Properdin Is Critical for Antibody-Dependent Bactericidal Activity against *Neisseria gonorrhoeae* That Recruit C4b-Binding Protein

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Gonorrhea, a sexually transmitted disease caused by Neisseria gonorrhoeae, is an important cause of morbidity worldwide. A safe and effective vaccine against gonorrhea is needed because of emerging resistance of gonococci to almost every class of antibiotic. A gonococcal lipooligosaccharide epitope defined by the mAb 2C7 is being evaluated as a candidate for development of an Ab-based vaccine. Immune Abs against N. gonorrhoeae need to overcome several subversive mechanisms whereby gonococcus evades complement, including binding to C4b-binding protein (C4BP; classical pathway inhibitor) and factor H (alternative pathway [AP] inhibitor). The role of AP recruitment and, in particular, properdin in assisting killing of gonococci by specific Abs is the subject of this study. We show that only those gonococcal strains that bind C4BP require properdin for killing by 2C7, whereas strains that do not bind C4BP are efficiently killed by 2C7 even when AP function is blocked. C3 deposition on bacteria mirrored killing. Recruitment of the AP by mAb 2C7, as measured by factor B binding, occurred in a properdin-dependent manner. These findings were confirmed using isogenic mutant strains that differed in their ability to bind to C4BP. Immune serum that contained bactericidal Abs directed against the 2C7 lipooligosaccharide epitope as well as murine antigenococcal antiserum required functional properdin to kill C4BP-binding strains, but not C4BP-nonbinding strains. Collectively, these data point to an important role for properdin in facilitating immune Ab-mediated complement-dependent killing of gonococcal strains that inhibit the classical pathway by recruiting C4BP.

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Neisseria gonorrhoeae (the gonococcus) is the causative agent of gonorrhea, a sexually transmitted disease that causes considerable morbidity worldwide. In 2009, 301,174 cases of gonorrhea were reported in the United States (http://www.cdc.gov/std/stats09/gonorrhea.htm), although the Centers for Disease Control and Prevention estimates that the true incidence may be about twice the number of reported cases (1). Gonorrhea elicits a wide variety of clinical syndromes at local genital sites, ranging from uncomplicated lower genital tract infection (cervicitis and urethritis) to upper tract endometritis in women and occasionally epididymitis in men. In some instances, N. gonorrhoeae disseminate, causing tenosynovitis, septic arthritis, and/or papulopustular skin lesions (2–5). Over the years, N. gonorrhoeae has demonstrated a remarkable capacity to develop resistance to almost every class of antibiotic used to treat infection (6). Thus, there is an urgent need to develop a safe and effective vaccine against this disease.

Prior efforts to develop vaccines against N. gonorrhoeae have not come to fruition because the Ags selected showed extreme variability across strains as in the case of pilin and porin (Por), which may limit protection to a limited repertoire of strains (7, 8). Abs directed against outer membrane proteins following natural infection, such as opacity protein and porin B (PorB), may provide protection against homologous or closely related strains, but again, antigenic variability of these proteins precludes eliciting cross-protective Abs against diverse strains (9–11). In contrast, Abs elicited against antigenically conserved targets on N. gonorrhoeae, such as reduction modifiable protein, are often either nonbactericidal or even subversive (blocking Abs) (12–16).

Lipooligosaccharide (LOS) is an important gonococcal virulence factor and plays a key role in several aspects of gonococcal pathogenesis, including but not limited to resistance to complement, adhesion, and entry into cells and recognition by the innate immune system (17–26). Abs directed against LOS activate the complement system that result in direct killing of N. gonorrhoeae (27), which has prompted efforts to evaluate LOS as a vaccine candidate. Abs against LOS may protect against reinfection with the homologous strain, as suggested in the male urethral experimental gonococcal infection model; those males who showed a ≥4-fold rise in anti-LOS IgG were more resistant to reinfection with the homologous strain than volunteers who did not mount an anti-LOS response (28). Prior work in our laboratory has identified an LOS epitope that is recognized by a bactericidal mAb called 2C7 (29) as a potential vaccine candidate and have prompted efforts to develop a mimotope of the 2C7 LOS epitope as an approach to circumvent the potential toxicity of the LOS molecule (30, 31).

The complement system forms an important line of defense against Neisserial infections. The complement cascade comprises three major pathways called the classical, lectin, and alternative pathways (AP), all of which converge at the level of C3 (32, 33). Although an intact classical pathway of complement is required for complement-dependent killing of N. gonorrhoeae (34, 35), the role of the AP, in particular properdin, in facilitating killing by
specific Abs remains unclear. Properdin functions as a positive regulator of the AP by virtue of its ability to stabilize C3 convertase (C3bBb) and prolong its life to 1.5 min by 5–10-fold (36). The secondary granules of polymorphonuclear neutrophils (PMNs) are the principal reservoir of properdin (37). Cervical secretions contain an intact complement system (38), the result of local synthesis of AP proteins by cervical epithelial cells (39) and exudation of serum components into the cervical lumen (40). A requirement for properdin in bacterial killing could suggest an important role for PMNs in determining the efficacy and efficiency of complement activation and killing of gonococci. In this study, we sought to examine the role of the AP and properdin in determining the bactericidal efficacy of Abs directed against the 2C7 epitope, which is present on diverse gonococcal strains. Studies used a murine mAb directed against the 2C7 epitope and were also performed using specific immune human serum derived from a gonococcal vaccine trial that contained excess Ab against the 2C7 epitope.

