The in vitro antibacterial activity of ceftriaxone against *Streptococcus pyogenes* is unrelated to penicillin-binding protein 4 *

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(Received 9 February 1993; revision received 2 April 1993; accepted 12 April 1993)

Abstract: The in vitro activities of penicillin and ceftriaxone were compared against 29 strains of *Streptococcus pyogenes* with the result that ceftriaxone showed greater activity than penicillin. The morphological changes induced by 1/2 and 1× MIC concentrations of penicillin and ceftriaxone, respectively, were very similar using scanning electron microscopy. Competitive binding studies using 'cold' penicillin or ceftriaxone as inhibitors of radiolabeled penicillin binding demonstrated that ceftriaxone had a very low affinity for penicillin binding protein (PBP) 4 compared to that of penicillin. Since ceftriaxone had greater antibacterial activity, this suggests that PBP 4 may not be important to the in vitro activity of ceftriaxone. In contrast, the IC₅₀ for ceftriaxone was much lower (> 200 fold) for PBPs 2 and 3 compared to PBP 4, suggesting greater avidity of these high molecular mass PBPs for ceftriaxone. These data may at least in part explain the superior in vitro activity of ceftriaxone compared to penicillin against *S. pyogenes*. These data, together with the observation that PBP 1 was saturated at a lower concentration of penicillin than any of the other PBPs, suggest that the inhibition of PBPs 1, 2, and 3 mediates the bactericidal activity of beta-lactam antibiotics against group A streptococci.

Key words: Ceftriaxone; *Streptococcus pyogenes*; Penicillin-binding proteins

Introduction

Ceftriaxone, like penicillin, has high antibacterial activity against *S. pyogenes* in vitro [1–3], and, because of its unusually long half-life [4], ceftriaxone may be a potentially useful antibiotic in the treatment of streptococcal infections [5]. We propose that the superior in vitro activity of ceftriaxone may be related to its unique binding properties to streptococcal PBPs. This report investigates the in vitro activity of penicillin and ceftriaxone against 29 clinical isolates of *S. pyogenes* and describes morphological alterations induced by them. In addition, the pattern of PBPs of strain ATCC 12384 was investigated to determine

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* Part of this work was presented at the 32nd Interscience Conference on Antimicrobial Agents and Chemotherapy, 1992, Anaheim, CA, Abstract number 1041.
Table 1
Antimicrobial susceptibility of 29 strains of *Streptococcus pyogenes*

<table>
<thead>
<tr>
<th></th>
<th>MIC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>MIC&lt;sub&gt;90&lt;/sub&gt;</th>
<th>Range</th>
<th>MBC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>MBC&lt;sub&gt;90&lt;/sub&gt;</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin</td>
<td>0.015</td>
<td>0.030</td>
<td>0.004–0.03</td>
<td>0.030</td>
<td>0.060</td>
<td>0.008–0.06</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>0.008</td>
<td>0.015</td>
<td>0.004–0.015</td>
<td>0.015</td>
<td>0.030</td>
<td>0.004–0.06</td>
</tr>
</tbody>
</table>

Susceptibility was determined by the microbroth dilution method and expressed in μg/ml.

Fig. 1. Morphological changes induced by penicillin and ceftriaxone. Mid-log phase cultures of *Streptococcus pyogenes* ATCC 12384 were exposed to either 1/2 MIC (A) or 1 MIC (B) of penicillin, and 1/2 MIC (C) or 1 MIC (D) of ceftriaxone for 2 h before being prepared for SEM observation. The insert shows the untreated control. The original magnification for all are 10000×.

Table 2
Characteristics of PBPs in *Streptococcus pyogenes* ATCC 12384

<table>
<thead>
<tr>
<th>PBP</th>
<th>Molecular mass (kDa)</th>
<th>% of penicillin bound</th>
<th>S&lt;sub&gt;50&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt; (pen-G)</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt; (pen-G)</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (ceftriaxone)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>95</td>
<td>5.6</td>
<td>0.007</td>
<td>0.090</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>83/82</td>
<td>59.8</td>
<td>0.037</td>
<td>0.165</td>
<td>0.108</td>
</tr>
<tr>
<td>3</td>
<td>76</td>
<td>10.7</td>
<td>0.025</td>
<td>0.168</td>
<td>0.146</td>
</tr>
<tr>
<td>4</td>
<td>48</td>
<td>20.6</td>
<td>0.041</td>
<td>0.359</td>
<td>&gt; 32</td>
</tr>
<tr>
<td>5</td>
<td>42</td>
<td>3.3</td>
<td>ND</td>
<td>0.215</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>a</sup> S<sub>50</sub> is the lowest concentration of labelled penicillin required to saturate 50% of an individual PBP.

