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A Novel Interaction of Outer Membrane Protein A with C4b Binding Protein Mediates Serum Resistance of Escherichia coli K1

Nemani V. Prasadarao,2* Anna M. Blom, † Bruno O. Villoutreix, ‡ and Linette C. Linsangan*

Escherichia coli is an important pathogen that causes meningitis in neonates. The development of bacteremia preceding the traversal across the blood-brain barrier is a prerequisite for this pathogen that obviously must survive the bactericidal activity of serum. Here we report that outer membrane protein A (OmpA) of Escherichia coli contributes to serum resistance by binding to C4b binding protein (C4bp), a complement fluid phase regulator. C4bp contains seven identical α-chains and one β-chain linked together with disulfide bridges. We found that OmpA binds the α-chain of C4bp, which is composed of eight homologous complement control protein (CCP) modules. Binding studies using mutants of recombinant C4bp that lack one CCP at a time suggest that CCP3 is the major site of interaction with OmpA. Furthermore, we demonstrate that the N terminus of OmpA interacts with C4bp. Binding of C4bp to OmpA is not significantly inhibited in the presence of either C4b or heparin and is not salt sensitive, implying that it is hydrophobic in nature, suggesting a novel interaction between OmpA and C4bp. A compelling observation in this study is that synthetic peptides corresponding to CCP3 sequences block the binding of C4bp to OmpA and also significantly enhance serum bactericidal activity. The Journal of Immunology, 2002, 169: 6352–6360.

E vading host defense mechanisms is a special property of many pathogenic bacteria that allows them to survive and multiply in the blood. Considering that in the case of Escherichia coli K1 infection a certain threshold of bacteremia is required to cause meningitis in neonates, it is obvious that E. coli must avoid complement attack. Previous studies have indicated that the K1 capsular polysaccharide, which is a polymer of sialic acid, is necessary for the survival of E. coli in the blood (1). Weiser et al. (2) subsequently showed, using OmpA− and OmpA+ E. coli strains, that outer membrane protein A (OmpA)3 confers serum resistance both in vivo and in vitro; however, the mechanisms by which E. coli avoids serum bactericidal activity are not known.

The complement system is the first line of defense against pathogenic micro-organisms. Activation of this system results in opsonization of the micro-organism for phagocytosis and formation of the membrane attack complex. Excessive activation of complement is inhibited by various regulatory proteins, both cell bound and fluid phase, which protect the host from attack by its own complement. The complement regulatory proteins include soluble plasma proteins, C4b binding protein (C4bp) and factor H (FH), and several membrane proteins.

Neisserial pathogens have been studied extensively for their serum resistance mechanism (3). The sialylation of lipooligosaccharides has been implicated as a mechanism of unstable serum resistance in Neisseria gonorrhoeae, in which sialic acid binds to FH, a critical regulator of the alternative pathway of complement. In contrast to unstable serum resistance, several gonococcal strains remain serum resistant under reduced sialylation. This is termed stable resistance and has been shown to depend on the binding of FH to Por1A (4). The binding of FH is confined to loop 5 of Por1A, and the addition of loop 5 peptide in a serum bactericidal assay enhances the killing of an otherwise completely serum-resistant gonococcal strain (5). Furthermore, Por1A as well as Por1B bind to C4bp, and blocking of this interaction renders the bacteria serum sensitive (6). Finally, type IV pili of Neisseria also bind C4bp; thus, gonococci have several mechanisms that act cooperatively to mediate serum resistance (7). C4bp is present in human plasma at concentrations of 200–250 μg/ml and is present in plasma in several forms, the major one composed of seven identical 70-kDa subunits (α-chains) and one 45-kDa subunit (β-chain) (8). Most C4bp in blood exists in complexes with protein S (C4bp-PS), a component of the vitamin K-dependent protein C anticoagulant system, and interacts with the β-chain of C4bp. Many isolates of Streptococcus pyogenes have also been reported to bind C4bp (9). In this species the NH2-terminal, highly variable region of several members of the M family proteins is responsible for binding to C4bp. In addition, C4bp binding has been demonstrated in all clinical isolates of Bordetella pertussis expressing filamentous hemagglutinin (10, 11).

Since the classical pathway is crucial in initiating complement deposition on pathogenic bacteria, regulation of this pathway is an efficient means for bacteria to evade initial host defense by serum complement depots (12, 13). E. coli K1 has been shown to activate the classical complement pathway via 018 LPS (14); however, E. coli still survive in the blood by avoiding complement attack. In the present study we demonstrate that OmpA, which is a 35-kDa

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3 Abbreviations used in this paper: OmpA, outer membrane protein A; AHS, adult human serum; AT, anti-thrombin; C4bp, C4b binding protein; C4bp-PS, C4bp complex with protein S; CCP, complement control protein (domain); FH, factor H; F5, fluorescein amine.
highly conserved protein among Gram-negative bacteria, of E. coli K1 contributes to serum resistance. Its N-terminal domain, encompassing aa residues 1–177, crosses the membrane eight times, forming anti-parallel β-strands. The four extracellular loops are mobile and are not well defined in the crystal structure (15). Our study demonstrates that C4bp binds to the N-terminal loops of OmpA, which leads to a decrease in serum killing, and that the interaction is confined to CCP3 of the α-chain of C4bp. Another salient feature of this study is that the synthetic peptides that represent sequences from the CCP3 domain of C4bp inhibit OmpA-C4bp interaction and increase serum bactericidal activity.