Materials and Methods

**Strains**

Strains used in this study were 15253 (41, 42), 252 (40), and 442089 (43, 44), all of which express PorB1A molecules and strains FA1090 (45) and 24-1 (46) (both express PorB1B molecules). N. gonorrhoeae strains were chosen based on their ability to: 1) resist killing (100% survival) in 16.7% pooled nonimmune normal human serum (NHS) used in this study; and 2) be killed ≥50% when 5 μg/ml mAb 2C7 was added to NHS. With the exception of strain 24-1, all of the wild-type strains fulfilled these criteria. 24-1 is sensitive to the bactericidal action of NHS but develops resistance to complement-mediated killing when grown in media supplemented with 5′-cyclohexyladenosine-3′,5′-cyclic monophosphate (cAMP) (CAMP-NANA) (47). Resistance of sialylated 24-1 is related to its ability to bind to human Fc and downregulate the AP of complement (47). Note that strain 252 is killed by select normal human sera, but none of the individual sera that were pooled in this study as a source of complement had intrinsic bactericidal activity against this strain. A mutant derivative of strain 15253 (lgtG::ermR) called 15253 ΔlgtG that does not bind mAb 2C7 has been described previously (48).

An isogenic mutant derivative of strain 252 that differed only in its PorB molecule, called 252/PorFA19, was created. Stain FA6546, a derivative of C4b-binding protein (C4BP)-binding and serum-resistant strain FA19 (provided by Drs. P. Frederick Sparling and Christopher Elkins, University of North Carolina, Chapel Hill) had been created by reintroducing the FA19 porB into the FA19 background using plasmid pUNCH62 (49). This mutant strain possesses the chromophenolase resistance marker ∼900 bp 3′ to porB. Chromosomal DNA extracted from FA6546 was used to transform strain 252, and transformants were selected on GC agar supplemented with 2 μg/ml chloramphenicol. porB genes of transformants were sequenced, and two clones that: 1) contained the entire FA19 porB sequence; and 2) also bound to mAb 2C7 were selected for further study.

**Sera and complement reagents**

Sera obtained from three healthy adult human volunteers (NHS) were pooled, aliquoted, and stored at −80°C until used. The use of these sera was approved by the Committee for the Protection of Human Subjects in Research at the University of Massachusetts Medical School. All subjects who donated blood for this study provided written informed consent. None of the sera, used individually or pooled, killed any of the gonococcal strains in a serum bactericidal assay that contained 16.7% serum. Further, the addition of 5 μg/ml mAb 2C7 to 16.7% serum mediated ≥50% killing of test strains as indicated above. Factor B-depleted serum, C2-depleted serum, purified factor B, and purified C2 were all purchased from Complement Technologies (Tyler, TX). In some experiments, complement activity of NHS was destroyed by heating at 56°C for 30 min. Sera from adult male volunteers immunized with a gonococcal outer membrane preparation enriched for porin (PorB1B), but also containing 2C7 epitope bearing LOS, taken as part of a 1985 vaccine and challenge trial (50, 51), were tested for bactericidal activity against strain 15253 and its IgG deletion mutant (15253 ΔIgG) that did not express the 2C7 epitope. Four (of seven tested) sera killed the parent but not the mutant strain; one of these sera was selected for use because it displayed the greatest change in Ab titer (from corresponding preimmunization serum) directed against all of the strains used in this study.

In experiments to measure the effects of anti-factor Bb to block AP activation in zymosan (see below), the classical and lectin pathways of complement were blocked by adding EGTa (10 mM) and MgCl2 (10 mM) to NHS (NHS-Mg/EGTA).

**Abs**

mAb 2C7 [IgG3A (51, 52)] is directed against an epitope that comprises at a minimum a lactose extension off heptose (H) and a lactose residue on HII of LOS. In addition, N-acylation of LOS appears to be required for mAb 2C7 binding (29). mAb 2C7 binding is not affected by HepI glycan extensions beyond the proximal lactose substitution (51).

The function of the AP in serum was blocked by the addition of an anti-factor Bb mAb (catalog number A227; Quidel, San Diego, CA) used at a final concentration of 125 μg/ml. Properdin function was blocked with an anti-properdin mAb (anti-properdin mAb #1; catalog number A233) used at a final concentration of 50 μg/ml (53). C7 function in NHS was blocked by adding anti-C7 mAb (catalog number A221; Quidel) at a concentration of 100 μg/ml. Blockade of C7 function was confirmed by loss of hemolytic activity of NHS as measured using the Total Hemolytic Complement Kit (The Binding Site, Birmingham, U.K.). Azide (0.1%), present in the commercial anti-properdin, anti-factor Bb, and anti-C7 mAb preparations, was removed by spin-concentration and dialyzed against PBS/0.1% BSA using a 30-kDa cutoff Amicon Ultra-15 centrifugal filter device (Milli-pore). Polyclonal goat anti-human C3 antisera was from Complement Technologies, and goat polyclonal anti-human factor B was from Quidel. An Ab preparation directed against a membrane preparation derived from strain FA1090 was kindly provided by Dr. Ann E. Jerse (Uniformed Services University of the Health Sciences, Bethesda, MD).

