

3.4. Ag85A gene-transduced DC immunization can generate the antigen-specific CD4$^+$ and CD8$^+$ T cells

In previous section, we examined immune responses of splenocytes derived from Ag85A DC-immune mice in response to PPD or purified Ag85A protein. CD4$^+$ T cells are speculated to respond to these exogenous antigens which should be presented on MHC class II molecules on APCs. We next examined whether Ag85A gene-transduced DC immunization is capable of inducing the antigen-specific CD4$^+$ or CD8$^+$ T cells. CD4$^+$ and CD8$^+$ T cells in the spleens of Ag85A gene-transduced DC-immune mice were prepared with magnetic beads. They were cultured with Ag85A gene-transduced DCs and examined IFN-γ amounts in the culture supernatants. As shown Fig. 4, CD4$^+$ T cell- or CD8$^+$ T cell-enriched splenocytes of Ag85A gene-transduced DC-immune mice produced IFN-γ in the presence of Ag85A gene-transduced DCs. In this experiment, CD4$^+$ T cell-enriched splenocytes of control DC-immune mice also produced IFN-γ in the presence of Ag85A gene-transduced DCs, although the amounts were lower than those by CD4$^+$ T cell-enriched splenocytes of Ag85A gene-transduced DC-immune mice (Fig. 4). It may be caused by bovine serum

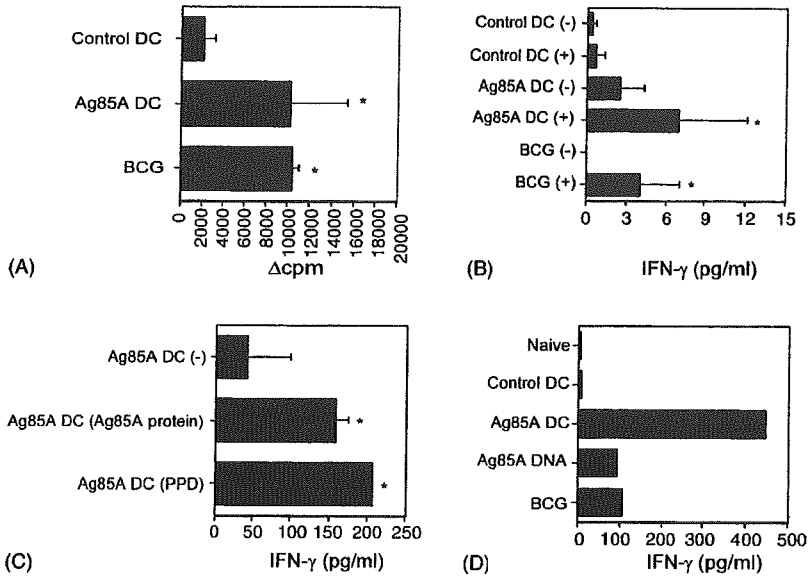

Fig. 3. (A) PPD-specific splenocyte proliferation of mice immunized with DCs transduced with Ag85A gene. BALB/c mice were immunized with Ag85A gene-transduced DCs two times at a 1-week interval. The data of mice immunized with BCG twice were also shown as controls. Spleen cells of the immunized mice were harvested 1 month after the last immunization and cultured in vitro in the presence or absence of 5 μg/ml of PPD for 48 h and pulsed with 0.5 μCi [methyl-^{3}H] thymidine for last 12 h. The values represent Δcpm (the value after in vitro stimulation in the presence of PPD subtracted by the value in the absence of PPD). The mean ± S.D. of quintuplicate determinations of a representative experiment from three independent experiments, are shown. Asterisks indicate statistically significant ($p < 0.001$) compared with the value of control untransduced DC immune mice (Control DC). (B) PPD-specific IFN-γ production from spleen cells of Ag85A gene-transduced DC-immune mice. BALB/c mice were immunized with Ag85A gene-transduced DCs two times at a 1-week interval. The data of mice immunized with BCG twice were also shown as controls. Spleen cells of the immunized mice were harvested 1 month after the last immunization and cultured in vitro in the presence or absence of 10 μg/ml of PPD for 72 h. The supernatants were harvested and assayed for IFN-γ with ELISA. The mean ± S.D. of five independent experiments are shown. Asterisks indicate statistically significant ($p < 0.001$) compared with the value of control untransduced DC-immune mice in the absence of PPD [Control DC(−)]. (C, D) Ag85A-specific IFN-γ production from spleen cells of mice immunized with Ag85A gene-transduced DCs. (C) BALB/c mice were immunized with Ag85A gene-transduced DCs twice at a 1-week interval. Spleen cells of the immune mice were harvested 1 month after the last immunization and cultured in vitro in the presence of 5 μg/ml of Ag85A protein or 10 μg/ml of PPD for 72 h. The supernatants were harvested and assayed for IFN-γ with ELISA. The mean ± S.D. of three independent experiments are shown. Asterisks indicate statistically significant ($p < 0.04$) compared with the value without Ag85A protein or PPD [Ag85A DC (−)]. (D) BALB/c mice were immunized with control untransduced DCs (Control DC), Ag85A gene-transduced DCs (Ag85A DC), Ag85A expression plasmid DNA (Ag85A DNA), or BCG. Spleen cells of the immunized mice were harvested 1 month after the last immunization and cultured in vitro in the presence of 5 μg/ml of Ag85A protein for 72 h. The supernatants were harvested and assayed for IFN-γ with ELISA. Average values from two independent experiments are shown.

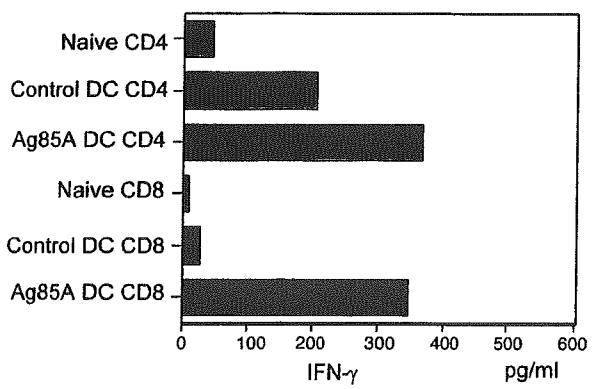

Fig. 4. Ag85A gene-transduced DC immunization elicits the antigen-specific CD4$^+$ and CD8$^+$ T cells. CD4$^+$ and CD8$^+$ T cell-enriched spleen cells of control untransduced DC-, or Ag85A gene-transduced DC-immune mice were cultured with in vitro-prepared Ag85A gene-transduced DCs for 4 days and the supernatants were examined for IFN-γ with ELISA. Naïve BALB/c mice were also examined as controls.

proteins contained in culture medium for DCs. Immunization with DCs taken up the proteins may induce CD4$^+$ T cells specific to these proteins, which would lead to the relatively high background value.

