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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 IS6110 clustering rather than ethambutol resistance.”

For DNA sequencing of the *embB* gene (primer set includes forward, 5'-CGGCATGCGCCGGCTGATTC, and reverse, 5'-TCCACAGACTGGCGTCTGCTG) 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-

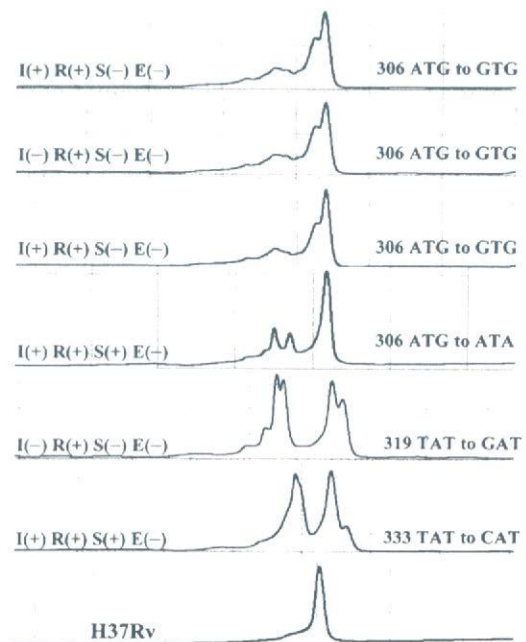


FIG. 1. *embB* DHPLC pattern of six EMB-susceptible isolates from Henan Province, China. I, isoniazid; R, rifampin; S, streptomycin; E, EMB; +, resistant; -, susceptible.

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

EMB MIC ($\mu\text{g/ml}$)	No. of isolates with:			No. of resistant isolates at indicated drug concn ($\mu\text{g/ml}$) ^a									Total no. of isolates with <i>embB</i> mutation and resistance to other drug(s) (n = 74)
	No <i>embB</i> mutation	<i>embB</i> mutation in codon 306	<i>embB</i> mutation at another position	INH			RIF			OFX			
				≤ 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.

ected 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 $\mu\text{g/ml}$ was taken as a standard for EMB resistance (and the World Health Organization suggests that 2 $\mu\text{g/ml}$ 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 EMB at concentrations ranging from 0.25 to 20 $\mu\text{g/ml}$, 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 *embB* 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

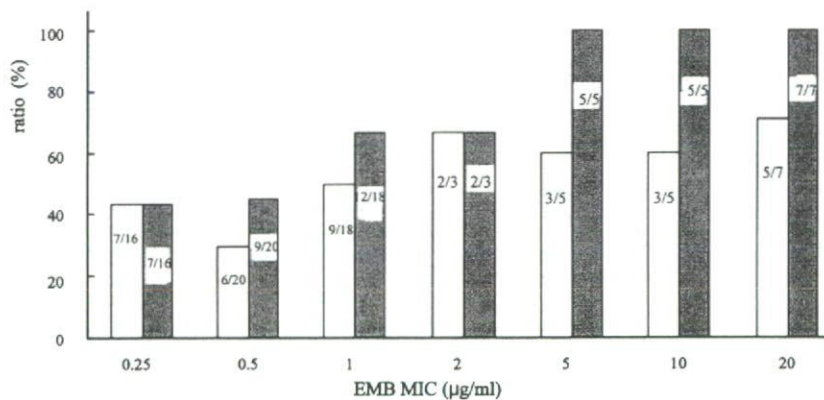


FIG. 2. Distribution of *embB* mutation rates among isolates at different EMB concentrations. White bars indicate the *embB* mutation rates, and shaded bars show the multidrug resistance rates among isolates for each EMB MIC. Numbers inside the bars represent numbers of *embB*-mutated (or multidrug-resistant)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

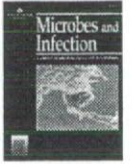
^a INH, isoniazid; RIF, rifampin; OFX, ofloxacin.

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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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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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.

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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 *rrsA*, *rrsB* and *rrsC*, 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)
<i>rpsL</i> X80124	F 5'-ATG CCA ACC ATC CAG CAG CT R 5'-ACC GCG GAT GAT CTT GTA GC	300	65.8
<i>rrs</i> BX842576			
<i>rrsA</i> (530 loop)	F 5'-GAT GAC GGC CTT CGG GTT GT R 5'-TCT AGT CTG CCC GTA TCG CC	238	63.2
<i>rrsB</i> (912 loop)	F 5'-GTA GTC CAC GCC GTA AAC GG R 5'-AGG CCA CAA GGG AAC GCC TA	240	62.3
<i>rrsC</i> (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 *TaqCycle* 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 *rpsL*88 mutation (AAG → AGG, Lys → Arg) and 81 (70.4%) had an *rpsL*43 (AAG → AGG, Lys → Arg). Seven of the isolates with an *rpsL*43 mutation harbored a second mutation in *rrs* and *rpsL* genes: one isolate had *rpsL*39 (ACC → ACT, no amino acid change), one had *rpsL*71 (GGC → AGC, Gly → Ser), one had *rrs*462 (T → G, 645 A deletion), one had *rrs*513 (A → C), one had *rrs*1400 (A → G) and two had *rrs*1401 (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 *rrsA* 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.

