Properdin Can Initiate Complement Activation by Binding Specific Target Surfaces and Providing a Platform for De Novo Convertase Assembly

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Complement promotes the rapid recognition and elimination of pathogens, infected cells, and immune complexes. The biochemical basis for its target specificity is incompletely understood. In this report, we demonstrate that properdin can directly bind to microbial targets and provide a platform for the in situ assembly and function of the alternative pathway C3 convertases. This mechanism differs from the standard model wherein nascent C3b generated in the fluid phase attaches nonspecifically to its targets. Properdin-directed complement activation occurred on yeast cell walls (zymosan) and Neisseria gonorrhoeae. Properdin did not bind wild-type Escherichia coli, but readily bound E. coli LPS mutants, and the properdin-binding capacity of each strain correlated with its respective serum-dependent AP activation rate. Moreover, properdin: single-chain Ab constructs were used to directly serum-dependent complement activation to novel targets. We conclude properdin participates in two distinct complement activation pathways: one that occurs by the standard model and one that proceeds by the properdin-directed model. The properdin-directed model is consistent with a proposal made by Pillemer and his colleagues >50 years ago. The Journal of Immunology, 2007, 179: 2600–2608.

Complement is an ancient and vital part of innate immunity (1). It marks targets for immune clearance and lysis, triggers inflammatory reactions, and instructs the adaptive immune response (2). Complement is activated by three pathways, with each responding to a different set of activators. The complement classical pathway (CP) is initiated by Ag:Ab complexes and the lectin pathway (LP) is initiated by lectin:polysaccharide interactions. The alternative pathway (AP) is engaged independently of the CP and LP by a variety of microbial and molecular surfaces but the basis for its relatively general specificity is not fully understood. Because the AP has been strongly implicated in many types of human disease including Neisseria infection (3–5), age-related macular degeneration (6–9), glomerulonephritides (10), and inflammatory arthritis (11), it is important to clarify this point.

The AP was described first by Louis Pillemer and his collaborators (12) over 50 years ago. The centerpiece of their study was the serum protein properdin. In their report, Pillemer et al. (12) provided evidence that properdin:target complexes directed Ab-independent complement activation. At the time, most all of immunology dealt with Ab reactions of great specificity, while the properdin system was relatively nonspecific and one of the first examples of “natural immunity” (13). Thus, amid much publicity, the properdin system was received with great interest. Soon, however, the properdin work encountered formidable objections, notably those of Nelson (14), who showed that properdin’s “activity” could be due to Ab contaminants. With Pillemer’s tragic death (15), properdin lost its strongest advocate and was largely dismissed by the scientific community (16). The properdin system was reborn as the AP >20 years later (16), but the properdin protein itself was cast in a relatively nonspecific role (17).

The C3 convertases are serine proteases that cleave the serum protein C3 at a single site, generating C3b and C3a. C3a is an anaphylatoxin that stimulates local inflammatory processes, while nascent C3b covalently attaches nonselectively to surfaces via its highly reactive thioester moiety. Membrane-bound C3b promotes immune clearance and cell lysis. In the long-standing “textbook” model for the initiation of the AP, nascent C3b generated by the slow nonspecific fluid-phase activation of C3 covalently attaches to nearby targets (18–20). Target-bound C3b then associates with factor B and the resulting C3bB complex is cleaved by factor D. Two factor B fragments are generated, with the Ba fragment released and the Bb fragment remaining as part of the AP C3 convertase, C3bBb. The C3bBb complex is relatively unstable, with a t1/2 of 90 s under physiological conditions (21, 22). Properdin associates with C3bBb, resulting in a 5- to 10-fold more stable complex (17). By this model, target specificity is largely determined by the action of regulatory proteins that suppress complement activation on host tissues by inactivating bound C3b and resulting convertases. Although this mechanism can be demonstrated in vitro (23, 24), it does not provide a comprehensive accounting for in vivo target specificity (25). Recently, we observed that properdin bound to a biosensor surface serves as a platform for in situ convertase assembly (26). These new results raise again the possibility that properdin:target complexes initiate AP activation. In this report, we re-examine this issue.
Table I. *E. coli* K12 strains used in this study

| Strain (Reference) | CGSC No. | LPS Genotype (Phenotype) | Properdin Binding | AP Kinetics |
|--------------------|----------|-------------------------|-------------------|-------------|
| W1485S (43)        | 5024     | WT                      | 0                 | Slow        |
| D31 (44)           | 5165     | rfa-229 (O-Ag)          | +                 | Intermediate|
| D31m3 (44)         | 5166     | rfa-229, galU (Core oligosaccharide) | + + +   | Rapid       |
| D31m4 (44)         | 5167     | rfa-229, rfa-230 (Core oligosaccharide) | + + +   | Rapid       |
| ED3867 (53)        | 6989     | WT                      | + + +             | Slow        |
| ED3867 (53)        | 6990     | rfaU (Core oligosaccharide) | + + +   | Rapid       |
| RC703              | 5352     | WT                      | 0                 | Slow        |

* E. coli Genetic Stock Center.

Materials and Methods

**Proteins, Abs, and sera**

Properdin, C3b, factor B, and factor D were obtained from CompTech, formerly Advanced Research Technologies. The properdin was analyzed by SDS gel electrophoresis followed by Coomassie-staining and formed a single band of ~53 kDa; no other bands were visible. Mouse anti-human properdin mAb (IgG1 κ binding factor B and the Bb fragment; catalog no. A227) were purchased from Quidel. The mouse anti-human C3d mAb (IgG1 κ binds C3, C3b, and C3d) used for the experiments described in Figs. 3, 5, and 7 was obtained from Antibody Shop (catalog no. HAV 003-05). The mouse anti-human C3d mAb (IgG1 κ binds C3b, C3d, and C3d) used for the experiments described in Fig. 6 was obtained from Quidel (catalog no. A207). FITC-labeled goat anti-mouse IgG was obtained from Sigma-Aldrich. FITC-labeled goat anti-mouse C3 was obtained from Cappel. C3-depleted human serum was obtained from Quidel. C7-depleted human serum was obtained from CompTech. Properdin-deficient and C7-deficient human sera were gifts from P. Densen (University of Iowa, Iowa City, IA). Normal human serum was obtained from healthy volunteers and prepared as described (27).

