ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 1985, p. 921–924
0066-4804/85/060921-04$02.00/0

Vol. 27, No. 6

Alteration of Ribosomes and RNA Polymerase in Drug-Resistant Clinical Isolates of *Mycobacterium tuberculosis*

TAKESHI YAMADA,* AKIHISA NAGATA, YASUKO ONO, YASUHIKO SUZUKI, AND TAKAHISA YAMANOUCHI

Research Institute for Microbial Diseases, Osaka University, Yamada-Oka 3-1, Suita City, Osaka 565, Japan

Received 20 December 1984/Accepted 22 March 1985

The biochemical mechanism of resistance to kanamycin, viomycin, and rifampin in five clinical isolates of *Mycobacterium tuberculosis* was studied. Resistance to viomycin and kanamycin was attributed to altered ribosomes, whereas resistance to rifampin was attributed to an alteration of RNA polymerase. Ribosomal resistance was, however, not the only way of expressing resistance to viomycin and kanamycin.

The majority of drug-resistant, gram-negative and gram-positive bacilli isolated from patients are known to possess plasmids which confer drug resistance, and a recent report has suggested that ribosomal resistance to streptomycin (SM) is a significant cause of high-level resistance among clinical enterococcal isolates (3).

The contribution of altered RNA polymerase to rifampin (RIF) resistance has been suggested with in vitro-developed mutants of *Mycobacterium smegmatis* (17) and *Mycobacterium bovis* BCG (4). Ribosomal resistance to kanamycin (KM) and viomycin (VM) was demonstratd with in vitro-developed drug-resistant mutants of *M. smegmatis* (12–15). The cell-free polypeptide-synthesizing system derived from a SM-resistant mutant isolated from *Mycobacterium tuberculosis* H37Rv was shown to be resistant to SM (9).

With respect to clinical isolates of mycobacteria, a few reports have suggested the involvement of plasmids in the resistance of *Mycobacterium intracellulare* to drugs (2, 6, 8), and one report indicated that a strain of *M. tuberculosis* isolated from a patient possessed an enzyme that inactivated KM (5). Further studies are necessary to obtain a general view of the biochemical mechanism of drug resistance in clinical isolates of pathogenic *M. tuberculosis*. In this paper, we present the results of biochemical analysis of six *M. tuberculosis* strains isolated from patients.

MATERIALS AND METHODS

Reagents and antibiotics. The sources of reagents, VM, KM, and SM used in this study are as described previously (12). RIF was purchased from Sigma Chemical Co.

Strains and culture. The drug-susceptible and -resistant strains of *M. tuberculosis* were obtained from inpatients at Toneyama National Hospital, Osaka, Japan, from H. Maeda and Y. Yamamura. Of 20 strains, all except strain TY0 were resistant to all or some of the drugs SM, KM, VM, capreomycin, RIF, isoniazid, *p*-aminosalicylic acid, ethionamide, cycloserine, and ethanbutol. Five of the drug-resistant strains were chosen randomly for the study. Each strain was purified as follows. The organisms were cultured in Kirchner liquid medium (16) at 37°C. Portions of the culture were streaked on 30 ml of whole egg medium consisting of 200 ml of whole egg, 1 g of potassium phosphate (monobasic), 1 g of sodium glutamate, 6 ml of glycerol, 6 ml of 2% malachite green, and 100 ml of distilled water. After incubation for more than 2 weeks, isolated colonies were obtained. These procedures were repeated twice. The drug-susceptible strain TY0 and drug-resistant strains TY4, TY6, TY8-1, TY8-2, and TY18 were thus obtained. TY8-1 and TY8-2 were isolated from the same patient. Each of the other strains was isolated from a different patient. Strains TY4, TY6, TY8-1, and TY8-2 were further purified by cultivation in Kirchner liquid medium containing VM at a concentration of 10 to 20 μg/ml, after they were confirmed to be VM-resistant isolates. Sauton medium, used for harvesting the cells, contained 4.0 g of asparagine, 2.8 g of sodium citrate, 0.5 g of potassium phosphate (dibasic), 0.5 g of magnesium sulfate, 0.05 g of ammonium ferric citrate, and 60 ml of glycerol in 1,000 ml (pH 7.2).

