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Recombinant BCG Tokyo (Ag85A) protects cynomolgus monkeys (*Macaca fascicularis*) infected with H37Rv *Mycobacterium tuberculosis*

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Summary
One tuberculosis vaccine candidate that has shown a promising degree of protective efficacy in guinea pigs is recombinant BCG Tokyo (Ag85A)(rBCG-Ag85A[Tokyo]). As a next step, cynomolgus monkeys were utilized because they are susceptible to *Mycobacterium tuberculosis* and develop a continuous course of infection that resembles that in humans both clinically and pathologically. The recombinant BCG vaccine was administered once intradermally in the back skin to three groups of cynomolgus monkeys, and its protective efficacy was compared for 4 months with that of its parental BCG Tokyo strain. Vaccination of the monkeys with the rBCG-Ag85A[Tokyo] resulted in a reduction of tubercle bacilli CFU ($p<0.01$) and lung pathology in animals challenged intratracheally with 3000 CFU H37Rv *M. tuberculosis*. Vaccination prevented an increase in the old tuberculin test after challenge with *M. tuberculosis* and reaction of *M. tuberculosis*-derived antigen. Thus, it was shown in monkeys that rBCG-Ag85A[Tokyo] induced higher protective efficacy than BCG Tokyo. This warrants further clinical evaluation.

Introduction

Tuberculosis (TB) still remains a major health threat affecting millions of people worldwide. The only TB vaccine currently available is *Mycobacterium bovis* BCG. However, the efficacy of BCG against adult pulmonary TB still remains controversial.[1–4] Thus, development of a better TB vaccine is urgently required to counteract the global threat of TB.

We have previously reported the protective efficacy of a TB DNA vaccine (Ag85A) and a recombinant strain BCG Tokyo (Ag85A) in small-animal models challenged with *M. tuberculosis* Kurono strain.[5,6] We found that recombinant BCG

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Tokyo was better than Ag85A DNA in terms of protective efficacy against *M. tuberculosis*.[2] The spleen tissues from guinea pigs vaccinated with rBCG-Ag85A[Tokyo] or Ag85A DNA expressed IFN-γ and IL-2 mRNA at significantly high levels.[6] This finding prompted us to explore further the efficacy of rBCG-Ag85A[Tokyo] in cynomolgus monkeys. We chose cynomolgus monkeys because this animal is reportedly protected more efficiently than rhesus monkeys by BCG vaccination.[7] Previous studies have shown that whereas the rhesus macaque is highly susceptible to *M. tuberculosis*, the closely related cynomolgus macaque is more resistant.[8–10] Cynomolgus monkeys are more efficiently protected by BCG vaccination than rhesus monkeys and therefore afford a good experimental model for the evaluation of new TB vaccine candidates.

Several TB vaccines are currently being tested using various models[11,16–19] and several recent reviews on TB vaccines have been published.[12–15] These include recombinant BCG vaccine expressing 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). However, there have been few reports on the efficacy of TB vaccine candidates in cynomolgus monkey models due to lower availability of monkey P3 facilities. Vaccination of cynomolgus monkeys with Ag85B-ESAT-6 reportedly induces protective immune responses.[20] DNA vaccine (HSP65+IL-12/HVJ) as well as 72f recombinant BCG provide better protective efficacy in cynomolgus monkeys.[21] In order to find a better TB vaccine, it is progression to the primate model after positive results in the small animal models. In the present investigation, we examined the efficacy of rBCG-Ag85A[Tokyo] in cynomolgus monkeys, and found that it induced higher protective efficacy than BCG Tokyo.

Materials and methods

Construction of recombinant BCG Tokyo (rBCG-Ag85A[Tokyo])

The Ag85A gene was amplified by PCR and subcloned into the pCR4 vector. The presence of the Ag85A gene was then confirmed by DNA sequencing. The gene was inserted into the pBBN vector (Ag85A-HA) possessing a hemagglutinin (HA) tag at its 5′ end. At this stage, the Ag85A-HA was expressed in *Escherichia coli*, and then the Ag85A-HA gene was introduced into the downstream region of the pHPS integration vector. The vector was then electroporated into BCG Tokyo. The resulting transformants (rBCG-Ag85A[Tokyo]) were cultured individually and the content of the extracted lysate that contains Ag85 protein was confirmed by western blotting.[6]

Bacterial strain

M. tuberculosis H37Rv (ATCC 25618) was passed through mice and grown once in 7H9 liquid medium before titration and storage in aliquots at −85 °C. The culture strain was filtered through a membrane filter (4-μm pore size; Millipore, Bedford, MA, USA) before use to ensure even dispersal.

Monkeys

A total of 18 cynomolgus male monkeys (*Macaca fascicularis*) (5–7 kg, 6–8 years old) were used. All animals were housed at the animal biosafety level (ABSL) 3 facility of Wuhan University, Wuhan, China. The animals were studied in groups of six. Before the start of the studies, all animals were examined clinically and radiologically, and tuberculin skin-tested. For intratracheal challenge, animals were anesthetized with ketamine. Prior to commencement, experiments were reviewed and approved by the Wuhan University ethics committee.

Inoculation of monkeys

The monkeys were randomly assigned to three groups. Group 1 (6 monkeys) received one intradermal injection of 2×10^6 CFU/ml rBCG-Ag85A[Tokyo]. Group 2 received one intradermal injection of 2×10^6 CFU/ml BCG Tokyo. Group 3 comprised 6 unvaccinated monkeys that received physiological saline as a control.

Intratracheal infection of monkeys

Eight weeks after vaccination, the animals were challenged by intratracheal instillation of 1 ml (3000 CFU) of H37Rv *M. tuberculosis*. All animals were challenged on the same day with the same preparation, and were then observed for 4 months after infection. As PPD did not give better positive results to the monkeys, old tuberculin was used. The old tuberculin test (Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan) was carried out 1, 2 and 3 months after infection. Briefly, 0.1 ml of old tuberculin solution was injected intradermally into the left palpebral skin and 0.1 ml saline was injected intradermally into the right palpebral skin. Two days later, swelling and redness on both sides were compared. When the diameter of redness is more than 10 mm, it was judged as +, and when more than 11 mm, it was judged as 2+. All animals were housed in animal biosafety level (ABSL) 3 facilities.

Animal care

After infection, animals were observed daily by the animal caretakers for changes in behavior, eating and coughing. Weight, erythrocyte sedimentation rate (ESR) and temperature were recorded at times of blood sampling. Body temperature was measured rectally.

Immunological examination

Blood from the femoral vein was used to obtain serum. TB Dot assay (Shanghai Upper Biotech and Pharma Co., Shanghai, China) was carried out according to the instruction sheet provided by the manufacturer. Briefly, two drops of blocking buffer were spotted on a TB blot membrane previously coated with 38-kDa *M. tuberculosis*-derived

antigen.[22] Then, 40 μl serum was added and semi-dried. Thereafter, six drops of washing buffer were added and semi-dried. Then, two drops of gold-labeled anti-human antibody solution were added and semi-dried. Finally, six drops of washing buffer were added and allowed to dry completely. When a reddish spot appeared, it was judged as positive and when no reddish spot appeared, it was judged as negative.

Bacterial enumeration

Just after death of the unvaccinated monkeys, 10 pieces from the upper and lower lobes of lungs, and also spleen tissue, about 0.5 cm^3 in size were taken randomly. For the vaccinated monkeys, similar samples were taken randomly at necropsy. After being weighed, the samples were combined, homogenized and diluted with physiological saline. For the vaccinated groups, pyrazinamide (200 μg/20 μl) was added to determine BCG Tokyo-derived colonies (background count). Pyrazinamide kills *M. tuberculosis*, but does not kill BCG Tokyo. Triplicate 10-fold dilutions were incubated for 4 weeks in 1% Ogawa solid slant agar and the number of colonies was counted. To examine *M. tuberculosis*-derived colonies, the background count was subtracted from the number of colonies. The lung and spleen tissues were weighed and the results were expressed as CFU ± SD/whole organ.

Histopathology

Necropsies were undertaken on unvaccinated monkeys just after death and on vaccinated monkeys after euthanasia. The removed organs were fixed with 15% formalin for 10 days. Tissue sections from paraffin blocks containing lung, spleen, hilar lymph nodes and liver were stained with hematoxylin and eosin or the Ziehl–Neelsen method for acid-fast bacilli. The severity of pulmonary lesions was judged independently by two experts (I.S. and T.U.).

Statistical analysis

We performed analysis of variance (ANOVA) for repeated measurements using the baseline results at screening as a covariate on log-transformed data to compare between groups.

Results

Clinical course

The monkeys vaccinated with rBCG-Ag85A[Tokyo] or parental BCG Tokyo and their non-vaccinated controls were infected intratracheally with H37Rv *M. tuberculosis*. No coughing was observed in the animals after challenge. None of the vaccinated animals gained weight during the infection period. Their ESR was within the normal range (1–2 mm/h). None of the non-vaccinated animals showed an appreciable increase in body weight. Two of the monkeys (17 and 18) showed a gradual decrease in weight (50 g). The other four non-vaccinated control animals showed a severe decrease in weight of 550–800 g, and their ESR was higher than the reference value (55, 10, 8 and 10 mm/h)(Table 1).

On radiographs of the chest, the non-vaccinated animals exhibited early development of multilobar pneumonia in the right lung and rapid progression to bilateral pneumonia. Lobar consolidation and atelectasis in the involved lungs and hilar lymphadenopathy were observed frequently in the non-vaccinated groups (Table 1).

In the vaccinated groups, pneumonia was slight to mild (Figure 1).

Immunological responses

Two immunological methods (the old tuberculin test and serum TB diagnosis) were utilized to clarify the severity of *M. tuberculosis* infection. In the vaccinated monkeys 2 months after infection, the old tuberculin test gave a positive result (+). In the non-vaccinated animals, the reaction was strongly positive (2+) and marked palpebral reddish skin swelling was observed.

The TB dot assay gave a negative result in all vaccinated and non-vaccinated animals 1 month after infection. However, 2 months after infection, the result was positive in the vaccinated monkeys, and strongly positive (2+) in the non-vaccinated monkeys (Table 1).