**Bacteria**

*Neisseria gonorrhoeae* strain N401 (recA6, kan); Ref. 28), a gift from M. Koovery (University of Oslo, Oslo, Norway) was used throughout. Rec A6 is an isopropyl β-d-thiogalactoside-inducible allele of rec A. In this study, rec A was not expressed, minimizing antigenic variation. Plates made with GC agar supplemented with 1% IsoVitaLEX (BD Biosciences) were inoculated with *N. gonorrhoeae* from frozen stock and incubated for 18–20 h at 37°C in 5% CO2 before use. In some cases, 24-h cultures were transferred to fresh plates and grown 18–20 h. Bacteria were washed in Mg2+/H926237°C, diluted 20-fold in fresh medium, and allowed to grow 1.5 h before use. In some experiments, 24-h cultures were transferred to fresh plates and grown 18–20 h. Bacteria were washed in Mg2+/H926237°C, and resuspended to an OD595 of 0.5 (~5 × 108/ml). They were treated in the same buffer with various human complement proteins including properdin (10 μg/ml), C3b (10 μg/ml), factor B (10 μg/ml), and factor D (1 μg/ml) for 30 min at 37°C and washed twice in this buffer between incubations. In some experiments, *Neisseria* were first incubated with properdin, washed twice, and then incubated in 5% properdin-deficient human serum in Mg2+/H926237°C. Under these buffer conditions, AP activation is permitted but CP and LP activation are excluded. In some experiments, the 5% properdin-deficient serum was supplemented with purified properdin (10 μg/ml). The data presented in each panel of Fig. 3 is representative of at least four independent experiments.

*Escherichia coli* K-12 strains (Table I) were obtained from the *E. coli* Genetic Stock Center (Yale University, New Haven, CT). *E. coli* cultures were grown on agar plates or in Luria-Bertani medium overnight at 37°C, diluted 20-fold in fresh medium, and allowed to grow 1.5 h before use. Bacteria were suspended and washed in Mg2+/H926237°C EGTA buffer, diluted to an OD95 of 0.5 (~5 × 108/ml), and treated with a properdin and/or human normal or C7-deficient serum and analyzed by FACS as described for *Neisseria*. Strains were initially screened for properdin-binding capacity by incubating the bacteria with properdin at 10 μg/ml for 10 min at 37°C. Strains that bound properdin but were later found to bind properdin within 1 min at 37°C. The FACS data presented in each panel of Fig. 7 and summarized in Table I is representative of at least three independent experiments.

For Western analysis, 500–μl reactions were conducted in 5% serum, Mg2+/H926237°C EGTA buffer, washed twice in Mg2+/H926237°C EGTA buffer, and resuspended in a mixture of 25 μl of SDS running buffer and 5 μl of reducing solution (Pierce). Samples were incubated at 95°C for 10 min and run on 8% SDS gels (Novex). Western blots were prepared and then probed with goat anti-human C3 polyclonal Ab (1/5000 dilution; CompTech) followed by HRP-conjugated rabbit anti-goat polyclonal Ab (1/5000 dilution; Jackson ImmunoResearch Laboratories). The data presented in the Western analysis of Fig. 8 is representative of three independent experiments.

**Rabbit and sheep erythrocytes**

Rabbit erythrocytes (E1) and sheep erythrocytes (E2) were purchased from the Colorado Serum Company, washed in Mg2+/H926237°C, and resuspended at 109/ml. They were treated in the same buffer with various human complement proteins including properdin (3 μg/ml), C3b (10 μg/ml), factor B (10 μg/ml), and factor D (1 μg/ml) for 30 min at 37°C and washed twice in buffer between incubations. The data presented in each panel of Fig. 5 is representative of at least four independent experiments.

**Cells**

Human 293T kidney cells (CRL-11268; American Type Culture Collection) were used for protein generation. They were maintained in DMEM (Mediatech) containing 10% FCS (Harlan Breeders). Media were supplemented with L-glutamine (2 mM final concentration; Sigma-Aldrich), non-essential amino acids (BioWhittaker), and penicillin and streptomycin (10 U/ml and 100 μg/ml; Cellgro, Mediatech). The hybridoma cell line BRIC 256 was maintained in RPMI 1640 containing all the supplements mentioned for the DMEM medium described above and additionally supplemented with 1 mM sodium pyruvate and 0.05 mM 2-ME (Sigma-Aldrich).

**Construction of scFv-properdin that binds mouse erythrocytes**

The human properdin coding sequence (minus the signal peptide region) was amplified by PCR from a human U937 cDNA library provided by J. Verbsky (Medical College of Wisconsin, Milwaukee, WI). Its 1356-bp sequence (flanked with the BstWI and Xhol restriction enzyme cutting sites at the 5′ and 3′ termini, respectively) is identical to the published sequence that was derived from the same cell line (30). The 735-bp anti-mouse glycophorin A (GPA) (m)scFv sequence flanked by BstWI restriction sites was derived directly from st-DAF (31). The NH2-(m)scFv-Properdin-COOH construct was prepared from the st-DAF plasmid and the above properdin and (m)scFv-coding segments in the following steps: 1) the st-DAF plasmid was digested with BstWI and Xhol, releasing a 960-bp DAF fragment and a 735-bp scFv fragment but retaining the signal peptide sequence; 2) the two excised fragments were then replaced by the properdin sequence at the BstWI and Xhol sites; 3) the intermediary plasmid (designated pNCIThProp) was then cut at the BstWI site (between the signal peptide and properdin coding sequences) and the (m)scFv fragment (above) was introduced in the appropriate direction at that site. The mature plasmid was designated pNScThProp.

