ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 1996, p. 2452–2454 Vol. 40, No. 11
0066-4804/96/$04.00+0

Correlation of Molecular Resistance Mechanisms and Phenotypic Resistance Levels in Streptomycin-Resistant *Mycobacterium tuberculosis*

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Received 20 May 1996/Returned for modification 25 July 1996/Accepted 15 August 1996

Quantitative susceptibility testing of clinical isolates of streptomycin-resistant *Mycobacterium tuberculosis* demonstrated that there is a close correlation between the molecular resistance mechanism and the in vitro activity of streptomycin: mutations in *rpsL* were mainly associated with high-level resistance, mutations in *rrs* were associated with an intermediate level of resistance, and streptomycin-resistant isolates with wild-type *rpsL* and *rrs* exhibited a low-level resistance phenotype. Investigations of streptomycin-resistant isolates with wild-type *rpsL* and *rrs* revealed that (i) there is no cross-resistance to other drugs and (ii) a permeability barrier may contribute to resistance, because resistance was significantly lowered in the presence of a membrane-active agent.

Streptomycin, an aminocyclitol glycoside antibiotic, is commonly used for the treatment of tuberculosis. Its mechanism of action presumably is by interfering with translational proofreading, resulting in an inhibition of protein synthesis (3, 15, 17). Mutations associated with streptomycin resistance in *Mycobacterium tuberculosis* have been identified in the genes encoding 16S rRNA (*rrs*) and ribosomal protein S12 (*rpsL*) (2, 4–7, 10, 11, 13, 14). Most of the mutations in *rrs* map to the 530 region, a region which interacts with ribosomal protein S12 (5). Different hypotheses which link the conformational structure of the 530 region to translation fidelity have been put forward (1, 16, 18).

Approximately, one-third of drug-resistant clinical isolates were found to lack changes in *rpsL* or *rrs* (5, 6, 10, 13), indicating that alternate mechanisms of streptomycin resistance exist in *M. tuberculosis*. Drug-modifying enzymes or efflux mechanisms, both frequent causes of bacterial drug resistance, have not been observed in *M. tuberculosis*. Analysis of in vitro drug-resistant mutants of *M. tuberculosis* has suggested that a permeability barrier may contribute to drug resistance (19), prompting us to study membrane-active agents, such as Tween 80, along with the antibiotic in growth inhibition studies (8, 12).

The purpose of the study described here was (i) to characterize the level of resistance associated with the different molecular mechanisms and (ii) to investigate whether a permeability barrier contributes to drug resistance in streptomycin-resistant isolates with wild-type *rpsL* and wild-type *rrs*.

MATERIALS AND METHODS

The collection of strains studied included 4 streptomycin-susceptible isolates and 19 streptomycin-resistant isolates. The strains represent clinical isolates obtained from patients with tuberculosis. Resistance mutations were identified by sequence analysis of the complete coding region of *rpsL* and by screening the relevant regions of the 16S rRNA (corresponding *Escherichia coli* positions 1 to 120, 380 to 550, and 850 to 950) as described previously (5); for an overview of the strains investigated, see Table 1. The strains were propagated on Löwenstein-Jensen medium and were clonally purified by repeated (three times) single-colony isolation on Middlebrook 7H10 medium supplemented with 10% OADC (oleic acid-albumin-dextrose-citrate; Difco, Augsburg, Germany). All of the drug-resistant strains were found to be genetically stable and did not revert on repeated subculture in drug-free medium. Stock solutions of streptomycin (Sigma, Deisenhofen, Germany) were prepared in aqua dest, and were stored at −20°C. Kanamycin, gentamicin, amikacin, and paromomycin were obtained from Sigma.

The MICs of streptomycin were determined in quadruplicate by using four single colonies of each strain grown to the required density. A 0.1-ml aliquot of a suspension was used for inoculation; the suspension of the test organisms was adjusted to give heavy growth on the drug-free plate. The medium for drug susceptibility testing was Middlebrook 7H10 supplemented with OADC with and without Tween 80 at 0.06%. Streptomycin was added at the concentrations tested (0.06, 0.125, 0.25, 0.50, 1.0, 2.0, 6.0, 12.5, 25.0, 50.0, 250, 500, and 1,000 mg/liter) to the sterile, liquified medium at 43°C immediately before the medium was poured into the plates. The incubation temperature for growth was 37°C. Growth was judged visually and was recorded semiquantitatively, as follows: +++, heavy growth; ++, moderate growth; +, poor growth; ±, barely visible growth; −, no growth. The drug concentration which resulted in barely visible or no growth compared with the heavy growth on the antibiotic-free plate was considered the MIC. Resistance to streptomycin is defined as a MIC of >2 mg/liter (9).