MICs. MICs were determined in Kirchner liquid medium. The concentrations of antibiotics used were 1.25, 2.5, 5.0, 10, 20, and 40 μg/ml. A 0.10-mg amount (wet weight) of each strain was inoculated into 5 ml of the medium. The extent of growth was examined after 2 weeks of incubation at 37°C. The lowest concentration of antibiotic for which no visible growth was observed was expressed as the MIC.

Ribosomes and supernatant fluid. Organisms were cultured on the surface of Sauton liquid medium at 37°C without shaking. They were harvested by filtration on filter paper (Toyo, Tokyo, Japan) before the growth reached maximum after about 3 weeks. After being washed with 10 mM Tris-hydrochloride buffer (pH 7.8) containing 60 mM ammonium chloride, 6 mM 2-mercaptoethanol, and 10 mM magnesium acetate, the cells were suspended in twice their wet weight of the same buffer and disrupted by sonic oscillation for 1 min. The experimental conditions for sonication were as described previously (13). Sonication was repeated three more times at 4 to 13°C. Cell debris was removed by centrifugation at 20,000 × *g* for 20 min. The resulting crude extract was centrifuged in the same way one more time. Ribosomes and the supernatant fluid were separated by centrifuging the crude extract at 104,500 × *g* for 120 min. The supernatant fraction was dialyzed against 10 liters of the same buffer.

Poly(U)-directed polyphenylalanine synthesis. Experimental conditions for polyphenylalanine synthesis were as described previously with a few modifications (10, 13). The standard reaction mixture (0.1 ml) contained the following: 95 mM Tris-hydrochloride (pH 7.8), 5.8 mM 2-mercaptoethanol, 7.5 mM phosphoenolpyruvate trisodium salt, 0.9 mM adenosine triphosphate, 0.028 mM guanosine triphosphate, 0.1 mM [^{14}C]phenylalanine (specific activity, 100 μCi/5.5 μmol), 48 mM NH_4Cl; 1.5 μg of phosphoenolpyruvate

* Corresponding author.

kinase, 10 μl of supernatant fluid (absorbance at 280 nm, 20), 3 A_{260} units of ribosomes, 100 μg of polyuridylate [poly(U)], 50 μg of *Escherichia coli* transfer RNA, and 10 mM magnesium acetate. After incubation at 37°C for 45 min, the reaction was stopped by adding 1 ml of 10% trichloracetic acid (TCA), and the mixture was incubated for an additional 30 min at 90°C. The resulting acid-insoluble material was collected on membrane filters (glass-fiber paper, Whatman paper chromedia, GE 83; Whatman Inc.). Radioactivity was counted in a liquid scintillation spectrometer.

Purification of RNA polymerase. The supernatant fraction was loaded on a DE52-cellulose column (bed volume, 20 ml) equilibrated with TGED buffer (50 mM Tris-hydrochloride [pH 7.9], 0.5 mM EDTA, 25% [vol/vol] glycerol, 1 mM dithiothreitol). The column was washed with 40 ml of TGED buffer containing 0.1 M NaCl and then eluted with 80 ml of a linear gradient (0.1 to 0.6 M NaCl in TGED buffer) at a flow rate of 1.5 ml/30 min. The activity of RNA polymerase in each fraction was determined by using calf thymus DNA as a template. The fractions containing RNA polymerase were collected and dialyzed against 10 mM Tris-hydrochloride buffer (pH 7.8) containing 10 mM magnesium-acetate, 0.1 mM EDTA, 1 mM dithiothreitol, 0.2 M NH_4Cl, and 5% glycerol. The sample was loaded on 11 ml of glycerol gradient (15 to 35%) in the same buffer. After centrifugation at 37,000 rpm in a Beckman SW41 rotor for 16 h at 4°C, 0.2-ml fractions were collected from the bottom of the tube. The fractions containing RNA polymerase were collected and then dialyzed against 10 mM Tris-hydrochloride buffer (pH 7.8) containing 10 mM magnesium-acetate, 0.1 mM dithiothreitol, 0.1 M NH_4Cl, 50% (vol/vol) glycerol, and 0.1 mM EDTA. The enzyme was stored at −20°C until use.