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

rrsC: Three (2.6%) isolates were found to have *rrsC* 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 *rrsA* 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 *rrsA* gene. No mutation was found in *rpsL*, *rrsB* or *rrsC*.

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,

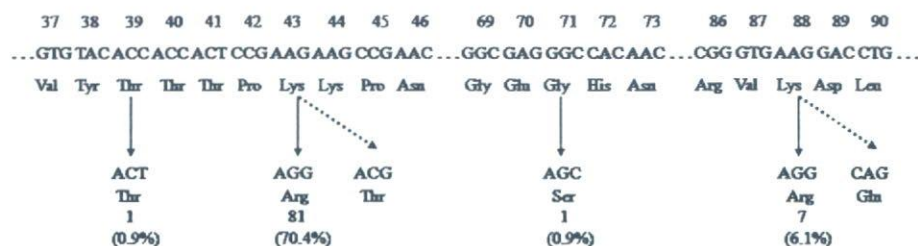


Fig. 1. Nucleotide sequence and missense mutations in the *rpsL* gene. —→ shows the mutations found in this work. - - - → shows mutations from other reports.

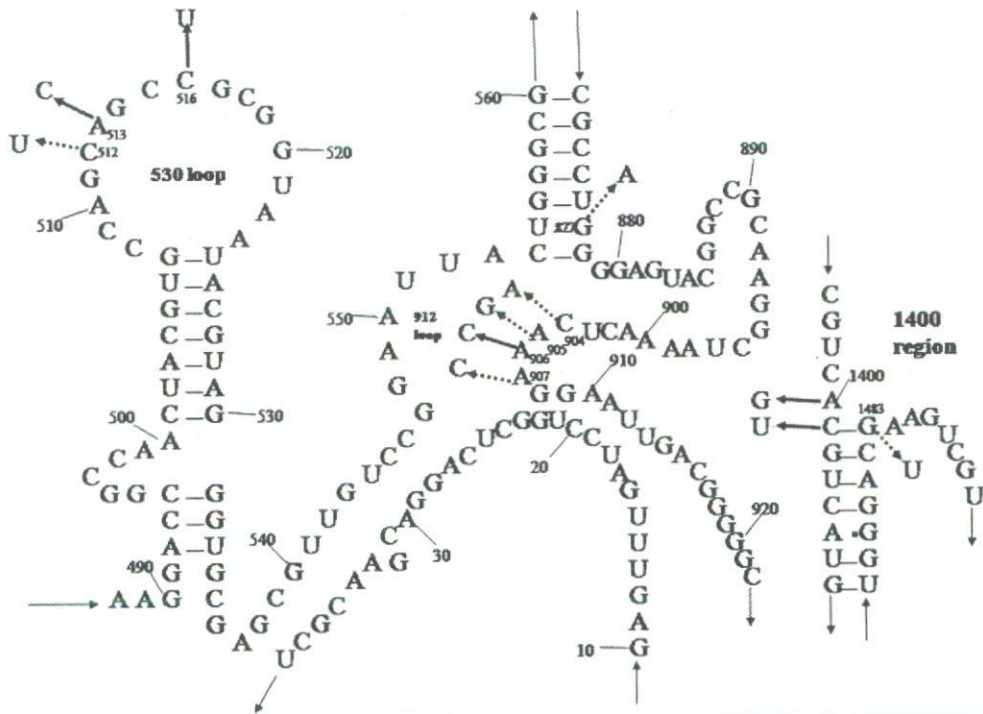


Fig. 2. Mutations located in the 530 loop, the 912 loop and the 1400 region in 16S rRNA associated with streptomycin resistance. The figure is based on a model structure of *M. tuberculosis* 16S rRNA [15]. —→ shows the mutations found in this work. - - - → shows mutations from other reports.

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 *rrsA* gene mutation, one type of *rrsB* gene mutation and two types of *rrsC* 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.

