

Binding of the Complement Inhibitor C4bp to Serogroup B *Neisseria meningitidis*¹

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Neisseria meningitidis (meningococcus) is an important cause of meningitis and sepsis. Currently, there is no effective vaccine against serogroup B meningococcal infection. Host defense against *neisseriae* requires the complement system (C) as indicated by the fact that individuals deficient in properdin or late C components (C6-9) have an increased susceptibility to recurrent neisserial infections. Because the classical pathway (CP) is required to initiate efficient complement activation on *neisseriae*, meningococci should be able to evade it to cause disease. To test this hypothesis, we studied the interactions of meningococci with the major CP inhibitor C4b-binding protein (C4bp). We tested C4bp binding to wild-type group B meningococcus strain (H44/76) and to 11 isogenic mutants thereof that differed in capsule expression, lipo-oligosaccharide sialylation, and/or expression of either porin (Por) A or PorB3. All strains expressing PorA bound radiolabeled C4bp, whereas the strains lacking PorA bound significantly less C4bp. Increased binding was observed under hypotonic conditions. Deleting PorB3 did not influence C4bp binding, but the presence of polysialic acid capsule reduced C4bp binding by 50%. Bound C4bp remained functionally active in that it promoted the inactivation of C4b by factor I. PorA-expressing strains were also more resistant to C lysis than PorA-negative strains in a serum bactericidal assay. Binding of C4bp thus helps *Neisseria meningitidis* to escape CP complement activation. *The Journal of Immunology*, 2005, 174: 6299–6307.

Neisseria meningitidis is a Gram-negative bacterium that causes meningitis and/or sepsis. The incidence of severe meningococcal disease varies from 1 to 50 cases per 100,000. The reported mortality rate in meningococcal meningitis is 1–10% and in sepsis is 20–40%, and up to 20% of survivors suffer from neurological sequelae (1). Based on the chemical composition of the capsular polysaccharide, meningococci are divided into 13 serogroups. Serogroup B is one of the most common serogroups encountered in clinical infections in industrialized countries, and efficient vaccines against this serogroup have not yet been developed. The serogroup B capsular polysaccharide consists of homopolymers of $\alpha(2\rightarrow8)$ sialic acid, which is identical to sialic acid in human fetal brain tissue (neural cell adhesion molecule) and is poorly immunogenic (2). In addition to the capsular polysialic acid, sialic acid also occurs as a terminal sugar substitution on the lacto-*N*-neotetraose lipo-oligosaccharide (LOS)⁴ of serogroup B meningococci (3).

Meningococci express several outer membrane proteins. Class 1 protein or porin (Por) A is a 45-kDa membrane protein, which structurally belongs to the Por superfamily and consists of 16 transmembrane segments with 8 surface-exposed loops (4). PorA sequence shows some variability among different meningococcal strains, which is mainly confined to the surface-exposed loops 1 and 4 (variable region) (4). The expression of class 2 protein (PorB2) or class 3 protein (PorB3) is mutually exclusive, i.e., only one of these two Por molecules is expressed on any given meningococcal strain. PorB is a 34- to 37-kDa protein with a similar transmembrane loop structure as PorA (4–6). Serotype classification of meningococci is based on mAb reactivity with the PorB molecule, and serosubtyping is based on the PorA mAb reactivity. PorB2 and PorB3 bear sequence similarity with *Neisseria gonorrhoeae* Por1B and Por1A, respectively (7). There is no homologue of PorA expressed on gonococci (8).

The complement system (C) is important in the defense against *N. meningitidis* and *N. gonorrhoeae*. Individuals deficient in properdin or one of the terminal C components (C6, C7, C8, or C9) have an increased susceptibility to recurrent or atypical neisserial infections. It has been suggested that sialic acid in the capsule and/or LOS may prevent the activation of the alternative pathway (AP) of C on the meningococcal surface (9, 10). Disease-causing serogroup B meningococci produce more capsular polysialic acid than avirulent strains (9). Although it has been observed that polymeric sialic acid (colominic acid resembling serogroup B polysialic acid) does not increase the binding affinity between C3b and the C regulator factor H, it is possible that terminally exposed sialic acids, particularly on LOS, could increase the binding of factor H to C3b deposited on the surface of the bacterium (11). However, direct evidence of the contribution of factor H to meningococcal serum resistance is still lacking.

The C system may become activated through the classical pathway (CP) during meningococcal infections. This could be due to

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⁴ Abbreviations used in this paper: LOS, lipo-oligosaccharide; Por, porin; C, complement system; AP, alternative pathway of complement; CP, classical pathway; C4bp, C4b-binding protein; CCP, complement control protein; VBS, veronal-buffered

saline; HIS, heat-inactivated normal human serum; NHS, normal human serum; wt, wild type; MBL, mannose-binding lectin; OMV, outer membrane vesicle.

natural Abs, cross-reactive Abs developed as a result of colonization with meningococci or against colonizing apathogenic neisserial species, or to specific Abs raised during infections or following vaccination (12). The mechanisms of CP activation and regulation on the meningococcal surfaces have not yet been thoroughly studied.

C4b-binding protein (C4bp) is a fluid-phase regulator of the classical and lectin pathways of C. The CP is activated when C1q comes into contact with surface-bound or complexed IgG, IgM, or C-reactive protein. The contact leads to sequential conformational changes in C1q and the protease C1r that cleaves C1s. The activated C1s proteolytically cleaves C4. C4b binds C2, which is also cleaved by C1s, thereby generating the C3 convertase C4b2a. C4bp acts by preventing the assembly and accelerating the decay of the C4b2a complex. It also acts as a cofactor for factor I in the cleavage and inactivation of C4b (13). C4bp is an \approx 500-kDa protein, which consists generally of seven α -chains and of one β -chain (13). The α -chains consist of eight and the β -chain of three complement control protein (CCP) domains, which are linked together by disulfide bridges at their C-terminal domains. By electron microscopy, C4bp has a spider-like structure, with extended polypeptide chains. The C4b-binding site on C4bp has been localized to CCPs 1–3 of the α -chain (14). The same CCPs also contain the heparin binding sites (13, 14).

Some microbes have been shown to bind C4bp to their surfaces. Examples include group A streptococci, *Escherichia coli*, and *Bordetella pertussis* (15–17). For group A streptococci, it has been shown that the binding of C4bp is of physiological importance (18). Gonococcal Por1A and Por1B both bind C4bp (19). Meningococcal PorB3 and PorB2 bear sequence similarities with gonococcal Por1A and Por1B, respectively, but binding of C4bp to meningococci has not been reported thus far.

In the present investigation, we studied the interactions of meningococci with the major CP inhibitor C4bp. We found that meningococci bind C4bp, and the expression of PorA is required for C4bp binding. Binding was sensitive to ionic strength. Importantly, bound C4bp remained functionally active and inhibited C activation on the meningococcal surface, thus contributing to the serum resistance of meningococci.