**Construction of scFv-properdin that binds human erythrocytes**

The scFv with specificity for human GPA was generated essentially as described (31) from the mouse hybridoma cell line BRIC 256 (32), provided by R. Mushens (International Blood Group Reference Laboratory, Bristol, U.K.). This line was chosen because it secretes an IgG1 mAb that recognizes a blood group-independent epitope on human GPA (33) and is therefore broadly applicable. Total RNA was isolated (RNeasy; Qiagen). Reverse transcription, followed by PCR (RT-PCR), was conducted using the SMART technology (BD Clontech) using primer combinations described previously (34). The resulting H and L chain variable cDNA fragments were subcloned into pCR2.1-TOPO (Invitrogen Life Technologies). The 744-bp cDNA sequence; 2) the two excised fragments were then replaced by the properdin sequence at the BstWI and Xhol sites; 3) the intermediary plasmid (designated pNCIThProp) was then cut at the BstWI site (between the signal peptide and properdin coding sequences) and the (m)scFv fragment (above) was introduced in the appropriate direction at that site. The mature plasmid was designated pNScThProp.

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Production and characterization of recombinant proteins

Recombinant protein was prepared by transient expression in 293T cells using Invitrogen Life Technologies Opti-mem serum-free medium and TransIT-293 (MIR2700; Mirus) transfection reagent, as per the manufacturer’s instructions. To obtain concentrated protein stocks, the supernatants were applied to centrifugal filter devices with a 10-kDa molecular cut-off (Centricron Plus-20; Millipore). Concentration of the recombinant forms of human properdin, (m)scFv-P and (hu)scFv-P, was initially determined by ELISA using goat anti-human properdin polyclonal Ab (1/1000) for capture, anti-human properdin mAb (1/5000) and peroxidase-conjugated donkey anti-mouse IgG polyclonal Ab (1/3000) for detection. Native properdin compared with scFv-properdin A and B, are representative of at least four independent experiments.

Mouse erythrocytes

For these experiments, C8-depleted and normal human serum (NHS) were first absorbed 4 times with 4% (v/v) of washed, packed mouse erythrocytes (Ehu) for 15 min at 4°C to remove Abs directed against Ehu surface Ags (36). The serum from normal individuals exhibited only minimal background activity in CP-mediated lysis assays of mouse E. Absorbed sera were immediately frozen at ~80°C.

C57BL/6 wild-type (WT) mice were used as an erythrocyte (Ehu) source. Blood was collected from the tail vein using heparinized glass capillaries. Procedures involving mice were approved by the local Animal Studies Committee and conducted in accordance with the guidelines for the care and use of laboratory research animals established by the National Institutes of Health.

In the experiment shown in Fig. 6, A and B, 4 μl of mouse whole blood (~4 × 10⁷ RBC) was incubated with 1.0 ml of the filtered, scFvP transfection supernatant for 2 h at room temperature. Control Ehu were treated for 2 h at room temperature with supernatants from nontransfected cells. Ehu samples were washed, resuspended in FACS buffer, and analyzed for properdin binding by flow cytometry. We estimated ~1000 copies scFvP/E by comparing fluorescence values to those of human decay accelerating factor (DAF) on human E (31) and assuming ~3300 DAF/human erythrocytes (Ehu) (37). Some Ehu samples were also treated with 10% C8-depleted serum for 30 min at 37°C, washed, and analyzed for the deposition of complement fragments by flow cytometry. Lysis was observed when the NHS was used in place of the C8-depleted serum. The data presented in Fig. 6, A and B, are representative of at least four independent experiments.

Human erythrocytes

Ehu were obtained from healthy volunteers according to the Washington University Medical Center Human Studies Committee guidelines. In Fig. 6, C and D, 10 μl of washed, packed Ehu were incubated with 1.0 ml of the filtered, transfection supernatant for 2 h at room temperature. Control Ehu were treated for 2 h at room temperature with supernatants from nontransfected cells. Ehu samples were washed, resuspended in Mg²⁺ EGTA buffer, and analyzed for properdin binding by flow cytometry. Copy number per E (~4500 scFvP/E) was determined as for Ehu. Ehu were treated with 20% autologous human serum for 60 min at 37°C, washed, and analyzed for the deposition of complement activation products by flow cytometry. The data presented in Fig. 6, C and D, are representative of at least four independent experiments.

Zymosan

Zymosan (Sigma-Aldrich) was prepared as described (38). Particles were counted with a Coulter counter (6.55 × 10⁸ particles/ml), and stored at 4°C until use. Zymosan was diluted in Mg²⁺ EGTA buffer to ~1.7 × 10⁶ particles/ml before treatment with various human complement proteins including properdin (10 μg/ml), C3b (10 μg/ml), factor B (10 μg/ml), and factor D (1 μg/ml) for 30 min at 37°C and washed twice in the same buffer before incubations. In some experiments, zymosan was first incubated with properdin, washed twice, and incubated in 5% properdin-deficient human serum for 10 min at 37°C. The data presented in Fig. 4 are representative of three independent experiments.

Flow cytometry

Erythrocytes, bacteria, or zymosan were washed and then incubated for 20–30 min at 4°C with a primary mAb (1/400). After washing, FITC-conjugated secondary Abs were added (1/100) and the incubation continued for an additional 15–20 min at 4°C. The Ab staining was conducted either in Mg²⁺ EGTA buffer (bacteria, zymosan, Ehu and Ehu) or in PBS supplemented with 1% heat inactivated FCS (Ehu and Ehu). The E. coli experiments of Fig. 7E were performed with a single FITC-conjugated goat anti-human polyclonal IgG (Cappel Laboratories) incubated for 20 min at 4°C (1/1000). Cells were washed and analyzed by flow cytometry (FACScan or FACScalibur; CellQuest software; BD Biosciences).

Results

A model for the assembly of the AP convertases

The standard model for the initiation of the AP C3 convertase (C3bBbP) on a target surface (Fig. 2A) begins with nascent C3b generated nonspecifically in the fluid phase. Nascent C3b features a highly reactive thioester that can form a covalent bond with a nearby target surface. Once a C3b-target complex is established, it binds factor B, the resulting C3bB complex is cleaved by factor D yielding C3bBb, and then the C3bBb complex is stabilized by

FIGURE 1. Western blot analysis of scFv-properdin chimeric proteins. A, Native properdin compared with scFv-properdin construct which reacts with mouse GPA. B, Native properdin compared with scFv-properdin which binds to human GPA. Positions of molecular mass markers with their respective molecular masses (kilodaltons) are indicated.