**Flow cytometry**

Flow cytometry was used to determine the concentration of anti-Bb Ab required to block C3 deposition on zymosan using anti-human C3c conjugated to FITC (Biodesign/Meridian Life Science, Saco, ME). C3 deposition on zymosan either in the absence or presence of increasing concentrations of anti-factor Bb mAb was measured using previously described methods (53). Briefly, NHS-Mg/EGTA (final concentration of 15% [v/v]) containing concentrations of anti-factor Bb mAb ranging from 0–500 μg/ml was added to fixed 5 × 107 zymosan particles suspended in HBSS containing 10 mM MgCl2 and 10 mM EGTA for 30 min at 37°C. The final volume of the reaction mixture was 100 μl. Total C3 deposited on zymosan was detected using anti-C3c-FITC (reacts with C3b and iC3b). Data were collected on an FACScanCalibur instrument (BD Biosciences, Franklin Lakes, NJ) and analyzed using the FlowJo analysis software program (version 7.2.4; Tree Star, Ashland, MA).

**Serum bactericidal assays**

Serum bactericidal assays were performed as described previously (54), with minor modifications. Bacteria that had been harvested from an overnight culture on chocolate agar plates were repassaged onto fresh chocolate agar and allowed to grow for 6 h at 37°C in an atmosphere containing 5% CO2. Bacteria were then suspended in HBSS containing 1 mM MgCl2 and 0.15 mM CaCl2 (HBSS++) for use in serum bactericidal assays. Strain 24-1 was sialylated by growth to the exponential phase in gonococcal liquid media supplemented with CAMP-NANA (2 μg/ml) as described previously (47). Approximately 2000 CFU gonococci were incubated with serum (concentration specified for each experiment) that had been blocked, either in the presence or absence of mAb 2C7. The initial reaction volumes were maintained at 150 μl, with the exception of experiments to measure the rate of bacterial killing in which the initial reaction volume was 450 μl. Aliquots of 25-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.

**Western blotting**

Gonococcal lysates in NaPAGE LDS Sample Buffer (4×) (Invitrogen) were separated on NaPAGE 12% Bis-Tris (Invitrogen) gels using Novex MES running buffer (Invitrogen) followed by transfer to an Immobilon PVDF membrane (Millipore) (55). Membranes were blocked with PBS/1% milk and probed with tissue-culture supernatants that contained mAb 2C7. mAb 2C7-reactive LOS bands were disclosed with anti-mouse IgG conjugated to alkaline phosphatase followed by the addition of BCIP/NBT-Purple Liquid Substrate (Sigma-Aldrich). The specificity of IgG in the immune serum of mice immunized with FA1090 membrane preparations was determined by incubating Western-blotted membrane preparations and bac-
terial lysates of FA1090 and 252 with a 1:1000 dilution of the immune serum, followed by disclosing the mouse IgG-reactive bands with anti-mouse IgG alkaline phosphatase.

Whole-cell ELISA

C3 deposition on and factor B binding to bacteria were measured by whole-cell ELISA as described previously (40, 56, 57). Briefly, $2 	imes 10^8$ organisms in HBSS ++ were incubated with NHS (final concentration 16.7%) that contained either no anti-complement component mAbs or contained anti-C7 alone or anti-factor Bb or anti-properdin (same concentrations used for bactericidal assays) in a reaction volume of 100 μl and then incubated for 10 min at 37˚C. This time point was chosen because maximal killing was observed at 10 min in this study (see Results) and based on complement deposition on gonococci in previously published data (40). Reactions were stopped after 10 min by washing three times with ice-cold HBSS containing 5 mM PMSF at 4˚C. Organisms were resuspended in 200 μl of the same buffer and 50 μl each sample applied per well of a 96-well U-bottom polystyrene microtiter plate (Dynatech Laboratories, Chantilly, VA) and incubated for 3 h at 37˚C. Plates were washed with PBS containing 0.05% Tween 20. Primary Abs (goat anti-human C3 and goat anti-human factor B) were diluted in PBS and secondary Abs diluted in PBS-0.05% Tween 20 prior to use. To ensure similar capture of bacteria incubated under different conditions, we measured the amount of gonococcal H.8 Ag (58, 59) expression using mAb 2-8C-4-1 (57), followed by anti-mouse IgG-alkaline phosphatase conjugate.

Results

Characterization of mAb 2C7 binding to the selected serum-resistant N. gonorrhoeae strains

A simplified schematic of the organization of LOS glycans on gonococcal LOS is provided in Fig. 1A. The phase-variable genes involved in gonococcal LOS biosynthesis are indicated in gray italic font (Fig. 1A). Expression of lactose on both HepI and HepII (indicated by the gray shaded box) is required for mAb 2C7 reactivity with gonococcal LOS (Fig. 1A). Glycan extensions beyond the lactose residue on HepI do not affect 2C7 binding (29). Thus, although expression of LgtG is required for mAb 2C7 binding, phase variations of lgtA, lgtC, and lgtD do not affect 2C7 reactivity. mAb 2C7 reactivity with the LOS of the strains used in this study is shown by Western blot in Fig. 1B, and, as expected, LOS phase variation resulted in variations in the pattern of mAb 2C7-reactive bands across strains. The lgtG deletion mutant of strain 15253 (15253ΔlgtG, Fig. 1B) that does not bind to mAb 2C7 was used as a negative control.

