COLISTIN-MEDIATED ACTION OF NEOCARZINOSTATIN, ACTINOMYCIN AND MIKAMYCIN ON *ESCHERICHIA COLI*

Sir:

The antimicrobial spectra of neocarzinostatin (NCS), an antitumor protein, is confined to a limited number of Gram-positive bacteria. Among them, *Sarcina lutea* PCI-1001 has been known to be most sensitive to NCS[1]. NCS inhibits the DNA synthesis of *S. lutea* in a specific manner[2,3]. Gram-negative organisms are generally resistant to NCS, but, according to personal communication of Y. ONO, *Escherichia coli* can be rendered susceptible to NCS after exposure to ethylenediaminetetraacetic acid disodium salt (EDTA). Recently, a mutant of *Shigella* was isolated which is more sensitive to NCS than *S. lutea*[4]. The EDTA-treated *E. coli* and the mutant of *Shigella* were found to be susceptible not only to NCS but also to actinomycin D (AcM), suggesting an involvement of membrane permeability in the inhibition by these peptide antibiotics. The present study was conducted along KAWAMATA's observation that colistin (CL), an antibiotic which induces morphological disorders on the cell surface of Gram-negative organisms, can render *E. coli* to be sensitive to AcM[5]. When *E. coli* was treated simultaneously with NCS and a subinhibitory dose of CL, the growth of *E. coli* was completely inhibited. In parallel experiments, *E. coli* exposed to the same subinhibitory dose of CL was found to become susceptible to AcM and mikamycin (MK).

E. coli was used throughout this experiment. Growth was pursued either by means of viable count of turbidimetry. The subinhibitory concentration of CL used in this experiment was 0.5 mcg/ml which was almost $^1/_{20}$ of the minimum inhibitory concentration. The logarithmically growing *E. coli* cells were distributed in 8 ml of glucose-SIMMON's medium in an L-shaped culture tube and the cell concentration was adjusted to 9×10^5 cells per ml. Then 1 ml of CL and 1 ml of either NCS or AcM or MK solution were added to give a final volume of 10 ml. The growth of *E. coli* is not influenced by a single administration of each of 0.5 mcg/ml of CL, 100 mcg/ml of NCS (Fig. 1a), 10 mcg/ml of AcM (Fig. 1b) and 10 mcg/ml of MK (Fig. 1c). On the other band, the growth of *E. coli* was inhibited by NCS, AcM and MK in the combination with CL (Fig. 1a, b, c).

Fig. 1. Combination effect on the growth of *E. coli* between CL and NCS (a) or CL and AcM (b) or CL and MK (c).

The over-night culture of *E. coli* in glucose-SIMMON's medium was diluted with the same medium, incubated for 3 hours at 37°C and then diluted again to give 9×10^5 cells/ml. At 0 time, 0.5 mcg/ml of CL and indicated concentrations of NCS, AcM and MK were added. At various times, 1 ml aliquot of culture was harvested and viable count was made on a nutrient agar plate.

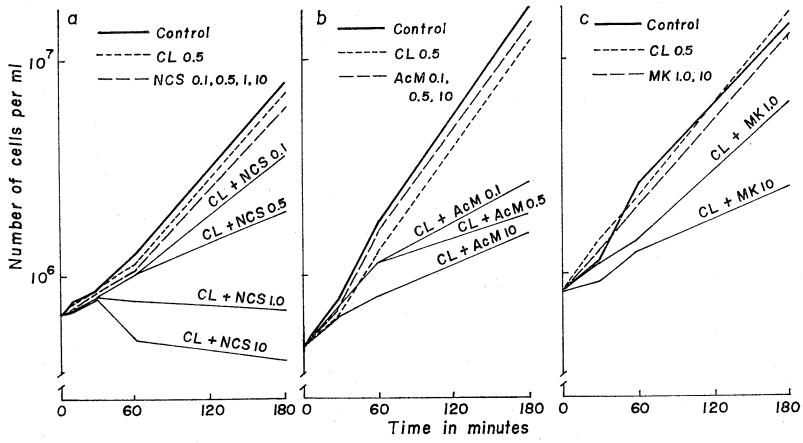


Fig. 2. Effect of CL and NCS (2 a) or CL and AcM (2 b) or CL and MK (2 c) on the incorporation of radioactive precursor into macromolecule synthesis.

