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Inhibition of the Classical Pathway of Complement by Meningococcal Capsular Polysaccharides

Sarika Agarwal,¹ Shreekant Vasudhev,¹ Rosane B. DeOliveira, and Sanjay Ram

Almost all invasive Neisseria meningitidis isolates express capsular polysaccharide. Ab is required for complement-dependent killing of meningococci. Although alternative pathway evasion has received considerable attention, little is known about classical pathway (CP) inhibition by meningococci, which forms the basis of this study. We engineered capsulated and unencapsulated isogenic mutant strains of groups A, B, C, W, and Y meningococci to express similar amounts of the same factor H–binding protein (fHbp; a key component of group B meningococcal vaccines) molecule. Despite similar anti-fHbp mAb binding, significantly less C4b was deposited on all five encapsulated mutants compared with their unencapsulated counterparts (p < 0.01) when purified C1 and C4 were used to deposit C4b. Reduced C4b deposition was the result of capsule-mediated inhibition of C1q engagement by Ab. C4b deposition correlated linearly with C1q engagement by anti-fHbp. Whereas B, C, W, and Y capsules limited CP-mediated killing by anti-fHbp, the unencapsulated group A mutant paradoxically was more resistant than its encapsulated counterpart. Strains varied considerably in their susceptibility to anti-fHbp and complement despite similar Ab binding, which may have implications for the activity of fHbp-based vaccines. Capsule also limited C4b deposition by anti–porin A mAbs. Capsule expression decreased binding of an anti-lipo polysaccharide IgM mAb (∼1.2- to 2-fold reduction in fluorescence). Akin to observations with IgG, capsule also decreased IgM-mediated C4b deposition when IgM binding to the mutant strain pairs was normalized. In conclusion, we show that capsule polysaccharide, a critical meningococcal virulence factor, inhibits the CP of complement.

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C omplement-dependent bactericidal activity is an established correlate of protection against meningococcal disease. Intrathecal serotherapy with equine anti–meningococcal antiserum was shown to be beneficial in meningococcal meningitis as far back as the early 1900s by Flexner and Jobling (1, 2), which suggested a role for Ab in host defenses against invasive disease. Elegant studies by Goldschneider et al. (3) firmly established a role for Ab in protection against invasive meningococcal infection. They reported an inverse relationship between the incidence of meningococcal disease and age-specific serum bactericidal activity against prototypic groups A, B, and C meningococcal strains (3). These findings were corroborated by a more recent study in the United Kingdom (4).

Whereas colonization of the human nasopharynx by Neisseria meningitidis is a relatively common event, invasive disease is rare (5). Previously “nonimmune” individuals may be protected against invasive meningococcal infection. They reported an inverse relationship between the incidence of meningococcal disease and age-specific serum bactericidal activity against prototypic groups A, B, and C meningococcal strains (3). These findings were corroborated by a more recent study in the United Kingdom (4).

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Materials and Methods

Bacterial strains and mutants

Isogenic mutant strains were derived from *N. meningitidis* strains A2594 (WUE2594), H44/76, C2120, and Y2225. Their relevant characteristics are listed in Table I. LOS sialylation was abrogated by deleting LOS sialyltransferase (*lst*) which encodes *lst* as described previously (18). Note that group A isolates do not sialylate their LOS unless supplied with cytidylenosinomphospho-N-acetyl neuraminic acid in growth media. To delete *lst* from group A strain 2594, *mynB* (its newly proposed designation is *cmyA*) (34) was deleted as described previously (18). The groups B, C, W, and Y isolates were rendered unencapsulated by deleting their respective polyosialyltransferase *siaD* genes (19, 33). These genes are now designated *siaB*, *siaC*, *siaE*, and *siaY*, respectively. (34, 35). *hfbp* from A2594, C2120, W171, and Y2225 was replaced with *Hfp* from H44/76 (variant 1 *hfbp*) as follows: (i) previously IgG alkaline phosphatase was used to construct a mutant of group Y strain Y2220, called Y2220 siaD44/76mynB43/76 (Hfp was previously called genome-derived *Neisseria* Ag 1870) (12), that expresses the full-length H44/76 Hfp (14). This mutant contains the tetracycline-resistance marker immediately 3′ to Hfp. DNA extracted from Y2220 siaD44/76mynB43/76 was used to transform encapsulated strains that lacked LOS sialylation (A2594, C2120 lst, W171 lst, and Y2225 lst) and unencapsulated/LOS unsialylated mutants A5094 mynB, C2120 siaD lst, W171 siaD lst, and Y2225 siaD lst. Tetracycline-resistant colonies were selected (the minimum inhibitory concentration of tetracycline for each strain was determined) and screened for binding to mAb JAR1 that is specific for variant 1 *hfbp* expressed by H44/76 (35). The *Hfp* of clones that bound to mAb JAR1 was sequenced to verify the presence of the entire H44/76 *hfbp* sequence. For simplicity, we hereon refer to the strains by their original serogroup (i.e., A, B, C, W, or Y) followed by Cap+ or Cap− to denote encapsulated and unencapsulated mutants, respectively. All mutants used in this study lack LOS sialic acid and express the same variant 1 *hfbp* molecule.