Gross pathology and histopathology of the vaccinated and non-vaccinated monkeys

At necropsy, all unvaccinated animals showed extensive bilateral lung pathology characterized by the presence of multiple granulomas. These granulomas showed conglomeration to larger caseous areas, especially in the hilar region. Granulomas were also present in the liver and spleen. In the vaccinated animals, a few small granulomas were evident, but these showed no caseous changes. Small liver granulomas were noted in two of the BCG Tokyo-vaccinated monkeys, but such granulomas were not observed in the recombinant BCG-vaccinated monkeys. Four of the non-vaccinated monkeys died of advanced TB 50, 67, 70 and 84 days after infection (Figure 2). These were necropsied just after death for further examination.

On microscopic examination, the non-vaccinated animals showed multifocal, coalescing granulomas with central necrosis and pronounced cellular infiltrates in the periphery (Figure 3). The vaccinated animals showed markedly less severe histopathology. In particular, the peripheral inflammatory cell infiltration was notably more pronounced in the unvaccinated than in the vaccinated animals. Histological examination of the animals that had received the recombinant BCG (Ag85A) showed almost normal lung tissue without granulomas in five of them. The remaining vaccinated animal showed a solitary small granuloma without central necrosis. Two BCG Tokyo-vaccinated animals showed a single small granuloma (Table 1).

Replication of tubercle bacilli in the lung and spleen tissues of vaccinated and non-vaccinated monkeys

At autopsy, 10 different pieces of lung and spleen tissue were taken for determination of CFU. Background culture

Table 1 Summary of the monkey experiments.

Monkey no.	Vaccination	Gross pathology	Lung histopathology	ESR (mm/h, 2 months)	Old tuberculin (2 months)	Serum diagnosis (1 month, 2 months)	Chest X-ray before death or necropsy
1	rec BCG	Lung, spleen, LN	Solitary granuloma	2	+	−, +	Slight pneumonia
2	rec BCG	Lung, spleen, LN	Pneumonia	1	+	−, +	Slight pneumonia
3	rec BCG	Lung, spleen, LN	Pneumonia	1	+	−, +	Slight pneumonia
4	rec BCG	Lung, spleen, LN	Pneumonia	1	+	−, +	Slight pneumonia
5	rec BCG	Lung, spleen, LN	Pneumonia	1	+	−, +	Slight pneumonia
6	rec BCG	Lung, spleen, LN	Pneumonia	1	+	−, +	Slight pneumonia
7	BCG Tokyo	Lung, spleen, LN	Pneumonia	1	+	−, +	Slight pneumonia
8	BCG Tokyo	Lung, spleen, LN	Pneumonia	1	+	−, +	Slight pneumonia
9	BCG Tokyo	Lung, spleen, LN	Pneumonia	1	+	−, +	Slight pneumonia
10	BCG Tokyo	Lung, spleen, LN, liver	Solitary granuloma	3	+	−, +	Slight pneumonia
11	BCG Tokyo	Lung, spleen, LN	Pneumonia	1	+	−, +	Slight pneumonia
12	BCG Tokyo	Lung, spleen, LN, liver	Solitary granuloma	2	+	−, +	Slight pneumonia
13	Saline	Lung, spleen, LN, liver	Miliary tuberculosis	55	2+	−, 2+	Severe pneumonia, consolidation
14	Saline	Lung, spleen, LN, liver	Miliary tuberculosis	10	2+	−, 2+	Severe pneumonia, consolidation
15	Saline	Lung, spleen, LN, liver	Miliary tuberculosis	8	2+	−, 2+	Severe pneumonia, consolidation
16	Saline	Lung, spleen, LN, liver	Miliary tuberculosis	10	2+	−, 2+	Severe pneumonia, consolidation
17	Saline	Lung, spleen, LN, liver	Miliary tuberculosis	5	2+	−, 2+	Severe pneumonia, consolidation
18	Saline	Lung, spleen, LN, liver	Miliary tuberculosis	6	2+	−, 2+	Severe pneumonia, consolidation

Criteria of old tuberculin test and serum diagnosis: +, positive; and 2+, strongly positive. rec BCG; recombinant BCG.

CFU of BCG Tokyo or rBCG-Ag85A[Tokyo] after pyrazinamide was added to the tissue homogenates was one or two. The lung tissue of animals vaccinated with recombinant BCG showed a significant 1000-fold decrease in the number of bacteria compared to the non-vaccinated animals ($p<0.01$). The number of CFU in BCG Tokyo-vaccinated animals after 16 weeks of infection was reduced 100-fold relative to that in the non-vaccinated animals ($p<0.01$). There was a statistically significant difference in the number of pulmonary CFU between recombinant BCG-vaccinated and BCG Tokyo-vaccinated animals ($p<0.01$) (Figure 4).

A similar tendency was also observed in the number of splenic CFU. The spleen tissues of animals vaccinated with recombinant BCG or BCG Tokyo showed a significant 1000-fold decrease in the number of bacteria compared to the non-vaccinated animals ($p<0.01$). However, there was no significant difference in the number of splenic CFU between recombinant BCG-vaccinated and BCG-vaccinated animals.

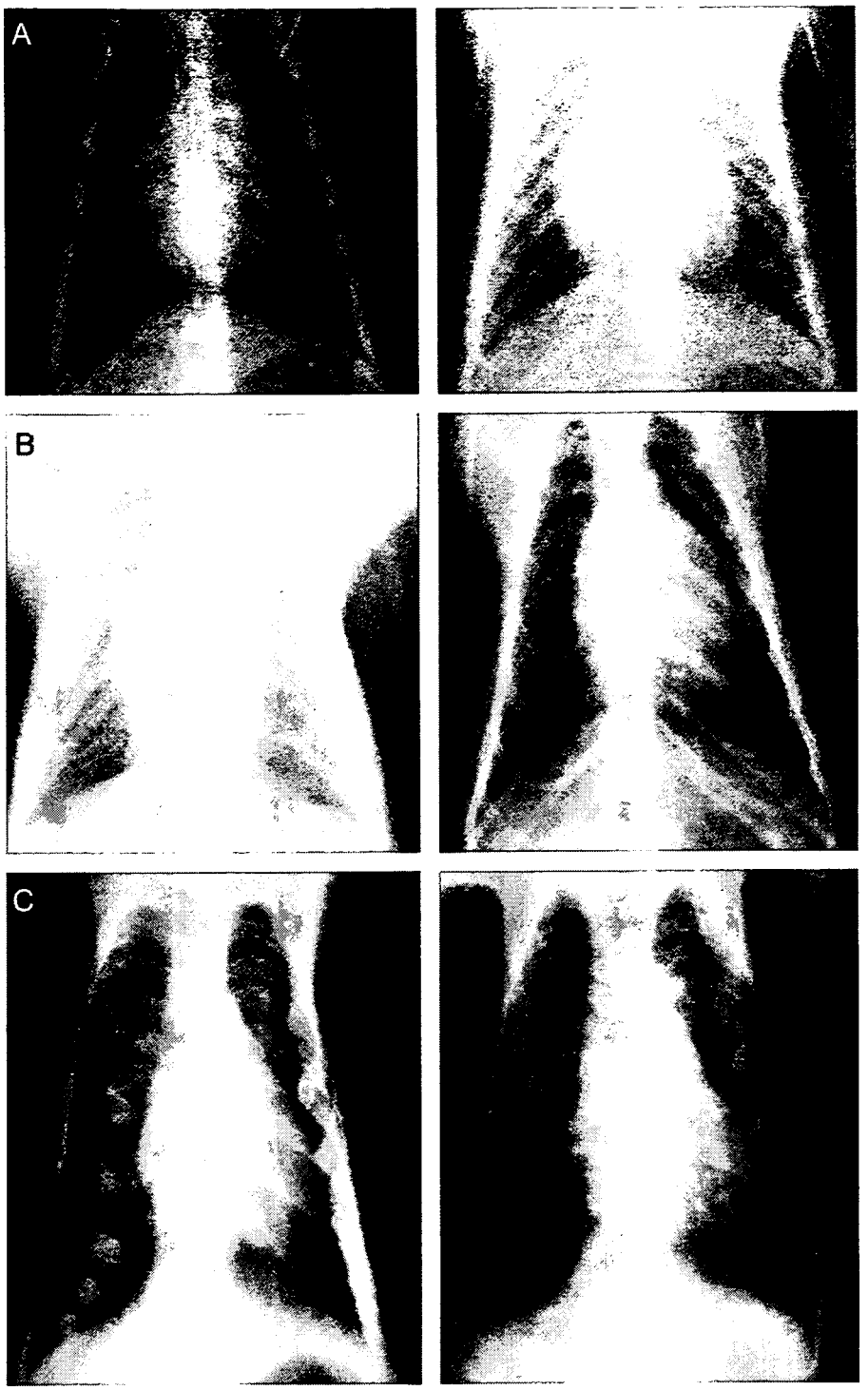

Figure 1 Chest radiologic examinations after challenge with H37Rv *M. tuberculosis*. (A) The monkey (No. 2) vaccinated with rBCG-Ag86A[Tokyo]. The chest X-ray picture was taken 1 day before necropsy. (B) The monkey (No. 9) vaccinated with parental BCG Tokyo. The chest X-ray picture was taken 1 day before necropsy. (C) The non-vaccinated monkey (No. 13) 2 days before death. After H37Rv challenge, the non-vaccinated monkeys rapidly developed extensive bronchopneumonia. Many nodular shadows (→) were recognized, but the vaccinated monkeys had negative chest X-ray findings 3 months after H37Rv challenge.

Discussion

In the present study, we have demonstrated that vaccination of cynomolgus monkeys with recombinant BCG (Ag85A)(rBCG-Ag85A[Tokyo]) induces protection against infection with H37Rv *M. tuberculosis*. In addition to measurement of protection in terms of reduction in bacterial number and/or lung pathology, we have also shown that recombinant BCG vaccination prevented the development of a number of important clinical and

immunological changes during infection. These changes included an increase of the ESR and the development of strong immune responses to a wide spectrum of mycobacterial antigens (old tuberculin). When we inoculated monkeys once with 2×10^6 CFU rBCG-Ag85A[Tokyo], there was a significant reduction of CFU in lung and spleen tissues compared to that in BCG Tokyo-inoculated monkeys. We showed for the first time that the H37Rv strain could also be used for intratracheal infection instead of the Erdman strain. Many researchers use the Erdman strain (1000 CFU or more) for optimal intratracheal infection.[10,16,17] We chose 3000 CFU as the dose for the H37Rv strain because it is less virulent than Erdman strain, and we were recommended to use H37Rv instead of the Erdman strain at the Animal Biosafety Level 3 Facility of Wuhan University.