Materials and Methods

Proteins and Abs

C4bp was purified from pooled human plasma as described previously (20). Protein S was removed during purification. The polymeric recombinant C4bp mutants lacking individual α -chain CCPs (Δ CCP1–8) were constructed and expressed as described previously (21). Human C4b, factor I, and the polyclonal rabbit anti-C4bp Ab were obtained from Calbiochem. Mouse mAbs against C4bp α -chain CCP1 (mAb 102), CCP1–2 (mAb 96), and CCP4 (mAb 67) were produced as previously described (22) and were a kind gift of Professor B. Dahlbäck (Lund University, Malmö, Sweden). Heparin from porcine intestinal mucosa and BSA were purchased from Sigma-Aldrich. C4bp, C4b, C3b, and the recombinant mutants of C4bp were radiolabeled with 125 I using the Iodogen method. Fab of mAb 102 were prepared with papain according to the protocol described in Ref. 23.

Bacterial strains

The meningococcal strains ($n = 12$) used in the initial studies were randomly collected clinical isolates from the blood and cerebrospinal fluid samples of patients with meningococcal septicemia and/or meningitis in the Helsinki University Central Hospital region. The capsular serogroups of these strains were determined at the National Public Health Institute (Helsinki, Finland). *N. gonorrhoeae* (ATCC no. 31426) and *Neisseria lactamica* (ATCC no. 49142) strains were tested as controls.

The serogroup B meningococcal strain H44/76 (B:15:P1.7.16) has been described previously (24). Mutants of this strain that lacked either PorA or PorB were provided by Dr. P. van der Ley (Netherlands Vaccine Institute, Bilthoven, The Netherlands). The PorA and PorB3 deletion mutants of strain H44/76 were isolated by using the Ab-dependent bactericidal activity

of C with mAbs Mn15A14H6 (against PorB3) and Mn5C11G (against PorA), respectively (25). Unencapsulated mutants were derived by insertional inactivation of the polysialyltransferase (*siaD*) gene with plasmid pH10 as described previously (26). Mutants that were unable to sialylate their LOS molecules were created by insertional inactivation of the α (2,3)-sialyltransferase (*lst*) gene with plasmid pCR-Script-*lst*/Kan as described previously (27). The characteristics of the strains used in this study are shown in Table I. The bacteria were grown overnight on chocolate agar plates in 37°C in an atmosphere containing 5% CO₂. One colony was inoculated into a brain-heart infusion broth and grown to mid-logarithmic phase at 37°C with shaking.

Direct 125 I-C4bp-binding assays

The different strains of meningococci were grown to mid-logarithmic phase in brain-heart infusion broth. The bacteria were washed with veronal-buffered saline (VBS) (142 mM NaCl, 1.8 mM sodium barbital, and 3.3 mM barbituric acid (pH 7.4)) containing 0.1% gelatin (GVB), and the concentration was adjusted to 2×10^{10} cells/ml in one-third GVB. Twenty microliters of the bacterial suspension were incubated for 30 min at 37°C with 125 I-labeled C4bp (initial specific activity 1.4×10^7 cpm/ μ g) or with the recombinant CCP deletion mutants of C4bp (specific activities ranging from 4.9×10^6 to 2.0×10^7 cpm/ μ g). Equimolar amounts of the proteins (30 nM) were used in the binding assays. In the inhibition assays, heparin (0–1000 μ g/ml), BSA (0–100 nM/assay), Abs against C4bp (0.1–10 μ g/ml), or unlabeled C4bp (0–100 nM/assay) were added to the reaction mixture. After incubation, the mixtures were centrifuged (10,000 \times g, 3 min) through 20% sucrose (BDH Laboratory Supplies) in one-third GVB. To separate the pellets, the bottoms of the tubes were cut, and radioactive counts in the pellets and supernatants were measured in a gamma counter. The ratios of bound (pellet) to total (pellet + supernatant) radioactivities were calculated.

Binding of C4bp from serum

Meningococci (2×10^{10} bacteria/ml, 1.8×10^9 bacteria/assay) diluted in VBS were incubated with 3% heat-inactivated serum (HIS) or with VBS for 30 min at +37°C. The bacteria were then washed five times with GVB. After washing, the bacterial pellet was resuspended in 50 μ l of 0.1 M glycine-HCl (pH 2.5) and incubated for 30 min at +37°C to elute the bacteria-bound serum proteins. After incubation, the bacteria were centrifuged down, and the supernatant was transferred to a new tube and neutralized with 1 M Tris-HCl (pH 9.5). Samples of the supernatants were run into 5% SDS-PAGE gel under nonreducing conditions. The proteins were transferred to a polyvinylidene fluoride membrane (Pall), and nonspecific binding sites were blocked with 3% fish gelatin. mAb 104 against CCP1 of C4bp (1 μ g/ml) and alkaline phosphatase-conjugated rabbit anti-mouse Ab (1:1000) (DakoCytomation) were used for the detection of C4bp in the eluates. The relative densities of the bands were quantified using the ImageQuant program.

Cofactor assay for C4b degradation

To analyze the functional activity of the bacteria-bound C4bp, a C4b cofactor assay was used. The bacteria (2×10^{10} bacteria/ml, 4×10^8 bacteria/assay) in one-third GVB were incubated with C4bp (200 ng/tube) for 30 min and washed. Factor I (400 ng/tube) and radiolabeled C4b (7 ng/tube) were added to the tubes and incubated for 60 min at 37°C. The

Table I. Relevant phenotypes of strain H44/76 and its mutants used in this study

Strain/Mutant	LOS Sialic			
	Capsule	Acid	PorA	PorB3
H44/76	+	+	+	+
H44/76 Δ PorA	+	+	–	+
H44/76 Δ PorB3	+	+	+	–
H44/76 <i>lst</i> –	+	–	+	+
H44/76 <i>lst</i> – Δ PorA	+	–	–	+
H44/76 <i>lst</i> – Δ PorB3	+	–	+	–
H44/76 <i>siaD</i> –	–	+	+	+
H44/76 Δ PorA <i>siaD</i>	–	+	–	+
H44/76 Δ PorB3 <i>siaD</i>	–	+	+	–
H44/76 <i>siaD</i> – <i>lst</i> –	–	–	+	+
H44/76 <i>siaD</i> – <i>lst</i> – Δ PorA	–	–	–	+
H44/76 <i>siaD</i> – <i>lst</i> – Δ PorB3	–	–	+	–

samples were run in a SDS-PAGE gel under reducing conditions, and C4b cleavage was analyzed after autoradiography by the cleavage of the C4b α -chain. ^{125}I -C4b incubated with C4bp and factor I was analyzed as a positive control and ^{125}I -C4b without C4bp and/or factor I as a negative control.