4. Discussion

One practical implication of the present findings is that that the DHPLC method has wide clinical application for *rpsL* and *rrsA* 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 *rrsA* 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

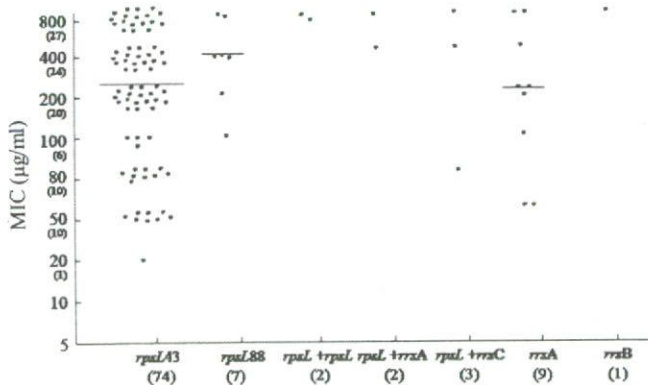


Fig. 3. Relationship between mutation types and MICs of 98 streptomycin-resistant isolates. Numbers in bracket indicate total number of clinical isolates harboring one type of mutation or one level of MIC. — indicates average MIC level in a group. No significant difference was found among *rpsL*43AAG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

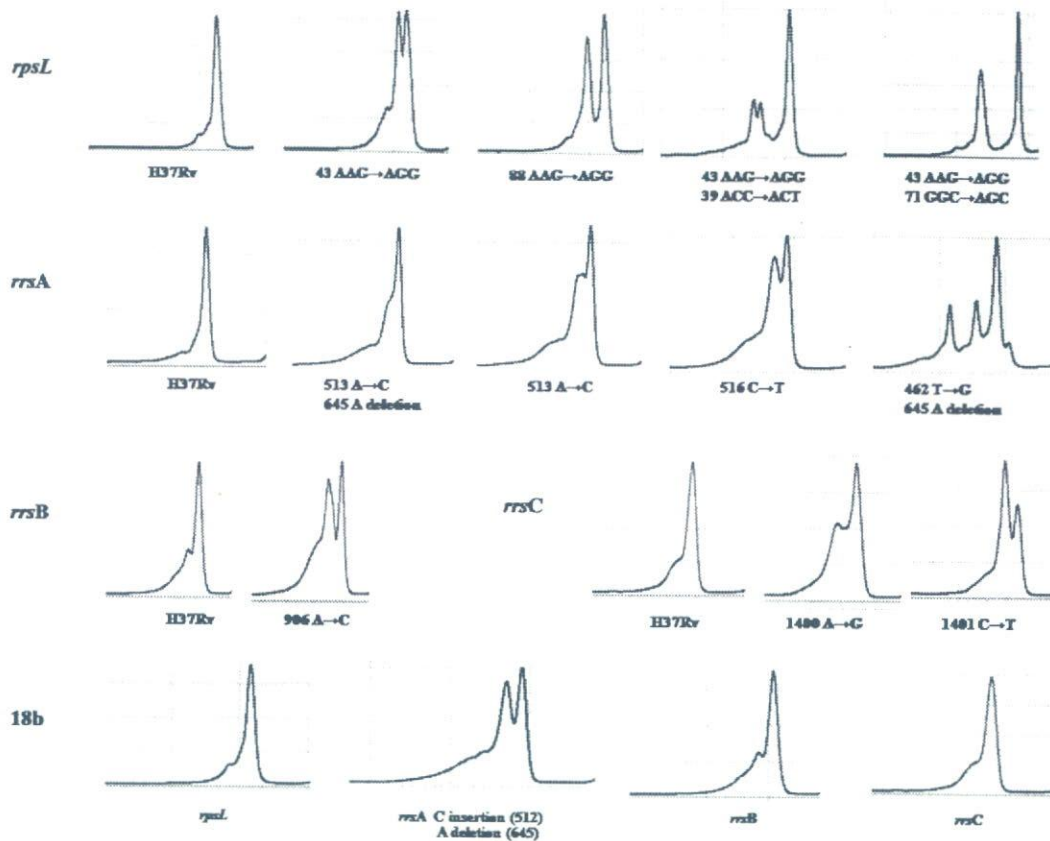


Fig. 4. DHPLC peak patterns of the *rpsL* and *rrs* genes of streptomycin-resistant clinical isolates and streptomycin-dependent strain b18.

reason is that streptomycin has been used for many years in China as an anti-TB drug. In China, Japan and other countries with a high frequency of *rpsL* and *rrs* mutation in streptomycin-resistant clinical isolates, it is anticipated that this DHPLC method will have a high predictive value.