Complement proteins

Purified C1 complex (200 μg/ml), C1q (1 mg/ml), and C4 (4 mg/ml) were from Complement Technology (Tyler, TX). Protease inhibitors in purified C1 (EDTA, benzamidine, and e-aminono-C-apicoid acid) were removed by dialysis at 4°C against PBS using an Amicon Ultra-4 centrifugal filter unit (30 kDa cutoff) prior to use.

IgG- and IgM-depleted serum

IgG and IgM were depleted from serum as described previously by passage over protein G–Sepharose and anti-human IgM agarose (36). Briefly, serum that was treated with EDTA (10 mM) to prevent complement activation and 1 M NaCl to minimize C1q loss during immunodepletion was passed over immobilized protein G and anti-human IgM at 4°C. The estimated amount of IgG and IgM in serum did not exceed the binding capacity of the columns. The fall-through was spin-concentrated and dialyzed against PBS with an Amicon Ultra-15 (30 kDa cutoff). Hemolytic activity was verified with a total hemolytic complement kit (The Binding Site, Birmingham, U.K.). IgG and IgM depletion was ascertained by dot blot assays on polystyrene membranes as described previously (36). Serum was stored in single-use aliquots at −70°C.

Abs and conjugates

Anti-variant 1 *hfbp* mAb JAR1 (mouse IgG3) has been described previously (35). For simplicity, JAR1 will henceforth be called anti-fHbp. Bacterial strains and mutants

Western blotting

C4b deposited on bacteria was analyzed by Western blotting as described previously (41, 42). Bacteria were incubated with anti-fHbp, C1, and C4 as described above, washed three times, and pellets were lysed with 4% LDS sample buffer containing 2-ME. The lysates were electrophoresed on 4–12% Bis-Tris gels and transferred to a polyvinylidene difluoride membrane by Western blotting. Blots were cut horizontally at the 50 kDa marker; proteins that migrated above this were probed with sheep anti-human C4 to detect adducts of the C4 α-chain covalently linked to its bacterial targets, whereas proteins migrating faster than the 50 kDa marker were stained with Coomassie blue, which served as a control to indicate equal loading of Cap+ and Cap− bacterial lysates.

Serum bactericidal assay

Serum bactericidal assays were performed as described previously (40). Bacteria that had been harvested from an overnight culture on chocolate agar plates were repassaged onto fresh chocolate agar and allowed to grow for 4–5 h at 37°C in a atmosphere containing 5% CO2 and then suspended in HBSS++ (Harbor Biologicals, Gaithersburg, MD). Overnight cultures of 2000 CFU *N. meningitidis* with anti-HPBP (0.25 μg/ml) were incubated with IgG/IgM-depleted serum (5%) that contained 100 μg/ml of the anti-factor Bb mAb, in a final reaction volume of 75 μl. This concentration of mAb and complement was determined following preliminary titration experiments. Aliquots of 12.5-μl reaction mixtures were plated onto chocolate agar in duplicate at the beginning of the assay (t0) and again after incubation at 37°C for 30 min (t30). Survival was calculated as the number of viable colonies at t30 relative to t0.

Results

**Capsule inhibits C4b deposition by anti-fHbp mAb JAR1**

To determine the effect of capsule on CP activation mediated by an anti-fHbp mAb, we replaced the fHbp molecule of the isogenic pairs of strains (A2594 and A2594 mynB, C2120 lst and C2120 siaD lst, W171 lst and W171 siaD lst, and Y2225 lst and Y2225 siaD lst) derived from the strains listed in Table I with the variant 1 *hfbp* molecule of strain H44/76. We elected to use mutants lacking LOS sialic acid (lst mutants) because LOS is a target for C4b deposition on the pathogenic neisseriae (41, 42), and LOS sialylation also limits C4b deposition on bacteria (42). Furthermore, deleting capsule (*siaD* mutants) on groups B, C, W, and Y isolates may “shunt” cytidylenosinomphospho-N-acetyl neuraminic acid from capsule biosynthesis on to LOS and result in a greater proportion of sialylated LOS on Cap+ strains (22). As shown in Fig. 1, all mutants bound similar amounts of anti-fHbp by flow cytometry.

