present data. The benefit of inhibition of IL-10 production for host defence has been previously demonstrated *in vivo*. IL-10-deficient mice displayed increased anti-mycobacterial immune responses and decreased bacterial burden (Murray & Young, 1999). In the absence of IL-10, antigen-specific memory T cells, which are efficiently produced by vaccination with BCG-SM for instance, may be fully activated for elimination of *M. leprae*. Although these are still preliminary findings, in one experiment BCG-SM more efficiently inhibited the multiplication of *M. leprae* in footpads of mice than in parent BCG. Therefore, BCG-SM may wipe out favourable conditions for the survival of *M. leprae*. The molecules that are present in the parental BCG and are associated with GM-CSF production remain undefined in the present study, but identification of these molecules may be useful to further enhance the T-cell-stimulating activity of BCG-SM. Also, the identification of such molecules may contribute greatly to the control of the pathogenic mycobacterial diseases using modified BCG.

In this study, we demonstrated that BCG-SM which can induce abundant GM-CSF production, may be more potent than parent BCG in immunostimulation and in the inhibition of IL-10 production, for preventing the survival of *M. leprae*.

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# Establishment of A Guinea Pig Model of Latent Tuberculosis with GFP-introduced *Mycobacterium Tuberculosis*

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There exists latent tuberculosis, in which small numbers of tubercle bacilli remain viable in the host without visible granulomatous lesions. As few data exist on the mechanisms of latent tuberculosis, it is important to examine latent tuberculosis in terms of pathogenesis and efficacy of chemotherapy. As a first step, we used green fluorescent protein (GFP)-introduced H37Rv *Mycobacterium tuberculosis* to establish latent tuberculosis in the guinea pig that provides one of the best animal models of tuberculosis. We inoculated the guinea pigs subcutaneously with 100 or 1,000 colony-forming unit (CFU) of tubercle bacilli. During the 300-day follow-up period after infection, there were no clinical signs of disease, suggesting a lack of visible granulomatous lesions. In fact, upon necropsy, no macroscopic tuberculous lesions were recognized, but histopathological examination of the lung, spleen and liver revealed microgranulomas consisting of epithelioid macrophages and lymphocytes without central necrosis. Importantly, photon imaging visualized granulomatous lesions corresponding to these histologically apparent microgranulomas. Tuberculin skin testing of infected guinea pigs showed strong positivity (≥ 10 mm induration) until the end of the experiments. Real-time PCR analysis showed a significant increase in the expression levels of interferon-$\gamma$, tumor necrosis factor-$\alpha$, interleukin-12, and inducible nitric oxide synthase mRNAs in infected lung tissues after 300 days ($P < 0.01$). As human samples are hardly available to study latent tuberculosis, our guinea pig model would be useful for examining the pathogenesis and molecular mechanisms of latent tuberculosis as well as for monitoring the results of chemotherapy with green fluorescence emission of tubercle bacilli.

——— GFP-H37Rv; latent infection; guinea pigs; *Mycobacterium tuberculosis*; photon imaging.
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Most cases of adult tuberculosis occur in individuals who have been previously exposed to *Mycobacterium* (*M.*) *tuberculosis* and have developed a localized and self-healing inflammatory reaction. In most of the individuals the inflammatory reaction is spontaneously contained; often it calcifies and persists for the remainder of the person's life (Medlar 1950). Small numbers of tubercle bacilli are thought to remain viable for life in a condition known as latent or dormant tuberculosis (Lurie 1950). However, it is almost impossible to understand the mechanism and pathogenesis of human latent tuberculosis because of the difficulty in obtaining suitable samples.

To overcome this difficulty several mouse models of latent tuberculosis have been proposed (McCune et al. 1956; Orme 1987; Phyu et al. 1998). Whether or not these murine models truly represent latent human tuberculosis remains controversial, but two models have been proposed: a low-dose model and the Cornell model. The low-dose model has the marked advantage of mimicking natural latency in the sense that it relies solely on the host immune response for control of the infection, but it has the disadvantage of a high bacillary burden that is unlike that found in human latent *M. tuberculosis* infection (Orme 1987). The drug-induced Cornell model of latent tuberculosis has the advantage of achieving very low or undetectable numbers of bacilli and maintaining those low levels for many weeks in a pattern analogous to that in human latent infection, but it has the disadvantage of artificially inducing latency. Therefore, Cornell model variants have been proposed (Scanga et al. 1999).

The guinea pig provides one of the best animal models for tuberculosis. Recently, a guinea pig model of latent or dormant infection with *M. tuberculosis* was created using a streptomycin-auxotrophic mutant of *M. tuberculosis* and streptomycin (Kashino et al. 2008). Although this model is relatively reproducible, it is complicated in that it uses *M. tuberculosis* 18b (Hashimoto 1955; Shi et al. 2007), which only a limited number of laboratories possess. We previously reported the photon imaging of pulmonary granulomas induced by *M. tuberculosis* H37Rv strain expressing green fluorescent protein (GFP) (Sugawara et al. 2006), showing that pulmonary granulomas more than 1 mm in diameter are localized clearly by the photon imager. We therefore considered it possible to detect latent granulomas

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by photon imaging.

To expand the limited amounts of data currently available on the mechanisms of latent tuberculosis in guinea pigs, we used GFP-H37Rv to develop a low-dose model of guinea pig *M. tuberculosis* latent infection. Upon necropsy, no macroscopic tuberculous lesions were recognized, but histopathological examination of organs revealed microgranulomas consisting of epithelioid macrophages and lymphocytes without central necrosis. Interestingly, the photon imager visualized granulomatous lesions that corresponded to the histologically apparent granulomas. Our guinea pig model should be useful for examining the molecular mechanisms of latent tuberculosis and for monitoring chemotherapy of latent tuberculosis utilizing green fluorescence emission.

## Materials and Methods

*Bacterial strain and animal infection*

*M. tuberculosis* H37Rv strain (ATCC 25618) was transformed with the heat shock protein (HSP) 60 promoter-GFP mut 3.1 M to obtain a stable GFP-H37Rv mutant (Sugawara et al. 2006). This mutant remains stable for a year after subcutaneous administration to C57BL/6 mice, and there is no significant difference in growth rate or virulence between H37Rv and GFP-H37Rv (Sugawara et al. 2006).

Outbred female Hartley guinea pigs (average weight 250 g) purchased from SLC Co., Shizuoka, Japan, were infected subcutaneously with 100 or 1,000 CFU of GFP-H37Rv (ten in each group). Ten uninfected outbred female Hartley guinea pigs were also used. All experiments were conducted in compliance with the Guidelines for the Animal Care and Use Committee of The Research Institute of Tuberculosis.

*Histopathology*

For histopathological examination, the guinea pig's lungs, spleen, hilar lymph nodes, and liver were removed and embedded in paraffin. Sections 4 $\mu$m thick were cut, formaldehyde-fixed, and stained with hematoxylin and eosin or by the Ziehl-Neelsen method for acid-fast bacilli (AFB) (Sugawara et al. 2004).

*Colony-forming unit (CFU)*

At different time points after infection (6 months and 10 months), animals were euthanatized and their lungs and spleen removed. Organ homogenates were prepared in phosphate-buffered saline (PBS) and plated at 10-fold serial dilution on 1% Ogawa slant media. The plated agar was incubated at 37°C for 4 weeks and the CFU were counted 4 weeks later (Yamada et al. 2001; Sugawara et al. 2006).

*Delayed type hypersensitivity reaction*

Tuberculin skin tests were performed by intradermal injection of pure protein derivative (PPD) (50 $\mu$l/guinea pig, 50 $\mu$g/1 mL, Japan BCG Labs., Tokyo, Japan) into a small shaved area of the skin of the back. Twenty-four hours later transverse diameters of induration reaction were measured in millimeters (Sugawara et al. 2006).