FIGURE 1. Specificity of mAb 2C7 and 2C7 reactivity against gonococcal strains used in this study. (A) A simplified schematic of glycan organization of gonococcal LOS. Lipooligosaccharide glycosyl transferase (lgt) genes that are involved in LOS biosynthesis and are subject to phase variation are indicated in gray italic font. The gray shaded box represents the minimum glycan extensions expressed by naturally occurring gonococcal strains that are required for mAb 2C7 binding. (B) Reactivity of mAb 2C7 with the LOS of gonococcal strains used in this study. Bacterial lysates were subjected to denaturing electrophoresis and Western blotting and membranes probed with tissue culture supernatants that contained mAb 2C7. Strain 24-1 was grown in media supplemented with CMP-NANA (2 μg/ml).

FIGURE 2. The AP of complement and properdin is required for killing of C4BP-binding strains of N. gonorrhoeae by mAb 2C7. (A) Survival of gonococcal strains was measured in serum bactericidal assays in the presence of NHS alone, NHS plus mAb 2C7, or NHS plus mAb 2C7 in which either factor B or properdin function was blocked with anti-factor Bb (α-fBb) or anti-properdin (α-P), respectively. Each data point represents the mean (± SD) of at least two independently repeated experiments. (B) C4BP binding to N. gonorrhoeae strains. N. gonorrhoeae strains 442089, 24-1 (LOS sialylated by growth in media containing CMP-NANA; labeled 24-1 sia+), FA1090, 15253, and 252 were incubated with 1, 3, or 10% NHS, and C4BP bound to the bacteria surface was measured by flow cytometry. One representative experiment of three independently performed experiments is shown. Numbers represent the average of the median fluorescence of each of the three experiments. *p < 0.001 (two-tailed Student t test) compared with the corresponding survival seen with mAb 2C7 + NHS.
mAb 2C7-dependent killing of select serum-resistant strains of N. gonorrhoeae requires properdin

We next asked whether the bactericidal activity of mAb 2C7 required participation of the AP of complement. To maintain activity of the classical (and lectin) pathways, but block activity of the AP, the function of factor B was blocked with an anti-factor Bb mAb. To determine the concentration of this mAb needed to fully block AP activation, we measured C3 deposition on zymosan, an AP-activating particle (60, 61), in the presence of Mg/EGTA-NHS that contained anti-factor Bb mAb at concentrations ranging from 62 to 500 μg/ml. Deposition of C3 was blocked (no increase in fluorescence over baseline) even at anti-factor Bb concentrations as low as 62.5 μg/ml (data not shown). Thus, an anti-factor Bb concentration of 125 μg/ml was chosen to ensure maximal AP blockade in subsequent experiments. The ability of the anti-properdin mAb (50 μg/ml) to limit C3 deposition on zymosan and on the surface of N. gonorrhoeae has been demonstrated previously (53).

As shown in Fig. 2A, all strains resisted killing by NHS alone (>100% survival at 30 min), but were killed >50% in the presence of 5 μg/ml of added 2C7. Blocking the AP with anti-factor Bb resulted in complete (>100%) survival of strains 442089, FA1090, and 15253, the C4BP-binding strains, but had little effect on the survival of strains 24-1 sia+ and 252 that did not bind C4BP. These results showed that Ab-mediated killing of some, but not all, serum-resistant strains of N. gonorrhoeae required an intact AP. Blocking properdin function with anti-properdin yielded similar results as with anti-factor Bb mAb, suggesting that the positive regulatory function of properdin on the AP was required for AB-dependent killing of select strains of N. gonorrhoeae (Fig. 2A). The negative control for this experiment included organisms incubated with mAb 2C7 and heat-inactivated NHS (no complement activity), which showed no killing (data not shown). In addition, the mutant strain 15253 ΔlgtG (does not bind to mAb 2C7) was not killed in a bactericidal assay that contained NHS (complement intact) and mAb 2C7 (data not shown).

We used factor B-depleted serum to confirm the results obtained with anti-factor Bb-blocking mAb (Table I). Note that the factor B-depleted serum alone (without added mAb 2C7) killed strain 252, so this strain could not be tested in this assay. Factor B-depleted serum supported significantly greater killing of 24-1 sia+ than the C4BP-binding strains (FA1090, 15253, and 442089) by mAb 2C7. Almost identical results were shown using properdin-depleted serum instead of factor B-depleted serum (24-1 sia+ showed only 9% survival, whereas C4BP binders FA1090, 15253, and 442089 survived 62, 69, and 107%, respectively).