3.5. *Ag85A gene-transduced DC immunization can generate the antigen-specific CTL*

Denis et al. [29] reported several candidate CTL epitopes on Ag85A in BALB/c mice. In order to identify minimal CTL epitopes on Ag85A in BALB/c mice, we examined IFN-γ production by spleen cells derived from Ag85A DNA vaccine-immune BALB/c mice in response to several candidate CTL epitope peptides. We chose these peptides because results in Denis et al. [29] indicate that 20-mer peptides containing these 9-mer peptides showed stimulatory effects on splenocytes from Ag85A DNA-immune BALB/c mice, and also these peptides showed high scores to bind H2-K^d or H2-L^d molecules in a computer algorithm for epitope prediction (SYFPEITHI; http://www.syfpeithi.de). We demon-

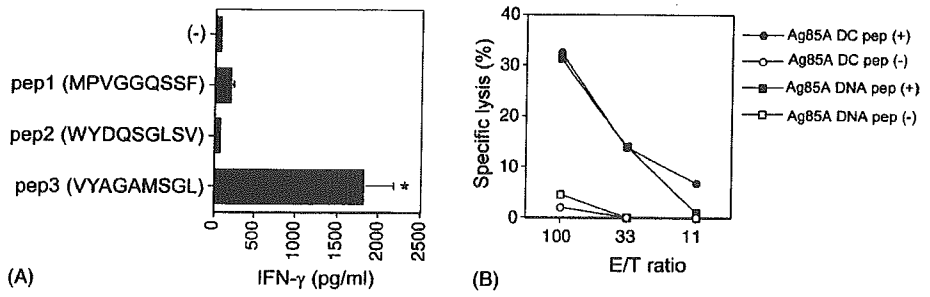

Fig. 5. Cytotoxic activity of Ag85A-transduced DC-immune splenocytes to VYAGAMSGL peptide-pulsed RAW264.7 cells. (A) IFN-γ production by spleen cells of BALB/c mice immunized with Ag85A plasmid DNA in the presence of candidate CTL epitope peptides. The spleen cells produced the significant level of IFN-γ only in the presence of VYAGAMSGL peptide. The mean ± S.D. of three independent experiments are shown. Asterisks indicate statistically significant ($p < 0.001$) compared with the value without any peptides [(−)]. (B) Spleen cells of Ag85A DC- or Ag85A plasmid DNA-immune mice (effectors) were incubated with the peptide-pulsed RAW264.7 cells (target cells) with the effector/target ratios (E/T ratio) indicated on the *x*-axis.

strated that only one peptide (VYAGAMSGL) among peptides examined was able to induce IFN-γ production by the spleen cells (Fig. 5A).

We next determined whether the peptide-specific CTL were generated following Ag85A gene-transduced DC vaccination. After in vitro restimulation of immune spleen cells with the peptide, spleen cells obtained from Ag85A gene-transduced DC-immune mice showed cytolytic activity to the peptide-pulsed RAW264.7 cells. The CTL activity was comparable to that by spleen cells from Ag85A DNA vaccine-immune mice (Fig. 5B). This result indicates that Ag85A gene-transduced DC immunization is capable of eliciting CTL specific for at least one CTL-epitope in Ag85A protein.

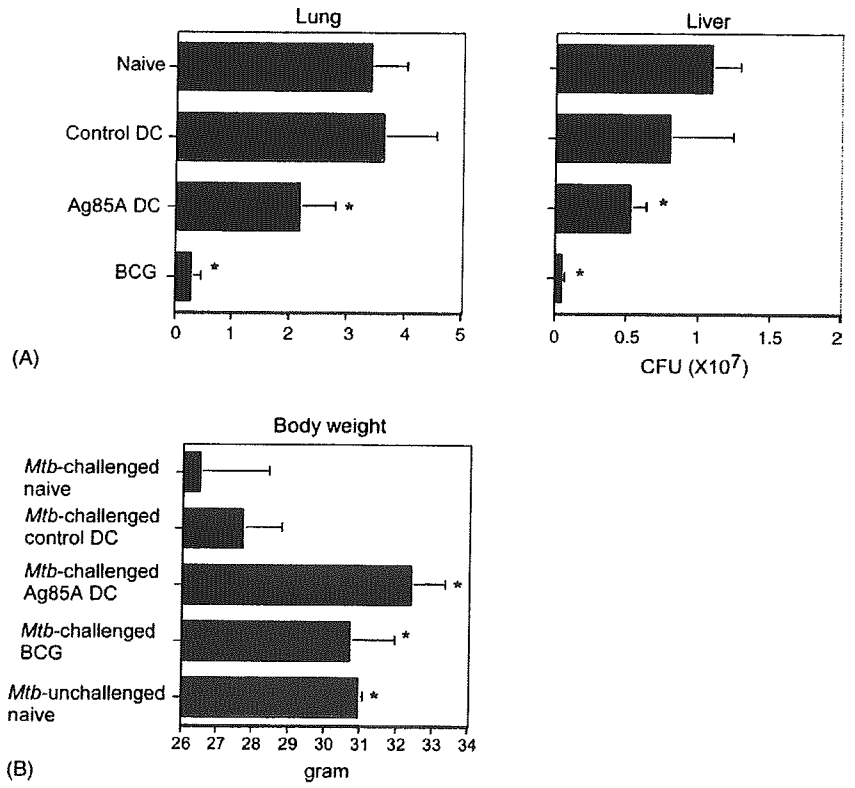

Fig. 6. (A) In vivo protective activity of mice immunized with DCs transduced with Ag85A gene against virulent *M. tuberculosis* challenge. BALB/c mice were immunized with Ag85A gene-transduced DCs twice at a 1-week interval. These mice were challenged i.v. with 5×10^5 CFU of live *M. tuberculosis* H37Rv. Numbers of the CFU in the lungs and the livers were determined 4 weeks later. The mean ± S.D. of five mice in each group are shown. Asterisks indicate statistically significant ($p < 0.05$) compared with the value of naïve mice. (B) Body weights of mice immunized with DCs transduced with Ag85A gene after virulent *M. tuberculosis* H37Rv challenge. Body weights of control untransduced DC-, Ag85A gene-transduced DC-, or BCG-immune mice or naïve mice were measured 4 weeks after i.v. challenge of *M. tuberculosis* H37Rv. Body weights of unchallenged naïve mice were also shown as controls. The mean ± S.D. of five mice in each group are shown. Asterisks indicate statistically significant ($p < 0.001$) compared with the value with *M. tuberculosis*-challenged naïve mice.

3.6. Ag85A gene-transduced DC immunization can provide moderate protective immunity against a subsequent challenge with viable *M. tuberculosis*

We finally evaluated the effects of Ag85A gene-transduced DC immunization on protective immunity against *M. tuberculosis* infection. Four weeks after i.v. injection with *M. tubeculosis* H37Rv, spleens, livers, and lungs were prepared from the immunized mice and the CFU of *M. tuberculosis* H37Rv in these tissues were evaluated. As shown in Fig. 6A, the CFU in lungs and livers of Ag85A gene-transduced DC-vaccinated mice, but not control untransduced DC-immune mice, were significantly lower than those of naïve mice ($p < 0.05$), although the difference was less than one log 10 order. But the CFU in spleens were not significantly different between Ag85A gene-transduced DC-vaccinated mice and naïve mice (data not shown). In addition, we evaluated body weights of the same mice as used in this challenge study. It is especially noteworthy that body weights of Ag85A gene-transduced DC-immune mice were as high as those of unchallenged naïve mice whereas naïve and control untransduced DC-immune mice showed significant loss of body weights (Fig. 6B).

4. Discussion

DCs have been shown to be the most powerful APCs that initiate the primary immune response. DC vaccines have been examined for the efficacy as vaccines against infectious diseases as well as cancer [6–13]. There are several strategies for using DCs as vaccines against intracellular bacteria, including ex vivo pulses with bacteria or bacterial antigens, or transfer of genes encoding antigens to DCs. Among them, retroviral transduction is advantageous for long-term antigen presentation in vivo, because the transgene integrates into the chromosome leading to gene expression throughout the life of the cell and its progeny [9]. In our previous work [14], we showed that immunization with DCs retrovirally transduced with a minimal CTL epitope derived from *Listeria monocytogenes* successfully induced the specific CTL and protective immunity against lethal listerial challenge. Here, we examined immunization with DCs retrovirally transduced with *M. tuberculosis*-derived Ag85A gene. The results shown here indicate that the DC immunization successfully induced the specific cellular immunity, including immune responses of $CD4^+$ T cells and $CD8^+$ CTL, as well as specific antibody responses. The de novo synthesized Ag85A proteins in DCs would be processed in MHC class I pathway to induce specific $CD8^+$ T cells. The Ag85A proteins are then secreted from DCs and would induce specific Abs. Specific $CD4^+$ T-cell responses to the proteins may be evoked through uptake of the secreted proteins by APCs or direct antigen presentation by Ag85A gene-transduced DCs. The conclusive description waits further analysis of the antigen presentation mechanisms in this system.

