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Mutation position and type of substitution in the β-subunit of the RNA polymerase influence in-vitro activity of rifamycins in rifampicin-resistant *Mycobacterium tuberculosis*

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Quantitative susceptibility testing for rifampicin, rifabutin and rifapentine of 36 *Mycobacterium tuberculosis* isolates with known sequences for the gene encoding for the RNA polymerase β-subunit (*rpoB*) revealed that both mutation position and type of amino acid substitution influence the in-vitro activity of rifamycins in rifampicin-resistant strains.

Introduction

In *Mycobacterium tuberculosis*, the substitution of a limited number of highly conserved amino acids encoded by the *rpoB* gene, which codes for the β-subunit of the RNA polymerase, was shown to be associated with rifampicin (RMP) resistance (Telenti *et al.*, 1993a), and was confirmed by studies of genetic complementation (Miller, Crawford & Shinnick, 1994). To date, no additional mechanisms of resistance, such as a permeability barrier (Hui, Gordon & Kajioka, 1977), have been shown to contribute significantly to RMP resistance in *M. tuberculosis*. Some RMP-resistant strains of *M. tuberculosis* remain susceptible to the spiro-piperidyl rifamycin rifabutin (RBU) (Della Bruna *et al.*, 1983; Dickinson & Mitchison, 1987), and some patients with RMP-resistant pulmonary tuberculosis have shown a favourable clinical and bacteriological response to this drug (Gillespie *et al.*, 1990; Hong Kong Chest Service/Tuberculosis Research Centre, 1992; Pretet *et al.*, 1992). We determined the MICs of RMP, RBU, and rifapentine (RPE) (Dickinson & Mitchison, 1987) for RMP-resistant strains of *M. tuberculosis* with known *rpoB* sequences to assess whether there was a correlation between the level of resistance to various rifamycins and specific mutations in the *rpoB* gene.

Material and methods

Strains

The following strains of *M. tuberculosis* were examined: H37 rv (Institut Pasteur 14001.0001), and 10 RMP-susceptible and 26 RMP-resistant clinical isolates. The latter strains have been characterised by partial sequencing of *rpoB*, and represented 14 RMP-resistance mutations (Telenti *et al.*, 1993*a*). All of the RMP-resistant strains exhibited resistance to one or more additional first-line antituberculosis drugs. When available three different isolates per mutation were examined.

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Antibiotics

RMP was provided by Ciba-Geigy Ag, Basle, Switzerland, RPE by Marion Merrell Dow Inc., Cincinatti, U.S.A., and RBU by Farmitalia Carlo Erba, Milan, Italy. RMP and RPE were dissolved in dimethyl-sulfoxide, and RBU in dimethyl-formamide. Stock solutions of each antibiotic (320 mg/L) were freshly prepared for each run in 0·067 M phosphate buffer, pH 7·0. Working solutions ranging from 0·015 mg/L to 8·0 mg/L were made by serial two-fold dilutions with distilled water.

MICs

The MICs of the test antibiotics were determined by a radiometric method (Heifets, 1991). Briefly, BACTEC 12B vials containing 4 mL 7H12 medium were inoculated with 0·1 mL of a subculture of the test organisms adjusted to McFarland 0·5 in distilled water, and then incubated at 37°C until the growth index (GI) reached 500–800. A 0·1 mL aliquot of this suspension was then inoculated into the BACTEC 12B vial with or without appropriate dilutions of the test antibiotic. To prepare a 1% control vial, 0·1 mL of the 100-fold dilution of the inoculum described above was inoculated into an antibiotic free 12B vial. The 12B vials were incubated at 37°C and GI readings were recorded daily, using the BACTEC 460 TB instrument (Johnston Laboratories, Inc., Sparks, MD, USA), until the 1% control vial reached a GI of $\geqslant 30$. 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, 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

