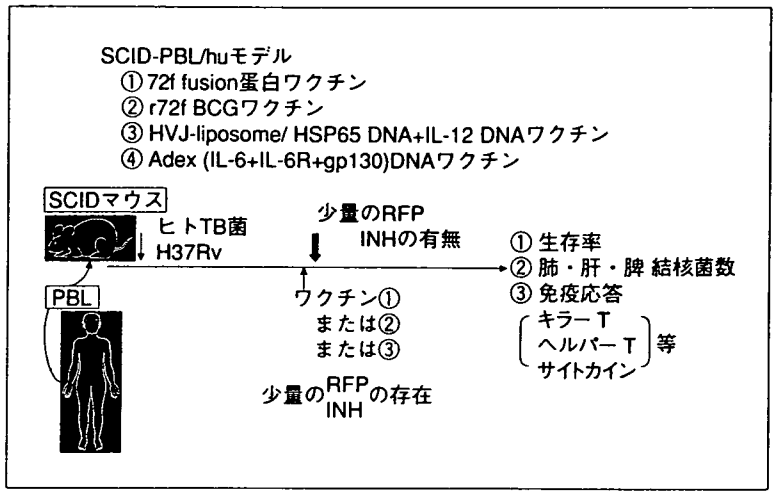

図5　新しい結核治療ワクチンの開発

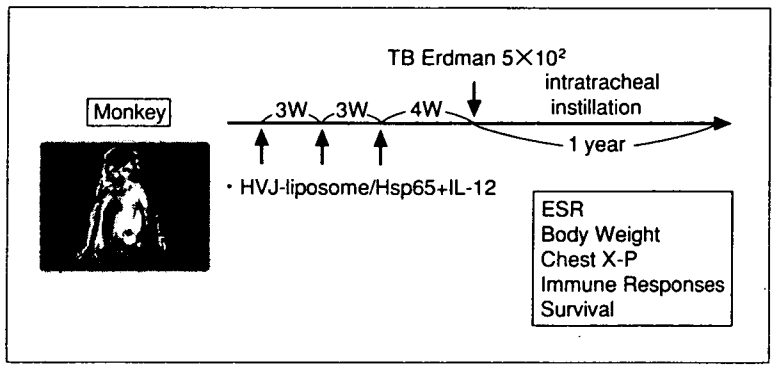

図6　プロトコール

表3　最先端の新しい結核ワクチン4種(WHO STOP TB Vacine Meeting)

1. HVJ-liposome/HSP65＋IL-12 DNAワクチン
(Okada M)
2. recombinant 85B BCGワクチン
(Horowitz)
3. 85B-ESAT6 fusion蛋白ワクチン
(Andersen P)
(モルモットではBCGワクチンよりも優れていない)
4. recombinant 72f fusion蛋白ワクチン
(Reed S, Skeiky Y, Gillis S)

合蛋白質サブユニットワクチンは，第Ⅰ相臨床試験が計画されている．さらに，われわれはHSP65 DNA＋IL-12 DNAワクチンと72f融合蛋白ワクチンやリコンビナント72f BCGワクチンを組み合わせ，きわめて強力なワクチン開発を目指している[5)~7)]．

このように，著者らはカニクイザルで結核感染後1年2か月経過観察し，コントロール群より著しく有効な，①HSP65 DNA＋IL-12 DNAワクチン，②r72f BCGワクチン，③BCG Tokyo＋72f fusion蛋白ワクチンを開発した．Ag85B-ESAT6融合蛋白質ワクチン，ワクシニアウイルスに85A DNAを導入したワクチンやr85B BCGもclinical trialの候補ワクチンであるが，もっとも切れ味のするどい臨床応用ワクチン候補の筆頭としてHSP65 DNA＋IL-12 DNAワクチンがあげられる(表3)[1)25)]．

〔WHO STOP TB Vaccine Group Meeting およびWHO STOP TB Partnership〕

これらの新しい結核ワクチンの開発研究が高く評価されWHO STOP TB PartnershipおよびWHO STOP TB Vaccine Group Meetingに選出された．HVJ-liposome/HSP65 DNA＋IL-12 DNA

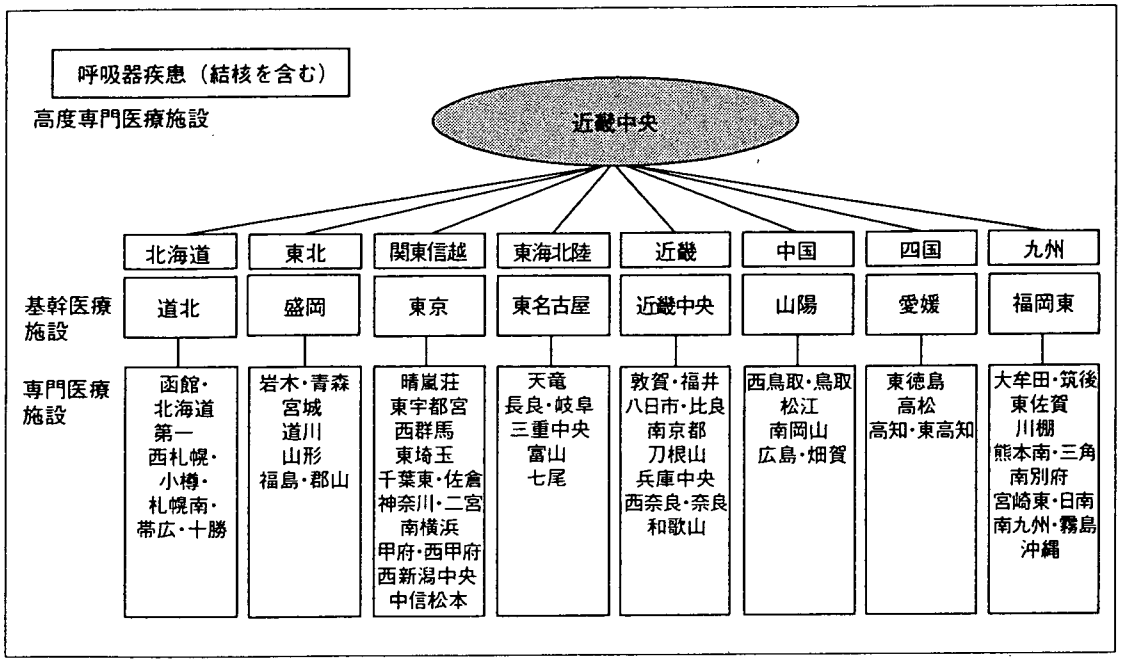

図7 国立病院機構政策医療呼吸器ネットワーク

ワクチンが高く評価された．当センターは呼吸器疾患(結核を含む)準ナショナルセンターであり，日本の結核患者数の約50%の診療を行っている政策医療呼吸器ネットワークを用い，サイトカインDNAワクチン(HSP65 DNA+IL-12 DNA)およびr72f BCGワクチンの臨床応用を計画している(表3)．

おわりに

当近畿中央胸部疾患センターは呼吸器疾患(結核を含む)準ナショナルセンターとなった．日本の結核患者数の43%の診断・治療を行っている，国立病院機構呼吸器専門54施設を統括し，国立病院機構政策医療呼吸器ネットワークを用い結核の新しい予防・治療法の確立が進展している(図7)．

HSP65 DNA+IL-12 DNAワクチンがBCGワクチンより1万倍強力な結核予防ワクチン効果を示す画期的な成果を得た．したがって，このワクチンを最優先で結核の発症予防や治療に対して臨床応用する計画が進んでいる．

