C1 Inhibitor Treatment Improves Host Defense in Pneumococcal Meningitis in Rats and Mice

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In spite of antibiotic treatment, pneumococcal meningitis continues to be associated with significant morbidity and mortality. The complement system is a key component of innate immunity against invading pathogens. However, activation of complement is also involved in tissue damage, and complement inhibition by C1 inhibitor (C1-inh) is beneficial in animal models of endotoxemia and sepsis. In the present study, we demonstrate classical pathway complement activation during pneumococcal meningitis in rats. We also evaluate the effect of C1-inh treatment on clinical illness, bacterial clearance, and inflammatory responses in rats and mice with pneumococcal meningitis. C1-inh treatment was associated with reduced clinical illness, a less-pronounced inflammatory infiltrate around the meninges, and lower brain levels of proinflammatory cytokines and chemokines. C1-inh treatment increased bacterial clearance, possibly through an up-regulation of CR3. Hence, C1-inh may be a useful agent in the treatment of pneumococcal meningitis.

In spite of antibiotic treatment, pneumococcal meningitis continues to be an infectious disease with significant morbidity and mortality [1]. The severity of the disease and the emergence and spread of penicillin-resistant Streptococcus pneumoniae [2] warrant the search for additional therapeutics.

Infection of the central nervous system (CNS) leads to a pathophysiological sequence of events that includes leukocytosis of cerebrospinal fluid (CSF) and the generation of inflammatory mediators. This local inflammatory response is important for the clearance of invading bacteria and contributes to the development of neuronal damage and cell death [1, 3, 4]. Inflammatory reactions result from the activation of cells and the release of mediators. Among these are components of the complement system, which constitutes an important part of the innate immune system. After activation of the complement system, biologically active peptides are generated that elicit a variety of proinflammatory effects. Major functions of complement in host defense against bacterial infections are opsonization, leukocyte recruitment, and activation and lysis of bacteria and cells. However, complement activation is also believed to contribute significantly to tissue damage in inflammatory diseases [5], including bacterial infection.

Although the CNS is considered to be an immune-privileged organ, the brain represents a significant site of complement synthesis [6]. All classical and alternative pathway complement components can be synthesized in the CNS [7], and functional receptors for complement proteins are present on several cell populations in the CNS [7–9]. Local activation of complement occurs both in experimental meningitis [10] and in CSF from patients with bacterial meningitis [11–13]. Activation of complement is regulated by soluble and membrane-associated regulatory proteins. Among these is C1 inhibitor (C1-inh), which regulates the initiation
phase of the classical pathway. C1-inh is a member of the serpin superfamily of protease inhibitors and is the only known inhibitor of C1r and C1s, components of the classical pathway of the complement cascade [5]. In addition, it is a major inhibitor of factor XII and prekallikrein of the contact system [14, 15]. C1-inh also exerts an effect independent of its protease inhibitory activity [16].

Beneficial effects of C1-inh treatment have been demonstrated in a number of preclinical models and human diseases [17], including sepsis [18–20]. In the present study, we evaluated the effect of C1-inh on the course of pneumococcal meningitis in rats and mice. Our results indicate that treatment with C1-inh improves the clinical course and outcome of experimental bacterial meningitis.

MATERIALS AND METHODS

Animals and reagents. Male Wistar rats (weight, 275–300 g) and 3–4-week-old male C57/Bl6 mice were purchased from Harlan. Pasteurized purified human C1-inh (Sanquin) was dialyzed and resolved in sterile PBS (pH 7.4), because the original buffer induced seizures in rats after intrathecal (itl) administration (unpublished observation). One unit equals the amount of C1-inh in 1 mL of normal human plasma [21]. All animal experimentation guidelines were followed.

Experimental design and models. S. pneumoniae serotype 6A, isolated from the CSF of an infected patient, was used to induce meningitis. Pneumococci were grown to the midlogarithmic phase at 37°C in brain-heart infusion (BHI) broth until an OD620 of 1.0 was achieved. Subsequently, the suspension was homogenized (unpublished observation). One unit equals the amount of C1-inh in 1 mL of normal human plasma [21]. All animal experimentation guidelines were followed.