When parental BCG Tokyo was used for vaccination, we found several grayish tubercles in the liver in two of six monkeys, but no such tubercles were evident in monkeys vaccinated with recombinant BCG. Moreover, there was a significantly lower number of CFU in lung tissues of monkeys vaccinated with recombinant BCG than in monkeys vaccinated with BCG Tokyo ($p<0.01$). Taken together, the results suggest that the recombinant BCG bearing the introduced Ag85A gene gives better protective efficacy than BCG Tokyo. However, to evaluate the efficacy of the Ag85A antigen carefully, it will be necessary to lower the dose of recombinant BCG because 2×10^6 CFU BCG Tokyo alone is still sufficiently effective.

TB dot assay, which targets the 38-kDa antigen from *M. tuberculosis*, gave a negative result 1 month after infection, but a positive one 2 months after infection. Therefore, care is needed when diagnosing TB in the early phase. The old tuberculin test may be more useful for early-phase TB diagnosis.

We selected Ag85A as a promising immunogen because the protein from *M. tuberculosis* induces significant humoral

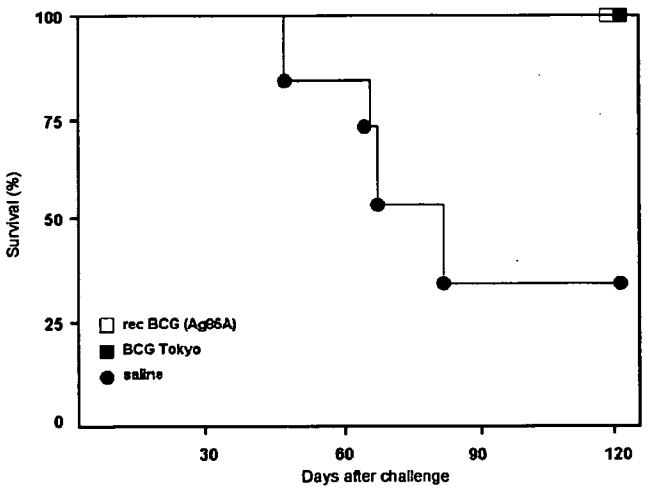

Figure 2 Mortality curve after challenge. Animals in groups of six were challenged by intratracheal inoculation with 3000 CFU of H37Rv. Non-vaccinated animals (six per group) were inoculated with saline.

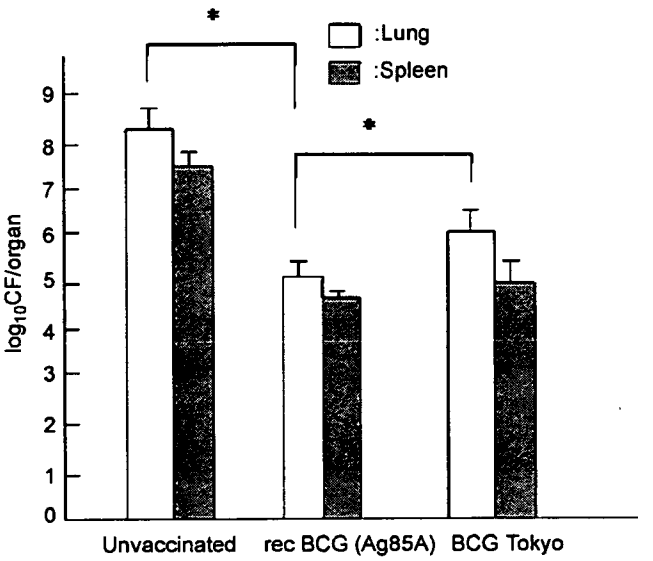

Figure 4 CFU counts in lung and spleen tissues of H37Rv *M. tuberculosis*-infected cynomolgus monkeys vaccinated with recombinant BCG Tokyo (Ag85A), and in non-vaccinated controls. *Statistical difference at $p<0.01$.

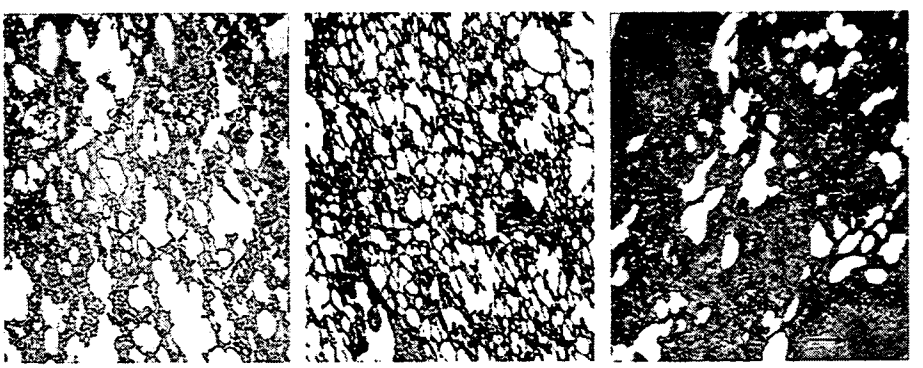

Figure 3 Histopathology of lung tissues from *M. tuberculosis*-infected monkeys vaccinated with rBCG-Ag85A[Tokyo] (A), BCG Tokyo (B) or non-vaccinated controls (C) at necropsy. The miliary granulomas with caseating necrosis are surrounded peripherally by a dense infiltrate of epithelioid cells and lymphocytes (C), but in the vaccinated monkeys interstitial pneumonia is evident (A and B) × 100. Hematoxylin and eosin stain.

and cell-mediated immune responses.[23,24] The expression levels of IFN-γ and IL-2 mRNAs were increased in spleen tissues from guinea pigs that had been vaccinated with parental BCG Tokyo, rBCG-Ag85A[Tokyo], and Ag85A DNA vaccine. Among them, the expression levels of IFN-γ and IL-2 mRNAs were the highest in rBCG-Ag85A[Tokyo].[6] Furthermore, the sera from the rBCG-Ag85A[Tokyo]-vaccinated guinea pigs reacted with Ag85A peptide we used in our previous study significantly (data not shown). We have shown previously that vaccination with Ag85A DNA twice by gene gun bombardment or with rBCG-Ag85A[Tokyo] once significantly reduced the severity of pulmonary pathology and the number of CFU in guinea pigs.[5,6] When the immunogenic synthetic Ag85A peptide was further used as a booster together with recombinant BCG (Ag85A), lung pathology was improved significantly, together with a significant reduction in the number of pulmonary CFU.[6] Although a single intradermal inoculation of 2×10^6 CFU BCG (Ag85A) was enough to induce protective efficacy in the present study, it would be desirable to use Ag85A peptide as a booster, Ag85B-ESAT-6 fusion protein and 72f fusion protein in combination with recombinant BCG Tokyo (Ag85A) to achieve much better protective efficacy.[6,18]

In summary, we have shown that vaccination of primates with rBCG-Ag85A[Tokyo] induces good protective immune responses. Using the macaque challenge model, further optimization of the dose and timing, and use of a booster, may well lead to levels of protection that are better than those achieved with BCG.

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Ethical Approval: Not required

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Lack of Correlation between *embB* Mutation and Ethambutol MIC in *Mycobacterium tuberculosis* Clinical Isolates from China▽

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Seventy-four *Mycobacterium tuberculosis* clinical isolates from China were subjected to drug susceptibility testing using ethambutol, isoniazid, rifampin, and ofloxacin. The results revealed that the presence of *embB* mutations did not correlate with ethambutol resistance but was associated with multiple-drug resistance, especially resistance to both ethambutol and rifampin.

Ethambutol (EMB), often used in combination with isoniazid, rifampin, and pyrazinamide, is a key drug of first-line antituberculosis treatment. EMB seems to exert its toxic effect by inhibiting the *embABC*-encoded proteins, and mutations in the *embB* gene appear to play a major role in the development of EMB resistance in *Mycobacterium tuberculosis* (13). The marked clinical association between *embB* codon 306 mutations and EMB resistance in *M. tuberculosis* at one time led to its proposal as a marker for EMB resistance in diagnostic tests (6, 8, 11, 15). However, discrepancies between the results of genotypic and phenotypic EMB resistance testing have raised questions about the accuracy of molecular assays based on the detection of point mutations in *embB* codon 306 for prediction of EMB resistance (3, 4, 7, 14). Hazbon et al. (3) has reported that for *embB* codon 306 mutations, there is "a novel association with broad drug resistance and IS*6110* clustering rather than ethambutol resistance."

For DNA sequencing of the *embB* gene (primer set includes forward, 5′-CGGCATGCGCCGGCTGATTC, and reverse, 5′-TCCACAGACTGGCGTCGCTG) from 141 EMB-resistant and 40 EMB-sensitive clinical isolates from Henan Province, China, ABI Prism Big Dye terminator sequencing kits were used. We found that 45.2% of EMB-resistant isolates harbored *embB* codon 306 mutations (ATG to ATA, GTG, ATT, CTG, or ATC [five types]) (12). We also found that 15% (6/40) of EMB-susceptible isolates had *embB* gene mutations (the breakpoint concentration of EMB is 2 μg/ml in L-J medium) in 2000. After a previous analysis by denaturing high-pressure liquid chromatography (DHPLC) of drug resistance genes in *M. tuberculosis* in our laboratory (9, 10), we went back to test these isolates and found that the DHPLC results for the *embB* gene were completely consistent with those of DNA sequencing. Figure 1 shows the DHPLC and DNA sequencing results for the six isolates that were EMB sensitive but harbored *embB* mutations. Results similar to those we obtained for streptomycin resistance were obtained (R. Shi, J. Zhang, C. Li, Y. Kazumi, and I. Sugawara, unpublished data). We also found that 22% (16/72 EMB-sensitive clinical isolates from an affiliated hospital of the Beijing Tuberculosis and Lung Tumor Research Institute) possessed *embB* mutations in 2006. We tested the EMB MICs for these 16 isolates, and all were less than 2 μg/ml, but the MIC for four isolates was 0.125 μg/ml (data not shown). We also tested 100 clinical isolates of *M. tuberculosis* from Henan Province, China (9 were isoniazid monoresistant, and all others were pansusceptible), and 200 clinical isolates from Fukujuji Hospital, Tokyo, Japan (7 were isoniazid monoresistant, 1 was rifampin monoresistant, and all others were pansusceptible), by the DHPLC method to screen the *embB* gene, but no mutation was found (DHPLC data not shown).