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Immunization with dendritic cells retrovirally transduced with mycobacterial antigen 85A gene elicits the specific cellular immunity including cytotoxic T-lymphocyte activity specific to an epitope on antigen 85A

Hideki Nakano[a], Toshi Nagata[b,*], Takafumi Suda[a], Takao Tanaka[c], Taiki Aoshi[b], Masato Uchijima[b], Sachiko Kuwayama[c], Noriko Kanamaru[c], Kingo Chida[a], Hirotoshi Nakamura[a], Masaji Okada[c], Yukio Koide[b]

[a] *Second Division, Department of Internal Medicine, Hamamatsu University School of Medicine, 1-20-1 Handa-yama, Hamamatsu 431-3192, Japan*
[b] *Department of Microbiology and Immunology, Hamamatsu University, School of Medicine, 1-20-1 Handa-yama, Hamamatsu 431-3192, Japan*
[c] *Clinical Research Center, National Hospital Organization Kinki-chuo Chest Medical Center, 1180 Nagasone-cho, Sakai, Osaka 591-8555, Japan*

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Abstract

In the present study, we evaluated antigen 85A (Ag85A) gene-transduced dendritic cells (DCs) vaccine against *Mycobacterium tuberculosis*. Murine bone marrow-derived DCs were retrovirally transduced with mycobacterial Ag85A gene and injected to BALB/c mice intravenously. The DC vaccine was capable of inducing purified protein derivative (PPD)- and the antigen-specific spleen cell proliferation and IFN-γ production from both $CD4^+$ and $CD8^+$ T cells in spleens of the immune mice. In addition, the DC vaccination induced cytotoxic T-lymphocytes (CTL) and IFN-γ-producing cells specific for a 9-mer CTL epitope on Ag85A molecule. This eliciting cellular immunity led to protection against wasting disease due to *M. tuberculosis* infection and induction of moderate bacterial clearance.

Keywords: Antigen 85A; Dendritic cells; *Mycobacterium tuberculosis*

1. Introduction

Tuberculosis (TB) remains one of most serious public health problems being prevailed worldwide along with AIDS and malaria, resulting in 8 million new cases and 2 million deaths each year [1]. The appearance of multidrug-resistant *Mycobacterium tuberculosis* strains has worsened the problem. The only TB vaccine currently available is the attenuated *Mycobacterium bovis* strain bacillus Calmette-Guérin (BCG), which has been reported to have a variable protective efficacy especially in adult TB [2]. Therefore, there remains an urgent need for more effective vaccines for TB [3].

Protection against intracellular bacteria such as *M. tuberculosis* critically depends on induction of cellular immune responses. Administration of soluble proteins would be insufficient to stimulate these responses. The reason why BCG vaccine has been utilized for decades is that the vaccine is able to induce specific cellular immunity although the efficacy is controversial as mentioned before. Immunization with dendritic cells (DCs) is one of promising strategies for eliciting effective cellular immunity against intracellular pathogens as DCs are the most potent antigen-presenting cells (APCs). DCs capture the pathogens or apoptotic cells. Then they migrate to regional lymphoid organs, where they present antigens to naïve T cells [4,5]. DCs possess the distinct ability to prime naïve helper T-lymphocytes (Th) and cytotoxic T-lymphocytes (CTL). Thus there has been much interest in the

* Corresponding author. Tel.: +81 53 435 2335; fax: +81 53 435 2335.
E-mail address: tnagata@hama-med.ac.jp (T. Nagata).

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use in immune modulation of infectious diseases and cancer. Vaccination with DCs pulsed with tumor-associated antigens has been shown to generate specific anti-tumor immunity in vivo in murine tumor models [6–9]. DC vaccination has been also examined in the field of infectious diseases [10–13]. We also showed that vaccination with DCs retrovirally transduced with a gene for a dominant CTL epitope derived from *Listeria monocytogenes* elicited significant protective immunity against lethal listerial challenge infection [14].

Promising candidate antigens for TB vaccines include antigen (Ag) 85 family molecules such as Ag85A or Ag85B, heat shock proteins such as Hsp60 and ESAT-6 (reviewed in [15]). We used Ag85A as a vaccine target in this study. Ag85A molecule is a mycobacterial major secreted protein which belongs to the Ag85 family consisting of three structurally related components, Ag85A (p32A; 32 kDa), Ag85B (p30, MPT59, α antigen; 30 kDa), and Ag85C (reviewed in [16]). The Ag85 family molecules are cross-reactive antigens and are highly conserved among *Mycobacterium* spp. The genes encode proteins with fibronectin-binding capacities [17] and mycolyltransferase activities, which are involved in the final stage of mycobacterial cell wall assembly [18]. Ag85A protein was reported to stimulate B- and T-cell responses in TB patients and immunization with Ag85A protein induced the protective immunity against *M. tuberculosis* in guinea pigs [19]. In addition, reports of naked DNA vaccines against TB employing Ag85A gene have accumulated [20–24]. In addition, we reported recently that vaccination with attenuated *Listeria* carrying Ag85A expression plasmid elicited significant protective immunity against *M. tuberculosis* challenge [25]. More recently, vaccination with Ag85A-expressing vaccinia virus was shown to be effective in boosting antimycobacterial immunity in human trial [26]. According to these reports, Ag85A molecule seems to be one of the most promising candidates for future subunit TB vaccines.

In the present study, we developed a retrovirally transduced DC vaccine expressing Ag85A, and assessed its ability to generate the antigen-specific cellular immunity and to induce protective immunity against murine *M. tuberculosis* infection.

2. Materials and methods

2.1. Recombinant retroviral vector

BCG Ag85A gene was amplified from a plasmid, pMB49 [27] by PCR with following primers: 5′-ATAAGAATGCGGCCGCACCATGCAGCTTGTTGACAGG-3′ (forward primer) and 5′-ATAGTTTAGCGGCCGCTGTTCGGAGCTAGGCGC-3′ (reverse primer) (underlined letters indicate NotI sites). These PCR fragments were digested with NotI and inserted into a NotI site of pMX [28]. The nucleotide sequence designed in the plasmid was confirmed by DNA sequencing using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). Large-scale purification of the plasmid was conducted using the Qiagen Plasmid Mega Kit System (Qiagen, Chatsworth, CA) and endotoxin was removed by Triton X-114 phase separation. Retroviral supernatant was generated by transfection of pMX-Ag85A proviral construct into Phoenix ecotropic packaging cell line [purchased from American Type Culture Collection (Manassas, VA) and used with the permission of Dr. GP Nolan (Stanford University School of Medicine, Stanford, CA)].

2.2. Reverse transcription (RT)–PCR analysis for Ag85A gene detection

Bone marrow-derived DCs transduced with pMX-Ag85A were harvested and total RNA was prepared from the cells by Isogen RNA extraction solution (Nippon Gene, Tokyo, Japan). The single-stranded cDNA was synthesized with Molony murine leukemia virus reverse transcriptase (Life Technologies, Gaithersburg, MD) and then used for PCR analysis. The images were recorded using AE-6900M densitograph (ATTO, Tokyo, Japan). Primers used for Ag85A gene detection are: 5′-AGGCCAACAGGCACGTCAA-3′ (forward primer) and 5′-ACATGTCGGAGGCCTTGTA-3′ (reverse primer). As a control, the same RT–PCR was performed with primers for glycelaldehyde-3-phosphate dehydrogenase (G3PDH).

2.3. Mice

BALB/c mice were purchased from SLC Japan (Hamamatsu, Japan). These mice were maintained in a specific-pathogen-free condition at the Experimental Animal Institute, Hamamatsu University School of Medicine. All mice used in this study were between 8 and 14 weeks of age. All animal experiments were performed according to the Guidelines for Animal Experimentation, Hamamatsu University, School of Medicine.