Assessment of the in vitro effect of C1-inh on S. pneumoniae. To assess the effect of C1-inh on pneumococcal growth, bacterial growth was compared in the absence and presence of C1-inh. Pneumococci were grown in BHI broth with or without C1-inh (20 U/mL) at 37°C while the rate of increase in optical density was monitored. Suspensions were plated in serial dilutions onto blood agar plates, and colonies were counted after incubation for 18 h at 37°C.

Quantification of bacterial outgrowth. Serial dilutions in sterile isotonic saline were made of CSF and blood and were plated onto blood agar plates. Plates were incubated for 18 h at 37°C, after which colonies were counted.

Cytokine and complement assays. Levels of murine cytokines (KC, macrophage inflammatory protein–2, interleukin [IL]–1α, IL-1β, IL-6, IL-18, and tumor necrosis factor [TNF]–α) were measured using commercially available ELISAs in accordance with the recommendations of the manufacturer (R&D Systems). Plasma, brain homogenate, and CSF levels of exogenously administered C1-inh were measured using a nephelometer (Nephelometer Analyzer; Behringwerke) and are expressed as milligrams per liter. Functional C1-inh levels were...
RESULTS

Histopathologic analysis. Brains were fixed in 10% buffered formalin; after paraffin embedding, 4-μm sections were stained with hematoxylin-eosin. All slides were coded and semi-quantitatively scored by a pathologist without knowledge of the experimental group.

Immunohistochemical analysis. Cryostat sections (8 μm) were cut serially, put on gelatin-coated slides, and air dried overnight in a container with silica gel. Immunohistochemical analysis was done after a 10-min fixation in dehydrated acetone to examine the presence of CD11b/CRI3 and C1q. The monoclonal antibodies used were the anti-rat ED8 (murine IgG1), which is directed against CD11b/CRI3 and was generated at the Department of Molecular Cell Biology, Vrije Universiteit Medical Center (Amsterdam, the Netherlands) [29], and rabbit antihuman C1q, which cross-reacts with rat C1q (AO136; DAKO). Antibodies and conjugates were diluted in 0.1 mol/L Tris buffer (pH 7.6) with 0.1% bovine serum albumin (BSA; Organon Technika) and were used at optimal final dilutions of 1:250 for ED8-biotin and 1:100 for AO136. In control slides, incubation with the primary antibody was omitted. All incubations were conducted horizontally at room temperature. After incubation with the first antibody for 60 min, the slides were rinsed in Tris buffer (pH 7.6) with 0.1% BSA, incubated with conjugate/0.1% normal rat serum for 60 min, and washed again with Tris/BSA buffer. As conjugates, streptavidin–alkaline phosphatase–conjugated rabbit anti–mouse IgG (DAKO) and alkaline phosphatase–conjugated goat anti–rabbit IgG (DAKO) were used. Alkaline phosphatase activity was demonstrated by incubation in naphthol AS-BI phosphate substrate in 0.1 mol/L Tris buffer (pH 8.7) for 10 min. Sections were rinsed in 0.1 mol/L Tris (pH 7.6), counterstained with hematoxylin, and mounted in VectaMount (Vector Laboratories). Multiple serial sections from each rat were compared to ensure that representative slides were assessed.

Statistical analysis. Results are expressed as means ± SEs. Differences between groups were evaluated using the Mann-Whitney U test. P < .05 was considered to indicate statistical significance.

RESULTS

Rat Model

Development of meningitis. Within 48 h after bacterial challenge, all rats inoculated with pneumococci developed meningitis, characterized by positive CSF cultures, leukocytosis of CSF (>5 × 10⁴ cells/mL), and an inflammatory infiltrate (consisting mainly of neutrophils) around the meninges. Control rats, which received 100 μL of PBS (i.e., no pneumococci), had low CSF leukocyte counts (1.3 ± 0.5 × 10⁴ cells/mL), no bacterial growth on CSF culture, and normal brain histologic results.