(2 a) CL and NCS, (2 b) CL and AcM, (2 c) CL and MK were added to the bacterial suspension at 0 time. One ml of cultures were taken 5 minutes before the indicated time and exposed to (2 a) 0.01 μC/ml of ^{3}H-TdR, (2 b) 0.01 μC/ml of ^{3}H-UdR and (2 c) 0.02 μC/ml of ^{3}H-Leucine respectively for 10 minutes at 37℃ followed by the addition of 100-fold cold TdR or UdR. At the end of labelling, the cultures were immediately chilled in an ice-bath and one drop of 30% perchloric acid was added together with 1% bovine serum albumin. Cells collected by centrifugation were dissolved in 10% ammonia and dried on metal planchet for counting.

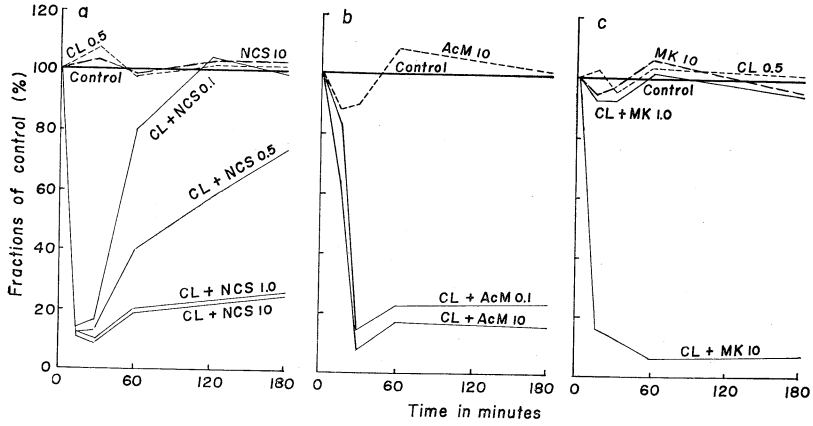
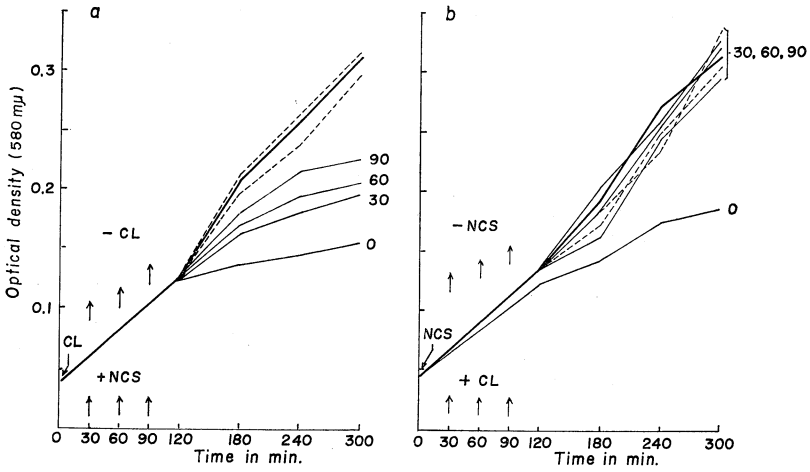


Fig. 3. Effect of pretreatment of CL and NCS on the growth of *E. coli*

The cultures under the procedure described in Fig. 1 were treated with 0.5 mcg/ml of CL at 0 time. After incubation for the indicated periods, the bacterial cells were harvested and washed by centrifugation with sterlized saline followed by addition of 10 mcg/ml of NCS. Bacterial growth was measured by turbidimetry at 580 mμ in Shimazu spectronic photometer. Arrows and numbers show time of pretreatment with CL and followed by the replacement with NCS after removal of CL (3 a). In Fig. 3b, first NCS was added at 0 time and then CL was added at the indicated time after removal of NCS. Arrows and numbers show time of removals of NCS and additions of CL (3 b).



