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EFFECT OF EDTA ON ANTIBACTERIAL ACTIVITY OF GRAMICIDIN S

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Abstract

The effect of EDTA on the antibacterial activity of gramicidin S (GS) for *E. coli* has been studied. The antibacterial activity of GS for *E. coli* enhanced remarkably and the amount of ^{14}C-labeled GS adsorbed on the cells increased in the presence of EDTA in the medium. Therefore, it is concluded that EDTA falls the cell wall permeability barrier for GS. On the other hand, EDTA did not have a pronounced effect on the action of GS for *B. subtilis*.

Gramicidin S (GS) is an antibiotic with a cyclo structure (-Val-Orn-Leu-D-Phe-Pro-Val-Orn-Leu-D-Phe-Pro-) which is produced by certain strains of *Bacillus brevis*. GS is known to be bacteriostatic and bactericidal for numerous Gram-positive bacteria, but less active against Gram-negative ones (*1, 2*). In a previous paper, we described that GS molecules were adsorbed on the cell membrane and GS prevented the functioning of the cell membrane (*3*). The bacteria dies when destraction of cell membrane extends over the whole cell membrane, because most of the cell membrane is covered with GS molecules at the minimum inhibitory concentration (MIC) (*4*). GS is adsorbed rapidly on the cells of Gram-positive bacteria such as *Bacillus subtilis,* but not easily adsorbed on the cells of Gram-negative bacteria such as *Escherichia coli* (*4*). However, the amount of GS adsorbed on the spheroplast membrane increased remarkably compared with natural *E. coli* cells. It is concluded that the poor GS adsorption on *E. coli* cells may be due to the permeability barrier of the *E. coli* cell wall. Consequently, the antibacterial spectra of GS on Gram-positive and Gram-negative bacteria are accounted for by a difference in the cell wall permeability.

When *B. subtilis* cells are treated with lysozyme, the cell walls are digested and the cells are transformed to protoplasts (*5*). On the other hand, the cell walls of *E. coli* are not digested with lysozyme alone. Gram-negative bacteria have outer membrane which consists of protein, lipid and lipopolysaccaride. Lysozyme must be unable to penetrate the

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Abbrebiations: GS, gramicidin S; MIC, minimum inhibitory concentration

outer membrane. When Gram-negative bacteria cells are treated with lysozyme in EDTA solution, the cell walls are digested with lysozyme and the cells are transformed to spheroplasts (*6*). EDTA presumably falls the permeability barrier of outer membrane. If Gram-negative bacteria are incubated with GS in EDTA solution, GS may be able to penetrate outer membrane easily and the antibacterial activity of GS may increase. In this paper, we examined the effect of EDTA on the action of GS for *E. coli* cells.

Materials and Methods

Bacillus subtilis IFO 3007 and *Escherichia coli B* were used for antibacterial experiments. The bacteria were grown in a medium of polypeptone-meat extract-yeast extract at 30°C (*4*). The cell culture was diluted with the medium to 2.7×10^8 cells/ml (cell suspension). The filter used was glass fiber filter, Whatman GF/F. ^{14}C-Labeled GS (282 dpm/μg) was prepared previously (*3*). Scintillator (Scintisol EX-H) was purchased from Wako Pure Chemical Industries Co.

Antibacterial Activity—EDTA was dissolved in the medium (E-medium) and GS was dissolved in E-medium. Various amounts of GS solution were placed in test tubes, made up to 1 ml with E-medium, and the cell suspension (1 ml) was added. After incubation for 8 hr at 30°C, distilled water (1 ml) was added, and the absorbance at 620 nm was measured.

Time Course of Adsorption of Labeled GS on Cells—A mixture of 18 ml of labeled GS solution [6 μg/ml of 0.1% E-medium (*E. coli*) and 1 μg/ml of 0.1% E-medium (*B. subtilis*)] and 18 ml of the cell suspension was incubated at 30°C, and 5 ml of the samples were taken at intervals, filtered, and the cells on the filter were washed twice with physiological saline solution (2.5 ml). The filter was placed in a vial, dried *in vacuo*. Scintillator was added to the vial, and radioactivity was measured.

Effect of Concentration of Labeled GS on Its Adsorption on E. coli Cells—Labeled GS was dissolved in 0.1% E-medium. Various amounts of labeled GS solution were placed in test tubes, made up to 3 ml with 0.1% E-medium and 3 ml of the cell suspension was added. After incubation for 40 min at 30°C, 5 ml of the reaction mixtures were filtered and the cells on the filter were treated as described above.

Adsorption of Labeled GS on Heated Cells—Fifteen ml of the cell suspension of *E. coli* was heated for 10 min in boiling water. A mixture of 8 ml of labeled GS solution (5 μg/ml of 0.1% E-medium) and 8 ml of the heated cell suspension was incubated at 30°C, and 5 ml of the reaction mixtures were taken at intervals and treated as described above.

