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Bactericidal Activity of Trimethoprim Alone and in Combination with Sulfamethoxazole on Susceptible and Resistant *Escherichia coli* K-12

SEBASTIAN G. B. AMYES

Department of Bacteriology, University of Edinburgh Medical School, Edinburgh EH8 9AG, United Kingdom

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The combined effects of trimethoprim and sulfamethoxazole on the viability of *Escherichia coli* K-12 and resistant strains possessing resistance plasmids were examined in minimal medium. When methionine, glycine, and adenine were present, sulfamethoxazole could enhance trimethoprim activity against *E. coli* K-12 so that the combination was bactericidal. However, this enhancement occurred over a narrow range of trimethoprim concentrations (0.04 to 0.2 mg liter^{-1}) and only when the sulfamethoxazole concentration was more than 10 times that of trimethoprim. Under certain conditions, sulfamethoxazole enhanced trimethoprim bactericidal activity against *E. coli* K-12 carrying plasmid R1 at concentrations of sulfamethoxazole far below those required to inhibit the organism, but there was no such enhancement with the same host containing the SSu plasmid. Similar differences were found with strains possessing trimethoprim resistance plasmids R483 and R751. Sulfamethoxazole can promote a bactericidal response with trimethoprim in *E. coli* K-12 and some of its resistant derivatives, but only under a narrow range of concentrations.

Trimethoprim (Tp) has been marketed in combination with sulfamethoxazole (Sx) in the United Kingdom for 13 years. The reasons for combining Tp with Sx are threefold (12): (i) the two drugs together have been reported to show a synergistic effect in vitro which is greater than would be expected from the additive effect of the two drugs (12), (ii) the two drugs, when administered together, delay the emergence of resistant bacteria (12); and (iii) the two drugs used individually are bacteriostatic, but in combination they are bactericidal (12). Recently, these reasons for combining Tp with Sx have been questioned (18). The demonstration of synergy between Tp and Sx has often been reported in vitro (10, 11, 14), but has never been shown in vivo. However, it has been reported that Tp and Sx in combination perform better clinically than Tp alone in the treatment of urinary tract infections (17), but in this study the populations being treated by Tp and the Tp-Sx combination were not comparable. On the other hand, other workers have reported that there is no clinical advantage of combining Tp with Sx over Tp alone for the treatment of urinary tract infections (8, 23–25). The combination of Tp and Sx has been shown to delay the emergence of resistant bacteria in laboratory isolates (14). However, it is more difficult in the absence of extensive controlled surveys to determine whether this occurs in clinical strains. Studies on such strains are further complicated by the presence of resistance plasmids (R-plasmids) conferring resistance to both Tp and Sx. However, in Finland, where Tp has been used extensively without Sx, the incidence of Tp resistance among enterobacteria is no higher than in other European countries, where only the combination has been given (3, 19, 22).

The bacteriostatic nature of Tp has been shown to be very dependent on the composition of the medium (4). We have shown previously with *Escherichia coli* strain 114 that, when methionine, glycine, and the purine adenine are present in the medium, Tp is bactericidal (4). This effect is similar to "thymineless death" and can be reversed by the presence of thymine derivatives (4). The effect of methionine, glycine, and adenine is to preserve the integrity of the tetrahydrofolic acid pool during Tp treatment (4). This allows protein synthesis to occur, which is essential for the "death" of the organism (7).

This communication reports on an investigation of the in vitro antibacterial effects of combinations of Tp and Sx on *E. coli* K-12 and resistant derivatives possessing R-plasmids.