Materials and Methods

Bacterial strains, culture conditions, and chemicals

All strains used in this study were derived from a cerebrospinal fluid isolate of E. coli K1 strain RS218 (serotype O:113:H17) as described previously (16), and their characteristics are depicted in Table I. Bacteria were grown in brain heart infusion broth (Difco, Detroit, MI) with appropriate antibiotics at the following concentrations: rifampin, 100 μg/ml (E44 and E58); tetracycline, 12.5 μg/ml (E91); and ampicillin, 100 μg/ml (E105 and E111). Normal adult human serum (AHS) was obtained from 10 healthy individuals. Sera were pooled, aliquoted, and stored at −70°C until further use. For some experiments, AHS was incubated at 56°C for 30 min to yield heat-inactivated (72°C) serum.

Proteins, peptides, and Abs

The purification of human C4bp-PS, FH, and the generation of recombinant human C4bp (rC4bp) and eight rC4bp molecules lacking individual CCPs were described previously (17, 18). Vitrineconectin from adult human serum was purified as described previously (19). Peptide sequences that represent portions of CCP3 and CCP8 domains of C4bp were synthesized on an automatic peptide synthesizer. The sequences of the peptides are as follows: CCP3-1, 123 DIERYGRRHG 139; CCP3-2, 141 EENFYAYGF 149; CCP3-3, 156 DPRSFSLLGH 164; CCP3-4, 172 ENETIGVWRP 181; CCP8-1, 437 RKPELVNGR 445; CCP8-2, 449 DKDYQVPEN 458; CCP8-3, 464 DSGYGVQPG 473; and CCP3-4, 480 NRTWYPEVPK 489. Polyclonal Abs to C4bp, C4bp, C1q, and FH were obtained from Calbiochem (San Diego, CA). The mAbs 104 and 67 against C4bp were raised by Dr. B. Dahlback (Lund University, Malmo, Sweden). Polyclonal anti-OmpA Abs that recognize the N-terminal portion of OmpA (OmpA-N-Ab) were generated as previously described (16), and polyclonal anti-OmpA Ab (OmpA-C-Ab) that reacts to the C-terminal portion of OmpA was obtained from Dr. M. Inouye (Robert Wood Johnson Medical School, Piscataway, NJ).

Flow cytometry

We used flow cytometry for quantitation of binding of C4bp to E. coli. OmpA+ and OmpA- E. coli strains were grown overnight in appropriate antibiotics. The cells were centrifuged and washed twice with HBSS at room temperature. The bacteria were resuspended in HBSS, and the OD600 was adjusted to $1 \times 10^9$ CFU/ml. The bacterial suspension (140 μl) was incubated with AHS (60 μl) for 1 h at 37°C. The suspension was centrifuged at 8000 × g for 3 min, and the pellet was resuspended in HBSS. The suspension was further incubated with either anti-C4bp or anti-FH Abs (0.2 μg/ml) at room temperature for 30 min, followed by washing four times with HBSS. The bacterial pellets were further incubated with FITC-conjugated secondary Abs for 30 min at room temperature, washed, and resuspended in PBS. Flow cytometric analysis was conducted on a BD Biosciences (Mountain View, CA) instrument using CellQuest software.

Table I. Characteristics of various E. coli strains

<table>
<thead>
<tr>
<th>E. coli Strain</th>
<th>Relevant Characteristics</th>
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<tbody>
<tr>
<td>E44</td>
<td>Spontaneous Rif' mutant of RS218, LPS, K1</td>
</tr>
<tr>
<td>E58</td>
<td>E44 OmpA::TnPhoA' Truncated OmpA'</td>
</tr>
<tr>
<td>E91</td>
<td>E44 OmpA' Tet</td>
</tr>
<tr>
<td>E105</td>
<td>E91 pUC19 with ompA, OmpA+ Amp'</td>
</tr>
<tr>
<td>E111</td>
<td>E91 pUC19, OmpA+ Amp'</td>
</tr>
<tr>
<td>HB101</td>
<td>OmpA+', LPS, K12</td>
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Biotinylation of C4bp proteins and incubation with bacteria

The proteins to be biotinylated (C4bp-PS and rC4bp, 2 mg each) were added to 0.1 M sodium bicarbonate buffer (pH 8.0) containing 0.5 mg/ml NHS-LC-Biotin (Pierce, Rockford, IL) at a final protein concentration of 2 mg/ml. The mixture was incubated on ice for 1 h, followed by extensive dialysis against PBS, and was concentrated using Centricron tubes (Millipore, Bedford, MA; 10-kDa cut-off). The biotinylated proteins (2–5 μg) were incubated with the bacterial pellet from a 5-ml overnight culture in a volume of 0.5 ml at 37°C on a rotator for 1 h. The bacteria were then centrifuged, and the pellets were washed three times with PBS containing 0.1% Triton X-100. After a final wash, the bound proteins were released with Laemmli buffer in the presence of β-ME and analyzed by SDS-PAGE. The separated proteins were transferred to nitrocellulose, and immunoblotting was conducted using streptavidin coupled to peroxidase. The protein bands were visualized by ECL reagent (Amersham Biosciences, Piscataway, NJ).