2.4. Peptides and protein

Lyophilized peptides were purchased from Invitrogen Corporation (Carlsbad, CA). The three Ag85A CTL-epitope candidate peptides are synthesized based on Denis et al. [29]. They are, pep1 (MPVGGQSSF; corresponding to amino acid residues (aa) 70–78 of Ag85A which is predicted to bind H2-L^d according to SYFPEITHI computer algorithm [http://www.syfpeithi.de]), pep2 (WYDQSGLSV; aa 60–68 of Ag85A predicted to bind H2-K^d), and pep3 (VYAGAMSGL; aa 144–152 of Ag85A predicted to bind H2-K^d). The purity of peptides was confirmed by mass spectrometry. All peptides were dissolved in 5% dimethyl sulfoxide in distilled water to a concentration of 1 mM and were stored at −80 °C until used. Purified recombinant (r) Ag85A protein was kindly provided by Dr. John T. Belisle

(Colorado State University, Fort Collins, CO) through the NIH, NIAID Contract NO1 AI-75320 entitled "Tuberculosis Research Materials and Vaccine Testing".

2.5. Culture of bone marrow-derived DCs and transduction with retrovirus

Bone marrow-derived DCs were cultured using a method described by Inaba et al. [30] with some modifications as in our previous work [14]. To determine the phenotype of cultured DCs, we stained them with PE-, or FITC-conjugated monoclonal antibodies (mAbs) against cell surface molecules [CD40, CD80, CD86, H2-A^d (all from BD Biosciences, San Diego, CA)] and analyzed using EPICS Profile-II (Beckman Coulter, Fullerton, CA). Transduction of retroviruses was also carried out as in our previous work [14]. Briefly, 1×10^6 bone marrow-derived DCs were cultured in RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum (RPMI/10FCS) for 48 h and resuspended in 1 ml of the retroviral supernatant supplemented with 8 μg/ml polybrene (Sigma Chemical Co., St. Louis, MO), 1000 units/ml of murine rGM-CSF, and 1000 units/ml of murine rIL-4. These cells were centrifuged at $2500 \times g$ at 32 °C for 2 h. After centrifugation, cells were cultured in RPMI/10FCS in 5% CO_2 atmosphere. The transduction process was repeated on days 3 and 4.

2.6. Immunization

After washing twice in phosphate-buffered saline (PBS), 1×10^5 transduced DCs in 0.2 ml of PBS were injected intravenously into mouse twice at a 2-week interval. As a control, mice were also immunized with 2×10^6 CFU of BCG (substrain Tokyo; Japan BCG Inc., Tokyo, Japan) subcutaneously twice at a 2-week interval. In some experiments, 2 μg of Ag85A expression plasmid (pCI-Ag85A) was immunized with Helios gene gun system (Bio-Rad Laboratories, Hercules, CA) four times at 1-week intervals as in our previous work on MPT51 molecule [31].

2.7. Detection of PPD-, or Ag85A-specific antibodies (Abs) by ELISA

The 96-well ELISA plates (EIA/RIA plate A/2; Costar, Cambridge, MA) were coated with 25 μg/ml of purified protein derivative (PPD; Japan BCG Inc., Osaka, Japan) or 5 μg/ml of purified Ag85A protein at 4 °C overnight, washed with PBS containing 0.05% Tween 20 (PBS/Tween), and blocked with 30% Block Ace (Dainippon Seiyaku, Tokyo, Japan) solution in PBS at 37 °C for 2 h. After washing, the sera diluted with RPMI1640 medium were added to the plates and incubated at 4 °C overnight. After washing, horseradish peroxydase (HRP)-conjugated goat anti-mouse IgG antibodies (Abs) were added to the plates at room temperature for 2 h. After washing, the bound HRP-conjugated Abs were detected by HRP substrate reagent (Techne, Minneapolis, MN). The absorbance at 450 nm was determined with EZS-ABS microplate reader (Iwaki, Tokyo, Japan).

2.8. Lymphocyte proliferation assay

Spleen cells were prepared from the immune mice and treated with Tris-buffered 0.83 M NH_4Cl buffer for 1 min at room temperature to remove red blood cells. Then the spleen cells (5×10^5 per well) were incubated for 48 h at 37 °C in 96-well round-bottom tissue culture plates (Greiner Bio-One GmbH, Frickenhausen, Germany) in the presence or absence of 5 μg/ml of PPD (Japan BCG Inc.). The de novo DNA synthesis was assessed by adding 0.5 μCi/well of [methyl-^{3}H] thymidine (6.7 Ci/mmol; ICN Biochemicals, Irvine, CA) for the last 12 h of culture. The cultured cells were harvested onto glass fiber filters, and the radioactivity was counted by a liquid scintillation counter (LSC-5100; Aloka, Tokyo, Japan). The [methyl-^{3}H] thymidine incorporation was calculated in counts per minute (cpm).

2.9. Quantification of IFN-γ by sandwich ELISA

Pools of spleen cell suspensions (2×10^6 ml^{-1}) from groups of mice immunized with DCs were cultured in RPMI/10FCS in 24-well plates in the presence of PPD (Japan BCG Inc.) (10 μg/ml), Ag85A protein (5 μg/ml), or peptides (5 μM) at 37 °C in 5% CO_2 atmosphere. The culture supernatants were harvested after 5 days, aliquoted, and stored at −20 °C until assayed for IFN-γ. Concentration of IFN-γ in the culture supernatants was determined by sandwich ELISA as described in our previous work [31]. Briefly, the 96-well ELISA plates (EIA/RIA plate A/2; Costar) were coated with 2 μg/ml of capture Ab (anti-murine IFN-γ mAb, R4-6A2; BD Biosciences) at 4 °C overnight and washed with PBS/Tween and blocked with PBS/Tween containing Block Ace (Dainippon Seiyaku) at 37 °C for 2 h. After washing, the culture supernatants to be tested and serially diluted IFN-γ standard solutions were added to the plates and incubated at 4 °C overnight. After washing, 0.5 μg/ml of detection Ab (biotinylated anti-murine IFN-γ mAb, XMG1.2; BD Biosciences) was added to the plates. The plates were incubated at room temperature for 2 h and washed. The plates were then added with 0.1 μg/ml of HRP-conjugated streptavidin (Vector laboratories Inc., Burlingame, CA) and incubated at room temperature for 30 min. After washing, bound HRP-conjugated streptavidin was detected by HRP substrate reagent (Techne). The absorbance at 450 nm was determined with EZS-ABS microplate reader (Iwaki).

2.10. Preparation of $CD4^+$ and $CD8^+$ T cell subsets from immune splenocytes with Ag85A gene-transduced DCs

Spleen cells were prepared from the immune mice and treated with Tris-buffered 0.83 M NH_4Cl buffer for 1 min

at room temperature to remove red blood cells. Then they were washed twice with RPMI 1640 medium. $CD4^+$ and $CD8^+$ T cell subsets were prepared from spleen cells of immune mice using murine $CD4^+$ or $CD8^+$ T cell isolation kit according the manufacturer's instruction (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany). The $CD4^+$ or $CD8^+$ T cells (1×10^6 cells) and Ag85A gene-transduced DCs (1×10^5 cells) were cultured in 96-well round-bottom tissue plates (Greiner Bio-One GmbH) for 4 days. The culture supernatants were harvested and stored at −20 °C until assayed.

