

Phage Lytic Enzyme Cpl-1 for Antibacterial Therapy in Experimental Pneumococcal Meningitis

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Treatment of bacterial meningitis caused by *Streptococcus pneumoniae* is increasingly difficult, because of emerging resistance to antibiotics. Recombinant Cpl-1, a phage lysin specific for *S. pneumoniae*, was evaluated for antimicrobial therapy in experimental pneumococcal meningitis using infant Wistar rats. A single intracisternal injection (20 mg/kg) of Cpl-1 resulted in a rapid (within 30 min) decrease in pneumococci in cerebrospinal fluid (CSF) by 3 orders of magnitude lasting for 2 h. Intraperitoneal administration of Cpl-1 (200 mg/kg) led to an antibacterial effect in CSF of 2 orders of magnitude for 3 h. Cpl-1 may hold promise as an alternative treatment option in pneumococcal meningitis.

Streptococcus pneumoniae causes severe infectious diseases, including pneumonia, sinusitis, otitis media, bronchitis, septic arthritis, endocarditis, and sepsis. It has also been shown to be the leading cause of bacterial meningitis (47%), in a survey study conducted in the United States [1]. Despite emerging vaccination strategies and the development of new antibiotics, pneumococcal meningitis (PM) is still associated with a high mortality rate, ranging from 19% to 26% [1]. Prompt and adequate antimicrobial therapy reduces fatality rates to <10% in infants and children [2].

Antibiotic-resistant strains have emerged because of the genetic plasticity of *S. pneumoniae* and the high exposure to anti-

biotic environments. Replacement by nonvaccine serotypes under vaccination pressure has been demonstrated [3].

Phage lytic enzymes have recently been proposed for the reduction of nasopharyngeal carriage of *S. pneumoniae* [4–6]. Cpl-1, a lytic enzyme from the phage Cp-1 [7, 8], has been shown to kill in vitro all tested serotypes of *S. pneumoniae*. By virtue of its choline-binding domain, it displays an exquisite specificity toward *S. pneumoniae* and can act synergistically with penicillin and/or gentamicin in vitro [9]. Furthermore, Cpl-1 has successfully been tested as a therapy in rodent models of pneumococcal sepsis [4, 6] and endocarditis [10]. It can also prevent otitis media caused by colonizing *S. pneumoniae* [11]. Here, we report on the efficiency of Cpl-1 as an antimicrobial agent in an infant-rat model of PM.

Methods. An established model of PM in infant rats was used [12, 13]. The animal studies were approved by the Animal Care and Experimentation Committee of Canton Bern, Switzerland, and National Institutes of Health guidelines for the performance of animal experimentation were followed. Wistar rats (Charles River Laboratories) were infected on postnatal day 11 by intracisternal (ic) injection of 10 μ L of a log₁₀ 5.7 \pm 0.3 cfu/mL suspension of a penicillin-susceptible clinical isolate of *S. pneumoniae* (serogroup 3) [12, 13] in 0.85% NaCl saline solution.

To produce recombinant Cpl-1, this enzyme, a muramidase of the *S. pneumoniae*-specific lytic phage Cp-1, was expressed in *Escherichia coli* DH5 α (pJML6), purified to homogeneity using DEAE column chromatography, and suspended in enzyme buffer (50 mmol/L phosphate buffer [pH 7.0] with 1 mmol/L EDTA and 1 mmol/L dithiothreitol). The specific activity, defined elsewhere [5], of a freshly produced and purified batch was \sim 1 U/ μ g. Endotoxin was removed with ActiClean Etox (Sterogene Bioseparations).

The treatment regimen was as follows. Eighteen hours after infection, cerebrospinal fluid (CSF) was harvested by ic puncture and cultured quantitatively to document meningitis. Immediately afterward, Cpl-1 was injected ic (600 U in 30 μ L [i.e., 20 mg/kg of body weight] n = 30) or intraperitoneally (ip; 6000 U in 300 μ L [i.e., 200 mg/kg of body weight]; n = 24). Untreated infected animals (n = 12) were injected ic with 30 μ L of sterile, pyrogen-free saline as control rats. Animals were killed at 30 min (n = 8 for ic and n = 4 for ip), 1 h (n = 7 for ic and n = 5 for ip), 2 h (n = 4 for ic and n = 5 for ip), 3 h (n = 4 for ic and n = 5 for ip), and 4 h (n = 7 for ic and n = 5 for ip), and samples of CSF and plasma were collected for determination of bacterial titers by serial dilution and plating on sheep blood agar

Received 20 August 2007; accepted 17 December 2007; electronically published 7 April 2008.

