Susceptibility of Spheroplast of *Proteus mirabilis* to Colistin in the Presence of Ethylenediaminetetraacetic Acid

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Received September 17, 1974

Colistin (CL) is one of a group of basic peptide antibiotics, named polymyxin.4) The antimicrobial spectra of CL are confined to some kinds of gram-negative bacteria, however the *Proteus* species has been known to be insensitive to it. It is well recognized that the primary site of action of CL is upon the cell membrane with *Escherichia coli*.2) On the other hand, CL shows synergism with sulfonamide to *Proteus mirabilis*.1) Although actinomycin has no effect on an intact cell of *E. coli*, it inhibits the growth and RNA synthesis of the ethylenediaminetetraacetic acid (EDTA)-treated cell.6) Consequently, it can be assumed that at least EDTA treatment increases the nonspecific permeability of *E. coli* and releases lipopolysaccharide-lipoprotein complex from the cell wall into the medium5,6) and that phosphatide synthesis of the cell membrane would be interfered slightly by sulfonamide.1)

The purpose of the present report is to study the susceptibility of spheroplasts of *Proteus* species to CL in the presence of EDTA.

*Proteus mirabilis* 129 was used throughout this experiment. 80 ml of a heart-infusion broth (Difco) culture of bacteria, growing logarithmically, were mixed with 20 ml of 50% sucrose solution containing 0.2% MgCl₂, 0.2% CaCl₂ and 5000 μg/ml of penicillin. After incubation for 16 hr at 37°C, the spheroplasts were collected by centrifugation at 1500 rpm for 10 min. The pellets were then resuspended in 0.01 M Tris-HCl buffer (pH 7.4) containing 0.2% MgCl₂, 0.2% CaCl₂ and 0.25 M sucrose. Appropriate concentration of the spheroplasts was made by diluting the suspension with the same buffer and the concentration was measured by a hemocytometer and adjusted to 5.5 x 10⁵/ml of spheroplasts.

Release of UV absorbing materials from CL and EDTA-treated spheroplasts and morphological changes on an electron microscope were investigated by the following two methods:

**UV absorption**

One ml of a various concentration of CL and 1.0 ml of 10⁻⁵ M EDTA were added to 8.0 ml of spheroplast suspension and incubated for 120 min at 37°C. In parallel experiments, *Proteus* exposed to the same dose of EDTA was found to be unsusceptible to CL. After incubation, the mixture was centrifuged and absorbance of the resulting supernatant was measured at 260 nm by a Beckman DSB 70 spectrophotometer. As shown in Fig. 1, the release of UV absorbing materials from spheroplasts could not be detected with 10 μg/ml of CL or 10⁻⁶ M EDTA alone, but was detectable with 10 μg/ml of CL in the presence of EDTA (10⁻⁶ M) and markedly with 100 μg/ml of CL in the presence of EDTA. At 4°C, no release of UV absorbing materials was detected. The release of UV absorbing materials is dependent on the incubation periods and it seems similar to the case of *E. coli*, which is treated with CL.7) The relation between the release of UV absorbing materials and the viability of CL and EDTA-treated spheroplasts clearly indicates a direct correlation. The UV absorbancy was gradually increased with incubation periods and at the same time the viability was decreased gradually.

**Electron-microscopy**

Specimens were prepared by the conventional fixation technique and were shadowed with carbon, then, examined with a Hitachi HU-12 electron microscope. As shown in Fig. 2, when spheroplasts were treated with 100 μg/ml of CL together with EDTA (10⁻⁶ M) at 37°C for 10 min (Fig. 2E) and 39 min (Fig. 2F), the rupture of the cell was generally observed. On the other hand, in the case of the treatment with CL (100 μg/ml) or EDTA (10⁻⁶ M) alone no burst was detected (Fig. 2C)

![Fig. 1. Release of UV Absorbing Substances from *Proteus mirabilis*.](image)

Symbols: ○—○, Control; ○—○, EDTA only; ○—○, CL only; ■—■, A various conc. (μg/ml) of CL and with EDTA (10⁻⁶ M).
or 2D). It has been reported that in the treatment of E. coli with polymyxin E a number of bleb were seen on the cell surface.3,8) However, in the case of CL and EDTA-treated spheroplasts of Proteus mirabilis no bleb formation was seen on the cell membrane, but a number of bursted cell were observed.

In conclusion, CL alone does not affect the spheroplasts of Proteus mirabilis yet it does affect the spheroplasts in the presence of EDTA. It seems that CL with EDTA increases the permeability of spheroplasts by prolonged incubation at 37°C and finally disrupts the cell.