2.11. CTL assay

Eight weeks after the last immunization, immune spleen cells were cocultured in 12-well plates at density of 2×10^7 cells/well for 5 days with 2×10^7 cells/well syngeneic splenocytes that had been pretreated with 100 μg/ml of mitomycin C and pulsed with 1 μM of Ag85A pep3 peptide (VYAGAMSGL) for 1 h at 37 °C. Each well received also 10 units/ml of human rIL-2 (Hoffmann-La Roche, Nutley, NJ). Cell-mediated cytotoxicity was measured using a conventional ^{51}Cr release assay. The target cells used in this study were RAW264.7 (mouse macrophage cell line; $H2^d$) pulsed with 1 μM of the peptide for 1.5 h at 37 °C. Target cells at a concentration of 1×10^4 cells/well were incubated for 5 h in triplicate at 37 °C with serial dilutions of effector cells, and the specific lysis was determined as calculated by the formula: percent specific lysis = [(experimental cpm − spontaneous cpm)/(total cpm − spontaneous cpm)] × 100.

2.12. Bacterial infection and evaluation of protective ability of Ag85A gene-transduced DC vaccine

Immunized BALB/c mice were infected with 5×10^5 CFU of *M. tuberculosis* H37Rv i.v. 2 months after the last immunization. Mice were sacrificed 4 weeks later and the bacterial numbers in the spleens, livers, and lungs were counted in CFU on Middlebrook 7H11 plates (BD Biosciences).

2.13. Statistical analysis

Data from multiple experiments were expressed as mean ± standard deviations (S.D.). Statistical analyses were performed with the StatView-J5.0 statistics program (SAS Institute Inc., Cary, NC). Data were analyzed by Fisher's protected least significant difference (PLSD).

3. Results

3.1. Retroviral transduction of bone marrow-derived DCs and expression of Ag85A gene in the cells

DCs were generated from murine bone marrow by culturing with rGM-CSF and rIL-4, as previously described [30]. DCs transduced with Ag85A-encoding retrovirus (Ag85A gene-transduced DCs) and control untransduced DCs expressed similar amounts of CD40, CD80, CD86, and MHC class II molecules (data not shown), indicating that retroviral transduction to DCs did not affect the phenotype of the DCs.

In order to confirm the expression of Ag85A gene in transduced DCs, RT–PCR was performed. As shown in Fig. 1A, an Ag85A gene-specific band was detected in the retrovirus-transduced DCs, but not in control untransduced DCs, indicating Ag85A gene expression in the transduced DCs.

We next examined the antigen presentation capacity of Ag85A gene-transduced DCs. When Ag85A gene-transduced DCs or untransduced DCs were incubated with spleen cells derived from Ag85A DNA vaccine-immune mice, Ag85A-transduced DCs, but not untransduced DCs rendered the spleen cells to produce IFN-γ (Fig. 1B), indicating that Ag85A gene-transduced DCs were capable of presenting the antigen (Ag85A) to T cells.

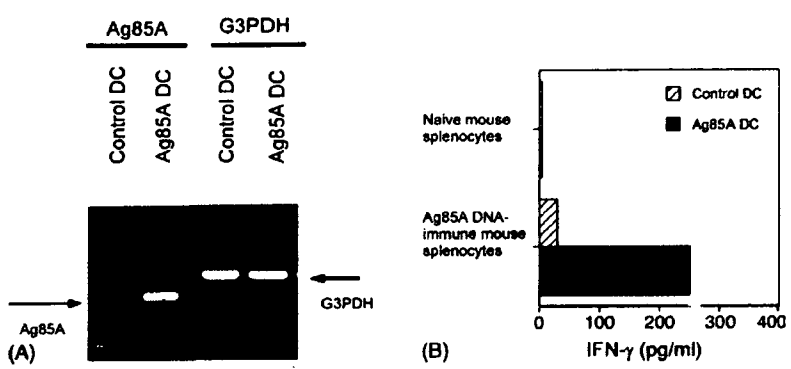

Fig. 1. Ag85A gene expression in Ag85A-transduced DCs and antigen presentation capacity of the cells. (A) Ag85A gene expression by DCs transduced with pMX-Ag85A. DCs were transduced with Ag85A-expressing retrovirus and harvested to prepare total RNA. Ag85A gene expression was evaluated by RT–PCR with Ag85A-specific primers. (B) Antigen presentation capacity of DCs transduced with Ag85A-expressing retrovirus. DCs transduced with or without Ag85A-expressing retrovirus were incubated with spleen cells of Ag85A DNA-immune mice or naïve mice for 2 days and the culture supernatant was examined for IFN-γ amounts with ELISA. Average values from two independent experiments are shown.

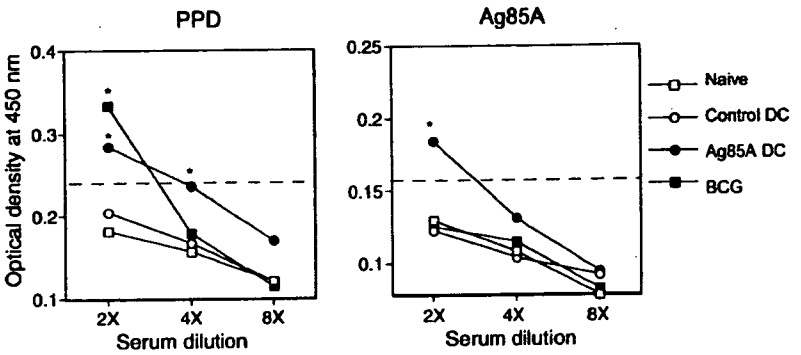

Fig. 2. Detection of PPD- and Ag85A-reactive Abs in the sera of Ag85A gene-transduced DC-immune mice. The sera of control untransduced DC-, Ag85A gene-transduced DC-, or BCG-immune mice or naïve mice were examined for binding to PPD (left panel) or purified Ag85A protein (right panel) by ELISA. The mean 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

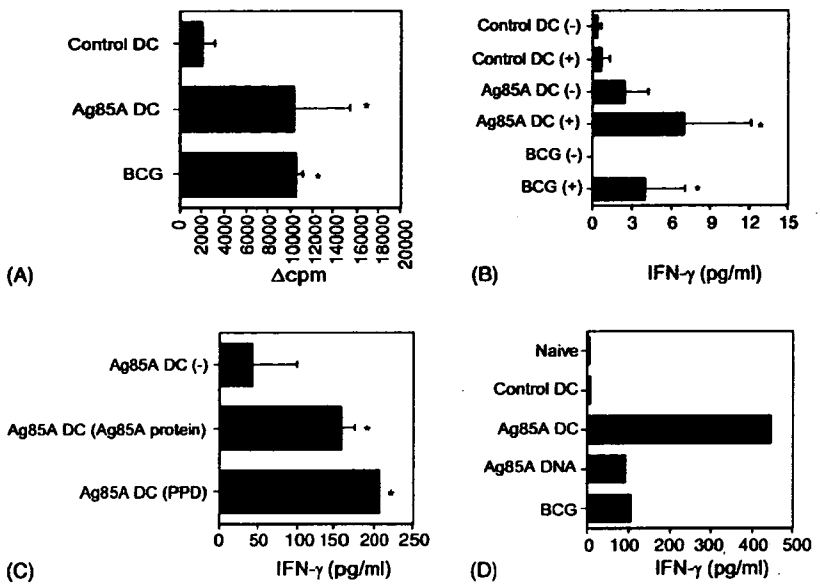

Fig. 3. (A) PPD-specific splenocyte proliferation of mice immunized with DCs transduced with Ag85A gene. BALB/c mice were immunized with Ag85A gene-transduced DCs two times at a 1-week interval. The data of mice immunized with BCG twice were also shown as controls. Spleen cells of the immunized mice 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.