Serum bactericidal assay

To examine the significance of PorA expression to serum resistance, we studied the survival of the meningococcal strains in serum. Normal human serum (NHS) was obtained from healthy laboratory personnel ($n = 9$) with no known history of neisserial disease. For the bactericidal assay, meningococci were grown in brain-heart infusion broth to mid-logarithmic phase and washed with VBS. Bacteria were suspended in VBS to a concentration of 1×10^4 bacteria/ml. Seventy-five-microliter portions of the bacterial suspensions were mixed with serum (final concentration 10%) diluted in VBS (75 μl) with or without Fab 102 (final concentration 50 nM). The suspension was incubated at 37°C with shaking. Aliquots were collected at 0, 30, and 60 min and cultured overnight on chocolate agar plates at 37°C and 5% CO_2 . Incubation in HIS was tested as a control. Survival was expressed as percentage of bacteria surviving in serum relative to survival in HIS at the corresponding time points or as percentage of bacteria surviving in the presence of Fab 102 in comparison to survival in serum in the absence of Fab 102.

Mapping of the C4bp binding sites on PorA by peptide scanning

For peptide scanning of potential C4bp binding sites, we chose surface-exposed loops of meningococcal PorA and PorB and gonococcal Por1A and Por1B. The selected sequences are listed in Table II. Twenty-amino acid long peptides with 2-aa transitions and 18-aa overlaps were used. The peptides were synthesized as spots onto polyethylene glycol-derivatized cellulose membranes (AIMS Scientific Products) using the peptide scanning instrument AutoSpot Robot ASP222 (Abimed Analysen-Technik). The membrane was incubated with radiolabeled C4bp (1×10^6 cpm). After washing, binding was detected by exposure on a phosphor imager plate and Fujifilm BAS 2500 instrument (Fuji Photo Film).

Results

PorA expression influences C4bp binding to meningococcal strains

We observed that several meningococcal strains bound the CP C inhibitor C4bp (Fig. 1). Clinical isolates of serogroup A and B meningococci (4×10^8 cells/assay) were incubated (30 min, 37°C) with radiolabeled C4bp in one-third GVB. *N. gonorrhoeae* (ATCC no. 31426) was used as a positive control, and *N. lactamica* strain (ATCC no. 49142) was tested as a representative nonpathogenic neisserial strain. C4bp was found to bind to several meningococcal strains (binding percentages ranging from 12 to 45%), although none of the meningococcal strains tested bound C4bp as strongly as the gonococcal strain (56%). Negligible C4bp binding (0.9%) was seen to *N. lactamica*.

We proceeded to test the binding of C4bp to the serogroup B meningococcal strain H44/76 and its 11 derivative mutants, which lacked either PorA or PorB or LOS sialic acid and/or capsule (Table I). As shown in Fig. 2, ^{125}I -C4bp bound to all strains expressing PorB3, but the binding to strains lacking PorA was much weaker. Deleting PorA from the wild-type (wt) strain and the strain with unsialylated LOS (*lst*⁻) decreased the binding of C4bp by 76 and 72%, respectively. However, deleting PorA from the unencapsulated mutant strains (*siaD*⁻ and *lst*⁻/*siaD*⁻) did not lead to as marked a decrease in C4bp binding (45 and 27% decrease, respectively) as seen with the encapsulated strain. These data suggest that PorA is necessary for the binding in encapsulated strains, but the loss of capsule may alter the surface architecture and possibly reveal additional ligands for C4bp. The capsule itself prevented the binding of C4bp by 50, 76, and 41% compared with the wt,

Table II. Amino acid sequences of meningococcal and gonococcal porins used in the peptide mapping analysis

Meningococcal PorA (GenBank accession no. X79056.1)	
Loop 1	GNNIQLQLTEQPSKQVGNKVTGKSRIRTKINDFGSFKG
Loop 2	AGEFGTLRAGRVANQFDDASQGI
Loop 3	NNDVASQLGIFKRHDDMP
Loops 4 and 5	VPAQNSKSAYTPAYVDEQSKYHAAVVGKPGSDVYYAGLNYK NGGFAGNYAFKYARHANVGRDAFELFLLGSGSDQAKGTDPLNH GLNLALAAQLDLSENADK
Loop 6	DFIERGKKGENTSYS
Meningococcal PorA (GenBank accession no. X81111.1)	
Loop 4	VPAQNSKSAYTPATYTVDSGCVTPVPAVVGKPGSDVYYAG
Loop 5	AGNYAFKYAKHANVGRDAFNLFLGRIGEDDEAKGTDPL
Meningococcal PorB (GenBank accession no. PH0224)	
Loop 1	TSRSVFHQNGQVTEVTATGIVDLGSK
Loops 2 and 3	KGGFGKLRVGRNLNSVLKDTGDIWPWDSKSDYLGVNKAIEPEARL
Loops 4 and 5	ALNDNAGRHNSESYHAGFNKNGGFFVQYGGAYKRHHQ VQEGLNIEKY
Loop 6	VSGYDNDALYASVAVQQQDAKLTDASN
Loop 7	KGLVDDADIGNE
Loop 8	QEGKGENKFVATAAS
Meningococcal PorB (GenBank accession no. X96496.1)	
Loop 6	DAKLTEENY
Loop 7	KGSFDATNYNND
Loop 8	SKFVSTAG
Gonococcal porin 1B (GenBank accession no. AF044793.1)	
Loop 1	TYRSVEHTDGKVSQVETGSEIADFGSKI
Loops 2 and 3	TIRAGSLNSPLKNTKDNVNAWESGKFTGNVLEISGMAKREHRY
Loops 4 and 5	APKDNSSNGESYHVLNLYQNSGFPAQYAGLQRYGEGT KKIEYDGGQAYSMPSLFVEKI
Loop 6	ALYVSVAAQQQDAKLYGATRVN
Loop 7	KGTVDSANHDNT
Loop 8	ADKIVSTASA
Gonococcal porin 1A (GenBank accession no. AF044796.1)	
Loop 5	EYEHQFYYSIPSLLEKLE
Meningococcal PorA (GenBank accession no. X78802.1)	
Loop 1	GRNYQLQLTEAQAANGGASQVVKTKAKSRIRTKISDFGFSFI
Loop 4	VPAQNSKSAYKPAYWTTVNTGSAATTFVPAVV