Potential conflicts of interest: none reported.

Presented in part: 44th Interscience Conference on Antimicrobial Agents and Chemotherapy, Washington, DC, 30 October–2 November 2004 (abstract B-940).

Financial support: Swiss National Science Foundation (grant 31-116257); US Public Health Service (grant AI-057472 VAF).

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The Journal of Infectious Diseases 2008; 197:1519–22

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0022-1899/2008/19711-0007\$15.00

DOI: 10.1086/587942

plates. Subsequently, the concentration of Cpl-1 was determined in the samples after centrifugation at 4°C for 5 min at 10,000 g. The volume of CSF that can be obtained from infant rats is subject to variation because of the individual clinical course of disease. Therefore, we were unable to both determine titers and conduct Western blot analysis at all time points. When the health status of an animal worsened rapidly because the biological activity of Cpl-1 waned and the bacteria reestablished infection or because multiple ic injections caused clinical deterioration, it was euthanized for ethical reasons.

The lytic activity of Cpl-1 against the pneumococcal isolate was tested in vitro by incubating 1×10^7 cfu of *S. pneumoniae* with 30 U of Cpl-1 at room temperature in a total volume of 300 μ L. At 30 s and at 10 min after the start of the incubation, 10 μ L was taken and immediately diluted 1:100 in ice-cold saline. Further decimal dilutions were also performed with ice-cold saline.

The amount of Cpl-1 in CSF at different time points after injection was assessed by Western blotting and spot densitometry. Briefly, proteins in 15 μ L of CSF or 1 μ L of plasma were separated on a 12.5% SDS-polyacrylamide gel. Cpl-1 in serial dilutions was added as standards. After transfer of the proteins on a polyvinylidene fluoride membrane (Immobilon-P; Pierce), Cpl-1 was detected as a 39-kDa band with a rabbit polyclonal antibody and an enhanced chemiluminescence detection kit (Supersignal West Pico; Pierce). CSF or plasma from at least 2 animals was analyzed at each time point during the first 8 h after Cpl-1 administration. A 1-phase exponential decay curve was fitted to calculate the half-life, by means of GraphPad Prism (version 4.03; GraphPad Software).

All statistical analyses were performed using GraphPad Prism. For the difference in bacterial titers between different treatment regimens, 2-way analysis of variance was performed. Comparison between groups was then performed with Bonferroni posttests. Because titers at 3 h were not determined for ic control rats, this time point was excluded from the statistical analyses.

Results. The ability of Cpl-1 to kill the *S. pneumoniae* strain used in this study was tested in vitro. Incubation of 1×10^7 cfu of *S. pneumoniae* with 30 U of Cpl-1 for 30 s and 10 min, respectively, decreased bacterial titers to below the detection limit ($<1 \times 10^3$ cfu/mL) for both time points tested. This is in agreement with the killing rate observed for other strains [6].

Cpl-1 was then evaluated as a candidate for the treatment of experimental PM. After a single ic injection of 600 U (600 μ g) of Cpl-1, bacterial titers quickly decreased over the first 4 h, whereas treatment with buffer had no effect (figure 1). Starting at 30 min and for the next 2 h, CSF titers in rats treated with Cpl-1 were at or below the detection limit. Soon after, pneumococci started to multiply again in CSF (figure 1). The amount of Cpl-1 in each sample was calculated by densitometric scanning of Western blots, using a standard curve of defined amounts of Cpl-1 (figure 2). Cpl-1 was consistently detected in CSF during the first 2 h after ic injection of 600 U in 2 independent experi-