In this work, we identified a minimal CTL epitope on Ag85A molecule in BALB/c mice. We showed here that immunization with DCs retrovirally transduced with Ag85A gene could efficiently induce the CTL activity specific to a peptide in Ag85A molecule, VYAGAMSGL. Denis et al. [29] showed that vaccination of BALB/c mice with Ag85A plasmid DNA induced the CTL activity against target cells pulsed with at least three 20-mer peptides in Ag85A. We, however, observed CTL activity only to VYAGAMSGL-pulsed target cells. Generally, the number of the dominant CTL epitope in one protein is small (one or two). In our previous work for identifying T-cell epitopes on MPT51 molecule derived from *M. tuberculosis*, we only identified one dominant CTL epitope on the protein in BALB/c mice [30]. We therefore speculated that the peptide (VYAGAMSGL) is the dominant CTL epitope on Ag85A molecule in BALB/c mice. The peptide was highly predicted to bind to H2-K^d molecule in an MHC-binding peptide prediction algorithm [the binding score in SYFPEITHI (http://www.syfpeithi.de) is 25 and that in RANKPEP (http://www.mifoundation.org/Tools/) is 102.0].

Ag85A gene-transduced DC immunization was able to induce PPD- and Ag85A-specific immune responses. The immunization, however, led to the moderate level of protection against virulent *M. tuberculosis* challenge. Body weights of *M. tuberculosis*-challenged mice appeared to indicate that Ag85A gene-transduced DC immunization was very effective (Fig. 6B), but the immunization was not so strikingly effective in terms of clearance of *M. tuberculosis* from tissues (Fig. 6A). It seems to be a good possibility that the DC immunization was able to induce granuloma formation which restricts *M. tuberculosis* growth and at the same time permits persistence of *M. tuberculosis*. In addition, several other factors would be also speculated. First, the amount of DCs immunized to the mice may be critical. Indeed, when we immunized mice with 5×10^5 DCs instead of 1×10^5 DCs, we observed much more bacterial burden in tissues in the immune mice after *M. tuberculosis* challenge (data not shown). Too much immunization of DCs augmented T-cell response against pathogens including the IFN-γ production by T cells, but that may not be favorable for the protective capacity of the DC immunization. González-Juarrero et al. [32] reported that intranasal immunization with lung-derived DCs pulsed with Ag85A protein elicited IFN-γ production by $CD4^+$ T cells but showed exacerbation in terms of the protective capacity against *M. tuberculosis* infection. The exacerbation was attributed to florid pulmonary inflammatory responses by the DC immunization. Further assessment of optimal DC dosage to be immunized and careful examination of tissue pathology would be necessary. Second, condition of DCs to be vaccinated may be also important. In this work and our previous work [14], we used DCs incubated with medium supplemented with GM-CSF and IL-4, but we did not treat the DCs with maturation-inducing reagents, such as lipopolysaccharide or CpG oligodeoxynucleotides. We chose this condition because we think that DCs maturate after the injection into mice. Indeed, in our previous work [14],

immunization with DCs which were not treated with such reagents successfully induced protective immunity against *L. monocytogenes*. The culture condition of DCs which is most optimal for immunization should be clarified in further studies. In addition, the expression level of Ag85A in the transduced DCs may not have been strong enough to induce more protective immunity, although Ag85A gene expression was observed in RT–PCR analysis.

We also evaluated the prime-boost regimen, namely, the regimen in which, mice were primed with Ag85A gene-transduced DC vaccine and boosted with BCG injection. Our data showed that the protocol was not effective compared with two BCG injection protocol in terms of *M. tuberculosis* clearance from tissues after the intravenous challenge (data not shown). Several investigators evaluated the regimen in which DNA immunization was used for priming and BCG vaccination for boosting. Ag85B DNA vaccination followed with BCG vaccination has been shown to be more effective than BCG immunization alone in protecting against *M. tuberculosis* infection [33,34]. However, Skinner et al. [35] reported that priming with Ag85A/ESAT-6 fusion DNA vaccination and boosting with BCG vaccination augmented antigen-specific IFN-γ-producing T cell number, but did not increase the protective efficacy of BCG against *M. tuberculosis*. Skinner et al. [35] pointed out several possible reasons including the difference of BCG strains used. A variety of factors must be considered for the successful prime-boost regimen.

Taken together, we showed here that immunization of DCs retrovirally transduced with Ag85A gene was able to elicit specific cellular immune responses containing $CD4^+$ and $CD8^+$ T-cell responses as well as specific Ab production. During this study, we identified a minimal CTL epitope on Ag85A molecule in BALB/c mice. But the responses lead to only a moderate level of protective immunity. Further study is clearly necessary to improve the effectiveness of DC vaccines against TB.

Acknowledgments

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IS*6110* DNA fingerprinting analysis of individually separated colonies of *Mycobacterium tuberculosis*

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KEYWORDS
IS*6110* RFLP;
Spoligotyping;
Individually separated colony

Summary There are no data so far that show IS*6110* restriction fragment length polymorphism (RFLP) patterns of individually separated tuberculosis bacilli from clinical isolates, and their alterations during follow-up surveys. We picked 20–60 tuberculosis clones from clinical isolates under anti-tuberculosis medication, and individually analysed their DNA fingerprinting patterns using IS*6110* RFLP as well as spoligotyping as a second typing.

The study using cloned bacilli of *Mycobacterium tuberculosis* showed that clinical isolates contained several clones with different DNA fingerprints and that their band patterns altered weakly but distinctly during follow-up surveys. However, there was no significant difference in the fingerprinting patterns when clinical isolates were to RFLP without separating to subjected/individual colonies.

In view of the IS*6110* RFLP of individually separated tuberculosis bacilli, we have now speculated several possibilities: (1) that clones with different DNA fingerprints exist in clinical isolates; (2) that IS*6110* RFLP patterns of the materials depend on the population of the original clone and the variants having DNA fingerprints different from the original pattern; and (3) that their band patterns are influenced not only by the stability of the original germ having its own fingerprint, but also by the fragility of the new clones.

Introduction

IS*6110* DNA fingerprinting of *Mycobacterium tuberculosis* is one of the powerful tools for transmission analysis of tuberculosis.[1–4] Clinical isolates from patients with tuberculosis have been considered to contain clonally expanded *M. tuberculosis*.

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Therefore, it is generally accepted that their DNA fingerprint patterns are derived from their original clones. The estimated average half-life of the band of IS*6110* DNA fingerprinting is approximately 3.2–3.7 years.[5–7] Thus, the DNA band patterns of *M. tuberculosis* obtained from sputa may alter slowly but distinctly. There are no data so far that show the DNA fingerprinting of each clonally separated *M. tuberculosis* from sputa, and their alterations during follow-up surveys. In order to elucidate this point, we analysed the IS*6110* DNA fingerprinting, as well as spoligotyping as a second typing, of the single-cloned *M. tuberculosis*.

Material and method

Patients

Since 2001, all *M. tuberculosis* complex isolates in Osaka Prefectural Habikino Hospital (since October 2003, renamed as Osaka Prefectural Medical Center for Respiratory and Allergic Diseases) have been subjected to IS*6110* DNA fingerprinting.[8] Four patients with lung tuberculosis were selected in this study because their present illness and the drug susceptibilities of their bacilli were intensively investigated. The drug susceptibility profiles of cases 1–4 were recorded, and the corresponding clinical isolates were stored. Case 1 suffered from primary rifampicin-resistant tuberculosis. He was given a 6-month standard short course therapy and was initially cured. However, tuberculosis relapsed 30 months later. Cases 2 and 3 had multi-drug resistant (MDR) tuberculosis. Tuberculosis of case 4 was streptomycin (SM) resistant. After a standard short course of anti-tuberculosis therapy, the disease of case 4 relapsed again. In case 2, interestingly, the susceptibility to rifampicin transiently increased, and then decreased again. In cases 3 and 4, transient alterations were observed in IS*6110* restriction fragment length polymorphism (RFLP) patterns during follow-up surveys, which disappeared finally. Brief summaries of these patients are described in Table 1.

