Protective Role of Complement C5a in an Experimental Model of *Staphylococcus aureus* Bacteremia

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Key Words

Bacteremia · Complement C5a · Infection, susceptibility ·
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Abstract

The complement system is a key component of the innate immune response. Here, we have examined the role of complement anaphylatoxin C5a in an experimental model of *Staphylococcus aureus* bacteremia. Our data provide compelling evidence for a protective role of C5a during staphylococcal bloodstream infection.

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Introduction

Staphylococcus aureus is an important human pathogen that causes a wide spectrum of clinical manifestations, ranging from superficial cutaneous infections to life-threatening systemic diseases. The impact of *S. aureus* infection on human health has dramatically increased as a result of its remarkable ability to become resistant to antibiotics [1]. Given the increasing number and severity of *S. aureus* infections worldwide [2], it is important to understand the pathophysiology of these infections.

The complement system is a fundamental component of the immune response to infection with many pathogens [3]. Activation of complement by microbes proceeds through a sequence of proteolytic activation events, that results in the formation of bacterium-bound opsonins and the anaphylatoxins C3a, C4a and C5a [3, 4]. C5a is regarded as the most potent chemoattractant for neutrophils and monocytes, contributing to a rapid mobilization of phagocytic cells to the site of infection to promote pathogen clearance [5]. In addition, C5a stimulates oxidative metabolism, enhances expression of adhesion molecules in neutrophils, and activates the coagulation pathway [4].

In the context of *S. aureus* infections, mice deficient in the production of complement C5 have been reported to have decreased pulmonary clearance of *S. aureus* after aerosol infection [6]. In addition, it has been shown that deletion of C5aR, a receptor for C5a, predisposed mice to colonization with staphylococci in the lungs [7]. However, little is known about the role of C5 during bloodstream infections, one of the most life-threatening diseases caused by *S. aureus* [8]. The present study was designed to evaluate the role of complement factor C5 and its cleavage product C5a for host defense during staphylococcal bacteremia using an experimental murine model of infection.

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Methods

Mice

Inbred, pathogen-free, 8- to 12-week-old C57BL/6 JolaHsd, DBA/2 OlaHsd, and B6D2F1/OlaHsd-F1-hybrids (generated after crossing female C57BL/6 with male DBA/2 mice) were purchased from Harlan Winkelmann (Borchen, Germany). Congenic C5-deficient B10.D2-Hc0 H2d/SnJ and the corresponding C5-sufficient B10.D2-Hc1 H2d/SnJ mice were purchased from Jackson Laboratory (Bar Harbor, Maine, USA). All experiments were approved by the appropriate national ethical board (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, Oldenburg, Germany).

Infection Model

Mice were inoculated intravenously with 4×10^7 CFU of *S. aureus* SH1000 in 0.2 ml PBS, as described previously [9]. For determination of bacterial loads, infected mice were sacrificed by CO₂ asphyxiation, their kidneys removed, homogenized in PBS and bacterial colonies were enumerated after plating serial dilutions onto blood agar.

Serum Adoptive Transfer

DBA/2 mice were intravenously injected (200 μ l/mouse) 30 min prior to infection with serum from C57BL/6 mice that was either left untreated (complement-sufficient serum) or heat-inactivated at 60°C for 30 min (complement-deficient serum).

Treatment with Recombinant C5a (rC5a)

DBA/2 mice were injected intravenously with 1 dose (2 μ g) of murine rC5a (R&D Systems, Wiesbaden, Germany; diluted in 200 μ l PBS + 0.1% BSA) 30 min prior to infection. Control mice were injected intravenously with 200 μ l of PBS + 0.1% BSA. In some experiments, DBA/2 mice received 2 additional doses of 2 μ g rC5a given intraperitoneally at 6 and 12 h following bacterial inoculation.