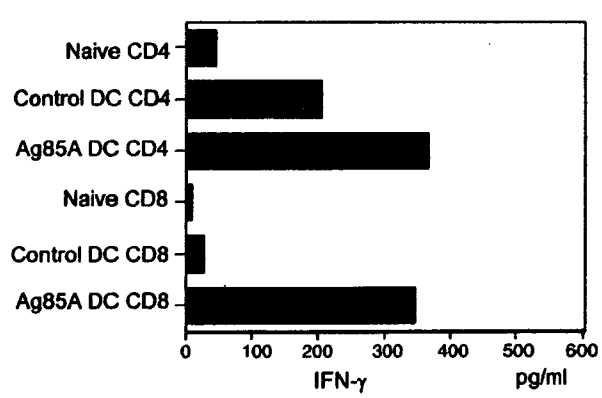

Fig. 4. Ag85A gene-transduced DC immunization elicited the antigen-specific CD4$^+$ and CD8$^+$ T cells. CD4$^+$ and CD8$^+$ T cell-enriched spleen cells of control untransduced DC-, or Ag85A gene-transduced DC-immune mice were cultured with in vitro-prepared Ag85A gene-transduced DCs for 4 days and 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-

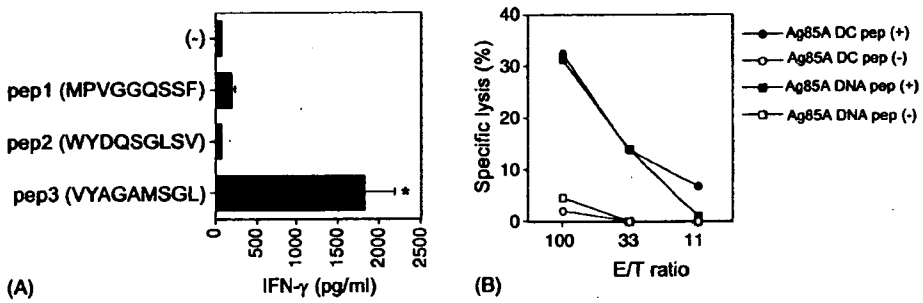

Fig. 5. Cytotoxic activity of Ag85A-transduced DC-immune splenocytes to VYAGAMSGL peptide-pulsed RAW264.7 cells. (A) IFN-γ production by spleen cells of BALB/c mice immunized with Ag85A plasmid DNA in the presence of candidate CTL epitope peptides. The spleen cells produced the significant level of IFN-γ 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.

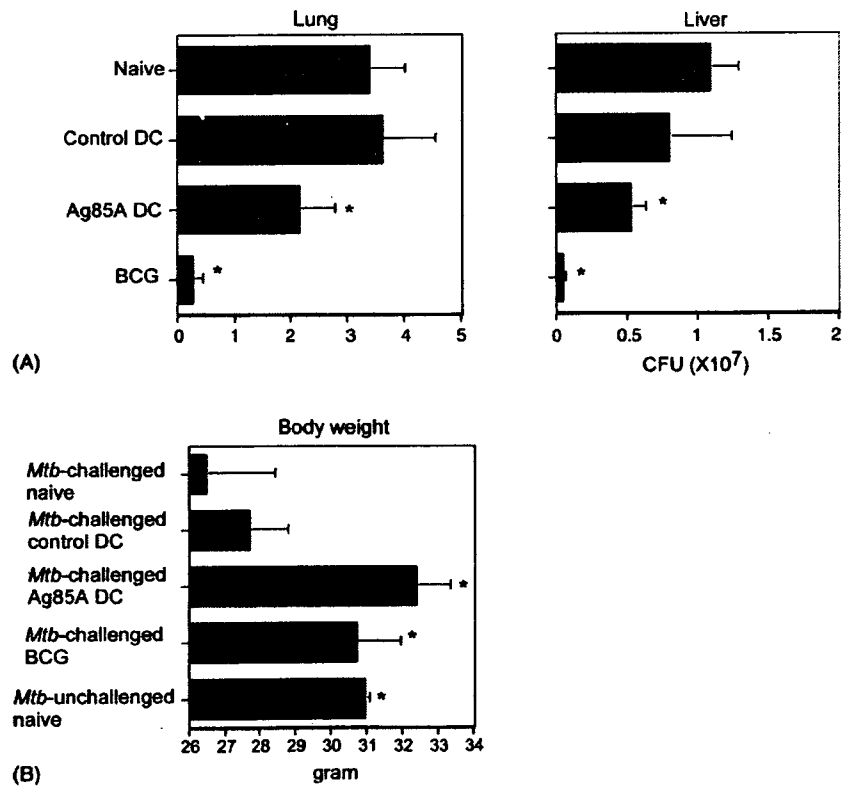

Fig. 6. (A) In vivo protective activity of mice immunized with DCs transduced with Ag85A gene against virulent *M. tuberculosis* challenge. BALB/c mice were immunized with Ag85A gene-transduced DCs twice at a 1-week interval. These mice were challenged i.v. with 5×10^5 CFU of live *M. tuberculosis* H37Rv. 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 $H2\text{-}K^d$ 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.

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The Molecular Epidemiology of Ethambutol-Resistant *Mycobacterium tuberculosis* in Henan Province, China

Isamu Sugawara*, Koji Otomo, Hiroyuki Yamada, Guobin Wang[1], Changmei Du[1], Ruiru Shi[1] and Guolong Zhang[1]

Mycobacterial Reference Center, The Research Institute of Tuberculosis, Tokyo 204-0022, Japan and [1]Henan Chest Hospital, Zhengzhou, Henan 450003, China

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The incidence of tuberculosis (TB) in China is high, according to the Nationwide Random Survey for the Epidemiology of Tuberculosis, 1990, conducted by the Beijing Tuberculosis and Thoracic Tumor Research Institute (Tongzhou, Beijing, China). It is important to obtain fundamental data about drug-resistant TB in China to enable successful treatment of this disease. In 1994, WHO launched the global drug resistance surveillance (DRS) project. Henan province was chosen as the first site in China for collection of data for the DRS in accordance with WHO/IUATLD guidelines. Thirty counties in Henan province were selected as survey sites. The samples chosen comprised 1,372 cases of TB, including 916 new cases and 456 relapsed cases. The enrollment period of the TB patients was from April 1 to December 31, 1996. Only genotypic detection of ethambutol (EMB) resistance was performed due to tight restrictions on the research budget; there have been few reports on EMB resistance involving large numbers of *Mycobacterium tuberculosis* isolates (1-4). As the *embB* operon, a gene cluster of *M. tuberculosis*, is involved in resistance to EMB (5,6), the study focused on the detection of a point mutation in *embB* codon 306 by DNA sequencing.

The samples of 171 *M. tuberculosis* isolates were recovered from 30 counties in Henan province. The sex ratio (M:F) of the TB patients was 2.2:1, and the mean age was 43.7 years. The mycobacteria were recovered from diseased patients with a variety of distinct clinical manifestations, including pulmonary and extrapulmonary infections. Seventeen reference strains (15 resistant and 2 sensitive) were provided by the Korean Institute of Tuberculosis, Seoul, Korea. The sample included 133 EMB-resistant and 38 EMB-susceptible isolates. The isolates were initially tested for EMB susceptibility in routine diagnostic laboratories by the proportion method with Middlebrook 7H10 medium. The critical concentration of EMB was 2 μg/ml.

