PREVENTION OF PENICILLIN-INDUCED LYSIS OF *STAPHYLOCOCCUS AUREUS* BY CELLULAR LIPOTEICHOIC ACID

HIDEKAZU SUGINAKA, MASAHIRO SHIMATANI, *MICHIO OGAWA and SHOZO KOTANI

Department of Microbiology and Oral Bacteriology, Osaka University Dental School, and *Second Department of Surgery, Osaka University Medical School, Kita-ku, Osaka 530, Japan

(Received for publication October 20, 1978)

The lysis of *Staphylococcus aureus* FDA 209P induced by benzylpenicillin was completely inhibited by cellular lipoteichoic acid isolated from an homologous organism. The cells prevented from penicillin-induced lysis were in static state and did not lose viability. The lipoteichoic acid inhibited either extracellular autolysin activity in culture supernatant or autolysis of whole cells in exponential phase of *S. aureus*. These results indicate that the prevention of penicillin-induced lysis of *S. aureus* by the lipoteichoic acid was brought about by the inhibition of autolytic activity of the organism.

It has been well known that β-lactam antibiotics are specifically and irreversibly bound to their target sites on the cytoplasmic membrane of Gram-positive[1~4] and Gram-negative bacteria[2,3,5], where they inhibit cross-linking reaction in peptidoglycan synthesis[6~11]. The target sites are the penicillin binding proteins, transpeptidase and D-alanine carboxypeptidase[12]. Until recently, the bacteriolysis has been assumed to be induced by the binding of β-lactam antibiotics to the penicillin binding proteins, indicating that the lysis was a consequence of the inhibition of cell wall biosynthesis[2,13].

Recent investigations[14~16] have focused attention on bacterial autolysin and its inhibitor, lipoteichoic acid, and have revealed that the lipoteichoic acids from several bacteria strongly inhibit the autolytic enzyme activity[17,18] and the autolyses of walls[19] and whole cells[20] of some Gram-positive bacteria such as *Streptococcus pneumoniae* and *Str. faecalis*.

This report deals with the prevention of penicillin-induced lysis of *Staphylococcus aureus* by the cellular lipoteichoic acid isolated from an homologous organism. In addition, the mechanisms of bactericidal action of penicillin will be discussed.

Materials and Methods

Organism and Antibiotic

The study has been performed with *Staphylococcus aureus* FDA 209P. Benzylpenicillin was obtained from Meiji Seika Ltd., Japan. The minimal inhibitory concentration (MIC) for this organism was 0.0125 μg/ml.

Preparation of Cellular Lipoteichoic Acid

The organism was grown in Trypticase soy broth (BBL) at 37°C with shaking, and the cells were harvested at the late exponential phase. After 3 washes with distilled water, the cell pellet was sedimented by centrifugation and the wet pellet was suspended in 20 volumes of chloroform – methanol (2: 1, by volume) and stirred vigorously to extract lipid at room temperature for 2 hours. The defatted cell pellet was suspended in 5 volumes of distilled water followed by the addition of the same volume of 95% phenol, and stirring at room temperature for 3 hours. To remove phenol, the aqueous phase of the phenol extract was extracted 3 times with chloroform and concentrated by evaporation. The

concentrate was fractionated on a Sepharose 6B column as reported previously[19,21]. The main effluent fractions (Kd=0.18) containing glycerol were combined and concentrated.

Chemical analyses revealed the presence of glycerol, phosphorus and fatty acid ester of the isolated lipoteichoic acid in a molar ratio of approximate 1: 1: 0.07. This lipoteichoic acid reacted with antibody specific for polyglycerol phosphate, and was capable to sensitize the red blood cells (unpublished data). Concentration of the lipoteichoic acid was determined on the basis of its glycerol contents[22].

Measurement of Growth

Exponential growing cultures were transferred to a fresh Trypticase soy broth and benzylpenicillin or the lipoteichoic acid was added to a final concentration of 40 ng/ml or 20 nmol/ml, respectively. They were grown with shaking at 37°C together with a control culture which had received no additions. At the time indicated by the arrows in Fig. 1, the lipoteichoic acid of the same concentration was added to the cultures, and they were further incubated. The measurement of growth was carried out by following the rate of change in turbidity of a suspension with a Shimadzu Bausch & Lomb Spectronic 20 Colorimeter (Shimadzu Seisakusho, Japan) at 550 nm. At the same time, the viability of cells at various incubation time was determined by the method of plating serial dilutions on Trypticase soy agar plate.