FIGURE 2. Two models are presented for the initiation and assembly of the AP C3 convertase on a target surface. A, Assembly is initiated via nascent C3b (C3b*) which is generated by nonspecific fluid-phase activation of C3 and then covalently binds to nearby surfaces. B, Assembly is initiated via properdin, which binds targets directly and then recruits fluid-phase C3b (nascent C3b is not required). In each case, the Ba fragment is released when factor B is cleaved by factor D.
properdin (C3bBbP). Recently, we observed that properdin attached to a biosensor surface binds C3b and that treatment of the resulting C3bP complexes with factor B and factor D leads to the formation of C3bBbP (26). This process provided a new order for C3bBbP assembly (Fig. 2B) and raised the possibility that properdin:target complexes could initiate AP activation.

**Properdin initiates complement activation on Neisseria**

Complement-deficient individuals are vulnerable to Neisseria infections (3–5), with meningococcal disease frequencies 6000-fold greater than in normal individuals (39). Those with properdin deficiencies suffer mortality rates of 34–63%. From our model of properdin function (Fig. 2B), we would predict that properdin binds to the Neisseria surface, initiating assembly of the AP C3 convertase, and promotes the opsonization and clearance or lysis of the pathogen (see also Ref. 40). To test this possibility, we examined the capacity of properdin to interact with Neisseria. As demonstrated in Fig. 3A, properdin binds WT Neisseria gonorrhoeae. C3b binds Neisseria, but only if the bacteria were pretreated with properdin (Fig. 3B). Surface-bound C3bBbP is generated when the bacteria-C3bP complexes are treated with factor B and factor D (Fig. 3C). These results demonstrate that properdin can initiate assembly of AP convertases on the Neisseria surface via bacterium-P and bacterium-C3bP intermediates. Importantly, Neisseria treated with properdin-deficient serum, under conditions that permit AP activation but exclude CP and LP activation, undergo low levels of C3 opsonization whereas Neisseria pretreated with properdin (forming bacterium-P complexes) become highly opsonized (Fig. 3D). C3 deposition achieved in the later case was comparable to that obtained with properdin-deficient serum reconstituted with purified properdin, and with serum bearing normal properdin levels (data not shown). These experiments demonstrate that Neisseria:properdin complexes promote the serum- and AP-dependent opsonization of the pathogen.

**Properdin initiates complement activation on zymosan**

In their original studies, Pillemer and his collaborators (12) incubated human serum with zymosan, an insoluble yeast cell wall preparation, and eluted the properdin protein from the resulting activation complexes. The remaining serum was depleted of complement activity that could be reconstituted by addition of the properdin protein or the zymosan activation complexes. In their straightforward interpretation, the authors proposed that properdin combined with zymosan to activate complement. Later, it was concluded that additional serum factors were required to form the zymosan:properdin complexes (19). We have re-examined this issue and show by FACS analysis that purified properdin does indeed bind zymosan (Fig. 4A) and that the zymosan:properdin complexes bind C3b more readily than do the untreated zymosan particles (Fig. 4B). Moreover, zymosan:properdin complexes promote complement activation in properdin-deficient human serum under conditions that permit AP activation but exclude CP and LP activation (Fig. 4C). These results support Pillemer’s original explanation of properdin function although they do not preclude activation of complement via the standard AP model.

**Effects of properdin on erythrocyte target specificity**

The E$_R$ and E$_K$ are targets commonly used to study AP activation (19). Our observations with Neisseria and zymosan (above), suggested to us that E$_K$:properdin complexes might also initiate AP activation. Thus, we treated E$_K$ with native properdin, analyzed the result by FACS, and observed that properdin bound E$_K$ surfaces (Fig. 5A). E$_K$, the classic nonactivator of the AP (19), did not bind properdin (Fig. 5B). C3b bound E$_K$ pretreated with properdin (E$_K$P), but did not bind E$_K$ alone (Fig. 5C). Treatment of E$_K$:C3b, factor B, and factor D led to the generation of E$_K$:C3bBbP complexes (Fig. 5D). These results demonstrate that properdin can initiate assembly of AP convertases, in this case on the E$_K$ surface.

**Directing complement activation with properdin**

The above experiments establish that properdin:target complexes can direct the in situ assembly and function of C3 convertases (C3bBbP). In principle, the AP could be activated wherever properdin is bound (26, 41). To test this prediction, we constructed a bifunctional recombinant protein consisting of a human properdin chain polypeptide, NH2-(mouse)scFv-(hu) properdin-COOH (or scFvP), bound EM, as shown by FACS analysis (Fig. 6A). Our rationale was to use this reagent to “tag” a target with properdin and to determine whether it would activate the alternative pathway.
To that end, EM were pretreated with scFvP and incubated with C8-depleted human serum in Mg\(^{2+}\)/H\(_{1100}\) EGTA buffer. As predicted, the EM-scFvP complex promoted deposition of C3 fragments on the EM surface (Fig. 6B). Deposition was dependent on the surface dosage of scFvP. Relatively low levels of complement-dependent C3 deposition occurred on mouse E that were not coated with scFvP. This activity could be attributed to the nonspecific AP initiation model. Additional experiments indicated mouse E bound little human properdin directly. No deposition was evident when reactions used heat-inactivated serum. In other experiments, if C8-depleted serum was replaced with absorbed normal human serum, properdin-tagged EM directed C-dependent cell lysis (data not shown).

A scFvP construct was also prepared to target EHU in a totally autologous system. In these experiments, in which EHU and serum were derived from the same individual, erythrocytes carried the human complement regulators CD59, DAF, and complement receptor 1 (CR1), which in this case would be expected to actively inhibit C3 deposition and hemolysis. An scFv domain was generated from a mAb that recognizes human GPA (32). The scFv-coding region was combined with human properdin-coding region to form a cDNA that encodes a NH\(_2\)-(hu)scFv-(hu)properdin-COOH single-chain polypeptide, similar in design to the mouse-scFvP protein (above). The scFv protein bound E\(_{HU}\) (Fig. 6C) and the E\(_{HU}\)-scFvP complex promoted C3b deposition in the presence of autologous human serum (Fig. 6D). E\(_{HU}\) did not bind human properdin directly (data not shown). C3 opsonization was lower than in the case of E\(_{M}\), probably due to the presence of human complement regulators DAF and CR1 on the E\(_{HU}\) surface. Similarly, the E\(_{HU}\) did not lyse, likely due to the presence of CD59, which inhibits the membrane attack complex.

