

Linkage Specificity and Role of Properdin in Activation of the Alternative Complement Pathway by Fungal Glycans

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ABSTRACT Fungal cell walls are predominantly composed of glucans, mannans, and chitin. Recognition of these glycans by the innate immune system is a critical component of host defenses against the mycoses. Complement, an important arm of innate immunity, plays a significant role in fungal pathogenesis, especially the alternative pathway (AP). Here we determine that the glycan monosaccharide composition and glycosidic linkages affect AP activation and C3 deposition. Furthermore, properdin, a positive regulator of the AP, contributes to these functions. AP activation by glycan particles that varied in composition and linkage was measured by C3a generation in serum treated with 10 mM EGTA and 10 mM Mg²⁺ (Mg-EGTA-treated serum) (AP specific; properdin functional) or Mg-EGTA-treated serum that lacked functional properdin. Particles that contained either β 1 \rightarrow 3 or β 1 \rightarrow 6 glucans or both generated large and similar amounts of C3a when the AP was intact. Blocking properdin function resulted in 5- to 10-fold-less C3a production by particulate β 1 \rightarrow 3 glucans. However, particulate β 1 \rightarrow 6 glucans generated C3a via the AP only in the presence of intact properdin. Interestingly, zymosan and glucan-mannan particles (GMP), which contain both β -glucans and mannans, also required properdin to generate C3a. The β 1 \rightarrow 4 glycans chitin and chitosan minimally activated C3 even when properdin was functional. Finally, properdin binding to glucan particles (GP) and zymosan in serum required active C3. Properdin colocalized with bound C3, suggesting that in the presence of serum, properdin bound indirectly to glycans through C3 convertases. These findings provide a better understanding of how properdin facilitates AP activation by fungi through interaction with the cell wall components.

IMPORTANCE Invasive fungal infections have increased in incidence with the widespread use of immunosuppressive therapy and invasive procedures. Activation of the complement system contributes to innate immunity against fungi by generating chemoattractants that recruit white blood cells and by coating the pathogen with complement fragments that “mark” them for phagocytosis. The fungal cell wall activates complement in an antibody-independent manner through the alternative pathway (AP). Properdin is a positive regulator of the AP. This study elucidates how the specificity of cell wall glycan linkages affects AP activation and the role properdin plays in this process. Particulate β 1 \rightarrow 3 glucans activated the AP even in the absence of properdin, while β 1 \rightarrow 6 glucans required properdin for AP activation. In contrast, the β 1 \rightarrow 4 glycans chitin and chitosan failed to activate the AP. These findings enhance our mechanistic understanding of how fungi activate complement and have implications for the use of glycans in biomedical applications.

Received 4 August 2011 Accepted 8 August 2011 Published 30 August 2011

Citation Agarwal S, et al. 2011. Linkage specificity and role of properdin in activation of the alternative complement pathway by fungal glycans. *mBio* 2(5):e00178-11. doi:10.1128/mBio.00178-11.

Editor Liise-ann Pirofski, Albert Einstein College of Medicine

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Complement activation, a critical component of host defenses against fungal infection (1–3), can proceed by three well-defined pathways. Classical pathway activation is mainly initiated when antibody is complexed to antigens. The lectin pathway shares structural similarities to the classical pathway but is triggered by binding to mannose and ficolins. The third complement activation pathway, the alternative pathway (AP), does not require antibody or lectins for its initiation. Rather, initiation of the AP occurs by spontaneous low-rate hydrolysis of the thioester in C3, which results in continuous supply of C3(H₂O) in solution (4). The half-life of the activated C3 thioester has been estimated to be ~60 μ s (5). Binding of factor B to C3(H₂O) and cleavage of bound factor B to Bb by the enzyme factor D lead to the formation of the

initial AP C3 convertase [C3(H₂O)Bb]. This fluid-phase C3 convertase can cleave C3 to release C3a and C3b, and the latter can covalently bind to microbial surfaces. Binding of factor B to surface-bound C3b in the presence of factor D leads to the formation of an AP C3 convertase (C3b,Bb) that cleaves additional C3 molecules. This positive C3 amplification loop is a key feature of the AP. The AP C3 convertase C3b,Bb has a half-life of only 1.5 min. Properdin carries out the important function of binding to and stabilizing the C3b,Bb complex, thereby increasing its half-life 5- to 10-fold (6). Properdin is the only known positive regulator of the complement system.