In this study, DNA sequencing and the DHPLC method were applied to investigate the molecular mechanism of streptomycin resistance in *M. tuberculosis* clinical isolates from China. The results revealed that 85.2% of streptomycin-resistant isolates had mutation in the *rpsL* or *rrs* gene. This rate is much higher than those of 56–68% reported in America [10,11], 48% in Germany [12], 60% in France [13], 52% in Poland [14], 24% in Mexico [15], and 77.8% in Japan [16], and indicates the different geographic distribution of *rpsL* and *rrs* mutations among streptomycin-resistant *M. tuberculosis* isolates, those from China showing the highest frequency. There is a possibility that a high transmission frequency could be the cause for the high frequency of *rrs* and *rpsL* mutations in our isolates compared to those found in previous reports [10–16]. It was found in this study that *rpsL* played a more important role (76.5%, 88/115) than the *rrs* gene (*rrsA*: 9.6%, 11/115) in the mechanism of streptomycin resistance, and no *rpsL* or *rrs* mutation was found among 100 streptomycin-susceptible clinical isolates, strongly confirming and extending the findings of other investigators [11–16]. The relative proportions of *rpsL* versus *rrs* mutations in the

streptomycin-resistant isolates are similar compared with other studies [11–16]. No relationship between mutation type and resistance level was found in this study, thus differing from the reports of Cooksey [5] and Bottger [17]. The reason may be related to the difference in areas from which the strains were derived, similarly to the differences in the geographic distribution of mutation types discussed above. It is also possible that there are other reasons for no relationship between MIC and mutation type. Unknown mechanisms may exist between different strain families of *M. tuberculosis*.

In this study, *rpsL* mutations were found to occur predominantly at codon 43 (91%, 81/88 isolates). Codon 88 mutation played a minor role (9%, 7/88 isolates). These results largely confirm the findings of other researchers [21]. Two new point mutations found in this study, at codon 39 (ACC → ACT) with no amino acid substitution, and at codon 71 (GGC → AGC, Gly → Ser), both compounded with codon 43 AAC → AGG, seemed to be of only minor importance. Previously reported mutations involving codon 43 AAC → ACG (Lys → Thr) and codon 88 AAC → CAG (Lys → Gln) were not found [21]. This is because they have been proved to be restrictive mutations leading to fitness cost and show attenuated virulence. Only non-restrictive mutations such as codon 43 AAC → AGG (Lys → Arg), which has unaltered virulence properties, can be widely transmitted and finally dominate in clinical isolates [22]. In this study, a total of 15 isolates

(13%) were found to have *rrs* mutation. In contrast to most bacteria that have multiple copies of the *rrs* gene, *M. tuberculosis* and other slow-growing mycobacteria have only one copy [4,21]. Mutations in the *rrs* gene, which encodes the loops of 16S rRNA, the highly conserved 530 loop, the 912 loop and the 1400 region that interact with the S12 ribosomal protein, constitute an easily selected resistance site. In *rrsA*, 513 A → C, 516 C → T, 464 A → C mutations were demonstrated in these isolates from China, while 512 C → T mutation and 491 C → T polymorphism [15,23] were not found. Since only one isolate carried *rrsB* mutation and three *rrsC*-mutated isolates were all compounded with *rpsL* codon 43 AAG → AGG mutation concurrently, it can be concluded that *rrsB* and *rrsC* mutations are not common, while the majority of *rrs* point mutations producing streptomycin resistance in *M. tuberculosis* occur in *rrsA*, which encodes the 530 loop of 16S rRNA. Two isolates had identical *rpsL* codon 43 mutation plus *rrsC* codon mutation. There is a possibility of transmission for the two isolates each with two identical mutations, although no data are available. It is reported that *rrsC* 1400 A → G mutation is the main mechanism involved in resistance to other aminoglycosides (amikacin and kanamycin) in *M. tuberculosis* [24]. It is interesting that the double mutations result in a higher MIC, although the numbers are small. Further study will be required to clarify the mechanism to undergo double mutations. In this study, 14.8% (17/115) of streptomycin-resistant isolates revealed no mutation in the *rpsL* or *rrs* gene. This observation implies that there is at least one additional mechanism conferring streptomycin resistance, and that future molecular genetic studies should be aimed at identifying the gene(s) involved. Recently, Okamoto *et al.* [25] reported that mutations within the *gidB* gene, which encodes a conserved 7-methylguanosine methyltransferase specific for the 16S rRNA, played a role in the mechanism of streptomycin resistance. In fact, two of the 17 clinical isolates in this study that were streptomycin-resistant but with no *rpsL* or *rrs* mutation were found to harbor *gidB* gene mutation (DHPLC results not shown), and the results were also confirmed by DNA sequencing and therefore further study is needed. Apart from this, a growth inhibition experiment [17] has revealed that membrane-active agents such as Tween 80 are capable of reducing the level of resistance significantly by approximately 10-fold, supporting a hypothesis that membrane permeability may play a role in streptomycin resistance. It seems entirely possible that a combination of different resistance mechanisms may operate in a drug-resistant clinical isolate. Therefore, for example, a permeability barrier would be expected to increase the level of resistance of isolates with an altered *rpsL* or *rrs* gene. Other factors, for example a membrane efflux pump [26,27], may have some relationship to streptomycin resistance. In order to further explore the molecular mechanism of streptomycin, we have also sequenced the streptomycin-dependent strain 18b. As is the case for *E. coli*, *M. tuberculosis* also has three streptomycin phenotypes: sensitive, resistant and dependent. Streptomycin-dependent strain 18b was isolated in Japan in 1955 by Hashimoto [28], and in 1995 Cole [29] found that it possessed