Deglycosylation of C4bp

Recombinant C4bp (200 μg) was treated with 5 U of peptide N-glycosidase F in 50 mM sodium phosphate buffer (pH 7.8) at 37°C overnight (20). The protein was purified by gel filtration over Sephacryl S-300 HR. The peak containing C4bp was concentrated and tested for deglycosylation by blotting with wheat germ agglutinin, followed by anti-wheat germ agglutinin Ab immunoblotting.

Bactericidal activity

The susceptibility of various E. coli strains to complement-mediated killing was determined using nonimmune AHS at a final concentration of 40%. The bacteria (10⁸) were suspended in HBSS containing CaCl₂, followed by addition of AHS, and were incubated for 1 h with rotation. Routinely, heat-inactivated AHS (56°C for 30 min) was included as a control. In some experiments AHS was incubated with 100 nM EGF-T for 15 min before performing the bactericidal assays. Various Abs and C4bp peptides were incubated with the bacteria in separate experiments at room temperature for 30 min before the addition of AHS to examine their effect on the bactericidal activity. Synthetic OmpA peptides were incubated with AHS at room temperature for 30 min before adding them to the bacteria. The effects of these reagents on bacterial survival were examined by the colony count method.

Heparin-Sepharose column chromatography of serum proteins

The AHS containing protease inhibitors was passed through a heparin-Sepharose column for a period of 2 h at 4°C. The column was then thoroughly washed with PBS, and bound proteins were eluted with PBS containing varying concentrations of NaCl. The proteins from each fraction were dialyzed against PBS and concentrated using Centricon tubes.

Labeling of peptides with fluorescein amine

The synthetic peptides were labeled using a fluorescein amine (FS) labeling kit (Panvera, Madison, WI). The labeling reagent is a succinimidyl ester of fluorescein, which selectively forms an amide bond with N-terminal amino group of peptides or proteins at neutral pH; this reagent reacts more rapidly and forms a more stable derivative than FITC. Briefly, each peptide (500 μg) was resuspended in coupling buffer and chilled to 4°C for 10 min. Ice-cold FS in DMSO (20 μM/20 μl) was added to the peptide solution and incubated at 37°C for 1 h. Following incubation, excess FS was quenched by addition of 1 M Tris-HCl (pH 8.0), and the mixture was incubated for an additional 30 min at room temperature. A portion of fluoresceinated peptide was purified by TLC on silica gel plates using n-butanol/acetic acid/water (4:1:1) as an irrigation solvent. The plates were dried, and fluoresceinated peptides were visualized under UV light. Each peptide band, which runs very close to the origin of application, was excised from the plate and then eluted from the silica gel with 50 mM Tris-HCl, pH 8.0. Each peptide (10 μg) was separately incubated with either E. coli strain E44 or E91 (1 ml culture pellet) at 4°C for 1 h, followed by extensive washing with Tris-HCl, and was subjected to flow cytometry as described above.

Results

OmpA increases E. coli resistance to serum bactericidal activity

Weiser et al. (2) previously showed that expression of OmpA on E. coli enhances serum survival. The study used E44 and a TnPhoA mutant of E44, E58, in which TnPhoA was inserted at the 3' end of the ompA gene. Strain E58 has been classified as an OmpA deletion mutant by Western blot analysis using OmpA-C-Ab.
OmpA is not sufficient to confer serum resistance, perhaps due to the disturbed orientation of OmpA. The HB101 strain, used as a control, barely survived. In addition, incubation of E44 with heat-inactivated serum (to denature the complement) resulted in the loss of bactericidal activity. To further confirm that OmpA expression is important for the serum resistance capacity of E. coli, E91 was complemented with the ompA gene in a pUC19 plasmid (E105) and subjected to AHS bactericidal activity. As a control, E91 containing vector alone (E111) was used. Introduction of OmpA in E91 restored the serum resistance capacity of E91 to the level of E44. In contrast, E91 with only vector was not able to survive in a similar fashion in AHS. In addition, AHS did not show any bactericidal activity toward E91 in the presence of 100 nM EGTA, suggesting that the classical complement pathway might be responsible for the serum killing activity. To further confirm the role of the classical pathway, AHS-treated E44 was subjected to flow cytometry using anti-C1q Ab, and the results showed that a significant amount of C1q, a marker for the classical complement pathway, had been deposited (Fig. 1B). E91 had been coated with similar amounts of C1q (data not shown). Taken together, these data indicate that although the classical pathway is activated, intact OmpA expression may contribute to the increased serum resistance of OmpA+ E. coli compared with OmpA− E. coli.