Properdin is found in serum at a concentration of 4 to 25 μ g/ml (7–11). Properdin is stored in neutrophils as secondary

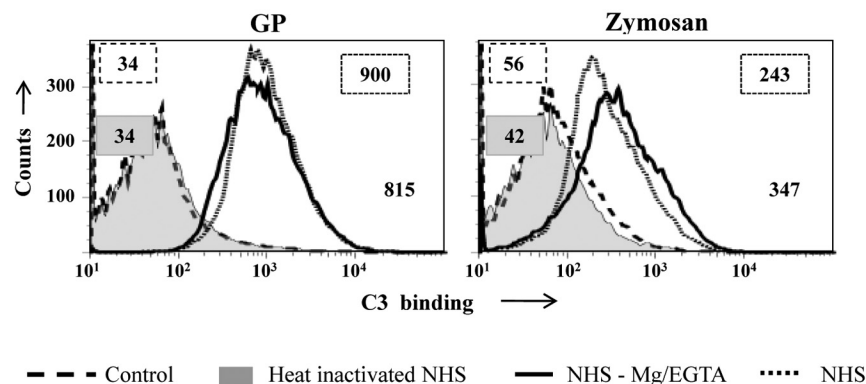


FIG 1 The alternative pathway (AP) mediates C3 deposition on glucan particles (GP) and zymosan. Total C3 deposited on GP and zymosan was measured by flow cytometry. GP and zymosan were incubated with either normal human serum (NHS) (all complement pathways active; dotted black lines), NHS treated with 10 mM EGTA and 10 mM Mg^{2+} (NHS-Mg/EGTA) (only AP active; solid black line), or heat-inactivated NHS (no active complement; solid grey histograms). The final concentration of serum in all reaction mixtures was 40%. Total C3 (C3b and iC3b) deposited on the glycan surface was measured by flow cytometry using sheep polyclonal anti-human C3c conjugated to FITC. The dashed black line represents a control where particles were incubated with FITC conjugated C3c antibody alone (human serum was omitted from the reaction mixture). The x axis represents fluorescence on a \log_{10} scale, and the y axis shows the number of events (counts). The numbers adjacent to the histograms represent the median fluorescence intensity of C3 binding. The results of one representative experiment of two reproducibly repeated experiments are shown.

granules and is released upon cell stimulation (12), which appears to play a key role in determining local AP activity (10). Individuals with properdin deficiency are predisposed to severe invasive meningococcal infections, often with a higher mortality than healthy individuals (13–15).

Although interspecies and intraspecies variations are common, most fungal cell walls feature an inner layer composed of β -glucans and chitin, and the inner layer is wholly or partially masked by covalently linked cell surface mannoproteins (16–18). Mannans consist of polymers of mannose added to proteins via N- or O-linkages. The β -glucan layer predominantly comprises β -1,3 linkages, with a minority (9 to 20%) of glucans linked by a β -1,6 bond (18, 19). Glucan particles (GP), highly purified *Saccharomyces cerevisiae* cell walls composed of β -1,6 branched β -1,3 glucans have been suggested as an antigen delivery vehicle because their polysaccharide composition provides receptor-targeted delivery to phagocytic antigen-presenting cells and their hollow and porous structure allows for high antigen loading (20, 21). β -Glucan preparations derived from fungi such as *Saccharomyces cerevisiae* have a record of safety in both preclinical and human trials (22–24). These findings support the clinical development of glucan particles as a vaccine delivery platform and immunoadjuvant.