*Immunohistochemistry*

Immunohistochemistry was performed with avidin-biotin-peroxidase complex (ABC), as described in detail previously (Hsu et al. 1981). Anti-BCG antibody (Dakopatts, Copenhagen, Denmark) was used at a final concentration of 10 $\mu$g/mL (Sugawara et al. 1998) to visualize BCG-related antigens. For negative-control slides, all these steps were repeated, but with non-immune serum substituted for the primary antibody.

*Real-time PCR*

The remaining portions of the right lower lobes of the lungs and of the spleen were used for reverse transcription (RT) polymerase chain reaction (PCR) analysis to examine the expression levels of several cytokine mRNAs during GFP-H37Rv infection. These samples were snap-frozen in liquid nitrogen and stored at –85°C until use. RNA extraction was performed as described previously (Yamada et al. 2005). Briefly, the frozen tissues were homogenized in a microcentrifuge tube with an autoclaved disposable 1,000-$\mu$L tip cooled down at 4°C by being dipped in liquid nitrogen. The homogenates were then treated with 1 mL of TRIzol reagent (Invitrogen Japan Co., Tokyo, Japan), as specified by the manufacturer. After RNA isolation, the total RNA concentration was measured with a spectrophotometer, and the agarose gel electrophoresis pattern of the total RNA was examined. The total RNAs were reverse-transcribed into cDNA with Moloney murine leukemia virus reverse transcriptase (Invitrogen). ABI TaqMan Gene Expression Assay was used for relative quantification of the expression of interferon (IFN)-$\gamma$, tumor necrosis factor (TNF)-$\alpha$, interleukin (IL)-10, IL-12, and inducible nitric oxide synthase (iNOS) mRNAs. A TaqMan Rodent glyceraldehydes-3-phophate dehydrogenase (GAPDH) Control Reagents set was used for normalization for data analysis. Real-time RT-PCR was performed according to the instructions for the ABI PRISM 7900HT Sequence Detection System (Applied BioSystems Inc., California, USA). Data were analyzed by the $\Delta\Delta C_T$ method using the ABI PRISM Sequence Detection System software package (version 2.1; Applied BioSystems) on a Windows 2000 operating system. The results obtained from GFP-H37Rv-infected and control guinea pigs were expressed relative to those from uninfected guinea pigs, which were calibrated against the expression of an internal control gene (GAPDH) (Yamada et al. 2007).

*Photon imaging*

Guinea pigs infected with GFP-H37Rv were scanned for green fluorescence by using a $\Phi$ imager (photon imager, Biospace Mesures, Paris, France; or NightOwl LB 981 Molecular Light Imager, Berthold Technologies, Germany). This imager is based on a third-generation GaAs intensified charge-coupled device (ICCD) camera that allows real-time photon counting over a wide spectral range (wavelength 400 to 900 nm). The imager uses an ICCD chip to amplify every photon up to $10^6$ light spots in a detector. The detection conditions of this imager were as follows: spatial resolution, equivalent to 1,080 × 1,440 pixels CCD; dynamics, 2,000 counts pixel$^{-1}$ min$^{-1}$; excitation wavelength, 485 nm; emission wavelength, 535 nm (Sugawara et al. 2006; Hyoudou et al. 2007).

*Statistical analysis*

All values were expressed as means ± S.D. and compared by using Student's *t*-test. For all statistical analyses, the level of statistical significance was set at $P < 0.01$.

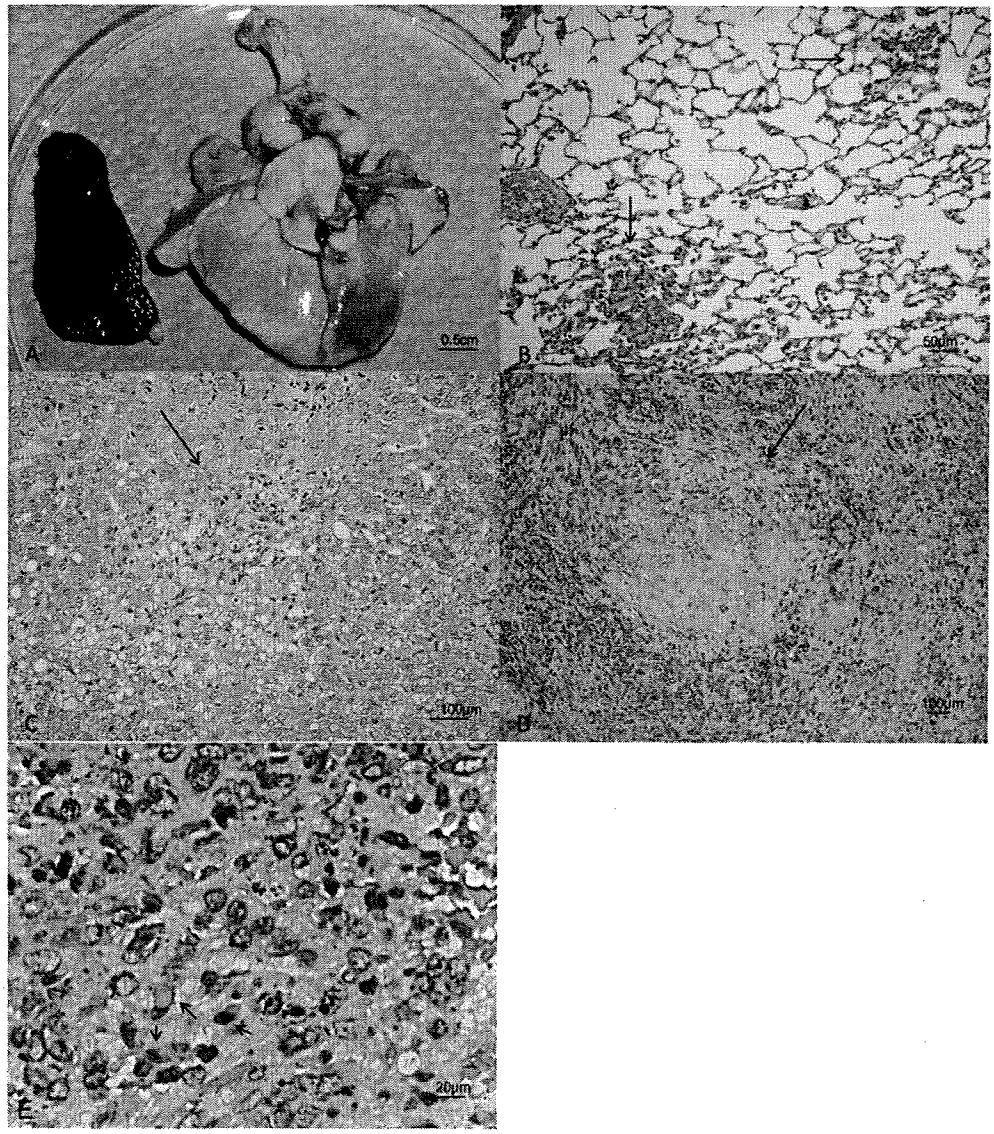


Fig. 1. Pathology of lung, spleen, and liver tissues from guinea pigs infected with GFP-H37Rv. A, Macroscopy of infected guinea pig organs; B, Lung tissue; C, Liver tissue; D, Spleen tissue; E, Epithelioid macrophages (→) immunostained with anti-BCG polyclonal antibody and counter-stained with hematoxylin. Arrow (→) indicates the microgranuloma in B, C, and D. Hematoxylin eosin stain × 100.