a novel mutation in the 530 loop of the 16s rRNA: insertion of an additional cytosine between 512 C and 513 A. In the present study, we found a second mutation in its 16s rRNA: a 645 A deletion. How streptomycin has helped to stabilize the conformational structure of 16s rRNA and S12 ribosomal protein and eventually become a necessary component for the process of translation is not clear. The mechanism responsible for the streptomycin dependence of 18b still remains to be discovered.

Acknowledgments

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**Retinoic Acid Therapy Attenuates the Severity
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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 $\mu\text{g}/100\text{ g}$ 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 IFN γ 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 TNF α and IL-1 β 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 IFN γ -induced IFN regulatory factor (IRF)-1 transcription factor by affecting multiple components of the IFN γ -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, bronchoalveolar 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/CrlCrj), 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 Acrodisc 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 IFN γ (Rn00594078_m1), TNF α (Rn00562055_m1), inducible nitric oxide synthetase (iNOS) (Rn00561646_m1), IL-1 β (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 TGF β (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 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 bronchoalveolar 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/δ T-cell). Thereafter, the cells were fixed in 2% paraformaldehyde/PBS, examined with a FACS (FACSCAN, BD), and

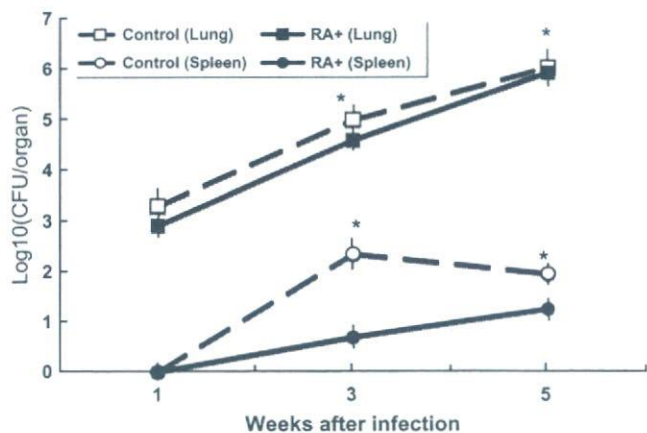


FIGURE 1 RA administration (RA+) significantly decreased lung and spleen CFU in *M. tuberculosis* H37Rv-infected rats. Values are means \pm SE, $n = 3$. *Different from RA+ at that time, $P < 0.005$.

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

Statistical methods. Values are means \pm 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. 2A vs. 2D) and fewer acid-fast bacilli in the lesions than control rats (Fig. 2C vs. 2F). In control rat lung tissue, typical granulomas were formed, where many lymphocytes surrounded epithelioid cells (Fig. 2D,E). Although typical

granulomas were formed similarly in the lungs of RA-treated rats (Fig. 2A,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 IFN γ and iNOS mRNA differed with RA treatment at 5 wk, when expression was highest. For TNF α and IL-1 β mRNA, expression was affected by RA at both 3 wk and 5 wk. Expression of IL-2, IL-12 β R, 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 IFN γ , TNF α , iNOS, IL-1 β , 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 TGF β 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 IFN γ , TNF α , 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 $\times 135$ for A and D, $\times 270$ for B and E. C and F show Ziehl-Neelsen staining, magnification $\times 600$.

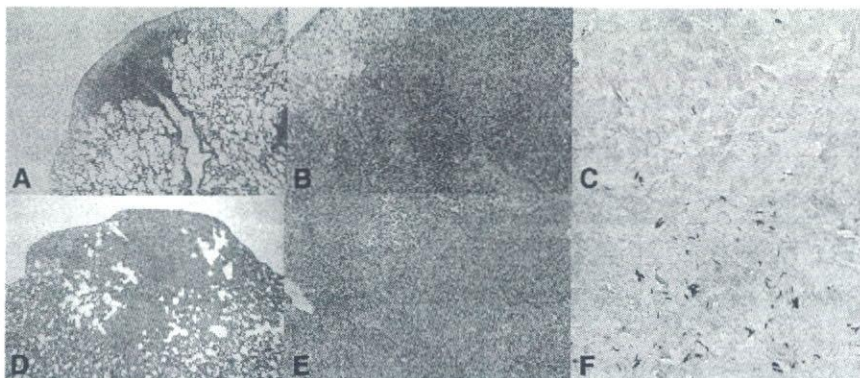


TABLE 1 Flow cytometric analysis of lung BAL cells from *M. tuberculosis* H37Rv-infected RA-treated (RA+) and control (RA-) rats 3 and 5 wk postinfection¹

