

INCREASE OF CARDIOLIPIN CONTENT IN *STAPHYLOCOCCUS AUREUS* BY THE USE OF ANTIBIOTICS AFFECTING THE CELL WALL

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Effect of antibiotics affecting cell wall synthesis on phospholipid composition in *Staphylococcus aureus* 209P was examined. Each antibiotic was added in the middle exponential growth phase and the growth was followed turbidimetrically. Penicillin, fosfomycin, cycloserine, moenomycin and cefazolin caused a leveling off of turbidity and growth to cease without lysis. Enramycin and bacitracin were bacteriolytic. Bacteriolytic antibiotics caused a greater increase of cardiolipin content than those that were non-bacteriolytic. The amount of phosphatidylglycerol decreased in proportion to the increment of cardiolipin content. Since bacteriolytic antibiotics bind to undecaprenol, the role of cardiolipin was discussed in relation to the mechanism of synthesis of cell surface materials.

The phospholipid composition of plasma membrane tends to be altered in response to a structural change in the cell wall. A previous report showed that stable L-forms of *Staphylococcus aureus* contained more cardiolipin than the parent strain, while other phospholipids did not show remarkable change.¹⁾ In autoplasts of *S. aureus*, cardiolipin was nearly half of the total phospholipids.²⁾ Similarly, L-forms of *Streptococcus pyogenes*, *Escherichia coli*, *Proteus mirabilis* and *Pseudomonas aeruginosa* contained more cardiolipin than their parent strains.

Antibiotics which affect the synthesis of cell wall caused an increase of cardiolipin content in *Bifidobacterium bifidum*, *S. aureus* and *E. coli*.³⁻⁵⁾ It is of particular interest to examine whether the increase of cardiolipin content accounts for the alteration of phospholipid metabolism coupled with the stage in the peptidoglycan synthetic pathway. In this study, seven antibiotics which have different acting sites were chosen. These are penicillin, fosfomycin, cycloserine, moenomycin, cefazolin, bacitracin and enramycin.⁶⁻¹¹⁾ This paper describes alteration of phospholipid composition of *S. aureus* after treatment with these antibiotics.

Materials and Methods

Antibiotics

Antibiotics were kindly provided by the following companies: benzylpenicillin, fosfomycin and cycloserine; Meiji Seika Kaisha, Ltd., Tokyo; moenomycin; Hoechst Japan Limited, Tokyo; cefazolin; Fujisawa Pharmaceutical Co., Ltd., Osaka; bacitracin; Ono Pharmaceutical Co., Ltd., Osaka; enramycin; Takeda Chemical Industries, Ltd., Osaka. Each potency was as follows; benzylpenicillin 1,600 u/mg; fosfomycin, 758 µg/mg; cycloserine, 1,000 µg/mg; moenomycin, 980 µg/mg; cefazolin, 944 µg/mg; bacitracin, 68 u/mg; enramycin, 1,050 µg/mg.

Organism and Culture Condition

Staphylococcus aureus 209P (FDA) was used throughout this study. The organism was cultured in nutrient broth (Nissui, Tokyo, Japan) at 37°C with reciprocal shaking in a T-shaped tube and an inoculum size of 0.5%. The growth was monitored at 30 minutes intervals by measuring optical density at 650 nm with a Spectronic 20A (Shimadzu, Japan). Antibiotics were added to the cultures at the mid-

dle exponential phase (O.D. 0.8). Antibiotics dissolved in 0.85% NaCl solution were prepared immediately before adding to the cultures. Enramycin was added to the culture as a powder.

Analysis of Lipids

Cells were harvested at 0, 90 and 180 minutes after the addition of antibiotics and collected by centrifugation at $8,000 \times g$ for 10 minutes. The cells were washed twice with cold 0.85% NaCl solution. Then the wet weight was measured after centrifugation at $12,000 \times g$ for 10 minutes. Total lipids were extracted with 45 volume of chloroform - methanol (2:1, v/v) for 15 hours at room temperature and were purified by the method of FOLCH *et al.*¹²⁾ Phospholipids were analyzed by two dimensional Silica Gel G (E. Merck, Darmstadt) thin-layer chromatography. The solvent system was CHCl_3 - MeOH - H_2O (65: 25: 4, v/v/v) in the first development and CHCl_3 - MeOH - 7N NH_4OH (60: 35: 4.5, v/v/v) in the second. Phospholipid phosphorus was determined by the method of KATES *et al.*¹³⁾ The weight of each phospholipid was calculated by multiplying the amount of phosphorus by 25.