OmpA+ E. coli binds to two human serum proteins

To understand the mechanism of serum survival of E. coli, we next examined whether OmpA+ E. coli binds to any AHS proteins compared with OmpA− E. coli strains. OmpA+ E. coli, when incubated with biotinylated serum proteins, bound to three proteins with apparent molecular sizes of 65–70, 45–50 kDa, and, to a lower extent, 50–55 kDa (Fig. 2). In contrast, OmpA− E. coli bound only the 50–55-kDa protein, suggesting that OmpA may be specifically responsible for interacting with the two former serum proteins. However, HB101, a laboratory E. coli strain that also contains OmpA on its surface, showed only weak binding to both serum proteins.

Based on the molecular masses of OmpA-bound proteins, we speculated that they could be vitronectin (~70 kDa) and antithrombin (AT; ~50 kDa), which are known to bind heparin. Thus, AHS was subjected to heparin-Sepharose chromatography, and the bound proteins were eluted with increasing concentrations of NaCl. Each fraction contained several proteins, as observed by SDS-PAGE, followed by Coomassie staining (gel not shown), which were then biotinylated and incubated with OmpA+ and OmpA− E. coli. No proteins bound to OmpA+ E. coli from the 0.1-M NaCl fraction, whereas two proteins (~70 and ~45–50 kDa), similar to the serum proteins, bound to AHS eluted from the 0.2- to 0.4-M NaCl fractions (Fig. 2B). The binding was significantly greater in the 0.2-M fraction than in the other fractions. Since these two proteins bound heparin, we examined whether OmpA interacts at the heparin binding region of these proteins. Interestingly, incubation of E44 with AHS in the presence of excess amounts of heparin did not block the binding of serum proteins to OmpA+ E. coli (Fig. 2B, lane 1). These results suggest that the two heparin-binding proteins interact with OmpA+ E. coli via an interaction site localized away from the heparin binding site.

C4bp binds directly to OmpA+ E. coli, and protein S does not influence the binding

Since heparin-Sepharose-bound proteins were observed to interact with OmpA+ E. coli, we examined whether vitronectin and AT

![FIGURE 1. Survival of E. coli in normal adult human serum and binding of C1q. A, Various bacterial strains were incubated with 40% AHS at 37°C for 1 h, followed by enumeration of bacteria on blood agar plates. In some experiments either heat-inactivated (HI) or EGTA-pretreated (AHS+EGTA) serum was used. The results are expressed as the percent survival relative to that of E44 grown in medium alone and as an average of four separate experiments. Error bars indicate the SD. The difference in killing of the OmpA− E. coli strain compared with the OmpA+ E. coli strain was statistically significant (p < 0.03, by paired t test). B, OmpA+ and OmpA− E. coli strains were incubated with AHS for 1 h at 37°C. The bacteria were washed with PBS and incubated with anti-C1q Ab for 1 h at room temperature, followed by thorough washing and further incubation with FITC-conjugated secondary Ab for 30 min at room temperature. The bacteria were washed again and subjected to flow cytometry. As the control, primary Ab was omitted. Only results for E44 strain are presented.](http://www.jimmunol.org/)

![FIGURE 2. Binding of AHS proteins to OmpA+ E. coli. A, Biotinylated AHS (0.5 ml) was incubated with different bacterial strains (10⁶ bacteria) for 1 h at 37°C, followed by extensive washing with PBS. The bound proteins were eluted with Laemmli buffer, separated by SDS-PAGE, and transferred to nitrocellulose. The proteins were identified by probing with streptavidin-peroxidase followed by ECL reagent. B, Biotinylated AHS (0.5 ml) was incubated with 250 μg of heparin on ice for 30 min before incubation with E44. In some experiments NaCl fractions from a heparin-Sepharose column were biotinylated and incubated with E44 for 1 h at 37°C. E44-bound proteins were eluted and analyzed as described. Molecular mass markers (in kilodaltons) are indicated to the right.](http://www.jimmunol.org/)
bound to the bacteria. Purified vitronectin (5 μg) and proteins from the 0.2-M NaCl fraction of the heparin-Sepharose column were incubated with E44 as described, followed by immunoblotting with anti-vitronectin Ab. The results show that vitronectin did not bind E44, and the protein (∼70-kDa) binding to E44 from the 0.2-M fraction is not vitronectin (Fig. 3A). Similarly, analysis of biotinylated AT binding to OmpA+/E. coli showed no association with the bacteria, suggesting that heparin binding proteins from serum other than vitronectin and AT are interacting with E44. C4bp, a key soluble phase classical pathway regulatory protein, is also known to bind heparin. Therefore, we tested whether plasma-purified C4bp-PS is able to interact with OmpA+/E. coli strains. As shown in Fig. 3B, biotinylated C4bp-PS binds E44 in significantly greater quantities than it does E91. HB101 bound with far less efficiency to C4bp-PS despite the presence of OmpA. It seems that the orientation of OmpA on the HB101 strain is different from that of OmpA present on the E44 strain. In agreement with this concept, the ompA gene from the HB101 strain can complement the OmpA+/E. coli strain, suggesting that OmpA might acquire a special three-dimensional structure only on E44 due to the presence of other molecules. This particular orientation may enable E. coli K1 to interact with serum proteins and confers serum resistance to complement.