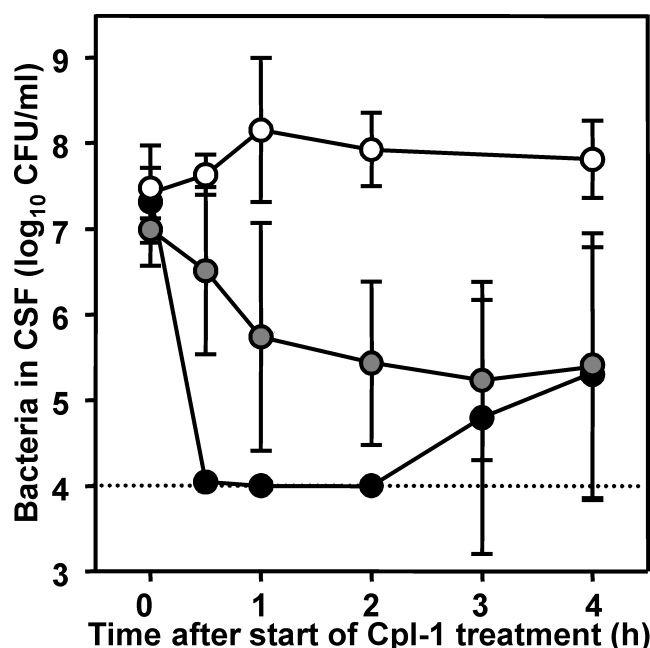


Figure 1. Effect of intracisternal (ic) and intraperitoneal (ip) administration of Cpl-1 on cerebrospinal fluid (CSF) bacterial titers. CSF bacterial titers were unaffected by ic application of saline (white circles). Injection of Cpl-1 ic (600 μ g; black circles) resulted in a rapid (within 30 min) decrease in bacterial titer by 3 orders of magnitude. Injection of Cpl-1 ip (6 mg; gray circles) led to the clearance of pneumococci from the CSF by 2 orders of magnitude until 3 h after administration. The limit of detection for the determination of CSF bacterial titers was 1×10^4 cfu/mL of undiluted CSF (dashed line). Two-way analysis of variance revealed that both treatment and time after treatment were significant sources of variation ($P < .0001$). Bonferroni posttests showed significant differences between treatment groups ($P < .05$) for the 0.5-, 1-, 2-, and 4-h time points for all except control ic vs. Cpl-1 ic at 0.5 h and Cpl-1 ic vs. Cpl-1 ip at 4 h.

ments, (figure 2C). The half-life of Cpl-1 in CSF was estimated to be ~ 16 min (figure 2A). Thus, the regrowth of bacteria 4 h after ic Cpl-1 administration was due to the short biological half-life of Cpl-1 in CSF.

In a second approach that more closely modeled an envisioned clinical application, Cpl-1 was administered by ip injection (200 mg/kg of body weight). Western blot analysis of plasma samples at different time points after infection demonstrated that Cpl-1 was continuously released in the bloodstream over the first 4 h after injection, and concentrations remained stable between 50 and 60 μ g/mL (figure 2B and 2C). During the same time, the corresponding bacterial titers in blood were below the detection limit (1×10^3 cfu/mL). Importantly, a marked reduction in bacterial titer in CSF was observed after ip injection of Cpl-1 (figure 1, gray circles). After 2 h, titers decreased from a mean \pm SD of 6.99 ± 0.11 to 5.23 ± 0.6 \log_{10} cfu/mL, representing a drop of 98%. This antibacterial effect was detectable in CSF for as long as 4 h after initiation of ip therapy

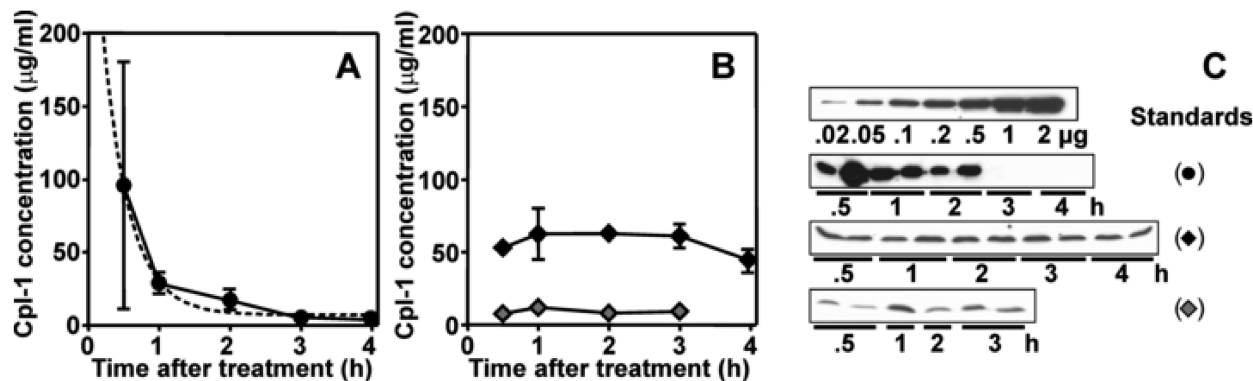


Figure 2. Assessment of Cpl-1 in cerebrospinal fluid (CSF) and plasma by Western blot. *A*, Decrease in Cpl-1 concentration in the CSF after intracisternal (ic) administration of 600 µg (black circles). The half-life was determined to be ~16 min by fitting a 1-phase exponential decay curve (dashed line). *B*, Concentration of Cpl-1 in serum (black diamonds) and CSF (gray diamonds) after intraperitoneal (ip) application of 6 mg. Cpl-1 concentrations remained stable for the time period tested. *C*, Anti-Cpl-1 Western blots of standards and samples used to perform the densitometric determination of Cpl-1 concentrations shown in panels *A* and *B*.