Deciphering the mechanistic basis for how fungal cell walls activate complement is important not only for understanding host defenses and fungal pathogenicity but also for the safe and effective design of fungal glycan-based biotherapies. More than 25 years ago, Czop and Austen (25) demonstrated that aggregated β -glucans derived from yeast were potent activators of the human AP. In this study, we have extended those observations and have defined the role of properdin in activating the AP in a linkage-specific manner. These studies provide important mechanistic insights into the role of properdin in complement activation on fungal glucans, which could affect downstream events in the immune response, such as leukocyte recruitment, neutrophil activation, and T cell skewing.

RESULTS

C3 is deposited on GP through the AP. Complement-dependent C3 fragment deposition on glucan particles (GP) (contains both $\beta 1 \rightarrow 3$ and $\beta 1 \rightarrow 6$ D-glycosidic linkages) was measured by flow cytometry. The alternative pathway (AP) (normal human serum [NHS] treated with 10 mM EGTA and 10 mM Mg^{2+} [Mg-EGTA-NHS]) accounted for C3 deposition at 30 min; addition of the lectin and classical pathways (NHS) did not increase C3 deposition (Fig. 1, left panel). These results were similar to those observed with zymosan, which is known to activate C3 through the AP (26) and used here as a positive control. Although zymosan displayed the same results qualitatively, less C3 deposition was observed (Fig. 1, right panel). To ensure that chelating Ca^{2+} with EGTA did not affect the assays, we also used C2-depleted serum (AP alone, functional) and confirmed the results (data not shown); Mg-EGTA-treated serum was employed for the remainder of the study.

Properdin facilitates AP-mediated C3 deposition on GP.

Properdin is the only known positive regulatory protein of the AP (6), and we speculated that it facilitated deposition of C3 fragments on GP. We used confocal microscopy to assess whether C3 and properdin localized as predicted by the traditional model of properdin binding to alternative pathway C3/C5 convertases (26–28). As a prelude to confocal microscopy experiments, we examined several concentrations of Mg-EGTA-NHS with functioning properdin or Mg-EGTA-NHS where properdin function was blocked with monoclonal antibody (MAb) A233 (Quidel), and we found that a serum concentration of 40% (vol/vol) (40% NHS), which had been optimized for measuring C3 deposition by fluorescence-activated cell sorting (FACS), was also suitable for confocal experiments. GP or zymosan was incubated with either 40% NHS treated with 10 mM EGTA and 10 mM Mg^{2+} (40% NHS-Mg-EGTA) or 40% human properdin-depleted serum, and the reactions were stopped at 10, 15, 20, and 30 min by adding EDTA. C3 deposition was detected using fluorescein isothiocyanate (FITC)-conjugated anti-human C3c, and bound properdin was detected using anti-human properdin followed by anti-mouse IgG conjugated to Alexa Fluor 647. At 10 min, discrete foci with colocalization of C3 and properdin were observed on both GP and zymosan particles (Fig. 2A). With time, C3 deposition spread over the entire surfaces of the particles, while properdin binding continued at discrete locations of C3b binding (Fig. 2A and B, merged columns). In the absence of properdin, the amount of C3 fragments deposited on both GP and zymosan particles was greatly reduced (Fig. 2B shows minimal C3 deposition at 30 min), highlighting the importance of properdin in facilitating C3 deposition on these particles.

Properdin binds to GP only in the presence of C3 in the context of human serum. Previous studies have shown that native forms of purified properdin can bind directly to certain AP activator surfaces, including zymosan in non-serum-containing sys-