Several studies have shown that the bacterial polysaccharide capsule containing sialic acids is an important virulence factor for a number of pathogens for evading host defense mechanisms. Sialic acids have been shown to bind FH, another critical regulator of the complement system in the blood (4). Since the E. coli used in this study contains K1 polysaccharide made up of polysialic acid residues, we have used purified FH to examine its potential interaction with E. coli K1. Interestingly, FH binds with slightly greater efficiency to OmpA+/E. coli strains compared with E44 (Fig. 3B). We next attempted to determine whether the proteins that bound OmpA+/E. coli strains from AHS could be C4bp by preincubating the bacteria with excess amounts of nonbiotinylated C4bp-PS (25 μg), followed by incubation with biotinylated AHS. The bound proteins were released and analyzed as described above. C4bp-PS pretreatment completely abolished the binding of AHS proteins to OmpA+/E. coli, suggesting that the protein bound to OmpA+/E. coli is indeed C4bp (Fig. 3C). To further confirm these results, FACS analysis using anti-C4bp Ab was performed. As shown in Fig. 4, E44 bound significantly more C4bp compared with E91. We have also analyzed FH binding to these strains, and the results are in concurrence with the results of Western blots showing that E91 bound FH slightly better than E44.

Next, to assess the influence of protein S on the binding of C4bp to OmpA+/E. coli strain, rC4bp (polymerized six α-chains without β-chain and protein S) was used in the binding assays. We found that rC4bp binds in a fashion similar to C4bp-PS (Fig. 3B). The rC4bp also blocked the binding of AHS proteins to E44 when bacteria were pretreated with excess amounts of protein (Fig. 3C). These results suggest that protein S does not influence the binding of rC4bp to OmpA+/E. coli and that the binding region for OmpA on C4bp is localized within the α-chain.

To further characterize the interaction of rC4bp with OmpA, we tested whether increasing salt concentrations had any influence on the binding. In our experiments OmpA+/E. coli suspended in PBS containing 0.1% BSA, 0.1% Tween 20, and various concentrations of NaCl ranging from 50 to 500 mM were incubated with biotinylated rC4bp. After 2 h of incubation at 37°C and washing with PBS, the bound rC4bp was analyzed as described above. As shown in Fig. 5, binding of rC4bp to OmpA+/E. coli was not reduced even at high NaCl concentrations. Thus, the rC4bp-OmpA interaction seems to primarily rely on hydrophobic contacts, with only minor contributions from electrostatic forces.
CCP3 contributes to the binding of rC4bp to OmpA

We determined the region of human C4bp that bound OmpA using rC4bp and eight mutant rC4bp proteins, each lacking one CCP at a time. The characterization of these recombinant proteins was described previously (17). Equal amounts of biotinylated CCP mutant proteins were incubated with OmpA+ E. coli, and the bound proteins were analyzed by SDS-PAGE. As shown in Fig. 6A, deletions of CCP3 and CCP8 of rC4bp affected the binding considerably. Particularly rC4bpΔCCP3 molecules showed a dramatic reduction of affinity to OmpA. Other deletions did not significantly affect the binding, suggesting that CCP3 and CCP8 modules could be important for the interaction with OmpA+ E. coli.

We have previously shown that OmpA interacts with N-acetylglucosamine 1,4-N-acetylgalactosamine epitopes of HBMEC glycoproteins for invasion of the blood-brain barrier (20). Therefore, OmpA could be interacting with the N-linked oligosaccharides of rC4bp. Consensus sequences for N-glycosylation are present at residues N173 (CCP2-CCP3 interface area), N458, and N-480 (both in CCP8; see Fig. 7a) (21). To assess the potential role of glycosylation, rC4bp was deglycosylated using peptide N-glycosidase F and was incubated with E44. Although the binding was slightly affected by deglycosylation (Fig. 6B), it was not completely abrogated, suggesting that N-glycosylation plays a minimal role in the interaction. In addition, we examined the effect of mAb 104, which recognizes CCP1, on the binding of rC4bp to OmpA. This Ab has been shown to inhibit the interaction of C4b to C4bp, and this interaction was, in turn, mapped to CCP1 to -3 (22). Pre-incubation of this Ab with rC4bp partially blocked the binding of rC4bp to OmpA+ E. coli. Densitometric analysis of the respective bands indicated a 20% decrease in the binding of rC4bp to OmpA.