MATERIALS AND METHODS

Organisms and plasmids. *E. coli* K-12, a prototrophic strain susceptible to both Tp and Sx, and four drug-resistant derivatives containing plasmids were

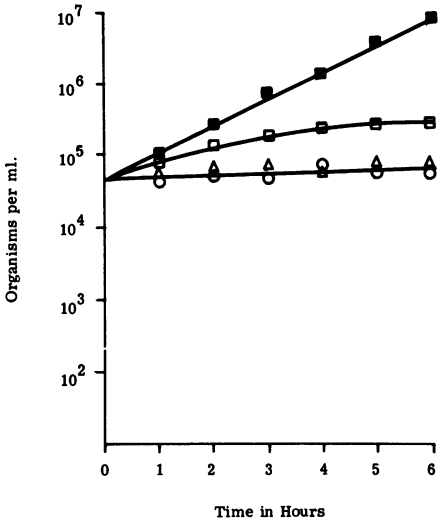

FIG. 1. Effect of Tp and Sx on the viability of *E. coli* K-12 in DM. An exponential culture was diluted into prewarmed medium containing (■) no additions; (△) Tp (5 mg liter^{-1}); (□) Sx (100 mg liter^{-1}); or (○) Tp (5 mg liter^{-1}) and Sx (100 mg liter^{-1}).

used. The Sx-resistant strain K-12 possesses either the R-plasmid R1, which confers ampicillin, chloramphenicol, and streptomycin resistance in addition to Sx resistance (27), or the plasmid SSu, which confers streptomycin resistance in addition to Sx resistance (13). The Tp-resistant K-12 strains possessed either the R-plasmid R751, which confers only Tp resistance, or R483, which confers streptomycin resistance as well as Tp resistance (21).

Media. Davis-Mingioli (DM) minimal medium (15) was used. It was made up as described by Smith (29), and methionine, glycine and adenine (each at 50 μg/ml) were added as described (29).

Reagents. Methionine and glycine (Sigma Chemical Co., Poole, United Kingdom) were sterilized in solution by membrane filtration. Adenine (Koch-light, Colnbrook, United Kingdom) was sterilized by autoclaving. Sx and Tp (as Tp lactate) were gifts from the Wellcome Foundation Ltd., Beckenham, United Kingdom, and the Tp test results are expressed in terms of the concentration of the Tp base.

Effects of drugs. Cultures were grown overnight at 37°C in DM medium. Exponential phase cultures were prepared by subculturing a stationary phase culture into prewarmed DM medium at 37°C and incubating the culture until the logarithmic phase was reached. The logarithmic phase culture was diluted into DM medium containing methionine, glycine, adenine, Tp, and Sx where appropriate. The cultures were incubated at 37°C, and the viable count was estimated by plating suitable serial dilutions on Oxoid MacConkey agar. The plates were incubated at 37°C for 18 h, and the resultant colonies were counted.

RESULTS

Effect on Tp and Sx on *E. coli* K-12. *E. coli* K-12 was subcultured into DM medium containing either Tp or Sx, but no further supplements. In both cases, there was no loss of viability of the organism during 6 h of incubation (Fig. 1). When the two drugs were present together at the same concentrations there was no greater loss of viability than with Tp alone (Fig. 1). The experiment was repeated with DM medium containing methionine, glycine, and adenine. Under these circumstances, Tp concentrations above 0.2 mg liter^{-1} were bactericidal. When lower Tp concentrations were used (0.1 mg liter^{-1}) the drug was bacteriostatic, and at concentrations below 0.04 mg liter^{-1}, the drug had no effect (Fig. 2a). When methionine, glycine, and adenine were added to DM medium, there was no effect on the response of strain K-12 to Sx. Even at higher Sx concentrations (100 mg liter^{-1}) no loss of viability was found (Fig. 2b).

E. coli K-12 was subcultured into DM medium containing methionine, glycine, adenine, and both Tp and Sx. The Sx concentration was 20 times that of Tp. When a Tp concentration of 0.04 mg liter^{-1} was used with 0.8 mg of Sx per liter, the combination was bactericidal during the 6-h incubation (Fig. 3). Tp at 0.04 mg liter^{-1} did not inhibit growth of the organism (Fig. 3), and Sx at 0.8 mg liter^{-1} was bacteriostatic (Fig. 2b). Similarly, when Tp (0.1 mg liter^{-1}) was combined with Sx (2 mg liter^{-1}), they were bactericidal (Fig. 3) even though the individual drugs were bacteriostatic at these concentrations. The bactericidal effect of the Tp (0.1 mg liter^{-1})–Sx (2.0 mg liter^{-1}) combination was similar to that of Tp alone at 0.4 mg liter^{-1} (Fig. 2a).