Cell subpopulation	Relative intensity			
	3 wk after infection		5 wk after infection	
	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}

Cytokine or enzyme	Intensity (relative to GAPDH)	
	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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Involvement of Caspase-9 in the Inhibition of Necrosis of RAW 264 Cells Infected with *Mycobacterium tuberculosis*[▽]

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In order to know how caspases contribute to the intracellular fate of *Mycobacterium tuberculosis* and host cell death in the infected macrophages, we examined the effect of benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethane (z-VAD-fmk), a broad-spectrum caspase inhibitor, on the growth of *M. tuberculosis* H37Rv in RAW 264 cells. In the cells treated with z-VAD-fmk, activation of caspase-8, caspase-3/7, and caspase-9 was clearly suppressed, and DNA fragmentation of the infected cells was also reduced. Under this experimental condition, it was found that the treatment markedly inhibited bacterial growth inside macrophages. The infected cells appeared to undergo cell death of the necrosis type in the presence of z-VAD-fmk. We further found that z-VAD-fmk treatment resulted in the generation of intracellular reactive oxygen species (ROS) in the infected cells. By addition of a scavenger of ROS, the host cell necrosis was inhibited and the intracellular growth of H37Rv was significantly restored. Among inhibitors specific for each caspase, only the caspase-9-specific inhibitor enhanced the generation of ROS and induced necrosis of the infected cells. Furthermore, we found that severe necrosis was induced by infection with H37Rv but not H37Ra in the presence of z-VAD-fmk. Caspase-9 activation was also detected in H37Rv-infected cells, but H37Ra never induced such caspase-9 activation. These results indicated that caspase-9, which was activated by infection with virulent *M. tuberculosis*, contributed to the inhibition of necrosis of the infected host cells, presumably through suppression of intracellular ROS generation.

Tuberculosis caused by *Mycobacterium tuberculosis* is still a serious threat to human health at the global level. It has been estimated that one-third of the world's population are infected, and 8 million people develop active tuberculosis every year (15, 29). A number of studies have been carried out to identify the pathogenic determinants of *M. tuberculosis*, and various candidate molecules that may contribute to mycobacterial virulence have been reported (7). However, the molecular mechanisms for the virulence still remain unclear.

Macrophages play a role in the first line of host defense against bacterial infection by exerting microbicidal activity and contribute to the development of protective T cells as antigen-presenting cells through production of cytokines, including interleukin-12 (IL-12) and IL-18 (26). However, *M. tuberculosis* is capable of modulating such host response and survives inside macrophages (15). Therefore, some type of host response in the infected cell itself is necessary to control the replication of *M. tuberculosis* in the initial phase of infection. There are several reports indicating that induction of early death of infected cells is an important and alternative strategy for host defense against *M. tuberculosis*. For instance, it has been shown that macrophages go into apoptosis upon infection with *M. tuberculosis* in a caspase-dependent manner, resulting in the suppression of intracellular bacterial replication, and that ar-

rest of macrophage apoptosis conversely enhances bacterial growth (22, 28). Furthermore, it has been reported that the apoptotic vesicles formed in the infected macrophages have an important role in transporting the mycobacterial antigen to dendritic cells and developing cellular immunity against *M. tuberculosis* (25). These results suggest that apoptosis of the infected cells constitutes an important part of the host resistance and affects the fate of intracellular *M. tuberculosis*. To date, the intracellular cascade of apoptosis has been characterized well and various caspases are known to be involved in apoptosis induction (21).

Caspases are synthesized as biologically inactive precursors and converted into active forms by sequential proteolytic cleavage. The activation process is regulated by various intracellular components and is under strict control. Upon apoptosis, which is a form of innate immunity against bacteria, however, it appears that *M. tuberculosis* exerts resistance by modification of the activation cascade of caspases in the cells where it resides. Sly et al. have recently reported that virulent *M. tuberculosis* strains cause less apoptosis than attenuated strains by induction of macrophage antiapoptotic *mcl-1* gene expression (28). Balcewicz-Sablinska et al. have also shown that *M. tuberculosis* H37Rv inhibits apoptosis of infected macrophages by IL-10-dependent release of a soluble tumor necrosis factor (TNF) receptor that inactivates TNF- α (2). These findings suggest that though apoptosis is coupled with killing of intracellular *M. tuberculosis*, the bacterium possesses a virulence-associated ability to evade apoptosis.