Determination of C5a Serum Concentration

Serum C5a levels were determined by ELISA. Immuno-Maxi-Sorb 96-well microtiter plates (Nunc, Wiesbaden, Germany) were coated overnight at 4°C with 50 μ l of 4 μ g/ml purified rat anti-mouse C5a IgG (BD Pharmingen, San Diego, Calif., USA). Wells were blocked with 1% skim milk in PBS containing 0.05% Tween-20 (Merck, Darmstadt, Germany) and incubated for 2 h at room temperature. After 3 washes with PBS, serum samples or recombinant mouse C5a (R&D Systems, Minneapolis, Minn., USA) at concentrations of 400 ng/ml to 12.5 ng/ml (50 μ l/well diluted in PBS containing 0.1% skim milk and 0.05% TWEEN-20) were added and incubated at 37°C for 2 h. After 3 washes, 50 μ l biotinylated rat anti-mouse C5a antibody (BD Pharmingen, San Diego, Calif., USA) was added at 2 μ g/ml for 2 h at 37°C, followed by 3 washing steps and 1 h incubation with 1:5,000 diluted streptavidin-peroxidase (Acris, Hiddenhausen, Germany). After 3 final washes, plates were colorimetrically developed with *o*-phenylenediamine dihydrochloride (Sigma-Aldrich, Taufkirchen, Germany) as described in the manufacturer's instructions, and reaction stopped with 0.5 M sulfuric acid, prior to measuring the absorbance at 490 nm. All samples were analyzed in duplicate and results calculated from the means of duplicate sample analysis.

Histopathological Examination

Kidneys of mice were fixed in 10% buffered neutral formalin solution, embedded in paraffin, and then cut into 3- μ m-thick sections. Sections were stained with hematoxylin and eosin and analyzed using an Olympus Bx51 microscope.

Statistics

Statistical analysis was performed using GraphPad Prism 4.0. All data are presented as mean $\pm$ SD. Comparison between 2 groups was made by unpaired t test. Comparison of survival curves was performed by use of Log-rank (Mantel Cox) test (minimum $n = 9$ mice). p values of ≤ 0.05 were considered significant.

Results and Discussion

We have recently shown that different mouse strains vary in their degree of susceptibility to intravenously inoculated *S. aureus* [9]. Susceptible mice develop severe infection associated with high bacterial loads in kidneys and 100% mortality within 1–3 days after intravenous inoculation with 4×10^7 CFU of *S. aureus* strain SH1000. Interestingly, the most susceptible strains (A/J and DBA/2) have a genetic deficiency that results in the impaired production of complement C5 [10]. This is of particular interest, since human individuals deficient in complement C5 have been shown to be strongly predisposed to recurrent infections caused by *S. aureus* [11]. It has also been reported that individuals heterozygous for C5 deficiency exhibit half-normal levels of C5a, but are nevertheless healthy individuals with no notable infectious episodes [12]. To determine whether this was also the case in the murine system, we compared the serum levels of the complement C5 degradation product, C5a, in F1 (C5^{+/-}) progeny resulting from crossing C57BL/6 (C5^{+/+}) with C5-deficient DBA/2 (C5^{-/-}) mice at 24 h after bacterial inoculation. Similar to humans, the levels of C5a in infected F1 (C5^{+/-}) mice were significantly lower than those measured in C57BL/6 (C5^{+/+}) animals (fig. 1a). C5a in serum of infected DBA/2 (C5^{-/-}) mice were below detection levels (fig. 1a). Furthermore, F1 (C5^{+/-}) mice were significantly more resistant to *S. aureus* infection than DBA/2 (C5^{-/-}) mice, as shown by a better capacity to control bacterial growth in the kidneys (fig. 1b) and improved survival (fig. 1c). These results indicate that the generation of C5, at least to half-normal levels, may have an impact on the resistance of mice to staphylococcal bloodstream infection.