An intact classical pathway of complement is required for mAb 2C7-mediated killing

A requirement of the AP for manifestation of bactericidal activity of mAb 2C7 against select gonococcal strains raised the possibility that AB-dependent AP activation (34) may have been fully responsible for killing of bacteria. To address this possibility, strains 24-1 sia+ and FA1090 were incubated with mAb 2C7 in the presence of C2-depleted human serum (classical and lectin pathways blocked, AP intact). No killing of either strain (>100% survival) was seen. Reconstitution of C2-depleted serum with physiological concentrations of C2 restored killing by mAb 2C7 (92.5 and 83% killing of strains 24-1 sia+ and FA1090, respectively). Controls included C2-depleted serum alone and depleted serum reconstituted with C2; neither control showed killing (>100% survival). To underscore the importance of an intact classical pathway in mediating killing by specific Ab, we tested wild-type strain 24-1 (unsialylated) that is extremely sensitive to killing by NHS (46, 47). Incubating this strain with mAb 2C7 and C2-depleted serum together also resulted in >100% survival. As expected, killing was restored (0% survival) when purified C2 was added to the reaction mixture. These data suggest that an intact classical pathway is required for 2C7-dependent bactericidal activity; recruitment of the AP alone is insufficient to support gonococcal killing under these conditions.

Table I. Factor B in killing of N. gonorrhoeae by mAb 2C7

<table>
<thead>
<tr>
<th>Strain</th>
<th>Percent Survival [Mean (Range)]</th>
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<tbody>
<tr>
<td>24-1 sia+ (C4BP+)</td>
<td>117 (4) 6 (0) 73.2 (2)</td>
</tr>
<tr>
<td>FA1090 (C4BP+)</td>
<td>110 (2) 70 (2) 18 (2)</td>
</tr>
<tr>
<td>15253 (C4BP+)</td>
<td>105 (2) 61.7 (3) 16.8 (6)</td>
</tr>
<tr>
<td>442089 (C4BP+)</td>
<td>107.5 (3) 107 (6) 50.5 (3)</td>
</tr>
</tbody>
</table>

*Factor B (B)-depleted serum (B-dep) reconstituted with purified human FB (200 μg/ml).
Strains that require properdin for mAb 2C7-dependent killing bind the classical pathway inhibitor C4BP

Direct complement-dependent killing of N. gonorrhoeae requires an intact classical pathway (34, 35). Active inhibition of the classical pathway, as occurs on strains that bind to the classical pathway inhibitor C4BP (43), may reduce the ability of the classical pathway alone to support bactericidal activity, although small amounts of active C3b deposited on organisms are sufficient to amplify the AP. We reasoned, therefore, that killing of C4BP-binding strains may require participation of the AP. Properdin is critical for stabilization of the positive-feedback loop of the AP and therefore is likely to be critical for killing. In contrast, Ab-independent killing, via the classical pathway, of strains that do not bind to C4BP should be unimpeded. We speculated that strains that bound C4BP were killed by mAb 2C7 via the AP, which required properdin. C4BP binding to strains 15253, 442089, and FA1090 in 1, 3, and 10% human serum (Fig. 2B) (43) correlated with a properdin requirement for 2C7-mediated killing of these strains (Fig. 2A), whereas strains 252 and 24-1 sia+ that bound C4BP minimally or not at all were killed in the absence of properdin (Fig. 2A).

Properdin does not alter the rate of killing by mAb 2C7

Although 2C7-mediated killing of strains 252 and 24-1 sia+ at 30 min in the presence or absence of properdin was similar, we asked whether properdin influenced the rate of bacterial killing as may be expected if the AP was amplifying Ab-dependent Neisserial killing to a significant extent (62). Blocking properdin function did not alter the kinetics of killing of strain 252 by mAb 2C7. Both in the presence and absence of properdin, bactericidal activity was almost complete within 10 min, suggesting that properdin did not augment the rate of killing of a strain that bound minimal levels of C4BP (data not shown) and that bacterial killing of these strains was less influenced by the AP.

Maximal C3 deposition on C4BP-binding gonococci requires an intact AP

Having shown that killing of C4BP-binding N. gonorrhoeae by mAb 2C7 required properdin and an otherwise intact AP, we next determined the roles for factor B and properdin in C3 fragment deposition on and factor Bb binding to gonococci by whole-cell ELISA. A 10-min incubation period was chosen based on the killing kinetics presented above and our previous studies of complement activation on N. gonorrhoeae (40). In these experiments, a function-blocking anti-C7 mAb was added to NHS to avoid a possible effect from bacterial killing and membrane lysis. When compared with NHS, the addition of anti-C7 to NHS did not affect C3 or factor Bb binding to bacteria; therefore, only data with NHS plus anti-C7 are shown for simplicity. Shown in Fig. 3 (top panel) and in accordance with the results of bactericidal assays presented in Fig. 2A, blocking factor B or properdin function did not affect mAb 2C7-mediated C3 deposition on the C4BP nonbinding strains 24-1 sia+ and 252, but significantly reduced mAb 2C7-dependent C3 deposition on the C4BP binders (FA1090, 15253, and 442089). Controls in which bacteria were incubated with heat-inactivated NHS or with buffer alone showed OD405nm readings <0.1 (data not shown).

Addition of mAb 2C7 to NHS resulted in a marked increase in factor Bb binding to all strains (Fig. 3, bottom panel), suggesting that this Ab recruited the AP on all strains, even though only C4BP-binding strains required the AP for killing (Fig. 2A). Of note, blocking properdin function resulted in almost complete loss of detectable factor Bb binding to all strains to levels comparable to those seen when factor B function was blocked, suggesting a key role for properdin in AP recruitment by mAb 2C7.