Because we found that the N-terminal portion of OmpA interacts with the CCP3 and/or CCP8 regions of rC4bp, it was of interest to define potential recognition sites on the C4bp molecule. To this end we defined solvent-exposed regions on a three-dimensional model structure for C4bp (Fig. 7A) and used synthetic peptides from both CCP3 and CCP8 to study their effects on the binding of rC4bp to E44. A total of eight peptides (four from each CCP) were synthesized, and each peptide (50 μM) was preincubated with E44 on ice for 30 min, followed by incubation with biotinylated C4bp. As shown in Fig. 8, peptides CCP3-1, CCP3-3, and CCP3-4 completely abolished the binding of rC4bp, whereas CCP3-2 did not show any effect on the interaction. Interestingly, except for peptide CCP8-2, which showed marginal inhibition, all other peptides derived from CCP8 were ineffective in blocking the binding of rC4bp to E44. To examine whether the inhibition was due to direct binding of the peptides to OmpA+ E. coli, the synthetic peptides were labeled with FS and examined for their binding capacity by flow cytometry. As shown in Fig. 9, peptides CCP3-1, -3, and -4 bound E44 significantly more compared with E91 (one picture is shown as a representative for all; Fig. 9, A and B). CCP3-2 peptide and all CCP8 peptides showed very weak or negligible binding.
no binding (Fig. 9C), suggesting that the inhibitory effect of CCP3 peptides that impede the rC4bp-OmpA\(^+\) \textit{E. coli} interaction is due to direct binding to OmpA.

**CCP3 of C4bp binds to the N-terminal part of OmpA**

To identify the C4bp binding site on OmpA, we first examined the effect of OmpA-N-Ab on the binding. The anti-Ab significantly blocked the binding of C4bp-PS to E44, whereas neither OmpA-C-Ab nor control Ab showed such inhibition (Fig. 10), suggesting that C4bp-PS binds to the N-terminal portion of OmpA. To further confirm that hypothesis, we used two synthetic peptides, N (Asn\(^{27}\)-Gly-Pro-Thr-His-Glu\(^{32}\)) and G (Gly\(^{65}\)-Ser-Val-Glu-Asn\(^{69}\)), from N-terminal loops 1 and 2 of OmpA in the C4bp-PS binding assays. Our previous studies by computer simulation (23) showed that the N-acetylglucosamine 1,4 N-acetylglucosamine epitopes of HBMEC glycoproteins interact with these sequences. In addition, these peptides significantly inhibited the \textit{E. coli} invasion of human brain microvascular endothelial cells (16). As expected, these peptides (200 µM) also blocked the binding of C4bp-PS to E44, whereas, peptide H (His\(^{19}\)-Asp-Thr-Gly\(^{22}\)), a control peptide sequence present on loop 4, did not have any effect on the interaction (Fig. 10). These results suggest that C4bp-PS binds OmpA near or at the N-terminus.

**Inhibition of C4bp binding to OmpA increases the bactericidal activity of serum**

Our studies to date have suggested that OmpA of \textit{E. coli} is responsible for the binding of C4bp, a phenomenon that might contribute to serum resistance of \textit{E. coli}. To further investigate this point, we examined whether inhibition of C4bp interaction with OmpA\(^+\) \textit{E. coli} increases the bactericidal activity of serum. First, we verified the effects of various Abs that block the binding of rC4bp on the bactericidal activity. OmpA-N-Ab and OmpA-C-Ab were incubated with OmpA\(^+\) \textit{E. coli} on ice before the bactericidal assays. OmpA-N-Ab contributed to a significant increase in the bactericidal activity of serum.

**FIGURE 8.** Effects of CCP3 synthetic peptides on the binding of rC4bp to OmpA\(^+\) \textit{E. coli}. Various synthetic peptides (50 µM) generated from the sequences of CCP3 and CCP8 of C4bp were incubated with OmpA\(^+\) \textit{E. coli} for 1 h on ice, followed by incubation with biotinylated rC4bp. The bound rC4bp was eluted and analyzed as described in \textit{Materials and Methods}.

**FIGURE 9.** Binding of CCP3 synthetic peptides to OmpA\(^+\) \textit{E. coli}. FS-labeled synthetic peptides (5 µg each) were incubated with either OmpA\(^+\) \textit{E. coli} or OmpA\(^-\) \textit{E. coli} for 1 h on ice, washed, and subjected to flow cytometry. Untreated bacteria were used as a negative control. A representative picture is shown for E91 treated with various peptides (A); E44 treated with CCP3-4 for CCP3-1, -3, and -4 (B); or E44 treated with CCP3-2 for CCP3-2 and CCP8-1, -2, -3, and -4 (C).
FIGURE 10. Inhibition of C4bp-PS binding to OmpA+ E. coli by anti-OmpA Ab and synthetic peptides that represent N-terminal domains of OmpA. OmpA+ E. coli was incubated with either OmpA-N-Ab or control Ab (cAb) on ice for 1 h before the addition of biotinylated C4bp-PS. In other experiments synthetic peptides (200 μM) were incubated with 5 μg of C4bp-PS for 1 h on ice before addition to the bacteria. In addition, E. coli strain E58, which expresses truncated OmpA without the C-terminal portion, was also incubated with rC4bp. The bound proteins were analyzed by immunoblotting with streptavidin-peroxidase, followed by ECL reagent.