Effects of Tp combined with various concentrations of Sx. When Sx was added at the same concentration as Tp (0.04 mg liter^{-1}) (Fig. 4), it did not affect the viability of strain K-12. Only at Sx concentrations 10 times that of Tp was a bactericidal effect found. The bactericidal effect was maximal when the Sx concentration was 20 times that of Tp; an Sx concentration 100 times the Tp concentration did not increase the bactericidal effect.

Effect of Tp and Sx on *E. coli* K-12 carrying Sx R-plasmids. Tp and Sx can have a synergistic action on *E. coli* strains possessing certain R-plasmids carrying Sx resistance (2). The viability of *E. coli* K-12 possessing either R1 or the SSu plasmid was followed in DM medium containing methionine, glycine, and adenine plus increasing concentrations of Tp. Both strains behaved in almost the same way as *E. coli* K-12 (results not shown). The minimum inhibitory concentration of Sx for both strains was greater than 2 g liter^{-1}. However, when Tp and Sx were added together, the response of the two strains to the combination was very different (Fig. 5). Low concentrations of Tp and Sx had a bactericidal

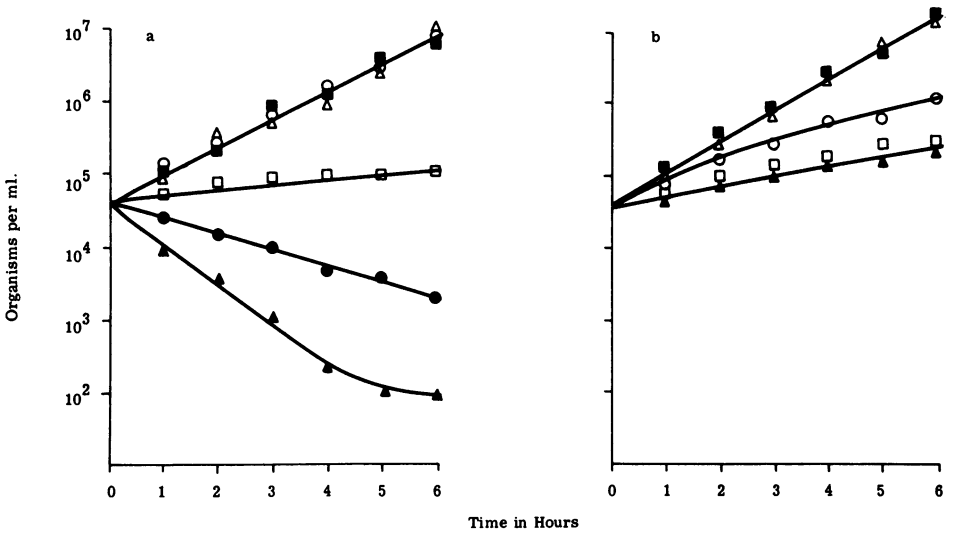

FIG. 2. Effect of Tp and Sx on the viability of *E. coli* K-12 in DM supplemented with methionine, glycine, and adenine (50 mg liter^{-1} each). An exponential culture was diluted into prewarmed medium containing the following antimicrobial drugs. In (a) Tp was used at 0 (■), 0.02 (○), 0.04 (△), 0.1 (□), 0.2 (●), and 0.4 (▲) mg liter^{-1}. In (b) Sx was used at 0 (■), 0.4 (△), 0.8 (○), 2.0 (□), and 100 (▲) mg liter^{-1}.

effect on the R1-containing strain, where identical concentrations of each drug alone had no inhibitory effect (Fig. 5a). On the other hand, the SSu-containing strain was uninhibited by similar combinations of Tp and Sx. Indeed, when the Sx concentration was increased to 100 mg liter^{-1} the drugs were still unable to affect the viability (Fig. 5b). When 10 Sx R-plasmids isolated from clinical strains within this laboratory were tested in this manner, 5 behaved in a manner similar to R1, and the rest behaved in a manner similar to SSu.

