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Neisseria meningitidis and *Escherichia coli* are protected from leukocyte phagocytosis by binding to erythrocyte complement receptor 1 in human blood☆

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ABSTRACT

The initial interaction of Gram-negative bacteria with erythrocytes and its implications on leukocyte phagocytosis and oxidative burst in human whole blood were examined. Alexa-labeled *Escherichia coli*, wild-type H44/76 *N. meningitidis* and the H44/76*lpxA* lipopolysaccharide (LPS)-deficient mutant were incubated with whole blood using lepirudin as anticoagulant which has no adverse effects on complement. Bacteria free in plasma, bound to erythrocytes or phagocytized by granulocytes and monocytes were quantified using flow cytometry. The effects of the C3 inhibitor compstatin, a C5a receptor antagonist (C5aRa) and a complement receptor 1 (CR1)-blocking antibody (3D9) were examined. Most bacteria (80%) immediately bound to erythrocytes. The binding gradually declined over time, with a parallel increase in phagocytosis. Complement inhibition with compstatin reduced erythrocyte binding and bacterial C3 opsonization. In contrast, the C5aRa efficiently reduced phagocytosis, but did not affect the binding of bacteria to erythrocytes. The anti-CR1 blocking mAb dose-dependently reduced bacterial binding to erythrocytes to nil, with subsequent increased phagocytosis and oxidative burst. LPS had no effect on these processes since similar results were obtained using an LPS-deficient *N. meningitidis* mutant. *In vivo* experiments in a pig model of sepsis showed limited binding of bacteria to erythrocytes, consistent with the facts that erythrocyte CR1 receptors are absent in non-primates and that the bacteria were mainly found in the lungs. In conclusion, complement-dependent binding of Gram-negative bacteria to erythrocyte CR1 decreases phagocytosis and oxidative burst by leukocytes in human whole blood.

1. Introduction

E. coli and *N. meningitidis* are important Gram-negative pathogens causing sepsis (Martin et al., 2003; Stephens et al., 2007). These bacteria activate complex inflammatory pathways, involving both the innate and the adaptive immune systems (Castellheim et al., 2009). Complement activation is a key feature important for activating the defense mechanisms opsonophagocytosis (Castellheim et al., 2009; Mollnes et al., 2002) and serum bactericidal activity. It also exerts potent inflammatory effects in sepsis through the release of anaphylatoxins, including C5a (Ward, 2004), and excessive complement activation in meningococcal diseases is related to disease severity (Brandtzaeg et al., 1989). *N. meningitidis* activates complement mainly through the alternative and lectin pathways, whereas the classical pathway is only slightly activated (Sprong et al., 2003). In contrast, *E. coli* mainly activates the alternative pathway (Mollnes et al., 2002). The opsonization of the bacterial surface with complement components, such as C1q, C3 and C4, are important for bacterial recognition by the

Abbreviations: C5aR, C5a receptor; C5aRa, C5a receptor antagonist; CR1, complement receptor 1; IC, immune complex; MBL, mannose-binding lectin.

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immune system (Castellheim et al., 2009). In addition, ficolins (Matsushita and Fujita, 2002), mannose-binding lectin (MBL) (Jack et al., 2005), properdin (Hourcade, 2006) and Igs may function as opsonins. The complement-opsonized bacteria are recognized by the immune system and binding to specific receptors such as complement receptor 1 (CR1) occurs (Birmingham and Hebert, 2001). CR3 or CD11b/CD18 is important in the phagocytosis (Mollnes et al., 2002) of bacteria by blood leukocytes. In the fluid phase, the anaphylatoxin C5a is released and binds to specific receptors on various cells, such as granulocytes, monocytes and endothelial cells (Lee et al., 2008). Interestingly, the inhibition of the anaphylatoxin C5a or its receptors has been reported to greatly enhance the survival of sepsis in animal models (Parrish et al., 2008; Ward, 2004). The C3 convertase inhibitor compstatin was also recently shown to decrease *E. coli*-induced coagulation activation in a sepsis model in baboons (Silasi-Mansat et al., 2010). These observations indicate that complement inhibitors are potential therapeutic agents for sepsis.

Erythrocytes are generally viewed as simple hemoglobin carriers involved in oxygen transport in humans. However, in 1930, using serum-opsonized trypanosomes, it was first observed that erythrocytes may bind microorganisms and play a role in the pathogenesis of bloodstream infections (Duke and Wallace, 1930). Later, Nelson demonstrated that opsonized particles and Gram-positive pneumococci bind to erythrocytes in a complement-dependent manner (Nelson, Jr., 1953). Subsequently, erythrocyte CR1 was identified as the high-affinity binding site of C3b, although it can also bind iC3b, C4b, iC4b (Cooper, 1969), C1q and MBL with lower affinity (Ghiran et al., 2000), linking complement-coated bacteria to erythrocytes (Birmingham and Hebert, 2001). CR1 is a large transmembrane glycoprotein, consisting of several homologous motifs (Birmingham and Hebert, 2001). It is expressed in varying numbers from approximately 100 to 1000 per human erythrocyte (Birmingham and Hebert, 2001) and distributed in clusters on the erythrocyte membrane after ligation (Ghiran et al., 2008; Paccaud et al., 1988). CR1 is also found on human monocytes, granulocytes (Ross et al., 1978) and B lymphocytes (Birmingham and Hebert, 2001). Immune complexes (ICs) opsonized with C3b and C4b bind erythrocyte CR1; they can then be cleared from the circulation and destroyed in the liver and spleen (Cornacoff et al., 1983; Schifferli et al., 1988). Another interesting feature of CR1 is its ability to inhibit complement activation by functioning as a cofactor of factor I that cleaves C3b and C4b into inactive forms (Iida and Nussenzweig, 1981). Through binding C3b and C4b, CR1 also accelerates the decay of the alternative (Fearon, 1979) and classical pathway C3 convertases (Iida and Nussenzweig, 1981). Many studies have examined the binding of ICs to erythrocyte CR1 and its involvement in IC clearance (Birmingham and Hebert, 2001). However, the roles of complement in the binding of different Gram-negative bacteria to erythrocytes and in leukocyte phagocytosis in human whole blood using lepirudin as an anticoagulant, have not been studied.

