An electronmicroscope study of the effect of sulphadiazine and trimethoprim on *Enterobacter cloacae*

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Summary. Electronmicroscopy of thin sections of log phase cells of *Enterobacter cloacae* NCTC 10005 grown for 4 h in the presence of sulphadiazine 250 µg/ml, trimethoprim 12.5 µg/ml or the combination of sulphadiazine 250 µg/ml plus trimethoprim 12.5 µg/ml indicated that both agents caused marked morphological damage even though the MIC of sulphadiazine for the *E. cloacae* strain was > 3000 µg/ml. The damage took the form of electron-transparent areas devoid of ribosomes in the cytoplasm and detachment of the outer membrane. The latter was most marked with trimethoprim, which also caused damage to the cytoplasmic membrane. It is postulated that the synthesis of the peptidoglycan layer was affected by the antimetabolites since the morphological effects were strikingly similar to those caused by treatment of *E. cloacae* with disodium edetate plus lysozyme. Viable counts of cultures undergoing the same treatments as those prepared for electronmicroscopy indicated that although sulphadiazine merely partially inhibited growth it nevertheless enhanced the bactericidal action of trimethoprim over a 5-h period.

Introduction

We have presented evidence recently that sub-inhibitory concentrations of trimethoprim and sulphonamides mutually enhance their uptake by bacteria, even when the bacteria are resistant to one member of the combination.¹⁻⁴ Thus a strain of *Enterobacter cloacae* for which the MIC of sulphadiazine was > 3000 µg/ml, and that of trimethoprim was 15 µg/ml, was inhibited by a combination of sulphadiazine 100 µg/ml and trimethoprim 7.5 µg/ml. The synergy correlated with markedly increased uptake of the two compounds. This provided an explanation for antibacterial synergy between sulphonamides and trimethoprim alternative to the sequential blockade of folate synthesis. Similar increases in the combined uptake of sulphadiazine and trimethoprim were obtained with two strains of *Pseudomonas aeruginosa* that were resistant to high concentrations of both antibacterial agents used alone.¹

The purpose of the present investigation was to investigate the morphological effects of sulphadiazine and trimethoprim on log phase cells of *E. cloacae*, to clarify further our earlier findings.¹⁻³ An additional objective was to determine whether the sulphadiazine could contribute to the bactericidal action of the combination against a strain that was highly resistant to the sulphonamide. It is well known that sulphonamides contribute to the bacteriostatic action of

sulphonamide-trimethoprim combinations against sensitive bacteria, but there is considerable uncertainty as to whether they contribute to bactericidal synergy, especially if the bacteria are sulphonamide resistant.⁵⁻⁸

Materials and methods

E. cloacae NCTC 10005 was obtained from the National Collection of Type Cultures, Colindale Avenue, London. Sulphadiazine, trimethoprim and *p*-aminobenzoic acid were from Sigma.

Sample preparation

From an 18-h broth culture of *E. cloacae* 1 ml was inoculated into 99 ml of Iso-Sensitest Broth (Oxoid) at 37°C in a 250-ml conical flask. The culture was shaken at 100 oscillations/min in a water bath at 37°C for 4 h; 10-ml quantities of this log phase culture were then inoculated into 250-ml conical flasks containing 90 ml of Iso-Sensitest broth plus sulphadiazine 250 µg/ml, or trimethoprim 12.5 µg/ml, or both. A flask containing 90 ml of Iso-Sensitest broth without an antibacterial agent was used as the control. These four flasks were incubated at 37°C for 4 h in a water bath with shaking (100 oscillations/min).

Electronmicroscopy

The cultures were centrifuged at 6000 *g* for 15 min at 4°C, and the supernate was removed. The pellet was

Fig. 1. Ultrathin sections of log phase *E. cloacae* NCTC 10005 grown in Iso-Sensitest broth. **a, b**, control (no antibacterial agent); **c, d**, bacteria exposed to sulphadiazine 250 µg/ml for 4 h. Bar = 1 µm (**a, c**); 0.5 µm (**b, d**).

fixed in glutaraldehyde 8% v/v in 0.1 M phosphate buffer (pH 7.2) for 1 h, and then in glutaraldehyde 4% v/v in 0.1 M phosphate buffer (pH 7.2) for 4 h at 4°C. After washing in the same buffer the bacteria were resuspended in OsO₄ (Emscope, Watford) 1% w/v for 1 h at room temperature. They were then washed three times by centrifugation and resuspension in distilled water. The final pellet was resuspended in a small volume of warm agarose 2% w/v, poured on to a glass slide and allowed to cool. When set, small pieces of gel containing suspended bacteria were cut out and dehydrated through a graded series of ethanol solu-

tions. After embedding in Araldite (Agar Scientific Ltd, Stansted, Essex) thin sections were cut with a diamond knife on a Reichert Ultracut E ultramicrotome, stained with uranyl acetate and lead citrate, and examined in a Philips EM301 electron microscope operating at 80 kV.

Treatment with disodium edetate plus lysozyme

From a log phase culture of *E. cloacae*, 10-ml volumes were inoculated separately into three 250-ml conical flasks each containing 90 ml of IsoSensitest

Fig. 2. Ultrathin sections of log phase *E. cloacae* NCTC 10005 grown in Iso-Sensitest broth containing: **a, b**, trimethoprim 12.5 µg/ml; **c, d**, sulphadiazine 250 µg/ml and trimethoprim 12.5 µg/ml. Bar = 1 µm (**a, c**); 0.25 µm (**b**); 0.5 µm (**d**).

broth at 37°C and incubated for 4 h in a water bath with shaking at 100 oscillations/min. The bacteria were then centrifuged and the cell pellets were washed with HEPES buffer (pH 7.2) and resuspended in the same volume of buffer solution. Lysozyme was added to give a concentration of 50 µg/ml followed by disodium edetate to a concentration of 0.1 mM. The action of disodium edetate was quenched after 45, 60 and 90 s, respectively, by the addition of sufficient MgCl₂ to achieve a concentration of 40 mM. After the lysozyme had reacted with the cells for 4 min at 20°C, the suspensions were centrifuged and prepared for electronmicroscopy as described above.