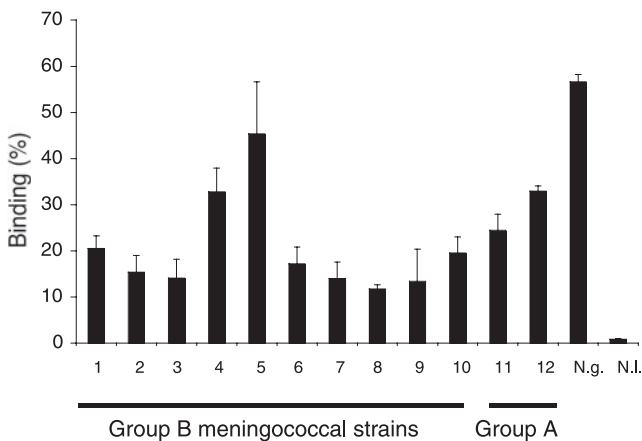


FIGURE 1. Binding of C4bp to clinical isolates of meningococci. Twelve cerebrospinal fluid or blood isolates of meningococci (samples 1–10, serogroup B; samples 11 and 12, serogroup A) were grown to log phase and incubated with radiolabeled C4bp. Binding was determined as percentage of bound radioactivity vs offered radioactivity. The binding of ^{125}I -C4bp to meningococci varied between 11 and 45%. *N. gonorrhoeae* (N.g.) was tested as a positive control (binding 56%). Binding to *N. lactamica* (N.I.), representing a nonpathogenic *Neisseria*, was <1%.

wt Δ PorA mutant, and wt Δ PorB mutant. However, even in the unencapsulated strains, loss of PorA resulted in a clear decrease in the binding of C4bp. In a marked contrast, deletion of PorB or the absence of LOS sialylation had no effect.

The binding of C4bp to meningococci is salt sensitive

The binding experiments thus far were performed in hypotonic buffer containing 50 mM salt (one-third GVB). To study the effect of salt concentration on the C4bp-meningococcus interaction, we increased the concentration of NaCl in the incubation buffer. Bacteria (4×10^8 bacteria/assay) and ^{125}I -C4bp were incubated in 30–100% GVB, and the binding was measured as described above. The binding decreased with increasing amount of salt in the incubation buffer (Fig. 3A), and an $\approx 90\%$ reduction in binding was seen at physiologic osmolarity. This suggested that the binding of C4bp to meningococci was ionic in nature. These data are consistent with our inability to detect C4bp binding to meningococci under iso-osmolar conditions (28).

Inhibition by heparin

Heparin binds to CCP1–3 of C4bp, with CCP2 as the most important domain required for this interaction (21). Using the direct binding assay, we tested whether the presence of heparin influenced the binding of C4bp to meningococci. Wild-type H44/76 meningococci were incubated with ^{125}I -C4bp in the presence of heparin (0–1000 $\mu\text{g}/\text{ml}$). As seen in Fig. 3B, heparin inhibited the binding of C4bp to meningococci in a dose-dependent fashion. Fifty percent inhibition of C4bp binding was observed at a heparin dose of 100 $\mu\text{g}/\text{ml}$.

Binding is inhibited by unlabeled C4bp

We tested the specificity of the C4bp binding to meningococci by adding increasing amounts of unlabeled C4bp or BSA (0–100 nM concentration) to the reaction mixture of wt meningococci and ^{125}I -C4bp. In Fig. 3C, it can be seen that the addition of unlabeled C4bp inhibited the binding of ^{125}I -C4bp but the addition unlabeled BSA did not. This suggests that the binding of C4bp to meningococci is specific and that the number of binding sites on the bacterial surface is limited.

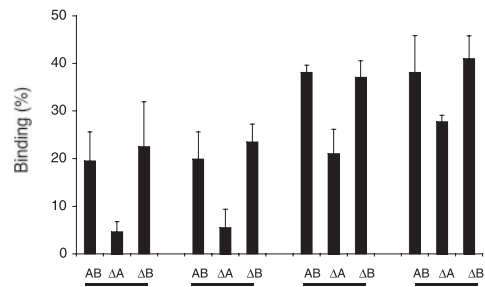


FIGURE 2. PorA is important for the binding of C4bp to meningococci. To determine the meningococcal structures important for binding of C4bp, various mutants of the wt H44/76 group B strain were analyzed: mutants with no terminal sialic acid on LOS (*lst* $^-$) or no capsule (*siaD* $^-$) and a mutant with a combined lack of capsule and terminal sialic acid of LOS (*siaD* $^-$ /*lst* $^-$). In addition, mutants of these strains with deletions of either PorA or PorB (Δ PorA and Δ PorB) were also tested. ^{125}I -C4bp was incubated with the mutant meningococcal strains (4×10^8 bacteria/assay) in one-third GVB, and binding was calculated as a percentage of bound radioactivity vs total radioactivity. Binding of C4bp to strains with PorA deletion was weaker than to strains expressing PorA. Strains with PorB deletion in general bound C4bp as well as the strains expressing both PorA and PorB. Strains without capsule showed an increased binding of C4bp, suggesting that the capsule could sterically hinder the interaction between C4bp and meningococci. Also in the unencapsulated mutants, decreased binding of C4bp to strains with PorA deletion was seen. Mean \pm SD values ($n = 4$) are shown. Δ A = Δ PorA, PorA-negative strains; Δ B = Δ PorB, PorB-negative strains.

Meningococci bind C4bp from serum

Meningococci were incubated with 3% HIS, and after washing, the bound serum proteins were eluted from the bacterial surface with 0.1 M glycine-HCl (pH 2.5). The eluates were run into a SDS-PAGE gel under nonreducing conditions, transferred onto a membrane, and C4bp was detected with a mAb. As can be seen in Fig. 4, wt (lane 2) and PorA and PorB deletion mutants (lanes 4 and 6, respectively) bound C4bp from serum. However, the binding by the PorA deletion mutant was weaker than by the wt and the PorB deletion mutant. The relative band densities were 100, 41, and 132% for the wt, PorA deletion mutant, and PorB deletion mutant, respectively. The eluates from bacteria incubated with VBS (lanes 3, 5, and 7) showed no reactivity. Thus, meningococci are able to bind C4bp from 3% serum.

Binding site on C4bp

To map the binding site on C4bp for meningococci, we analyzed recombinant mutants of C4bp with deletions of individual CCP domains. Wild-type meningococci were incubated with equimolar amounts (30 nM) of radiolabeled C4bp or the deletion mutants. The deletion of CCP2 and CCP3 decreased the binding of C4bp to meningococci by 70 and 59%, respectively (Fig. 5A). The deletion of CCP6 reduced the binding by 46%, but no other deletion resulted in such a marked decrease in binding of C4bp.

