Pneumococcal LytA Autolysin: a Potent Therapeutic Agent in Experimental Peritonitis-Sepsis Caused by Highly β-Lactam-Resistant Streptococcus pneumoniae

Running title: Activity of lysins in experimental pneumococcal sepsis

Violeta Rodríguez-Cerrato¹, Pedro García², Lorena Huelves¹, Ernesto García², Gema del Prado¹, Matilde Gracia¹, Carmen Ponte¹, Rubens López², Francisco Soriano¹*. ¹Department of Medical Microbiology and Antimicrobial Chemotherapy, Fundación Jiménez Díaz, Madrid, Spain. ²Department of Molecular Microbiology, Centro de Investigaciones Biológicas (CSIC), Ramiro de Maeztu 9, 28040 Madrid, Spain.

¹Corresponding author. Department of Medical Microbiology and Antimicrobial Chemotherapy, Fundación Jiménez Díaz, Avenida de Reyes Católicos 2, 28040 Madrid, Spain. Phone: +34-91-544-73-87. Fax: +34-91-549-47-64. E-mail: fsoriano@fjd.es
ABSTRACT

The *in vitro* and *in vivo* antipneumococcal activities of the main pneumococcal autolysin (LytA), and Cpl-1, a lysozyme encoded by phage Cp-1, were studied. Intraperitoneal therapy with LytA or high-dose Cpl-1 remarkably reduced peritoneal bacterial counts (>5-log_{10} CFU/ml) versus controls. After intravenous injection, LytA was the most effective treatment.
New therapeutic strategies are being sought for combating infections caused by antibiotic-resistant *Streptococcus pneumoniae*. Lytic enzymes produced by bacteria and phages may be useful alternatives for the treatment of pneumococcal infections. The LytA amidase is the main pneumococcal autolysin (3, 6), and is responsible for the lytic effects of penicillin and other cell wall inhibitors (5, 10, 11). Cpl-1 is a lysozyme encoded by the pneumococcal phage Cp-1 (1, 2, 4).

The aim of this study was to compare the in vitro and in vivo activity of purified LytA and Cpl-1 with that of cefotaxime (CTX) against a β-lactam-resistant (penicillin MIC, 2 µg/ml) meningeal pneumococcal isolate (strain MJD3693).

The MICs of LytA, Cpl-1, and CTX for MJD3693 strain were, respectively, 16, 32, and 4 µg/ml, as determined by using broth microdilution methodology (7). LytA and Cpl-1 were overproduced and purified by affinity chromatography in DEAE-cellulose (8). One unit (U) of enzymatic activity (5) was defined as the amount of enzyme that catalyzed the hydrolysis of 1 µg (∼715 net cpm) of [methyl-³H]choline-labeled pneumococcal cell walls (6) in 10 min at 37ºC. Time-kill experiments were performed in three separate assays by exposing early-log-phase cultures of MJD3693 to 50 µg/ml of LytA, Cpl-1, or CTX in tubes with either cation-adjusted Mueller-Hinton broth with 4% lysed horse blood (CA-MHB-LHB) or phosphate-buffered saline pH 7.0 (PBS). Such concentration was over 12-fold greater than the CTX MIC, but only 1.6- and 3-fold higher than the MIC of Cpl-1 and LytA, respectively. After incubation at 35ºC, bacterial titers were compared at 1, 3, and 5 h (Table 1). The killing effect of the enzymes was very rapid and similar in both media, and their antipneumococcal activity was remarkably higher than that of CTX, particularly in PBS. In CA-MHB-LHB at 1 and 3 h, LytA and Cpl-1 were significantly more effective than CTX (P ≤ 0.018), although after 5 h incubation, only LytA demonstrated a significantly higher activity.
than CTX ($P = 0.004$). In PBS, LytA and Cpl-1 exerted a profound bactericidal effect at all times whereas, as expected, the effect of CTX in PBS was quite poor (Table 1).