Classical pathway activation during experimental meningitis. To assess the induction of classical pathway activation of the complement cascade, C1q was stained on brain tissue of S. pneumoniae-inoculated and control (saline-injected) rats. Control rats did not show C1q staining. However, C1q depositions were clearly visible around the meninges after the induction of meningitis (figure 1), indicating activation of the complement cascade via the classical pathway.

C1-inh activity in CSF after iv and itl administration. To evaluate local availability of C1-inh in the CNS after iv and itl administration, C1-inh was measured in CSF and brain homogenates. C1-inh antigen and activity could be detected in CSF of rats treated both iv and itl (table 1). CSF, brain homogenate, and plasma levels of C4b/c, a classical pathway component downstream of C1r and C1s, were measured to evaluate the effect of C1-inh on the local complement activation. CSF C4b/c levels were increased in rats with meningitis but were strongly reduced in rats that received C1-inh itl, indicating efficient inhibition of classical pathway activation locally in the CSF under these conditions (table 1). Plasma C4b/c levels were also significantly diminished in rats treated iv and itl.

Reduced clinical illness in C1-inh treated rats. None of the study groups showed signs of clinical illness during the first 8–14 h after inoculation. Thereafter, signs of systemic toxicity developed, including lethargy, piloerection, loss of appetite, and weight loss. Treatment with itl C1-inh significantly improved clinical scores. Rats treated iv also showed fewer signs of illness than untreated rats; however, this difference did not reach statistical significance (figure 2A).

Figure 1. Up-regulation of C1q during pneumococcal meningitis in rats. Representative slides of staining of C1q with the monoclonal antibody A0136 are shown. A, Brain parenchyma and meninges of a control rat (injected with saline) (original magnification, ×40). B, Brain parenchyma and meninges of a rat inoculated with Streptococcus pneumoniae (original magnification, ×40). Samples were collected 48 h after inoculation.
Table 1. C1 inhibitor (C1-inh) and C4b/c levels in cerebrospinal fluid (CSF), brain, and plasma.

<table>
<thead>
<tr>
<th>Antigen and activity, location</th>
<th>Treatment group</th>
<th>Intravenous treatment</th>
<th>Intrathecal treatment</th>
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<tbody>
<tr>
<td></td>
<td>Untreated</td>
<td>0.56 ± 0.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.07 ± 0.04</td>
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<tr>
<td></td>
<td>C1-inh antigen</td>
<td></td>
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<tr>
<td>CSF</td>
<td>ND</td>
<td>0.62 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.51 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Brain</td>
<td>ND</td>
<td>14.6 ± 1.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.85 ± 0.04&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Plasma</td>
<td>ND</td>
<td></td>
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</tr>
<tr>
<td>C1-inh activity</td>
<td>CSF</td>
<td>0.29 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.05 ± 0.03</td>
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<tr>
<td></td>
<td>Brain</td>
<td>0.13 ± 0.006&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.49 ± 0.02&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>Plasma</td>
<td>9.55 ± 0.79&lt;sup&gt;0&lt;/sup&gt;</td>
<td>0.75 ± 0.09&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>C4b/c</td>
<td>CSF</td>
<td>2.40 ± 0.53</td>
<td>1.65 ± 0.59</td>
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<tr>
<td></td>
<td>Brain</td>
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<tr>
<td></td>
<td>Plasma</td>
<td>32.75 ± 2.12</td>
<td>23.5 ± 1.31&lt;sup&gt;c&lt;/sup&gt;</td>
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NOTE. Data are mean ± SE C1-inh antigen levels (in micrograms per liter), C1-inh activity, and C4b/c levels (percentage of the amount generated in normal rat serum by incubation with heat-aggregated IgG) in CSF (pooled CSF from 2 rats), brain homogenates, and plasma from untreated rats and rats treated with C1-inh intravenously or intrathecally, collected 48 h after intrathecal inoculation with 5 × 10<sup>6</sup> cfu of Streptococcus pneumoniae. n = 8 rats/group. ND, not detectable.