In the present study, we therefore examined the interaction of *E. coli* and *N. meningitidis* with erythrocytes and how the interaction affects phagocytosis in a human whole-blood model. The roles of membrane lipopolysaccharide (LPS) and bacterial opsonization in the initial binding of *E. coli*, wild-type *N. meningitidis* H44/76 with LPS and the LPS-deficient H44/76*lpxA* mutant to erythrocyte CR1 were examined. The specific thrombin inhibitor lepirudin was used as anticoagulant because it does not affect complement activation, in contrast to calcium-binding anticoagulants and heparin (Mollnes et al., 2002). Our data shed new light on the interaction of Gram-negative bacteria with various blood cells and indicate that initial binding of the bacteria to erythrocytes reduces phagocytosis and oxidative burst by leukocytes in human whole blood.

2. Materials and methods

2.1. Equipment and reagents

All equipment, including polypropylene tubes (Nalgene NUNC, Roskilde, Denmark) and tips used in the whole-blood experiments, was endotoxin-free. Phosphate buffered saline (PBS) with or without Ca^{2+} and Mg^{2+} was obtained from Life Technologies (Paisley, UK). Lepirudin (Refludan®) was obtained from Hoechst (Frankfurt am Main, Germany). Protein G Spin Kit columns (0.2 mL) for antibody purification were obtained from Thermo Fisher Scientific (Pierce, Rockford, IL). Burst test and Phago test kits were obtained from ORPEGEN Pharma (Heidelberg, Germany). LDS-751, Alexa 488, a BacLight green kit for the direct fluorescent staining of unlabeled bacteria, and dimethylsulfoxide (DMSO) were obtained from Invitrogen Molecular Probes (Eugene, OR). Zymosan A, EDTA and bovine serum albumin were obtained from Sigma–Aldrich (St. Louis, MO).

2.2. Monoclonal antibodies and inhibitors

Mouse anti-human CR1 blocking mAb (clone 3D9) which inhibits the binding of CR1 to C3b/C4b has been extensively characterized previously (O'Shea et al., 1985). Using protein G columns, the mAb 3D9 was purified from 50 μL of sterile ascites fluid containing approximately 1 g/L mAb. The concentration of the purified 3D9 IgG1 antibody in the eluate (0.46 g/L) was measured at 280 nm using a SmartSpec™Plus Spectrophotometer from Bio-Rad (Hercules, CA). An isotype-matched mouse anti-human IgG1 control mAb (clone BH1) was purchased from Diatec. Antibodies were tested for LPS contamination using a chromogenic Limulus Amebocyte Lysate (LAL) assay (QCL-1000) from BioWhittaker (Walkersville, MD). When necessary, LPS was removed from the mAbs using END-X B15 from Associates of Cape Cod Inc. (East Falmouth, MA), and final LPS concentrations in the low pg/mL range were obtained. Compstatin is a 13-amino acid cyclic peptide that binds C3 and inhibits the cleavage of C3. We used the compstatin analogue Ac-IT-NH2, which is 264 times more active than the parent peptide I[CVVQDWGH HRC]T-NH2. Both compstatin and the control peptide IAVVQ DWGHHRAT-NH2 were synthesized as previously described (Katragadda et al., 2006). The cyclic hexapeptide AcF[OPdChaWR], a C5a receptor antagonist (C5aRa), was synthesized as previously described (Mastellos et al., 2001). The murine anti-human mAbs anti-C2 and anti-factor D, as well as the isotype-matched murine control mAb G3-519, were previously described in detail (Lappegard et al., 2005; Brekke et al., 2007).

2.3. Bacterial strains and counting

The *E. coli* strain LE-392 (ATCC 33572) was obtained from the American Type Culture Collection (Manassas, VA). *E. coli* was grown overnight on a Lactose dish, and 5-10 colonies were transferred to LB medium (1% tryptone, 0.5% (w/v) yeast extract and 1% NaCl) from Becton Dickinson (Sparks, MD) and grown overnight. The bacteria were harvested and washed once, using Dulbecco's PBS without Ca^{2+} and Mg^{2+} by centrifugation for 10 min at 3220 × *g* (4 °C). Subsequently, the bacteria were aliquoted, heat-inactivated for 1 h at 60 °C and stored at −80 °C. A frozen ampoule was thawed at ambient temperature and was washed six times with PBS and centrifuged for 10 min at 3220 × *g* (4 °C) to remove extracellular LPS. Bacteria for Alexa staining were separated, whereas the rest were washed three more times. For counting, the bacteria were stained for 5 min using SytoBC (Invitrogen Molecular Probes) and were counted in Truecount tubes (Becton-Dickinson) using a FACScalibur or a LSRII flow cytometer with FACSDiva software (Becton-Dickinson). The heat-inactivated *E. coli* bacteria were