The plasma pharmacokinetics of purified LytA and in vivo efficacy of the compounds were studied using adult Swiss mice with the approval of our Ethics Committee. Plasma pharmacokinetics of LytA was determined using a solution of 3.1 mg/ml (specific activity $5.9 \times 10^5$ units (U)/mg). After single intravenous (iv) doses (via the tail vein) of LytA (25 mg/kg) to uninfected mice, blood samples were obtained from three animals per group sacrificed at 5, 15, 60, and 120 min, and plasma enzyme activity was measured. The mean plasma area under the enzymatic activity-time curve was 3,440 U min µl$^{-1}$ with an intercept value of 101 U/µl. The plasma half-life of LytA was 22.5 min, similar to that of Cpl-1 (20.5 min) (4) and longer than that of CTX (ca. 12 min) (9).

To induce the peritonitis-sepsis model, mice were intraperitoneally inoculated with 0.25 ml of the pneumococcal suspension ($7.3 \pm 0.3 \log_{10}$ CFU per mouse). This inoculum was 100% lethal within 1-3 days and provided a detectable number of bacteria in the peritoneal cavity and blood. Treatments started 1 h after bacterial challenge as single 0.25-ml doses by intraperitoneal (ip) or iv administration. Ten groups of infected mice (5-6 animals per group) were used; half of them were treated by either via. Mice were treated with LytA (1.7 mg; 57 mg/kg; specific activity $8.9 \times 10^5$ U/mg), Cpl-1 (1.4 mg; 47 mg/kg; specific activity $9.8 \times 10^5$ U/mg) (“low-dose” regimen), Cpl-1 (3.3 mg; 110 mg/kg; specific activity $6 \times 10^5$ U/mg) (“high-dose” regimen), or CTX (12 mg; 400 mg/kg); control mice received sterile saline. Four hours after injection the animals were sacrificed and blood and peritoneal lavage fluid specimens were processed for bacterial counting. The lower limit of organism quantification in these studies was 10 CFU/ml.
For analytical purposes, specimens with <10 CFU/ml were arbitrarily assigned a value of 1 CFU/ml.

Figure 1 and Table 2 summarize the efficacy of agents to reduce bacterial loads in the peritoneal lavage fluid and blood after ip and iv administration of the different agents. At 5 h post-challenge, the bacterial titers in the peritoneal fluid and blood of control animals were $7.4 \pm 0.6$ and $6.5 \pm 0.7 \log_{10} \text{CFU/ml}$, respectively.

The bacterial counts diminished significantly following the ip administration of all agents (versus controls). The most important effect was observed with LytA, although a profound killing effect was also seen after ip administration of Cpl-1 either at high or low dose. The mice treated intraperitoneally with CTX experienced the smallest antibacterial effect in the peritoneum. After the iv administration of LytA, CTX, or the high-dose Cpl-1, the bacterial titers in the peritoneal fluid were significantly diminished with respect to the controls. Intravenous LytA had a more potent effect at the peritoneum than either of the two Cpl-1 iv regimens.

Intraperitoneal administrations of LytA, high dose Cpl-1 or CTX all significantly reduced the bacterial load in the blood; indeed, 3 out of 5 mice treated with LytA and 2 out of 5 mice treated with CTX had no detectable bacteremia (Fig. 1). Intravenous LytA had the highest bactericidal effect in blood. The antipneumococcal activity of all the agents tested was superior when administered intraperitoneally than when injected intravenously, although the differences were only significant for both doses of Cpl-1 and bacterial load in peritoneal fluid and blood ($P < 0.001$) and for LytA and bacterial load in peritoneal fluid ($P < 0.01$).

The reduction in the peritoneal and blood colony counts achieved by ip LytA reflects the intrinsic activity and bactericidal capacity of LytA. The two Cpl-1 regimens tested were also more effective than CTX when the ip route was used. The results comparing...
the bacterial killing achieved with the iv and ip routes suggest that LytA is acceptably distributed throughout the circulatory system and enters the peritoneal cavity, a highly vascularized site.

To our knowledge, the present study is the first to treat with LytA a β-lactam-resistant pneumococcal peritonitis-sepsis in mice. These results may therefore represent a step forward towards the potential use of enzybiotic therapy.