## Results

*Infection of guinea pigs with GFP-H37Rv, and macroscopic and microscopic pathology*

To begin characterizing the infection caused by the GFP-H37Rv strain, the guinea pigs were inoculated subcutaneously with very low doses of tubercle bacilli (100 or 1,000 CFU) and then followed up for 10 months. First, we monitored for external clinical changes related to tuberculosis in guinea pigs (lethargy, scuffed fur, foot-dragging walk, weight loss). Over the 10-month period, the guinea pigs inoculated with 100 CFU GFP-H37Rv showed none of the above-mentioned signs except gradual increase of body weight and no changes in overall behavior. The guinea pigs inoculated with 1,000 CFU GFP-H37Rv showed no scuffing of the fur, foot-dragging walk, or lethargy for the first 8 months, but thereafter a foot-dragging walk appeared. No loss of body weight was observed in this group; all 10 infected guinea pigs (1,000 CFU inoculation) steadily gained weight throughout the experiment, and no deaths occurred.

There were no macroscopic lesions suggestive of tuberculosis at 10 months in 100 CFU-inoculated group (Fig. 1A). However, histopathological examination showed microgranulomas in the lungs in 100 CFU-inoculated group, the liver parenchyma, and the lymph follicles of the spleen (Fig. 1B-D). Only one tubercle bacillus was found in one lung tissue section stained by Ziehl-Neelsen stain for acid-fast bacilli. Immunohistochemistry using anti-BCG polyclonal antibody demonstrated BCG antigens in the epithelioid macrophages that constituted the microgranulomas (Fig. 1E).

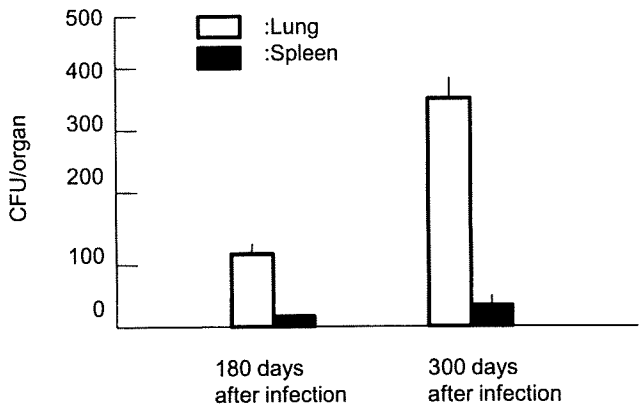


Fig. 2. Persistence of viable GFP-H37Rv strain in the tissues of infected guinea pigs. CFU counts were made on lung and spleen tissues 180 and 300 days after infection (three per group).

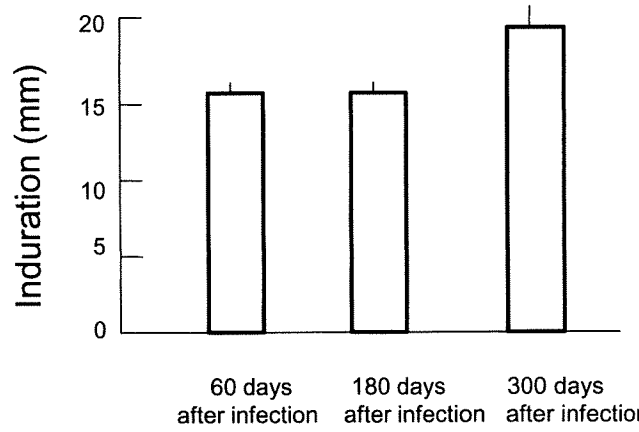


Fig. 3. PPD skin testing of guinea pigs infected with GFP-H37Rv.
Skin tests were performed 60, 180, and 300 days after infection (three per group), and the diameter of the induration was measured in mm.

*Burden of mycobacteria in guinea pig tissues after subcutaneous infection with GFP-H37Rv*

To confirm that infection of guinea pigs with the GFP-H37Rv resulted in latent infection, 180 and 300 days after infection CFU counts were made in the pulmonary and splenic tissues of guinea pigs that had been inoculated with 100 CFU GFP-H37Rv. At 180 days after infection the pulmonary CFU was 121 ± 10, and 300 days after infection it was 360 ± 15 (Fig. 2). The splenic CFU counts 180 and 300 days after infection were 10 ± 1 and 25 ± 2, respectively.

*PPD skin testing*

We used the tuberculin purified protein derivative (PPD) skin test, because it is used worldwide as an adjunctive test for the diagnosis of both latent and active tuberculosis. All infected guinea pigs were strongly positive in all PPD tests, which were performed at 60 days (16.3 ± 1.1 mm diameter), 180 (16.7 ± 1.3 mm), and 300 days (19.7 ± 1.4 mm) after infection (Fig. 3). No induration reaction was recognized in the skin of uninfected control guinea pigs ($n = 5$).

*Photon imaging of organs from guinea pigs*

To localize the lesions suggestive of the presence of GFP-H37Rv, the major organs were serially cut to a thickness of 5 mm and subjected to photon imaging. Strong red signals of varying intensities were recognized in the serially cut lung and liver tissues from infected guinea pigs (Fig. 4A and 4B). Strong red signals were also detected in the spleen. The locations of the strong signals corresponded to the granulomas (proliferative inflammation) found on histopathological examination (see Fig. 1).

*Real-time PCR analysis of infected pulmonary tissues*

Data obtained by real-time PCR analysis of infected pulmonary tissues were expressed as relative intensities. In the lung tissues of uninfected guinea pigs, expression levels of IFN-$\gamma$, TNF-$\alpha$, IL-10, IL-12, and iNOS mRNAs were very low (< 0.1 as relative intensity). At 180 days after infection, there were no statistically significant differences in mRNA expression between uninfected and infected guinea pigs (Table 1). At 300 days after infection the intensities were minimal, but significant differences in IFN-$\gamma$, TNF-$\alpha$, IL-12, and iNOS mRNA expression were recognized between uninfected and infected guinea pigs ($P < 0.01$).

## Discussion

In this study we have reported the successful establishment of latent *M. tuberculosis* infection in guinea pigs. In human tuberculosis, exogenous reinfection and reactivation of *M. tuberculosis* are major problems that require resolution, but in order to do so, the pathogenesis of latent human tuberculosis needs to be clarified. Because we did not have an animal model suitable for the investigation of latent tuberculosis, it was of great importance to develop one in order to study the pathogenesis and molecular mechanisms of latent infection.

Several murine models of latent infection have already been reported (McCune et al. 1956; Orme 1987; Phyu et al. 1998). In a model known as the Cornell model, mice are inoculated intravenously with viable tubercle bacilli and the resulting infection is treated for 12 weeks with anti-mycobacterial drugs. From this time point on, no tubercle bacilli can be cultured from the organs of these animals for several months. Administration of cortisone 2 to 3 months after interruption of the antibiotic therapy reverses this condition, and tubercle bacilli can then be cultured from lungs and spleens of more than 50% of the animals. The Cornell model is therefore not so highly reproducible and has no standardized protocol for establishment of latency. Although variant Cornell murine models have been proposed, their reproducibility is still low and many parameters (inoculating dose, infection period, antibiotics, and duration

