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Contribution of the complement Membrane Attack Complex to the bactericidal activity of human serum

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ABSTRACT

Direct killing of Gram-negative bacteria by serum is usually attributed to the Membrane Attack Complex (MAC) that is assembled upon activation of the complement system. In serum bactericidal assays, the activity of the MAC is usually blocked by a relatively unspecific method in which certain heat-labile complement components are inactivated at 56 °C. The goal of this study was to re-evaluate MAC-driven lysis towards various Gram-negative bacteria. Instead of using heat-treatment, we included the highly specific C5 cleavage inhibitor OmCI to specifically block the formation of the MAC. Using a C5 conversion analysis tool, we monitored the efficacy of the inhibitor during the incubations. Our findings indicate that 'serum-sensitive' bacteria are not necessarily killed by the MAC. Other heat-labile serum factors can contribute to serum bactericidal activity. These unidentified factors are most potent at serum concentrations of 10% and higher. Furthermore, we also find that some bacteria can be killed by the MAC at a slower rate. Our data demonstrate the requirement for the use of specific inhibitors in serum bactericidal assays and revealed that the classification of serum-sensitive and resistant strains needs re-evaluation. Moreover, it is important to determine bacterial viability at multiple time intervals to differentiate serum susceptibility between bacterial species. In conclusion, these data provide new insights into bacterial killing by the humoral immune system and may guide future vaccine development studies for the treatment of pathogenic serum-resistant bacteria.

1. Introduction

Mammalian immune protection against Gram-negative bacteria depends on the bactericidal action of the Membrane Attack Complex (MAC), a ring-structured pore-forming complex that disintegrates bacterial membranes (Joiner et al., 1984; Taylor, 1983). The MAC is the final activation product of the complement cascade, a family of serum proteins that circulate in the blood as inactive precursors (Walport, 2001). Upon contact with bacteria, complement proteins organize into a proteolytic cascade that functions to label bacteria for phagocytosis, release anaphylatoxins and directly kill the target cell (Ricklin et al., 2010; Walport, 2001). MAC formation is initiated when the C5 convertase cleaves C5 into the anaphylatoxin C5a and C5b. C5b binds C6 and C7, exposing a hydrophobic site in C7 allowing binding of C5b67 to the bacterial membrane. Subsequent attachment of C8 induces binding and polymerization of 12-18 C9 molecules to form the membrane-perturbing structure known as the MAC (Frank et al., 1987; Müller-Eberhard, 1986). This complex can effectively kill Gram-negative bacteria whereas Gram-positive bacteria are resistant to MAC killing.

The importance of the MAC in host defense against Gram-negatives is reflected by recurrent infections in patients with deficiencies of the MAC proteins (Skattum et al., 2011) and by paroxysmal nocturnal hemoglobinuria (PNH) patients that are treated with Eculizumab, a humanized antibody directed towards C5 thereby inhibiting C5 proteolysis and MAC formation. Eculizumab treatment is effective but leads to an increased risk of infections with *Neisseria meningitidis* and therefore patients need to be vaccinated prior to treatment (Mckeage, 2011). Moreover, the non-lytic MAC (terminal complement complex, TCC, or C5b-9 complex) was found to have additional roles in host defense as an inflammatory mediator. C5b-9 complexes can bind to endothelial cells which stimulates a pro-inflammatory status (Tedesco et al., 1997) and these complexes can induce neutrophil chemotaxis (Dobrina et al., 2002).

Serum bactericidal assays are widely used to determine the sensitivity of micro-organisms towards the complement system or to assess the efficacy of serum antibodies in vaccine development

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Table 1
Bacterial strains and growth conditions NB, nutrient broth; NA, nutrient agar; LB, lysogeny broth; LA, lysogeny agar; TSB, tryptic soy broth; TSA, tryptic soy agar; THB, Todd Hewitt broth; THA, Todd Hewitt agar.

Strain	Growth media	Culture conditions
B. cepacia ATCC 25417	NB/NA	37 °C, aerobic
E. cloacae IVC106	NB/NA	37 °C, aerobic
E. coli BL21	LB/LA	37 °C, aerobic
E. coli MG1655	LB/LA	37 °C, aerobic
K. pneumoniae 13-591402-02	NB/NA	37 °C, aerobic
N. meningitidis Hb-1	TSB/GC agar + vitox	37 °C, anaerobic
P. mirabilis ATCC 43071	TSB/TSA	37 °C, aerobic
P. aeruginosa PA01	THB/THA	37 °C, aerobic

against Gram-negative bacteria (Granoff, 2009). Bactericidal activity of the serum can be easily determined *in vitro* by mixing bacterial cells with human serum. Bacterial killing is analyzed after 30–60 min by enumeration of viable bacterial cells. Bacteria that are killed within this time period are designated to be 'serum-sensitive' while resistant bacteria are called 'serum-resistant'. In order to discriminate the activity of the MAC from other serum components, the complement system is blocked by pre-treatment of serum at 56 °C for 30 min. This ensures that heat-labile factors C2 and factor B (FB) are inactivated (Joisel et al., 1983), and that complement activation is prevented at a very early stage in the cascade. Although heat is routinely used to assess the contribution of complement to bacterial killing, we questioned whether heat-treatment of serum only inactivates complement or also other bactericidal serum components. We here performed systematic analyses to re-evaluate the contribution of the MAC in the bactericidal activity of human serum towards various Gram-negative bacteria. We show that heat-treatment of serum is unreliable for specific evaluation of the MAC. Using the complement C5 inhibitor OmCI (Coversin) (Nunn et al., 2005), we find that several clinically relevant Gram-negatives are killed by serum components other than the MAC. Furthermore, we demonstrate that some 'serum-resistant' bacteria can still be killed by the MAC but at a slower rate.

