Effects of mucopolysaccharides on penicillin-induced lysis of Staphylococcus aureus

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Summary. Effects of four mucopolysaccharides and dextran sulphate on penicillin-induced lysis of Staphylococcus aureus FDA 209P were studied. Heparin and dextran sulphate inhibited lysis, whereas hyaluronic acid enhanced it. Chondroitin sulphates A and C had no effect. Incubation of S. aureus suspended in 0-03 M phosphate buffer (pH 7-0) with dextran sulphate inhibited autolysis of the bacteria, whereas incubation with hyaluronic acid enhanced autolysis. Both extracellular and cell-associated autolysin activities of S. aureus were suppressed by dextran sulphate and high concentrations of heparin. The addition of hyaluronic acid enhanced autolysin activity. The release of lipoteichoic acid (LTA), a modulator of autolysin activity, from penicillin-treated bacteria was inhibited by heparin and dextran sulphate. However, hyaluronic acid had no effect on release of LTA.

These results suggest that inhibition of penicillin-induced lysis of S. aureus by heparin results mainly from inhibition of LTA release while dextran sulphate inhibits both autolysin activity and LTA release. Hyaluronic acid appears to enhance penicillin-induced lysis through activation of the autolysins.

Introduction

Infected lesions contain inflammatory exudates, leukocytes, and many other substances which may influence the antibacterial activity of antibiotics. Inflammatory exudates are rich in mucopolysaccharides and other anionic polyelectrolytes. Heparin, one such mucopolysaccharide, is used therapeutically as an anticoagulant; chondroitin sulphate is used as an accelerator of wound healing. It has been reported that leukocyte extracts and some cationic substances activate the autolytic systems of Staphylococcus aureus, whereas anionic polyelectrolytes such as heparin, dextran sulphate, suramine polyglutamic acid, and Liquoid (sodium polyanethol sulphonate) markedly inhibit autolysis (Ginsberg et al., 1976, 1982; Ne’eman et al., 1979). Because penicillin lyses bacteria by inhibition of cell-wall synthesis and activation of autolytic enzymes (Rogers, 1967; Tomasz et al., 1970; Rogers and Forsberg, 1971; Tomasz, 1974; Garcia et al., 1982), mucopolysaccharides and other anionic polyelectrolytes, which inhibit autolysis, may affect the activity of penicillin. Therefore, we investigated the effects of mucopolysaccharides and dextran sulphate on lysis of S. aureus by penicillin.

Materials and methods

Reagents

Hyaluronic acid, chondroitin sulphates A and C, and heparin were obtained as sodium salts from Nakarai Chemicals Ltd, Kyoto, Japan. Dextran sulphate was obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden.

Effects of mucopolysaccharides and dextran sulphate on penicillin-induced lysis

Exponentially growing cultures of S. aureus FDA 209P in Trypticase Soy Broth (TSB; BBL Microbiology Systems, Cockeysville, MD, USA) were transferred to fresh broth and mucopolysaccharide or dextran sulphate was added to achieve the required concentration. These cultures and a control culture without additive were grown at 37°C with shaking. The rate of change in turbidity during growth was measured at 660 nm in a spectrophotometer (100-10, Hitachi, Tokyo, Japan). When the absorbance reached 0.20 OD units, benzylpenicillin (Meiji Seika Ltd, Tokyo, Japan) was added to the cultures to achieve a concentration of 0.1 µg/ml (2 MIC) and they were further incubated. The numbers of colony forming units (cfu) at the time of antibiotic addition and after incubation for 4 h were determined by plating serial dilutions of the culture on to Trypticase Soy Agar.
Measurement of autolysis of whole cells

Exponentially growing cells of *S. aureus* were thoroughly washed, suspended in 0.03 M phosphate buffer (pH 7.0) to a final concentration of 0.25 mg (wet weight)/ml, and incubated at 37°C. The change in turbidity of the suspension in the presence and absence of various concentrations of mucopolysaccharides or dextran sulphate was monitored spectrophotometrically at 600 nm. A suspension of heat-killed *S. aureus* was included as a negative control.