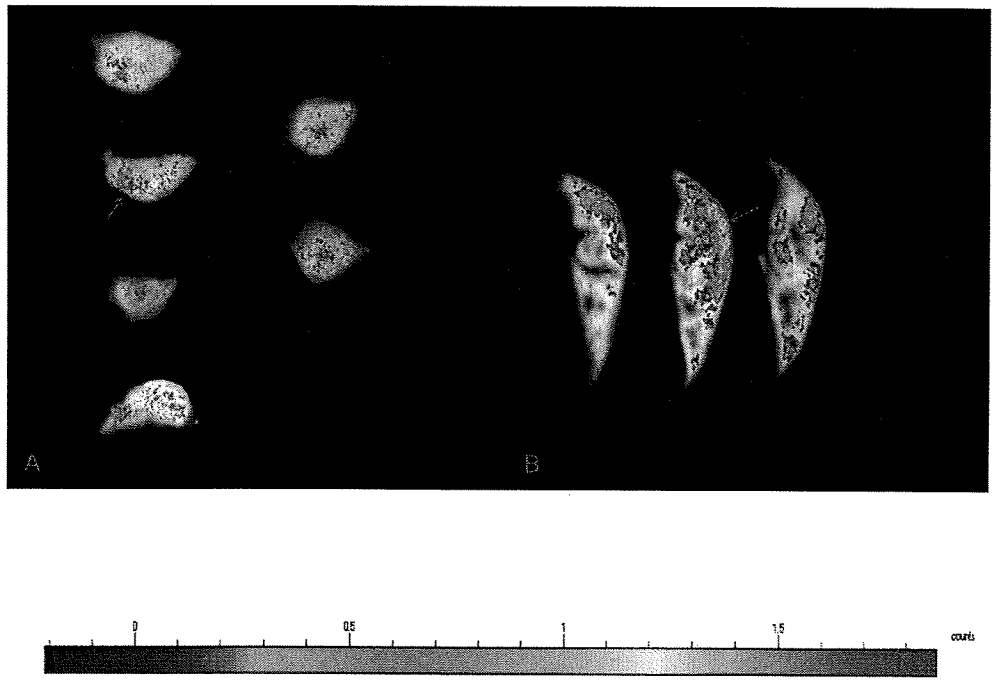


Fig. 4. Visualizing granulomas in the lung and liver of infected guinea pigs.
Small pulmonary (A) and liver (B) granulomas were visualized by photon imaging. Granulomatous lesions are visible as strong red signals ( → ).

Table 1. Expression profiles of IFN-$\gamma$, TNF-$\alpha$, IL-10, IL-12, and iNOS mRNAs in the lungs of infected guinea pigs.

| | IFN-$\gamma$* | TNF-$\alpha$ | IL-10 | IL-12 | iNOS |
|---|---|---|---|---|---|
| 180 days after infection | 1.12 | 1.20 | 0.95 | 1.05 | 1.17 |
| 300 days after infection | 1.74+ | 1.89+ | 1.02 | 1.79+ | 2.09+ |

The guinea pigs were inoculated with 100 CFU GFP-H37Rv. *In the lung tissues of uninfected guinea pigs, expression levels of IFN-$\gamma$, TNF-$\alpha$, IL-10, IL-12, and iNOS mRNA were very low (< 0.1 as relative intensity).

+Statistically significant ($P < 0.01$).

of treatment) are involved in the induction of latency (Scanga et al. 1999).

In low-dose murine models, mice are aerogenically or intraperitoneally infected with a low dose of *M. tuberculosis*. The quiescent phase of the infection is maintained for a relatively long time, after which the infection begins to reactivate and the mice succumb to tuberculosis (Orme 1987). This low-dose murine model has the important advantage of tracing natural latency in the sense that it relies solely on the host immune response for control of the infection. However, it has the disadvantage of a high bacillary burden, unlike that found in human latent *M. tuberculosis* infection.

A guinea pig model of *M. tuberculosis* latent or dormant infection was therefore proposed recently (Kashino et al. 2008). This model employs a streptomycin-auxotrophic mutant of *M. tuberculosis* (*M. tuberculosis* 18b) and streptomycin. However, this model is not still reproducible, because $10^7$ CFU of *M. tuberculosis* 18b are injected intravenously and the guinea pigs are then inoculated with streptomycin for 3 months. To overcome this difficulty, we created a low-dose model by injecting guinea pigs subcutaneously with 100 or 1,000 CFU GFP-H37Rv. Ten months later, no mycobacterial lesions were observed macroscopically, but all of the tuberculin skin tests conducted during the follow-up period gave positive results. However, the lymphocytes from the hilar lymph nodes of the latent guinea pig tuberculosis did not respond to Ag85A peptides in vitro (data not shown). It was not easy to detect granulomas histopathologically, but use of a photon imaging technique clearly localized the granulomatous lesions in the organs. The 1,000-CFU subcutaneous model was not suitable for latent infection because the tubercle bacilli were reactivated 10 months later and many granulomatous lesions were recognizable in the major organs. Conversely, the 100-CFU model was favorable in that only microgranulomas were observed histologically. To date we have followed up the 100-CFU group for more than 1 year; the guinea pigs are all still alive and their tuberculin skin tests still give positive results.

To shed light on the molecular mechanisms of latent tuberculosis, we examined the expression of cytokine and iNOS mRNAs. At 180 days after infection there were no significant differences in mRNA expression between uninfected and latently infected guinea pigs, but significant changes in expression of IFN-$\gamma$, TNF-$\alpha$, IL-12, and iNOS mRNAs were recognized between uninfected and latently infected animals at 300 days after infection. These results indicate that the immune network (Th2 cytokines and the cytokines related to Th2 cells) was stimulated minimally but effectively in latent infection.

Our animal model should provide useful clues for studying the pathology of adult latent tuberculosis. Humans and rabbits infected with *M. tuberculosis* develop cavities in the lungs, whereas guinea pigs do not (Dannenberg 2006). After reactivation of latent infection or exogenous reinfection with *M. tuberculosis*, caseous lesions and cavities may develop. Further study will be required to elucidate the mechanisms involved. Our model may also be of help for studying new tuberculosis vaccines and drugs for latent tuberculosis. As mentioned above, it is difficult to detect microgranulomas, but they were clearly detectable with a high-sensitivity photon imager. If a drug or vaccine being tested were effective in latent tuberculosis, then no fluorescent signals would be detected. Our model is therefore an attractive alternative for use in the development of anti-tuberculosis vaccines in the context of presensitization of animals.

In summary, we have established a guinea pig model of latent tuberculosis. Our guinea pig model can be used widely for studies of the immunology, molecular biology, and chemotherapy of latent tuberculosis.

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**Short Communication**

# Tuberculosis Complicated by Diabetes Mellitus at Shanghai Pulmonary Hospital, China

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**SUMMARY**: An association between diabetes mellitus (DM) and tuberculosis (TB) has been implied for a long time. We previously reported that KDP type 1 diabetic rats and GK type 2 diabetic rats are highly susceptible to *Mycobacterium tuberculosis* infection. As a next step, we conducted a retrospective analysis of 2,141 patients with pulmonary TB newly diagnosed during the period from 2008 to 2009 to evaluate the influence of DM on the drug response rate and the long-term relapse rate of TB. There were 203 DM patients with TB (type 1 DM, 7 [3.4%]; type 2 DM, 196 [96.6%]). The TB relapse rate (2 years after discharge) was higher in DM patients than in non-diabetic patients (20% versus 5.3%). The frequency of multidrug-resistant-TB among DM patients with TB was higher than that among TB patients (17.7% versus 8.4%, $P < 0.01$). These results suggest that the period of TB treatment should be prolonged, and that in the meantime the blood glucose level should be maintained within a reference value range.

Diabetes mellitus (DM) is a high-risk factor for tuberculosis (TB). An association between diabetes and TB has been implied for centuries. In the late 17th century, Morton recognized a link between diabetes and TB (1). Since then, a body of clinicoepidemiological data has been accumulated (2-7). However, there is no definitive evidence of an association between DM and TB. Patients with DM have been noted to have impaired granulocyte chemotaxis, phagocytosis, bactericidal activity and superoxide production (8,9). In order to obtain conclusive evidence of the link between DM and TB, we have employed two kinds of rat diabetes models, the Komeda diabetes-prone (KDP) type 1 rat model and the Goto-Kakizaki (GK) type 2 rat model, and have reported that both type 1 and type 2 diabetic rats are highly susceptible to *Mycobacterial tuberculosis* (10,11). Although there are a few reports on the association between TB and DM in Asia (7,12,13), no such report has come from China. Therefore, we focused on TB patients with DM in Shanghai, China. A total of 2,141 TB patients were hospitalized between April 2008 and March 2009, of whom 1,464 (68.4%) were smear- and culture-positive, and 20.2% were smear-negative and culture-positive. The male to female ratio was 3.2:1. All were farmers, salaried persons or unemployed, with an average age of 42.7 years. This project was approved by the ethics committee of Shanghai Pulmonary Hospital, China.