RNA synthesis in a cell-free system. For the in vitro assay of transcription catalyzed by purified RNA polymerase, the reaction mixture (0.105 ml) contained 40 mM Tris-hydrochloride buffer (pH 7.8), 6 mM 2-mercaptoethanol, 60 mM NH_4Cl, 10 mM magnesium acetate, 0.2 mM Na_2EDTA, 2.2 mM ATP, 0.3 mM each of GTP, CTP, and [^{3}H]UTP (0.4 mCi/μmol), 1 mM K_2HPO_4, 4 μg of plasmid colicine E1 DNA, and 1 μg of RNA polymerase. After incubation of the reaction mixture at 37°C for 20 min, the reaction was halted by treatment with 5% trichloroacetic acid solution at 0°C for 30 min, and the reaction mixture was filtered slowly through glass-fiber paper. The residue on the filter was washed with 5% trichloroacetic acid solution, dried, and counted in a scintillation counter. For the preparation of plasmid DNA, a cleared lysate was prepared according to the method of Clewell and Helinski (1). The plasmid DNA was extracted with chloroform-isoamyl alcohol (24:1), precipitated with ethanol, and purified by ethidium bromide-CsCl density gradient centrifugation. The purified DNA was dialyzed against 10 mM Tris-hydrochloride (pH 7.8) containing 1 mM Na_2EDTA.

RESULTS

MICs of various antibiotics. MICs of VM, KM, SM, and RIF for strains TY0, TY4, TY6, TY8-1, TY8-2, and TY18 are shown in Table 1. The drug-susceptible strain TY0 was inhibited by SM, KM, VM, and RIF at concentrations of 1.25, 2.5, 5, and 1.25 μg/ml, respectively. Strains TY8-1 and TY8-2, which were isolated from the same patient, responded differently to the antibiotics. Strain TY8-2 was resistant to KM, whereas strain TY8-1 was susceptible to KM. This observation indicates that strains varying in susceptibility may exist in an individual patient. All of the strains were analyzed biochemically.

TABLE 1. MICs of antibiotics for clinical isolates of *M. tuberculosis*[a]

Strain	MICs (μg/ml)			
	SM	KM	VM	RIF
TY0	1.25	2.5	5	1.25
TY4	20	>40	40	10
TY6	2.5	2.5	40	1.25
TY8-1	2.5	2.5	40	2.5
TY8-2	1.25	>40	40	1.25
TY18	2.5	2.5	5	40

[a] Experimental procedures were as described in the text.

Characterization of strain TY4. A poly(U) system containing ribosomes and supernatant fluid from strain TY0 was prepared and tested for susceptibility to VM, KM, and SM. Poly(U)-directed polyphenylalanine synthesis was almost completely inhibited by VM at 1 μg/ml, and approximately 80% of polypeptide synthesis was inhibited by KM at 5 μg/ml. On the other hand, SM was not found to inhibit poly(U)-directed polyphenylalanine synthesis, for an unknown reason, although it was active in the culture (Table 1). Therefore, our efforts were focused on determining the susceptibility of the system to VM and KM. A similar system from strain TY4 was tested, and the susceptibility to the drug was compared to that from strain TY0. The in vitro system from strain TY4 was found to be resistant to these antibiotics (data not shown).

To determine whether the drug resistance was due to an alteration of ribosomes or of the supernatant fraction, these two components were exchanged between strains TY0 and TY4. Each system was tested for susceptibility to VM and KM. The results (Table 2) showed that drug resistance was determined by the ribosome source. The system composed of susceptible ribosomes and resistant supernatant fluid was inhibited strongly by 1 μg of VM per ml, as was the system in which both supernatant fluid and ribosomes were susceptible (homologous susceptible). On the other hand, the system composed of resistant ribosomes and susceptible supernatant fluid was resistant to VM, as was the system in which both supernatant fluid and ribosomes were resistant (homologous resistant). Similarly, the susceptibility of each system to KM was determined. It can be seen that an alteration of ribosomes is responsible for KM resistance.