Collection of bacilli of *M. tuberculosis*

The studied bacilli of *M. tuberculosis*, grown from sputa, were frozen and stored at −80 °C. The storage of *M. tuberculosis* and analysis of its DNA were performed after obtaining written informed consent.

Single colony isolation

The frozen *M. tuberculosis* stocks were thawed and cultured on 7H11 agar medium. As many as possible resulting colonies were individually picked (up to 60 colonies) and cultured in 3 ml of liquid medium for 3–4 weeks. The growing cells were cultured onto the slopes of Ogawa medium.

DNA extraction

Bacilli of *M. tuberculosis* were harvested from the slopes of Ogawa medium, placed into 0.75 ml of buffer (0.3 M Tris–HCl, 0.1 M NaCl, 6 mM EDTA) with 25% (v/v) of 0.1 mm glass beads, vortexed by a bead beater (Biospec products) for 200 s at 4600 rpm and centrifuged at 5000 rpm for 5 min. The supernatant was collected and the DNA was extracted by the standard phenol–chloroform extraction method.

IS*6110* RFLP analysis

The IS*6110* DNA fingerprinting was performed according to a standardized protocol.[9] Briefly, genomic DNA was digested with restriction endonuclease *Pvu*II (Wako junyaku, Japan), electrophoresed through a 0.8% agarose gel, and vacuum blotted onto a nylon membrane.[10] The molecular size standard used was a mixture of *Hind*III-digested fragments of bacteriophage λDNA and *Hae*III-digested φx174 DNA (5 ng of each digest). After blotting, the membranes were hybridized with a

Table 1 Patient characteristics.

	Sex	Age (years)	Drug resistance
Case 1	Male	39	RFP
Case 2	Female	76	INH, RFP, EB, KM, CS, TH, LVFX
Case 3	Male	61	INH, RFP, EB, SM, KM, CS, TH, LVFX, EVM
Case 4	Male	61	SM

INH: isoniazid; RFP: rifampicin; EB: ebuthol; SM: streptomycin; KM: kanamycin; CS: cycloserine; TH: ethionamide; LVFX: levofloxacin; EVM: emviomycin.

digoxigenin-labelled IS*6110* probe. Hybridization and colorimetric detection were performed in hybridization bags. The restriction fragment patterns revealed with IS*6110* were captured on 256 grey-scale tagged image file format images at 150 dots per square inch by flat bed scanning. The images were compared with each other using the computer program Gel-Compar (Applied Maths).

Spoligotyping

The clones of *M. tuberculosis* with less than five IS*6110* RFLP bands were subjected to the second typing, spoligotyping. Spoligotyping membranes were purchased from Isogen bioscience BV (Bilthoven, The Netherlands). The procedures were carried out according to the manufacturer's instructions.

Results

In case 1, there was no difference in the DNA fingerprinting pattern of *M. tuberculosis* between the first and the recurrent stage when clinical isolates were subjected to IS*6110* RFLP analysis without cloning. However, the RFLP analysis of individual colonies isolated from clinical isolates revealed that clones having different fingerprints had existed even at the first stage (Fig. 1). Such variants were also observed in those at the recurrent stage, although the number was smaller

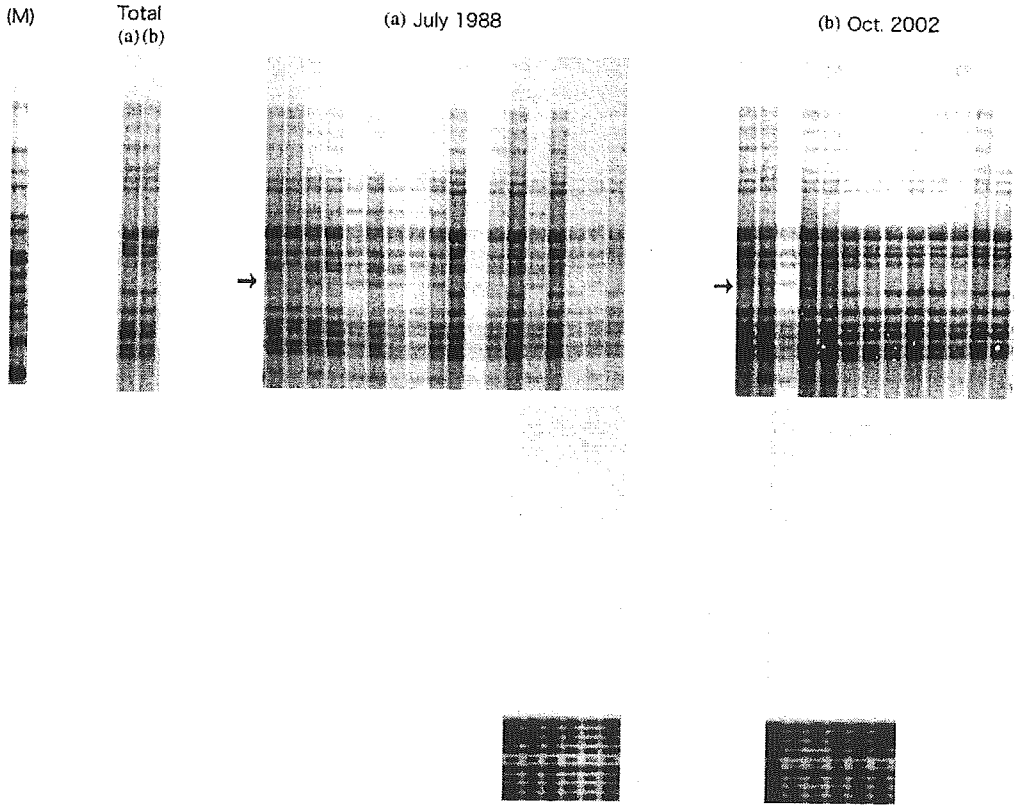

Figure 1 The IS*6110* RFLP patterns of clinical isolates and individually separated colonies in case 1. Symbol (M) indicates the IS*6110* fingerprinting of 37Ra. The upper and lower columns show the IS*6110* RFLP fingerprint and the corresponding spoligotyping, respectively. (a) and (b) in the upper left part indicate the fingerprints of whole clinical isolates. (a) July 1998 and (b) October 2002 in the upper right part show the IS*6110* RFLP of individually separated colonies corresponding to the date: (a) before the primary medication and (b) before the secondary medication after relapse. There are colonies of *M. tuberculosis* in sputa showing weakly altered patterns in the IS*6110* DNA fingerprint. In the IS*6110* DNA fingerprint of the colonies separated from sputa collected (a) before and (b) after anti-tuberculosis medication, there is an additional band (arrow) in the separated tuberculosis DNA fingerprint (a). However, the band is not seen in RFLP of the whole isolates (b), because the population of the bacilli with the additional band is smaller than that of those without the band. There was no difference in spoligotyping patterns among these colonies.