**Propertin binds E. coli and Salmonella typhimurium LPS mutants**

Experiments designed to identify the specific targets recognized by properdin were begun. We observed that properdin did not bind WT strains of E. coli K12 (Table I) or S. typhimurium (our unpublished observations). However, the major surface component of the enteric bacteria, LPS, differs significantly from its Neisseria counterpart lipo-oligosaccharide; although LPS is composed of three major regions: a lipid A anchor, a “core” oligosaccharide, and a highly repeated penta-saccharide, known as the O-Ag (42), lipo-oligosaccharide is made up of only the lipid A anchor and the core oligosaccharide. Thus, we also examined “rough” LPS mutants that lack the O-Ag and all or part of the core oligosaccharide. Interestingly, properdin bound all E. coli (Table I) and S. typhimurium (our unpublished results) rough mutants tested, suggesting that properdin recognizes a common bacterial surface component that is readily exposed in the case of Neisseria but masked by the O-Ag in the WT (“smooth”) enteric bacteria.
Properdin-binding capacity of several bacterial strains correlates with their respective serum-dependent AP activation rate

If properdin initiates the AP in vivo, then we would expect that, in human serum, targets that bind properdin would activate the AP more readily than targets that do not bind properdin. We tested this hypothesis with several *E. coli* strains that differed markedly in their capacities to bind properdin (above and Table I). We included W1485, the strain was used by Schreiber et al. (43) in a detailed study of the bactericidal effects of the AP including the kinetics of serum-dependent C3 deposition. W1485 is a smooth strain which features the WT LPS structure and does not bind properdin (Fig. 7A). D31 is a rough strain which features reduced O-Ag (44) and binds properdin, D31m3 is a rough strain which features LPS that is truncated in the core oligosaccharide region (44). D31m3 binds properdin most readily of the three strains (Fig. 7A). We incubated the three bacteria strains for up to 10 min at 37°C in 5 or 20% human serum under buffer conditions that permit AP activation but exclude CP and LP activation, and then analyzed them by flow cytometry for C3 deposition. We found that at 5% serum levels (Fig. 7, B–D), W1485, which did not bind properdin, induced C3 deposition slowly with a lag of ~5 min. These observations are consistent with those of Schreiber et al. (43). In contrast, D31m3, which bound purified properdin most readily, induced C3 deposition rapidly. In that case, activation did not appear to require the capacity of properdin to partially stabilize the AP C3 convertase, because C3bBb on its own has a half-life of ~90 s, and the rapid reaction was evident within 1 min (data not shown). Finally, D31, which bound intermediate levels of properdin, induced C3 deposition with intermediate kinetics. These experiments were repeated with two additional WT (smooth) strains that did not bind properdin and two additional mutant (rough) strains that bound properdin readily and comparable results were obtained: the strains that bound properdin activated the AP more quickly than those that did
FIGURE 8. E. coli strains D31m3 and W1485 were incubated with 5% NHS in Mg²⁺ EGTA buffer for the indicated times. Cells were then washed, solubilized under reducing conditions, and subjected to SDS gel electrophoresis followed by Western blot using anti-C3 polyclonal Ab. Purified C3b was included as a control along with molecular weight markers. The high molecular weight forms (indicated as HMW) that appear at later time points are likely generated by the covalent attachment of nascent C3b* to surface macromolecules (52).

not bind properdin (Table I). Similar observations were made when the strains were treated at 20% serum levels (Fig. 7E).

E. coli were also treated with 5% human serum as above, and C3 deposition was analyzed by SDS-PAGE under reducing conditions followed by Western blot (Fig. 8). In the case of D31m3, surface-bound C3b was seen by 2 min, evident by its 110-kDa α’ fragment and 75-kDa β subunit. By 10 min, C3b was gone, having been converted to iC3b. This is confirmed by the loss of α’ and the appearance of its 68-kDa α1 and 37-kDa α2 cleavage products. Thus, for the D31m3 strain, C3 convertase formation and function occurred rapidly. In contrast, in the case of W1485, C3 deposition occurred relatively slowly: C3b was not seen at 2 min but was evident at 10 min, along with iC3b. These results, obtained also with C7-deficient serum (data not shown), are consistent with the kinetics of C3 deposition observed by FACS analysis (above). Together, these experiments demonstrate that E. coli that do not bind properdin, activate the AP rapidly, while E. coli that do not bind properdin, activate the AP slowly.

Discussion

The experiments described here present strong evidence that properdin can play a major role in complement activation. By this view, properdin can recognize targets directly and form a platform for the de novo assembly of the AP C3 convertases. This properdin-directed mechanism differs greatly from the standard model of AP C3 convertase assembly, and properdin function in particular, whereby initiation occurs via nascent C3b generated in the fluid phase that attaches nonspecifically to potential targets and properdin simply stabilizes the assembled convertase. The properdin-directed model was first suggested by experiments performed with purified proteins (Figs. 3, A–C, 4, A and B, 5, and Ref. 26), which demonstrated the possibility of assembling a convertase beginning with properdin-target surface. Further observations summarized below are predicted by, or are consistent with, the properdin-directed model but are inconsistent with, or unexpected by, the standard model: 1) Neisseria:properdin complexes (Fig. 3D) and zymosan:properdin complexes (Fig. 4C) incubated with properdin-deficient serum are intermediates in the formation of functional target:convertase complexes. Likewise, RBCs “tagged” with properdin via scFv triggered AP-dependent complement activation in human serum (Fig. 6). These findings strongly support a key step in the properdin-directed model that has no place in the standard model (Fig. 2). 2) Properdin mediates the noncovalent association of C3b with a variety of AP targets independently of factor B and factor D (Figs. 3–5), a point that is predicted by the properdin-directed model and unexpected by the standard view. 3) In the experiments performed with the E. coli panel (Figs. 7, 8, and Table 1), for each strain, properdin-binding capacity correlated with rate of AP-dependent C3 deposition. This result is predicted by the properdin-directed model but unexpected from the role of properdin in the standard model. 4) In the experiments performed with E. coli LPS mutants (Figs. 7, B–D, and 8), C3 deposition occurs on properdin-binding surfaces much faster than can be explained by the role of properdin in the standard model, but is consistent with the properdin-directed model.