<sup>b</sup> IC<sub>50</sub> is the lowest concentration of unlabelled penicillin or ceftriaxone which will inhibit 50% of subsequent binding of labelled penicillin at the concentration of 2 μg/ml.

ND, not determined.
if differences in biological activity could be explained by an alternative pattern of PBPs.

**Materials and Methods**

Twenty-eight clinical isolates of *S. pyogenes* and ATCC 12384 were included in this report. The inoculum was prepared from late log phase cultures and adjusted to 1 x 10^6 cfu/ml. Following inoculation, MICs and MBCs were determined using the microbroth dilution method in Todd-Hewitt broth (THB) [6] incubated for 18 h at 37°C in 5% CO_2. The MIC and MBC were defined using standardised criteria [7]. Penicillin V was obtained from Dr. Larry Blaszczak (Eli Lilly and Company, Indianapolis, IN) and iodo-destannylation of penicillin V was performed as previously reported [8]. In terms of PBP patterns, [125I]penicillin V has shown identical binding activity to 3H benzylpenicillin [9].

*S. pyogenes* ATCC 12384 grown in the presence of penicillin or ceftriaxone at concentrations equal to 1/2 and 1 x the MIC were dehydrated in increasing concentration of ethyl alcohol and critical point dried on cellulose filters, and then ion-coated before being observed under SEM [10].

Bacterial membrane fractions from ATCC 12384 strain were prepared as previously reported [11,12] except that the cells were ruptured in a Bead-Beater (Biospec Products, Bartlesville, OK). For PBP saturation studies (S_50), membrane samples (40 μl) containing a total of 200 μg of protein were incubated with 10 μl of various concentrations of [125I]penicillin V. For PBP inhibition studies (IC_50), membrane samples (40 μl) containing a total of 200 μg of protein were incubated with 10 μl of various concentrations of non-radiolabelled penicillin or ceftriaxone followed by [125I]penicillin V, in a 37°C water bath for 10 min. The reactions were stopped by adding 12.5 μl of 5 x stop buffer (3.8% Tris, 10% SDS, 0.005% bromphenol blue, 50% glycerol (v/v), 25% beta-mercaptoethanol (v/v), pH 7.0) and then immediately boiled for 5 min. Proteins were separated by 10% SDS-PAGE. Gels were stained, dried and then exposed to Kodak X-OMAT film.

**Fig. 2.** Fluorography of penicillin-binding proteins in *S. pyogenes*. Membrane proteins (200 μg) were incubated with a series of concentrations of [125I]penicillin V (A), and unlabelled penicillin (B) or unlabelled ceftriaxone (C) for 10 min before addition of 2 μg/ml of [125I]penicillin V followed by SDS-PAGE separation and fluorography. The concentrations of [125I]penicillin (A) or unlabelled penicillin (B) and unlabelled ceftriaxone (C) were as follows (from lane a): (A) 2.0, 1.0, 0.5, 0.125, 0.06, 0.03, 0.015, 0.004, 0.001, and 0.00025 μg/ml; (B) 32, 16, 4, 1.0, 0.25, 0.06, 0.015, 0.004, 0.001, and 0.00025 μg/ml; (C) 256, 64, 32, 8, 2, 0.5, 0.125, 0.03, 0.008, 0.002, and 0.0005 μg/ml. The numbers 1 to 5 refer to PBP 1 to 5, respectively.
at room temperature for 48–72 h. The density of PBPs was measured by microdensitometry.

**Results**

The MICs of penicillin G, and ceftriaxone for *S. pyogenes* 12384 were both 0.015 μg/ml. MBCs were 0.03 μg/ml for ceftriaxone and 0.06 μg/ml for penicillin. The MIC\textsubscript{50}, MIC\textsubscript{90} and MIC range, for penicillin G, and ceftriaxone against 29 strains of *S. pyogenes* are presented in Table 1.

After exposure to either penicillin or ceftriaxone at concentrations equal to 1/2 × MIC or 1 × MIC for 1 h, no lysis of bacteria was observed using SEM; however, both penicillin and ceftriaxone caused cells to become irregular and enlarged compared to the untreated control (Fig. 1).