Results and Discussion

As shown in Table I, antibacterial activity of GS on *E. coli* enhanced remarkably in the presence of EDTA compared with in the absence of EDTA. The MIC of GS for *E. coli* in the presence of 0.01% EDTA was close to the MIC of GS for *B. subtilis* (2.5 μg/ml and 1.5 μg/ml respectively). On the other hand, antibacterical activity of GS on *B. subtilis* did not vary in both cases.

Fig. 1 shows that the amount of labeled GS adsorbed on *E. coli* cells increases until 40

Table I. Effect of EDTA on antibacterial activity

Bacteria	Concentration of EDTA (%)	MIC (μg/ml)
E. coli	0	5< <10
	0.01	1< <2.5
	0.05	1< <2.5
	0.25	1.5< <2.5
B. subtilis	0	1< <1.5
	0.05	1< <1.5

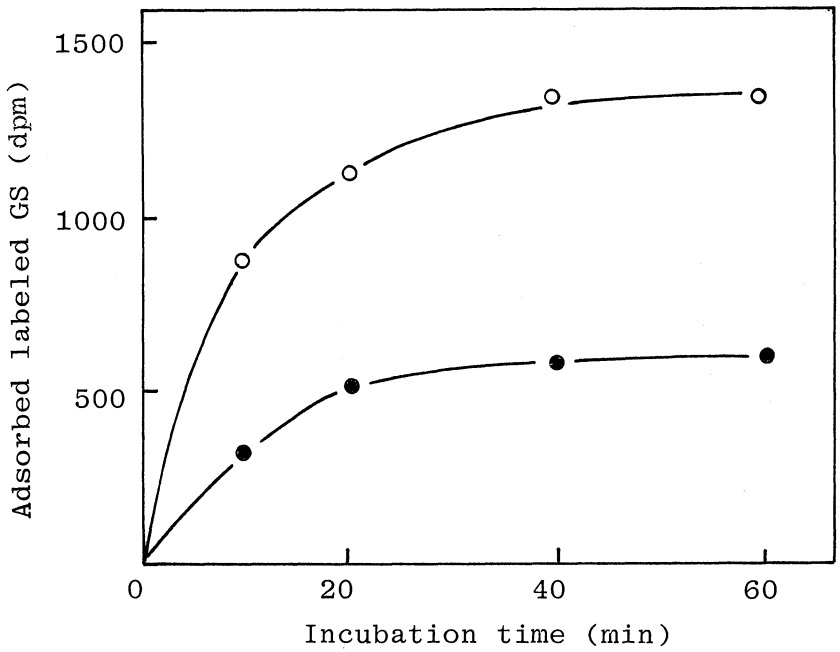

Fig. 1 Time course of adsorption of labeled GS on *E. coli* cells. absence of EDTA (●); presence of EDTA (○).

min and does not change up to 60 min. Therefore, in subsequent experiments, an incubation time of 40 min was employed. The amount of GS adsorbed on *E. coli* cells in the presence of EDTA increased twofold compared with the amount in the absence of EDTA. The amounts of labeled GS adsorbed on *B. subtilis* cells were same in both cases (Fig. 2).

As shown in Fig. 3, when EDTA was absent in the medium, labeled GS was not easily adsorbed on *E. coli* cells at lower concentrations, but the amount adsorbed increased above 7 μg/ml, and the cells were temporarily saturated with GS at 10 μg/ml, which is the MIC of GS for *E. coli.* On the other hand, when 0.05% EDTA was presented in the medium, the adsorption curve of labeled GS on *E. coli* cells were similar to that of labeled GS on *B. subtilis* (*2*). As the concentration of labeled GS increased, the amount adsorbed on *E. coli* cells increased discontinuously, producing a curve which had three plateaus. The cells were temporarily saturated with GS at the concentration of 2.5~3 μg/ml, which is the MIC

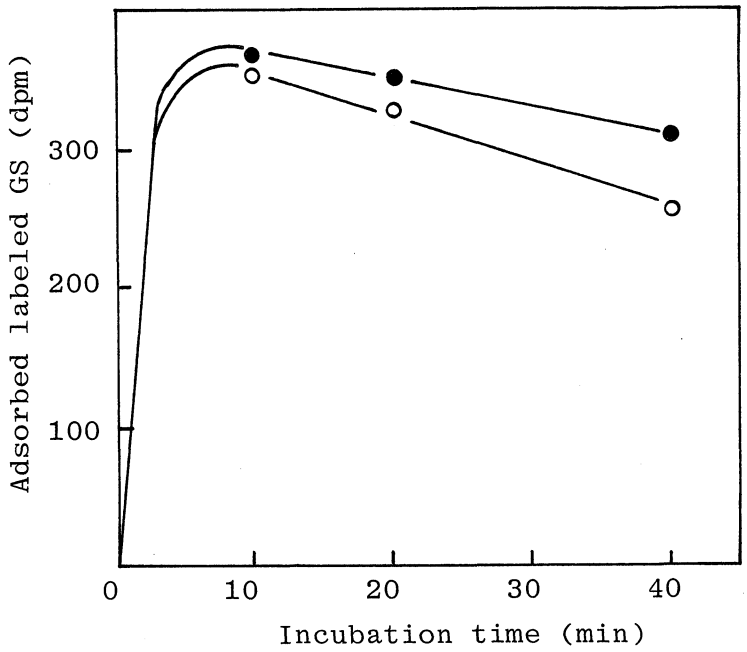

Fig. 2 Time course of adsorption of labeled GS on *B. subtilis* cells. absence of EDTA (●); presence of EDTA (○).