To confirm this assumption, we determined whether treatment of DBA/2 (C5^{-/-}) mice with serum from a C5-sufficient strain (C57BL/6) could enhance their resis-

Fig. 1. Differential susceptibility of C57BL/6 (C5^{+/+}) mice, DBA/2 (C5^{-/-}) mice and their F1 (C5^{+/-}) progeny to *S. aureus* bloodstream infection. Levels of C5a in serum (a) and bacterial loads in kidneys (b) of C57BL/6 (C5^{+/+}) (black bar), DBA/2 (C5^{-/-}) (white bar), and F1 (grey bar) mice at 24 h after intravenous inoculation with *S. aureus*. Each bar represents the mean $\pm$ SD of determination of a minimum of 3 (a) or 5 (b) mice/group. c Survival curves of C57BL/6 (C5^{+/+}), DBA/2 (C5^{-/-}), and F1 (C5^{+/-}) mice after intravenous inoculation with 4×10^7 CFU of *S. aureus*. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

tance to *S. aureus* infection. For this purpose, DBA/2 (C5^{-/-}) mice were adoptive transferred with complement-sufficient (normal serum obtained from C57BL/6 mice) or complement-deficient (heat-inactivated serum obtained from C57BL/6 mice) serum prior to infection with *S. aureus*. Our results show that DBA/2 mice receiving complement-sufficient serum survived significantly longer (fig. 2a) and exhibited significant lower bacterial loads in the kidneys at 24 h of infection (fig. 2b) than DBA/2 mice either untreated or treated with complement-deficient serum. In good correlation to these data, examination of kidney tissue sections obtained from *S. aureus*-infected mice at 24 h after bacteria inoculation revealed a diffuse and confluent inflammation with extensive areas of tissue destruction containing abundant bacteria in the kidneys of infected DBA/2 mice receiving complement-deficient serum (fig. 2c, arrow on panel ii). In contrast, no signs of histological tissue damage were detected in the kidney sections of mice receiving comple-

ment-sufficient serum (fig. 2c, panel iii). Therefore, adoptive transfer of complement C5 from C57BL/6 serum resulted in delayed development of pathology and enhanced the resistance of DBA/2 (C5^{-/-}) mice to *S. aureus* infection.

The activation of C5 during infection results in the generation of the degradation products C5a and C5b [3–5]. The anaphylatoxin C5a is a potent chemoattractant for phagocytic cells and exerts an immunoregulatory role during inflammation [3–5], while C5b is involved in the formation of the membrane attack complex (MAC) consisting of C5b, C6, C7, C8 and C9. As the activity of MAC is largely limited to Gram-negative bacteria [3], we hypothesized that the protective role of C5 during *S. aureus* bacteremia is entirely mediated by C5a. Indeed, complement C6-deficient (C6^{-/-}) C57BL/6 mice, which are incapable of forming MAC, were as resistant to *S. aureus* as wild type mice (online suppl. fig. S1, available at www.karger.com/doi/10.1159/000247157).

Fig. 2. Effect of treatment with complement-sufficient serum, complement-deficient serum or rC5a in the response of DBA/5 ($C5^{-/-}$) mice to intravenous *S. aureus* infection. **a** Survival of either untreated DBA/2 ($C5^{-/-}$) or DBA/2 ($C5^{-/-}$) mice after passive transfer with 200 μ l of either complement-sufficient or complement-deficient serum after intravenous infection with *S. aureus*. **b** Bacterial loads in kidneys of the above-mentioned mice at 24 h of infection (minimum $n = 6$ mice). **c** Histological examination of kidney tissue obtained from either uninfected DBA/2 ($C5^{-/-}$) mice (panel i) or *S. aureus*-infected DBA/2 ($C5^{-/-}$) animals treated with either complement-deficient (panel ii) or complement-sufficient (panel iii) serum. The black arrow indicates abscess formation containing abundant bacteria. **d** Bacterial loads in kidneys of DBA/2 ($C5^{-/-}$) mice treated with either 1 dose of rC5a 30 min prior to bacterial inoculation ($n = 14$), or 3 consecutive doses of rC5a (30 min prior to and 6 and 12 h after bacterial inoculation; $n = 4$) at 24 h after intravenous infection with *S. aureus*. Each bar represents the mean $\pm$ SD. ** $p < 0.01$; *** $p < 0.001$.