The in vitro RNA-synthesizing system was examined for susceptibility to RIF. The results are presented in Table 3. RNA synthesis was almost completely inhibited by 1 and 5 μg of RIF per ml in the system containing RNA polymerase from the susceptible strain, whereas no significant inhibition by the same concentrations of the antibiotic was seen in the system derived from strain TY4. We concluded that an alteration of RNA polymerase in strain TY4 is responsible for the resistance to RIF.

Characterization of strain TY6. Strain TY6 was susceptible to RIF and KM but resistant to VM (Table 1). The location of the resistance to VM was examined in the poly(U)-directed polyphenylalanine-synthesizing system. Ribosomes and supernatant fluid were exchanged in various combinations. The effects of VM and KM on the systems were tested, and the results are presented in Table 2. The homologous system from susceptible strain TY0 was inactive in the presence of 1 μg of VM per ml, whereas less inhibition in the similar system from resistant strain TY6 occurred in the presence of the same concentration of VM. From the results with interchanging ribosomes and supernatants, it was concluded that an alteration of ribosomes was responsible for

TABLE 2. In vitro susceptibility of *M. tuberculosis* to drugs in the poly (U) system[a]

Source of:		Drug concn (μg/ml)		cpm (%) incorporated[b] by the following resistant strain:			
Rb	Sup	VM	KM	TY4	TY6	TY8-2	TY8-1
S	S	0	0	13,844 (100)	10,956 (100)	7,633 (100)	22,422 (100)
		1	0	252 (2)	320 (3)	228 (3)	428 (2)
		0	5	2,785 (20)	1,298 (12)	1,527 (20)	3,927 (18)
R	R	0	0	16,370 (100)	12,964 (100)	6,877 (100)	15,124 (100)
		1	0	7,997 (49)	4,598 (35)	1,078 (16)	994 (2)
		0	5	17,255 (105)	2,014 (16)	2,059 (30)	2,439 (18)
S	R	0	0	16,421 (100)	7,592 (10)	6,528 (100)	8,305 (100)
		1	0	414 (3)	199 (3)	169 (3)	188 (2)
		0	5	3,525 (21)	1,010 (13)	1,306 (20)	1,568 (18)
R	S	0	0	12,746 (100)	8,332 (100)	5,396 (100)	22,270 (100)
		1	0	5,105 (40)	3,222 (39)	748 (14)	1,771 (8)
		0	5	13,004 (102)	1,150 (14)	1,673 (31)	3,417 (15)

[a] Experimental procedures were as described in the text. S and R are susceptibility and resistance, respectively. S, strain TY0. R, strains, TY4, TY6, TY8-2, and TY8-1. Rib and Sup are ribosome and supernatant fractions, respectively.

[b] The radioactivity (100 to 200) in the absence of poly(U) was subtracted from the radioactivity of each reaction. Values given are averages of duplicates. An incorporation value of 100% corresponds to that observed in respective systems in the absence of VM. Incorporation on susceptible ribosomes from TY0 in the presence of 1 μg of VM per ml and 5 μg of KM per ml ranged from 0 to 3% and 12 to 20% of the control, respectively.

VM resistance. In contrast, each system was equally inhibited by 5 μg of KM per ml, as expected from the results in Table 1.

Characterization of strains TY8-2 and TY8-1. Strain TY8-2 was susceptible to RIF, but resistant to VM and KM (Table 1). Indeed, RNA polymerase derived from strain TY8-2 was as susceptible to RIF as was that from susceptible strain TY0 (data not shown). Ribosomes and supernatant fluid were exchanged between susceptible strain TY0 and resistant strain TY8-2, and the effect of VM and KM on poly(U)-directed polyphenylalanine synthesis was examined. The results are presented in Table 2. Polypeptide synthesis in the system consisting of ribosomes from strain TY0 and supernatant fluid from strain TY8-2 was almost completely inhibited at a VM concentration of 1 μg/ml, as was the homologous susceptible system. In contrast, the system consisting of sensitive supernatant fluid and ribosomes from strain TY8-2 was resistant to inhibition by VM, as was the homologous resistant system. The degree of resistance, however, was lower than that of strain TY4 and strain TY6. Regarding the KM sensitivity, no clear-cut result was obtained, though a tendency for KM resistance to be associated with ribosomes from the resistant strain was reproducibly observed.