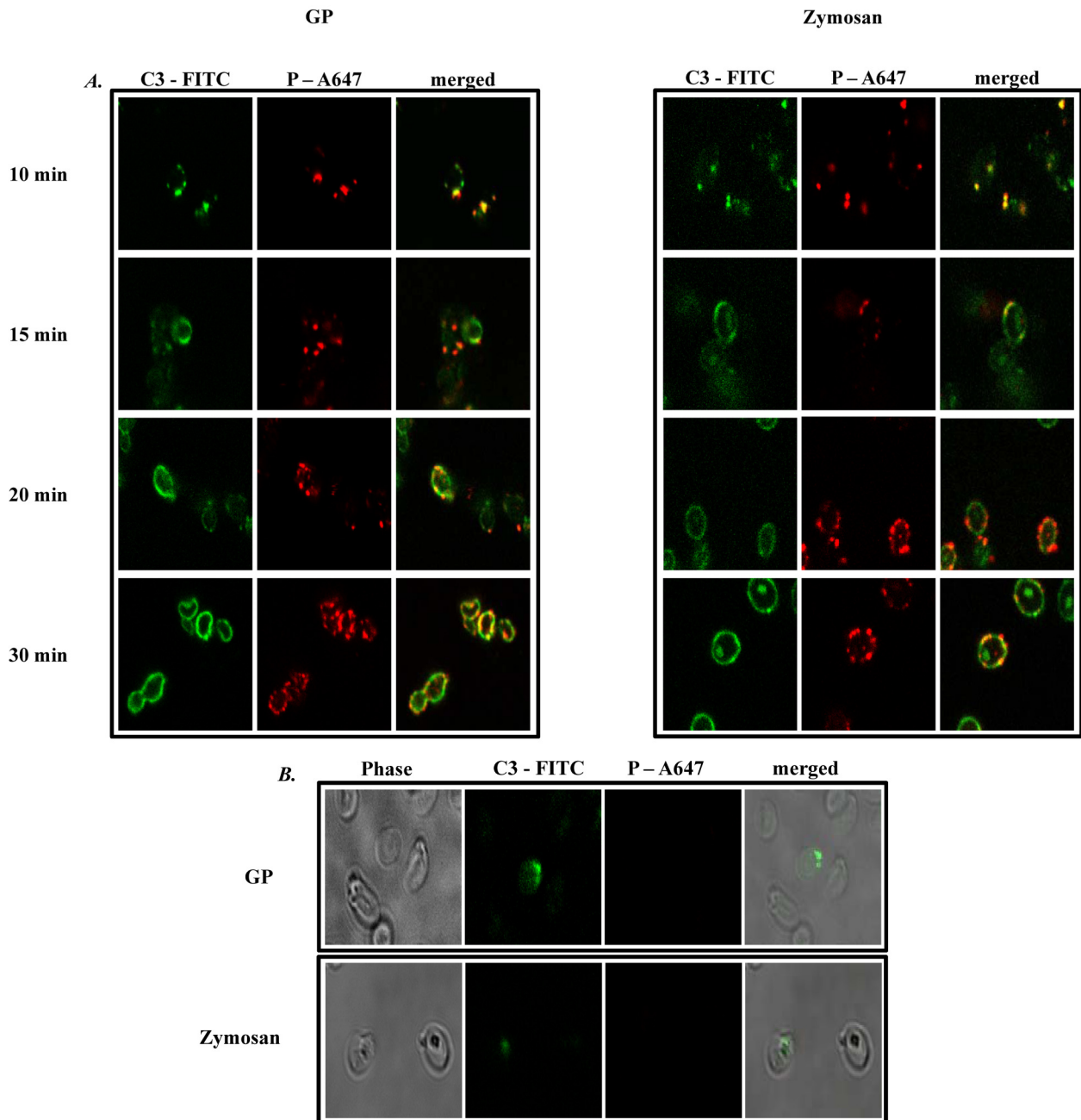


FIG 2 The kinetics and location of C3 deposition and properdin binding on GP and zymosan particles by confocal microscopy. (A) GP and zymosan particles were incubated with 40% NHS–Mg-EGTA (40% NHS treated with 10 mM EGTA and 10 mM Mg^{2+}), and the reaction was stopped by adding 10 mM EDTA at the indicated time points. C3 deposited on particles was detected with sheep polyclonal anti-human C3c conjugated to FITC (green) (C3 - FITC columns), and properdin (P) binding was detected with an antiproperdin MAb (A235; Quidel MAb 235) followed by Alexa Fluor 647-labeled anti-mouse IgG (red) (P - A647 columns). The merged columns are the merged images of C3 and properdin binding. (B) Properdin is required for maximal C3 deposition on GP and zymosan. GP and zymosan were incubated with 40% serum (Mg-EGTA-treated serum depleted of properdin). C3 and P binding were detected as described above for panel A. The phase-contrast images are shown in the leftmost column, and the phase-contrast and C3 images were merged and are shown in the rightmost column. The images were taken using a 63 \times lens and imaged with a Leica TCS SP2 AOBS laser scanning confocal microscope (Leica, Wetzlar, Germany).