Every series of EMB susceptibility tests included the two drug-susceptible reference strains of *M. tuberculosis*. The results of EMB susceptibility tests on the clinical isolates were cross-checked at the Korean Institute of Tuberculosis, with 98% of the results confirmed (laboratory accuracy: 90.8%). Seven hundred and five of the 1,372 isolates were resistant to one of the anti-TB drugs, isoniazid, rifampicin, EMB and streptomycin, by drug susceptibility testing. Ninety-four of the 916 new cases (10.3%) and 93 of the 456 retreated cases were EMB-resistant (20.4%). The 133 EMB-resistant *M. tuberculosis* isolates were investigated by EMB susceptibility testing for *embB* point mutations.

Next, DNA from the *M. tuberculosis* isolates was purified as described previously (7). Primer sets were designed to amplify regions of the *embB* gene; the amplification size was 260 bp (8). Five microliters (300 ng) of genomic DNA was used as the template for amplification in 100 μl of reaction mixture. Reaction mixtures were subjected to PCR in a thermal cycler (Perkin Elmer, Fremont, Calif., USA) as follows: 93°C for 3 min followed by 35 cycles at 93°C for 1 min, annealing at 65°C for 1 min, and an extension step at 72°C for 2 min. Final extension was done at 72°C for 10 min. Efficient amplification was confirmed by gel electrophoresis on 12% polyacrylamide gels. Care was taken to prevent false results due to amplicon contamination. Direct sequencing of amplified PCR products was performed with an ABI PRISM (Model 377; Perkin Elmer). Because the previous reports suggested that mutations at codon 306 of the *embB* gene (the gene encoding arabinosyl transferase) were important in EMB resistance, a 260-bp *embB* region was sequenced in 133 EMB-resistant and 38 EMB-susceptible organisms. A major point mutation was detected in codon 306 with five different kinds of base changes. Five distinct missense mutations were identified in codon 306 (ATG, Met): GTG (Val), ATA (Ile), ATT (Ile), CTG (Leu) and ATC (Ile) (Table 1). Three point mutations were detected in codons 319 and 333 (TAT,Tyr): TGT, GAT and, CAT (Cys). When only epidemiologically distinct organisms were considered, 45.2% of EMB-resistant mycobacteria had an amino acid change in the region of *embB* studied, and most replacements (72%) occurred at position 306. We investigated the relationship between multi-

Table 1. Characteristics of clinical isolates of *M. tuberculosis* with *embB* mutations

Frequency (n)	Base change	Amino acid change
43	ATG→GTG	306 Met→Val
7	ATG→ATA	306 Met→Ile
5	ATG→ATT	306 Met→Ile
3	ATG→CTG	306 Met→Leu
2	ATG→ATC	306 Met→Ile
1	TAT→TGT	319 Tyr→Cys
1	TAT→GAT	319 Tyr→Cys
1	TAT→CAT	333 Tyr→Cys

*Corresponding author: Mailing address: Mycobacterial Reference Center, The Research Institute of Tuberculosis, 3-1-24 Matsuyama, Kiyose, Tokyo 204-0022, Japan. Tel: +81-424-93-5075, Fax: +81-424-92-4600, E-mail: sugawara@jata.or.jp

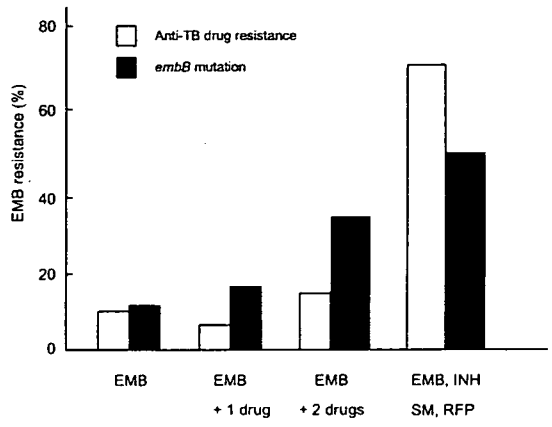

Fig. 1. The relationship between ethambutol to anti-TB drug(s) as evaluated by drug susceptibility testing and *embB* mutation.
EMB, ethambutol; INH, isoniazid; SM, streptomycin; RFP, rifampicin.

drug resistance (MDR) and *embB* mutation (Fig. 1) and detected an *embB* mutation in 9.0% of *M. tuberculosis* isolates resistant to EMB alone and in 17% of *M. tuberculosis* isolates resistant to EMB and any one of the other drugs. A higher frequency of *embB* mutations was detected in *M. tuberculosis* isolates resistant to EMB and any two drugs (38%) and in *M. tuberculosis* isolates resistant to EMB, isoniazid, streptomycin and rifampicin (53%).

Molecular epidemiological analysis of EMB resistance was carried out on *M. tuberculosis* isolates from randomly selected TB patients in Henan province, China. Resistance to the anti-TB drugs isoniazid, rifampicin, streptomycin and EMB was detected by drug susceptibility testing in 705 out of 1,372 patients tested (51.4%). MDR occurred in 320 of the 1,372 cases (23.4%). This prevalence is significantly higher than that reported previously in Latvia, Thailand, Mozambique and Uganda by WHO (9). EMB resistance occurred in 187 of the 1,372 cases (13.6%). Ninety-four of 916 new TB cases (10.5 %) were EMB-resistant. This tempted us to explore the frequency of EMB resistance at the molecular level, as there have been few reports on the prevalence of EMB resistance in large samples of *M. tuberculosis* isolates. Point mutations of embB, the gene encoding arabinosyl transferase, were detected in 60 (45.2%) of the 133 frozen samples of tubercle bacilli. The MIC of these EMB-resistant clinical isolates was >20 μg/ml.

As shown in Table 2, there have been several previous reports on EMB resistance at the molecular level. Substitutions at codon 306 in the gene *embB* are the most common mutations found in EMB-resistant *M. tuberculosis*, occurring in 48.3 - 69% of resistant clinical isolates. The results of this study show a relatively low level of EMB resistance (45.2%), although the sample size is the largest of the five reports (133 cases). The *embB* mutation at codon 306 was most common, but three point mutations were found at other codons. Thus, it is possible that an *embB* mutation at another codon also confers EMB resistance.

Sreevatsan et al. (1) reported a point mutation at codon 630 (Thr → Ile). We could not detect this mutation in this study, but *embB* point mutations were detected at codons 319 (Tyr → Cys) and 333 (Tyr → Cys) instead. Further studies are required to determine the importance of such point mutations in EMB resistance.

Mokrousov et al. (4) reported a high frequency of *embB* codon 306 mutations (31.2%) in 154 EMB-susceptible isolates,

Table 2. Frequency of *embB* mutations according to published reports

EMB-resistant isolates	EMB-sensitive isolates	Reference
11/16 (69%)	0/3	Sreevatsan et al., 1997 (1)
52/75 (68%)	0/33	Ramaswamy et al., 2000 (2)
12/21 (57%)	0/5	Lee et al., 2002 (3)
14/29 (48.3%)	48/154 (31.2%)	Mokrousov et al., 2002 (4)
60/133 (45.2%)	3/38 (7.9%)	Current report

whereas other researchers reported no *embB* mutation in EMB-susceptible strains (1-3). The cause of this large discrepancy is unknown, but in this study, the *embB* mutation was detected in three out of 38 EMB-susceptible isolates (7.9%). This mutation was also confirmed by temperature-mediated heteroduplex analysis performed by denaturing high-performance liquid chromatography to identify sequence polymorphisms (10,11). EMB susceptibility testing is currently being performed, because there is a possibility that these three isolates are EMB-resistant. It is interesting that the *embB* mutation occurs more frequently in strains that are resistant to the four anti-TB drugs than in strains that are resistant to EMB only (Fig. 1). Cross-resistance to the four anti-TB drugs may occur, although the drug targets are clearly different from each other.