Collectively, these data suggest that unimpeded classical pathway activation on C4BP nonbinders was sufficient for maximal C3 deposition and killing of these strains even in the absence of AP activation (factor B function blocked) or when the AP lacked function.
of its positive regulator properdin. In contrast, C4BP-binding gonococcal isolates required an intact AP for mAb 2C7-mediated killing. Further, properdin played a critical role in mAb 2C7-mediated recruitment of the AP and assembly of C3 convertases on gonococci.

Isogenic mutant strains confirm the association between C4BP binding and the requirement of properdin for Ab-dependent killing

In addition to their ability to bind to complement inhibitors, additional factors such as LOS structure can modulate serum resistance of *N. gonorrhoeae* (22, 23, 63). To provide further evidence linking C4BP binding to the requirement for properdin for Ab-mediated killing, we created isogenic mutants that differed in their ability to bind C4BP by replacing the PorB.1A molecule in strain 252 (binds C4BP minimally) (Fig. 2B) with the PorB.1A molecule of strain FA19 that binds C4BP strongly (43). The resulting mutant, called 252/PorFA19, showed no change in the 2C7 binding profile compared with the parent strain, 252 (Fig. 4B). As expected, the 252/PorFA19 mutant strain also bound C4BP (Fig. 4A). Shown in Fig. 4C, C4BP binding to strain 252/PorFA19 resulted in resistance to killing by mAb 2C7 when properdin function was blocked. A second 252/PorFA19 clone yielded similar results (data not shown). Similar to results seen with the anti-properdin mAb, blocking factor B function with the anti-Bb mAb yielded a mean bacterial survival of 66% of strain 252/PorFA19 (results omitted from the graph for simplicity), thereby demonstrating the absolute need for properdin for recruitment of the AP and killing of C4BP-binding gonococci. Similar to strain 252/PorFA19, the C4BP-binding strain FA1090, used as a control, showed restored survival when properdin function was blocked, whereas mAb 2C7-mediated killing of the C4BP nonbinding parent strain 252 was unaffected by loss of properdin function (Fig. 4C). The effects of blocking factor B on survival of control strains 252 and FA1090 under similar conditions are shown in Fig. 2A. C3 deposition on strain 252/PorFA19, measured by whole-cell ELISA, showed that blocking properdin function resulted in a significant decrease in C3 deposition on the organism (Fig. 4D). This stood in contrast to the parent strain 252, in which the total amount of C3 deposition was not affected by blockade of the AP. Blocking factor B function reduced C3 deposition to levels similar to those seen when properdin function was blocked (mean [±SD], OD$_{405\text{nm}}$ 0.8 [±0.05]).

Taken together, these results strongly suggest that killing of *N. gonorrhoeae* strains that bind to C4BP by specific anti-LOS Ab requires participation of the AP. Further, properdin plays a key role in facilitating AP recruitment to mediate bactericidal activity.

**Requirement for properdin for killing by human Ab elicited by vaccination with a gonococcal vaccine preparation**

The studies described above were performed with a murine bactericidal mAb against LOS. To address the applicability of the results to human immune Ab against LOS, we asked whether properdin was required for killing by immune serum obtained from a human volunteer and administered a gonococcal outer membrane-derived vaccine candidate. The vaccine candidate used was enriched with PorB.1B, derived from a gonococcal strain called 2399, but the candidate also contained other components of the outer membrane that were immunogenic including LOS (50, 51). The PorB.1B Ab response was restricted to that of the homologous strain (2399) used to prepare the vaccine candidate; no cross-reacting PorB.1B Abs to any of the PorB Ags present in the strains used in this study were detected by Western blotting (data not shown). However, in several vaccinees, the resultant bactericidal Ab response was directed principally against the LOS-derived 2C7 epitope.

We used such a vaccinee serum in this study (2C7 epitope-specific Ab was 3.61 μg/ml, which was 91% of the total LOS Ab [3.95 μg/ml]) (51), in which bactericidal activity depended solely on LgtG expression of the target organism as evidenced by complete killing (no survival) of 15253, but no killing (±100% survival) of 15253 ΔlgtG (shown in Fig. 5A).

Consistent with the presence of 2C7 epitope-specific bactericidal Abs, the vaccinee serum also killed strains FA1090, 252, and sialylated 24-1 (sia+), all displaying the 2C7 epitope (Figs. 1B, 5B). In accordance with results using mAb 2C7, the immune human serum also required functional properdin to kill strains FA1090 and 15253 (both bound C4BP), but not 252 or 24-1 sia+ (bound C4BP minimally or not at all) (Fig. 5B). Survival of 252 and 24-1 sia+ in the presence of the human immune serum when factor B function was blocked (no AP activity) were similar to results seen when properdin was blocked (mean survival of 252 and 24-1 sia+ in two independent experiments was 48.5 and 1.6%, respectively, demonstrating that killing of the C4BP nonbinders by specific human anti-LOS Ab did not require any input from the AP). Survival of the C4BP-binding strains ≥100% when only the positive regulatory function of the AP was blocked with anti-

![FIGURE 5. Properdin is required for killing of C4BP-binding strains of *N. gonorrhoeae* by immune human Ab directed against the 2C7 LOS epitope.](http://www.jimmunol.org/...)