We also examined the binding site on C4bp by using mAbs directed against CCP1 (mAb 102), CCP1–2 (mAb 96), or CCP4 (mAb 67). Wild-type meningococci were incubated with ^{125}I -C4bp and varying amounts (0.1–10 $\mu\text{g}/\text{ml}$) of the mAbs. In Fig. 5B, binding is expressed as percentage of binding without Ab. mAbs against CCP1 and CCP1–2 of C4bp dose dependently inhibited the binding of ^{125}I -C4bp to meningococci, whereas the mAb against CCP4 had no significant effect on the binding. A polyclonal rabbit anti-C4bp Ab was also tested in this setting. The binding of C4bp to meningococci was inhibited by the polyclonal Ab at dilutions 1/300–1/10,000 (data not shown).

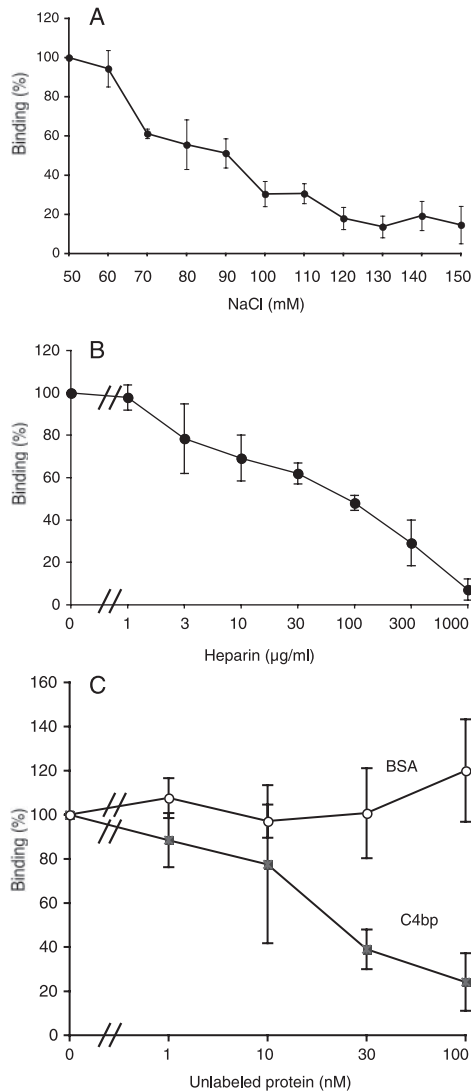


FIGURE 3. The effect of increasing ionic strength, heparin, and unlabeled C4bp on the binding of ^{125}I -C4bp to meningococci. *A*, Radiolabeled C4bp was incubated with wt serogroup B meningococci (H44/76) in veronal buffer with varying amounts of NaCl. The binding is shown as percentage of the binding seen at 45 mM NaCl in veronal buffer \pm SD. The binding of C4bp to meningococci was primarily seen under hypotonic conditions and was weak at 150 mM NaCl. *B*, Inhibition of binding of C4bp to meningococci by heparin. Wild-type meningococci were incubated with ^{125}I -C4bp and increasing amounts of heparin (0–1000 $\mu\text{g}/\text{ml}$) in 1/3 GVB. Binding is expressed as a percentage of ^{125}I -C4bp binding without heparin. Heparin dose dependently inhibited the binding of C4bp to meningococci. Fifty percent inhibition of binding was seen at 100 $\mu\text{g}/\text{ml}$ heparin, and binding was almost completely abolished when 1 mg/ml heparin was present. *C*, The binding of ^{125}I -C4bp is inhibited by unlabeled C4bp. Meningococci were incubated with ^{125}I -C4bp in the presence of varying amounts of unlabeled C4bp or BSA (0–100 nM) in one-third GVB. The addition of unlabeled C4bp resulted in a diminished binding of radiolabeled C4bp to meningococci while the addition of BSA had no effect.

Functional activity of meningococcus-bound C4bp

C4bp acts as a cofactor for factor I in the cleavage of C4b. Using the cofactor assay, we studied whether C4bp bound to meningococcal surface remained functionally active in promoting C4b cleavage. Group B meningococci (1×10^8 bacteria in one-third GVB) were incubated with C4bp and washed, after which factor I and radiolabeled C4b were added. After incubation, the samples were run in a SDS-PAGE gel under reducing conditions, and the

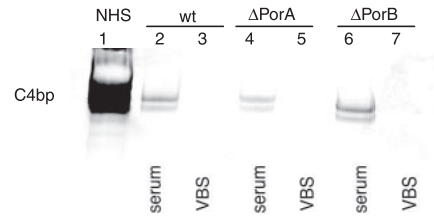


FIGURE 4. Meningococci bind C4bp from serum. Meningococci (wt, PorA-negative, and PorB-negative mutants) were incubated in 3% serum. Bacteria were then washed, and bound serum proteins were eluted with acidic glycine-HCl. Eluates were run into an SDS-PAGE gel under non-reducing conditions and immunoblotted against C4bp using a mAb. Meningococci were capable of binding C4bp from the serum, and the binding was stronger to the PorA-expressing strains (lanes 2 and 6) than to the PorA-negative strain (lane 4). The C4bp band density for the PorA-negative mutant (lane 4) was 41 and 31% of the C4bp band densities for the wt and the PorB-negative mutants, respectively. Elutions from bacteria incubated with VBS were negative for C4bp (lanes 3, 5, and 7).

cleavage of C4b was visualized by autoradiography. In Fig. 6, lanes 1 (wt) and 3 (PorB deletion mutant) show that C4b is cleaved, and the C4d fragment of the α' -chain becomes visible. In contrast, no appearance of C4d is seen when ^{125}I -C4b is incubated with factor I and the C4bp-treated, PorA-negative mutant (lane 2). Therefore, the PorA-positive strains bind enough functionally active C4bp to promote C4b cleavage. When PorA is deleted, no cleavage of C4b occurs.

Because C4bp is also a weak cofactor for factor I-mediated cleavage of C3b, we tested with wt meningococci whether meningococcus-bound C4bp would be active in this respect. Meningococci were incubated with C4bp and washed, and factor I and ^{125}I -C3b were added. Cleavage was analyzed by autoradiography as described above. Similarly to ^{125}I -C4b, ^{125}I -C3b became cleaved by C4bp bound to wt meningococci (data not shown).

Serum bactericidal assays

We next tested whether the expression of PorA conferred protection against direct serum bactericidal killing. For this, we used a serum bactericidal assay where PorA-positive and -negative meningococcal strains were incubated in 10% NHS. A potentially interfering aspect is the fact that some meningococcal strains also bind fH (Ref. 28 and our unpublished observations), which may render the interpretation of the serum sensitivity assays more difficult. We tried to overcome this by using the *lst⁻* strain, which lacks the LOS sialic acid that could be important for the fH binding. Fig. 7A shows the results of the survival of *lst⁻* and *lst⁻* ΔPorA strains in 10% human serum. The *lst⁻* strain expressing PorA survived better in 10% serum than the *lst⁻* strain lacking PorA. At 60 min, the survival of the PorA-positive meningococci was 95% compared with 39% of the PorA-negative bacteria. In contrast, the deletion of PorB did not influence the survival (data not shown).

