Ristocetins, Inhibitors of Cell Wall Synthesis in Staphylococcus aureus*

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Inhibition of bacterial cell wall synthesis is an important mechanism of action of antibacterial agents. Bacterial cell walls are chemically and morphologically distinct from any structures found in animal cells. Inhibition of synthesis of these structures results, therefore, in selective toxicity. The antibacterial action of penicillins (1, 2), n-cycloserine (3, 4), bacitracin (5), and novobiocin (6) is accompanied by inhibition of cell wall synthesis and, in the case of n-cycloserine, the identification of two enzymatic reactions sensitive to n-cycloserine and uniquely found in animal cells. Inhibition of synthesis of these structures are chemically and morphologically distinct from any structures found in bacteria provides a relatively complete explanation of its antibacterial activity (7).

Many of these laboratory studies have been carried out with Staphylococcus aureus. After the emergence of drug-resistant strains of S. aureus in infected humans, a search for new antibiotic substances resulted in the isolation of vancomycin (8) and ristocetin (9). Both of these have been widely used in the treatment of staphylococcal infections. Investigation of the mechanism of action of these two substances has resulted in the finding that both of them selectively inhibit bacterial cell wall synthesis at levels which are comparable with those required for antibacterial activity. While these experiments were in progress, two other laboratories reported similar experiments with vancomycin (10, 11) and, hence, only the results obtained with ristocetin will be reported here.

EXPERIMENTAL PROCEDURE

The organism employed was S. aureus, strain Copenhagen. Measurement of uridine nucleotide accumulation (12), isolation and degradation of accumulated nucleotides (2, 12-14), and isotopic measurements of cell wall synthesis (15) were carried out by techniques described previously. In the isotope experiments the amounts of C14-lysine and P32-inorganic phosphate employed were approximately half of those used previously. Briefly, cells in logarithmic growth were exposed to antibiotic for 5 minutes, after which isotopes were added. After 15 additional minutes (i.e. less than one division time) cells were harvested and fractionated (15). Isotope incorporation into various cell fractions was compared with that in a parallel culture to which no isotope was added.

A commercial preparation, Spontin (Abbott Laboratories), which contains both ristocetin A and ristocetin B, was employed in initial studies. Later pure ristocetin A, ristocetin B, and partial acid hydrolysis products of each of these pure substances were also tested. The hydrolysis products were isolated after treatment with 1 N H2SO4 at 100° for 10 minutes, which liberates various sugars and an amine from these antibiotics (16).

RESULTS

Uridine Nucleotide Accumulation as Function of Time and Ristocetin Concentration—Accumulation of UDP-acetylamino sugar compounds (believed to be precursors of the cell wall) was induced by Spontin (the commercial preparation containing both ristocetin A and ristocetin B), as measured colorimetrically. The minimal concentration required for nucleotide accumulation was similar to the minimal concentration which inhibited growth of a 10% inoculum (Fig. 1). Similar data were obtained with ristocetin A, ristocetin B, and partial acid hydrolyses of each of these substances (Fig. 1). Accumulation of nucleotides began promptly on addition of ristocetin and reached a maximum at 2 hours (Fig. 2).

Isolation and Identification of Accumulated Nucleotides—One liter of culture at half-maximal growth was treated with ristocetin (50 µg per ml) for 2 hours. The cells were collected by centrifugation, washed, and extracted with hot water. After cooling to 0-3°, trichloroacetic acid (5% final concentration) was added and insoluble material was removed by centrifugation. Nucleotides adsorbed onto charcoal and recovered by elution with ammoniacal ethanol. After the volume had been reduced to 1 ml, the eluate was subjected to two-dimensional paper chromatography, 5% of the total eluate, corresponding to 50 ml of original culture, on each sheet (Fig. 3). The pattern of nucleotides observed was virtually identical with that obtained with penicillin (14). Compounds found in the two prominent nucleotide areas were recovered by elution. Paper chromatography of 0.1 µmole of each compound after acid hydrolysis indicated the presence of only alanine in Compound 1 and of alanine, glutamic acid, and lysine in Compound 2.

Compound 1 contained, per µmole of uridine, 2.11 µmoles of

1 We are very grateful to Dr. J. R. Schenck of Abbott Laboratories for supplying Spontin, pure ristocetins A and B, and their partial acid hydrolysis products for these studies.

2 Minimal growth-inhibitory concentrations, measured by tube dilution with a 10% inoculum of cells, were: ristocetin A, 15 µg per ml; ristocetin B, 15 µg per ml; hydrolysate of ristocetin A, 5 to 15 µg per ml; hydrolysate of ristocetin B, 5 to 15 µg per ml. These values cannot be compared directly to similar data obtained by measurement of nucleotide accumulation (Fig. 1). The latter data are necessarily obtained with a 50% inoculum and the minimal inhibitory level increases with inoculum size.
organic phosphate, 1.03 μmoles of acetylmuramic acid, and 0.59 μ mole of alanine, all of which was the L isomer. This material is presumably a mixture of UDP-GNAc-lactic acid and UDP-GNAc-lactyl-L-Ala, both of which accumulate in the presence of penicillin (17). It occupies the position of this mixture in the two-dimensional paper chromatogram. The separation of these two compounds is difficult, however, and has not been attempted in the present study.

