STUDIES ON THE SELECTIVITY OF ACTION OF COLISTIN, COLISTIN NONAPEPTIDE AND COLISTIN HEPTAPEPTIDE ON THE CELL ENVELOPE OF *ESCHERICHIA COLI*

MIKIKO ITO-KAGAWA and YASUO KOYAMA

Kayaku Antibiotics Research Laboratory Ltd., Funado 2-8-16, Itabashi-ku, Tokyo, Japan

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KOIKE *et al.* reported that a cationic acylpeptide antibiotic, colistin (polymyxin E) caused the formation of blebs on the outer layer of the Gram-negative bacterial cell envelope[1]. They have also reported that colistin affects the cytoplasmic membrane, leads to leakage of cytoplasmic 260 nm-absorbing materials and has specific bursting-activity on spheroplasts of Gram-negative bacteria[2]. On the other hand, COOPERSTOCK showed with Limulus assay that endotoxic lipopolysaccharide (LPS), which was located in the cell envelope, was inactivated with colistinmethate derived from colistin sulfate[3].

Successively, the authors[4,5] reported that colistin nonapeptide was obtained from the hydrolysate of colistin after treatment with the proteolytic enzyme, ficin, and that colistin was cleaved selectively by colistin-inactivating enzyme, colistinase, to yield colistin heptapeptide. These peptides exhibited no antibiotic activity. However, recently, VAARA and VAARA reported that mixtures of polymyxin B nonapeptide and a hydrophobic antibiotic had a synergistic effect against *Escherichia coli*[6]. SIXL and GALLA demonstrated by fluorescense polarization measurements that colistin nonapeptide bound to phosphatidic acid in the cell membrane and, consequently, led to a rigidification of the cell membrane[7].

The present study reports the investigation, using colistin nonapeptide and colistin heptapeptide, of the importance of the cyclic peptide moiety and the tripeptide side chain of colistin in the action of colistin on the cell envelope of *E. coli* NIHJ.

E. coli NIHJ, used as the test organism in this study, was inoculated into 10 ml of medium in a test tube and incubated overnight at 37°C. The medium had the following composition per liter: glucose 1.0 g, Casamino Acids 1.0 g, K_2HPO_4 7.0 g, KH_2PO_4 2.0 g, $MgSO_4 \cdot 7H_2O$ 0.1 g, $(NH_4)_2SO_4$ 1.0 g, sodium citrate 0.5 g, L-tryptophan 20 mg.

Erythromycin, rifampicin and novobiocin were obtained from Boehringer Mannhein GmbH, West Germany. Fusidic acid, cloxacillin and ficin (EC 3.4.22.3) were obtained from Sigma Chemical Co., U.S.A. Lipopolysaccharide w from *E. coli* 0111: B4 was obtained from Difco Laboratories, U.S.A. "PYRODICK", which was used for colorimetric determination of endotoxin, was obtained from Seikagaku Kogyo Co., Ltd., Tokyo.

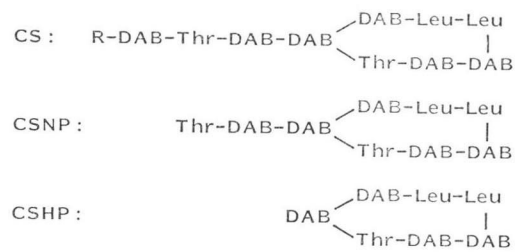
Colistin nonapeptide (CSNP) was prepared by the method of CHIHARA *et al.* using ficin[4], and colistin heptapeptide (CSHP) using colistinase by the method of the authors[5].

Minimum inhibitory concentration (MIC) was determined by a dilution method using tissue culture plates with 96-wells (Falcon, catalog No. 3072; Becton Dickinson Labware). Culture medium (200 μl) containing the indicated amount of each antibiotic and CSNP or CSHP was pipetted into each well of a tissue culture plate. An aliquot (10 μl) containing 2×10^4 bacterial cells per ml suspended in a fresh medium was added to each well. The plates were covered, mixed on a plate mixer and incubated at 37°C for 18 hours. The lowest antibiotic concentration that completely inhibited growth was defined as the minimum inhibitory concentration (MIC).

Fig. 1 shows chemical structures of colistin (CS), colistin nonapeptide (CSNP) and colistin heptapeptide (CSHP).

Minimum inhibitory concentrations of five

Fig. 1. Chemical structures of colistin (CS), colistin nonapeptide (CSNP) and colistin heptapeptide (CSHP).



Colistin A: R = 6-Methyloctanoic acid
Colistin B: R = Isooctanoic acid
DAB: 2,4-Diaminobutyric acid

Table 1. MIC (μg/ml) of various antibiotics against *E. coli* NIHJ in the presence of colistin nonapeptide (CSNP).