Measurement of Autolysis of Whole Cells and Extracellular Autolysin Activity from Culture Supernatant

The exponential growing cells of *S. aureus* were thoroughly washed, whole cells were suspended in 0.03 M phosphate buffer (pH 7.0) to a final concentration of about 0.25 mg/ml, and incubated at 37°C. The loss of turbidity of the suspension was followed at 550 nm to measure autolysis of whole cells. The inhibition of the autolysis by the lipoteichoic acid was determined by addition of various concentrations of the lipoteichoic acid to the above suspensions.

Extracellular autolysin of *S. aureus* was obtained as follows. Solid ammonium sulfate was added to the culture supernatant of exponential culture to 0.75 saturation. The resulting precipitate was dissolved in 0.01 M phosphate buffer (pH 7.0). The solution was dialyzed against the same buffer and the fraction was used as a source of extracellular autolysin. The autolysin activity was determined after addition of the autolysin fraction (final about 400 μg/ml protein) by following the rate of decrease in turbidity of a suspension of heated cells of *Micrococcus lysodeikticus* NCTC 2665 (final 0.25 mg/ml) in 0.03 M phosphate buffer (pH 7.0)[23]. The cells were used as a substrate because of high sensitivity to the autolysin of *S. aureus* (unpublished data).

Results

Prevention of Penicillin-induced Lysis by Cellular Lipoteichoic Acid

The results in Fig. 1 show the effect of the lipoteichoic acid on penicillin-induced lysis. The addition of a final concentration of 40 ng/ml of benzylpenicillin to growing culture of *S. aureus* FDA 209P caused marked bacteriolysis (center). This concentration of benzylpenicillin had maximal effect on the lysis of this strain. To initiate cell lysis at this time (2-hour incubation), additional lipoteichoic acid was added to a final concentration of 20 nmol/ml which inhibited lysis completely (center). The prevention of the lysis by the lipoteichoic acid was observed even when lysis had already developed, and the reduction in turbidity ceased at the time the lipoteichoic acid was added (center). On the other hand, the lipoteichoic acid had no effect at all on growth (right), and the change in turbidity was identical to control (left).

Effects of Benzylpenicillin and Cellular Lipoteichoic Acid on Viability of *S. aureus* Cells

Results are presented in Table 1 showing that *S. aureus* cells which had been prevented from penicillin-induced lysis shown in Fig. 1 did not lose viability. The addition of the lipoteichoic acid

Fig. 1. Effects of benzylpenicillin and lipoteichoic acid on the growth of *S. aureus*.

Exponentially growing culture of *S. aureus* FDA 209P was divided into 5 portions. Control culture (left) received no additions: Three cultures (center) received benzylpenicillin to a final concentration of 40 ng/ml at zero time on the graph. Two of these received 20 nmol/ml of the lipoteichoic acids at the time indicated by arrows. Remaining one culture received no lipoteichoic acid: The last culture (right) received to a final concentration of 20 nmol/ml of the lipoteichoic acid at zero time.

Abbreviation: PCG; benzylpenicillin, LTA; lipoteichoic acid.

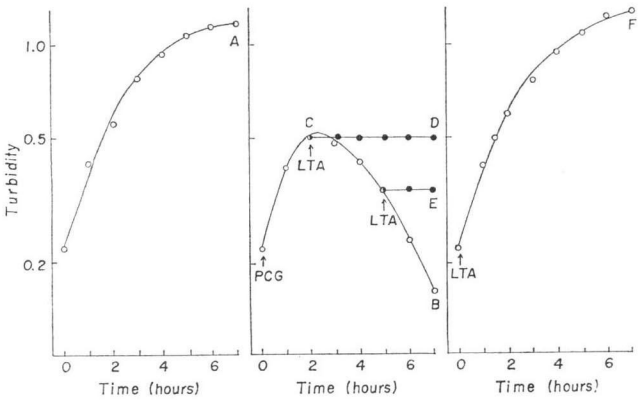


Fig. 2. Effects of lipoteichoic acid on autolysis of *S. aureus* cells.