<sup>a</sup> P < .05 vs. untreated rats (Mann-Whitney U test).
<sup>b</sup> P < .005 vs. untreated rats (Mann-Whitney U test).
<sup>c</sup> P < .01 vs. untreated rats (Mann-Whitney U test).

Increased bacterial clearance in rats treated with C1-inh. S. pneumoniae colony-forming units were significantly lower in CSF and blood in rats treated itl with C1-inh than in untreated rats (4.2 ± 1.6 × 10<sup>6</sup> cfu/mL in untreated rats vs. 0.2 ± 0.05 × 10<sup>4</sup> cfu/mL in itl-treated rats for CSF [P = .03]; 2.0 ± 1.2 × 10<sup>4</sup> cfu/mL in untreated rats vs. 0.3 ± 0.1 × 10<sup>4</sup> cfu/mL in itl-treated rats for blood [P = .04]). Rats treated iv with C1-inh also showed a reduction in bacterial outgrowth, compared with untreated rats; however, these differences did not reach statistical significance (4.2 ± 1.6 × 10<sup>6</sup> cfu/mL in untreated rats vs. 1.1 ± 0.6 × 10<sup>6</sup> cfu/mL in iv-treated rats for CSF [P = .08]; 2.0 ± 1.2 × 10<sup>4</sup> cfu/mL in untreated rats vs. 0.6 ± 0.6 × 10<sup>4</sup> cfu/mL in iv-treated rats for blood [P = .8]).

Inflammatory responses in rats treated with C1-inh. An influx of leukocytes is an important characteristic of meningitis. Histopathologic evaluation revealed a dense inflammatory infiltrate around the meninges in control rats (figure 3A) that was less pronounced in rats treated iv (figure 3B) and itl (figure 3C) with C1-inh. Leukocyte counts in CSF were strongly diminished in C1-inh–treated groups (figure 3D). The cellular influx consisted mainly of neutrophils; C1-inh treatment did not alter the composition of CSF leukocytes.

Up-regulated CR3 in rats treated with C1-inh. The induction of meningitis was associated with an up-regulation of CR3; surprisingly, the up-regulation was enhanced in rats treated with C1-inh (figure 4). This up-regulation was not restricted to the meninges but was also visible, to a lesser extent, in the brain parenchyma and indicated an up-regulation of the receptor itself in rats treated with C1-inh.

Mouse Model

Information obtained from animal models of bacterial meningitis strongly depends on the site of inoculation and the animal used [1, 30]. To obtain further proof for a protective effect of C1-inh in pneumococcal meningitis, we evaluated C1-inh treatment in a different model of this disease. For this, we used our established meningitis model in young mice, in which infection is induced by intranasal (inl) inoculation [22, 24, 25]. The route of infection in this murine model, in which bacteria cross the blood-brain barrier (BBB), adequately mimics the characteristics of the disease in humans.

At 72 h after inoculation, 75% of untreated mice showed bacterial growth in blood cultures, compared with 50% of mice treated with C1-inh. Similarly, 72 h after bacterial challenge, 61.5% of untreated C57Bl/6 mice had developed meningitis, whereas only 38.5% of C57Bl/6 mice treated with C1-inh had developed meningitis. In other words, comparable proportions of bacteremic mice developed meningitis in both groups, indicating a decreased systemic susceptibility to pneumococci after inl challenge but unaltered passage through the BBB.