(A) Bactericidal activity of gonococcal vaccine-elicited human immune serum (directed against the 2C7 LOS epitope) requires expression of lactose on HepII (Fig. 1A). Strain 15253 and its isogenic LgtG deletion mutant (15253 ΔlgtG) were incubated with NHS alone (indicated as 0 on the graph) as a constant source of complement and with increasing concentrations of heat-inactivated vaccine-elicited human immune serum (complement inactivated, but Abs intact) and bacterial survival following a 30-min incubation period was measured. (B) Properdin-dependent killing of C4BP-binding *N. gonorrhoeae* by vaccine-elicited human immune serum. C4BP-binding strains FA1090 and 15253 and minimal C4BP-binding strains 252 and sialylated 24-1 (24-1 sia+) were incubated with NHS alone, NHS + immune human serum (final concentration 6.7%), or NHS + immune human serum + o-P (anti-properdin mAb). Bacterial survival was measured following incubation of reaction mixtures for 30 min at 37°C. Each data point represents the mean (±SD) of two separate experiments.
properdin obviated the need for testing survival of these strains when the entire pathway was rendered nonfunctional with anti-Bb.

**Properdin is required for maximal bactericidal activity against C4BP-binding gonococci of a polyonal murine anti-outer membrane vesicle Ab**

The data thus far have focused on the requirement of properdin for the bactericidal activity of anti-LOS Abs, in particular Abs directed against the 2C7 epitope. To determine whether properdin was required for killing activity of Abs directed at potential bactericidal Ab-reactive targets other than LOS, we tested complement-dependent killing mediated by a murine polyclonal Ab raised against a membrane preparation of FA1090 (Fig. 6A shows a silver stain of the immunogen). The broad cross-reactivity of the elicited murine Abs against the membrane preparation as well as whole-cell lysates of FA1090 and 252 was determined by Western blot, shown in Fig. 6A. The antiserum reacted strongly with a variety of proteins, but only weakly against LOS. In the presence of an intact complement cascade, the polyclonal anti-membrane Ab was bactericidal against strain FA1090 (the homologous strain) and to a similar extent against heterologous strain 252 (Fig. 5A, solid bars). Akin to observations with anti-LOS Abs, the addition of the anti-properdin mAb to block properdin function resulted in increased survival of C4BP binding strain FA1090 (Fig. 5B, left panel), but had no effect on survival of 252 (Fig. 5B, right panel). Thus, the critical role for properdin in maximizing Ab-mediated bactericidal activity against C4BP-binding gonococcal strains likely extends to Ags other than LOS.

**Discussion**

In addition to its pivotal role in protection against Neisseria infections (64), complement also plays an important role in innate immune defenses against several other bacterial pathogens. Over 50 y ago, Roantree and Rantz (65) reported that Gram-negative bacteria isolated from the bloodstream were almost always resistant to the killing activity of complement. Defects in Ab and/or early components of the classical pathway that impair opsonophagocytosis predispose to recurrent respiratory infections and sepsis caused by bacteria such as *Haemophilus influenzae* and *Streptococcus pneumoniae* (66, 67). The link between deficiencies of the alternative and terminal pathways of complement and recurrent, disseminated meningococcal disease is well established (66, 67).

Previous studies have shown that the classical pathway of complement is essential to mediate complement-dependent killing of *N. gonorrhoeae* (34, 35). Gonococcal strains that are otherwise sensitive to killing by nonimmune normal human serum (all pathways of complement intact) survive when the classical (and lectin) pathway is blocked with Mg/EGTA-NHS (to selectively chelate Ca2+) (34) or with serum that lacks C1q (35), while permitting activation of the AP. However, such strains can be killed when the AP alone is selectively blocked, as with factor B-depleted serum (35). *N. gonorrhoeae* have evolved several mechanisms to resist killing by human complement. Effective strategies used by *N. gonorrhoeae* to escape killing by complement include binding to human complement inhibitors such as factor H and C4BP through their PorB molecule (43, 44). Sialylation of gonococcal LOS that expresses the lacto-N-neotetraose structure also enhances factor H binding to bacteria.

The rapid and widespread development of antibiotic resistance by *N. gonorrhoeae* (6, 68) has severely limited therapeutic options, and there is an urgent need to develop safe and effective vaccines that prevent gonococcal carriage and disease. Ab-based vaccines against *N. gonorrhoeae* could depend on efficient complement activation to either mediate direct killing through membrane attack complex insertion or promote opsonophagocytosis to protect the host. To effectively activate complement on the bacterial surface, vaccine-induced Abs need to overcome the complement inhibitory effects of molecules such as factor H and C4BP that bind to the gonococcal surface. mAb 2C7 kills gonococci that inherently resist killing by complement (Fig. 2A) (51) via any of several mechanisms (31, 51) and is therefore an attractive vaccine candidate. Abs elicited by immunization of mice with a peptide that mimics the 2C7 epitope bind the 2C7 epitope on gonococcal LOS and kill gonococci for which LOS displays the epitope (31). Binding of mAb 2C7 to gonococcal LOS requires expression of LgtG, which is encoded by lgtG that contains a poly-C tract and may therefore be subject to phase variation (48). Nevertheless, >90% of minimally passaged clinical isolates of *N. gonorrhoeae* bind to 2C7 (52), suggesting an important, but undefined role for