Compound 2 (1.7 μmoles) was subjected to acid hydrolysis, and the phosphate, amino sugar, and amino acids present were separated on a column of Dowex 50W-H+ (8% cross-linked, 200 to 400 mesh). The column was 0.9 cm × 55 cm. For each 2 residues of phosphate in the column eluate, 2 residues of D-alanine and 1 residue each of L-alanine, glutamic acid, and lysine were found (Table I). The lower molar ratio of muramic acid observed (Table I) is due to some destruction during acid hydrolysis and corresponds to previous experience. The amino acids were identified by their positions in the ion exchange chromatogram, by paper chromatography in 1-butanol-acetic acid-water (3:1:1) and isobutyric acid-NH₂OH (5:3), and, in the case of alanine, by determination of d-alanine with d-amino acid oxidase and of L-alanine with L-alanine-α-ketoglutarate transaminase (Table I). By these methods the nucleotide is identical with UDP-GNAc-lactyl-L-Ala-d-Glu-l-Lys-d-Ala-d-Ala, which also accumulates in the presence of penicillin (17).

Effects of Ristocetin in Incorporation of C¹⁴-Lysine and of P³²-Inorganic Phosphate into Cell Wall—Under conditions in which ristocetin inhibited incorporation of these isotopes into the cell wall by greater than 95%, incorporation into the tri-

<table>
<thead>
<tr>
<th>Compound isolated</th>
<th>Micromoles found</th>
<th>Ratio</th>
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<tbody>
<tr>
<td>Phosphate</td>
<td>3.53</td>
<td>2.1</td>
</tr>
<tr>
<td>Amino sugar</td>
<td>0.99</td>
<td>0.6</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>1.66</td>
<td>(1.0)</td>
</tr>
<tr>
<td>Alanine (total)</td>
<td>4.80</td>
<td>2.9</td>
</tr>
<tr>
<td>d-Alanine</td>
<td>3.24</td>
<td>2.0</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>1.82</td>
<td>1.1</td>
</tr>
<tr>
<td>Lysine</td>
<td>1.84</td>
<td>1.1</td>
</tr>
</tbody>
</table>
Table II

Effect of ristocetins on incorporation of isotopes into various cell fractions

Spontin, the commercial preparation containing both ristocetin A and ristocetin B, was used in this experiment. Antibiotic and isotopes were added to 400 ml of logarithmic phase cultures as described previously (15). Cell walls and trichloroacetic acid precipitates of the soluble cell contents (protein and nucleic acid) were prepared, purified, and counted (15). Data are expressed as counts per minute per mg of cell wall peptide or protein in each fraction. Similar data are obtained if the results are expressed as total counts per minute in each fraction.

<table>
<thead>
<tr>
<th></th>
<th>Cell wall</th>
<th>Protein and nucleic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P32-Phosphate</td>
<td>C14-Lysine</td>
</tr>
<tr>
<td>Control</td>
<td>180,000</td>
<td>19,600</td>
</tr>
<tr>
<td>+ Spontin, 150 µg per ml</td>
<td>4,180</td>
<td>692</td>
</tr>
<tr>
<td>Per cent inhibition</td>
<td>98</td>
<td>97</td>
</tr>
</tbody>
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chloroacetic acid precipitate of the cell contents (which contains both protein and nucleic acid) continued at a normal rate (Table I).

Discussion

Ristocetin A and ristocetin B can thus be added to the list of antibiotics which inhibit bacterial cell wall synthesis. These substances, like penicillins, bacitracin, novobiocin, and vancomycin, inhibit at a late stage in cell wall synthesis, and although superficially, by the technique employed here, they have similar loci of action (i.e. the same cell wall precursors accumulate with each of these substances), it is unlikely that their precise mechanisms of action are identical. Bacitracin and the penicillins are both peptide antibiotics whereas novobiocin is related chemically to dicoumarol and belongs to a heterogeneous group chemically. The ristocetins and vancomycin, on the other hand, are complex oligosaccharides which contain amino acids. In the case of ristocetins, even shorter oligosaccharide chains prepared by acid hydrolysis exhibited both growth inhibition and inhibition of cell wall synthesis. In view of the complexity of cell wall synthesis, it would be surprising if each of these substances interfered with this process in exactly the same manner. It is possible, for example, that some of these substances interfere in some manner with synthesis of the primary polysaccharide backbone of the cell wall (a polymer of N-acetylglosamine and N-acetylmuramic acid), whereas others interfere with the formation of the peptides and their cross-links. An experimental approach to this problem, however, will require much more information about the mechanisms of cell wall synthesis than is presently available.

Summary

Ristocetin A, ristocetin B, and partial acid hydrolys products of these compounds, like several other antibiotics, induce accumulation of cell wall precursors (uridine diphosphoacetylmuramyl-peptide compounds) in Staphylococcus aureus. Moreover, studies with isotopes indicate that inhibition of cell wall synthesis is a selective effect of ristocetins and may, therefore, represent their primary mechanism of antibacterial activity.

References

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