In line with the results in rat experiments, C1-inh treatment in mice was also associated with lower bacterial counts in CSF (figure 5). Hence, in our mouse model, C1-inh treatment resulted in not only a reduction of the percentage of mice that...
C1-Inh Treatment in Bacterial Meningitis

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Figure 3. Less-pronounced inflammatory infiltrate and diminished leukocyte influx after treatment with a C1 inhibitor (C1-inh). Shown are representative histologic sections of brain of untreated rats (A), rats treated intravenously (iv) with C1-inh (B), and rats treated intrathecally (itl) with C1-inh (C) at 48 h after inoculation with $5 \times 10^6$ cfu of *Streptococcus pneumoniae*. Hematoxylin-eosin staining is shown (original magnification, $\times 40$). D, Leukocyte counts of cerebrospinal fluid (CSF) of untreated rats (black bar), rats treated iv with C1-inh (gray bar), and rats treated itl with C1-inh (white bar) at 48 h after intrathecal inoculation with $5 \times 10^6$ cfu of *S. pneumoniae*. Data are means ± SEs of 8 rats/group. $P$ values are for the comparison with untreated rats.

developed meningitis after IT inoculation but also diminished outgrowth of pneumococci in CSF in mice with meningitis. No significant difference was found in bacterial counts in blood ($2.4 \pm 0.9 \times 10^4$ cfu/mL in untreated mice vs. $2.9 \pm 2.6 \times 10^4$ cfu/mL in mice treated with C1-inh [not significant]). In addition, mice treated with C1-inh displayed a less pronounced inflammatory infiltrate around the meninges than did untreated mice (data not shown). Leukocyte counts in CSF were lower in mice treated with C1-inh than in untreated mice; however, the difference did not reach statistical significance ($4.3 \pm 1.5 \times 10^4$ cells/mL in untreated mice vs. $3.3 \pm 2.1 \times 10^4$ cells/mL in mice treated with C1-inh). All measured cytokines were less up-regulated in mice treated with C1-inh than in untreated mice except TNF-α; however, the difference in IL-1β concentrations did not reach statistical significance (table 2).

DISCUSSION

The principal finding of this study is that treatment of pneumococcal meningitis with C1-inh improves the course and outcome of the disease in rats and mice. We used a murine model, in which the strength lies in the resemblance to the route of infection in humans, as well as a rat model, to be able to evaluate considerable amounts of CSF. Moreover, models using direct inoculation into the CSF, as in our rat model, are considered by some investigators [1] to be more appropriate to study the characteristics of meningeal inflammation during meningitis, because the systemic immune response may be more pertinent than CNS inflammation to the outcome in models in which meningitis develops after bacteremia. In both species, using different models of meningitis, treatment with
Figure 4. Increased expression of CR3 during meningitis in rats treated with a C1 inhibitor (C1-inh). Shown are representative slides of CR3 staining with the monoclonal antibody ED8 in brain parenchyma and meninges of a control rat (injected with saline; A), a rat inoculated with *Streptococcus pneumoniae* (B), a rat inoculated with *S. pneumoniae* and treated intravenously with C1-inh (C), and a rat inoculated with *S. pneumoniae* and treated intrathecally with C1-inh (D). Brain tissue was collected 48 h after inoculation. Original magnification, ×40.

C1-inh reduced the outgrowth of pneumococci in CSF and the associated inflammatory response in the brain. The confirmation of these findings in 2 different animal models strengthens the conclusions about the potential beneficial effects of C1-inh treatment.

The BBB is a unique functional part of the vasculature within the CNS that contributes to the maintenance of homeostasis. Thus, attempts to interfere with the local inflammatory reactions in the CNS may fail after systemic administration because of the impermeability of the BBB. We have demonstrated that C1-inh antigen and activity are present in the CSF after iv administration, despite its molecular weight of ∼105 kDa [15]. Because the assays used for these measurements are specific for human C1-inh, it is unlikely that local synthesis of endogenous C1-inh accounted for this increase in CSF levels. Local intracerebral intervention in the host response by systemically administered C1-inh seems feasible during the acute phase of this disease, presumably because of the enhanced permeability of the BBB, which is an important characteristic of bacterial meningitis.