In the present study, 74 clinical isolates from the Beijing Tuberculosis and Lung Tumor Research Institute were sub-

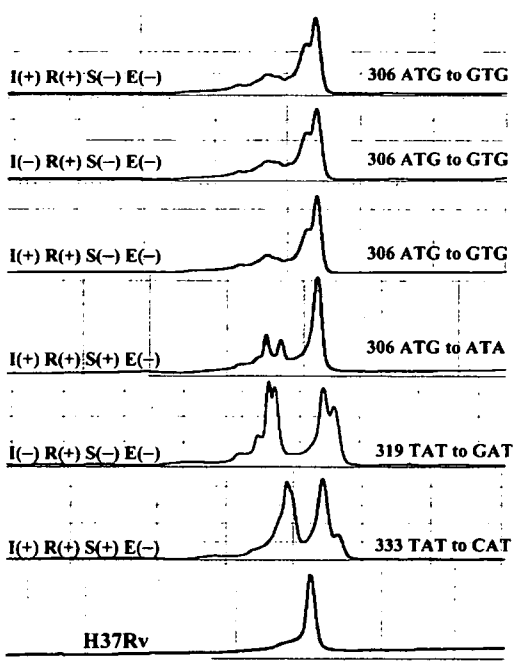

FIG. 1. *embB* DHPLC pattern of six EMB-susceptible isolates from Henan Province, China. I, isoniazid; R, rifampin; S, streptomycin; E, EMB; +, resistant; −, susceptible.

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TABLE 1. Correlation of *embB* mutation and resistance to four drugs for 74 clinical isolates from Beijing, China

EMB MIC (μg/ml)	No. of isolates with:			No. of resistant isolates at indicated drug concn (μg/ml)[a]									Total no. of isolates with *embB* mutation and resistance to other drug(s) (n = 74)
				INH			RIF			OFX			
	No *embB* mutation	*embB* mutation in codon 306	*embB* mutation at another position	≤1	1 to 10	≥10	≤50	50 to 400	≥400	≤4	4 to 16	≥16	
0.25	9	6	1	14	2	0	9	4	3	12	3	1	16
0.5	14	6	0	14	5	1	9	6	5	17	1	2	20
1	9	9	0	14	3	1	7	7	4	12	4	2	18
2	1	2	0	1	2	0	1	0	2	2	1	0	3
5	2	3	0	2	1	2	4	1	0	3	1	1	5
10	2	3	0	2	1	2	2	3	0	4	1	0	5
20	2	5	0	1	0	6	1	2	4	4	1	2	7

[a] INH, isoniazid; RIF, rifampin; OFX, ofloxacin.

jected to testing for drug susceptibility (in L-J medium) to EMB, isoniazid, rifampin, and ofloxacin, and analysis of their respective resistance genes, *embB*, *katG*, *rpoB*, and *gyrA*, was done by DHPLC and DNA sequencing (9, 10, 11). Variable-number tandem repeat analysis was also performed using 12 standard loci of mycobacterial interspersed repetitive units reported by Mazars et al. (5). Among the 74 isolates, 14 were pansusceptible and no *embB* mutations were found. When a MIC of more than 2 μg/ml was taken as a standard for EMB resistance (and the World Health Organization suggests that 2 μg/ml in L-J medium is the MIC), 17 isolates were found to be resistant, of which 65% (11/17) showed *embB* mutations, while 56% of EMB-susceptible isolates (24/43) revealed *embB* mutations. Of these latter 24 isolates, 21 were multidrug resistant, and 3 were monoresistant to rifampin at a high level. Three isoniazid-sensitive isolates were found to possess *embB* mutations, while seven isolates showing a high level of isoniazid resistance had no *embB* mutations. None of the rifampin-sensitive isolates were found to have *embB* mutations, while most of the isolates showing high rifampin resistance harbored *embB* mutations. The variable-number tandem repeat analysis results revealed that the *embB* mutations were clustered (data not shown). Table 1 shows the correlation of an *embB* mutation and resistance to four drugs at different concentrations for the 74 clinical isolates. DHPLC data and DNA sequencing results for the *katG*, *rpoB*, and *gyrA* genes are omitted. As shown in Fig. 2, *embB* mutations were distributed among isolates with susceptibility or resistance to EMBat concentrations ranging from 0.25 to 20 μg/ml, but the isolates exhibited multidrug resistance, indicating that the presence of mutations in *embB* codon 306 is not applicable for prediction of EMB resistance in clinical *M. tuberculosis* isolates. A chi-square test revealed that the presence of an *embB* mutation was strongly correlated with rifampin resistance and an increased frequency of resistance to other drugs, whereas it showed no correlation with isoniazid resistance (Table 2).

In summary, our data reveal no evidence of *embB* mutations in pansusceptible clinical isolates. An *emb* mutation was restricted to EMB-susceptible strains that were already resistant to other antituberculosis drugs. There was no strong relationship between the presence of an *embB* mutation and the EMB MIC. Our results support the findings of Hazbon et al. (3) that an *embB* mutation is strongly associated with resistance to an increased concentration of drugs. It is speculated that the development of *embB* mutations may predispose an isolate to the development of resistance to multiple antibiotics and may increase the ability of these multiple-drug-resistant clinical isolates to be transmitted between subjects. Our data also suggest that an *embB* mutation has a strong relationship to rifampin resistance but no relationship to isoniazid resistance. Although

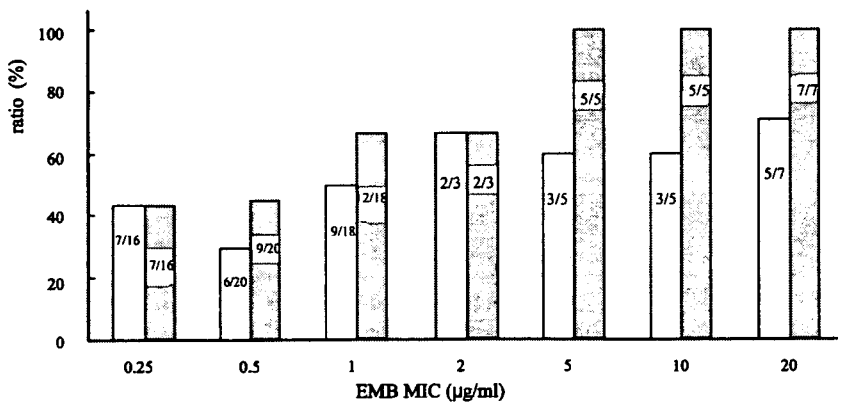

FIG. 2. Distribution of *embB* mutation rates among isolates at different EMB concentrations. White bars indicate the *embB* mutation rates, and shaded bars show the multidrug resistance rates among isolates for each EMB MIC. Numbers inside the bars represent numbers of *embB*-mutated (or multidrug-resistant) isolates relative to the total number of isolates at each MIC.

TABLE 2. Relationship between *embB* mutation and resistance to isoniazid, rifampin, and ofloxacin and to increasing concentrations of drugs

Drug resistance[a]	Total no. of isolates	No. (%) of isolates with *embB* mutation:		*P* (chi-square test)
		Present	Absent	
Resistant to				
INH alone	54	31 (57)	23 (43)	0.10
>1 μg/ml	13	6 (46)	7 (54)	0.71
>10 μg/ml				
RIF alone	52	35 (67)	17 (33)	1.27×10^{-5}
>50 μg/ml	35	26 (74)	9 (26)	1.46×10^{-8}
>250 μg/ml				
OFX alone	52	32 (62)	20 (38)	0.007
>2 μg/ml	21	14 (67)	7 (33)	0.008
>8 μg/ml				
INH and RIF	47	32 (70)	15 (30)	1.137×10^{-5}
Any single drug	4	1 (25)	3 (75)	0.25
Any two drugs	15	4 (27)	11 (73)	0.03
All three drugs	42	30 (71)	12 (29)	2.03×10^{-5}
Pansusceptible	14	0 (0)	14 (100)	

[a] INH, isoniazid; RIF, rifampin; OFX, ofloxacin.

there was also a significant correlation with ofloxacin resistance, it is entirely possible that this may have been an indirect association, as ofloxacin is a second-line antituberculosis drug and in China it is usually used in place of rifampin when rifampin resistance becomes evident. The precise mode of action of EMB and the molecular basis of resistance are not fully understood. The effects of EMB are pleiotropic, and several hypotheses have been proposed for its mode of action (2). Inhibition of cell wall biosynthesis may not play an important role, and inhibition of RNA metabolism may be partly responsible (1, 2). The present study did not provide firm evidence to allow a conclusion to be drawn as to whether there is a close relationship between an *embB* gene mutation and rifampin resistance because of the limited number of samples and the assay system used. However, our findings suggest that studies of interrelationships among mechanisms of antituberculosis drugs and drug resistance genes would be a fruitful area of research.

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Retinoic Acid Therapy Attenuates the Severity of Tuberculosis While Altering Lymphocyte and Macrophage Numbers and Cytokine Expression in Rats Infected with *Mycobacterium tuberculosis*[1–3]

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Abstract

Because retinoic acid (RA) exerts a stimulatory effect on macrophages and tubercle bacilli target alveolar macrophages, the therapeutic potential of RA was examined in rats with tuberculosis. In the main study, 15 rats were randomized to treatment with oil (control) or RA, 100 μg/100 g body weight per dose, given 3 times weekly for 3 and 5 wk after infection with *Mycobacterium tuberculosis* strain *H37Rv*. There was a significant difference in the severity of tuberculosis histopathology between control and RA-treated rats, and oral administration of RA decreased the number of colony-forming units (CFU) in both lung and spleen at 3 and 5 wk after H37Rv infection ($P < 0.005$). CD4-positive and CD8-positive T cells, natural killer cells, and CD163-positive macrophages increased ($P < 0.05$) in the infected lung tissues of RA-treated rats. Expression of IFNγ and inducible nitric oxide synthetase messenger RNA (mRNA) was higher in the infected lung tissues of RA-treated rats than in control rats. Alveolar macrophages from rats treated in vivo with RA and infected in vitro with *M. tuberculosis* showed significantly higher expression of TNFα and IL-1β mRNA than macrophages in control rats. To our knowledge, this is the first reported study to demonstrate that orally administered RA significantly inhibits the in vivo growth of *M. tuberculosis* and the development of tuberculosis. J. Nutr. 137: 2696–2700, 2007.