In addition to apoptosis, it has been shown that *M. tubercu-*

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lisis triggers necrosis of infected macrophages. Unlike apoptosis, it appears that necrosis does not interfere with the survival of intracellular *M. tuberculosis*. Moreover, it is supposed that *M. tuberculosis* ultimately escapes macrophages by inducing necrosis, and necrotic cell death provides the nutrient source for *M. tuberculosis* in granuloma (30). Park et al. have shown that virulent clinical strains rapidly grow inside macrophages and induce necrosis of infected macrophages (20). Hsu et al. have demonstrated that an attenuated mutant of *M. tuberculosis* H37Rv failed to induce necrosis of infected macrophages (14). These results suggest that virulence of *M. tuberculosis* is associated with the ability to manipulate not only apoptosis but also necrosis of infected macrophages. However, little is known about the regulatory mechanism of apoptosis and necrosis or the relationship between *M. tuberculosis*-induced caspase activation and the fate of intracellular bacteria.

In this study, we employed various caspase inhibitors and examined their effects on the intracellular growth of a virulent H37Rv strain in macrophage-like RAW 264 cells. Unexpectedly, it was found that inhibition of caspases resulted in the necrosis of H37Rv-infected cells and our analysis revealed that the activation of caspase-9 is involved critically in the inhibition of necrosis. Furthermore, we found that H37Ra did not induce either necrosis of infected cells or activation of caspase-9. It was suggested that virulent *M. tuberculosis* strains avoid excessive necrosis of infected host cells by inducing caspase-9 activation.

MATERIALS AND METHODS

Reagents. Benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethane (z-VAD-fmk; an inhibitor of various caspases) and acetyl-Tyr-Val-Ala-Asp-chloromethane (a caspase-1 inhibitor) were purchased from Peptide Institute, Inc. (Osaka, Japan). Other inhibitors, including benzyloxycarbonyl-Val-Asp(OMe)-Val-Ala-Asp(OMe)-fluoromethane (a caspase-2 inhibitor), benzyloxycarbonyl-Asp(OMe)-Gln-Met-Asp(OMe)-fluoromethane (a caspase-3 inhibitor), benzyloxycarbonyl-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethane (a caspase-8 inhibitor), benzyloxycarbonyl-Leu-Glu(OMe)-His-Asp(OMe)-fluoromethane (a caspase-9 inhibitor), and benzyloxycarbonyl-Phe-Ala-fluoromethylketone (z-FA-fmk; an inactive caspase inhibitor analogue), were purchased from Sigma Aldrich (St. Louis, MO), Merck Biosciences, Inc. (San Diego, CA), Techne Corporation (Minneapolis, MN), R & D Systems, Inc. (Minneapolis, MN), and Calbiochem (San Diego, CA), respectively. 3(2)-*t*-Butyl-4-hydroxyanisole (BHA) and 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) were obtained from Wako Pure Chemical Industries (Osaka, Japan) and Molecular Probes (Eugene, OR), respectively. Rabbit anti-mouse caspase-9 antibody was obtained from Cell Signaling Technology, Inc. (Danvers, MA).

Bacteria. The *M. tuberculosis* H37Rv and H37Ra strains maintained in our laboratory were grown at 37°C to mid-log phase in Middlebrook 7H9 broth (Becton Dickinson Microbiology Systems, Sparks, MD) supplemented with 0.5% albumin, 0.2% dextrose, 3 µg/ml catalase, and 0.2% glycerol. H37Rv was harvested and stirred vigorously with glass beads to disperse the bacterial clumps and stood for 30 min. An upper part of the suspension without visible clumps was collected and stored at -80°C in aliquots. After being thawed, the bacterial suspension was centrifuged at 150 × *g* for 3 min to remove clumps, and only the upper part of the suspension was used for the experiments to ensure an even infection of each cell. Viable bacteria were enumerated by plating the diluted suspension on Middlebrook 7H10 agar plates containing 50 µg/ml oleic acid, 0.5% albumin, 0.2% dextrose, 4 µg/ml catalase, and 0.85 mg/ml sodium chloride and counting the number of colonies 3 weeks after incubation at 37°C.

Measurement of intracellular bacterial growth. RAW 264 cells were seeded in 24-well microplates at 1.0×10^5 cells/well and incubated for 12 h at 37°C in 5% CO₂ in a culture medium consisting of RPMI 1640 medium supplemented with 10% fetal bovine serum and 5 µg/ml of gentamicin. Cells were washed and infected with 5×10^5 CFU of H37Rv for 4 h. After three washes with the culture medium for removal of extracellular bacteria, the cells were cultured for 7 days in the presence or absence of various caspase inhibitors and/or BHA. Cells were

lysed in 0.05% Triton X-100 solution, and the number of viable bacteria in each well was determined by plating the lysate on Middlebrook 7H10 agar plates. In one experiment, thioglycolate-induced peritoneal macrophages (1.0×10^5 cells) were infected with H37Rv and the intracellular bacterial number was determined 7 days later.