tems (29). Because it has been suggested that a serum component(s) may interfere with binding of properdin directly to alternative pathway activator surfaces (30), we questioned whether properdin could bind directly to GP and zymosan particles in the context of serum. C3-depleted serum was used to eliminate C3 deposition on GP and zymosan to examine whether pro-

perdin would bind directly to GP and zymosan surfaces [or possibly through a serum component(s) distinct from C3]. There was no detectable properdin binding to GP or zymosan by FACS analysis in C3-depleted serum over baseline fluorescence levels seen with properdin-depleted serum (Fig. 3A). Reconstitution of C3-depleted serum with physiological concentrations of purified

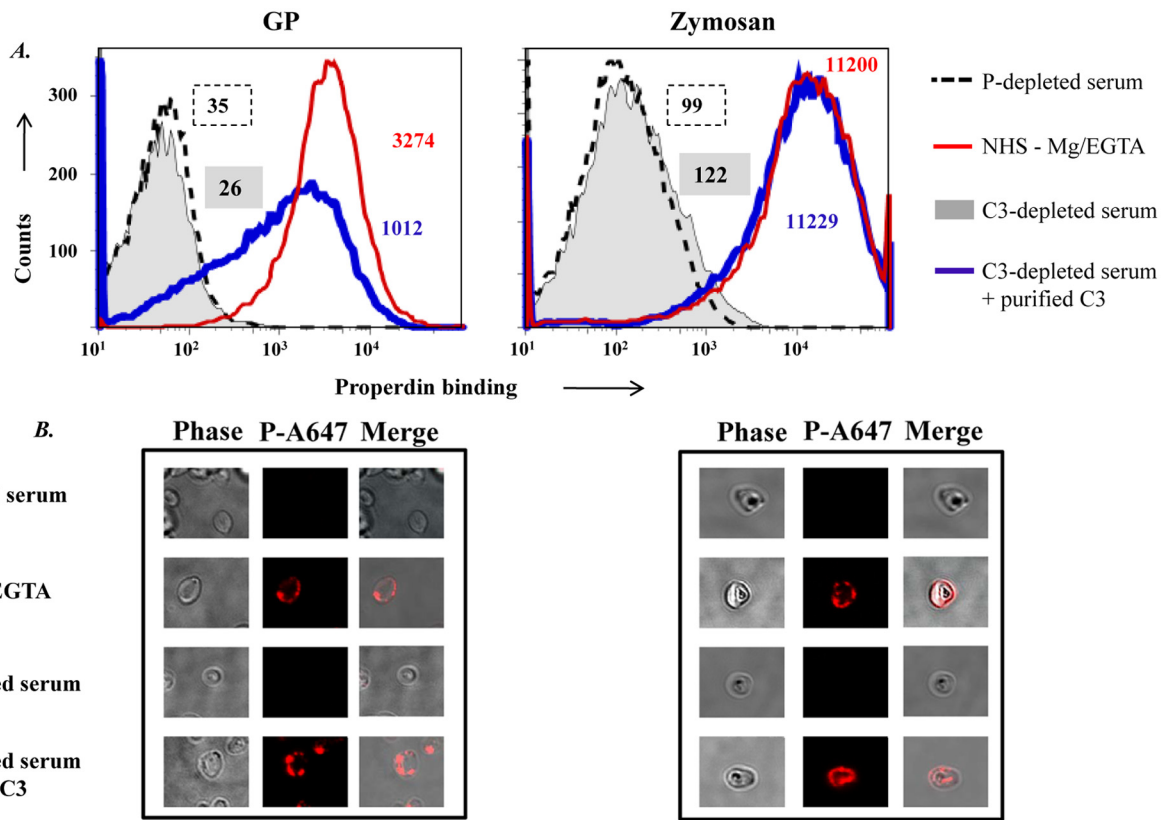


FIG 3 Properdin in serum binds to GP and zymosan particles only in the presence of active C3. Properdin binding to GP and zymosan was detected following incubation of the particles with C3-depleted serum and C3-depleted serum reconstituted with purified C3. The final concentration of serum in all reaction mixtures was 40%. Normal human serum (NHS) and properdin-depleted (P-depleted) serum were used as positive and negative controls for properdin binding, respectively. (A) Glycan-bound properdin was detected by flow cytometry using an antiproperdin MAb (A235; Quidel) followed by anti-mouse IgG conjugated to Alexa Fluor 647. In all graphs, the x axis shows fluorescence on a log₁₀ scale, and the y axis shows the number of events. Numbers adjacent to the histogram represent the median fluorescence intensity of properdin binding. (B) Properdin binding to GP and zymosan by confocal microscopy. Using conditions described above for panel A, the particles were stained for properdin using an antiproperdin MAb and Alexa Fluor 647. The phase-contrast images, properdin staining (red) (middle column), and merged images of the phase-contrast and properdin images are shown in the right-hand column. The images were collected and analyzed as described in the legend to Fig. 2. The results of one representative experiment of two separate and reproducibly repeated experiments are shown.

C3 (1 mg/ml) restored properdin binding, measured by flow cytometry (Fig. 3A). These results were confirmed using confocal microscopy (Fig. 3B), where properdin bound to GP and zymosan occurred only when active C3 was present in the serum, not when C3-depleted serum was used. These data provide evidence that properdin in human serum does not bind directly to GP or zymosan but associates with alternative pathway C3/C5 convertases that bound to GP and zymosan.