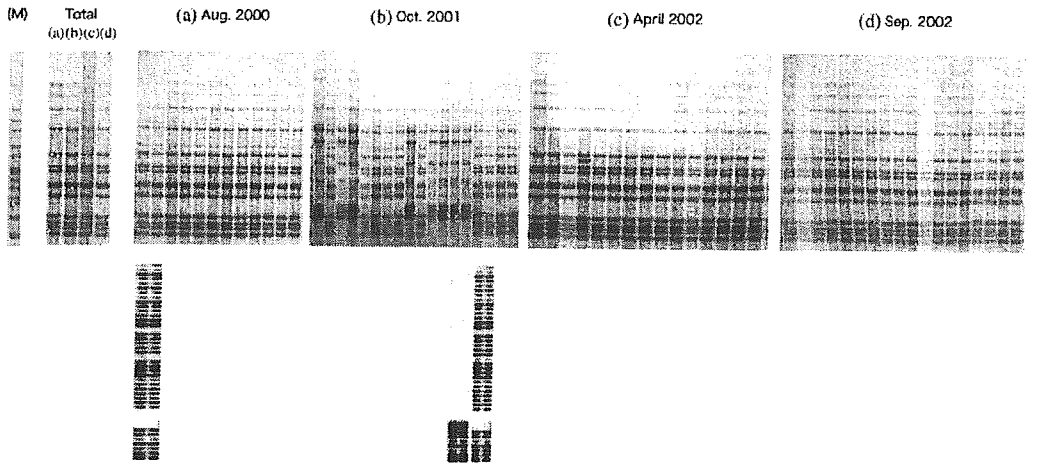

Figure 2 The IS*6110* RFLP pattern of clinical isolates and individually separated colonies in case 2. Symbol (M) indicates the IS*6110* fingerprinting of 37Ra. The upper and lower columns show the IS*6110* RFLP fingerprint and the corresponding spoligotyping, respectively. (a), (b), (c) and (d) in the upper left part indicate the fingerprints of whole clinical isolates. (a) August 2000, (b) October 2001, (c) April 2002 and (d) September 2002 in the upper right part show the IS*6110* RFLP of individually separated colonies corresponding to the date. IS*6110* RFLP of individual colonies separated during follow-up surveys showed weak but distinct alterations among these patterns. These changes are not recognized in the analysis using total isolates without further cloning. From the point of view of the separated DNA fingerprints, the IS*6110* DNA fingerprints drastically altered. According to spoligotyping, the colony with different bands belonged to the Beijing family. It disappeared in the next analysis.

than that at the first stage. Spoligotyping analysis showed that there was no difference in the pattern of colonies between the first and the recurrent stage, indicating that the variants had been derived from the original clone.

Case 2 was a patient with MDR tuberculosis. During follow up, drug susceptibility of his bacilli showed interesting alterations: the susceptibility to rifampicin was low at first, was transiently elevated, and then became low again. DNA fingerprint analysis of picked clones in follow-up surveys revealed that several clones with drastically changed fingerprints had appeared (Fig. 2). However, no difference occurred in RFLP analysis during follow-up surveys when tested using whole clinical isolates without cloning.

These findings shown in IS*6110* RFLP analysis were also reflected on spoligotyping analysis (Fig. 2). The new clone appearing during follow-up survey, a member of the so-called Beijing family, exhibited good susceptibility to rifampicin, while the original clone was rifampicin resistant. These results strongly suggest that dual infection of *M. tuberculosis* with a different susceptibility to rifampicin occurred during therapy, and that the newly infecting bacilli were eliminated in this case.

In cases 3 and 4, tuberculosis was MDR and SM resistant, respectively. In IS*6110* DNA fingerprint analysis, transient alterations were observed during follow-up surveys, which disappeared finally. In a single colony analysis of cases 3 and 4, a clone with a new band appeared in the clinical isolates at relapse and then vanished. Finally, the fingerprint of the bacilli did not show any change in its pattern (Fig. 3(A) and (B)). Similarly, spoligotyping as a second typing showed that there was no difference in the pattern between original clones and those obtained after relapse (Fig. 3(A)), suggesting that such a variant, derived from the original clone, might be able to alter the DNA fingerprint patterns of the whole clinical isolate only if it occupied a major proportion in the materials.

Discussion

There has been no article describing IS*6110* DNA fingerprinting of clonally expanded tuberculosis bacilli from clinical isolates and its alteration during follow-up survey. The IS*6110* RFLP of clinical isolates has been considered to represent the sum of several DNA fingerprints of clones included in the clinical isolates. Our study using IS*6110* RFLP showed that DNA fingerprints of clinical isolates obtained from whole sputa consisted of bands showing differences in intensity. Low intensity bands were sometimes found in IS*6110* RFLP analysis. These bands may reflect the small population of bacilli with different DNA

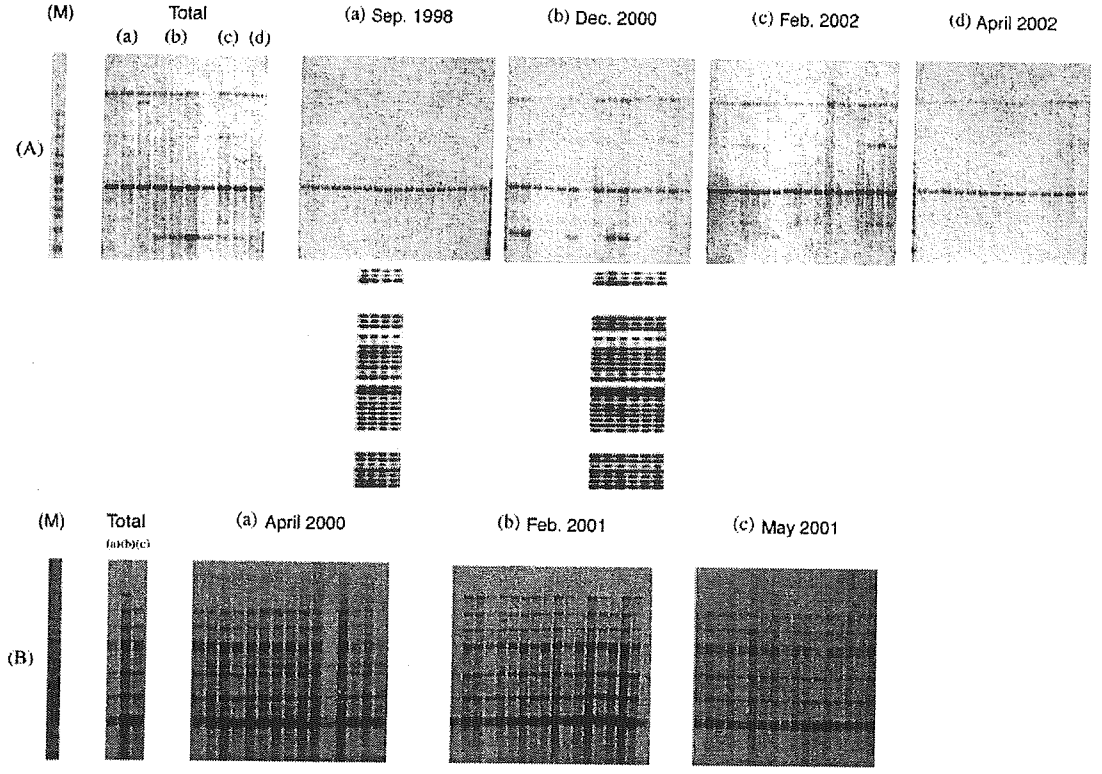

Figure 3 The IS*6110* RFLP pattern of clinical isolates and individually separated colonies in case 3 (A) and case 4 (B). In (A) and (B), symbol (M) indicates the IS*6110* fingerprinting of 37Ra. The upper and lower columns show the IS*6110* RFLP fingerprint and the corresponding spoligotyping, respectively. In (A), (a), (b), (c) and (d) in the upper left part indicate the fingerprints of whole clinical isolates. (a) September 1998, (b) December 2000, (c) February 2002 and (d) April 2002 in the upper right part show the IS*6110* RFLP of individually separated colonies corresponding to the date. In (B), (a), (b) and (c) in the upper left part indicate the fingerprints of whole clinical isolates. (a) April 2000, (b) February 2001 and (c) May 2001 in the upper right part show the IS*6110* RFLP of individually separated colonies corresponding to the date: (a) before the first standard short course medication for tuberculosis, (b) before the secondary medication after relapse, and (c) during the secondary medication. The IS*6110* DNA fingerprinting of clinical isolates shows that one band split into two bands, and then returned to one band. Data of the IS*6110* RFLP from the individually separated colonies showed that the clinical isolate with two bands consisted of clones with one band and of those with two bands. There was no difference in spoligotyping patterns among these colonies.

fingerprints. These tiny bands were not due to bacterial contamination in our laboratory, because spoligotyping confirmed that these new clones are identical with the original clones with respect to the other bands, and they have not been found in the other tests using IS*6110* RFLP analysis (data not shown).