Among these TB patients, 203 (9.5%) were complicated by DM (type 1, 7; type 2, 196). Of these, 114 patients were smear- and culture-positive and 47 were smear-negative and culture-positive. The blood glucose range of these patients was 200-700 mg/dl. After the DM patients had been hospitalized for 2 to 4 weeks, those with type 1 DM were treated by subcutaneous injection of recombinant insulin, and those with type 2 DM received oral medication including metformin hydrochloride and gliclazide. Thereafter, they received standardized regimens consisting of isoniazid, rifampicin, pyrazinamide, ethambuthol or streptomycin. We then examined the drug susceptibility of *M. tuberculosis* isolates cultured from the TB patients with DM by the proportional method (14). As shown in Table 1, 36 (17.7%) of the patients with DM had multidrug-resistant (MDR)-TB, the proportion being significantly higher than that of non-diabetics with TB (9.3%) ($P < 0.01$). Furthermore, the proportion of MDR-TB patients was much higher in the group with poorly controlled DM (32 versus 4). Of 167 patients who had received standardized chemotherapy, 20 (12.0%) relapsed within 2 years after being discharged from the hospital. There are several reasons that the incidence of MDR-TB and the relapse rate are higher in patients with DM than in non-diabetics. First, the DM patients have moderate to severe TB, and it takes longer period to treat them sufficiently with chemotherapy. Their drug compliance is often poor, and they suffer adverse reactions more frequently. They take anti-TB drugs irregularly. Second, some patients cannot pay for their medical expenses, and so their chemotherapy has to be discontinued for short periods of time until they earn enough money to restart the chemotherapy. Third, some patients do not take anti-DM drugs regularly, and their blood glucose levels are not well controlled, until eventually their TB becomes refractory. Lastly, these DM patients may be reinfected with new MDR-TB strains due to their lowered immunity.

Table 1. Drug sensitivity of tubercle bacilli cultured from TB patients with DM

| | Drug-susceptible | MDR[1)] | Total |
|---|---|---|---|
| Diabetics ($n$ = 203)[2)] | 82.3% | 36 (17.7%) | 100% |
| FBS ≧ 200 mg/dl | | 32 (15.7%) | |
| 126 ≦ FBS ≦ 199 | | 4 (2.0%) | |
| Non-diabetics ($n$ = 1,938) | 90.7% | 9.3% | 100% |

[1)]: $P < 0.01$.
[2)]: The detailed profiles of 203 diabetics and 1,938 non-diabetics are as follows: 67 (FBS ≧ 200 mg/dl) (51-78 years old, man:woman = 5.5:1), 136 (126 ≦ FBS ≦ 199) (30-82 years old, man:woman = 5.5:1), and 1,938 (FBS≦125) (2-96 years old, man:woman = 3:1).
MDR, multidrug-resistant; FBS, fasting blood sugar (mg/dl).

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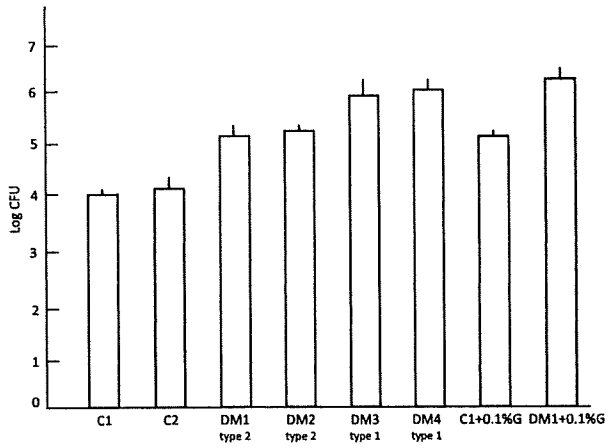


Fig. 1. The effect of serum from DM patients on growth of *M. tuberculosis* H37Rv. C, healthy volunteer; DM, diabetes mellitus; G, glucose.

There have been two reports of pulmonary TB complicated by DM in Japan (12,13). Kameda et al. analyzed 116 TB patients with DM among 644 TB patients. MDR-TB patients with DM accounted for 6.0% of the total, which was a significantly lower proportion than that in our present series. Their relapse rate within 30 months after discharge was 10.3%, which was not significantly different from the result in our series. Wada et al. also reported 54 TB patients with DM among 620 patients with TB, and the relapse rate within 24 months after discharge was 11.1%, compared with 1.3% in non-diabetics. This difference was statistically significant ($P < 0.01$). Thus, it is worthwhile to examine the TB relapse rate in diabetics on the basis of large samples.

We have recently reported that 0.1% glucose increased mycobacterial growth in vitro and that insulin treatment resulted in a significant reduction of tubercle bacilli in infected KDP rats (13). Therefore, it is useful to examine the effects of serum samples from DM patients on mycobacterial growth in vitro. We collected two serum samples from healthy subjects (C1 and C2), two samples from type 2 DM patients (DM1 and DM2) and two samples from type 1 DM patients (DM3 and DM4). Blood glucose levels and immunoreactive insulin (IRI) levels in DM1, DM2, DM3 and DM4 were 520 mg/dl and 5 $\mu$U/ml, 660 mg/dl and 5 $\mu$U/ml, 610 mg/dl and <1 $\mu$U/ml, and 705 mg/dl and <1 $\mu$U/ml, respectively. The reference value ranges of fasting blood glucose and IRI were 100-125 mg/dl and 5-15 $\mu$U/ml, respectively, in this hospital. The patients' serum (0.5 ml each) was added to 0.5 ml of 7H9 liquid medium and cultured in the presence of *M. tuberculosis* H37Rv (1,000 CFU) for 1 week. Thereafter, serially diluted samples were cultured on 1% Ogawa solid agar slants in triplicate, and the colonies that appeared were counted 4 weeks later. In some experiments, 0.1% glucose was added to C1 and DM1 serum samples. As shown in Fig. 1, the growth of tubercle bacilli was facilitated in the patients' sera. Moreover, the sera from type 1 DM patients enhanced mycobacterial growth significantly ($P < 0.01$). When 0.1% glucose was added to C1 and DM1 serum samples, the growth of tubercle bacilli was better facilitated ($P < 0.01$). Although the sample numbers were small, the results suggested that glucose stimulates mycobacterial growth, whereas insulin reduces mycobacterial colonies.

We then conducted a retrospective analysis of 2,141 patients with pulmonary TB newly diagnosed during the period from 2008 to 2009 to evaluate the influence of DM (203 cases) on the drug response rate and the long-term TB relapse rate. The cases of TB complicated by DM showed a poor prognosis if relapse occurred within 2 years. Thus, it appears that a longer treatment period is required for TB patients with DM. At the same time, as there were more MDR-TB patients with DM in this series, there is a need to devise a new chemotherapy regimen to achieve a more effective treatment.

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# Cross-resistance of *Mycobacterium tuberculosis* isolates among streptomycin, kanamycin and amikacin

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Seventy-four streptomycin (SM)-resistant *M. tuberculosis* clinical isolates were subjected to cross-resistance drug testing against two major aminoglycosides, kanamycin (KM) and amikacin (AMK). Among them, 15 clinical isolates (20.3%) were resistant to both KM and AMK. Fifteen (80%) of 19 KM-resistant isolates were AMK-resistant. Fifteen SM, KM, and AMK resistant isolates harbored *rrs* mutation, but only two had *rrs* and *rpsL* double mutations. Low-level SM resistance was associated with *rpsL* mutation, whereas high-level SM resistance was linked to *rrs* mutation.