Detection of DNA fragmentation. Two days after infection at a multiplicity of infection (MOI) of 5, 5×10^6 cells were lysed in a lysis buffer consisting of 10 mM Tris-HCl (pH 7.6), 0.15 M NaCl, 5 mM MgCl₂, and 0.5% Triton X-100. Intact nuclei were collected by centrifugation at 1,000 × *g* for 5 min, suspended in 10 mM Tris-HCl (pH 7.6) buffer containing 0.4 M NaCl, 1 mM EDTA, and 1% Triton X-100, and centrifuged at 12,000 × *g* for 15 min to segregate the nucleoplasm from high-molecular-weight chromatin. The semipurified nucleoplasm was consecutively incubated at 37°C with 20 µg/ml of RNase for 1 h and 100 µg/ml of proteinase K for 2 h. DNA was extracted with the phenol-chloroform method and electrophoresed on a 1.4% agarose gel. After being stained with ethidium bromide, DNA was visualized on a UV transilluminator. Alternatively, oligonucleosomes were quantified by using a Cell Death Detection ELISA^{PLUS} kit (Roche Diagnostics, Penzberg, Germany) according to the manufacturer's protocol. The degree of DNA fragmentation was expressed as an arbitrary unit calculated by the following formula: arbitrary unit = (A_{405} of experimental group - A_{405} of negative control [medium only]) / (A_{405} of untreated cells - A_{405} of negative control).

Flow cytometric analysis. RAW 264 cells were collected 2 and 4 days after infection and washed with phosphate-buffered saline (PBS) containing 0.2% albumin. Cells were incubated with 0.2 mM propidium iodide (PI; Molecular Probes, Eugene, OR) for 10 min on ice in the dark, washed, and fixed with 1% paraformaldehyde in PBS. Fluorescence intensity was analyzed by FACSCalibur (BD Biosciences, San Jose, CA). In order to detect intracellular reactive oxygen species (ROS), RAW 264 cells infected with H37Rv 2 days before were incubated with 5 µM DCFH-DA for 15 min at 37°C. DCFH-DA diffused into cells and was hydrolyzed to DCFH (2', 7'-dichlorodihydrofluorescein). Cells were detached from culture plates, and the fluorescence intensity of DCFH, which was converted into oxidized form by intracellular ROS, was analyzed by FACSCalibur according to a method described previously (3).

Detection of LDH. RAW 264 cells and peritoneal macrophages were infected with H37Rv or H37Ra, and the culture supernatants were collected 2 and 4 days later. The amount of lactate dehydrogenase (LDH) released from the infected cells was measured using an LDH cytotoxicity detection kit (TaKaRa BIO Inc., Shiga, Japan). The percentage of LDH release was calculated according to the following formula: percent release = 100 × (experimental LDH release - spontaneous LDH release) / (maximal LDH release - spontaneous LDH release). A value for maximal LDH release was obtained from the supernatant of cells treated with 1% Triton X-100.

Transmission electron microscopy. RAW 264 cells were infected with H37Rv for 3 days. The cells were washed twice with PBS and once with 0.1 M cacodylic acid buffer and fixed with 2.5% glutaraldehyde in 0.1 M cacodylic acid buffer. After fixation, the cells were treated with 2% osmium tetroxide in 0.1 M cacodylic acid buffer, dehydrated by treatment with graded ethanol solutions, and embedded in Quetol-812 resin mixture-embedding media. The ultrathin sections were stained with uranyl acetate and lead citrate and examined with a JEOL model JEM-1200EX electron microscope. The percentage of cells undergoing apoptosis or necrosis was estimated by investigating the morphologies of 100 cells in each experimental group.

Measurement of caspase activities. RAW 264 cells were lysed 1 and 2 days after infection, and the caspase-8, caspase-3 and/or -7, and caspase-9 activities in the cleared lysate were measured by using Caspase-Glo 8, Caspase-Glo 3/7, and Caspase-Glo 9 assays (Promega Corporation, Madison, WI) according to the manufacturer's protocols.

Statistical analysis. Student's *t* test was used to determine the statistical significance of the values obtained, and *P* values of <0.05 were considered statistically significant.

RESULTS

Effect of z-VAD-fmk on intracellular growth of H37Rv. In order to determine the effect of inhibition of caspase activities on the intracellular growth of H37Rv in RAW 264 cells, we first infected the cells with H37Rv at an MOI of 5 in the presence or absence of z-VAD-fmk and monitored the number of intracellular bacteria. H37Rv replicated slowly in RAW 264 cells in the absence of z-VAD-fmk (Fig. 1). A similar pattern of