De Boer et al.[11] have reported that low intensity bands in IS*6110* RFLP reflect genetic heterogeneity of *M. tuberculosis* isolates. The results of our study confirmed their speculation that IS*6110* DNA fingerprinting patterns depend on the populations of the original and the variant clones of *M. tuberculosis*. Our present study using both IS*6110* RFLP and spoligotyping of individually separated clones further revealed that the variants originating from the original clone of *M. tuberculosis* have only weakly altered DNA fingerprints, resulting in no apparent difference in their band patterns when clinical isolates were tested without further cloning.

DNA fingerprints of individual clones are well conserved, therefore small but distinct alterations in their patterns and their disappearance during follow-up surveys strongly suggest that the DNA fingerprint patterns depend not only on the stability of the clone, but also on the fragility of the variants with new bands. There was no correlation between prevalence of IS*6110* RFLP patterns and drug susceptibility of each clone (data not shown). In case 2, clones with drastically changed fingerprints transiently appeared during follow-up surveys. The clones with altered fingerprints, which had resistance to isoniazid (INH) and

SM, but susceptibility to RFP, soon disappeared. The patient had been treated with INH alone when the IS*6110* RFLP alteration was found. Thereafter, the patient was given an additional treatment with ethionamide (TH). TH might eradicate the clone with a new band. In view of colony separation, additional infection or reactivation with *M. tuberculosis* strains with the different IS*6110* RFLP findings should be considered.

Conclusion

IS*6110* RFLP of *M. tuberculosis* separated from whole sputa represents the sum of DNA fingerprints of several clones included in the clinical isolates. The IS*6110* RFLP patterns depend not only on the stability of the original clone but also on the fragility of new clones.

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途上国におけるハンセン病の病理診断

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〔受付・掲載決定：2005年7月21日〕

キーワード：JICA、国際協力、病理検査、ミャンマー、免疫組織化学

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ハンセン病が多発している発展途上国では、ハンセン病の診断は臨床症状だけで行われることが多い。しかし、ハンセン病対策が進んで診断困難症例や治療困難症例が増えると、生検組織の病理診断が確定診断のために必要となる。本論文では、JICAミャンマー国ハンセン病対策基礎保健サービス改善プロジェクトの一環として、ミャンマーのハンセン病参照施設における病理診断システム再開と免疫組織化学導入の指導を2004年と2005年に実施した経験を述べ、途上国における技術援助のありかたを考察する。

1．はじめに　Introduction

2000年4月から5年間の国際協力機構（JICA）ミャンマー国ハンセン病対策基礎保健サービス改善プロジェクト（以下、JICAプロジェクト）の一環として、ハンセン病対策の質的向上のために、ヤンゴン総合病院特別皮膚科外来（以下、CSSC/YGH）検査部の機能強化が実施された。派遣期間は、1回目が2004年2月15日～3月13日で、2回目が2005年2月13日～3月4日であった。

この機能強化の実施に先立って、ミャンマーから研修のために来日して大学院博士課程で学んでいる病理医（Thida Aung）と一緒にヤンゴン総合病院病理部で作成された皮膚生検21症例のパラフィンブロックの品質を検討した。その結果、ブロックの条件が悪いために薄切が困難で、個々の細胞・組織を充分に認識できない標本がしばしば見られた。その例を写真1に示すが、細胞の特徴が区別できないために、診断が困難である。しかし、免疫組織化学（免疫染色）は良く染色され、抗原性は保持されていることが判明した。[1] これらの予備調査から、組織の固定には問題がないものの、組織のパラフィン包埋過程における脱水の不足やパラフィンの高温・長時間などが推測された。そこで、これらの点を中心に改善を行う必要があるだろうと予測して、出発準備を行った。

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2．ハンセン病対策における病理診断の位置づけと、病理診断のプロセス Role of pathological diagnosis in the leprosy control

一般に、途上国のハンセン病対策において、病理検査（病理診断）は以下のような役割を担っている。ハンセン病の少ない先進国では、菌検査、PCR検査などと並んで、病理検査は、診断確定のために不可欠の検査の一つである。しかし、途上国のハンセン病対策においては、病理検査（病理

診断）は以下のような位置づけがなされている。

1）ハンセン病が多発している場所・時期では、臨床症状のみでハンセン病の診断をつける。多少の誤診例があっても、公衆衛生対策上は大きな問題にはならない。（Policy of WHO）

2）しかし、ハンセン病対策が進むと、診断困難症例や治療困難症例が増え、鑑別診断が必要なことも増えてくる。その場合に、確定診断をつけるためには皮膚や神経の生検（バイオプシー）が有用である。（Final diagnosis by histopathology）

3）病理診断は材料費は高くないが人手がかかるため、先進国では高価である。しかし途上国では人件費が低いために比較的安価に行える。（Cost-effective in developing countries）

4）途上国では、診断を行う病理医が不足していることが多く、人材養成が必要なことが多い。（Need of pathologists training）

5）病理診断のプロセスについて Process of pathological diagnosis

臨床診断や治療経過の病理学的な確定が必要な場合には、具体的には以下のプロセスが必要となる。（i）適切な場所から生検を行ない、速やかに固定液に浸漬すること。（ii）診断が可能な良質の標本が安定して作成されること。（iii）訓練を受けた病理医が速やかに診断を行い、報告すること。

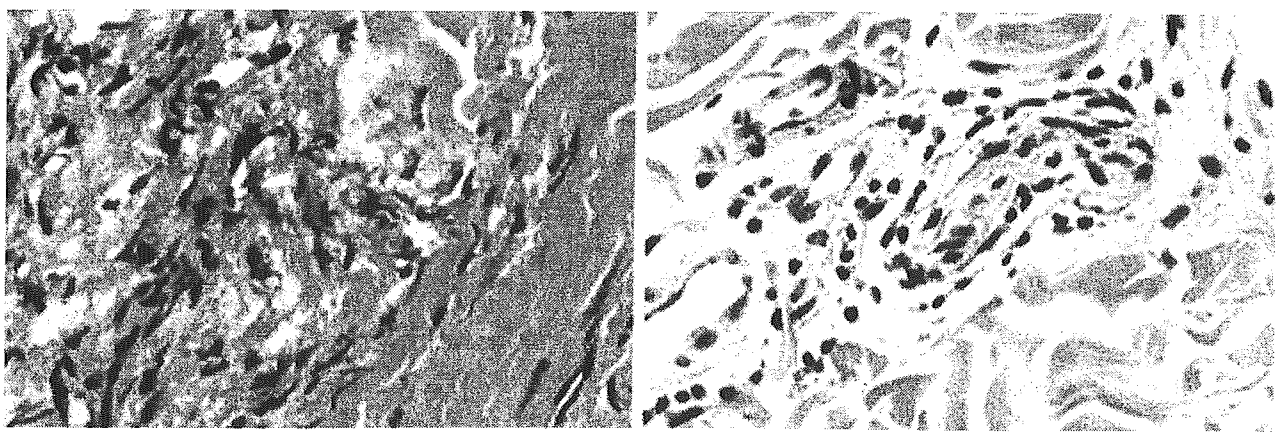
なお、病理医は一般的に、全身の外科・生検標本の診断、細胞診の診断、病理解剖などの訓練を受けているものの、皮膚病理と神経病理については特別な訓練が必要とされ、この2分野については皮膚科医や神経専門医が何らかのかたちで病理診断に関与することが少なくない。

3．ヤンゴン総合病院特別皮膚科外来検査部機能強化（2004年）
Histopathology service in the laboratory of Central Special Skin Clinic, Yangon General Hospital (2004)

3－1．ヤンゴン総合病院特別皮膚科外来の病理検査システムについて

20年前まではCSSC/YGHにおいて皮膚生検の病理標本作製と診断が行われていたが、当時の常勤医の退職後は、病理標本作製と診断はヤンゴン総合病院の病理部で実施され、その標本（プレパラート）を現在の常勤医である皮膚科医（Dr. Kyaw Kyaw）が確認するというシステムになっていた。しかし、上述したように標本の品質が一定しないために、しばしば病理診断に困難をきたしていた。