We propose that the AP can be initiated via two different mechanisms, one that is nonspecific and is described by the standard model, and a second that is directed to specific target surfaces by properdin and described here: 1) in the case of nonspecific initiation, nascent C3b* formed through fluid-phase C3 tickover becomes covalently attached to nonspecific target surfaces where it initiates the assembly of a C3 convertase (Fig. 2A). Once that convertase begins to function, activation can be propagated via the AP amplification loop. This model explains AP activation observed with targets that do not bind properdin. 2) In the case of properdin-directed initiation, properdin binds directly to a specific target surface where it provides a platform for the assembly of C3 convertases (Fig. 2B). Fluid-phase tickover provides C3b and iC3, a functional analog of C3b. In principle, target-bound properdin could also be a receptor for any available fluid-phase C3bB and C3bBb.

Properdin is a multivalent protein composed of identical subunits (45), which may enable it to bind strongly to ligands clustered on a microbial surface while interacting weakly with the same ligands in solution. By this reasoning, target recognition is based on multiple weak protein:ligand interactions. This model accounts for AP activation with targets that bind properdin such as Neisseria, the E. coli LPS mutants, and zymosan. It could also account for the sensitivity of properdin-deficient individuals to Neisseria. Results with the enteric bacteria suggest that properdin can recognize a common bacterial surface component that is readily exposed in the case of Neisseria but masked by the O-Ag in the WT enteric bacteria. We conclude that properdin-directed initiation is a major pathway to AP activation on targets that bind properdin.

Although most complement proteins are produced in the liver, properdin is synthesized instead by blood monocytes, blood T cells (both CD4⁺ and CD8⁺), and blood neutrophils (46). In the case of neutrophils, properdin is stored in secondary granules and released upon specific stimulation, including activation through LPS and chemokines/cytokines (47). This process would provide an influx of properdin, making the properdin-directed mechanism of AP activation particularly effective at sites of inflammation. It may also represent a major physiological instance of properdin-directed AP activation because the binding of bacteria to native properdin was not readily detected when bacteria were simply incubated in serum in the absence of complement activation (our unpublished results). Alternatively, in the presence of potentially inhibitory factors in serum, association of properdin directly to a target may be stabilized only if C3bBbP target is assembled. We are currently examining potential interactions among AP targets, neutrophils, properdin, and other serum components.

Pillemer and his collaborators (12) first described properdin and the alternative pathway over 50 years ago. Their primary observations were made with zymosan, an extract derived from yeast cell walls. The authors proposed that properdin combined with zymosan to activate complement, but later attempts to detect the binding of purified properdin to zymosan failed and the model was
abandoned (19). We have re-examined this issue and obtained strong evidence in support of the Pillemer model. It is likely that part of the apparent discrepancy lies in the complexity of properdin:target interactions: initially, relatively few properdin proteins may bind to a target, but they are sufficient in number to initiate the AP. Once the AP is activated (as in the incubation of zymosan with serum), many more properdin-binding sites are generated through C3b opsonization. We have examined the initial steps in this process using purified proteins and highly sensitive methods including surface plasmon resonance technology (26), mAbs, flow cytometry, and recombinant DNA technology. The tools that were available during Pillemer’s time were sufficient to detect zymosan:properdin complexes achieved via the full activation process, but not sensitive enough to detect the initial zymosan:properdin interaction. Many of the AP studies conducted since then were performed with E$_{R}$ or with WT E. coli, which do not bind properdin (Figs. 5B and 7A, Table I).

Factor H is a serum protein that suppresses the assembly and function of the AP convertases. Previous work demonstrated that surface sialic acid promotes local factor H function and that some surfaces that feature sialic acid (E$_{S}$) are less sensitive to the AP than others devoid of sialic acid (E$_{D}$). It is not surprising that E$_{R}$ both binds properdin and is devoid of sialic acid because E$_{D}$ are among the most AP sensitive of erythrocyte species. Conversely, it is not surprising that E$_{S}$ both binds properdin poorly and harbors surface sialic acid (23, 24). Our new results do not preclude the importance of factor H, but instead indicate that a positive activation mechanism is also at work. In principle, we might expect that pathogens would evolve to avoid or control properdin affinity while hosts would evolve to maximize properdin: pathogen recognition. However, the interplay of different bacterial structures with properdin, factor H, Ab, and other immunity factors is complex so that avoidance of properdin-directed complement activation could result in sensitivity to another pathway. This may account for the particular vulnerability of properdin-deficient individuals to relatively rare N. meningitidis serogroups (48).

The alternative pathway has been implicated in lupus nephritis, rheumatoid arthritis, antiphospholipid syndrome, ischemia/reperfusion injury, asthma, atypical hemolytic-uremic syndrome, type II membranoproliferative glomerulonephritis, spontaneous fetal loss, and macular degeneration (49). The role of properdin in these instances now must be reconsidered. Properdin is stored in neutrophils at sites of infection would direct complement activation from neutrophils at sites of infection would direct complement activation appropriately to pathogen surfaces.

In summary, we present evidence that properdin:target interactions can initiate complement activation. Thus, properdin participates in two distinct activation pathways: one that occurs by the standard model and one that proceeds by a properdin-directed model. Our observations could account for the sensitivity of properdin-deficient individuals to Neisseria infections and we expect that other microbial targets also initiate complement activation via this mechanism. A reappraisal of properdin activity could shed new light on the evolution and function of the complement alternative pathway and its involvement in innate immunity and human disease.

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Disclosures

The authors have no financial conflict of interest.