Preparation of autolysins

Solid ammonium sulphate was added to the culture supernate of an exponential-phase culture of *S. aureus* to achieve 75% saturation. The resulting precipitate was dissolved in 0.01 M phosphate buffer (pH 7.0), dialysed against the same buffer and used as a source of extracellular autolysin. Cell-associated autolysin was prepared from exponential-phase bacteria harvested by centrifugation at 4°C and washed twice with saline. The cell pellet was extracted with ten times its volume of Triton X-100 2%; the suspension was centrifuged and the supernate was dialysed against distilled water. Solid ammonium sulphate was added to the dialysed supernate to achieve 75% saturation. The precipitate was collected by centrifugation, dissolved in 0.01 M phosphate buffer (pH 7.0) and dialysed against the same buffer. This fraction was used as a source of cell-associated autolysin. The protein concentration was measured by the method of Lowry et al. (1951).

Autolysin activity was determined with heated cells of *Micrococcus lysodeikticus* NCTC 2665 as substrate. The autolysin fraction containing protein 300 µg/ml (extracellular autolysin) and 50 µg/ml (cell-associated autolysin) was incubated with *M. lysodeikticus* (0.5 mg dry weight/ml) suspended in 0.05 M phosphate buffer (pH 7.0), and the change in turbidity of the suspension was monitored spectrophotometrically.

Release of 

\[ ^{14}C \] -glycerol labelled lipoteichoic acid from staphylococci

\[^{14}C\] -glycerol (11.8 mCi/mmmole) (New England Nuclear, IL, USA) was added to an early exponential-phase culture of *S. aureus* to achieve a concentration of 2 µCi/ml and incubated with shaking. The cells were harvested in the late exponential phase by centrifugation and washed twice in TSB. The washed radiolabelled cells were resuspended in fresh TSB (final turbidity of the cultures: 0.2 absorbance unit at 600 nm), and incubated at 37°C with benzylpenicillin (final concentration 0.1 µg/ml) and a mucopolysaccharide or dextran sulphate. A negative control culture that contained no mucopolysaccharide or dextran sulphate and a positive control with penicillin were included. A 0.4-ml sample was removed 30 min after the addition of penicillin and centrifuged at 9000 g for 5 min. The supernate (0.2 ml) was removed, dissolved in 10 ml of ACS II (Aqueous Counting Scintillant; Amersham, IL, USA) and analysed in a liquid scintillation counter (type LSC-903, Aloka, Tokyo, Japan). The release of lipoteichoic acid (LTA) was determined as the cpm of the supernate.

Combined antibacterial effect of penicillin with heparin, dextran sulphate or hyaluronic acid on *S. aureus*

The minimum inhibitory concentration (MIC) of penicillin alone and in combination with heparin, dextran sulphate or hyaluronic acid against *S. aureus* was determined by a broth dilution method. The concentrations of heparin and hyaluronic acid was in the range 100-1000 µg/ml and that of dextran sulphate 10-100 µg/ml. Each concentration was tested with serial dilutions of penicillin and an inoculum of *S. aureus* of 10^6 cfu/ml. The MIC was defined as the lowest concentration of penicillin at which there was no turbidity on visual inspection after overnight incubation at 37°C.

Results

Effects of mucopolysaccharides on penicillin-induced lysis

Addition of benzylpenicillin (final concentration 0.1 µg/ml; 2 MIC) to a growing culture of *S. aureus* caused marked bacteriolysis (fig. 1). Addition of heparin or dextran sulphate inhibited the penicillin-induced lysis in a dose-dependent fashion, whereas lysis in the presence of hyaluronic acid was accelerated. Chondroitin sulphates A and C had no effect on lysis by penicillin. Inhibition of lysis by heparin was observed only when it was added before penicillin. However, dextran sulphate inhibited lysis even when it was added 30 min after penicillin (data not shown). Heparin, dextran sulphate, chondroitin sulphates, and hyaluronic acid had no effect on bacterial growth in the absence of penicillin (fig. 1).

The bactericidal effect of penicillin on *S. aureus* was slightly reduced in the presence of heparin or dextran sulphate and slightly increased in the presence of hyaluronic acid (table I). Microscopy of bacteria exposed to heparin or dextran sulphate with penicillin revealed considerable cell swelling in comparison with untreated cells (data not shown). Viability of *S. aureus* was not influenced by the addition of heparin, dextran sulphate, or hyaluronic acid alone.