2. Materials and methods

2.1. Sera, plasma, and proteins

Human serum was derived from 20 healthy volunteers, pooled, and prepared as described (Berends et al., 2013). Heat inactivated serum (30 min incubation in 56 °C water bath) or serum containing 20 μg/ml OmCI were used where indicated. The OmCI used in this study refers to pOmCI, a double mutant (N78Q/N102Q) expressed in *Pichia methanolica*. The mutations have no effect on the activity of OmCI. The mutant was constructed because native OmCI purified from tick salivary glands is not glycosylated and the conservative double mutations in pOmCI prevent glycosylation of the protein expressed in yeast. pOmCI was expressed and purified to homogeneity as described (Hepburn et al., 2007). Plasma was isolated from the blood of one healthy volunteer. Blood was anti-coagulated with 50 μg/ml lepirudin and centrifuged for 10 min, 2700 × *g* at 4 °C after which the plasma was collected from the supernatant. C3a was obtained from Complement Technology, Inc. Lysozyme, apo-, and holo-tranferrin were obtained from Sigma.

2.2. Bactericidal assays

Bacteria were grown to mid-log phase ($OD_{660} \sim 0.5$) from an overnight culture in conventional growth media as described in Table 1. Bacteria were diluted in sterile RPMI (Gibco) supplemented with 0.05% human serum albumin (HSA, Sanquin) to a concentration of $\sim 1 \times 10^4$ cfu/ml. Incubation of bacteria was performed with 50% serum or plasma, or lower concentrations as indicated, in sterile round-bottom 96-wells plates (Greiner) under shaking conditions for 1 h and 3 h. After incubation, samples were serially diluted in PBS and plated onto nutrient agar (NA) (*Klebsiella pneumoniae*, *Burkholderia cepacia*, *Enterobacter cloacae*), tryptic soy agar (TSA) (*Proteus mirabilis*), lysogeny agar (LA) (*Escherichia coli*), Todd Hewitt agar (THA) (*Pseudomonas aeruginosa*), or GC vitox agar (*N. meningitidis*) plates for enumeration of colony forming units (cfu) after overnight incubation. Serum bactericidal assays were performed in duplicate.

2.3. C5a analyses

Supernatants of the bactericidal assay were isolated after centrifugation and analyzed for the presence of C5a by a calcium mobilization assay as described previously (Berends et al., 2013). In short, U937 cells transfected with the C5a receptor (U937-C5aR) (Kew et al., 1997), were labeled with 2 μM Fluo-3-AM (Life technologies). Calcium mobilization was analyzed by measuring fluorescence 10 s before and 70 s after addition of the supernatants by flow cytometry (FACSVerse).

3. Results

3.1. C5 cleavage analysis in bactericidal assays

To get better insight into the generation of the MAC during bactericidal assays, we first developed a method that allows sensitive measurement of C5 cleavage. Complement activation on bacterial surfaces leads to formation of C5 convertase enzymes that cleave C5 into the small anaphylatoxin C5a (Gerard and Gerard, 1994) and the larger fragment C5b that initiates assembly of the bacteriolytic MAC (Müller-Eberhard, 1986). Thus, C5a is released into the fluid phase at the same time as the MAC assembles into bacterial membranes. We started by analyzing the amount of surviving bacteria after serum incubation of two model Gram-negative strains; one serum-sensitive strain, *E. coli* MG1655, and a strain that was described to be serum-resistant, *P. aeruginosa* PA01 (Wretlind et al., 1985). Since bacteria in their logarithmic growth phase represent a universal population and are in general more susceptible to the bactericidal effect of serum (Davis and Wedgwood, 1965; Rowley and Wardlaw, 1958), we performed all bactericidal analyses with log-phase bacteria. As expected, we found that the *E. coli* strain was directly killed by the lowest concentration of serum after 1 h (Fig. 1A), and that the *P. aeruginosa* strain was resistant towards 1 h exposure to serum (Fig. 1A). Killing of bacteria was verified by microscopy analysis of bacterial morphology, to confirm that the lack of cfu was not due to bacteria sticking to the plastic or clumping (data not shown). In parallel, we analyzed the supernatants of serum-incubated *E. coli* and *P. aeruginosa* for the presence of C5a by a calcium mobilization assay using U937-C5aR cells (Kew et al., 1997). This method is highly sensitive and more specific for C5a than commercially available C5a ELISA-kits; C3a and uncleaved C5 do not elicit any calcium mobilization signal (Fig. S1). We found that the amount of released C5a increases dose-dependently with increasing percentages of serum. Moreover, at longer incubation times, more C5a is generated. Heat inactivation of the serum at 56 °C abrogates this effect (Fig. 1B). Thus, we have developed a sensitive method to analyze C5 cleavage in bactericidal serum assays allowing us to specifically evaluate the role of the MAC.

3.2. OmCI inhibits C5 conversion in serum bactericidal assays

Then, we studied whether there are more specific methods to block MAC formation in serum that can be used as an alternative for heat inactivation. Heat treatment of serum at 56 °C is a