Bacteria were then incubated with anti-fHbp (10 μg/ml), purified C1 complex (7.5 μg/ml), and pure C4 (50 μg/ml) and C4b deposited on the bacterial surface was measured by flow cytometry. Significantly less C4b was deposited on each of the Cap+ strains compared with its corresponding Cap− mutant (Fig. 2A; representative histogram tracings are shown in Supplemental Fig. 1). C4b deposition was dependent on the presence of specific Ab, as revealed by minimal C4b deposition when bacteria were in-
cubated with pure C1 and C4 alone (Fig. 2B). Considerable variation in the amount of C4b deposited across the Cap mutants was noted; the least amount of C4b was deposited on the group A Cap mutant and intermediate amounts were deposited on the group B Cap mutant, whereas high levels of C4b were deposited on the groups C, W, and Y Cap mutants. Bacteria were then incubated with anti-fHbp and IgG/IgM-depleted normal human serum and C4b deposition was measured. With the exception of the group A mutant pair where similar and low amounts of C4b were deposited, inhibition of C4b deposition by capsular polysaccharide mirrored findings with purified C1 and C4 (Fig. 2C). Similar to observations in Fig. 2B, minimal C4b was deposited when anti-fHbp was excluded from the reaction mixture (≤10% of the fluorescence seen when anti-fHbp was present in the reaction mixture; data not shown). We also performed Western blotting analysis of bacteria that were incubated with anti-fHbp and purified C1 and C4 (Fig. 2D), which confirmed findings of the flow cytometry assays using a second independent method. Note that the targets for C4b deposited by anti-fHbp were LOS and opacity protein (Opa), as described previously in studies using normal human serum (41). Collectively, the data suggest that all five major meningococcal capsular polysaccharides interfere with C4b deposition mediated by an anti–fHbp mAb.

Capsule interferes with C1q engagement by anti-fHbp

C4b deposition on a surface is the result of several steps. C1 complex binding by Ab induces a conformational change in C1q, which results in autoactivation of C1r. Activated C1r then cleaves and activates C1s and the latter cleaves a 9-kDa fragment from the N terminus of the α-chain of C4, called C4a, which exposes an internal thioester bond in C4. Nucleophilic attack of the exposed and metastable thioester in the C4b fragment by hydroxyl or amino groups leads to formation of covalent ester or amide bonds, respectively (43, 44).

The ability of anti-fHbp to engage C1q represents an early step in Ab-mediated CP activation and was studied next. As shown in Fig. 3A (Supplemental Fig. 2 shows representative histograms), capsule expression interfered with C1q binding by anti-fHbp. The amount of C1q bound also varied greatly across Cap mutants and showed a statistically significant positive correlation with the amount of anti-fHbp–mediated C4b deposition (Fig. 3B). Collectively, these data show that despite similar anti-fHbp binding, the ability of anti-fHbp to activate the CP and deposit C4b varies across strains.

Effects of capsular polysaccharide expression on CP-mediated killing of meningococci by anti-fHbp

We next determined the ability of anti-fHbp to mediate complement-dependent bactericidal activity through the CP. Anti–factor Bb mAb (100 μg/ml) was added to IgG/IgM-depleted serum to block the alternative pathway of complement (40). As shown in Fig. 4, the groups B, C, W, and Y encapsulated mutants were significantly more resistant to CP-mediated killing by the anti–fHbp mAb. Paradoxically, the Cap group A mutant was more resistant than the isogenic Cap + mutant. Controls where bacteria were incubated with complement (IgG/IgM-depleted serum) alone resulted in 120–200% survival in every instance (data not shown).
Capsule inhibits C4b deposition by anti-PorA mAbs

To ensure that the effects of capsular polysaccharide on CP activation was not specific for anti–fHbp Ab, we next measured C4b deposition mediated by anti-PorA mAbs that were available against four of the strains used in this study (groups A, B, C, and Y). Whereas capsule expression did not affect binding of the mAbs to the bacterial surface (Fig. 5A), decreased C4b deposition was noted on the Cap + mutants (Fig. 6A). The differences were the least with the group A mutant pair (~1.2-fold) and the highest (~2-fold) with the group B strain pair.

To determine whether capsule interfered with C4b deposition by IgM mAb 3F11, we first “normalized” the amount of 3F11 binding to the Cap + mutants (Fig. 6A). The differences were the least with the group A mutant pair (~1.2-fold) and the highest (~2-fold) with the group B strain pair.