The washed cells from exponential culture of *S. aureus* FDA 209P were incubated with various concentrations of the lipoteichoic acid (5, 10, and 20 nmol/ml). Control culture was incubated without the lipoteichoic acid. Assays were carried out as described in Materials and Methods. Data are expressed as percent reduction of turbidity of a cell suspension.

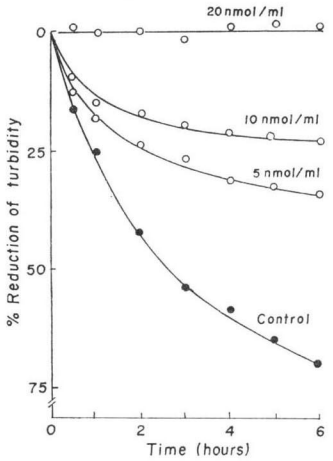


Table 1. Effects of benzylpenicillin and lipoteichoic acid on viability of *S. aureus*.

Sample*	Additions	Viable cell number (cells/ml)
A	None	4.0×10^9
B	PCG —	2.0×10^6
C	PCG LTA	2.2×10^8
D	PCG LTA	1.2×10^8
E	PCG LTA	2.0×10^7
F	LTA —	2.5×10^9

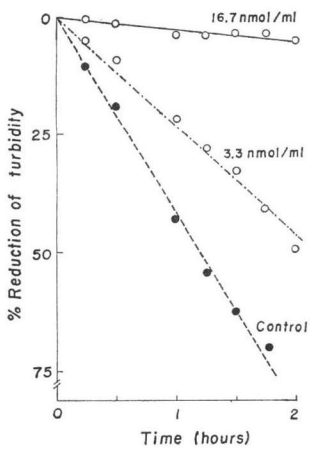
* corresponding to those in Fig. 1.

Abbreviation: PCG; benzylpenicillin, LTA; lipoteichoic acid.

inhibited penicillin-induced cell lysis (C, D), and the viable cell number was almost the same for (C) and (D), indicating the cells were in static state. On the contrary, viable cell number was markedly decreased when the lipoteichoic acid was not added (B). Viability of *S. aureus* was not influenced by the addition of the lipoteichoic

Fig. 3. Effects of lipoteichoic acid on extracellular autolysin activity against *M. lysodeikticus* cells.

The heated cells of *M. lysodeikticus* NCTC 2665 were incubated with various concentrations of the lipoteichoic acid (3.3, and 16.7 nmol/ml). Control received no addition. Assays were carried out as described in Materials and Methods. Data are expressed as percent reduction of turbidity of a cell suspension.



acid alone (F), and the viable cell number was almost the same as control culture (A). When the lipoteichoic acid was added after cell lysis initiated, the viable cell counts were decreased in proportion to loss of turbidity (E).

Inhibition of Autolysis and Extracellular Autolysin Activity by Cellular Lipoteichoic Acid

Effect of the lipoteichoic acid on autolysis of washed *S. aureus* FDA 209P cells is shown in Fig. 2. Decrease in turbidity with time was observed when the lipoteichoic acid was not added. The autolysis was inhibited with increase in concentration of the lipoteichoic acid and 20 nmol/ml of the lipoteichoic acid inhibited completely the cell lysis of *S. aureus*.

The addition of the extracellular autolysin to the suspension of *M. lysodeikticus* cells caused the cell lysis as shown in Fig. 3. Complete inhibition was observed when 16.7 nmol/ml of the lipoteichoic acid was added.

Thus the lipoteichoic acid isolated from *S. aureus* inhibited both cell-associated and extracellular autolysin activities of an homologous organism.

Discussion

Bacterial autolysin is implicated in elongation, cell division and separation of bacterial surface growth[24~26]. Recently, it was reported that the lytic effect of penicillin was due to the action of autolytic enzyme(s) in cell[14,15], and that the autolysin activity was inhibited by the lipoteichoic acid in some species of Gram-positive bacteria[18~20]. Lipoteichoic acid is water soluble, high molecular weight substance attached to the cytoplasmic membrane of many Gram-positive bacteria with its lipid portion and consists of substituted polyglycerol phosphate chains jointed covalently to glycolipid[27~29]. The chemical structure of the lipoteichoic acid from *S. aureus* was reported by DUCKWORTH *et al.* in 1975, but little is known about the physiological function of the lipoteichoic acid of *S. aureus*, and its role in the inhibition of cell lysis.