**Keywords**: Amikacin, Cross-resistance, Kanamycin, MDR-TB, Streptomycin

Streptomycin (SM) is one of the aminoglycosides and is used as a first-line anti-tuberculosis (anti-TB) drug. Its structure is o-2-deoxy-2-(methylamino)-α-L-glucopyranosyl-(1→2)-o-5-deoxy-3-C-formyl-α-L-lyxofuranosyl-(1→4)-N,N'-bis(aminoiminomethyl)-D-streptamine. Earlier in a study, we used 115 streptomycin (SM)-resistant clinical isolates from Beijing, China, of which 85.2% harbored *rpsL* or *rrs* mutation, while *rpsL* mutation (76.5%) dominated among the isolates[1]. Among them, 45 clinical isolates were resistant to SM at more than 100 μg/mL and regarded as high-level SM-resistant. These 115 SM-resistant clinical isolates were collected from local farmers (treated previously with several anti-TB drugs) from all over China and sent to Beijing Tuberculosis and Lung Tumor Research Institute for further survey. There are reports that kanamycin (KM) and/or amikacin (AMK) are used as second-line anti-TB drugs for patients with MDR-TB and KM and AMK kill SM-resistant isolates[2].

KM and AMK are aminoglycosides with the structures o-3-amino-3-deoxy-α-D-glucopyranosyl-(1→6)-o-[6-amino-6-deoxy-α-D-glucopyranosyl-(1→4)]-2-deoxy-D-streptamine and o-3-amino-3-deoxy-α-D-glucopyranosyl-(1→6)-o-[6-amino-6-deoxy-α-D-glucopyranosyl-[1→4]]-N1-(4-amino-2-hydroxy-1-oxobutyl)-2-deoxy-D-streptamine, respectively. It indicates that SM, KM and AMK have similar structures. Moreover, KM and AMK are used as second-line anti-TB drugs for patients with MDR-TB, but there is no report on cross-resistance among SM, KM and AMK based on large clinical samples[3]. In view of this, the present study was undertaken to elucidate the cross-resistance among SM, KM, and AMK.

Seventy-four streptomycin (SM)-resistant and 11 SM-sensitive clinical isolates of *M. tuberculosis* from China were considered for the study. The 74 patients with SM-resistant TB had been treated with several anti-TB drugs, but had no previous history of KM and AMK treatment. There were no mono-resistant SM clinical isolates. Several isolates did not grow well and hence, were omitted from this study. Minimum inhibitory concentrations (MICs) of SM, KM, and AMK were detected by absolute concentration method in L-J medium at 1, 2, 5, 10, 20, 40, 80, 100, 200, 400, 800 and 1,000 μg/mL (Ref. 1). Isolates with a MIC exceeding 10 μg/mL are defined as SM-resistant[4,5]. However, for KM and AMK resistance, MIC was 20 μg/mL (Refs 4, 6).

Forty-five isolates (61%) were resistant to SM at more than 100 μg/mL and referred to as high-level SM resistance (Fig. 1). Twelve isolates had a MIC of less than 40 μg/mL. On the other hand, there were 19 KM-resistant isolates, and 15 of them were resistant to concentrations exceeding 100 μg/mL. There were 15 AMK-resistant isolates, of which 9 were resistant at more than 100 μg/mL of concentration. There were 15 isolates resistant to both KM and AMK, and 9 (60%) of them were resistant to concentrations exceeding 100 μg/mL (Table 1).

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It has been considered worth to examine these 15 SM, KM and AMK resistant clinical isolates genetically in terms of *rpsL* and *rrs* mutations[6,7]. We utilized a denaturing HPLC (DHPLC) system to detect point mutation of target genes as reported previously[1,8]. All the isolates displayed *rrs* mutation and three of them had *rpsL* mutation (Table 2)[9]. The most common *rrs* mutation ($^{513}$A→C) was found in 7 isolates. Interestingly, high-level SM resistance was closely associated with both KM and AMK resistance [15/45 (33%)]. The target genes of SM were *rpsL* and *rrs*, while a target gene of KM and AMK was *rrs*. As all SM-resistant clinical isolates possessed *rrs* mutation, they were also resistant to KM and AMK. Among 11 SM-sensitive isolates, only two were resistant to KM and AMK. Thus, there were cases that KM or AMK could not be used as a second-line drug for MDR-TB patients with high-level SM resistance.

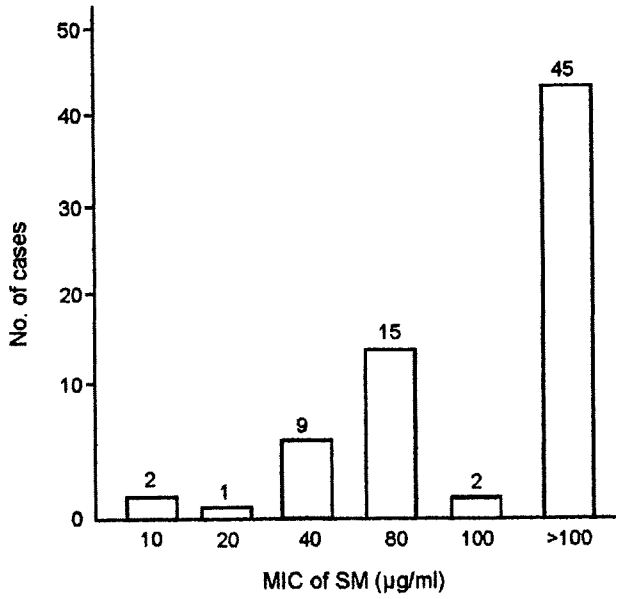


Fig. 1—Frequency of 74 SM-resistant clinical isolates at different MIC.

Table 1—Cross-resistance of 74 SM-resistant *M. tuberculosis* clinical isolates to KM and/or AMK

| Drug resistance | No. of clinical isolates |
|---|---|
| Resistance to SM | 74 |
| High-level SM resistance | 45 (61%) |
| Resistance to KM | 19 (25.75%) |
| High-level KM resistance | 15 |
| Resistance to AMK | 15 (20.3%) |
| High-level AMK resistance | 9 |
| Resistance to KM & AMK | 15 (20.3%) |
| High-level KM & AMK resistance | 9 |

SM- streptomycin; KM- kanamycin; and AMK- amikacin

Table 2—*rpsL* and *rrs* mutation of 15 SM-, KM- and AMK-resistant *M. tuberculosis* clinical isolates

| Clinical isolate No. | *rpsL* mutation | *rrs* mutation |
|---|---|---|
| 1 | codon 43 (AAG→AGG) | $^{513}$A→C |
| 2 | none | 645deletion |
| 3 | none | $^{513A}$→C |
| 4 | none | $^{513}$A→C |
| 5 | none | $^{513}$A→C |
| 6 | none | $^{513}$A→C |
| 7 | none | $^{513}$A→C |
| 8 | none | $^{516}$C→T |
| 9 | none | $^{464}$A→C |
| 10 | none | $^{906}$A→C |
| 11 | codon 43 (AAG→AGG) | $^{1400}$A→G |
| 12 | codon 43 (AAG→AGG) | $^{1401}$C→T |
| 13 | none | $^{1401}$C→T |
| 14 | none | $^{513}$A→C |
| 15 | none | $^{513}$A→C |

SM- streptomycin; KM- kanamycin; and AMK- amikacin

In the present study, it was found that MDR-TB cases were not only confined to China, but also found three SM-, KM- and AMK-resistant cases in Osaka, Japan. There were not many such cases in Japan (Personal communication with Dr. K. Tsuyuguchi, Japan).

In conclusion, it seems that there are few reports on no cross-resistance between SM and KM or SM and AMK, as long as we searched in the literature[2,5]. It is concluded that high-level SM resistance is closely linked to mutation of *rrs*, which is a target gene of KM and AMK, whereas low-level SM is linked to *rpsL* mutation.