A radiometric method was used to determine the MICs of the other drugs investigated (9). In brief, BACTEC 12B vials containing 4 ml of 7H12 medium were inoculated with the test organism and were incubated at 37°C until the growth index (GI) reached 500 to 800. A 0.1-ml aliquot of this suspension was then inoculated into a BACTEC 12B vial with or without appropriate dilutions of the test antibiotic. To prepare a 1% control vial, 0.1 ml of a 100-fold dilution of the inoculum described above was inoculated into an antibiotic-free BACTEC 12B vial. The BACTEC 12B vials were incubated at 37°C, and GI readings were recorded daily by using the BACTEC 460 TB instrument (Johnston Laboratories, Inc., Sparks, Md.) until the 1% control vial reached a GI of ≥30. When the daily increases in the GI of the antibiotic-containing vial and its final GI reading were lower than those for the 1% control vial, the antibiotic was considered to have inhibited more than 99% of the bacterial population, and this concentration was defined as the MIC.

RESULTS AND DISCUSSION

The collection of streptomycin-resistant clinical isolates of *M. tuberculosis* studied included eight strains with an altered *rpsL*, six strains with point mutations in the 530 region of the 16S rRNA gene (*rrs*), and five strains with wild-type *rpsL* and *rrs*. Mutations in *rpsL* were predominantly associated with a high-level resistance phenotype. While a replacement of Lys by Arg at amino acid position 43 invariably resulted in MICs of >1,000 mg/liter, an alteration at position 88 (Lys→Arg) gave

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TABLE 1. Comparison of genotype and antimicrobial susceptibility test results

Strain	Genotype			Phenotype (streptomycin MIC [mg/liter])	
	Drug susceptibility[a]	Mutations	Substitutions	Without Tween	With Tween
2742/95	Sm^s	Wild type	Wild type	1.0–2.0	0.5–1.0
2744/95	Sm^s	Wild type	Wild type	1.0–2.0	0.5–1.0
2529/95	Sm^s	Wild type	Wild type	1.0–2.0	0.5–1.0
2082/95	Sm^s	Wild type	Wild type	1.0–2.0	0.5–1.0
4649/83	Sm^r	*rpsL*	43-Lys→Arg	>1,000	>1,000
4513/83	Sm^r	*rpsL*	43-Lys→Arg	>1,000	>1,000
5141/83	Sm^r	*rpsL*	43-Lys→Arg	>1,000	>1,000
3626/83	Sm^r	*rpsL*	43-Lys→Arg	>1,000	>1,000
11966/89	Sm^r	*rpsL*	88-Lys→Arg	250–500	250–500
3555/83	Sm^r	*rpsL*	88-Lys→Arg	500–1,000	250–500
K8/94	Sm^r	*rpsL*	88-Lys→Arg	>1,000	>1,000
K11/94	Sm^r	*rpsL*	88-Lys→Arg	>1,000	>1,000
4362/83	Sm^r	*rrs*	523-A→C[b]	50–250	25–50
5127/85	Sm^r	*rrs*	523-A→C[b]	250–500	25–50
K4/94	Sm^r	*rrs*	523-A→C[b]	50–250	12.5–25
3976/83	Sm^r	*rrs*	522-C→T[b]	50–250	12.5–25
3601/84	Sm^r	*rrs*	526-C→T[b]	50–250	25–50
K3/94	Sm^r	*rrs*	526-C→T[b]	250–500	25–50
3564/83	Sm^r	Wild type	Wild type	25–50	2.0–6.0
3660/83	Sm^r	Wild type	Wild type	25–50	2.0–6.0
3694/83	Sm^r	Wild type	Wild type	25–50	2.0–6.0
4931/83	Sm^r	Wild type	Wild type	25–50	2.0–6.0
4308/95	Sm^r	Wild type	Wild type	25–50	2.0–6.0

[a] Sm^s, streptomycin susceptible; Sm^r, streptomycin resistant.
[b] Corresponding *E. coli* position.

more heterogeneous MICs, ranging from 250 to >1,000 mg/liter (Table 1).

The six strains with alterations of *rrs* had three different mutations: C to T at position 522 (522-C→T), 523-A→C, and 526-C→T. MICs ranging from 50 to 500 mg/liter were found; MICs greater than 500 mg/liter were never observed. The somewhat heterogeneous MICs were not associated with different mutations, because different MICs were observed for strains with identical genetic alterations (for strains 4362/83, 5127/85, and K4/94, 523-A→C; for strains 3601/84 and K3/94, 526-C→T).

Determination of the MICs for five streptomycin-resistant strains with wild-type *rpsL* and *rrs* revealed that all these isolates showed a low-level resistance phenotype, with MICs ranging from 25 to 50 mg/liter. For control susceptible isolates, the MICs were between 1.0 and 2.0 mg/liter.