(figure 1, gray circles). The reduction of bacterial titers in CSF is concomitant to the presence of Cpl-1 in the same compartment. In contrast to CSF concentrations after ic injection, Cpl-1 showed a prolonged presence for up to 3 h after ip injection of 200 mg/kg, ranging from 7 to 12 µg/mL of CSF (figure 2*B* and 2*C*).

Discussion. Here, we demonstrated the ability of Cpl-1, a phage lytic enzyme, to rapidly kill *S. pneumoniae* in an infant rat model of bacterial meningitis when injected ic or ip. A single ic injection of Cpl-1 decreased CSF bacterial counts to below the detection limit as early as 30 min after injection. This effect was evident for up to 3 h after administration, after which time bacteria in CSF were detected again. The antibacterial effect of Cpl-1 is temporally defined by the 16-min half-life in CSF. Increasing the bioavailability of Cpl-1 in CSF by multiple ic injections was attempted, but these repetitive injections proved to be excessively harmful to the animals (data not shown).

Cpl-1 has been investigated previously for in vivo therapeutic use in 2 murine sepsis models. In one model, mice were infected and Cpl-1 was injected intravenously [6]. In the other model, both the infection and Cpl-1 administration were performed by ip injection [4]. In both models, Cpl-1 was able to rapidly reduce blood titers. Furthermore, the half-life of Cpl-1 in plasma was estimated to be ~20 min [4], a value similar to what we observed in the CSF of infected animals. In contrast to these previous sepsis experiments, in which bacterial eradication was achieved only when Cpl-1 was administered within 4 h after infection, we initiated therapy only after clinical signs of PM had developed (i.e., at 18 h after infection) [6]. In the present study, the observed bacterial regrowth in the CSF after 4 h may have been due to *S. pneumoniae* transiently invading epithelial or endothelial cells [14] and thus being out of the reach of Cpl-1. Another possibility is that a single dose of Cpl-1 is not sufficient to contact

all the pneumococci in the CSF and that additional doses may be necessary for complete eradication.

Administration of Cpl-1 ip resulted in prolonged systemic release, as documented by a constant Cpl-1 level in plasma and CSF for a period of >4 h after injection. The amount of Cpl-1 in the CSF correlated with CSF total protein concentration (data not shown), suggesting that changes in the blood-brain barrier may have facilitated the diffusion of Cpl-1 from the blood. Administration of Cpl-1 ip (250 mg/kg) led to a Cpl-1 concentration that was sufficient to decrease CSF bacterial titers by ~98% over the first 4 h after administration. We speculate that the sustained release of biologically active Cpl-1 from the peritoneal cavity, in conjunction with the breakdown of the blood-brain barrier during meningitis, explains the extended presence of Cpl-1 in the CSF.

The data presented in this study identify Cpl-1 as a promising candidate for antibacterial therapy for several reasons. The presence of biologically active Cpl-1 in the CSF after ip application has not been demonstrated to date. This finding opens new doors for future investigations into the use of Cpl-1 in the therapy of invasive pneumococcal infections. It has been shown that Cpl-1 can act synergistically with Pal, another phage lytic enzyme [15], and (more recently) with penicillin or gentamicin [9]. Of special interest, Cpl-1 and penicillin showed synergy against a highly penicillin-resistant strain in vitro. With respect to emerging resistance, a combined treatment of Cpl-1 with conventional antibiotic therapy could therefore be beneficial. Furthermore, rapid bacterial clearance of the CSF by Cpl-1 in synergy with antibiotics may prove beneficial, because rapid CSF sterilization has been associated with an improved outcome of BM [2]. To our knowledge, the use of phage lysins to control bacterial infections of the central nervous system has never been demonstrated.

Given the emergence of antibiotic-resistant strains, considerable attention is given to alternative antibiotic therapy. In this context, phage lysins could represent alternatives for both prophylaxis (e.g., by selective reduction or eradication of carriage) and treatment by offering rapidity, safety, and lack of resistance.

Acknowledgment

We thank Jürg Kummer (Institute for Infectious Diseases, University of Bern) for excellent technical support.

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