As mAb 102 was shown to block the binding of C4bp to meningococci, we prepared Fabs of mAb 102 and tested whether the blocking of C4bp binding had any effect on the meningococcal survival. The Fab 102 fragments inhibited the binding of C4bp to meningococci in the direct binding assay (data not shown). In Fig. 7B, the survival of *lst⁻* and *lst⁻* ΔPorA strain in 10% serum in the presence of 50 nM Fab 102 was compared with the survival of the strains in serum without Fab 102. The survival of the C4bp-binding *lst⁻* strain decreased when the binding of C4bp was blocked by Fab 102. The survival of the *lst⁻* ΔPorA strain also decreased but

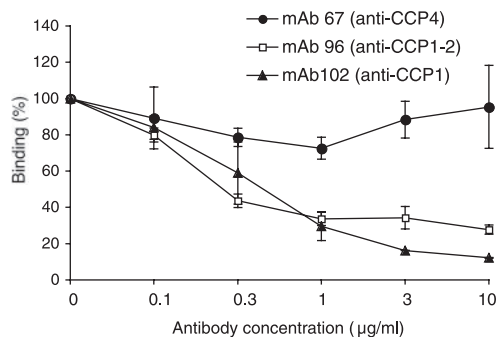
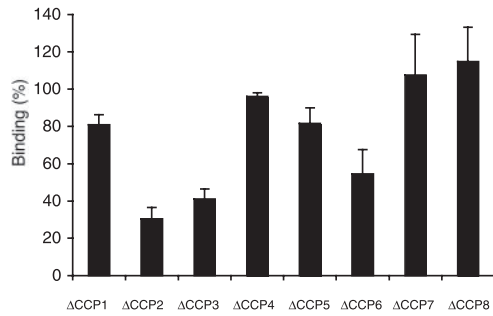


FIGURE 5. Localization of the meningococcus binding site on C4bp using C4bp deletion mutants and mAbs. *A*, H44/76 group B meningococci were incubated with radiolabeled C4bp or equimolar amounts (30 nM) of recombinant C4bp mutants with deletions of individual CCPs. Binding is expressed as percentage of the binding of whole plasma-derived C4bp. Deletion of CCPs 2 and 3 showed reduced binding (decrease of 70 and 59%, respectively). Deletion of CCP6 also decreased the binding but to a lesser extent (46% inhibition). *B*, Meningococci were incubated with ^{125}I -C4bp and increasing amounts (0.1–10 $\mu\text{g/ml}$) of mAbs against CCP1, CCP1–2, or CCP4. Binding is expressed as a percentage of the binding of C4bp without mAbs. Abs against CCP1 (mAb 102) and CCP1–2 (mAb 96) but not against CCP4 (mAb 67) inhibited the binding of C4bp to meningococci.

not as markedly. Thus, blocking of the binding of C4bp to meningococci increases their sensitivity to serum killing.

Putative C4bp binding site on PorA

Analyses with the H44/76 mutants suggested that PorA is important for C4bp binding. However, attempts to demonstrate a direct protein-protein interaction by ligand blotting failed, apparently because of the tight membrane-associated structure of PorA. Similarly, because of the strong hydrophobicity and membrane anchoring, surface plasmon resonance analysis could not be undertaken. To overcome this problem, we performed peptide mapping analysis by using only the extracellular regions of PorA. Peptides with amino acid sequences of the surface-exposed loops of meningococcal PorA and PorB proteins were synthesized for peptide spot analysis. Binding of C4bp was examined by incubating the peptide spot membrane with radiolabeled C4bp (Fig. 8A). ^{125}I -C4bp bound most strongly to two similar loop 1 sequences (KGKSRIRTK and KVTKAKSRIRTKI) (Fig. 8B). Both of these are rich in positively charged lysine and arginine residues. These sequences only partially overlap the loop 1 variable area. Strong binding was also seen to a loop 4 sequence SKYHAAVVGK, which is not as highly

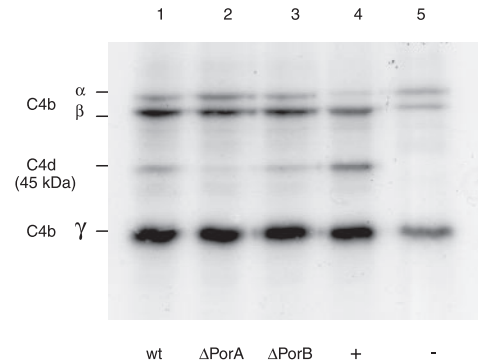


FIGURE 6. Analysis of the functional activity of meningococcal surface-bound C4bp. In the C4bp inactivation cofactor assay, meningococci were incubated with C4bp and washed. Factor I and ^{125}I -C4b were added, and after incubation, cleavage of C4b was visualized by autoradiography. Wild-type (H44/76) meningococci (lane 1) and the PorB deletion mutant (lane 3) bound C4bp, and C4b became cleaved as is evident by the appearance of the 45-kDa C4d band. No cleavage of C4b can be seen in lane 2 (PorA deletion mutant), indicating the importance of PorA for binding of C4bp. Lanes 4 and 5 are the positive (C4bp, factor I, and ^{125}I -C4b) and negative (^{125}I -C4b alone) controls, respectively. One representative experiment of three is shown.

charged. Weaker binding was seen to one loop 5 sequence of PorA and to one loop 3 and one loop 5 sequence of PorB. Thus, the peptide mapping suggested that C4bp has preferential binding sites on loops 1 and 4 of PorA.

Discussion

In this study, we show for the first time that serogroup B *N. meningitidis* binds the CP regulator C4bp. We found that PorA expression seems to be necessary for the binding, although we could not, probably because of the strong membrane association of PorA, demonstrate a direct protein-protein interaction. The binding is dependent on the ionic strength, and the bound C4bp remains functionally active as indicated by its ability to promote C4b inactivation by factor I.

For reasons not fully understood, humans are the only natural hosts of *N. meningitidis*. Up to 5–10% of adults are asymptomatic carriers of meningococci in their nasopharynx (1). It is estimated that pathogenic clones cause invasive disease in only 1% of individuals carrying these clones (1). However, the reasons why the same strain remains in the nasopharynx in one individual but penetrates the mucosa and causes systemic, sometimes fulminant, disease in another, are not understood.

It has earlier been shown that gonococci bind C4bp with their Por1A and Por1B proteins, thereby contributing to the serum resistance of gonococci (19). The gonococcal Por1 is homologous to meningococcal PorB (7). However, the deletion of PorB had no effect on the binding of C4bp to meningococci. Because gonococci do not express a PorA homologue, they may use PorB for C evasion. The reason why meningococcal PorB does not appear to contribute to C4bp binding is unknown but could be due to subtle sequence differences.