Introduction

All-*trans*-retinoic acid (RA)[6], an active form of vitamin A, has the ability to reduce human mortality through effects that are considered to be related to the immune system (1). Vitamin A deficiency results in multiple abnormalities of innate and adaptive immunity involving cell differentiation, hematopoiesis, and blood and lymphoid organ cell populations, and the organism's ability to respond to challenges by pathogens, antigens, and mitogens. In normal animals, RA has been shown to stimulate innate and adaptive immune responses (2–5). For example, aging rats chronically fed a marginal vitamin A diet had decreased numbers of peripheral blood mononuclear cells and the cell lytic efficacy of natural killer (NK) cells was reduced, as well as changes in the distribution and function of T cells, B cells, and NKT cells (6–8). RA regulates IFNγ-induced IFN regulatory factor (IRF)-1 transcription factor by affecting multiple components of the IFNγ-signaling pathways (9). RA is also used for the treatment of severe cystic acne, severe psoriasis, and acute promyelocytic leukemia, for which it induces differentiation (10).

Tuberculosis has been recognized as a major infectious disease worldwide; one-third of the world's population is infected with tubercle bacilli and is thus at risk for reactivation disease when immunity fails. Tubercle bacilli harbor and replicate in alveolar macrophages whose functions are affected by tubercle bacilli. RA also influences macrophage functions (8). RA is protective when added after infection at a pharmacologic concentration of 10^{-5} mol/L and when added before infection at a near-physiologic concentration of 10^{-7} mol/L (11). Synergistic actions of vitamin D and RA are able to downregulate tryptophan-aspartate-containing coat protein (TACO) gene transcription in human macrophages (12). It has also been reported that the entry and intracellular survival of *Mycobacterium tuberculosis* is significantly restricted in THP-1 macrophages exposed to chenodeoxycholic acid/RA (13).

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[3] Supplemental Tables 1 and 2 are available with the online posting of this paper at jn.nutrition.org.
[6] Abbreviations used: BAL, broncheoalveolar lavage; CFU, colony-forming unit; FACS, fluorescence-activated cell sorting; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IRF, IFN regulatory factor; mRNA, messenger RNA; NK, natural killer (cells); iNOS, inducible nitric oxide synthetase; RA, retinoic acid; TACO, tryptophan-aspartate-containing coat protein.
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Taken together, these findings suggest that RA may have therapeutic potential for tuberculosis. In this study, we examined the therapeutic efficacy of orally administered RA on development of rat tuberculosis after aerosol infection with *M. tuberculosis*.

Materials and Methods

Animals. Specific pathogen-free and viral antibody-free Wistar-Lewis rats (LEW/CrlCrlj), 7–10 wk old, were purchased from Charles River Laboratories, Japan. The rats were housed in a biohazard level 3 facility and consumed nonpurified diet (Rat chow II, Charles River Japan) and water ad libitum throughout the entire experimental period. All animal procedures were approved by the Ethical Committee of The Research Institute of Tuberculosis.

Experimental infections. M. tuberculosis H37Rv (ATCC27294) was grown in Middlebrook 7H9 broth with 0.05% Tween 80 for 2 wk. Then, the broth was filtered with an Acrodisk filter no. 4650 (pore size 5.0 μm, Pall) to disperse bacillary clumps. The filtered bacillary solution was then stored frozen at −80°C until use. The rats were infected via the aerosol route using a Glas-Col aerosol generator, in which the nebulizer compartment was filled with 5 mL of a suspension containing 10^7 colony-forming units (CFU) of H37Rv bacilli under conditions that would allow ~500 bacilli to be inhaled by each rat. Similar experiments were performed twice with similar results and 1 experiment is shown for the 2 studies. The number of viable bacteria in the lungs was determined at specific time points by plating 10-fold serial dilutions of individual partial organ homogenates on 1% Ogawa's medium and counting bacterial colonies after 4 wk of incubation at 37°C, as previously described (14).

Oral administration of vitamin A as RA. After aerosol infection with tubercle bacilli, all-*trans*-RA, the most bioactive form of vitamin A, was administered orally. The RA solution, ~5 g/L, was prepared by dissolving 12 mg RA in 120 μL ethanol and mixing with 120 μL of Tween 80 and 2.2 g corn oil, similar to the preparation described elsewhere (5). The RA stock solution and control solution without RA were stored at 4°C for as long as 2 wk. During the preparation of the RA dose, care was taken not to expose the pure compound or the dose preparation to bright light or air.

Oral administration was started from the day after infection (d1) and continued for 5 consecutive days (d5). From the week after infection until the end of wk 4, oral administration was performed 3 times per week on alternate days. The RA dose, which was equivalent to 100 μg RA/100 g body weight per dose, was given to rats ($n = 15$). For control rats ($n = 15$), the vehicle dose was given in the same manner as that for RA-treated rats. Oral administration was performed throughout the experimental period in a safety cabinet placed in a level 3 biosafety facility without illumination inside the cabinet, but with room illumination, to avoid degradation of the RA.

CFU assay. At 1, 3, and 5 wk after aerosol infection, groups of 3 rats were anesthetized with pentobarbital sodium, the abdominal cavities were incised, and exsanguination was carried out by splenectomy and transection of the left renal artery and vein. The lungs, spleens, and livers were excised and weighed. Part of the right lower lobes of the lungs and part of the spleen were weighed separately and used to evaluate the in vivo growth of *M. tuberculosis* (14). The lung and spleen samples for in vivo CFU assay were each homogenized with a mortar and pestle and then placed in test tubes and 1 mL of sterile saline was added to each sample. After homogenization, 100 μL of the homogenate was plated in 10-fold serial dilutions on 1% Ogawa slant medium. Colonies on the medium were counted after 4 wk of incubation at 37°C (14,15).

RNA extraction and real-time PCR. Another portion of the remaining right lower lobe of the lungs and the spleen were used for RT-PCR analysis to examine the expression levels of several cytokine messenger RNA (mRNA) in these samples during *M. tuberculosis* infection. These samples were snap-frozen in liquid nitrogen and stored at −85°C until use. RNA extraction was performed as described previously (14). Briefly, the frozen tissues were homogenized in a microcentrifuge tube with an autoclaved disposable 1000-μL tip cooled by dipping in liquid nitrogen. Then the homogenates were treated with 1 mL of TRIzol reagent (Invitrogen Japan, K.K.), as specified by the manufacturer. After RNA isolation, total RNA concentration was measured with a spectrophotometer and the agarose gel electrophoresis pattern of the total RNA was examined. The total RNA were reverse transcribed into cDNA with Moloney murine leukemia virus RT (Invitrogen). ABI Taqman Gene Expression Assay was used for relative quantitative measurement of the mRNA expression of IFNγ (Rn00594078_m1), TNFα (Rn00562055_m1), inducible nitric oxide synthetase (iNOS) (Rn00561646_m1), IL-1β (Rn00580432_m1), IL-2 (Rn00587673_m1), IL-4 (Rn01456866_m1), IL-6 (Rn00561420_m1), IL-10 (Rn00563409_m1), IL-12 p40 (Rn00575112_m1), IP-10 (CXCL-10, Rn00594648_m1), and TGFβ (Rn00572010_m1). A TaqMan Rodent 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). Data were analyzed by the $\Delta\Delta C_T$ method using to the ABI PRISM Sequence Detection system software package (version 2.1; Applied BioSystems) working on a Windows 2000 OS. The results obtained from RA-treated and control rats were expressed relatively, with expression in the targets compared with those of uninfected rats that were calibrated with the expression of an internal control gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (16).

Effect of in vitro RA treatment on bacterial burden and cytokine mRNA expression. Alveolar macrophages were prepared from uninfected rats to examine mRNA expression after RA treatment and H37Rv infection. After the rats had been anesthetized with pentobarbital, alveolar macrophages were obtained by bronchoalveolar lavage (17). Briefly, the trachea was cannulated and 2.5 mL of physiological saline was introduced. The saline was recovered using a 5-mL disposable syringe. After bronchoalveolar cells that were collected were cultured at 37°C on petri dishes for 6 h, the cells collected consisted of >99% macrophages, as assessed by cell morphology. The cell suspensions (1×10^7 cells) were plated in 50-cm^2 culture flasks and incubated in RPMI1640 medium supplemented with 10% FCS and 10 μL of 2 mmol/L RA for 24 h at 37°C in 5% CO_2 in air. For the control experiment, 10 μL of absolute ethanol was added to the culture instead of RA. Then, the cells were stimulated with live *M. tuberculosis* H37Rv with a multiplicity of infection of 50:1 for 18 h. The cells were transferred to a 50-mL centrifugation tube and the cells were recovered after centrifugation. TRIzol reagent was added to the pellets and total RNA were extracted. cDNA were prepared by RT and real-time PCR analyses were done using the cDNA according to the methods described previously (16). GAPDH mRNA expression of uninfected broncheoalveolar lavage (BAL) cells without RA treatment was used for calibration and GAPDH mRNA expression in each cell group was used for normalization.

Histopathological examination. For light microscopy, the left middle lobe of the lung was excised and fixed with a 20% formalin-buffered methanol solution, Mildform 20NM (containing 8% formaldehyde and 20% methanol) (Wako Pure Chemical), dehydrated with a graded ethanol series, treated with xylene, and embedded in paraffin. Sections 5 μm thick were cut from each paraffin block and stained with either hematoxylin and eosin or Ziehl-Neelsen stain for acid-fast bacilli (14,18).