Our molecular epidemiologic study revealed a very high incidence of MDR in Henan province, China. There are several possible reasons for the high incidence of MDR. First, the TB control program in Henan province is not operated efficiently due to the poor economic situation in that province. Second, there are no strict laws or regulations guarding against anti-TB drug abuse. Once patients feel better with anti-TB drugs, they stop taking them. Furthermore, anti-TB drugs can be bought at local drug stores without a prescription from medical practitioners. Finally, health education of the public and training of health workers are poor. In a few cases, anti-TB drugs were given to patients with non-TB respiratory diseases. The best treatment for TB patients is adoption of the directly observed therapy short course (DOTS) advocated by WHO (9).

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Newly Designed Primer Sets Available for Evaluating Various Cytokines and iNOS mRNA Expression in Guinea Pig Lung Tissues by RT-PCR

Hiroyuki YAMADA[1)], Tadashi UDAGAWA[1)], Satoru MIZUNO[1)], Kumiko HIRAMATSU[2)], and Isamu SUGAWARA[1)]

[1)]Pathology Division, Mycobacterium Reference Center, The Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association, 3–1–24 Matsuyama, Kiyose, Tokyo 204-8533, and [2)]Fourth Department of Internal Medicine, Nippon Medical School, 1–1–5 Sendagi, Bunkyou-ku, Tokyo 113-8602, Japan

Abstract: *Guinea pigs are often used as an animal model of human tuberculosis (TB). However, there are few methods available for pursuing the immunological processes involved in guinea pig TB. In this study, we developed for the first time systematic reverse transcription (RT)-PCR for evaluation of guinea pig mRNA expression. RT-PCR primer sets were newly designed for detection of cytokines and inducible nitric oxide synthase (iNOS) mRNA in guinea pig TB. Interferon (IFN)-γ, tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β, interleukin (IL)-1β, IL-2, IL-10, IL-12p40, granulocyte-macrophage-colony stimulating factor (GM-CSF) and iNOS mRNA expression were detected significantly and reproducibly when these primer sets were used. The data by real-time PCR were comparable with those of RT-PCR. We showed that these RT-PCR primer sets could be used to examine mRNA expression semi-quantitatively in guinea pig tissues, and conclude that these newly designed primer sets for conventional RT-PCR will be useful for studying the immunological processes in guinea pig tuberculosis experiments to investigate and evaluate efficacy of new vaccines or anti-mycobacterial drugs.*

Key words: *cytokine, guinea pig, real-time PCR, RT-PCR, tuberculosis*

Introduction

Tuberculosis (TB) is one of the most serious infectious diseases worldwide. About one-third of the world's population is infected with *Mycobacterium tuberculosis* and more than three million people succumb to this disease annually [21]. Consequently, extensive studies, using several animal models, have been undertaken to develop better TB vaccines and anti-mycobacterial drugs.

Among the animal models of TB used to date, guinea pig TB is thought to be one of the most useful because of its similarity to human TB [11]. However, because there are few immunological tools available for examining the pathological and immunological processes occurring in guinea pigs infected with *M. tuberculosis*,

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Address corresponding: H. Yamada, Pathology Division, Mycobacterium Reference Center, The Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association, 3–1–24 Matsuyama, Kiyose, Tokyo 204-8533, Japan

Table 1. Primer sequences of the murine and rat genes referred to in the design of the guinea pig primer set through sequence homology

Target (animal) [Accession no.]	Reference sequences (5'-3')[a] (S: Sense, A: Anti-sense)	Product length[b]
TGF-β (Mouse) [NM_011577]	S: CGG GGC GAC CTG GGC ACC ATC CAT GAC A: CTG CTC CAC CTT GGG CTT GCG ACC CAC	405 bp (2)
IL-2 (Mouse) [NM_008366]	S: CTT CAA GCT CCA CTT CAA GCT A: CCA TCT CCT CAG AAA GTC CAC	401 bp (3)
iNOS (Rat) [NM_012611]	S: CCA CAC ACT GGC CTC CCT CT A: TGG GCC TCA GCT TCT CAT CC	317 bp (2)

[a]Identical bases to the guinea pig primer are underlined. [b]The number of exon-intron boundaries in the amplified region is indicated in parentheses.

many investigators have used a mouse TB model for which immunological and molecular biological methodologies are available. This lack of suitable methodology has hampered immunological studies of guinea pig TB.

Bioassays for IFN-γ and TNF-α and northern blot analysis with limited cDNA probes have been used to examine the development of guinea pig TB [6, 7, 9, 12]. Although real-time RT-PCR has been used recently to examine gene expression quantitatively [1, 2, 8, 10], conventional RT-PCR is a more convenient method and uses cheaper reagents and devices [16–19, 22, 23]. In this study, new RT-PCR primer sets were developed for the detection of mRNA of several cytokines and other effector molecules in guinea pigs. Furthermore, real-time RT-PCR was also performed for some of the target genes and the results were compared with those of conventional RT-PCR. The results of RT-PCR with the newly designed primer sets correlated well with those of quantitative real-time RT-PCR and these primer sets can serve as cost-effective and convenient tools in conventional semi-quantitative RT-PCR.

Materials and Methods

Design of primer sets for RT-PCR

Two methods were used to design RT-PCR primer sets for investigating mRNA expression in guinea pig tissues. First, homology of mRNA sequences for cytokines and iNOS was determined among guinea pigs, mice and rats, because RT-PCR has been performed successfully with mRNA extracted from tissue samples of mice and rats using specific primer sets in our laboratory (Table 1) [16–19, 22, 23]. The sequences in guinea pig cytokine and iNOS mRNA that were homologous to sense or complementary to antisense primers for corresponding mouse or rat mRNA were identified. When there was sequence homology (or complementarity) to mouse or rat primers, primer sets were synthesized based on guinea pig mRNA sequences. RT-PCR was then carried out using these primer sets.

Alternatively, the primer construction program, Primer 3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi), was used with mRNA sequence information for guinea pigs collected from the Sequence Retrieval System at the Center for Information Biology and DNA Data Bank of Japan (DDBJ), National Institute of Genetics.

The sequences of these newly designed primer sets are shown in Table 2, and they were synthesized by Sigma Genosys Co., Ltd. (Sapporo, Japan). Among them, β-actin and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were used as positive and internal controls for RT-PCR. The sequence of the primer set for β-actin has already been published by Nishikawa *et al.* [13]. The GAPDH primer set was designed originally for humans, mice and rats by CLONTEC Laboratories, Inc. This primer set was also found to be useful for guinea pigs without any sequence modification, and a PCR product of the same length (452 bp) was obtained. In addition to this GAPDH primer set, an alternative primer set was designed that was specific for guinea pig GAPDH using the primer construction program, Primer 3.