Capsule inhibits CP activation by IgM directed against LOS

IgM is very efficient in fixing complement because a single IgM molecule can engage C1. However, IgM (Mr of ~900 kDa) is considerably larger than IgG (Mr of ~150 kDa) and the effects of capsule on binding of and complement activation by IgM directed against noncapsular membrane Ags are not clear and were investigated next.

Anti–LOS IgM mAb 3F11 directed against the unsialylated lacto-N-neotetraose structure that was elaborated by all strains used in this study was tested for its ability to bind to the Cap + and Cap − mutants. At all dilutions tested, we noted lower binding of mAb 3F11 to the Cap + mutants (Fig. 6A). The differences were the least with the group A mutant pair (~1.2-fold) and the highest (~2-fold) with the group B strain pair.

Discussion

A novel finding in this study was that all five major meningococcal capsules interfered with engagement of pure C1q by IgG Fc, which

FIGURE 2. Capsule expression limits anti–fHbp–mediated C4b deposition. (A) Bacteria were incubated with anti–fHbp (10 μg/ml), purified C1 complex (7.5 μg/ml), and pure C4 (50 μg/ml) and C4b deposited on bacteria (median fluorescence) was measured by flow cytometry. The control represents the fluorescence of a reaction mixture that lacked complement components. The median fluorescence of each reaction was recorded and each bar represents the mean (SEM) of three experiments (Supplemental Fig. 1 shows representative histograms). ***p < 0.001 (ANOVA). (B) C4b deposition on meningococci was Ab-dependent. Bacteria were incubated with C1 and C4 in the absence of anti–fHbp, and C4b deposition was measured by flow cytometry. Axes and controls are as described in (A) [note the magnified lower half of the y-axis compared with panel (A)]. (C) Capsule inhibits anti–fHbp–mediated C4b deposition in the presence of human serum. Isogenic capsule mutants were incubated with anti–fHbp, followed by the addition of IgG- and IgM-depleted human serum at a final concentration of 3.3%. Axes are as described above. *p < 0.05, **p < 0.01 (ANOVA). (D) Western blotting confirms capsule-mediated inhibition of C4b deposition and identifies LOS and Opa as targets for C4b deposited by anti–fHbp. Bacteria were incubated with anti–fHbp, C1, and C4 as described in (A). The ~87-kDa α′–chain of C4b binds to its bacterial targets covalently through ester or amide bonds. Electrophoresis under reducing conditions results in the ~87-kDa α′ migrating as a complex with its bacterial targets. Proteins were transferred to a polyvinylidene difluoride membrane by Western blotting to reveal C4b α′–bacterial target complexes. The locations of the ~90- and ~115-kDa complexes of (LOS plus C4b α′, labeled α′+LOS (~90 kD)) and (Opa plus C4b α′, labeled α′+Opa (~115 kD)) are indicated; the identity of these bands was defined previously (41). The lane marked C4 contains pure C4, and the positions of the intact 95-kDa α (labeled α (95 kD)) and 75-kDa β (labeled β (75 kD)) chains are shown. Control lanes contained bacteria alone (labeled W Cap − (alone)) or bacteria plus C1 and C4 with no added anti–fHbp. Proteins migrating below the 50 kDa marker were stained with Coomassie blue to ensure similar protein loading across the isogenic Cap + and Cap − mutants.
added to block alternative pathway activation. Percentage survival of bacteria at 30 min (mean ± SEM) of five separate experiments) is shown on the y-axis. Note the segmented y-axis for the group C strain. *p < 0.05, **p < 0.01, compared with the corresponding isogenic mutant (two-tailed t test).

The effects of meningococcal capsular polysaccharide on CP-mediated killing by anti-fHbp. The five Cap- meningococcal mutants were incubated with anti-fHbp (0.25 μg/ml) followed by the addition of serum immunodepleted of IgG and IgM (5%) to which anti–Bb mAb (100 μg/ml) was added to block alternative pathway activation. Percentage survival of bacteria at 30 min (mean ± SEM of five separate experiments) is shown on the y-axis. Note the segmented y-axis for the group C strain. *p < 0.05, **p < 0.01, compared with the corresponding isogenic mutant (two-tailed t test).
In a similar manner, capsule may also decrease engagement of the relatively large C1q molecule (∼460 kDa) by anti–fHbp Fc. C1q is formed by 18 polypeptide chains (6 A, B, and C chains) that are organized into six subunits, each comprising an A, B, and C chain. Engagement of C1q by IgG is a complex event. Recent data have shown that the IgG bound to surfaces form ordered Ab hexamers through noncovalent interactions of Fc, which results in binding of activated C1 (50). Furthermore, the interaction between the globular head of C1q and IgG interaction is ionic in nature (51), and interactions between charged amino acids contribute to C1q–IgG binding (52–54). How capsules interfere with C1q engagement by Ab remains unclear. Based on published literature, possibilities include restriction of IgG hexamer formation and/or interference of ionic interactions between C1q and IgG by the negatively charged capsules.