Effect of Tp and Sx on *E. coli* K-12 carrying Tp R-plasmids. The viability of *E. coli* K-12 pos-

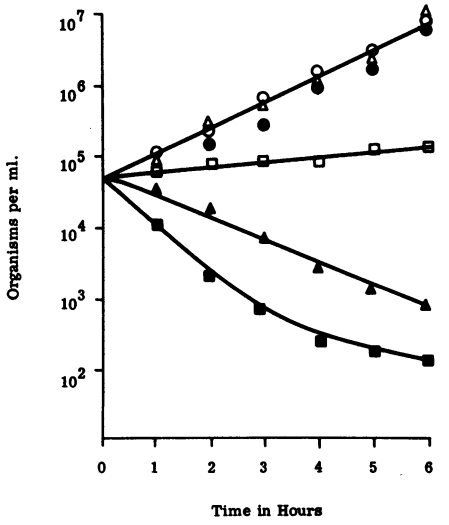

FIG. 3. Effect of Tp and Sx together on the viability of *E. coli* K-12 in supplemented DM. An exponential culture was diluted into prewarmed medium containing Tp at 0.02 (○), 0.04 (△), and 0.1 (□) mg liter^{-1} and Tp-Sx at 0.02 and 0.4 (●), 0.04 and 0.8 (▲), and 0.1 and 2 (■) mg liter^{-1}, respectively.

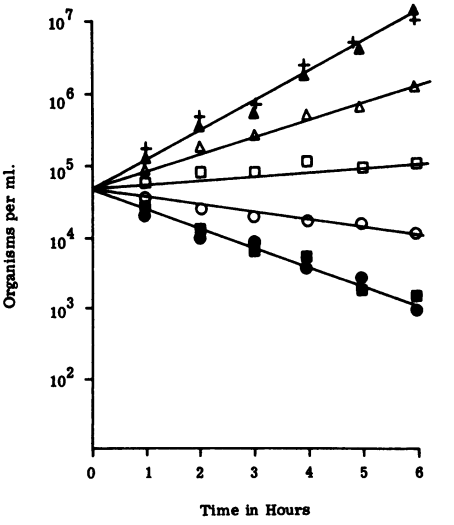

FIG. 4. Effect of Tp and various concentrations of Sx on the viability of *E. coli* K-12 in supplemented DM. An exponential culture was diluted into prewarmed medium containing Tp at 0.04 mg liter^{-1} and Sx at 0 (+), 0.04 (▲), 0.08 (△), 0.2 (□), 0.4 (○), 0.8 (●), and 4.0 (■) mg liter^{-1}.