Glycan linkage specificity and influence of properdin on AP-mediated release of C3a and C5a. GP are primarily composed of $\beta 1 \rightarrow 3$ and $\beta 1 \rightarrow 6$ glucan linkages (21, 31), while zymosan comprises $\beta 1 \rightarrow 3$ and $\beta 1 \rightarrow 6$ β -glucans and mannans (32, 33). In addition to $\beta 1 \rightarrow 3$ and $\beta 1 \rightarrow 6$ linkages, fungal glucans contain a small fraction of chitin composed of $\beta 1 \rightarrow 4$ glycosidic linkages (34–36). Both GP and zymosan also contain a small fraction of chitin. We examined whether glycan linkage specificity affected activation of the AP using glycan particles from an array of sources, which are listed in Table 1. In addition, three soluble glycans (dextran, laminarin, and mannan), representing several glycan linkages, were used as negative controls because soluble glycans are poor AP activators (25).

Activation of AP generates the C3 convertase C3b,Bb that cleaves the α chain of C3 and releases the N-terminal 9-kDa C3a fragment. Cleavage of the C3a fragment is accompanied by exposure of a labile internal thioester bond in the resulting α' chain of C3b. The C3b molecule must bind to a surface target (in this instance, the glycan particle) through a covalent ester or amide bond. Failure to do so results in hydrolysis of the internal thioester, leaving the C3b unbound in solution. Continued complement activation results in formation of the AP C5 convertase (C3bC3b,Bb) that cleaves C5 and releases the C5a fragment. We used enzyme-linked immunosorbent assay (ELISA) to measure the amounts of C3a and C5a generated by particulate and soluble glycans that activated the AP. Glucan particles that contained only $\beta 1 \rightarrow 3$ linkages (curdlan) or $\beta 1 \rightarrow 6$ linkages (pustulan) or both $\beta 1 \rightarrow 3$ and $\beta 1 \rightarrow 6$ linkages (GP and scleroglucan) were potent activators of AP, generating high levels of C3a and C5a (Fig. 4A and C). In contrast, particles that comprised only $\beta 1 \rightarrow 4$ linkages of amino *N*-acetylglucosamine and *N*-glucosamine (chitin and chitosan) failed to significantly activate AP above baseline levels. As expected, zymosan, a known AP activator that contains both β -glucans and mannans, generated high levels of C3a and C5a.