Another interesting finding was that the major targets for C4b deposition when Ab was specifically directed against fHbp were LOS and Opa, similar to that we have described previously when using a complement source devoid of alternative pathway activity (achieved in this instance by blocking factor B function) to inhibit results of serum bactericidal assays as they relate to CP activation. With the exception of the group A mutants, capsule expression decreased CP-dependent killing by anti-fHbp. The reason for the greater (and unexpected) resistance to killing of the Cap+ group A mutant relative to its Cap− counterpart (Fig. 4) remains unclear, but note that inhibition of C4b by group A capsule that was seen in the presence of anti-fHbp, pure C1, and pure C4 (Fig. 2A) was not seen in the presence of anti-fHbp and IgG/IgM-depleted serum (Fig. 2C). Complement deposition because greater expression of the target Ag would have permitted higher levels of Ab binding and more killing. As stated above, group C capsule regulates the alternative pathway, and upregulation of capsule could dampen alternative pathway amplification initiated by the CP. Activation of complement relatively distal to the membrane, which prevents insertion of bactericidal membrane attack complex, may have also contributed to serum resistance. These capsule “hyperproducers” were also resistant to killing by an anti-PorA mAb P1.5 (55). Although the reason cited was inhibition of the alternative pathway, an additional but not mutually exclusive explanation is capsule-mediated CP inhibition as we have reported in the present study. Whether complement activation by anticapsular Ab and Ab directed against membrane Ags differs quantitatively or qualitatively is an important consideration from the perspective of vaccine development.

An additional important finding that emerged from this study was the wide variation in the amount of C4b deposited by anti-fHbp across the five unencapsulated meningococcal mutants, despite similar amounts of Ab binding. Levels of Neisserial surface protein A expression (20) and different PorB alleles (19) are two variables that modulate alternative pathway activity, which necessitated the use of a complement source devoid of alternative pathway activity (achieved in this instance by blocking factor B function) to interpret results of serum bactericidal assays as they relate to CP activation. With the exception of the group A mutants, capsule expression decreased CP-dependent killing by anti-fHbp. The reason for the greater (and unexpected) resistance to killing of the Cap+ group A mutant relative to its Cap− counterpart (Fig. 4) remains unclear, but note that inhibition of C4b by group A capsule that was seen in the presence of anti-fHbp, pure C1, and pure C4 (Fig. 2A) was not seen in the presence of anti-fHbp and IgG/IgM-depleted serum (Fig. 2C). Complement deposition...
and bactericidal activity in the context of serum requires participation of several proteins of the complement cascade and is modulated by complement inhibitors, which underscores the importance of corroborating data obtained using pure complement components with serum. Variation in the amount of C4b deposited and CP-mediated killing across strains despite similar amounts of anti-fHbp binding has important implications for the activity and evaluation of efficacy of fHbp-based vaccines. As an example, these data suggest that binding of anti-fHbp Ab alone may not predict the amount of CP activation and/or complement-dependent bacterial killing on different strains. The factor(s) that contribute to variations in C4b deposition among the strains remain to be elucidated. An example of surface variation that lead to differences in complement activation was described by Brown et al. (56) where C2 cleavage varied markedly across human, guinea pig, and sheep erythrocytes that were all sensitized with similar amounts of C1 and C4.

Direct binding of C1 to several bacterial species can trigger CP activation independently of Ab (57–62). However, in this study only a low amount of C4b was deposited on meningococci that were incubated with C1 and C4 in the absence of Ab (Fig. 2B). These data are consistent with a requirement for Ab to kill meningococci (complement alone does not kill the bacteria), which forms the basis of evaluation of Ab responses to immunization by the serum bactericidal assay to predict protection against invasive disease (3).

In conclusion, we have shown that meningococcal capsular polysaccharides attenuate the CP of complement. These data highlight how this key virulence factor can modulate multiple facets of the complement cascade to subvert host immune responses.

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Disclosures
The authors have no financial conflicts of interest.

References
MENINGOCOCCAL CAPSULES INHIBIT THE CLASSICAL PATHWAY