ヤンゴン総合病院病理部の標本作製の改善が出来れば理想的であるが、JICAプロジェクトの規模をこえる課題であること、ミャンマー国ハンセン病対策の中央参照施設としてのCSSC/YGHの重要性を考えると、CSSC/YGHに独自の標本作製シス



A　　B

写真1．予備調査における病理標本の比較。ミャンマーで作成されたパラフィンブロックからの標本（A）では、細胞の区別ができないために正確な診断ができなかった。日本で作成されたブロック（B）では、神経線維やリンパ球が明確に区別できる。

テムを再建することは、適切であると考えられた。しかし、その場合でもヤンゴン総合病院病理部との良好な連携が保たれることが望ましい。

到着後、切片の薄切のためのミクロトームや免疫染色用の器具などの機材の供与が適切に行われていることを確認した。また、それらの基本的な使用方法についての指導を行った。特に脱水から包埋前までの処理を行うロータリープロセサーは20年以上使用していなかったものを調整して使用したが、乾期で湿度が低いこと、アルコールの純度が高かったことなどから、処理時間の調整とパラフィンの温度調整だけで、良好なパラフィンブロックを作製できるようになった。ミクロトームは替刃を使用することで高品質な切片が作製できた。通常染色のヘマトキシリン・エオジン（HE）染色とFite法抗酸菌染色には問題がなかった。

3－2．ハンセン病組織病理のための特殊染色の研修 Training on special stains for leprosy histopathology

らい菌特殊染色と神経染色のために、9名の受講生（医師5名うち3名は病理医、検査技師4名）に対して10日間の講習会（研修）を実施した。

表1の予定表に従って、ハンセン病の基礎、病理組織検査の重要性、ハンセン病の臨床と病理、ハンセン病の特殊染色概論、免疫組織化学（免疫染色）の基礎、免疫染色のために必要な器具の使用法、ハンセン病の診断に必要な免疫染色などについて講義と実習を行った（表2）。講義ではパワーポイントの資料を配布し、プロジェクターで投映して説明を行った。質問に対してはホワイト

Table 1. Schedule of "Training of Special Stains for Leprosy Histopathology"

	Morning session 1	**Morning session 2**	**Afternoon session 1**	**Afternoon session 2**
Day 1	Opening ceremony (Dr. Ishida) Introduction of leprosy (Dr. Goto)	Introduction of leprosy (Dr. Goto)	Clinical diagnosis and management of leprosy (Dr. Goto, Dr. Ozaki)	Basic histopathology staining techniques (Dr. Goto)
Day 2	Histopathology of leprosy, reactions and differential diagnosis (Dr.Goto)		*Clinical manifestation of leprosy and smear taking (at outpatient clinic)*	
			Acid-fast staining and evaluation of skin slit smears	
Day 3	Special stainings for leprosy (Dr.Goto)	Measurement and storage of solutions for IHC (Dr. Goto)	*Histopathology of typical cases of leprosy (microscopic observation and report writing)*	
			Preparation of buffers and other solutions for immunohistochemistry	
Day 4	Principle of immunohistochemistry (IHC) (Dr. Goto)	Immunohistochemistry for leprosy (Dr. Goto)	*Immunohistochemistry practice (1-1)*	
Day 5	*Immunohistochemistry practice (I-2)*		*Immunohistochemistry practice (I-3). Evaluation of results*	
Day 6	(Spare day)			
Day 7	*Histopathology of variation of leprosy (microscopic observation and report writing)*		Discussion of histopathology (Dr. Goto)	
	Cutting of paraffin sections		*Section cutting and stainingHE and Fite*	
Day 8	*Histopathology of leprosy reactions (microscopic observation and report writing)*		Discussion of histopathology (Dr. Goto)	
	Histopathology of HE and Fite. Preparation of solutions for immunohistochemistry		*Immunohistochemistry practice (2-1)*	
Day 9	*Histopathology of differential diagnosis of leprosy (microscopic observation and report writing)*		Discussion of histopathology (Dr. Goto)	
	Immunohistochemistry practice (2-2)		*Immunohistochemistry practice (2-3)*	
Day 10	*Histopathology of immunohistochemistry*	Topics and animal model of leprosy (Dr. Goto)	Summary discussion and evaluation of the course	Closing ceremony

Upper row, for medical doctors; Lower row, for medical technologists
Italic character shows practice

ボードを用いて説明を行った。実習ではまず講師が手技を1～2回行い、直後に参加者に繰り返してもらうことで、身体で覚えてもらうことを行った。具体的には、免疫組織化学染色を異なった抗体を用いて3回行い、手技を修得してもらった。また、組織切片の抗酸菌染色については、染色中に切片が剥離しない方法について議論し、潤滑油の混入を防ぐことと充分な伸展時間の設定で問題を解決した。この講習会は著者がそのほとんどを担当し、一部を、尾崎元昭とKyaw Kyawが担当した。

この研修でとくに留意したことは、以下の点である。（1）良い標本を作成することが、正確な診断を通じて患者のためにいかに重要であるかを強調することによって、技師の働く意欲を引き出す。（2）ハンセン病になじみのない一般病理医に、ハンセン病診療の実際を体験させることで、ハンセン病の病理診断に興味を持たせる。（3）免疫染色の理論を丁寧に解説し、一つ一つの処理過程の意味を充分に理解させる。（4）医師と技師とが一緒に研修することによって、チーム医療の重要性を認識させる。

この研修会では、免疫組織化学の経験のある病理医2名、初めての医師3名、病理組織専門技師1名、組織標本作製のトレーニング直後の技師1名、組織標本作製が初めての技師2名が、互いに教えあい、議論しながら、熱心に研修を受けたため、知識・技能の違いを超えて、9名が良いチームを作り出すことができた。特に、後半の実習では、指導者の指示を待つのではなく、自分達の判断で工夫と改善を行うようになった。

その結果、短期間の指導にもかかわらず、検査技師の積極的な活動によって、かなり高品質の組織標本（抗酸菌染色、らい菌の抗BCG抗体免疫組織化学、神経の抗S100抗体などによる免疫組織化学）の作製を行うことが可能となった。また、一般病理医のハンセン病病理診断能力の強化が得られた。

4．ヤンゴン総合病院特別皮膚科外来臨床病理サービス強化（2005年）
Reinforcement of clinical pathology in Central Special Skin Clinic, Yangon General Hospital (2005)

4－1．病理組織標本の品質向上と血液生化学検査の指導

1年前に実施したCSSC/YGH外来検査部機能強

表2．トレーニングコースの内容

分類	項目
全員に対する講義	ハンセン病概論 診断と治療 組織染色の基礎（良い標本の必要性） ハンセン病の病理診断と鑑別 ハンセン病の特殊染色 免疫組織染色の試薬調整と保管（マイクロピペットの使い方講習） 免疫組織化学の理論 ハンセン病の免疫組織化学 免疫組織化学の応用 最近の話題と動物モデル 総括討論
全員での実習	免疫組織化学実習1回 免疫組織化学の評価
医師に対する実習	ハンセン病の臨床と菌検査 典型的な症例の病理診断実習 非典型的な症例の病理診断実習 らい反応の病理診断実習 ハンセン病の病理学的鑑別診断実習
技師に対する実習	菌検査とその抗酸菌染色 免疫組織染色の緩衝液と染色液の作成 組織標本のブロックと切片作成 組織標本のＨＥと抗酸菌染色 免疫組織化学実習2回

化（らい菌特殊染色、神経染色）が現在も正常に機能しているのか、標本の質に問題がないかを調査した。その結果、高品質の組織切片が現在まで継続して作製されていることが確認できた。また、切片剥離防止のための薬液処理（シランコーティング）によってエオジン染色の不均一性が発生していることをつきとめ、改善を行った。