FACS analysis. Pulmonary mononuclear cells were isolated and stained for fluorescence-activated cell sorter (FACS) using reagents and methods similar to those described previously (14). After blocking with 5% bovine serum albumin, the cells were stained for 20 min at 4°C with monoclonal antibodies specific for various rat monocytic and lymphoid cells. These included phycoerythrin-conjugated OX62 (CD103, dendritic cell); fluorescein isothiocyanate-conjugated ED1 (CD68, dendritic cell/macrophage/monocyte); OX8 (CD8); OX52 (CD6, T lymphocyte), anti-rat W3/25 (CD4 T-cell); 10/78 (CD161, NK cells and T cell), R73 (α/β T-cell), and V65 (γ/δT-cell). Thereafter, the cells were fixed in 2% paraformaldehyde/PBS, examined with a FACS (FACSCAN, BD), and

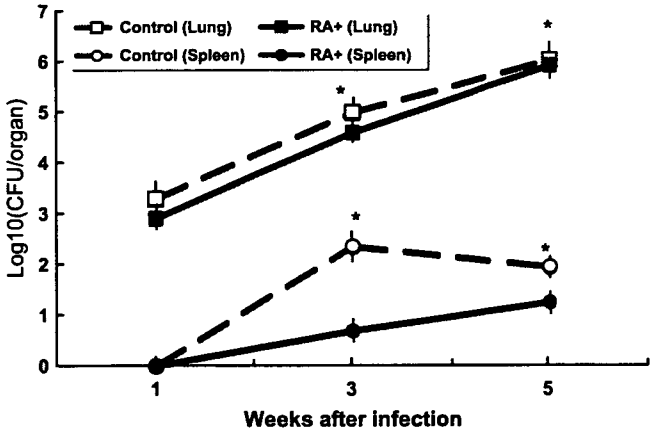

FIGURE 1 RA administration (RA+) significantly decreased lung and spleen CFU in *M. tuberculosis* H37Rv-infected rats. Values are means ± SE, $n = 3$. *Different from RA+ at that time, $P < 0.005$.

analyzed with Cell Quest software (Pharmingen). For ED1 immunostaining, the mononuclear cells (1×10^9/L) were made permeable with Leukoperm (Serotec) for 15 min before reaction with ED1 (19).

Statistical methods. Values are means ± SE for the number of replicates indicated. Differences between 2 groups were determined by Student's *t* test and differences between multiple groups were determined by ANOVA and a paired or unpaired *t*-test as a post hoc test. Differences of $P < 0.05$ were considered significant.

Results

Mycobacterial burden in the lungs and spleen. We examined the in vivo mycobacterial burdens of *M. tuberculosis*-infected rats with or without oral administration of RA. After H37Rv infection and 3 and 5 wk of oral administration of RA, the number of CFU in both the lungs and spleen was lower at each time shown ($P < 0.05$; **Fig. 1**) compared with the untreated group.

Light microscopic observation of infected lungs. Representative light micrographs of infected lung tissues obtained from RA-treated rats are shown in **Fig. 2**, *A–C* and from control rats in Fig. 2, *D–F*. Lung tissue of RA-treated rats had smaller lesion areas (Fig. 2*A* vs. 2*D*) and fewer acid-fast bacilli in the lesions than control rats (Fig. 2*C* vs. 2*F*). In control rat lung tissue, typical granulomas were formed, where many lymphocytes surrounded epithelioid cells (Fig. 2*D*,*E*). Although typical granulomas were formed similarly in the lungs of RA-treated rats (Fig. 2*A*,*B*), the granulomas were smaller and contained fewer cells than those in control rats.

Cell analysis by flow cytometry. To examine the cell populations comprising the lung lesions, we performed FACS analysis of the cells in lung homogenates. FACS analysis revealed that CD4-positive T cells, CD8-positive T cells, NK cells, α/β T cells, CD25-positive T cells, and CD163-positive monocyte/macrophages were higher ($P < 0.05$) in RA-treated rats compared with those in control rats 3 wk after infection (**Table 1**).

Cytokine and enzyme mRNA by real-time PCR. Next, we performed real-time PCR analysis using cDNA reverse transcribed from total RNA extracted from lung and spleen tissues of rats treated in vivo. The data thus obtained were expressed as an intensity relative to GAPDH. In the lung, expression of IFNγ and iNOS mRNA differed with RA treatment at 5 wk, when expression was highest. For TNFα and IL-1β mRNA, expression was affected by RA at both 3 wk and 5 wk. Expression of IL-2, IL-12βR, and IP-10 mRNA differed at either 1 wk or 3 wk, but not at 5 wk. Thus, RA affected several cytokines in the lung in temporally different patterns (**Supplemental Table 1**).

In contrast, in spleen tissues, expression of mRNA for IFNγ, TNFα, iNOS, IL-1β, and IL-1 in RA-treated rats was highest at 1 wk after infection and was more than double those of the control group, whereas expression of IL-6, IL-10, IL-12 p40, IP-10, and TGFβ mRNA in RA-treated rats peaked later after infection and RA did not alter expression (**Supplemental Table 2**).

Cytokines in infected alveolar macrophages. The level of mRNA was also determined in BAL cells collected from H37Rv-infected rats and treated with RA in vitro. For most cytokines, the level was low or undetectable in the absence of infection (data not shown). BAL macrophages treated with RA had higher levels of IFNγ, TNFα, iNOS, IL-10, and IP-10 mRNA relative to GAPDH mRNA than BAL cells cultured without RA (**Table 2**).

Discussion

In this study, we have shown that orally administered RA can inhibit the growth of *M. tuberculosis* in vivo. No previous report to our knowledge has described the effect of RA on the development of tuberculosis in vivo.

We utilized a Lewis rat tuberculosis model to study treatment with RA, because we had already established such a model (19) and also because rats have been used previously to examine the

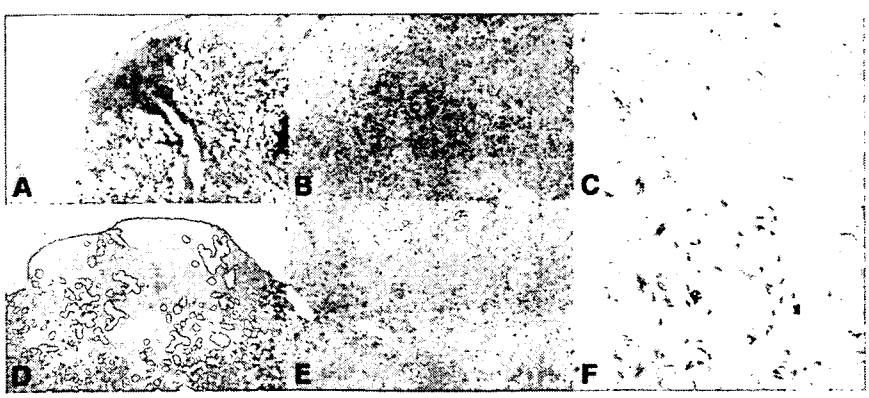

FIGURE 2 Histopathology of *M. tuberculosis* H37Rv-infected RA-treated rats (*A–C*) and H37Rv-infected control rats (*D–F*). RA-treated rats showed smaller lesion areas (*A*,*B*) and fewer acid-fast bacilli in the lesions (*C*) than control rats (*D–F*). *A*, *B*, *D*, and *E* show hematoxylin and eosin staining; magnification ×135 for *A* and *D*, ×270 for *B* and *E*. *C* and *F* show Ziehl-Neelsen staining, magnification ×600.

TABLE 1 Flow cytometric analysis of lung BAL cells from *M. tuberculosis* H37Rv-infected RA-treated (RA+) and control (RA−) rats 3 and 5 wk postinfection[1]

	Relative intensity			
	3 wk after infection		5 wk after infection	
Cell subpopulation	RA+	RA−	RA+	RA−
CD4	4.1 ± 0.2*	1.5 ± 0.1	7.2 ± 0.3	7.1 ± 0.3
CD8	3.1 ± 0.1*	2.1 ± 0.1	4.4 ± 0.1	7.3 ± 0.3
NK	4.6 ± 0.1*	1.9 ± 0.1	5.0 ± 0.1	5.6 ± 0.2
α/β T	5.1 ± 0.2*	2.2 ± 0.2	13.0 ± 0.7	12.2 ± 0.4
γ/δ T	0.3 ± 0.02	0.35 ± 0.03	0.4 ± 0.01	0.7 ± 0.03
CD25	0.9 ± 0.04*	0.2 ± 0.01	0.6 ± 0.03	0.7 ± 0.02
CD103	4.1 ± 0.2	4.2 ± 0.3	7.0 ± 0.5	10.0 ± 0.09
CD163	0.7 ± 0.05*	0.3 ± 0.01	0.8 ± 0.06	0.6 ± 0.02

[1] Values are means ± SEM, $n = 3$. *Different from RA at that time, $P < 0.05$.

biological functions of RA (10). When the CFU were counted in alveolar macrophages infected with H37Rv *M. tuberculosis* and in RA-treated alveolar macrophages infected with H37Rv *M. tuberculosis* in vitro, the number decreased significantly in RA-treated macrophages (data not shown). Two previous reports have provided evidence that RA stimulates macrophages to kill tubercle bacilli. First, RA has a protective effect when it is added at a pharmacologic concentration to cultured human macrophages after infection and at a physiologic concentration when it is added before infection (11). Furthermore, entry and intracellular survival of *M. tuberculosis* are significantly restricted in THP-1 human macrophage-like cells exposed to chenodeoxycholic acid and RA (13). It has been reported that iNOS plays an important role in killing tubercle bacilli (17). As RA itself does not induce iNOS expression in vitro, another mechanism for killing *M. tuberculosis* may exist. Recently, it was shown that TACO plays a crucial role in the entry/survival of *M. tuberculosis* within human macrophages (13). Because RA downregulates TACO gene transcription, TACO may be involved in killing *M. tuberculosis*. Further study will be required to clarify this possibility.