Antibiotic	Colistin nonapeptide (μg/ml)							
	0	0.16	0.32	0.63	1.25	2.5	5	10
Erythromycin	25	25	25	3.15	0.78	0.78	0.78	0.78
Novobiocin	12.5	12.5	12.5	6.25	3.13	1.56	1.56	1.56
Rifampicin	3.13	3.13	3.13	0.39	0.1	0.1	0.1	0.1
Fusidic acid	100	100	100	50	6.25	6.25	6.25	6.25
Cloxacillin	100	100	100	100	100	100	50	50

Table 2. MIC (μg/ml) of various antibiotics against *E. coli* NIHJ in the presence of colistin heptapeptide (CSHP).

Antibiotic	Colistin heptapeptide (μg/ml)				
	0	10	25	50	100
Erythromycin	25	25	25	6.25	1.56
Novobiocin	12.5	12.5	6.25	6.25	3.13
Rifampicin	3.13	3.13	3.13	0.78	0.2
Fusidic acid	100	100	100	100	50
Cloxacillin	100	100	100	100	100

Table 3. Inactivation of endotoxin (LPS) by colistin sulfate (CS), colistin nonapeptide (CSNP) and colistin heptapeptide (CSHP)

Test compound	Amount of CS, CSNP or CSHP	Residual LPS (ng/ml)	Inactivation (%)
CS	0	2.5	0
	10 μg/ml	0	100
	1 μg/ml	0.75	70
	100 ng/ml	1.8	28
	10 ng/ml	2.5	0
CSNP	0	2.5	0
	10 μg/ml	2.5	0
	1 μg/ml	2.5	0
CSHP	0	2.5	0
	10 μg/ml	2.5	0
	1 μg/ml	2.5	0

Endotoxin (LPS) prepared from *E. coli* 0111: B4 was used at a final concentration of 2.5 ng/ml.

antibiotics in the presence of varying amounts of colistin nonapeptide (CSNP) were examined against *E. coli* NIHJ. As shown in Table 1, synergistic effects were observed when each erythromycin, novobiocin, rifampicin or fusidic acid was added to the culture, whereas the β-lactam antibiotic, cloxacillin, was not effective. This result was similar to that of VAARA and VAARA in experiments using polymyxin B nonapeptide[6].

Subsequently, the same experiment was performed using cyclic colistin heptapeptide which lacks completely the side chain of colistin. Colistin heptapeptide gave a similar synergistic effect, but it was 100-fold less effective than colistin nonapeptide. This result is shown in Table 2.

From these results, it is suggested that the cyclic peptide moiety of colistin molecule effects the alteration of membrane permeability and subsequently facilitates entry of antibiotics except for cloxacillin, although itself it has no direct lethal action. It is assumed that the permeability-increasing action is attributable to the number of amino acids of the tripeptide side chain in colistin molecule, because colistin nonapeptide was more effective than colistin heptapeptide.

The reciprocity between the endotoxic lipopolysaccharide (LPS), which is located in the outer portion of the bacterial cell wall and colistin nonapeptide or colistin heptapeptide was investigated enzymatically using "PYRODICK" invented by HARADA *et al.*[8]. Colistin inactivated endotoxin from *E. coli* 0111: B4, whereas colistin nonapeptide and colistin heptapeptide did not (Table 3). From this result, it is confirmed that colistin interacts strongly with lipopolysaccharide and this function is attributable to the acyl moiety of the colistin molecule.

References

1) KOIKE, M.; K. IIDA & T. MATSUO: Electron microscopic studies on mode of action of polymyxin. J. Bacteriol. 97: 448~452, 1969
2) ITO, M. & Y. KOYAMA: Jolipeptin, a new peptide antibiotic. II. The mode of action of jolipeptin. J. Antibiotics 25: 309~314, 1972
3) COOPERSTOCK, M. S.: Inactivation of endotoxin by polymyxin B. Antimicrob. Agents Chemother. 6: 422~425, 1974
4) CHIHARA, S.; T. TOBITA, M. YAHATA, A. ITO & Y. KOYAMA: Enzymatic degradation of colistin, isolation and identification of α-*N*-acyl α,γ-diaminobutyric acid and colistin nonapeptide. Agric. Biol. Chem. 37: 2455~2463, 1973
5) ITO-KAGAWA, M. & Y. KOYAMA: Selective cleavage of a peptide antibiotic, colistin by colistinase. J. Antibiotics 33: 1551~1555, 1980
6) VAARA, M. & T. VAARA: Sensitization of Gram-negative bacteria to antibiotics and complement by a nontoxic oligopeptide. Nature 303: 526~528, 1983
7) SIXL, F. & H. GALLA: Calorimetric investigation of polymyxin binding to phosphatidic acid. Biochim. Biophys. Acta 693: 466~478, 1982
8) HARADA, T.; T. MORITA, S. IWANAGA, S. NAKAMURA & M. NIWA: A new chromogenic substrate method for assay of bacterial endotoxins using Limulus hemocyte lysate. Biochemical Applications of the Horseshoe Crab (Limulidae), p. 209, 1979