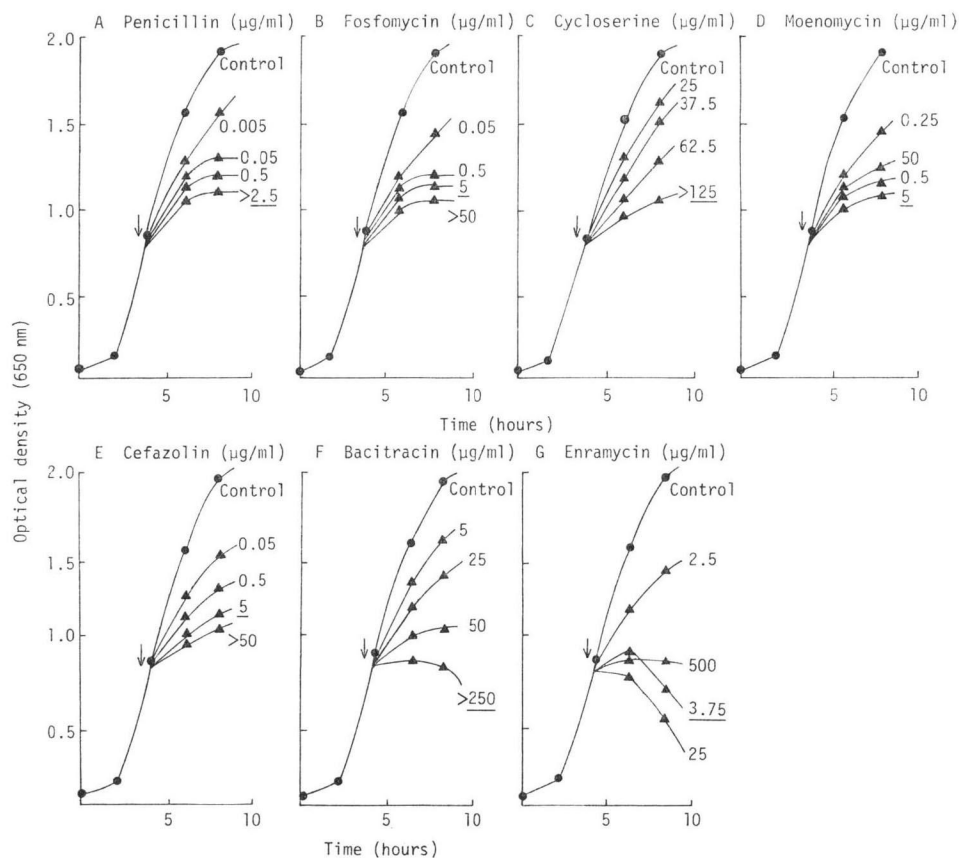
Results

Effects of Antibiotics on the Growth of *S. aureus*

Fig. 1A shows the growth curve of *S. aureus* in the presence of various concentrations of penicillin.

Fig. 1. Effects of various cell wall affecting antibiotics on the growth of *S. aureus*.

Various concentrations of antibiotics were added to the cultures at middle exponential phase (O.D. 0.8). Arrow indicates time of addition of each antibiotic. Numbers in figure indicate concentration ($\mu\text{g/ml}$) of the antibiotic. Lipid analysis was carried out at the underlined concentration.



The growth rate was reduced at the concentrations above 0.05 $\mu\text{g/ml}$ and it was inhibited completely at more than 2.5 $\mu\text{g/ml}$. The growth remained stationary during the 24 hours incubation without obvious cell lysis. Similar growth curves were obtained with various concentrations of fosfomycin, cycloserine, moenomycin and cefazolin (Fig. 1B & C, D, E). Completely inhibitory concentrations of fosfomycin, cycloserine and cefazolin were 5, 125 and 5 $\mu\text{g/ml}$ respectively. Moenomycin showed a zone phenomenon and caused the strongest cessation of growth at 5 $\mu\text{g/ml}$. Fig. 1F shows the growth curve of *S. aureus* with various concentrations of bacitracin. The lysis occurred at above 250 $\mu\text{g/ml}$. Enramycin gives quicker and stronger lysis than bacitracin at the concentration range from 3.75 to 100 $\mu\text{g/ml}$. Enramycin showed a zone phenomenon and it gave the strongest lysis at 25 $\mu\text{g/ml}$ (Fig. 1G). These antibiotics can be clearly divided into two groups; non-bacteriolytic and bacteriolytic.