Strain TY8-1 was resistant to VM but susceptible to KM and RIF. Ribosomes from strain TY8-1 were examined for susceptibility to KM and VM in the poly(U) system. The results are presented in Table 2. Poly(U)-directed polyphenylalanine synthesis with ribosomes from strain TY8-1 was strongly inhibited by 1 μg of VM per ml.

Characterization of strain TY18. Strain TY18 was susceptible to KM and VM but resistant to a high concentration of RIF (Table 1). RNA polymerase was purified from the strain and tested for susceptibility to RIF in the cell-free system. The results in Table 3 clearly show that the resistance is due to an alteration of RNA polymerase.

DISCUSSION

Despite a previous report that a KM-resistant strain of *M. tuberculosis* possesses the enzyme(s) for inactivating the drug by acetylation (5), there are good reasons to believe that chromosomal mutation and the resulting alteration of the cellular component might commonly be responsible for *M. tuberculosis* drug resistance in patients. First, a mutant resistant to multiple drugs can usually be isolated only after therapy with the individual drugs. Second, to acquire a plasmid conferring a drug-inactivating enzyme, tubercle bacilli carrying a plasmid must be newly inhaled (exogenous reinfection). However, such an exogenous reinfection cannot be assumed to be the usual case from the immunological standpoint. Rather, endogenous reinfection, which is caused by organisms which are present and have survived in the primary lesion of the lung of the patient, are considered to be common.

However, it must be stressed that arguments mentioned above do not exclude the possible involvement of R factors in some cases.

TABLE 3. In vitro susceptibility of purified RNA polymerase from *M. tuberculosis*[a] to RIF

Source of RNA polymerase	RIF concn (μg/ml)	Incorporation	
		cpm	%
TY0	0	2,688	100
	0.5	2,015	75
	1.0	298	11
	5.0	112	4
TY0	0	4,240	100
	0.1	2,394	56
	0.5	219	5
	1.0	188	4
TY4	0	4,765	100
	0.5	3,629	76
	1.0	5,414	113
	5.0	4,627	97
TY18	0	5,097	100
	1.0	5,079	100
	5.0	5,442	107
	10.0	5,603	110

[a] Experimental procedures were as described in the text.

In this paper, we present evidence that drug resistance was due to alterations of ribosomes and RNA polymerase. VM resistance of four isolates was due to altered ribosomes. The degree of VM resistance in vitro was different among the strains, although they were equally resistant to VM in the culture. A possible explanation for these observations might be that ribosomal alteration is not the only way to acquire VM resistance, and mutation in the gene coding for permeability to the drug may have occurred.

Genetic and biochemical analysis of the mutants from *M. smegmatis* revealed that there are two loci (*vicA* and *vicB*) for VM resistance which specify the structure of the ribosome, one for 30S and the other for 50S ribosomal subunits (7, 12, 13, 15). There is one more locus for VM resistance designated as *vicC* which might confer a change of permeability (11). These observations on *M. smegmatis* are consistent with the considerations discussed above regarding *M. tuberculosis*.

High-level resistance to KM was due to the alteration of ribosomes in the case of strain TY4. In contrast, ribosomes from strain TY8-2 were susceptible to KM in the cell-free system, whereas the strain was highly resistant to the drug in the culture. The experimental results suggest that either a change in permeability or acetylating enzyme may be responsible for KM resistance.

The ribosomal proteins were extracted from strain TY4 and analyzed by two-dimensional electrophoresis. At least two proteins were found to be altered. This data will be presented elsewhere.

Alteration of RNA polymerase was demonstrated in the system containing the highly purified enzyme. Therefore, there is no ambiguity regarding the existence of the drug-inactivating enzyme. The molecular characterization will be reported elsewhere.

We can therefore suggest that a variety of biochemical mechanisms may be concerned in the phenotypic expression of drug resistance in clinical isolates of *M. tuberculosis*. Furthermore, selection of multi-step mutations in patients during the course of chemotherapy can be proposed as a common mechanism.