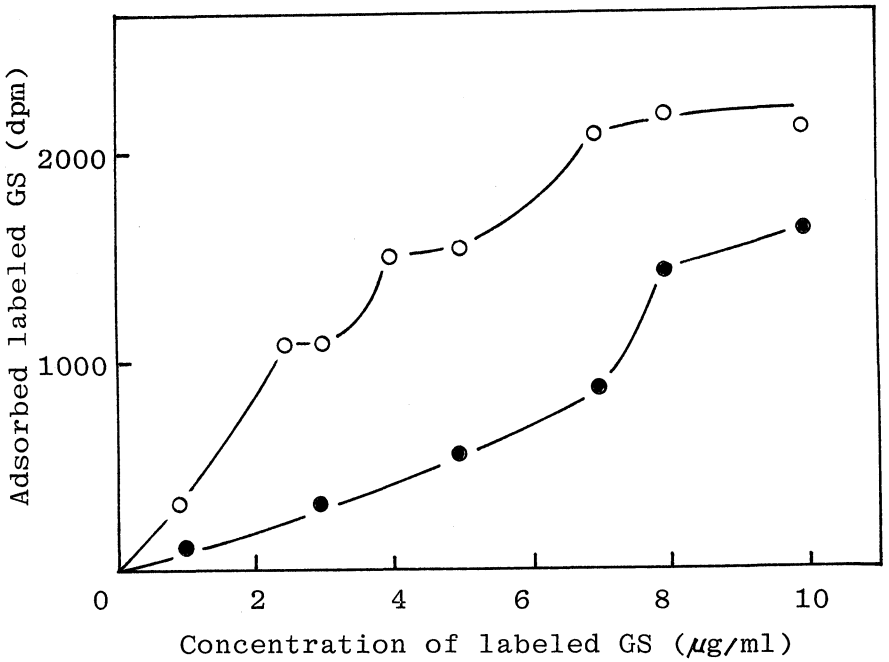

Fig. 3 Effect of concentration of labeled GS on its adsorption on *E. coli* cells. absence of EDTA (●); presence of EDTA (○).

of GS for *E. coli* in the presence of EDTA.

The results of these experiments show that EDTA falls the permeability barrier of outer membrane and increases the permeability of cell wall for GS. Then, the amount of labeled GS adsorbed on *E. coli* cells increases in the presence of EDTA and *E. coli* dies even at lower concentration of GS. The amount of labeled GS adsorbed on the heated *E. coli* cells in the absence of EDTA were approximately equal to that adsorbed on the cells in the presence of EDTA (Fig. 4). The permeability barrier for GS may be due to the active transport.

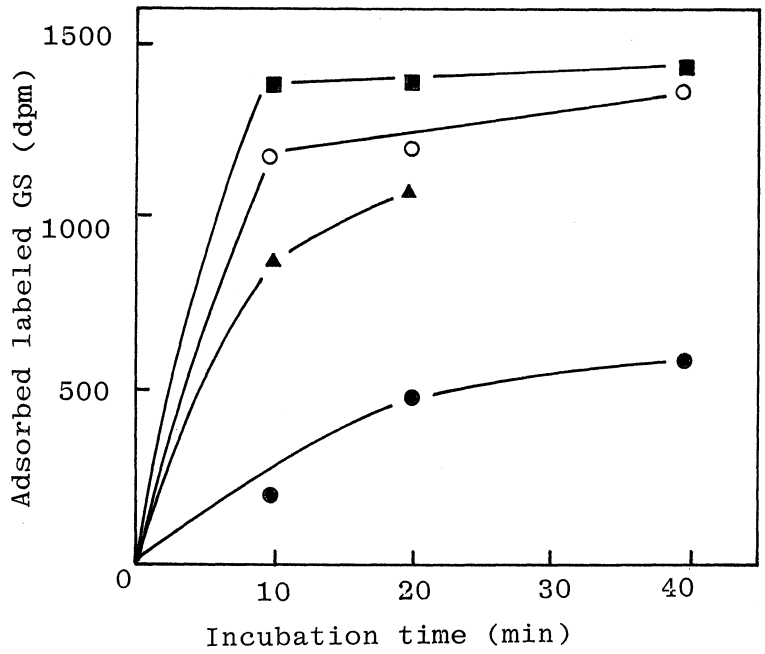

Fig. 4 Time course of adsorption of labeled GS on heated *E. coli* cells. natural cells in absence of EDTA (●); heated cells in absence of EDTA (○); natural cells in presence of EDTA (▲); heated cells in presence of EDTA (■).

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