serum killing activity of AHS (~80%) compared with either control Ab or OmpA-C-Ab (Fig. 11A). The killing of OmpA+ E. coli was significant even at 20% AHS concentration when the bacteria were pretreated with OmpA-N-Ab (data not shown). At a 20% serum concentration we did not observe significant bactericidal activity under normal conditions. Since the binding of OmpA-N-Ab could induce complement fixation via Fc portions of Ab, we also used Fab’ of the Ab to examine whether the enhanced serum killing is due to blocking of C4bp binding to E. coli. The serum bactericidal activity of AHS also increased ~60% in the presence of Fab’, suggesting that both blocking of C4bp to E. coli and complement activation by OmpA-N-Ab might be contributing to the enhanced bactericidal activity of AHS. It is interesting that mAb 104, which showed a partial inhibitory effect on the binding of C4bp to OmpA+ E. coli displayed a 10–20% increase in bactericidal activity. mAb 67 showed no effect on the bacterial killing. Incubation with various Abs alone did not alter OmpA+ E. coli survival. Surprisingly, these Abs also had some effect on OmpA+ E. coli. It is possible that these Abs bind OmpA+ E. coli nonspecifically and activate the classical pathway of complement.

Since the peptide sequences derived from both OmpA and C4bp inhibited the binding of C4bp to OmpA, we examined their effects on the bactericidal activity of AHS. Various peptides derived from C4bp were incubated with OmpA+ E. coli, whereas peptides derived from OmpA were incubated with AHS before carrying out the serum bactericidal activity assay. Peptides CCP1-3, CCP3-4, and CCP3-4 contributed to 50–70% more bactericidal activity than AHS alone, whereas CCP3-2 did not yield an increase in bacterial killing capacity (Fig. 11B). In contrast, the presence of other C4bp peptides had no effect on bactericidal activity. The C4bp peptides CCP3-1, CCP3-3, and CCP3-4 showed no enhancement of antibacterial activity in the absence of AHS. Similarly, OmpA peptides G and N showed ~40% enhancement of serum killing activity compared with peptide H. The C4bp peptides increased the sensitivity of OmpA+ E. coli for serum killing at a concentration of 100 μM, whereas OmpA peptides achieved a similar effect at 200 μM. All reagents (the blocking Abs or the peptides) had a profound serum killing capacity when the bacteria were incubated for longer times with AHS (2 h; data not shown). These results indicate that blocking the C4bp-OmpA interaction makes OmpA+ E. coli more susceptible to serum killing, which supports the idea that E. coli K1 evades serum bactericidal activity mostly by binding to C4bp, an important fluid phase regulator of the complement system.

Discussion

Complement activation is a crucial part of host defense against E. coli K1, a pathogen that causes meningitis in neonates, which must develop a strategy to subvert the complement attack to survive and multiply in the blood. However, the mechanisms of serum resistance in these strains are not known. Weiser et al. (2), using an OmpA+ E. coli strain, E58, have shown that OmpA expression is necessary for survival in serum, but our subsequent studies indicated that E58 still expresses truncated OmpA (16). Therefore, we used a true OmpA-negative E. coli strain and re-examined the serum bactericidal activity of AHS in the present study. Interestingly, OmpA+ E. coli, E91, which does not express OmpA on its surface showed survival rates similar to that of E58. It is possible that the orientation of OmpA on E58 may be disturbed due to lack of the C-terminal portion, such that its function is completely impaired. Conclusive evidence for the role of OmpA in serum resistance comes from the observation that E91 regains its survival
capability to the level of E44 when complemented with the ompA gene. HB101 strains, despite binding to minute quantities of C4bp to OmpA, could not survive well, suggesting that the orientation of OmpA on HB101 is not optimal for efficient binding of C4bp. Alternatively, resistance to the alternative pathway by binding to FH via sialic acids present on E. coli K1, which are absent on HB101, could provide a synergistic effect on the classical pathway for greater serum resistance capacity. In agreement with the concept that OmpA expression contributes to serum resistance, we showed a strong correlation of C4bp binding to OmpA to avoid serum killing. We also attempted to identify the binding site(s) for OmpA on the surface of C4bp by using both C4bp-PS and rC4bp, and the results suggested that OmpA binding is confined to α-chains. C4bp α-chains are also known to bind C4b (22), heparin (24), Bordetella pertussis (10), Streptococcus pyogenes (25), and serum amyloid P component (26, 27). We demonstrate here the specificity of the C4bp-OmpA interaction using several different experimental approaches. First, OmpA-N-Ab and the peptides corresponding to partial sequences of loops 1 and 2 of OmpA blocked the interaction. Second, the CCP3- and CCP8-deleted mutants of rC4bp could not bind OmpA, whereas other deletion mutants did not affect the binding. Synthetic peptides that represented portions of CCP3 blocked rC4bp binding to OmpA more efficiently than those of CCP8. This blocking effect was due to direct binding of the peptides to OmpA, suggesting that CCP3 contained a key binding site for OmpA. Interestingly, mAb 104, which is directed against CCP1 of the α-chain of C4bp, did not cause significant inhibition of the rC4bp-OmpA interaction. Yet, this Ab has been previously shown to fully block binding between CCP1 of the α-chain of C4bp and C4b (22), N. gonorrhoeae (7), and streptococcal M proteins (28), suggesting a novel site of interaction between OmpA and C4b. The moderate survival rates of OmpA-E. coli, E91, could be due to the presence of capsular polysaccharide K1, which is composed of sialic acid residues. Several reports have shown that the sialic acid expressed by many pathogenic bacteria binds to FH to avoid complement attack. Alternatively, binding of small quantities of C4bp from AHS to E91, as detected in the present study, might be another mechanism for survival of this strain despite the absence of OmpA. It could be that other molecules, such as S-fimbriae, also bind C4bp to a lesser extent. Such an interaction of C4bp with type IV pilus has been demonstrated in N. gonorrhoeae in addition to PorIB/1A, highlighting the role of more than one molecule binding to C4bp (7).