Next we investigated cross-resistance to other aminoglycosides using representatives from each streptomycin resistance group. The following isolates were included: strains 3660/83, 3564/83, and 3694/83 with wild-type *rpsL* and *rrs*; strains 4362/83, 5127/85, 3976/83, 3601/84, and K4/94 with mutations in *rrs*; strain 3626/83 with an altered *rpsL*; and strain 2744/95 as a control streptomycin-susceptible isolate. No cross-resistance to other aminoglycosides was found; the MICs were always in the range from 0.5 to 1.0 mg/liter for amikacin, 4.0 to 8.0 mg/liter for gentamicin, 1.0 to 4.0 mg/liter for kanamycin, and 0.2 to 0.8 mg/liter for paromomycin. In addition, the MICs of isoniazid, rifampin, ethambutol, and pyrazinamide for low-level streptomycin-resistant strains with wild-type *rpsL* and *rrs* (strains 3660/83 and 3694/83) were investigated and were compared with the MICs for the control susceptible isolates. No difference in MICs was found; the MICs were 0.05 to 0.1 μg/ml for isoniazid, 0.25 μg/ml for rifampin, 0.45 μg/ml for ethambutol, and 25 to 50 μg/ml for pyrazinamide.

Investigations on the resistance mechanism and the level of streptomycin resistance in *M. tuberculosis* have previously been scarce, and the results of different studies have in part been contradictory. In contrast to one report describing mutants with an altered *rpsL* at position 43 as high-level resistant and isolates with wild-type *rpsL* and *rrs* as low-level resistant (6), such a correlation was not found by other investigators (13). We attribute these contradictory findings to differences in the methods used for drug susceptibility testing (9). Our data demonstrate that the concentration of streptomycin at which growth inhibition was produced varied according to the resistance mechanism. The drug-resistant isolates could be classified into three categories according to the level of resistance. The experimental variability observed in MIC determinations did not mask the finding that the level of resistance reflected the underlying molecular mechanism of resistance. High-level resistant strains were those which could tolerate streptomycin concentrations above 500 mg/liter; this phenotype was found only for isolates with an altered *rpsL*. Intermediate-level resistant strains were those which could tolerate streptomycin at concentrations between 50 and 500 mg/liter; this phenotype was mainly associated with alterations in *rrs*. Low-level drug-resistant isolates were those which were susceptible to the antibiotic at concentrations between 25 and 50 mg/liter. This phenotype was found in isolates with wild-type *rpsL* and *rrs*.

Membrane-active agents such as Tween 80 are capable of reducing membrane barriers (8, 12, 19). The susceptibilities of our clinical isolates to streptomycin were investigated in the presence of Tween in the medium. Under these conditions, the MICs for the control susceptible isolates were only marginally affected and decreased from 1.0 to 2.0 mg/liter to 0.5 to 1.0 mg/liter. The presence of Tween did not significantly influence the MICs for isolates with an altered *rpsL*. The MICs for *rrs* mutants were lowered by approximately 2 dilution steps (Table 1). The most pronounced effect was observed in the low-level streptomycin-resistant isolates with wild-type *rpsL* and *rrs*. Although the addition of Tween did not result in a fully susceptible phenotype, the level of resistance decreased significantly by approximately 10-fold, i.e., from 25 to 50 mg/liter to 2 to 6 mg/liter, suggesting the possibility that an inaccessibility of the streptomycin target site in the cell, i.e., the ribosome, was overcome by Tween. The addition of Tween reinforced the correlation between the molecular resistance mechanism and the phenotypic resistance level: a MIC of <6.0 mg/liter was found in low-level-resistant isolates with wild-type *rpsL* and *rrs*, for isolates with a mutated *rrs* MICs ranged from 12.5 to 50 mg/liter, and for isolates with a mutated *rpsL* MICs were in excess of 250 mg/liter.

From our data we suggest that two mechanisms of resistance to streptomycin exist in clinical isolates of *M. tuberculosis*: a ribosomal one, which mediates high or intermediate levels of resistance, and a permeability barrier, which contributes to low-level resistance. The latter mechanism is an attractive hypothesis on the basis of our results with Tween, but it requires further investigations to be definitively established. In this regard it is curious that no cross-resistance to other aminoglycosides was found in the low-level streptomycin-resistant strains with a putative permeability barrier. It seems entirely possible that a combination of different resistance mechanisms may operate in a drug-resistant clinical isolate; thus, for example, a permeability barrier would be expected to increase the resistance level for isolates with an altered *rrs*. Although we note that, because of limitations with our assay system, we cannot

firmly answer this question, the results of the growth inhibition studies seem compatible with this hypothesis.

ACKNOWLEDGMENTS

We are indebted to our colleagues Gaby Pfyffer, Sabine Rüsch-Gerdes, and Knut Feldmann for providing drug-resistant clinical isolates. We thank S. Maibom for timely writing of the manuscript.

This study was supported in part by grants from the Bundesministerium für Bildung, Wissenschaft, Forschung und Technik (Verbund Mykobakterielle Infektionen), the Niedersächsischer Verein zur Bekämpfung der Tuberkulose e.V., and the Commission of the European Communities (Biomed 2, contract 951241). E.C.B. is a Hermann and Lilly Schilling professor.

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