We thank A. Burton for correcting the English version of the manuscript. This research was supported by grants from the Red Temática de Investigación Cooperativa Spanish Pneumococcal Infection Study Network (G03/103) (Ministerio de Sanidad y Consumo, Spain) and the program COMBACT Nuevas dianas para combatir a las bacterias patógenas (S-BIO-026-2006) (Consejería de Educación, Comunidad de Madrid, Spain). LH and GdP received scholarships from the Fundación Conchita Rábago; MG received a scholarship from the Fondo de Investigaciones Sanitarias, Ministerio de Sanidad y Consumo, Spain.

REFERENCES


TABLE 1. Time-kill experiments with the pneumococcal strain MJD3693 in either CA-MHB-LHB or PBS\footnote{CA-MHB-LHB, cation-adjusted Mueller-Hinton broth supplemented with 4% lysed horse blood; PBS, phosphate-buffered saline} after exposure to LytA, Cpl-1, or cefotaxime.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>CA-MHB-LHB</th>
<th>PBS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LytA</td>
<td>Cpl-1</td>
</tr>
<tr>
<td>1</td>
<td>–3.5 ± 0.0</td>
<td>–3.4 ± 0.9</td>
</tr>
<tr>
<td>3</td>
<td>–5.4 ± 0.1</td>
<td>–5.0 ± 0.8</td>
</tr>
<tr>
<td>5</td>
<td>–5.3 ± 0.2</td>
<td>–5.3 ± 0.7</td>
</tr>
</tbody>
</table>

\footnote{Differences of bacterial colony counts (expressed as log_{10} CFU/ml) between those found in enzyme- or antibiotic-containing tubes and those shown in control tubes at different time points. Values are expressed as mean ± SD from three experiments where the baseline (time 0) inoculum was 7.14 ± 0.03 log_{10} CFU/ml. LytA (specific activity 9.3 \times 10^5 U/mg), Cpl-1 (specific activity 9.8 \times 10^5 U/mg) and CTX were used at a final concentration of 50 µg/ml.}
**TABLE 2.** Decline of bacterial counts in peritoneal fluid and blood samples of animals with pneumococcal peritonitis-sepsis after 4 h post-therapy compared to those of controls

<table>
<thead>
<tr>
<th>Test group</th>
<th>Injection via</th>
<th>Mean (± SD) differences of bacterial counts (log$_{10}$ CFU/ml) $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(dose per mouse)</td>
<td>Peritoneal fluid</td>
</tr>
<tr>
<td>LytA (1.7 mg)</td>
<td>Intraperitoneal</td>
<td>$-5.4 \pm 0.6$ $^b$</td>
</tr>
<tr>
<td></td>
<td>Intravenous</td>
<td>$-3.5 \pm 0.9$ $^b$</td>
</tr>
<tr>
<td>Cpl-1 (1.4 mg)</td>
<td>Intraperitoneal</td>
<td>$-4.8 \pm 0.7$ $^b$</td>
</tr>
<tr>
<td></td>
<td>Intravenous</td>
<td>$-0.7 \pm 0.6$</td>
</tr>
<tr>
<td>Cpl-1 (3.3 mg)</td>
<td>Intraperitoneal</td>
<td>$-5.2 \pm 0.3$ $^b$</td>
</tr>
<tr>
<td></td>
<td>Intravenous</td>
<td>$-1.5 \pm 0.5$ $^b$</td>
</tr>
<tr>
<td>Cefotaxime (12 mg)</td>
<td>Intraperitoneal</td>
<td>$-3.5 \pm 0.6$ $^b$</td>
</tr>
<tr>
<td></td>
<td>Intravenous</td>
<td>$-3.1 \pm 1.3$ $^b$</td>
</tr>
</tbody>
</table>

$^a$ Differences in bacterial titers between treated animals and untreated controls.

$^b$ Statistically significant differences versus controls ($P < 0.05$).
FIG. 1. Antipneumococcal activity in the peritoneal fluid (A, C) and in the blood (B, D) of lytic enzymes or CTX 4 h after administration by the intraperitoneal (A, B) or intravenous (C, D) routes in mice with β-lactam-resistant S. pneumoniae peritonitis-sepsis. Each point represents one mouse; 5–6 animals per group; the mean ± SD for each group is also shown. In (B): 1 three animals treated with ip-LytA showed bacterial clearance from the blood; 2 two animals treated with ip-CTX also showed bacterial clearance in the blood. *Statistically significant difference compared to control.
IP THERAPY

IV THERAPY