The levels of TNFα, IL-1β, and iNOS mRNA expression are elevated in the lung tissues of rats with tuberculosis treated orally with RA and BAL cells from *M. tuberculosis*-infected rats treated with RA in vitro showed similar effects. These cytokines play important roles in the defense against tuberculosis (15,17,18). These molecules function as anti-tuberculosis immune factors. There is another possible mechanism related to defense against tuberculosis. IRF-1, a transcription factor and tumor suppressor

TABLE 2 Cytokine relative mRNA levels 18-h postinfection in control (RA−) and RA-treated (RA+) rat lung BAL cells infected in vitro with *M. tuberculosis* H37Rv[1,2]

	Intensity (relative to GAPDH)	
Cytokine or enzyme	RA−	RA+
IFNγ	ND	4.2 ± 0.3
TNFα	4.2 ± 0.3	29 ± 2*
iNOS	505 ± 46	2506 ± 225*
IL-10	105 ± 10	4102 ± 230*
IP-10 (CXCL10)	21 ± 2	118 ± 10*

[1] Values are means ± SEM, $n = 3$. *Different from RA−, $P < 0.05$.
[2] Uninfected cultures had low or nondetectable (ND) levels of these cytokines and are not shown.

involved in cell growth regulation and immune responses, has been shown to be induced by RA (9,20). We have also reported that *M. tuberculosis*-infected mice lacking IRF-1 expression die of disseminated tuberculosis (21). Thus, RA may regulate IFN-induced IRF-1 functions by affecting multiple components of the IFN-signaling pathways. In normal animals, RA has been shown to stimulate innate and adaptive immune responses (2–5). Thus, it is thought that RA stimulates immune cells to secrete cytokines and that its functions are multiple.

Histopathologically, there was a significant difference in the sizes of pulmonary granulomas between RA-treated and control rats in this study. RA has an inherent capacity to increase the constituent cells in granulomas, as evaluated by FACS. As stated above, RA may also be involved in killing tubercle bacilli (13). It has been suggested that RA, like vitamin D, may have some immunoprotective role against tuberculosis, as historically intimated by the regular use of vitamin A- and D-rich cod liver oil for the prevention of tuberculosis before the introduction of modern chemotherapy (11). RA-dependent downregulation of TACO, which has now been shown to be regulated by these nutrient metabolites, may be a promising new avenue for the treatment of tuberculosis.

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Original article

Detection of streptomycin resistance in *Mycobacterium tuberculosis* clinical isolates from China as determined by denaturing HPLC analysis and DNA sequencing

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Abstract

China is regarded by the World Health Organization as a major hot-spot region for *Mycobacterium tuberculosis* infection. Streptomycin has been deployed in China for over 50 years and is still widely used for tuberculosis treatment. We have developed a denaturing HPLC (DHPLC) method for detecting various gene mutations conferring drug resistance in *M. tuberculosis*. The present study focused on *rpsL* and *rrs* mutation analysis. Two hundred and fifteen *M. tuberculosis* clinical isolates (115 proved to be streptomycin-resistant and 100 susceptible by a routine proportional method) from China were tested to determine the streptomycin minimal inhibitory concentration (MIC), and subjected to DHPLC and concurrent DNA sequencing to determine *rpsL* and *rrs* mutations. The results showed that 85.2% (98/115) of streptomycin-resistant isolates harbored *rpsL* or *rrs* mutation, while *rpsL* mutation (76.5%, 88/115) dominated. MIC of 98 mutated isolates revealed no close correlation between mutation types and levels of streptomycin resistance. No mutation was found in any of the susceptible isolates. The DHPLC results were completely consistent with those of sequencing. The DHPLC method devised in this study can be regarded as a useful and powerful tool for detection of streptomycin resistance. This is the first report to describe DHPLC analysis of mutations in the *rpsL* and *rrs* genes of *M. tuberculosis* in a large number of clinical isolates.

Keywords: *Mycobacterium tuberculosis*; Streptomycin resistance; DHPLC; *rpsL*; *rrs*; MIC

1. Introduction

Tuberculosis has continued to be the most common infectious cause of death, and still has a serious impact medically, socially and financially [1]. Multidrug-resistant tuberculosis (MDR-TB), caused by tubercle bacilli that are resistant to at least isoniazid and rifampicin, is one of the most worrisome elements of the antibiotic resistance pandemic, because TB patients for whom treatment has failed have a high risk of death [1]. The global number of incident cases of MDR-TB in 2004 was estimated to be 424,203. Three countries, China, India and the Russian Federation, accounted for 261,362 MDR-TB cases, or 62% of the estimated global burden [2]. Very recently, XDR-TB has been proposed as a result of a global survey by the WHO, although its exact incidence is not known in the world. Streptomycin was the first antibiotic shown to be active against the etiologic agent of TB, *Mycobacterium tuberculosis*, and was used in control programs for many years. However, as a result of significant levels of resistance when streptomycin was used as monotherapy, and some side effects, streptomycin usage declined greatly in industrialized countries in the 1960s [3]. Recently, the emergence of

Abbreviations: MDR, multidrug-resistant; MIC, minimal inhibitory concentration; *M. tuberculosis*, *Mycobacterium tuberculosis*; TB, tuberculosis; Denaturing HPLC, denaturing high-performance liquid chromatography.

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strains of *M. tuberculosis* displaying resistance to some or all of the major anti-tuberculosis drugs (isoniazid, rifampicin, ethambutol, pyrazinamide and fluoroquinolones) has led to renewed interest in streptomycin and its derivatives, kanamycin and amikacin. However, in some countries including China, streptomycin has continued to be used commonly for tuberculosis treatment.

Streptomycin has been shown to interact directly with the 30S subunit of the ribosome, thereby interfering with protein biosynthesis [18]. The ribosome accuracy center is a highly conserved component of the translational apparatus, comprising a 16S rRNA domain and several polypeptides including the ribosomal protein S12. RNAs for ribosomal protein S12 (encoded by the *rpsL* gene) and 16S (encoded by the *rrs* gene) are the main targets of streptomycin [4]. It is now known that streptomycin binds tightly to the phosphate backbone of 16S rRNA in four different domains – helix 1, the 530 loop, the 912 loop and the 1400 region – thereby also forming both salt bridges and hydrogen bonds, and making contact with the S12 ribosomal protein [19,20], eventually leading to misreading of the genetic code during translation [20]. Mutations in genes encoding the rRNAs for ribosomal protein S12 (*rpsL* gene) and 16S (*rrs* gene, within which the parts encoding the 530 loop, the 912 loop and the 1400 region of 16S rRNA are named *rrs*A, *rrs*B and *rrs*C, respectively, in this work) lead to streptomycin resistance. This work was carried out to explore *rpsL* and *rrs* mutation in streptomycin-resistant clinical isolates from China.

There are several methods to detect various gene mutations. DNA sequencing is a good method for detecting mutation, but cannot be used routinely by many laboratories because of its relatively high cost. PCR-single-stranded conformational polymorphism (SSCP) is often used, but has a major disadvantage in that the technique is empirical and it is difficult to optimize the experimental conditions. Temperature-mediated heteroduplex analysis by denaturing high-performance liquid chromatography (TMHA-DHPLC) is a relatively new technique that uses heteroduplex formation between wild-type and mutated DNA strands to identify mutations. DHPLC was predicted to be a potentially useful genotypic screening method for gene mutations conferring drug resistance in *M. tuberculosis* [5–7] and it is cost-effective. We have developed the DHPLC method for detecting various gene mutations in *M. tuberculosis* [8,9]. The present study was focused on *rpsL* and *rrs* mutation analysis. The DHPLC method devised in this study can be regarded as a useful tool for clinical analysis of streptomycin resistance in tuberculosis. This is the first report to describe DHPLC analysis of mutations in the *rpsL* and *rrs* genes of *M. tuberculosis* in a large number of clinical isolates.

2. Materials and methods

2.1. Clinical isolates and drug susceptibility tests

M. tuberculosis H37Rv (ATCC 25618) was used as a reference strain. Streptomycin-dependent strain 18b was from the Mycobacterial Reference Center of The Research Institute of Tuberculosis. Two hundred and fifteen clinical isolates (115 proved to be streptomycin-resistant by a routine proportional method, and 100 streptomycin-susceptible) were collected from different patients with pulmonary tuberculosis (123 males and 92 females, aged 15–75 years) over a period of 3 years (2002–2004) at Beijing Tuberculosis and Thoracic Tumor Research Institute, China. Forty-three (37.3%) of 115 streptomycin-resistant isolates were MDR. MICs of streptomycin were detected by an absolute concentration method in L–J medium at concentrations of 1, 2, 5, 10, 20, 50, 80, 100, 200, 400, and 800 μg/ml.

2.2. DNA isolation and PCR amplification

Chromosomal DNA was extracted from *M. tuberculosis* H37Rv and clinical isolates by the method described previously [8,10]. For the *rpsL* gene, a 300-bp DNA fragment was generated by PCR with the primer set SM1, SM2. For the *rrs* gene, three primer sets were used to amplify regions corresponding to the 530 loop, 912 loop and 1400 region of 16S rRNA, respectively (Table 1). TaKaRa Ex *Taq* was the polymerase used for the PCR reaction.

2.3. DHPLC analysis

DNA from the reference strain was used for individual hybridization with each test isolate. DHPLC was performed with the WAVE DNA fragment analysis system (Transgenomic Inc.). The melting temperature for each gene is shown in

Table 1
Primer sets and predicted PCR products of *rpsL* and *rrs* genes

Accession number (GenBank)	Primer set (F: forward, R: reverse)	Size (bp)	Melting temperature (°C)
rpsL X80124	F 5′-ATG CCA ACC ATC CAG CAG CT R 5′-ACC GCG GAT GAT CTT GTA GC	300	65.8
rrs BX842576			
*rrs*A (530 loop)	F 5′-GAT GAC GGC CTT CGG GTT GT R 5′-TCT AGT CTG CCC GTA TCG CC	238	63.2
*rrs*B (912 loop)	F 5′-GTA GTC CAC GCC GTA AAC GG R 5′-AGG CCA CAA GGG AAC GCC TA	240	62.3
*rrs*C (1400 region)	F 5′-TTA AAA GCC GGT CTC AGT TC R 5′-TAC GCC CCA CCA GTT GGG GC	300	63.3

Table 1. The conditions for DNA hybridization and DHPLC analysis have been described in detail elsewhere [8].

2.4. DNA sequencing

rpsL and *rrs* gene PCR products of 115 streptomycin-resistant, and 100 streptomycin-susceptible clinical isolates, and the streptomycin-dependent strain 18b, were sequenced. The sequencing primer sets were the same as those used for PCR. After purification, the PCR product (5 ng) was used as a template for *Taq*Cycle Sequencing using ABI Prism Big Dye Terminator sequencing kits (Applied Biosystems). Cycle sequencing products were subsequently analyzed on an ABI PRISM 310 Genetic Analyzer (Perkin Elmer Applied Biosystems) [9].