Effects of Antibiotics on the Phospholipid Composition of *S. aureus*

Phospholipid compositions of antibiotic treated cultures are shown in Table 1. The concentration of each antibiotic used was determined according to the most effective dosage as shown by the results in Fig. 1. Cardiolipin content of untreated cells accounted for 5.8% of total phospholipid at the middle exponential growth phase (O.D. 0.8). After the incubation for 90 and 180 minutes, it increased slightly to 8.1 and 13.1% respectively. The total phospholipid content per wet weight of cells remained constant during the growth. When non-bacteriolytic antibiotics (penicillin, fosfomycin, cycloserine, moenomycin, cefazolin) were added, cardiolipin content increased gradually to 16.4~23.4% after 90 minutes and 19.5~27.2% after 180 minutes incubation. In the presence of bacitracin or enramycin, the increase of cardiolipin content was remarkable, and it came up to 55.2~71.0% as shown in Table 1. The decrease in the content of phosphatidylglycerol in proportion to the increment of cardiolipin content sug-

Table 1. Effects of antibiotics on the phospholipid composition of *Staphylococcus aureus*.

Antibiotics ($\mu\text{g/ml}$)	Minutes	Wet weight (g)	Total phospholipid (mg)	Origin (%)	Lysyl-phosphatidyl-glycerol (%)	Phosphatidyl-glycerol (%)	Cardiolipin (%)
Control	0	0.70	4.7	3.8	8.4	82.0	5.8
	90	1.40	13.1	6.1	10.0	75.8	8.1
	180	1.78	16.4	10.5	12.9	63.5	13.1
Penicillin (2.5)	90	0.73	6.2	3.2	10.9	68.8	17.2
	180	0.76	6.9	2.8	10.8	63.5	22.9
Fosfomycin (5)	90	0.83	7.4	4.7	15.0	62.0	18.4
	180	0.88	8.0	7.2	13.4	54.2	25.2
Cycloserine (125)	90	0.97	6.6	7.8	10.8	65.0	16.4
	180	1.00	7.0	4.4	13.2	55.2	27.2
Moenomycin (5)	90	0.81	6.1	6.2	10.2	60.3	23.4
	180	0.80	6.2	7.5	10.4	62.5	19.5
Cefazolin (5)	90	0.84	8.4	8.7	13.5	56.4	21.4
	180	0.85	7.9	3.3	15.7	59.4	21.6
Bacitracin (250)	90	0.74	5.9	7.4	12.5	24.8	55.2
	180	0.67	5.9	10.0	10.8	23.1	56.1
Enramycin (3.75)	90	0.68	6.0	2.3	11.1	20.9	65.8
	180	0.57	5.2	3.8	13.3	11.9	71.0

All values of wet weight and total phospholipid obtained from 400 ml cultures. Composition is expressed as percentage of phosphorus in total phospholipids.

gests that phosphatidylglycerol was converted to cardiolipin as a result of the treatment with antibiotics. The content of lysylphosphatidylglycerol remained constant.

Discussion

Bacteriolytic antibiotics caused a greater increase of cardiolipin content in growing *S. aureus* cells than did non-bacteriolytic antibiotics. This may well be explained by the different sites of action of the two classes of antibiotics on peptidoglycan synthesis. The non-bacteriolytic antibiotics inhibit the activity of enzymes in the synthesis of peptidoglycan and the bacteriolytic ones bind to carrier lipid (undecaprenol phosphate) or its derivative.⁹⁻¹¹⁾ The findings suggest that antibiotics binding undecaprenol phosphate increase cardiolipin content markedly.

Undecaprenol phosphate is a carrier lipid involved in peptidoglycan synthesis as well as teichoic acid synthesis. Phosphatidylglycerol is a precursor of poly(P-glycerol), which plays an important role in teichoic acid synthesis, and also is a precursor of cardiolipin.¹⁴⁻¹⁶⁾ When bacteriolytic antibiotics bind to undecaprenol phosphate, the biosynthesis of these materials for the cell wall would be affected. The increase of cardiolipin content may result from a relative activation of cardiolipin synthesis from phosphatidylglycerol by the inactivation of undecaprenol phosphate in the membrane.

Cell lysis is regulated by lipoteichoic acid which inhibits the activity of autolysin. Cardiolipin inhibits autolysin activity as well.¹⁷⁾ Bacteriolytic antibiotics may affect synthesis of lipoteichoic acid resulting in activation of cardiolipin synthesis. Previous studies on L-forms and autoplasts of *S. aureus* indicate that increased cardiolipin content plays an important role in compensating for changes in the rigidity of cell walls.^{1,2)} Consequently, the increase of cardiolipin during treatment of *S. aureus* with bacteriolytic antibiotics could be an adaptational reaction to prevent cell lysis by either inhibition of autolysin or increased rigidity of the cell membrane, or both.

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