Most of the mutations studied, that is those located at amino acid positions 513, 526 and 531 of RpoB, were associated with high level cross-resistance to RBU and RPE (MIC $\geqslant$ 4·0 mg/L). However, in the present study, five of 14 mutant alleles, at positions 511, 516, 518 and 522, were associated with MIC values between 0·25 and 1·0 mg/L RBU (Table). By comparing radiometrically determined MICs with the concentrations of RBU achievable in blood, *M. tuberculosis* strains were tentatively separated into the categories susceptible (MIC $\leqslant$ 0·12 mg/L), moderately susceptible (MIC 0·25 mg/L), resistant (MIC 0·5 mg/L), and very resistant (MIC > 0·5 mg/L) (Heifets, 1991). According to these criteria it appears that strains with mutations at amino acid position 511, 516, and 522 in the RpoB remain moderately susceptible to RBU, or exhibit low level resistance only. However, relying on serum concentrations when defining breakpoints for RBU is probably a conservative approach, since the achievable concentrations of this antibiotic are higher in tissue than in plasma (Della Bruna *et al.*, 1983). At present, clinical experience in the treatment of RMP-resistant tuberculosis with RBU is limited, but there is evidence of its beneficial effect in some patients with multidrug-resistant tuberculosis (Gillespie *et al.*, 1990; Pretet *et al.*, 1992). In one study, the initial responses in the two patients infected with RBU-susceptible *M. tuberculosis* were among the best, although rapid development of RBU resistance was seen (Hong Kong Chest Service/Tuberculosis Research Centre, 1992). Experimental studies in animals and controlled clinical trials

Table. Comparison of *rpoB* genotype and antimicrobial susceptibility test results for 36 isolates of *M. tuberculosis*.

Genotype			Phenotype		
mutation position[a]	amino acid substitution	isolates (*n*)	MIC (mg/L) rifampicin	rifabutin	rifapentin
Wild type	wild type	10	0·25–0·5	<0·015–0·125	<0·015–0·125
Leu 511	Pro	1	>8·0	0·5	>8·0
Gln 513	Leu	1	>8·0	>8·0	>8·0
Asp 516	Tyr	2	2·0	0·25	0·5
Asp 516	Val	3	⩾8·0	0·25–0·5	4·0–>8·0
Asn 518	deletion	1	>8·0	1·0	4·0
Ser 522	Leu	1	>8·0	0·5	8·0
His 526	Arg	2	>8·0	>8·0	>8·0
His 526	Asp	3	>8·0	>8·0	>8·0
His 526	Pro	2	>8·0	>8·0	>8·0
His 526	Tyr	3	>8·0	>8·0	>8·0
His 526; Val 498	Gln; Ala	2	>8·0	8·0	>8·0
Ser 531	Leu	3	>8·0	4·0–>8·0	>8·0
Ser 531	Trp	1	>8·0	8·0	>8·0
Ser 531	Tyr	1	>8·0	>8·0	>8·0

[a]Numbers correspond to *E. coli* RNA polymerase amino acid positions.

which address the efficacy of RBU in the treatment of infections caused by this subset of RMP-resistant strains are needed.

The second main observation of this study relates to the conventional understanding of the emergence of resistance to RMP. According to the current view, the development of resistance to RMP in *M. tuberculosis* is a single-step event leading to high-level resistance. Mutants arise spontaneously in strains not previously exposed to the antibiotic at a frequency of 10^{-7}–10^{-8} cells. Our results suggest, however, that not only with RBU but also with RMP, the level of resistance is dependent on the type of mutation involved. For example, substitution of aspartate for tyrosine at position 516 in two unlinked isolates only resulted in moderately susceptible strains with MICs of 2·0 mg/L. In contrast, three isolates with substitution of valine for aspartate at the same position exhibited high-level resistance with MICs above 8 mg/L.

The observed MIC values of RPE for the various mutant strains were similar to those observed with RMP.

Investigation of the RpoB substitutions associated with rifamycin resistance combined with the careful delineation of the phenotype by quantitative susceptibility testing may help in identifying new compounds that retain their efficacy in the presence of specific mutations, thus revealing new insights of the interactions between rifamycins and mycobacterial RNA polymerase. In addition, since the activity of RBU in RMP-resistant *M. tuberculosis* seemingly depends on both the mutation position and the type of substitution in *rpoB*, direct sequencing (Telenti *et al.*, 1993*a*) or polymerase chain reaction-single-strand conformation polymorphism analysis, which is a recently applied molecular tool for the direct detection of mutations in *rpoB* (Telenti *et al.*, 1993*b*), could predict moderate susceptibility or low level resistance to RBU within 24 h. The availability of this information at a very early stage of treatment might substantially influence the management of patients with multidrug-resistant tuberculosis.

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