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# Why does tuberculosis lead to specific inflammation ?

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Key words : genome, immunity, *M.tuberculosis*, specific inflammation, virulence

When *Mycobacterium tuberculosis* infects humans, about 20% of those infected actually develop tuberculosis (TB) [1]. In Japan, the incidence of TB in 2008 was 24,760 cases (19.4/100,000 persons) and the rate has been decreasing gradually, but is still higher than in the USA, Holland, and Belgium, for example. Histologically, tuberculosis displays exudative inflammation, proliferative inflammation and productive inflammation depending on the time course. In productive inflammation, granulomatous lesions with necrotic centers are formed. The typical granulomas consist of epithelioid macrophages, Langhans' multinucleated giant cells, lymphocytes and fibroblasts, and the process of their formation involves many cytokines, chemokines and transcription factors. These findings have been derived primarily from animal experiments utilizing an airborne infection apparatus. The conditions for airborne infection have been described in detail elsewhere [2]. This mini-review focuses on what has been found through animal experiments, and also indicates areas for which data are not currently available.

## 1. Inflammatory process of tuberculosis

Using animal experiments and an inhalation exposure system, the pathologic condition of the infected animals was followed up for one year. Exudative inflammation was observed for the first 10 days. Thereafter, granulomas, which corresponded to foci of proliferative inflammation, were formed. Cavity formation was not recognized in animal tuberculosis, except for rabbits [3].

## 2. Stages of experimental tuberculosis

Dr. Arthur Dannenberg was one of the pioneers of experimental tuberculosis research.

Using rabbit models, he described the pathology of tuberculosis in detail [3]. There are five stages: onset, symbiosis, early stages of caseous necrosis, interplay of cell-mediated immunity and tissue-damaging delayed-type hypersensitivity, and liquefaction and cavity formation. In stage 1, tubercle bacilli are usually destroyed or inhibited

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by the mature resident alveolar macrophages that ingest them. If bacilli are not destroyed, they grow and eventually destroy the alveolar macrophages. In stage 2, bacilli grow logarithmically within the immature nonactivated macrophages. These macrophages enter a tubercle from the bloodstream. This stage is termed symbiosis because bacilli multiply locally without apparent damage to the host, and macrophages accumulate and divide. In stage 3, the stage at which caseous necrosis first occurs, the number of viable bacilli becomes stationary because their growth is inhibited by the immune response to tuberculin-like antigens released from bacilli. Stage 4 is the stage that usually determines whether the disease becomes clinically apparent. Cell-mediated immunity plays a major role in this situation. The cytotoxic delayed-type hypersensitivity immune response kills these macrophages, causing enlargement of the caseous center and progression of the disease. If good cell-mediated immunity develops, a mantle of highly activated macrophages surrounds the caseous necrosis. In stage 5, bacilli evade host defenses. When liquefaction of the caseous center occurs, the bacilli multiply extracellularly, frequently attaining very large numbers. The high local concentration of tuberculin-like products derived from these bacilli causes a tissue-damaging delayed-type hypersensitivity response that erodes the bronchial wall, forming a cavity. Liquefaction and cavity formation do not occur at all in mice and rats.

## 3. Immune system involvement in experimental tuberculosis

T cells can be divided into two subsets, Th1 and Th2, on the basis of the cytokines they produce. In tuberculosis, Th1 plays a major role in defense against tuberculosis. Th1 cells suppress Th2 cells, and IFN-$\gamma$ down-regulates Th2 responses. We do not know the role played by B cells in tuberculosis. When activated, B cells increase the production of IFN-$\gamma$ by NK cells, and via antibody-dependent cell-mediated cytotoxicity (ADCC) they confer on NK cells the specific function of killing bacilli-laden macrophages. The exact role of CD25 T cells and $\gamma/\delta$ T cells remains unknown because the cell populations in the granulomatous lesions are very low. As long as NK T cell knockout mice are used, NK T cells do not play a major role in defense against TB [4].

## 4. Role of neutrophils in the initial phase of tuberculosis

The role of neutrophils in the development of tuberculosis remained unknown for a long time. We utilized lipopolysaccharide (LPS)-induced transient neutrophilia in the lungs. LPS (50$\mu$g/ml) was administered intratracheally to male Fischer rats [5], which were then infected with *M. tuberculosis* via an airborne route. Intratracheal injection of LPS significantly blocked the development of pulmonary granulomas and significantly reduced the number of pulmonary colony-forming units (CFU). Treatment with amphotericin B (an LPS inhibitor) or neutralizing anti-rat neutrophil antibody reversed the development of pulmonary lesions. LPS-induced transient neutrophilia prevented early mycobacterial infection. The timing of LPS administration was important. When given intratracheally at least 10 days after aerial infection, LPS did not prevent the development of tuberculosis. Neutrophils obtained by bronchoalveolar lavage killed *M. tuberculosis* bacilli. These results indicate clearly that neutrophils participate actively in defense against early-phase tuberculosis.

## 5. Characteristics of Langhans' multinucleated giant cells

When we infected IFN-γ knockout mice with avirulent H37Ra or BCG Pasteur, we found multinucleated giant cells in the granulomatous lesions. The lesions also contained tubercle bacilli and consisted of multinucleated cell clusters, being immunopositive with anti-Mac-3 antibody [6]. We subsequently infected many knockout mice with *M. tuberculosis*, but no Langhans' multinucleated giant cells were recognized. Thus it appears that formation of multinucleated giant cells requires optimal combinations and concentrations of various cytokines, and the level of IFN-γ, at least, has to be significantly low.

## 6. Significance of IFN-γ and TNF-α in protection against tuberculosis

The technique of gene targeting (knockout) has swept through biomedical research. IFN-γ, TNF-α, IRF-1, NF-IL6, NF-κB p50, STAT 1 and STAT 4 knockout mice succumbed to *M. tuberculosis* infection over time. There appears to be a cytokine and transcription factor hierarchy in experimental tuberculosis. The results indicate that these molecules play major roles in defense against the disease, IFN-γ and TNF-α being the leading players in this respect [7]. Fig. 1 shows the cytokine hierarchy in experimental tuberculosis.

## 7. Nitric oxide (NO) and apoptosis in tuberculosis

It is generally believed that nitric oxide (NO) plays a leading role in the killing of tubercle bacilli. In my experience, this is so in mouse and rat tuberculosis. However, it is ucertain whether NO produced by human mononuclear phagocytes can also kill tubercle bacilli. NO is regulated by inducible NO synthase (iNOS), and it has been reported that macrophages in the lungs of individuals with clinically active *M. tuberculosis* infection often express catalytically competent iNOS [8]. Of course, that does not exclude the possibility that other yet unknown molecules may be responsible for tubercle bacilli killing.

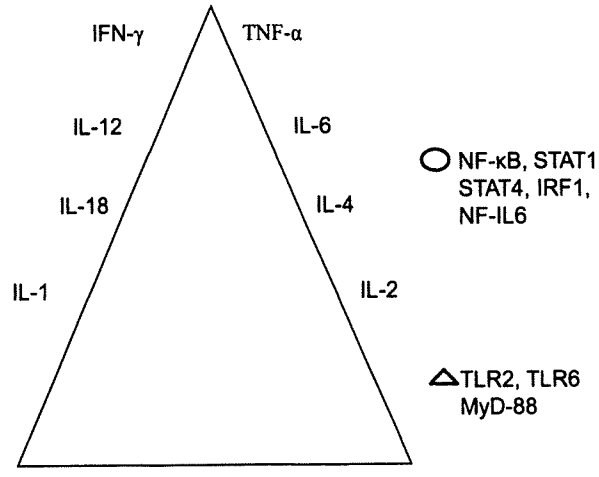


Fig. 1 Cytokine hierarchy in experimental tuberculosis.
IFN-γ and TNF-α are "grand champions" (*yokozuna* in Japanese) in defense against tuberculosis.
○ : important molecules, △ : less impotant molecules.