The interactions between meningococci and C are complex. As early as 1918, it was shown that C is required for the in vitro bactericidal activity of human serum against meningococci (29). Serogroup B meningococci are more resistant against C-mediated serum bactericidal activity than serogroup Y (30). However, group B strains are susceptible to phagocytosis after opsonization (30). Complement activation results in an increase of the anaphylatoxin C3a and C5a levels, which in concert with activated components of

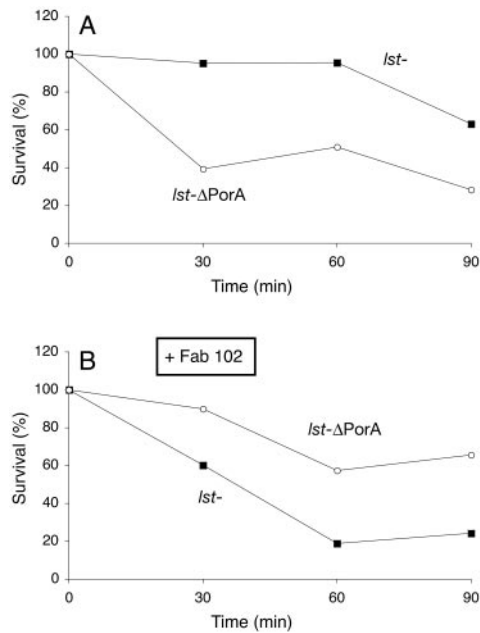


FIGURE 7. Sensitivity of PorA-expressing and PorA-negative strains to the bactericidal activity of serum. Sensitivity of meningococcal strains to C was tested using a bactericidal assay. Meningococcal strains were grown to log phase and incubated for indicated times with 10% serum at 37°C. Aliquots of the suspension were plated on chocolate agar plates and grown overnight at 37°C in 5% CO₂. *A*, Survival was determined by counting viable colonies and expressed as the percentage relative to survival in HIS at the corresponding time point. The encapsulated *Ist*⁻ strain expressing PorA survived better in 10% serum than the PorA-negative *Ist*⁻ΔPorA strain. *B*, The binding of C4bp to meningococci was blocked with Fab 102. Survival in the presence of Fab 102 in 10% serum is expressed as percentage of the survival in 10% serum without Fab. Inhibition of the binding of C4bp to *Ist*⁻ strain by Fab 102 decreased the survival of the strain. The survival of the PorA-negative *Ist*⁻ΔPorA strain was less affected by the blocking of C4bp binding.

the bradykinin system contribute to endothelial damage, vasodilatation, and capillary leakage. The level of C activation correlates with the severity of shock during meningococcal infection and also with the plasma levels of LOS (31–33). Both the AP and the CP are involved in C activation (34–36).

The significance of the lectin pathway in defense against meningococci is controversial. Some studies have shown that manose-binding lectin (MBL) deficiency is associated with an increased risk of meningococcal disease, whereas other studies have not demonstrated this (37, 38). MBL binds to serogroup B meningococcal mutants without LOS sialylation but not to the wt meningococci (39). MBL has also been shown to bind to serogroup C meningococci and increase C activation and C deposition on the meningococcal surface (40). In these studies, the major determinant of MBL binding to meningococcal surface was the structure and sialylation of LOS (39, 40). Recently, it was shown that MBL binds to PorB and Opa of serogroup C meningococci (41).

Meningococci release outer membrane vesicles (OMVs), or blebs, during infection (42). OMVs contain LOS and outer membrane proteins. LOS itself is a weak C activator, but the outer membrane proteins are strong activators (43). It has been suggested that C activation on the surface of meningococci occurs mainly through the CP, but activation in the fluid phase occurs through the AP, possibly on the surface of released OMVs (44). Complement activation on the OMVs does not lead to bacterial lysis but increases the inflammatory reaction and thus contributes to the development of shock.

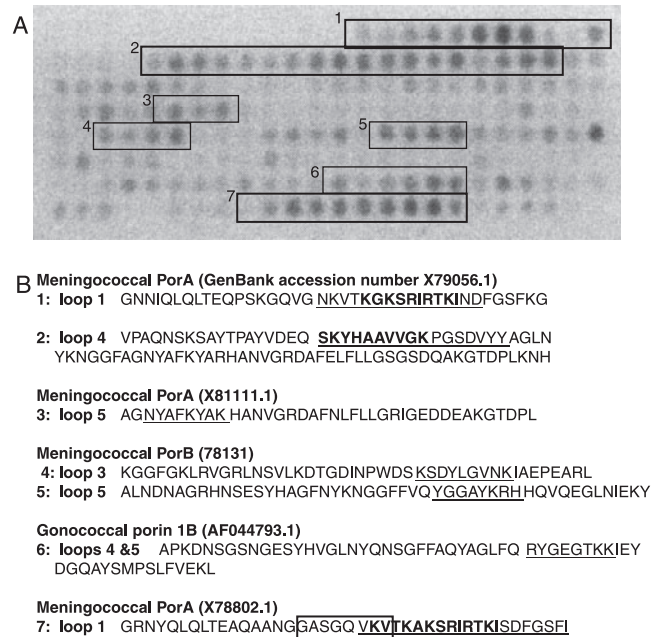


FIGURE 8. Peptide spot analysis of putative C4bp binding sites on meningococcal PorA. *A*, Twenty-amino acid peptides with 2-aa shifts of the surface-exposed regions of meningococcal PorA and PorB and gonococcal Por1A and Por1B were spotted on a cellulose membrane and incubated with ¹²⁵I-C4bp. After washing, binding was detected using a phosphor imager plate. The regions considered positive for binding are boxed. *B*, Putative C4bp-binding sequences on meningococcal and gonococcal porins. Residues common to all positive peptides are underlined. Stronger binding deduced from the peptide ligand blots is marked by bold letters, and weaker binding is marked with underlining. C4bp bound most strongly to two similar loop 1 sequences (KGKSRIRTK and KVTKAKSRIRTKI). The variable region of loop 1 is boxed in the sequence of one PorA. Strong binding was also seen to a loop 4 sequence SKYHAAVVGK. Weaker binding was seen to a loop 5 sequence of PorA and to loop 3 and loop 5 sequences of PorB.

CP activation on the meningococcal surface could be initiated through natural IgM Abs and also through cross-reactive Abs formed against nonpathogenic colonizing neisserial species and/or intermittently carried meningococcal strains. Also, certain enteric bacteria have capsules that are structurally and immunogenically related to the capsular polysaccharide of meningococci (1). Therefore, although the individual would have had no previous encounter with a specific meningococcal strain, the cross-reactive Abs could activate the CP on meningococci. MBL also binds to some meningococcal strains and initiates lectin pathway activation, which converges with the CP at C2 level. Therefore, it is important for meningococci to evade killing by the CP if they have to survive in the human host.