Determination of bacterial viability

Viable counts were determined by an overdried agar plate counting method. From a log phase culture 10-ml volumes were added to three flasks containing 90 ml of Iso-Sensitest broth with and without the appropriate antibacterial agent. Viable counts were determined after exposure to the antibacterial agents for 30 min, 1 h, 2 h, 3 h, 4 h and 5 h. *p*-Aminobenzoic acid 50 µg/ml was incorporated into the Iso-Sensitest agar to antagonise any sulphadiazine or trimethoprim which could have been present in low concentrations in the cells, since *p*-aminobenzoic acid at appropriate

concentrations not only blocks the antibacterial activity of sulphadiazine but also that of trimethoprim (unpublished results).

Results

Electronmicroscopy

Fig. 1 shows the appearance of normal log phase cells of *E. cloacae* NCTC 10005.

After exposure to sulphadiazine 250 µg/ml, at least 50% of the bacteria exhibited electron-transparent areas devoid of ribosomes (holes) clearly visible within the cytoplasm (fig. 1c). Also, the gap between the cytoplasmic and outer membranes appeared larger and less electron-dense (fig. 1d).

Approximately two-thirds of bacteria exposed to trimethoprim 12.5 µg/ml had holes similar to those seen in the sulphadiazine-treated bacteria (fig. 2a). In addition, there was partial detachment of the outer membrane from the cytoplasmic membrane in most of the bacteria. The membrane generally appeared to be unbroken although detached; however, where the holes were adjacent to the cytoplasmic membrane the membrane often appeared to be broken. Higher magnification showed that only certain areas of the cytoplasmic membrane remained connected to the outer membrane (fig. 2b).

When *E. cloacae* NCTC 10005 was exposed to the combination of sulphadiazine 250 µg/ml and trimethoprim 12.5 µg/ml all the bacteria exhibited damage (fig. 2c and d).

Fig. 3 shows that brief exposure to disodium edetate plus lysozyme had an effect on the morphology of the

Fig. 3. Ultrathin section of log phase *E. cloacae* NCTC 10005 treated with disodium edetate and lysozyme for 90 s. Bar = 1 µm.

Fig. 4. Viable counts of *E. cloacae* NCTC 10005 in Iso-Sensitest broth at 37°C. ○ Control; ■ sulphadiazine 250 µg/ml; □ trimethoprim 12.5 µg/ml; ▼ sulphadiazine 250 µg/ml plus trimethoprim 12.5 µg/ml.

E. cloacae NCTC 10005 strikingly similar to that of the antimetabolites.

Viable counts

Sulphadiazine 250 µg/ml slowed the replication of the inoculum of 10^7 cfu log phase *E. cloacae*/ml after several hours. Trimethoprim 12.5 µg/ml caused a 10-fold fall in viable count after 5 h, an effect that was enhanced in the presence of sulphadiazine 250 µg/ml (fig. 4). After 24 h the viable counts were 10^9 cfu/ml (sulphadiazine), 3×10^7 cfu/ml (trimethoprim), and 3×10^5 cfu/ml (sulphadiazine plus trimethoprim).

Discussion

Thin sections of *E. cloacae* NCTC 10005 exposed to sulphadiazine and trimethoprim or disodium edetate plus lysozyme showed electron-transparent areas devoid of ribosomes in the bacterial cytoplasm. Ciprofloxacin can cause similar damage to *Escherichia coli*.⁹ It may be assumed that protein synthesis would be affected by such damage.

Disodium edetate plus lysozyme causes hydrolysis of the peptidoglycan layer of gram-negative bacteria and morphological effects on *Pseudomonas fluorescens* similar to those shown here.¹⁰ The similarity between the effects of lysozyme and the antifolate drugs

suggests that trimethoprim, and to a lesser extent sulphadiazine, may have an additional effect on the production of peptidoglycan in *E. cloacae*. It is likely that the enhanced effect of the combination of sulphadiazine and trimethoprim is due to increased uptake into damaged bacterial cells as suggested previously.¹⁻⁴

Viable counts of cultures treated in exactly the same way as those from which the electronmicrographs were prepared indicate that, although sulphadiazine 250 µg/ml merely inhibited growth of log phase cells of *E. cloacae* over a 5-h period, it nevertheless increased the bactericidal activity of trimethoprim 12.5 µg/ml within 2 h and throughout the 5-h period. Moreover, inhibition by sulphadiazine and the bactericidal effect of trimethoprim were short in duration but the effect of the combination was longer lasting.

There is considerable controversy about the interaction between sulphadiazine and trimethoprim

against sulphadiazine-resistant cells⁶ and in conditions in which trimethoprim alone is able to halt bacterial growth completely.⁷ Brumfitt and Hamilton-Miller stated that "synergy does not occur with bacteria that are resistant to sulphonamides"⁶ and Greenwood stated "on theoretical grounds one would predict that sulphamethoxazole would be antagonized by the presence of inhibitory concentrations of trimethoprim".⁷ However, under the conditions reported here, the presence of sulphadiazine contributed to the overall activity of the combination of sulphadiazine with trimethoprim both against sulphadiazine-resistant cells and also in the situation in which trimethoprim alone had a bactericidal action. While these results cannot be extrapolated directly to the clinical situation they nevertheless provide experimental evidence of potential benefit from the combination of sulphadiazine with trimethoprim.

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