References

1. Volanakis, J. E. 1998. Overview of the complement system. In The Human Complement System in Health and Disease, 10 Ed. J. E. Volanakis and M. M. Frank, eds. Marcel Dekker, New York, pp 9–32.

2. Dempsley, P. W., M. E. Allison, S. Akkaraju, C. C. Goodnow, and D. T. Fearon. 1996. C3d of complement as a molecular adjuvant: bridging innate and acquired immunity. Science 271: 348–350.

3. Bracconier, J. H., A. G. Sjoholm, and C. Soderstrom. 1983. Fulminant meningococcal infections in a family with inherited deficiency of properdin. Scand. J. Infect. Dis. 15: 339–345.

4. Densen, P., J. M. Weiler, J. M. Griffiss, and L. G. Hoffmann. 1987. Familial properdin deficiency and fatal meningococcemia: correction of the bacterial defect by vaccination. N. Engl. J. Med. 316: 922–926.

5. Kolbke, K., and B. K. Reid. 1993. Genetic deficiencies of the complement system and association with disease–early components. Int. Rev. Immunol. 10: 17–36.

6. Kleinman, K. E., C. Zeiss, E. Y. Chew, J. Y. Tsai, R. S. Sackler, C. Haynes, A. K. Henning, J. P. Sangiovanni, S. M. Mane, S. T. Mayne, et al. 2005. Complement factor H polymorphism in age-related macular degeneration. Science 308: 385–389.

7. Edwards, A. O., R. Ritter, 3rd, K. J. Abel, A. Manning, C. Banuysen, and L. A. Farrer. 2005. Complement factor H polymorphism and age-related macular degeneration. Science 308: 421–424.

8. Haines, J. L., M. A. Hauser, S. Schmidt, W. K. Scott, L. M. Olson, P. Gallins, K. L. Spencer, S. Y. Kwan, M. Noureddine, J. R. Gilbert, et al. 2005. Complement factor H variant increases the risk of age-related macular degeneration. Science 308: 419–421.

9. Hageman, G. S., D. H. Anderson, L. V. Johnson, L. S. Hancos, A. J. Taiber, L. I. Hardisty, J. L. Hageman, H. A. Stockman, J. D. Borchardt, K. M. Gehrs, et al. 2005. A common haplotype in the complement regulatory gene factor H (H1F/CHF) predisposes individuals to age-related macular degeneration. Proc. Natl. Acad. Sci. USA 102: 7227–7232.

10. Pickering, M., C. H. Cook, J. Warren, A. E. Bygrave, J. Moss, M. J. Walport, and R. Botto. 2002. Uncertified C3 activation causes membranoproliferative glomerulonephritis in mice deficient in complement factor H. Nat. Genet. 31: 424–428.

11. Ji, H., K. Ohmura, U. Mahmood, D. M. Lee, F. M. Hofhuis, S. A. Bouchke, K. Takahashi, V. M. Holers, M. Walport, C. Gerard, et al. 2002. Arthritis critically dependent on innate immune system players. Immunity 16: 157–168.

12. Pillemer, L., B. Blum, J. H. Lepow, O. A. Ross, E. W. Todd, and A. C. Wardlaw. 1954. The properdin system and immunity. I. Demonstration and isolation of a new serum protein, properdin, and its role in immune phenomena. Science 120: 279–285.

13. Ratnoff, W. D. 1980. A war with the molecules: Louis Pillemer and the history of properdin. Perspect. Biol. Med. 23: 638–657.

14. Anderson, R. A., Jr. 1958. An alternative mechanism for the properdin system. J. Exp. Med. 108: 515–535.

15. Ecker, E. E. 1958. Louis Pillemer, 1908–1957. J. Immunol. 80: 415–416.

16. Lepow, J. H. 1980. Presidential address to American Association of Immunologists in Anaheim, California, April 16, 1980: Louis Pillemer, properdin, and scientific controversy. J. Immunol. 125: 471–475.

17. Fearon, D. T., and K. F. Austen. 1975. Properdin: binding to C3b and stabilization of the C3b-dependent C3 convertase. J. Exp. Med. 142: 856–863.

18. Lachmann, P. J., and N. C. Hughes-Jones. 1984. Initiation of complement activation. Springer Semin. Immunopathol. 7: 143–162.

19. Fearon, D. T. 1979. Activation of the alternative complement pathway. CRC Crit. Rev. Immunol. 1: 1–32.

20. Pangburn, M. K., and H. J. Muller-Eberhard. 1984. The alternative pathway of complement. Springer Semin. Immunopathol. 7: 163–192.

21. Medicus, R. G., O. Gotze, and H. J. Muller-Eberhard. 1976. Alternative pathway of complement: recruitment of precursor properdin by the labile C3/C5 convertase and the potentiation of the pathway. J. Exp. Med. 144: 1076–1093.