3. Results

3.1. rpsL mutations in clinical isolates

The results of DNA sequencing showed that all of the 100 streptomycin-susceptible isolates had wild-type *rpsL*. Of the 115 streptomycin-resistant isolates, 88 (76.5%) were found to have *rpsL* mutations, the majority at codon 43 (Fig. 1). Seven (6.1%) isolates rpsL88 mutation (AAG → AGG, Lys → Arg) and 81 (70.4%) had an rpsL43 (AAG → AGG, Lys → Arg). Seven of the isolates with an rpsL43 mutation harbored a second mutation in *rrs* and *rpsL* genes: one isolate had rpsL39 (ACC → ACT, no amino acid change), one had rpsL71 (GGC → AGC, Gly → Ser), one had rrs462 (T → G, 645 A deletion), one had rrs513 (A → C), one had rrs1400 (A → G) and two had rrs1401 (C → T) mutations. No compound mutation of codon 88 was found.

Codon 88 was the other mutation point. Seven (6.1%) isolates carried codon 88 mutation (AAG → AGG, Lys → Arg). No compound mutation of codon 88 was found.

3.2. rrs mutations in clinical isolates

No mutation was found among 100 streptomycin-susceptible isolates as evaluated by DNA sequencing. Of the 115 streptomycin-resistant clinical isolates, a total of 11 (9.5%) isolates had *rrs*A mutation (Fig. 2), among which two had compound mutation with *rpsL* codon 43 mutation as described above, and nine possessed mono-mutation. Among the mutation types, two isolates had 513 A → C and 645 deletion, five had 513 A → C, one had 516 C → T, and one had 464 A → C.

*rrs*B: Only one (0.9%) isolate showed *rrs*B 906 A → C mutation.

*rrs*C: Three (2.6%) isolates were found to have *rrs*C mutation (one with 1400 A → G, two with 1401 C → T), all of them compound with *rpsL* codon 43 mutation, as described above.

3.3. Streptomycin resistance gene mutation and MIC analysis

Fig. 3 shows the relationship between mutation types and MIC levels of 98 streptomycin-resistant isolates in which *rpsL* or *rrs* mutation was detected. Seventy-four isolates with *rpsL*43 AAG → AGG mutation were found at different MICs ranging from 20 to 800 μg/ml. The MICs of nine isolates with *rrs*A mono-mutation ranged from 50 to 800 μg/ml. There was no significant difference in MIC among the mutation types. These results revealed no close correlation between mutation type and streptomycin resistance level.

3.4. Mutations in streptomycin-dependent strain 18b

DNA sequencing results revealed one insertion of an additional cytosine residue between positions 512 and 513 in the 530 loop and one deletion of adenosine at position 645 in the *rrs*A gene. No mutation was found in *rpsL*, *rrs*B or *rrs*C.

3.5. DHPLC analysis

The results of DHPLC analysis were completely consistent with those of DNA sequencing. With *M. tuberculosis* H37Rv as a reference strain, DHPLC analysis revealed that all the streptomycin-susceptible isolates, and those isolates that were streptomycin-resistant but with no *rpsL* or *rrs* mutation, had a normal peak pattern, which was the same as that of H37Rv. All the 88 isolates that were found to carry *rpsL* mutation showed an aberrant DHPLC pattern. Four types of mutation in the *rpsL* gene were found by DNA sequencing, and four corresponding peak patterns were shown by DHPLC analysis (Fig. 4). Seventy-six codon 43 AAG → AGG mono-mutated isolates, seven codon 88 AAG → AGG mono-mutated isolates,

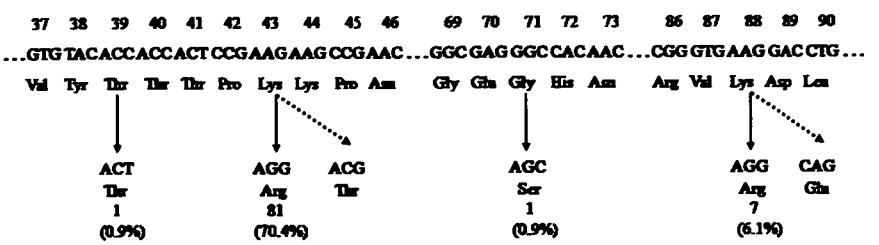

Fig. 1. Nucleotide sequence and missense mutations in the *rpsL* gene. ——▶ shows the mutations found in this work. - - - ▶ shows mutations from other reports.

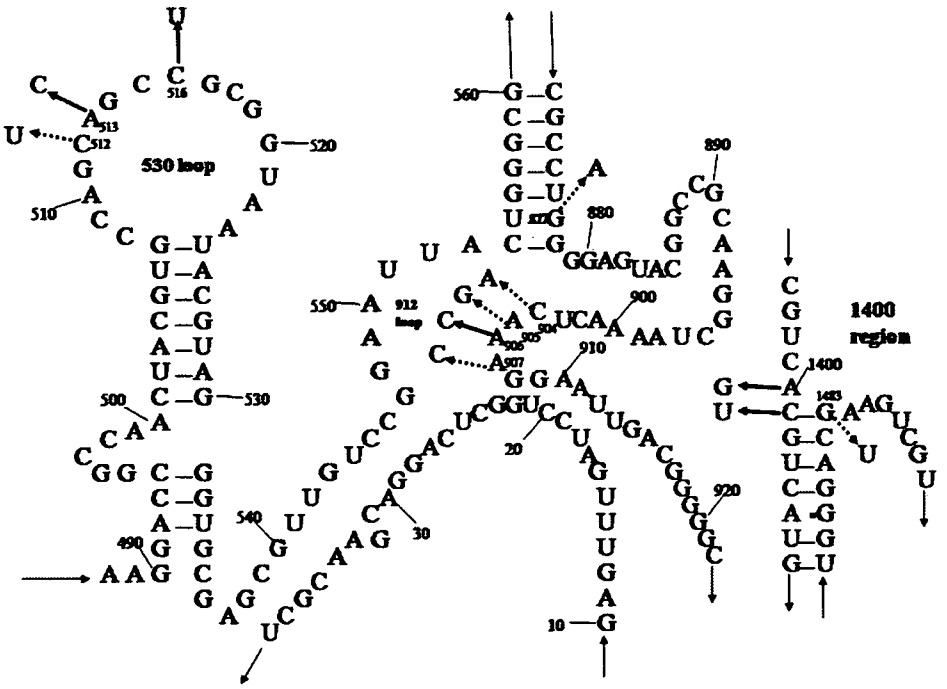

Fig. 2. Mutations located in the 530 loop, the 912 loop and the 1400 region in 16S rRNA associated with streptomycin resistance. The figure is based on a model structure of *M. tuberculosis* 16S rRNA [15]. ——▶ shows the mutations found in this work. - - - ▶ shows mutations from other reports.

one codon 43 AAG → AGG compound with codon 39 ACC → ACT mutated isolate, and one codon 43 AAG → AGG compound 71 GGC → AGG mutated isolate showed their own specific and aberrant DHPLC patterns, respectively.

Four types of *rrs*A gene mutation, one type of *rrs*B gene mutation and two types of *rrs*C gene mutation were found in this study. Their corresponding DHPLC patterns are shown in Fig. 4 and all of them differs from the susceptible reference strain, H37Rv. With regard to the streptomycin-dependent strain 18b, the results of *rpsL* and *rrs* gene DHPLC analysis are also shown in Fig. 4.

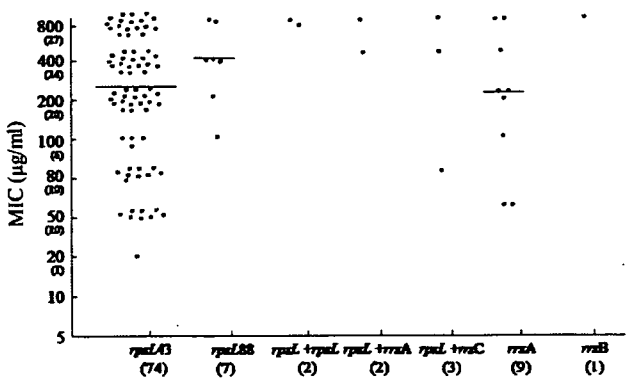

Fig. 3. Relationship between mutation types and MICs of 98 streptomycin-resistant isolates. Numbers in bracket indicate total number of clinical isolates harboring one type of mutation or one level of MIC. ——— indicates average MIC level in a group. No significant difference was found among *rpsL*43AAG ——▶ AGG, *rpsL*88AAG ——▶ AGG, *rrs*A mutation, *rrs*B mutation and three kinds of double mutations.

4. Discussion

One practical implication of the present findings is that that the DHPLC method has wide clinical application for *rpsL* and *rrs*A mutation analysis. Currently, control of MDR-TB and XDR-TB is a major issue throughout the world. It is useful to detect *rpsL* and *rrs* mutations in kanamycin- and amikacin-resistant *M. tuberculosis* strains. Kanamycin and amikacin are commonly used in second-line therapy of TB. Detection of drug-resistant phenotypes of *M. tuberculosis* using routine methods takes several weeks. The establishment of a rapid, simple and reliable method for detection of drug-resistant phenotypes of *M. tuberculosis* is one of the most urgent requirements for effective treatment of tuberculosis patients. Compared with DNA sequencing and drug susceptibility testing, DHPLC has been confirmed to be a simple, reliable and cost-effective method with high sensitivity and specificity, and has already been applied for detection of *rpoB* and *gyrA* gene mutation in order to predict rifampicin and fluoroquinolone resistance in *M. tuberculosis* [7–9]. This is the first report of the use of DHPLC for streptomycin resistance gene mutation analysis using a large series of clinical samples. The results of DHPLC are completely consistent with those of DNA sequencing: In all of the susceptible clinical isolates, no mutation was found; 84.3% of streptomycin-resistant clinical isolates revealed *rpsL* or *rrs*A mutation; one type of peak patterns corresponded to one specific mutation type. The DHPLC method devised in this study can be regarded as a useful and powerful tool for analysis of *rpsL* and *rrs* mutation in *M. tuberculosis*. It should be mentioned that the frequency of streptomycin resistance is very high in China. One obvious