## 8. Alveolar macrophages in tuberculosis development

When tubercle bacilli reach alveoli, they are phagocytosed by resident alveolar macrophages. Although tubercle bacilli are killed by alveolar macrophages, tubercle bacilli can also kill macrophages through an apoptotic process. What is the fate of tubercle bacilli once they enter the phagosomes of macrophages? We collected alveolar macrophages by bronchoalveolar lavage (BAL) from aerially infected guinea pigs. At 12 days after infection, one out of about 10,000 alveolar macrophages of various sizes contained many tubercle bacilli. This indicates that certain alveolar macrophages permit *M. tuberculosis* to replicate in the phagosomes, although most of the tubercle bacilli are killed by activated alveolar macrophages. It would be very interesting to examine the survival mechanism of *M. tuberculosis* at the single-cell level, but we still do not know why macrophages targeted by tubercle bacilli cannot kill bacilli. It is also known that macrophages possess an autophagy mechanism for removal of old organelles, in this case old infected phagosomes [9]. Autophagic pathways can overcome the trafficking block imposed by *M. tuberculosis*.

## 9. Exogenous reinfection and reactivation in tuberculosis

Two different types of TB infection are recognized among elderly persons: exogenous reinfection, and reactivation of the same latent tubercle bacilli. As TB is still prevalent in Japan (19.4/100,000 persons), reactivation is common in elderly persons with the disease. However, it is not easy to demonstrate, because obviously it is not possible to obtain samples of *M. tuberculosis* isolated 20 or 30 years previously from the same patients. In America most researchers believe that exogenous reinfection is common because the prevalence rate of TB is so low.

Tubercle bacilli remain intact in macrophages for a long time without manifestation of TB. It is therefore important to understand the immunological aspects of TB pathogenesis under conditions of such dormancy. Latent tuberculosis is currently a topic of active discussion. There are few data on the mechanisms of latent tuberculosis in humans, but an understanding of them is very important in terms of pathogenesis and chemotherapy. As a first step, we used GFP-H37Rv *M. tuberculosis* to establish latent tuberculosis in guinea pigs, which are among the best animals for creating models of tuberculosis. We inoculated the guinea pigs subcutaneously with 100 or 1,000 CFU of tubercle bacilli. During the 300-day follow-up period there were no clinical signs of disease. Upon necropsy no macroscopic lesions were recognized, but histopathological examination of major organs revealed microgranulomas consisting of epithelioid macrophages and lymphocytes without central necrosis. Interestingly, photon imaging visualized granulomatous lesions corresponding to these histologically apparent microgranulomas. Tuberculin skin testing of infected guinea pigs showed strong positivity ($\geq$10 mm induration) throughout the entire experiment. Real-time PCR showed a slight increase in the expression of IFN-$\gamma$, TNF-$\alpha$, IL-12, and iNOS mRNAs. Our guinea pig model should be useful for examining the pathogenesis and molecular mechanisms of latent tuberculosis, as well as for monitoring the results of chemotherapy [10].

## 10. Present status of tuberculosis vaccine

The efficacy of BCG against adult pulmonary tuberculosis still remains controversial. However, it is not so easy to find *M. tuberculosis*-derived immunogenic antigens suitable for TB vaccines because only a few such antigens have been detected so far. Several TB vaccines are currently being tested using various models [11, 12]. These include recombinant BCG vaccine expressing Ag85A, Ag85B, recombinant modified vaccinia virus Ankara expressing Ag85A, TB polyprotein vaccine, Mtb72f, ESAT-6 subunit vaccine, auxotrophic vaccines for TB, and recombinant BCG overexpressing major extracellular proteins (rBCG30). Several promising TB vaccine candidates may become available after verification in monkey studies. We propose conducting a global multicenter study of the TB vaccine thus chosen using the same experimental protocols, and selecting a promising one on the basis of consensus.

## 11. DNA genome of *M. tuberculosis*

The genome of *M. tuberculosis* H37Rv has been sequenced [13], and the length of the DNA is reported to be 4.04 Mbp. It was hoped that this would allow complete clarification of the functions of *M. tuberculosis*, but this still remains a distant goal. Seven percent of the genome encodes proteins of unknown function and 26% encodes conserved hypothetical proteins. At present, there are not many proteins that are immunogenic and thus suitable for TB vaccine design. Another important point is that *M. tuberculosis* does not possess toxins. This is in sharp contrast to *Listeria monocytogenes* (a type of intracellular pathogen), which possesses listeriatoxin. Different protein antigens may be clarified in the near future.

## 12. Cell wall components of *M. tuberculosis*

The cell wall of *M. tuberculosis* consists of lipid and sugar moieties. As *M. tuberculosis* induces granulomatous lesions, we have tried to find granulomatogenic substances derived from tubercle bacilli. When four kinds of mycolates derived from *M. tuberculosis* Aoyama B strain were introduced into the lungs via an airborne route, only trehalose 6,6'-dimycolate (TDM) and methyl ketomycolate induced pulmonary granulomas without central necrosis. The pulmonary granulomas consisted of epithelioid macrophages and T lymphocytes. Although these substances hardly induce immune responses, they are granulomatogenic [14].

## 13. Tuberculosis and high-risk factors

There are many high-risk factors for TB, including HIV, malnutrition, aging and poverty. Diabetes mellitus (DM) is also one such factor. Although there is a clinical association between DM and tuberculosis, no experimental evidence for this association exists. We attempted to clarify whether type 1 diabetic (KDP) and Goto Kakizaki type 2 diabetic rats were more susceptible to *M. tuberculosis* than non-diabetic wild-type (WT) rats. The infected diabetic rats developed large granulomas without central necrosis in their lungs, liver or spleen. This was consistent with a significant increase in the number of CFU of *M. tuberculosis* in the lungs and spleen ($p<0.01$). Insulin treatment resulted in significant reduction of tubercle bacilli in the infected KDP rats ($p<0.01$). Pulmonary IFN-$\gamma$, TNF-$\alpha$ and IL-1 $\beta$ mRNA levels were higher in the infected diabetic rats than

in WT rats. Alveolar macrophages from KDP rats were not fully activated by *M. tuberculosis* infection because they did not secrete nitric oxide (NO) that can kill *M. tuberculosis* ($p<0.01$). However, there was no significant difference in phagocytosis of tubercle bacilli by alveolar macrophages between KDP and WT rats. Taken together, it is appears that type 1 diabetic (KDP) and GK rats are more susceptible to *M. tuberculosis* that WT rats [15, 16]. Hypercholesterolemia and hyperlipidemia were not observed in these rats. We are now investigating the relationship between DM and TB exacerbation at TB specialist hospitals.

## 14. Concluding remarks

It now seems appropriate to point out several reasons why tuberculosis belongs to the category of specific inflammation. First, as mentioned above, *M. tuberculosis* does not possess toxin, while another typical intracellular pathogen, *Listeria monocytogenes*, does. Most would consider that toxins are responsible for acute inflammation. Second, the cell wall of *M. tuberculosis* consists of various sugars, polysaccharides and lipids. Generally, immune responses induced by these moieties are low, but some mycolic acid derivatives (trehalose dimycolate and methyl ketomycolate) are granulomatogenic factors. Lastly, the doubling time of *M. tuberculosis* is around 20-24 h, and it replicates very slowly, surviving in phagosomes of specific alveolar macrophages for a long time. We are currently planning a project to examine why *M. tuberculosis* replicates in certain alveolar macrophages at the single-cell level, a phenomenon that we have termed the death escape mechanism. It would also be interesting to examine the process (termed autophagy) by which infected macrophages remove old infected phagosomes.

Mycobacterial research is one of a number of fascinating areas that challenge us as professional researchers. I hope that the next generation of researchers will tackle many of the issues that still remain unresolved.

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