In our present study, the addition of the benzylpenicillin to *S. aureus* culture induced marked cell lysis as seen in many Gram-positive and negative organisms. The lysis was immediately and completely inhibited by the lipoteichoic acid of homologous organism. Furthermore, the measurement of viable cell counts revealed that the cells prevented from lysis by the lipoteichoic acid were viable and in static state. On the other hand, the growth of the organism did not change by the addition of the lipoteichoic acid alone, indicating that the lipoteichoic acid did not influence peptidoglycan synthesis. These facts suggested that the direct action of penicillin is only bacteriostatic and the cell lysis was caused by the activation of autolysin occurring at the same time. Recently, TOMASZ and WAKS[16] reported the marked release of the cellular lipoteichoic acid from penicillin-treated *Streptococcus pneumoniae* into the medium. We observed the same phenomenon in the addition of benzylpenicillin to *S. aureus* culture (unpublished data). It could be suggested that the bactericidal action of penicillin was brought about by the activation of autolysin(s) through the release of the cellular lipoteichoic acid, an autolysin inhibitor, in addition to the inhibition of peptidoglycan synthesis. Further aspects of the mechanisms of bactericidal action of penicillin will be discussed in the following paper, which will deal with our finding on relationship between penicillin-induced lysis and release of cellular lipoteichoic acid in *S. aureus*.

Acknowledgement

This work was supported in part by research grant (357150) from the Ministry of Education, Culture and Science of Japan.

References

1) LAWRENCE, P. J. & J. L. STROMINGER: Biosynthesis of the peptidoglycan of bacterial cell walls. XV. The binding of radioactive penicillin to the particulate enzyme preparation of *Bacillus subtilis* and its reversal with hydroxylamine or thiol. J. Biol. Chem. 245: 3653~3659, 1970

2) STROMINGER, J. L.; P. M. BLUMBERG, H. SUGINAKA, J. UMBREIT & G. G. WICKUS: How penicillin kills