TABLE 1 Glycans used in this study

Glycan	Species [source or reference(s)]	Composition and linkage
Particulate glycans		
Curdlan	<i>Alcaligenes faecalis</i> (Sigma)	$\beta 1 \rightarrow 3$ D-glucose
GP	<i>Saccharomyces cerevisiae</i> (21, 31)	$\beta 1 \rightarrow 3$ and $\beta 1 \rightarrow 6$ D-glucose and trace $\beta 1 \rightarrow 4$ D-N-acetylglucosamine
Scleroglucan	<i>Sclerotium glaucanicum</i> (Cargill)	$\beta 1 \rightarrow 3$ and $\beta 1 \rightarrow 6$ D-glucose
Pustulan	<i>Umbilicaria pustulata</i> (Calbiochem)	$\beta 1 \rightarrow 6$ D-glucose
Chitin	<i>Chionoecetes opilio</i> (Seikagaku)	$\beta 1 \rightarrow 4$ D-N-acetylglucosamine
Chitosan	<i>Chionoecetes opilio</i> (Primex)	$\beta 1 \rightarrow 4$ D-N-glucosamine
Zyimosan	<i>Saccharomyces cerevisiae</i> (Sigma)	β -Glucans, mannans, and trace $\beta 1 \rightarrow 4$ D-N-acetylglucosamine
GMP	<i>Saccharomyces cerevisiae</i> (31)	β -Glucans, mannans, and trace $\beta 1 \rightarrow 4$ D-N-acetylglucosamine
Soluble glycans		
Dextran	<i>Leuconostoc mesenteroides</i> (Sigma)	$\alpha 1 \rightarrow 6$ D-glucose
Laminarin	<i>Laminaria digitata</i> (Sigma)	$\beta 1 \rightarrow 3$ and $\beta 1 \rightarrow 6$ D-glucose
Mannan	<i>Saccharomyces cerevisiae</i> (Sigma)	$\alpha 1 \rightarrow 6$ D-mannose

The impact of C3 and properdin binding on GP (high C3, low properdin [Fig. 1 and 3]) and zyimosan (low C3, high properdin [Fig. 1 and 3]) was reflected in the generation of C3a and C5a by the AP with functional properdin. Zyimosan generated 78% of C3a and 93% of C5a production achieved by GP only in serum with

intact AP that contained active properdin (Fig. 4A and C). In accordance with previous work (25), none of the soluble glycans activated AP significantly above activation of AP by serum alone.

Next we determined whether AP activation mediated by glycans with different linkages required properdin. Interestingly,