Our data show that, although measured C1-inh levels are lower after itl treatment than after iv treatment, the effects of itl treatment are more pronounced. Because C1-inh concentrations were measured only at 48 h after administration, we postulate that the discrepancy between measured C1-inh levels and the associated effects is due to higher C1-inh levels in rats treated itl during an earlier phase of the infection. Consumption of C1-inh may explain a rapid decrease in local C1-inh levels.

Surprisingly, C1-inh treatment was associated with enhanced clearance of pneumococci in CSF and blood. Numerous studies have shown that complement contributes significantly to bacterial clearance by opsonization, chemotaxis, and lysis of cells [31], and the crucial role of complement in these actions is apparent in the striking susceptibility to bacterial infections of individuals who are genetically deficient in complement components [32]. Rabbits treated with cobra venom factor, which causes a continuous consumption of complement components, thereby depleting complement activity, demonstrated enhanced growth of pneumococci in CSF, because of their lack of opsonization [33]. However, complement activation may play a dual role in bacterial killing, given that impaired bacterial clearance after activation of the complement system has been reported elsewhere [34]. Immunohistochemical analysis showed an up-regulation of complement receptor 3 (CR3) in rats treated with C1-inh. The explanation for this finding is unclear at present.

However, CR3 is a multifunctional membrane protein that serves as both a membrane receptor that mediates phagocytosis and an adhesion molecule. CR3 binds with high avidity to opsonizing iC3b attached to microorganisms, but microorganisms can also directly interact with macrophage CR3, promoting phagocytosis in a C3-independent fashion [35, 36]. To
In addition to its antiinflammatory activities mediated via the inhibition of complement and contact system proteases, plasma C1-inh also plays a direct role in the modulation of leukocyte adhesion during inflammation. A direct role of C1-inh in leukocyte–endothelial cell adhesion has been recently demonstrated [16, 43] that is independent of protease inhibitory activity. In our models, C1-inh may interfere directly with selectin-mediated leukocyte adhesion, thereby hampering leukocyte migration.

Cytokine measurements in brain tissue revealed lower levels of proinflammatory cytokines and chemokines in mice injected with C1-inh. Previous studies have also demonstrated lower levels of proinflammatory cytokines and chemokines after C1-inh treatment; in a baboon model of Escherichia coli sepsis, C1-inh treatment was associated with diminished plasma concentrations of TNF-α, IL-6, IL-8, and IL-10 [44]. The attenuated up-regulation of these inflammatory mediators is in line with the diminished pleocytosis and lower bacterial counts but might also be due to a direct effect on the activation of leukocytes. Both in vivo and in vitro studies have demonstrated a key role for C5a in the generation of CXC and CC chemokines [45] and proinflammatory cytokines [46, 47] by macrophages and monocytes. Furthermore, iC3b and C3b have been shown to induce IL-1 synthesis or synthesis and secretion, respectively, in monocytes [48, 49], and C3a has been demonstrated to be capable of modulating monocyte IL-6 synthesis [50].

In conclusion, C1-inh treatment of experimental pneumococcal meningitis in rats resulted in reduced clinical illness, increased bacterial clearance, and less-severe inflammation, as reflected by decreased CSF pleocytosis and less-severe inflammatory infiltrate around the meninges. In a murine model, the favorable effect of C1-inh on antibacterial defense and brain inflammation was confirmed, and lower levels of proinflammatory cytokines and chemokines were observed. As such, C1-

![Figure 5. Diminished bacterial outgrowth in mice with meningitis after C1 inhibitor (C1-inh) treatment. Shown is bacterial outgrowth in cerebrospinal fluid of untreated mice (black bars) and mice treated intraperitoneally with C1-inh (white bars) at 72 h after intranasal inoculation with 8 × 10^8 cfu of Streptococcus pneumoniae and 180 U of hyaluronidase. Data are means ± SEs of 10–16 mice/group. The P value is for the comparison with untreated mice.](https://academic.oup.com/jid/article-abstract/196/1/115/842097)
inh may have promise as a useful clinical agent in the treatment of pneumococcal meningitis.

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References