bacteria: Progress and problems. Proc. Roy. Soc. London Ser. B 179: 369~383, 1971
3) SUGINAKA, H.; P. M. BLUMBERG & J. L. STROMINGER: Multiple penicillin-binding components in *Bacillus subtilis, Bacillus cereus, Staphylococcus aureus,* and *Escherichia coli.* J. Biol. Chem. 247: 5279~5288, 1972
4) SUGINAKA, H.: Comparison of the binding of penicillin G to staphylococcal L-form and its parent strain membranes. Antimicr. Agents & Chemoth. 9: 544~545, 1976
5) SUGINAKA, H.; A. ICHIKAWA & S. KOTANI: Penicillin-resistant mechanisms in *Pseudomonas aeruginosa*: Binding of penicillin to *Pseudomonas aeruginosa* KM 338. Antimicr. Agents & Chemoth. 7: 629~635, 1975
6) ARAKI, Y.; A. SHIMADA & E. ITO: Effect of penicillin on cell wall mucopeptide synthesis in an *Escherichia coli* particulate system. Biochem. Biophys. Res. Commun. 23: 518~525, 1966
7) IZAKI, K.; M. MATSUHASHI & J. L. STROMINGER: Glycopeptide transpeptidase and D-alanine carboxypeptidase: Penicillin-sensitive enzymatic reaction. Proc. Nat. Acad. Sci. U.S.A. 55: 656~663, 1966
8) MIRELMAN, D. & N. SHARON: Biosynthesis of peptidoglycan by a cell wall preparation of *Staphylococcus aureus* and its inhibition by penicillin. Biochem. Biophys. Res. Commun. 46: 1909~1917, 1972
9) SUGINAKA, H.; A. ICHIKAWA & S. KOTANI: Penicillin-resistant mechanisms in *Pseudomonas aeruginosa*: Effects of penicillin G and carbenicillin on transpeptidase and D-alanine carboxypeptidase activities. Antimicr. Agents & Chemoth. 6: 672~675, 1974
10) OKA, T.: Mode of action of penicillins *in vivo* and *in vitro* in *Bacillus megaterium.* Antimicr. Agents & Chemoth. 10: 579~591, 1976
11) PELLON, G.; C. BORDET & G. MICHEL: Peptidoglycan synthesized by a membrane preparation of *Micrococcus luteus.* J. Bacteriol. 125: 509~517, 1976
12) SPRATT, B. G. & A. B. PARDEE: Penicillin-binding proteins and cell shape in *E. coli.* Nature (London) 254: 516~517, 1975
13) BLUMBERG, P. M. & J. L. STROMINGER: Interaction of penicillin with the bacterial cell: Penicillin-binding proteins and penicillin sensitive enzymes. Bacteriol. Rev. 38: 291~335, 1974
14) TOMASZ, A.; A. ALBINO & E. ZANATI: Multiple antibiotic resistance in a bacterium with suppressed autolytic system. Nature (London) 227: 138~140, 1970
15) ROGERS, H. & C. W. FORSBERG: Role of autolysins in the killing of bacteria by some bacterial antibiotics. J. Bacteriol. 108: 1235~1243, 1971
16) TOMASZ, A. & S. WAKS: Mechanism of action of penicillin: Triggering of the pneumococcal autolytic enzyme by inhibitors of cell wall synthesis. Proc. Nat. Acad. Sci. U.S.A. 72: 4162~4166, 1975
17) HOLTJE, J. V. & A. TOMASZ: Lipoteichoic acid: A specific inhibitor of autolysin activity in *Pneumococcus.* Proc. Nat. Acad. Sci. U.S.A. 72: 1690~1694, 1975
18) CLEVELAND, R. F.; J. V. HOLTJE, A. J. WICKEN, A. TOMASZ, L. DANEO-MOORE & G. D. SHOCKMAN: Inhibition of bacterial wall lysis by lipoteichoic acids and related compounds. Biochem. Biophys. Res. Commun. 67: 1128~1135, 1975
19) CLEVELAND, R. F.; A. J. WICKEN, L. DANEO-MOORE & G. D. SHOCKMAN: Inhibition of wall autolysis in *Streptococcus faecalis* by lipoteichoic acid and lipid. J. Bacteriol. 126: 192~197, 1976
20) CLEVELAND, R. F.; L. DANEO-MOORE, A. J. WICKEN & G. D. SHOCKMAN: Effect of lipoteichoic acid and lipids on lysis of intact cells of *Streptococcus faecalis.* J. Bacteriol. 127: 1582~1584, 1976
21) COLEY, J.; M. DUCKWORTH & J. BADDILEY: Extraction and purification of lipoteichoic acids from gram-positive bacteria. Carbohyd. Res. 40: 41~52, 1975
22) DUCKWORTH, M.; A. R. ARCHIBALD & J. BADDILEY: Lipoteichoic acid and lipoteichoic acid carrier in *Staphylococcus aureus* H. FEBS Lett. 53: 176~179, 1975
23) SUGINAKA, H.; S. KASHIBA, T. AMANO, S. KOTANI & T. IMANISHI: Purification and properties of a staphylolytic factor produced by strain of *Staphylococcus epidermidis.* Biken J. 10: 109~120, 1967
24) ROGERS, H. J.: Bacterial growth and the cell envelope. Bacteriol. Rev. 34: 194~214, 1970
25) HARTMANN, R.; J. V. HOLTJE & U. SCHWARZ: Targets of penicillin action in *Escherichia coli.* Nature (London) 235: 426~429, 1972
26) SHOCKMAN, G. D.; L. DANEO-MOORE & M. L. HIGGINS: Problems of cell wall and membrane growth, enlargement and division. Ann. N.Y. Acad. Sci. 235: 161~197, 1974
27) KNOX, K. W. & A. J. WICKEN: Immunological properties of teichoic acids. Bacteriol. Rev. 37: 215~257, 1973
28) WICKEN, A. J. & K. W. KNOX: Lipoteichoic acids: A new class of bacterial antigen. Science 187: 1161~1167, 1975
29) LAMBERT, P. A.; I. C. HANCOCK & J. BADDILEY: Occurrence and function of membrane teichoic acids. Biochim. Biophys. Acta 472: 1~12, 1977