Multiple PBPs were present on the membranes of *S. pyogenes* ATCC 12384. The molecular mass and the binding of \([^{125}\text{I}]\text{penicillin V}\) by each of the PBPs in terms of the relative optical extinction properties of the autoradiographic bands of the film are shown in Table 2. The major bands binding penicillin were PBP 2 and PBP 4 (Table 2, Fig. 2A). The specificity of the binding of radio-labelled penicillin V was verified by incubating membrane proteins of strain ATCC 12384 with increasing concentrations of non-radiolabelled penicillin G for 10 min before reacting with a single concentration of \([^{125}\text{I}]\text{penicillin V}\) (Fig. 2B). The results showed that binding of radiolabelled penicillin V could be prevented by increasing concentrations of non-radiolabelled penicillin G. Non-radiolabelled ceftriaxone had a similar pattern of binding to these PBPs, but a much higher concentration of ceftriaxone was necessary to achieve a 50% inhibition of radiolabelled-penicillin V binding for PBP 4 (Table 2, Fig. 2C).

**Discussion**

The superior in vitro activity of ceftriaxone compared to penicillin against 29 strains of *S. pyogenes* is in agreement with a previous report [1]. Similarly, both penicillin and ceftriaxone induced morphological changes in *S. pyogenes* exposed to concentrations of either penicillin or ceftriaxone at or below the MIC, suggesting that these two beta-lactams may interact with the same targets (PBPs). This hypothesis is substantiated by studies demonstrating that a variety of beta-lactam antibiotics induce abnormal bacterial morphology [13] and that inhibition of different essential PBPs induces different morphological alterations [14]. In contrast, Lorian and Gemmell [13] stated that Gram-positive cocci develop similar morphological effects following exposure to a variety of beta-lactam antibiotics. This latter observation implies that there is little variation in the affinities of most beta-lactams for specific PBPs among Gram-positive cocci [13].

Multiple PBPs have been reported in *S. pyogenes* [11,12]. Interestingly, a comparative study of PBPs of *S. pyogenes* and its derived L-forms, in which cell wall is devoid, showed that PBPs 1 and 2 were absent in its L-forms, indicating that these two PBPs are major ones responsible for cell wall synthesis in this bacterium [12]. Our results showed that PBP 4 has a lower affinity for ceftriaxone than penicillin, and this lower affinity does not appear to alter its in vitro biological activity against *S. pyogenes*.

Knowledge of the functional role of specific PBPs in bacterial cell wall synthesis provides a biochemical basis for the morphological changes induced by beta-lactam antibiotics. PBPs are considered the targets of these antibiotics and they function as peptidoglycan transpeptidases and D,D-carboxypeptidases, both of which are necessary for the crosslinking of the cell wall [11]. The functional role for any of the PBPs of *S. pyogenes* has not been definitely determined. At concentrations below the MIC (0.015 μg/ml), penicillin binds to PBP 1 (Table 2). Furthermore, the competition data suggest that PBPs 1–3 appear more receptive to penicillin action than PBP 4 (IC\textsubscript{50} for Pen-G, Table 2), and that PBPs 2 and 3 may be the important targets for both ceftriaxone and penicillin. Since a 100-fold higher concentration of ceftriaxone compared to penicillin was necessary to cause a 50% inhibition (IC\textsubscript{50}) of labelled penicillin binding to PBP 4, this PBP is likely not...
essential for ceftriaxone inhibition of S. pyogenes multiplication. This is further supported by the observation that penicillin and ceftriaxone induce similar defects in the morphology of S. pyogenes and by the fact that the MIC$_{50}$ and MIC$_{90}$ values are lower for ceftriaxone than for penicillin. Avid binding of penicillin or ceftriaxone to PBPs 2 and 3 may be required for the morphological changes observed with these beta-lactams for S. pyogenes. In addition, PBP 2 demonstrates a lower IC$_{50}$ for ceftriaxone than for penicillin, suggesting that the avidity of PBP 2 for ceftriaxone may at least in part explain ceftriaxone’s greater biological activity.

Finally, PBPs 1, 2, and 3 are saturated (S$_{50}$) and inhibited (IC$_{50}$) at lower concentrations of penicillin than the other PBPs, suggesting that these PBPs are the most important in mediating bactericidal activity. This, coupled with the observation by others that L-forms lack PBPs 1 and 2, substantiates the concept that PBPs 1 and 2 are critical for the marked anti-bacterial properties of beta-lactam antibiotics against group A streptococcus.

Acknowledgements

This work was supported in part by a Merit Review grant from the Veteran Affairs and a grant from Hoffman-La Roche. We wish to thank J. Franklin Bailey for the excellent instructions of taking SEM photographs, Donald O. Chaffin for densitometry reading of PBP autoradiographic films, John Mangan and Michael Wyett for medical illustrations, and Kelly T. Thompson for word processing assistance.

References