Table 2. Guinea pig primer sets designed and/or used in this study

Target (Acc. No.)	Sequences (5'-3') (S: Sense, A: Anti-sense)	T_A & cycles[a]	Product Length
β-actin[b] (AF508792)	S: CCA ACT GGG ACG ACA TGG AG A: CGT AGC CCT CGT AGA TGG GC	65°C 30	279 bp
GAPDH[c] (AB060340)	S: ACC ACA GTC CAT GCC ATC AC A: TCC ACC ACC CTG TTG CTG TA	65°C 30	452 bp
GAPDH[c] (AB060340)	S: ACC ACA GTC CAT GCC ATC AC A: TGT CGC TGT TGA AGT CA	65°C 35	343 bp
IFN-γ[d] (AY151287)	S: GAC CTG AGC AAG ACC CTG AG A: GCC ATT TCG CCT GAC ATA TT	60°C 50	171 bp
TNF-α[d] (U39839)	S: ATC TAC CTG GGA GGC GTC TT A: GAG TGG CAC AAG GAA CTG GT	60°C 50	184 bp
TGF-β[e] (AF191297)	S: CGG GGC CTG GAC ACC AAC TAT TGC A: CTG CTC CAC CTT GGC TTT GCG GCC CAC	60°C 28	303 bp
IL-1β[d] (AF119622)	S: GGG CCT CAA GGG GAA TC A: GAG CAC CCC TTA GCG TGC TCT	65°C 50	430 bp
IL-2[e] (AB010093)	S: CTT CAA GCT CTC CAA AGC A A: CCA TCT CTT CAG AAA TTC CAC	60°C 60	353 bp
IL-10[d] (AF097510)	S: GGC ACG AAC ACC CAG TCT GA A: TCA CCT GCT CCA CTG CCT TG	60°C 50	367 bp
IL-12 p40[d] (AF097507)	S: TCT GAG CCG GTC ACA ACT GC A: AGG CGC TGT CCT CCT GAC AC	60°C 60	296 bp
iNOS[f] (AF027180)	S: GCA CAC GTT GGC TTC CCT CT A: TGG GCC AGT GCT TCT GAT TTT CC	65°C 40	456 bp
GM-CSF[d] (CPU46779)	S: CTG TGG TTT GCA GCA TCT GT A: GGG GCT CAA ACT GGT CAT AG	65°C 45	171 bp

[a]T_A & cycles indicate the annealing temperature and number of PCR cycles for each primer set, respectively. [b]The β-actin primer set was originally designed by Nishikawa *et al.* [13]. [c]The GAPDH primer set, which amplified the 452-bp product, was designed by CLONTEC Laboratories, Inc., and this primer set amplified the same 452-bp product in human, mouse and rat samples. The antisense primer of one primer set, which resulted in a 343-bp product, was specific for the guinea pig and was designed using the Primer 3 program. [d]Primer sets were designed using the Primer 3 program. [e]Primer sets were designed by referring to the mouse primer set. [f]Primer sets were designed by referring to the rat primer set.

Animals and experimental infection

Six-week-old female Hartley guinea pigs were purchased from Charles River Laboratory (Tokyo, Japan). Six-week-old female C57BL/6 mice and eight-week-old female Lewis rats were purchased from Japan SLC Co., Ltd. (Shizuoka, Japan). The animals were infected with the Kurono strain of *M. tuberculosis* (ATCC 35812) by placing them in the exposure chamber of an aerosol generator (Glas-Col, Inc., Terre Haute, Ind.) under conditions that would introduce about 100 bacteria into the lungs of each animal [16–19, 22, 23]. The animals were housed under biosafety level 3 conditions at an animal facility in The Research Institute of Tuberculosis and given rat, mouse or guinea pig chow and water *ad libitum*. Permission to conduct the animal experiment was given by the Animal Experiment Committee of the Research Institute of Tuberculosis.

A CFU assay in the guinea pigs was carried out to estimate the number of tubercle bacilli in lung and spleen tissues at the indicated time points. Three animals were used at each time point. Part of the right lung posterior lobe from each animal was excised and homogenized, plated on 1% Ogawa's egg medium to culture bacilli, cultured at 37°C for 4 weeks and the

colonies that appeared were counted [23]. The remaining part of the lung tissue was used for RNA extraction and histological examination. The results of the CFU assay were expressed as Log_{10} (mean CFU ± SD) of three animals sacrificed at two-week intervals from 1 week post-infection.

RNA isolation and purification

The animals were sacrificed at the indicated time points after aerosol infection. The infected lungs were excised and used for RT-PCR analysis to examine the mRNA expression levels of several cytokines. These tissue samples were snap-frozen in liquid nitrogen and stored at –85°C until use. RNA extraction was performed as described previously [16–19, 22, 23]. Briefly, the frozen tissues were homogenized in a microcentrifuge tube after being frozen in liquid nitrogen. The homogenates were then treated with total RNA isolation reagent, TRIzol (Invitrogen Japan Ltd., Tokyo, Japan), according to the manufacturer's instructions.

As little information was available on the genomic sequences from the guinea pig databank, there were no clues to indicate where and how many introns exist in the cytokine and iNOS genes. Purified mRNAs without any genomic DNA contamination were therefore required to obtain reliable results by RT-PCR. Oligotex™-dT30 <Super> mRNA purification kit (TAKARA BIO INC., Otsu, Shiga, Japan) was used to purify mRNA from total RNA, because only reverse-transcribed cDNAs had to be amplified.

cDNA synthesis

Purified mRNAs were reverse-transcribed into cDNA with M-MLV reverse transcriptase according to the manufacturer's instructions (Invitrogen Japan Ltd., Tokyo, Japan) except that the incubation temperature for the reverse transcription step was 42°C and the reaction time was 60 min.

PCR

PCR was performed using a mixture of equivalent amounts of cDNA of each sample (1 μl), 0.05 μl Takara EX Taq polymerase, 1.0 μl 10 × EX buffer, 0.8 μl dNTPs (Takara Bio Inc., Otsu Shiga, Japan), 0.5 μl each of gene-specific primer sets for β-actin, GAPDH, IFN-γ, TNF-α, TGF-β, IL-1β, IL-2, IL-10, IL-12p40, iNOS, and GM-CSF (Table 2), and finally 6.15 μl of distilled water (total volume: 10 μl in a 200-μl microcentrifuge tube). The sequences of the primer sets and annealing temperatures are shown in Table 2. The mRNAs purified with the Oligotex™-dT30 <Super> mRNA purification kit and not subjected to RT were used as negative controls in the PCR reaction to confirm that there was no genomic DNA contamination. All PCRs were performed as follows: denaturation at 94°C for 5 s, annealing at 60 or 65°C for 5 s, and extension at 72°C for 10 s, with the GeneAmp PCR System 9700 (Applied BioSystems, CA, USA). PCR products were visualized by ethidium bromide staining of 4% (w/v) SeaKem GTG agarose and NuSieve GTG (1:3) gels after electrophoresis.

Real-time RT-PCR

Taqman primer-probe sets for GAPDH, IFN-γ, TNF-α, and iNOS were designed using the Primer Express software package (version 2.0.0; Applied Biosystems) according to the manufacturer's guidelines, and synthesized by Sigma Genosys Co., Ltd. (Sapporo, Japan). The sequences of these primer-probe sets are shown in Table 3. Real-time RT-PCR was performed using cDNA synthesized as stated above with specific primer-probe sets according to the instructions for the ABI PRISM 7900HT Sequence Detection System (Applied BioSystems Inc.). Data analyses ($\Delta\Delta C_T$ analyses) were performed with the ABI PRISM Sequence Detection System software package (version 2.1; Applied BioSystems, California, USA) installed on Windows 2000 OS. The results were expressed as relative expression quantities of the targets compared to those of non-infected animals that were calibrated with the internal control gene expression (GAPDH).

Results

Determination of β-actin and GAPDH primer sets as internal and positive controls

The PCR products used as positive and internal controls, β-actin and GAPDH, are shown in Fig. 1. The PCR data (lane 1) for the β-actin gene using a primer set designed by Nishikawa *et al.* [13] showed that this primer set resulted in an amplified product of the expected length. Lane 3 shows the bands for the GAPDH PCR products and those obtained with primer sets de-