Effects of mucopolysaccharides on autolysis

The turbidity of suspensions of *S. aureus* fell with time, but the fall in turbidity was not observed with
Fig. 1. Effects of heparin (A), dextran sulphate (B), chondroitin sulphates A and C (C), and hyaluronic acid (D) on lysis of *S. aureus* by penicillin. Heparin, dextran sulphate, chondroitin sulphates, and hyaluronic acid were added at the start of the experiment; 1 h later penicillin was added (time 0) to achieve a concentration of 0.1 μg/ml. ○ = control; ● = penicillin alone; △ = mucopolysaccharides or dextran sulphate alone; △ = mucopolysaccharides, or dextran sulphate, and penicillin.
Table I. Effects of penicillin, heparin, dextran sulphate and hyaluronic acid on viability of \textit{S. aureus}

<table>
<thead>
<tr>
<th>Additions</th>
<th>Viable count (cfu/ml)</th>
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<tbody>
<tr>
<td></td>
<td>0 h</td>
</tr>
<tr>
<td>None</td>
<td>2.4 $\times 10^8$</td>
</tr>
<tr>
<td>Benzylpenicillin (0.1 $\mu$g/ml)</td>
<td>2.6 $\times 10^8$</td>
</tr>
<tr>
<td>Heparin (1.0 mg/ml)</td>
<td>3.1 $\times 10^8$</td>
</tr>
<tr>
<td>Heparin (1.0 mg/ml) + benzylpenicillin (0.1 $\mu$g/ml)</td>
<td>3.1 $\times 10^8$</td>
</tr>
<tr>
<td>Dextran sulphate (0.5 mg/ml)</td>
<td>2.5 $\times 10^8$</td>
</tr>
<tr>
<td>Heparin (1.0 mg/ml) + benzylpenicillin (0.1 $\mu$g/ml)</td>
<td>2.4 $\times 10^8$</td>
</tr>
<tr>
<td>Hyaluronic acid (1.0 mg/ml)</td>
<td>2.5 $\times 10^8$</td>
</tr>
<tr>
<td>Hyaluronic acid (1.0 mg/ml) + benzylpenicillin (0.1 $\mu$g/ml)</td>
<td>2.4 $\times 10^8$</td>
</tr>
</tbody>
</table>

Effects of mucopolysaccharides on autolysin activities

Extracellular or cell-associated autolysin added to a suspension of \textit{M. lysodeikticus} caused cell lysis (fig. 3). Dextran sulphate markedly inhibited both extracellular and cell-associated autolysin activities against \textit{M. lysodeikticus}. Heparin inhibited autolysin activities at a concentration of 8 mg/ml, but lower concentrations (≤ 4 mg/ml) had no effect. Hyaluronic acid accelerated autolysin activity.

Effects of mucopolysaccharides on the release of $[^{14}\text{C}]$-glycerol labelled LTA from \textit{S. aureus}

More than 90% of the $[^{14}\text{C}]$-glycerol labelled material released into the culture supernates was ascertained by the method of Kessler and Shockman (1979) to be polyglycerophosphate derived from LTA. Release of LTA from the untreated control culture was only 20% of that observed after exposure to penicillin for 30 min (fig. 4); there was no evidence of cell lysis at this time. Heparin or dextran sulphate inhibited the release of $[^{14}\text{C}]$-glycerol-labelled LTA from \textit{S. aureus} cells exposed to penicillin. Addition of heparin or dextran sulphate (final concentration 1 mg/ml) reduced the release of $[^{14}\text{C}]$-glycerol-labelled LTA to 60–70% of that induced by penicillin alone. In contrast, release of $[^{14}\text{C}]$-glycerol-labelled LTA was not influenced by hyaluronic acid.

Combined antimicrobial effect of penicillin with heparin, dextran sulphate or hyaluronic acid

The presence of heparin or dextran sulphate raised the MIC of penicillin for \textit{S. aureus}. Conversely, the addition of hyaluronic acid lowered the MIC (table II). Heparin, dextran sulphate and hyaluronic acid individually had no influence on bacterial growth.

Discussion

Lysis of gram-positive bacteria exposed to penicillin is caused by autolytic enzymes, the activity of which is triggered by release of an inhibitor from the bacterial cell wall (Tomasz, 1974; Tomasz and Waks, 1975; Garcia \textit{et al.}, 1982). In \textit{S. aureus} and other gram-positive bacteria, the inhibitor appears to be LTA (Cleveland \textit{et al.}, 1975, 1976a and b; Höltje and Tomasz, 1975; Suginaka \textit{et al.}, 1979a). Cardiolipin and other phospholipids can also

Heat-killed cells (fig. 2). Autolysis was inhibited by dextran sulphate and accelerated by hyaluronic acid; heparin had no effect (fig. 2). Chondroitin sulphates A and C also had no effect on autolysis (data not shown).
inhibit autolysin activity in *S. aureus* and *Streptococcus faecium* (Horne et al., 1977; Suginaka et al., 1979b).