Our studies showed that heparin treatment of AHS did not affect the C4bp-OmpA interaction, which suggests that the binding sites for heparin (positive amino acids on the interface between CCP1 and CCP2 as well as on CCP2) and OmpA on C4bp might be different. In addition, excess amounts of C4b could not impede the rC4bp-OmpA interaction. Several studies have indicated that a cluster of positively charged amino acids at the interface between CCP1 and CCP2 in C4bp is necessary for binding to C4b (22, 24). In contrast, the binding of rC4bp to OmpA was completely abolished by the deletion of CCP3, whereas no effect was observed by the deletion of CCP2. This is in agreement with the results of heparin and C4b inhibition studies, highlighting the discovery of a new binding site for OmpA on C4bp. This binding activity differs from the interactions of other bacterial proteins, which bind to the CCP1-CCP2 of C4bp (27). Minimal or no effect of salt on the C4bp-OmpA interaction also stands in sharp contrast to the C4b-C4bp binding that was completely abolished at 300 mM NaCl. Previous studies on the binding of N. gonorrhoeae porin 1B and pil also showed that 0.2 M NaCl significantly blocked their interaction with C4bp, highlighting the role of ionic interactions. In contrast, the high affinity binding of C4bp to CCP1 of streptococcal M proteins and to N. gonorrhoeae por1A was proposed to be hydrophobic in nature because it was insensitive to high ionic strength (5, 28). It is likely that the binding of C4bp to OmpA involves hydrophobic contacts and hydrogen bonds, while the C4b-C4bp interaction could be essentially dependent on salt bridges and long range attractive electrostatic forces.

Our data to date suggest that CCP3 is probably the key binding site for OmpA. The role of CCP8 is unclear, since we found that the mutant lacking CCP8 showed decreased binding ability for OmpA, but no peptides from CCP8 were able to inhibit the interaction. Thus, the role of CCP8 requires further investigations, which we will undertake in the future. It is interesting to note that both CCPs contain N-glycosylation sites. However, the slightly reduced binding of deglycosylated C4bp to OmpA indicates that the interaction is essentially mediated by amino acids. Collectively, our data strongly suggest that CCP3 is crucial to the interaction, because three synthetic peptides from CCP3 that cluster closely significantly blocked the interaction of C4bp with OmpA. In addition, the direct binding of CCP3-1, -3, and -4 peptides to OmpA+ E. coli compared with CCP3-2 and CCP8 peptides supports the idea that the CCP3 module forms the key binding site for OmpA.

A compelling observation of this study is the ability of CCP3-1, CCP3-3, and CCP3-4 peptides to enhance serum killing activity, which is in good agreement with the finding that these peptides efficiently blocked the binding of rC4bp to E. coli. Interestingly, none of the CCP8 peptides showed any effect on AHS bactericidal activity, suggesting that OmpA may not or may only minimally interact with this module. OmpA-N-Ab is the most potent Ab in enhancing the AHS bactericidal activity. In our earlier studies we developed Abs to OmpA, which recognize only the C-terminal portion of OmpA, by immunizing animals with UV-irradiated whole bacteria. Similarly, anti-OmpA Abs raised by several other investigators also preferentially recognize the C-terminal portion, indicating that the C-terminal region of OmpA might be more immunogenic than the N-terminus. In addition, the inability of OmpA-C-Ab and control Abs to increase serum sensitivity could be due to inefficient binding of these Abs to bacteria to initialize the classical pathway. Thus, the N-terminal portion of OmpA, at least for E. coli K1 could be a suitable target for the construction of an effective vaccine that nullifies the binding of C4bp to permit complement attack.

In conclusion, our studies have demonstrated that OmpA of E. coli contributes to serum resistance by avoiding complement attack via binding to C4bp. The N-terminal portions of OmpA and CCP3 of C4bp are involved in the interaction. Neither high ionic strength nor C4b/heparin could block the binding of C4bp to OmpA, highlighting a novel site of interaction at the surface of C4bp molecules. OmpA-N-Ab and synthetic peptides mimicking CCP3 regions enhance the serum sensitivity of E. coli, suggesting that these regions could be potential targets for novel therapeutic approaches.

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References

OmpA-C4bp INTERACTION MEDIATES SERUM RESISTANCE OF *Escherichia coli*