1年前に導入した免疫組織化学については、最近に染色された標本数例を観察したが、きちんと染色されており、大きな問題はないと考えられた。

さらに、昨年度に実施したトレーニングコースの成果として、技師が自ら顕微鏡で標本を観察する良い習慣が身に付いていた。標本と診断結果のファイリングシステムも出来ていた。

なお、血液生化学検査（肝機能・腎機能・ヘモグロビン・血糖など）のための遠沈器と分光光度計が今年度新たにJICAから供与されており、毎月約20症例の検査が行われていた。ハンセン病治療薬による副作用（貧血・肝障害・腎障害など）、治療中のらい反応に対してステロイドを投与する場合の血糖値モニターの意義、精度管理などについて、指導を行った。

4－2．顕微鏡デジタル写真装置の設置

CSSC/YGHの教育・研究機能の強化のために、既設の光学顕微鏡（オリンパスBH）に取り付けるデジタル写真装置（キャノンEOS Kiss Digitalとアダプター）を携行機材で持参して設置した。ところが、当初は期待された質の写真を撮影することができず、原因を調査したところ、顕微鏡の光学系のカビによることが判明した。現地のサービスマンでは内部のカビを除去することができなかったため、治具を調達してカビ取りを行い、画質は著明に向上した。撮影画像は既設のコンピュータに取り込んで、診断のみならず教育や研究にも活用されている。

4－3．病理組織診断の指導

CSSC/YGHのDr. Kyaw Kyawは皮膚病理学の教育を受けているが、皮膚の病理診断を専門とする病理医や皮膚科医が近くにいないため、診断困難例について相談できない状態であった。そこで、これらの症例についてディスカッション顕微鏡とデジタルカメラ画像を用いて診断についての議論を行った。

また、今年1月からの皮膚生検はほぼ全例について診断チェックを行った。なお、診断結果の記載方法については、短時間でもれなく系統的に観察するためのチェックシートシステムの導入を前年度に勧めていたが、わかりやすいチェックシートが作成されており、確実に診断が行われていた。ほとんどの症例について二人の診断は一致し、診断技術については問題ないと考えられた。

5．マンダレー特別皮膚科外来(MSSC)機能強化（臨床検査関連、2005年）
Clinical laboratory function of Mandalay Special Skin Clinic (2005)

MSSCは、北部ミャンマーにおけるハンセン病対策の中心であるが、菌検査以外の臨床検査がなされておらず、その機能強化が課題であった。ことに、皮膚生検がまだ実施されていないことが問題となった。MSSCの責任者であるDr. Maung Maung Htooは、一年前に上述した研修”Training on Special Stains for Leprosy Histopathology”に出席していたために既知で、円滑に仕事ができた。

CSSCでは昨年度に標本作製ができるようになっており、ここで採取した検体をヤンゴンに送れば診断がつくシステムとなっている。そこで、今回の滞在中に実際に皮膚生検を行い、今後も継続できるようにセットアップすることを目的とした。高圧滅菌器による生検器具の滅菌方法のテストを実施し、検査の適応になる患者があれば一緒に生検を行うことにしたが、滞在中には対象症例がなく、実際に生検を行うことはできなかった。さらに今後のフォローアップが必要と考えられた。

6．日本におけるミャンマーの病理医教育
Training of Myanmar's pathologist in Japan

ヤンゴン医科大学から病理医のDr. Thida Aungが2003年に来日し、鹿児島大学大学院博士課程で学んでいる。これまでも中国とパキスタンからの

留学生が同研究室で研究していたことがあり、スムーズに受け入れが行われた。病理学研究方法のトレーニングとして、多数症例の免疫染色の解析を行わせ、1編の英文原著論文執筆を終了させた。[2] 現在は、鹿児島大学病院での皮膚生検組織の検討、ハンセン病症例の免疫染色とPCRなどを、日本とミャンマーの両国で行っている。

7．考察　Discussion

今回のJICAプロジェクトでの臨床病理学の技術指導によって、20年ぶりにCSSC/YGH検査部で病理組織検査を再開でき、高水準の病理診断システムが構築された。病理組織検査は非常に高価ではないものの、高純度アルコールなどの試薬の定期的な補充が必要な検査法である。今回、ミャンマーでは現地で調達したアルコール（エタノール）、キシレン、蒸留水などの品質に問題がなかったために、高品質の標本の作製が可能であった。さらに、CSSC/YGHでは検査室の給電がかなり安定しており、電気冷蔵庫の停電による温度変化や、ロータリープロセサーが途中で停止することがなかったことも幸いであった。先進国では多くの検査関連機器がコンピュータ化されつつあるが、今回の供給機器はすべてアナログ制御であったことも、適正技術という面からは適切であった。染色試薬やガラスなども現地調達でき、技術の継続性という面からも大きな問題はないと考えられた。

当初述べたように、病理診断そのものはけっして最先端の検査方法ではないが、従来の一般染色（ＨＥ染色や抗酸菌染色）に比較的新しい技術である免疫組織化学を加えることによって、高感度かつ特異的にハンセン病の確定診断を行うことができる。免疫染色は先進国では普通に行われているが、途上国では抗体などの消耗品経費、微量天秤、マイクロピペット、pHメーター、高品質の蒸溜水、コールドチェーン（抗体などの低温輸送・保管システム）などの基盤整備がなければその実施は不可能である。器具については全てを揃えることができたので、今後も共同研究という形で試薬類を定期的に供給することによって、この技術を継続・発展させたい。

1992年にインドの国立研究所で病理技術指導を行った際には、高純度のエタノールが現地で調達できず、モレキュラーシーブで脱水を行うなどの苦労があったことや、頻回の停電で作業が中断したことと比較すると、作業は容易であった。また、知識を重視して実際の技術や技術者を低く見る傾向の強い国においては、日本式の技術指導が根付かない可能性があるが、ミャンマーではそのような傾向が見られず、良好な環境で技術指導を行うことができた。

現在ミャンマーでは、ハンセン病専門医（皮膚科医）一名がCSSC/YGHでの皮膚生検の病理診断を行っている。今回の技術協力によって免疫染色を含む高品質の標本作製がCSSC/YGHで可能になった。しかし、もしもこの専門家が種々の都合で今後この診断を行うことができなくなると、今回たちあげた病理組織標本作製・特殊染色システムが全く機能しなくなるおそれがある。今後の継続的病理診断システムのためには、ハンセン病の病理診断ができる複数の医師を養成すると共に、一般の病理医がハンセン病の組織診断を行う機会を作ること、また病理医とハンセン病専門医をつなぐ人材の育成が不可欠である。現在、鹿児島大学大学院でハンセン病の病理学の研究を行っているミャンマーの病理医Dr. Thida Aungがこの役割を充分に果たすことができるように引き続き教育を行う予定である。

また、ハンセン病は皮膚科のみならず、神経内科、耳鼻咽喉科、眼科などとの密接な関係が必要とされる疾患であるが、それらの分野でハンセン病に興味を持つ医師を養成し、良好な連携を構築することが望まれる。

2003年に国家レベルでの感染症としてハンセン病制圧に成功したミャンマー国において、ハンセン病対策に従事する医療従事者、患者・回復者に対して、「あなたがたの存在や仕事は重要で、国外からも常に関心と敬意を持ち続けている。」という事を伝える続けることは、彼らの活動意欲を維持・強化するために非常に重要である。今後のフォローアップ事業においても、この点が配慮されることが望まれる。

一方、研究協力を通じて、他の医療分野をリードする業績をハンセン病分野であげることができ

れば、ハンセン病対策を志す若い優秀な医療関係者を獲得できる可能性が高くなる。具体的な例としては、近い将来にこの国のインターネット環境が改善すれば、デジタル化した臨床画像や顕微鏡画像をインターネットで転送することによって（Telemedicine, Telepathology）、ミャンマー国内、ならびに日本・ミャンマー間でより高度の医療協力を行なうことが可能になるであろう。

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