In the present study, heparin and dextran sulphate were found to interfere with the bacteriolytic effect of penicillin on *S. aureus*, whereas hyaluronic acid accelerated the lytic effect. The effects of heparin and dextran sulphate in reducing the fall in turbidity of cultures of *S. aureus exposed to penicillin might have been exaggerated, because microscopy revealed swelling of the bacterial cells. Similarly, the falls in viable count might have been affected by inhibition of cell separation. Liquoid (sodium polyanethol sulphonate) which, like heparin and dextran sulphate suppresses autolysis of *S. aureus*, also inhibits cell separation (Wecke et al., 1986).

Inhibition of autolysis by dextran sulphate and acceleration of autolysis by hyaluronic acid seemed to occur through direct action on the autolysins. In contrast, inhibition of penicillin-induced lysis by heparin appeared to be due to inhibition of release of LTA. Thus, once release of LTA was initiated by exposure to penicillin, the addition of heparin did not inhibit lysis, whereas dextran sulphate did. Because hyaluronic acid had no effect on LTA release, acceleration of penicillin-induced lysis by this substance presumably resulted from enhancement of autolysin activity.

The finding that hyaluronic acid-enhanced lysis of *S. aureus* by penicillin conflicts with previous reports. Ginsberg *et al.* (1976, 1982) and Ne’eman (1979) reported that hyaluronic acid and other anionic polyelectrolytes markedly inhibited spon-
Fig. 4. Effects of heparin (Hp), dextran sulphate (Ds) and hyaluronic acid (Hyal) on the release of $^{14}$C-glycerol-labelled LTA from *S. aureus*. Data are expressed as percentage LTA released relative to the value for penicillin (PCG; final concentration 0.1 µg/ml) which was arbitrarily assigned a value of 100%. A = LTA release by heparin, dextran sulphate or hyaluronic acid alone. B = LTA released in the presence of penicillin and heparin, dextran sulphate, or hyaluronic acid.

**Table II.** Combined antibacterial effect of benzylpenicillin with heparin, dextran sulphate, or hyaluronic acid on *S. aureus*.

<table>
<thead>
<tr>
<th>Addition (µg/ml)</th>
<th>MIC of penicillin (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.05</td>
</tr>
<tr>
<td>Heparin 100</td>
<td>0.10</td>
</tr>
<tr>
<td>Heparin 500</td>
<td>0.15</td>
</tr>
<tr>
<td>Heparin 800</td>
<td>0.20</td>
</tr>
<tr>
<td>Dextran sulphate 10</td>
<td>0.10</td>
</tr>
<tr>
<td>Dextran sulphate 40</td>
<td>0.15</td>
</tr>
<tr>
<td>Dextran sulphate 60</td>
<td>0.20</td>
</tr>
<tr>
<td>Hyaluronic acid 100</td>
<td>0.02</td>
</tr>
<tr>
<td>Hyaluronic acid 200</td>
<td>0.015</td>
</tr>
<tr>
<td>Hyaluronic acid 300</td>
<td>0.01</td>
</tr>
<tr>
<td>Hyaluronic acid 400</td>
<td>0.01</td>
</tr>
</tbody>
</table>

taneous and induced lysis of *S. aureus*, and that staphylococci grown in the presence of anionic polyelectrolytes became highly resistant to lysis triggered by inducers of autolysis. These workers also showed that various cationic substances could activate the autolytic systems of *S. aureus*. The disparity between our results and those of others may reflect differences in the method of triggering bacteriolysis.

Acid mucopolysaccharides are important components of connective tissue; they have a negative charge at neutral pH and therefore act as anionic polyelectrolytes. Infected inflammatory lesions are rich in these substances. This study has shown that the antibacterial action of penicillin is influenced by several anionic polyelectrolytes and such effects should be taken into account during therapy. This might be particularly important when, for example, heparin is co-administered with penicillin.
REFERENCES


Ne’eman N et al. 1979 Effect of leukocyte hydrolases on bacteria. XIV. Bacteriolytic effects of human sera, synovial fluids, and purulent exudates on *Staphylococcus aureus* and *Streptococcus faecalis*: modulation by Cohn’s fraction II and by polyelectrolytes. *Inflammation* **3**: 379–394.