![FIGURE 6](image-url). Killing of C4BP-binding gonococcal strain FA1090 by anti-membrane preparation Ab requires functional properdin. (A) Broad cross-reactivity of murine antiserum raised against an FA1090 membrane preparation (Memb. prep.). The membrane preparation that was used as the immunogen (a silver stain of the same is also shown) and whole-cell lysates of FA1090 and 252 were separated on a 4–12% Bis-Tris gel and transferred to a polyvinylidene difluoride membrane that was then overlaid with a 1:1000 dilution of anti-FA1090 membrane Ab in PBS/0.05% Tween 20. Mouse IgG bound to bacterial Ags was disclosed with anti-mouse IgG alkaline phosphatase and substrate. Localization of IgG-reactive PorB.1B (faintly visualized only in the Memb. prep. and FA1090 lanes) and opacity protein (Opa) bands are indicated. LOS and LOS-reactive IgG bands are indicated with solid black dots. (B) Murine anti-membrane antiserum (dilutions ranging from 1:75 to 1:300) mediates complement-dependent killing of strains FA1090 (left panel) and 252 (right panel) in the presence (dashed lines) or absence (solid lines) of α-P (anti-properdin mAb) that blocks function of properdin; 16.7% NHS was used as a source of human complement. The 1:75 dilution experiment represents the mean (±SD) of three separately performed experiments; the 1:150 and 1:300 dilution experiments, the mean (±SD) of two experiments. *p < 0.005 (two-tailed Student t test).
lactose substitution of the HepII chain [a requirement for 2C7 epitope expression (29)] of gonococcal LOS.

A novel finding in this study was the requirement of the AP and, in particular, properdin for specific Ab-mediated complementdependent killing of *N. gonorrhoeae* strains that bind C4BP. Strains that bind C4BP and limit classical pathway activation require recruitment of the AP to assemble sufficient C3/C5 convertases for subsequent membrane attack complex (C5b-9) and bacterial killing. In the absence of the AP or when properdin is blocked, mAb 2C7 deposits only a small amount of C3 on strains such as FA1090, 442089, and 15253 because these strains actively regulate the classical pathway through recruitment of C4BP to their surfaces. However, an intact AP appears to effectively amplify the C3b that breaks through the barrier posed by C4BP and effect bacterial killing. The stabilizing effects of properdin on the AP C3 convertases are crucial to amplify C3 deposition and mediate bacterial killing. In contrast, strains that resist complement by mechanisms independent of C4BP binding and do not actively cleave C4b or dissociate the classical pathway convertase [C4b,2b according to newly proposed nomenclature (32)] do not require properdin for specific Ab-mediated bactericidal activity.

Importantly, the role of properdin in facilitating Ab-dependent killing was not restricted to murine mAb 2C7 but also extended to specific immune human serum that contained Abs directed against the 2C7-specific LOS epitope. These findings have implications for Ab-based vaccine development because PMNs are a major reservoir and source of properdin (37). In addition, properdin is synthesized by primary cervical epithelial cells (39) and would also be exuded into the cervical lumen from serum. The concomitant presence of neutrophils is likely to ensure high levels of properdin that may enhance complement activation on gonococci. Enhanced complement activation in the vicinity of PMNs may also facilitate opsonophagocytic disposition of organisms. As shown by Bracco-nier et al. (69), phagocytic killing was reduced in *S. pneumoniae* serotype 23F due to defective opsonization in the presence of properdin-deficient sera. Addition of native properdin to the cell-bound properdin-deficient sera restored opsonization of *S. pneumoniae* serotype 23F by primary cervical epithelial cells (39) and would also be exuded into the cervical lumen from serum. The concomitant presence of neutrophils is likely to ensure high levels of properdin that may enhance complement activation on gonococci. Enhanced complement activation in the vicinity of PMNs may also facilitate opsonophagocytic disposition of organisms. As shown by Bracco-nier et al. (69), phagocytic killing was reduced in *S. pneumoniae* serotype 23F due to defective opsonization in the presence of properdin-deficient sera. Addition of native properdin to the cell-bound properdin-deficient sera restored opsonization of *S. pneumoniae* serotype 23F by primary cervical epithelial cells (39) and would also be exuded into the cervical lumen from serum. The concomitant presence of neutrophils is likely to ensure high levels of properdin that may enhance complement activation on gonococci. Enhanced complement activation in the vicinity of PMNs may also facilitate opsonophagocytic disposition of organisms. As shown by Bracco-nier et al. (69), phagocytic killing was reduced in *S. pneumoniae* serotype 23F due to defective opsonization in the presence of properdin-deficient sera. Addition of native properdin to the cell-bound properdin-deficient sera restored opsonization of *S. pneumoniae* serotype 23F by primary cervical epithelial cells (39) and would also be exuded into the cervical lumen from serum.

In conclusion, we have demonstrated that both human and murine Abs that are directed against an LOS epitope that is currently being investigated as a potential vaccine candidate (31), as well as Abs raised against a gonococcal membrane preparation more generally, require a functional AP and, in particular, properdin to kill serum-resistant gonococcal strains that bind to the complement inhibitor C4BP. Strains that do not bind to C4BP are efficiently killed by specific Abs raised against a gonococcal membrane preparation via the classical pathway alone and do not require properdin. These findings provide insights into how complement is activated on *N. gonorrhoeae* by specific Ab and also shed light on the requirements for effective complement-dependent bactericidal activity by potential vaccine candidates.

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Disclosures

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