22. Pangburn, M. K., and H. J. Muller-Eberhard. 1986. The C3 convertase of the alternative pathway of human complement: enzymic properties of the bimolecular proteinase. Biochem. J. 235: 723–730.
23. Fearon, D. T. 1978. Regulation by membrane sialic acid of β1H-dependent de-
cay-dissociation of amplification C3 convertase of the alternative complement
pathway. Proc. Natl. Acad. Sci. USA 75: 1971–1975.
24. Pangburn, M. K., and H. J. Muller-Eberhard. 1978. Complement C3 convertase:
cell surface restriction of β1H control and generation of restriction on neuramin-
idase-treated cells. Proc. Natl. Acad. Sci. USA 75: 2416–2420.
25. Atkinson, J. P., and T. Farries. 1987. Separation of self from non-self in the
complement system. Immunol. Today 8: 212–215.
26. Hourcade, D. 2006. The role of properdin in the assembly of the alternative
pathway C3 convertases of complement J. Biol. Chem. 281: 2128–2132.
27. Spitzer, D., H. Hauser, and D. Wirth. 1999. Complement-protected amphotropic
retroviruses from murine packaging cells. Hum. Gene Ther. 10: 1893–1902.
28. Wolfgang, M., P. Lauer, H. S. Park, L. Brossay, J. Hebert, and M. Koomey. 1998.
PilT mutations lead to simultaneous defects in competence for natural transform-
tion and twitching motility in piliated Neisseria gonorrhoeae. Mol. Microbiol.
29: 321–330.
29. Whaley, K. 1985. Measurement of complement. In Methods in complement for
Clinical Immunologists. K. Whaley, ed. Churchill Livingstone, New York, pp
77–139.
30. Nolan, K. F., W. Schweable, S. Kaluz, M. P. Dierich, and K. B. Reid. 1991. Molecular
cloning of the cDNA coding for properdin, a positive regulator of the
alternative pathway of human complement. Eur. J. Immunol. 21: 771–776.
31. Spitzer, D., J. Unsinger, M. Bessler, and J. P. Atkinson. 2004. ScFv-mediated in
vivo targeting of DAF to erythrocytes inhibits lysis by complement. Mol. Immuno-
log. 40: 911–919.
32. Anstee, D. J., and P. A. Edwards. 1982. Monoclonal antibodies to human eryth-
rocytes. Eur. J. Immunol. 12: 228–232.
33. Gardner, B., S. F. Parsons, A. H. Merry, and D. J. Anstee. 1989. Epitopes on
sialoglycoprotein α: evidence for heterogeneity in the molecule. Immunology 68:
281–289.
34. Dubel, S., F. Breitling, P. Fuchs, M. Zewe, S. Götter, M. Welschof,
G. Moldenhauer, and M. Little. 1994. Isolation of IgG antibody Fv-DNA from
various mouse and rat hybridoma cell lines using the polymerase chain reaction
with a simple set of primers. J. Immunol. Methods 175: 89–95.
35. Hourcade, D. E., L. M. Mitchell, and T. J. Oglebsy. 1998. A conserved element
in the serine protease domain of complement factor B. J. Biol. Chem. 273:
25996–26000.
36. Gděit, U., S. Shohet, E. Kobrin, C. Stults, and B. Macher. 1988. Man, apes, and
Old World monkeys differ from other mammals in the expression of α-galactosyl
epitopes on nucleated cells. J. Biol. Chem. 263: 17755–17762.
37. Morgan, B. P., and S. Meri. 1994. Membrane proteins that protect against com-
plement lysis. Springer Semin. Immunopathol. 15: 369–396.
38. Foley, S., B. Li, M. Dehoff, M. Molina, and V. M. Holers. 1993. Mouse Cyr61/p65
is a regulator of the alternative pathway of complement activation. Eur. J.
Immunol. 23: 1381–1384.
39. Densen, P. 1989. Interaction of complement with Neisseria meningitidis and
Neisseria gonorrhoeae. Clin. Microbiol. Rev. 2(Suppl.): S11–S17.
40. Griffin, J. M., G. A. Jarvis, J. P. O’Brien, M. M. Eads, and H. Schneider. 1991.
Lysos of Neisseria gonorrhoeae initiated by binding of normal human IgM to a
hexosamine-containing lipooligosaccharide epitope(s) is augmented by strain-
specific, properdin-binding-dependent alternative complement pathway activa-
tion. J. Immunol. 147: 298–305.
41. Vuagnat, B. B., J. Mach, and J. M. Le Doussal. 2000. Activation of the alternative
pathway of human complement by autologous cells expressing transmembrane
recombinant properdin. Mol. Immunol. 37: 467–478.
42. Heinrichs, D. E., J. A. Yethon, P. A. Amor, and C. Whitfield. 1998. The assembly
system for the outer core portion of R1- and R4-type lipooligosaccharides of
Escherichia coli: the R1 core-specific β-glucosyltransferase provides a novel
attachment site for O-polysaccharides. J. Biol. Chem. 273: 29497–29505.
43. Schreiber, R. D., D. C. Morrison, E. R. Podack, and H. J. Muller-Eberhard. 1979.
Bactericidal activity of the alternative complement pathway generated from
eleven isolated plasma proteins. J. Exp. Med. 149: 870.
44. Boman, H. G., and D. A. Monner. 1975. Characterization of lipooligosaccharides
from Escherichia coli K-12 mutants. J. Bacteriol. 121: 455–464.
45. Smith, C. A., M. K. Pangburn, C. W. Vogel, and H. J. Muller-Eberhard. 1984.
Molecular architecture of human properdin, a positive regulator of the alternative
pathway of complement. J. Biol. Chem. 259: 4582–4588.
46. Schwaeble, W. J., and K. B. Reid. 1999. Does properdin crosslink the cellular and
the humoral immune response? Immunol. Today 20: 17–21.
47. Widhalmueller, U., B. Dewald, M. Thelen, M. K. Schafer, C. Stover, K. Whaley,
J. North, P. Eggleton, K. B. Reid, and W. J. Schwaeble. 1997. Properdin, a
positive regulator of complement activation, is released from secondary granules
of stimulated peripheral blood neutrophils. J. Immunol. 158: 4444–4451.
48. Sjoholm, A. G. 1991. Inherited complement deficiency states and disease.
Com-
mucosurface Immunol. 8: 341–346.
49. Thurman, J. M., and V. M. Holers. 2006. The central role of the alternative
complement pathway in human disease. J. Immunol. 176: 1305–1310.
50. Wipke, B. T., and P. M. Allen. 2001. Essential role of neutrophils in the initiation
and progression of a murine model of rheumatoid arthritis. J. Immunol.
167: 1601–1608.
51. Girardi, G., J. Berman, P. Redecha, L. Spruce, J. M. Thurman, D. Kraus,
J. P. O’Brien, M. C. Caroll, R. A. Wetsel, et al. 2003. Complement
C5a receptors and neutrophils mediate fetal injury in the antiphospholipid syn-
drome. J. Clin. Invest. 112: 1644–1654.
52. Law, S. K., and R. P. Levine. 1977. Interactions between the third complement
protein and cell surface macromolecules. Proc. Natl. Acad. Sci. USA 74:
2701–2705.
53. Beutin, L., and M. Achtman. 1979. Two Escherichia coli chromosomal cistrons,
sfrA and sfrB, which are needed for expression of F factor tra functions.
J. Bacteriol. 139: 730–737.