To provide direct evidence for a protective role of C5a during *S. aureus* bacteremia, DBA/2 ($C5^{-/-}$) mice were therapeutically treated with 1 or 3 doses of rC5a, and the bacterial loads in kidneys were determined 24 h after intravenous bacterial inoculation. As shown in figure 2d,

treatment with rC5a significantly improved control of bacterial growth in a dose-dependent manner.

Finally, as C56BL/6 and DBA/2 mice are genetically very heterogeneous, we used congenic B10.D2-Hc0 ($C5^{-/-}$) mice, which differ from B10.D2-Hc1 ($C5^{+/+}$) con-

Fig. 3. Response of congenic B10.D2-Hc0 (C5^{-/-}) and the corresponding B10.D2-Hc1 (C5^{+/+}) mice to intravenously inoculated *S. aureus*. **a** Survival of B10.D2-Hc0 and B10.D2-Hc1 after intravenous inoculation with 4×10^7 CFU of *S. aureus*. **b** Bacterial loads in kidneys of those mice at 24 h after bacterial inoculation. Each bar represents the mean $\pm$ SD of a minimum of 5 mice/group. ** $p < 0.01$.

trol animals at a small region of the genome containing a mutant allele at the C5 locus, to confirm the specific role of C5 in host response to *S. aureus* blood infection. Animals from both strains were intravenously inoculated with 4×10^7 CFU of *S. aureus* and lethality was recorded for a period of 14 days. All B10.D2-Hc0 (C5^{-/-}) mice succumbed by the third day after challenge, compared to 30% of the B10.D2-Hc1 (C5^{+/+}) animals, of which 40% were still alive at day 14 (fig. 3a). In conjunction with these data, the B10.D2-Hc0 (C5^{-/-}) mice were less capable than B10.D2-Hc1 (C5^{+/+}) animals of controlling bacterial growth, as shown by the significantly higher amount of *S. aureus* in the kidneys at 24 h of infection (fig. 3b). These results confirm that C5 per se exerts a major impact in host defense in this experimental model of *S. aureus* bacteremia. However, the observation that B10.D2-Hc1 (C5^{+/+}) mice are more susceptible to *S. aureus* than C57BL/6 (C5^{+/+}) animals also suggests the presence of additional genetic determinants in the B10.D2 background involved in its susceptibility to this pathogen.

Conclusions

Taken together, these data provide compelling evidence for a protective role of C5a during *S. aureus* bacteremia. C5 deficiency impairs host resistance to blood-borne *S. aureus*, resulting in uncontrolled bacterial growth in the kidneys, concomitant tissue damage and

death. The pathological effects of C5 deficiency can be restored by adoptive transfer of C5-sufficient serum or by exogenous administration of rC5a. These findings may be of high relevance for the development of new therapeutic agents against staphylococcal bloodstream infections.

Interestingly, blockade of C5a has been proposed as an attractive strategy for the treatment of sepsis in humans [13–15], when excessive production of C5a contributes to the immunopathogenesis (severity) of sepsis and septic shock by amplifying the inflammatory cascade, resulting in neutrophil dysfunction and vascular damage [16]. However, all these studies have been performed using experimental models of Gram-negative or polymicrobial sepsis (dominated by Gram-negative micro-organisms). Given that the frequency of Gram-positive bacterial infections such as *S. aureus* bloodstream infections as a cause of sepsis is increasing [17], it is evident that the risk-benefit relation of any new treatment strategy (e.g. blockade of C5a) should be carefully assessed for both Gram-positive and Gram-negative septicemias.

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