The effect on the macromolecular synthesis of the exponentially growing *E. coli* was studied by measuring incorporation of ^{3}H-TdR, ^{3}H-UdR and ^{3}H-leucine into DNA, RNA and protein with the respective combinations. One of the typical experiment is illustrated in Fig. 2. The incorporation of ^{3}H-TdR into DNA was inhibited immediately after the simultaneous addition of CL and NCS. No inhibitory effect was found after single administration of 0.5 mcg/ml of CL or 10 mcg/ml of NCS (Fig. 2a). Dose reponse of

the DNA synthesis was evident within the range of NCS concentrations used and the inhibition was complete with 1 mcg/ml of NCS (Fig. 2a). No inhibition by this combination was found on RNA and protein syntheses (not shown in the figure). As shown in Fig. 2b, the incorporation of ^{3}H-UdR into RNA was specifically inhibited after the addition of 0.5 mcg/ml of CL and 10 mcg/ml of AcM. As demonstrated in Fig. 2c, the simultaneous treatment of *E. coli* with CL and MK led to a specific inhibition of protein synthesis.

Further study was conducted to reveal the site of synergistic action of NCS and CL. When CL was added at 0 time and removed at 30, 60 and 90 minutes with substitution of NCS, the result shown in Fig. 3a was obtained. Pretreatment with CL followed by replacement with NCS resulted in the complete inhibition (Fig. 3a), but the reverse combination of NCS and CL did not result in such effect (Fig. 3b).

These finding together with above described specific inhibition of macromolecular syntheses lead to the conclusion that the cell surface of *E. coli* must have been altered by CL in order to become NCS permeable to cell surface.

It was also reported that degradation of DNA was caused by NCS in *S. lutea*[2]. On the basis of this description, ^{3}H-TdR prelabeled *E. coli* was treated simultaneously with CL and NCS and the fate of DNA was examined. As shown in Fig. 4, the radioactivity of acid-insoluble fraction of cells decreased when treated with 0.5 mcg/ml of CL and 10 mcg/ml of NCS. This effect was not found when 0.5 mcg/ml of CL or 10 mcg/ml of NCS was added separately. In Fig. 4, the effect of mitomycin C (MMC) was also illustrated[6].

In summary, NCS, AcM, MK which were originally ineffective in the inhibition of macromolecular syntheses became effective by combining with CL. The present results should be useful for the studies on the mode of action of NCS in *E. coli*.

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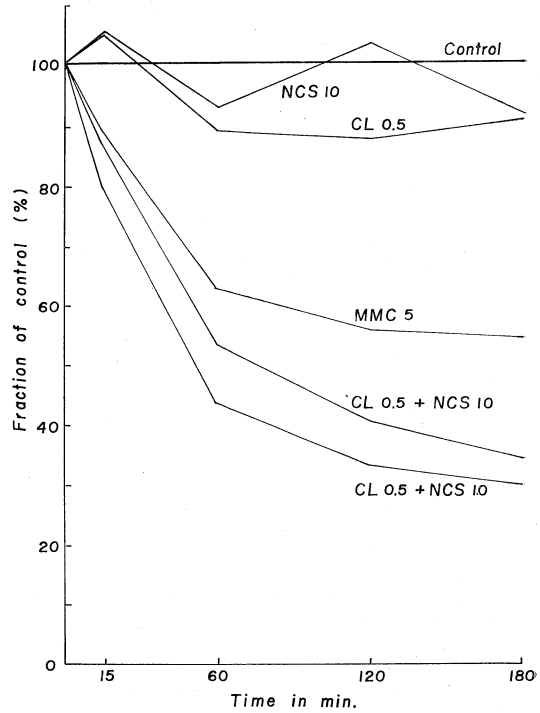
Fig. 4. Degradation of *E. coli* DNA when cells well treated simultanously with CL and NCS.

Exponentially growing *E. coli* B was pre-labelled with 0.01 μC/ml of ^{3}H-TdR for 2 hours at 37℃ and cells collected were washed three times by centrifugation with sterlized saline and reincubated in fresh medium without labelled precursor for 3 hours. Then a 100-fold amount of cold TdR and 0.5 mcg/ml of CL and a various amount of NCS and MMC were added and the cultures incubated at 37℃. At the indicated time, 1 ml of culture was taken from each culture and chilled immediately. To each culture, one drop of 1% bovine serum albumin and one-tenth volume of 60% perchloric acid were added and the mixture kept in an ice-bath for 30 minutes. After centrifugation at 3,000 rpm for 15 minutes, the precipitates were dissolved in 10% ammonia. Aliquots were neutralized with K_2CO_3 and dried on a metal planchet for counting.



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(Received October 11, 1971)

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