LITERATURE CITED

1. **Clewell, D. B., and D. R. Helinski.** 1969. Supercoiled circular DNA-protein complex in *E. coli*: purification and induced conversion to an open circular DNA form. Proc. Natl. Acad. Sci. U.S.A. **62**:1159–1166.
2. **Crawford, J. T., and J. H. Bates.** 1979. Isolation of plasmids from mycobacteria. Infect. Immun. **24**:979–981.
3. **Eliopoulos, G. M., B. F. Farber, B. E. Murray, C. Wennersten, and R. C. Moellering, Jr.** 1984. Ribosomal resistance of clinical enterococcal isolates to streptomycin. Antimicrob. Agents Chemother. **25**:398–399.
4. **Konno, K., K. Oizumi, and S. Oka.** 1973. Mode of action of rifampin on mycobacteria. Am. Rev. Respir. Dis. **107**:1006–1012.
5. **Mitsuhashi, S., T. Tanaka, H. Kawabe, and H. Umezawa.** 1977. Biochemical mechanism of kanamycin resistance in *Mycobacterium tuberculosis*. Microbiol. Immunol. **21**:325–327.
6. **Mizuguchi, Y., M. Fukunaga, and H. Taniguchi.** 1981. Plasmid deoxyribonucleic acid and translucent-to-opaque variation in *Mycobacterium intracellulare* 103. J. Bacteriol. **146**:656–659.
7. **Mizuguchi, Y., K. Suga, K. Masuda, and T. Yamada.** 1974. Genetic and biochemical studies on drug-resistant mutants in *Mycobacterium smegmatis*. Jpn. J. Microbiol. **18**:457–462.
8. **Mizuguchi, T., T. Udo, and T. Yamada.** 1983. Mechanism of antibiotic resistance in *Mycobacterium intracellulare*. Microbiol. Immunol. **27**:425–431.
9. **Shaila, M. S., K. P. Gopinathan, and T. Ramakrishnan.** 1973. Protein synthesis in *Mycobacterium tuberculosis* H37Rv and the effect of streptomycin in streptomycin-susceptible and -resistant strains. Antimicrob. Agents Chemother. **4**:205–213.
10. **Yamada, T., K. Kawaguchi, K. Masuda, K. Shoji, and M. Hori.** 1972. Inhibition of polyphenylalanine synthesis on ribosomes of mycobacteria by tuberactinomycin-N. Am. Rev. Respir. Dis. **106**:769–771.
11. **Yamada, T., K. Masuda, K. Kawaguchi, Y. Mizuguchi, and K. Suga.** 1976. Genetic and biochemical studies on drug-resistant *Mycobacterium smegmatis*. II. Analysis of drug-resistant ribosomes, p. 81–90. *In* E. Freerksen, I. Tarnok, and J. H. Thumim (ed.), Genetics of the *Actinomycetales*. Gustav Fischer Verlag, Stuttgart, Federal Republic of Germany.
12. **Yamada, T., K. Masuda, Y. Mizuguchi, and K. Suga.** 1976. Altered ribosomes in antibiotic-resistant mutants of *Mycobacterium smegmatis*. Antimicrob. Agents Chemother. **9**:817–823.
13. **Yamada, T., K. Masuda, K. Shoji, and M. Hori.** 1972. Analysis of ribosomes from viomycin-sensitive and -resistant strains of *Mycobacterium smegmatis*. J. Bacteriol. **112**:1–6.
14. **Yamada, T., K. Masuda, K. Shoji, and M. Hori.** 1974. Pleiotropic antibiotic resistance mutations associated with ribosomes and ribosomal subunits in *Mycobacterium smegmatis*. Antimicrob. Agents Chemother. **6**:46–53.
15. **Yamada, T., Y. Mizuguchi, K. H. Nierhaus, and H. G. Wittmann.** 1978. Resistance to viomycin conferred by RNA of either ribosomal subunit. Nature (London) **275**:460–461.
16. **Yamada, T., Y. Yamanouchi, Y. Ono, A. Nagata, T. Wakamiya, T. Teshima, and T. Shiba.** 1983. Antibacterial activity of palmitoyltuberactinamine N and di-β-lysylcapreomycin IIA. J. Antibiot. **36**:1729–1734.
17. **White, R. J., G. C. Lancini, and L. G. Silvestri.** 1971. Mechanism of action of rifampin on *Mycobacterium smegmatis*. J. Bacteriol. **108**:737–741.