Gonococci with sialylated LOS are resistant to C-mediated lysis and less susceptible to C-dependent phagocytosis (45). In meningococci, both the capsule and LOS are important factors for serum resistance (36, 46). The ability of serogroup B meningococci to resist high levels of complement (as may be encountered in bloodstream) requires capsule expression; the role of LOS sialylation in this situation is less well defined (27). Our data shows that LOS sialic acid did not contribute to the binding of C4bp to meningococci. In previous studies, LOS sialylation inhibited the binding of MBL to meningococci (39, 40). Capsulated mutants bound less C4bp than their unencapsulated counterparts, suggesting that the capsule may hinder C4bp binding to somatic targets, in this instance PorA. Despite binding less C4bp than unencapsulated

strains, encapsulated strains are more serum resistant, suggesting that capsular polysaccharide mediates resistance to C via a mechanism independent of C4bp binding. Deleting the capsule seemed to reveal new ligands for C4bp because the absence of PorA did not abrogate the binding of C4bp to the unencapsulated mutant strains (*siaD*⁻ and *siaD*⁻/*lst*⁻ mutants). All meningococcal strains that cause invasive disease are encapsulated. However, meningococci probably switch off their capsule expression to invade the mucosal epithelium and re-express the capsule when they enter the systemic circulation (47, 48). Therefore, meningococci also need to evade the host immune system in the temporary unencapsulated phase.

We could observe direct binding of C4bp by meningococci primarily under hypotonic conditions. In contrast, the Por molecules of serum-resistant gonococci can bind C4bp under iso-osmolar conditions. C4bp-binding gonococci are capable of surviving treatment by higher levels of C compared with unencapsulated meningococci. The differences in avidity of C4bp binding to these two neisserial species may, at least in part, account for their relative differences in serum resistance. However, we could show binding of C4bp from 3% serum to meningococci under physiological ionic strength conditions (Fig. 4).

The C inhibitory activity of C4bp requires CCPs 1–3 (13). With recombinant deletion mutants of C4bp, we found that CCPs 2 and 3 are needed for the PorA–C4bp interaction. Heparin inhibited dose dependently the binding of C4bp to meningococci. CCPs 1–3 of C4bp are needed for heparin binding, with CCP2 as the most important in this interaction (13). Thus, these results point at CCP2, and possibly CCP3, being crucial for the PorA binding. However, when mAbs against C4bp were used, Abs against CCP1 and CCP1–2 both inhibited the binding. This apparent discrepancy could be explained by steric hindrance caused by the Ab bound to CCP1, resulting in decreased exposure of the binding site on CCP2.

Based on the direct binding assays using deletion mutants of the H44/76 strains, PorA emerged as the putative C4bp ligand on meningococci. To verify that the ligand for C4bp is PorA, it would be necessary to directly demonstrate the protein-protein interaction. However, because PorA is an integral membrane protein with extracellular loops and transmembrane segments, it is difficult to study the interaction directly at the protein level using isolated proteins. No binding of radiolabeled C4bp to PorA (or PorB) run into a SDS-PAGE gel and transferred to a nitrocellulose membrane could be detected (data not shown). These results suggest that the binding of C4bp to PorA is dependent on the conformation and proper orientation of the PorA protein on the outer membrane of the meningococcus.

As an alternative approach to study the C4bp–PorA interaction, we used peptide mapping analysis. The peptide spot analysis revealed putative binding sites for C4bp on the meningococcal PorA protein. The strongest binding of ¹²⁵I–C4bp was seen to loops 1 and 4, i.e., the variable loops. However, on loop 1, the putative binding area was overlapping only partially the variable site (Fig. 8). The most prominent regions contained positively charged residues such as lysine and arginine. However, the putative region on loop 4 was rich in alanine and valine, small uncharged amino acids. PorA molecules form trimers, in which loops of each molecule come into contact with each other. Therefore, depending on the three-dimensional conformation of the loops in the PorA trimer, the putative binding sites can form one to six ligands for C4bp. The avidity of the binding interaction is increased by the redundancy of binding sites on C4bp: each of the seven α -chains could bind to PorA independently.

C4bp bound to the meningococcal surface remained functionally active as analyzed by the cofactor assay. C4bp acts as a cofactor for factor I in the cleavage of C4b, a function analogous to that of factor H of the AP in the cleavage of C3b. When C4bp binds to PorA on the meningococcal surface, CP activation is restricted. Because C4bp is also a weak cofactor for C3b cleavage, AP activation may also be affected. The C4bp-binding strains also survived better in the serum bactericidal assay we used. The difference between PorA-positive and -negative strains was clear and consistent. Also, the blocking of the binding of C4bp by using a Fab of the mAb 102 resulted in decreased survival of the PorA-expressing strain. This suggests that binding of C4bp is one of the factors affecting the serum resistance of group B meningococci.

Hazelzet et al. (33) have studied the plasma levels of C regulators in survivors and nonsurvivors of meningococcal infection. Lower C4bp serum levels significantly correlated with mortality during the first 24 h of infection. The difference could be due to a higher amount of bacteria, which may sequester C4bp, or to C consumption because of a strong C activation and C4b deposition caused either by released outer membrane vesicles or endothelial damage.

Protein S, which is required for the anticoagulant functions of protein C, forms an irreversible complex with C4bp in circulation (49). Only free protein S is functionally active (50). The plasma levels of proteins S and C are decreased during fulminant meningococcal infection (51). Also, meningococcal disease is associated with endothelial damage, hemorrhages, and microthrombi in small vessels. These events are of major importance in the development of skin necrosis and other sequelae of meningococcal disease. Therefore, the question whether the meningococcus–C4bp (or meningococcus–C4bp–protein S) interaction contributes to the dysfunction of the coagulation-anticoagulation pathways and the development of disseminated intravascular coagulation during fulminant meningococcal septicemia is intriguing.

In conclusion, we show in this study that serogroup B meningococci bind C4bp and the binding depends on the expression of the major outer membrane protein PorA. Complement activation is essential in the defense against neisserial infections, but in contrast, the degree of complement activation also correlates with the severity of the symptoms. Interestingly, C4bp circulates in complex with the anticoagulant protein S, and fulminant meningococcal disease is characterized by severe dysfunction of the anticoagulant activities. Additional studies on the role of the C4bp–meningococcus interaction on the development of fulminant meningococcal disease are warranted. We propose that binding of the classical and lectin pathway inhibitor C4bp is one of the complement evasion mechanisms of meningococci.

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Disclosures

The authors have no financial conflict of interest.

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