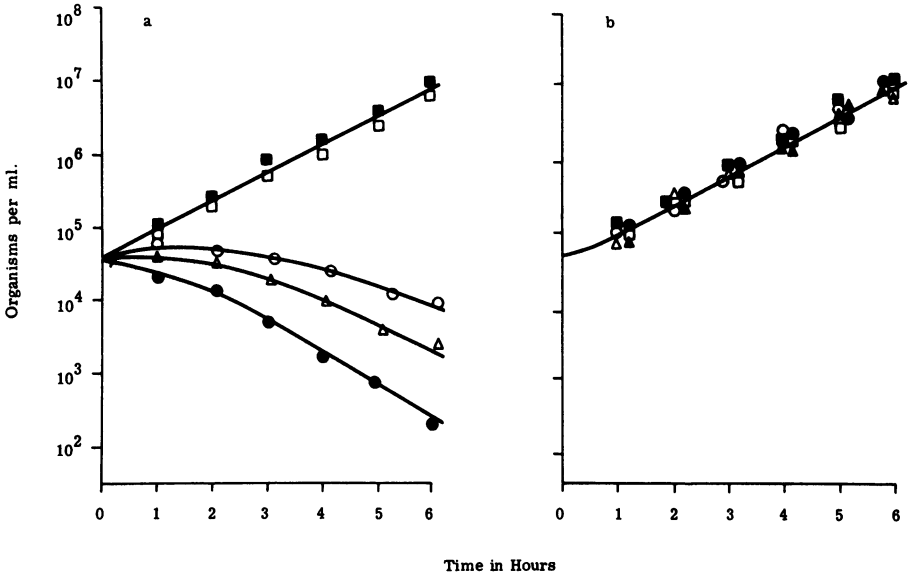

FIG. 5. Effect of Tp and Sx together on the viability of *E. coli* K-12 containing the Sx resistance R-plasmids (a) R1 and (b) SSu. Exponential cultures were diluted into prewarmed medium containing Tp at 0.04 mg liter^{-1} (■) or Sx at 100 mg liter^{-1} (□) and trimethoprim at 0.04 mg liter^{-1} plus Sx at 0.8 (○), 1.6 (△), 3.2 (●), and 100 (▲) mg liter^{-1}.

sessing either of the R-plasmids R483 and R751 was followed in DM medium supplemented with methionine, glycine, and adenine and containing Tp or Sx. The minimum inhibitory concentration of Tp for both strains was greater than 1 g liter^{-1}. Sx was bacteriostatic for both strains at the same concentrations as it was bacteriostatic for the susceptible *E. coli* K-12. However, the administration of the two drugs together gave different effects with these two R-plasmid strains. The drugs together had a bactericidal effect on the R751 containing strain, even in the presence of low concentrations of Tp (Fig. 6a). Conversely, the two drugs had a bacteriostatic effect on the R483-containing strain similar to that found when Sx was employed alone (Fig. 6b). Seven clinically isolated Tp R-plasmids, which did not confer Sx resistance (5), were tested in *E. coli* K-12 in this way, and they all behaved in the same way as R483.

DISCUSSION

The study of the synergistic action of antibacterial drugs by the use of kill curves (20) may be more predictive of the clinical effects than the checkerboard titration technique (16), and one of the original advantages for combining Tp with Sx was that both drugs are bacteriostatic when administered individually but bactericidal when used together (12). However, the effect of Tp on a susceptible *E. coli* strain is dependent on whether methionine, glycine, and adenine are present in the medium (4). When these supplements were not present, Tp was bacteriostatic. In the presence of these supplements, Tp was bactericidal for *E. coli* K-12 at concentrations above 0.2 mg liter^{-1}. At concentrations below this, Tp was either bacteriostatic or had no effect on viability. When Sx was added with Tp at these concentrations, the combination became bactericidal even when the individual drugs used alone were bacteriostatic. As the presence of methionine, glycine, and adenine has no effect on Sx action, the bactericidal effect is presumed to result from a potentiation of Tp activity by Sx.

These results may be applicable to the effect of Tp and Sx on *E. coli* in the urinary tract. It has been reported that the supplementation of minimal medium with methionine, glycine, and adenine can mimic urine as far as these drugs are concerned (30), as Tp can be bactericidal in urine (6). If this is so, the combination of Sx and Tp may give no greater effect than Tp alone. With *E. coli* K-12 the maximum bactericidal effect of Tp was obtained with Tp concentrations of 0.4 mg liter^{-1}; Sx is only able to promote Tp activity at Tp concentrations below this value. In the urinary tract, the expected level of Tp, after normal dosage, is between 30 and 150 mg liter^{-1} (9).

The ratio of the drugs in combination affects their joint activity. At an Sx/Tp ratio of 1:1, Sx is unable to potentiate the activity of Tp. Sx was only able to produce a bactericidal effect with Tp when an Sx/Tp ratio of greater than 10:1 was

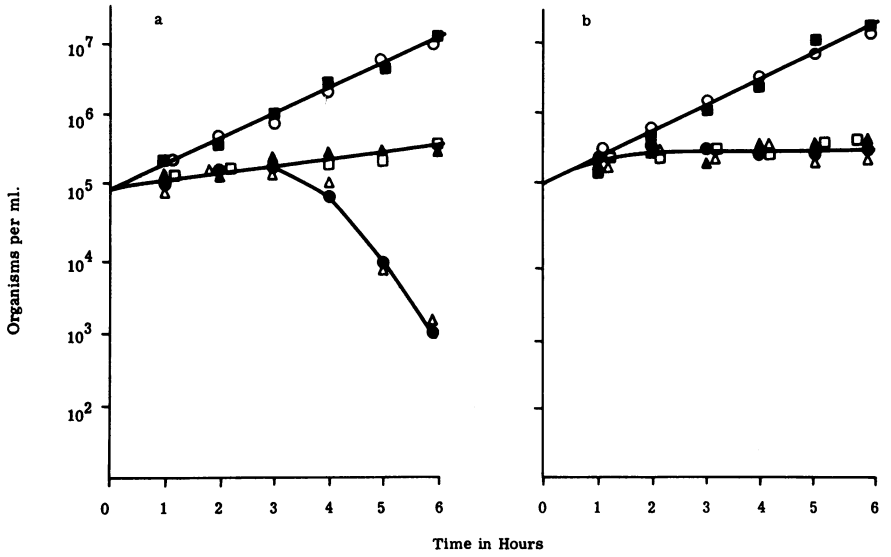

FIG. 6. Effect of Tp and Sx together on the viability of *E. coli* K-12 containing the Tp resistance plasmids (a) R751 and (b) R483. Exponential cultures were diluted into prewarmed medium containing Tp at 5 (■) or 50 (○) mg liter^{-1}, Sx at 100 (▲) or 1,000 (□) mg liter^{-1}, or Tp-Sx at 5 and 100 (●) or 50 and 1,000 (△) mg liter^{-1}, respectively.

used. In the urinary tract, for example, the Sx/Tp ratio is 2:1 (9). The findings presented here are in line with the clinical results that show no clinical advantage of combining Tp with Sx as compared with Tp alone for the treatment of *E. coli* infections of the urinary tract (1, 26).

With the strains possessing Sx R-plasmids, there were differing responses to the combination of Tp and Sx. In *E. coli* K-12 (R1), the potentiation of Tp by Sx occurs at concentrations of this drug far below those known to be inhibitory for this strain; such potentiation does not occur with the SSu-containing strain. A possible explanation for this difference may be found if Sx has a second site of action, namely, the target enzyme for Tp, dihydrofolate reductase, as suggested by Poe (28), and if the two plasmids employ different mechanisms for producing Sx resistance. Two types of Sx R-plasmid resistance mechanisms have been suggested (31). Plasmid SSu may resist Sx by coding for an impermeability barrier, in which case potentiation at low Sx concentrations would not be expected to occur as the drug is unable to act within the cell at any site. R1, on the other hand, may resist Sx by coding for an additional insusceptible target enzyme, dihydropteroate synthetase. In this case, the presence of the R-plasmid resistance mechanism would not affect the binding of Sx to its second target.

A similar difference in the response to Tp and Sx was found with the Tp R-plasmid-containing strains. Tp was effective against the R751-containing strain, at concentrations of this drug far below those known to be inhibitory, when Sx was present. The Tp-Sx combination, at similar concentrations, was not effective against the R483-containing strain. The reason for this difference is obscure. Both plasmids resist Tp by encoding an additional insusceptible dihydrofolate reductase (5). One difference between these resistance mechanisms is the specific activity of the enzyme encoded by the two plasmids. Plasmid R483 produces about 250 times the insusceptible dihydrofolate reductase that R751 produces (5), and this may explain the difference. On the other hand, the plasmid R751-encoded enzyme may itself possess a receptor that binds Sx in a way similar to that suggested for the bacterial enzyme (28).

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