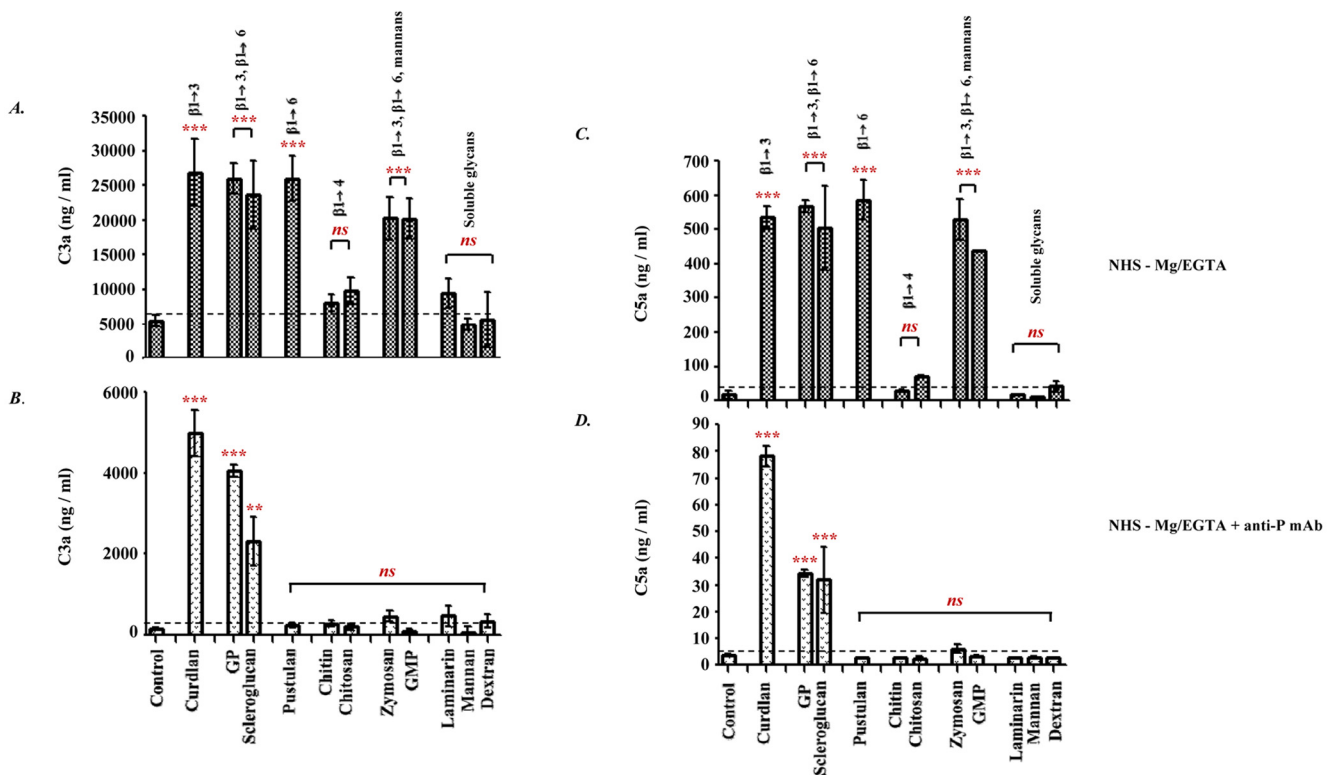


FIG 4 Role of properdin in alternative pathway activation by glycan particles. C3a or C5a resulting from 30 min of incubation of each of the particulate and soluble glycans listed in Table 1 in 40% NHS–Mg–EGTA with or without properdin. C3a or C5a concentrations (ng/ml; shown on the y axis) were measured in supernatants using MicroVue C3a or C5a enzyme immunoassay kits. (A) C3a generation by glycans incubated in NHS–Mg–EGTA (properdin intact). (B) C3a generation by glycans incubated in NHS–Mg–EGTA in the presence of an antiproperdin monoclonal antibody A233 that blocked functional properdin (anti-P mAb). (C and D) C5a generated by glycans incubated in 40% NHS–Mg–EGTA (C) and C5a generated by glycans incubated in 40% NHS–Mg–EGTA plus antiproperdin MAb (D). The broken horizontal line indicates baseline C3a or C5a generation by 40% NHS–Mg/EGTA alone (A and C) or 40% NHS–Mg/EGTA plus antiproperdin MAb alone (B and D) (also indicated by bars labeled “Control”). The particle linkages are indicated above the bars in panels A and C. Results are expressed as means \pm standard deviations (SD) (error bars) of two or three separate experiments; each reaction was performed in duplicate. Note the different scales of the y axes. Statistical analysis was performed using ANOVA. The statistical significance of the amounts of C3a and C5a generated was calculated between the amounts of C3a and C5a generated by NHS–Mg–EGTA in the presence or absence of functional properdin alone compared to the amounts of glycans incubated with corresponding serum. The statistical significance is represented in red above the bars as follows: ***, $P < 0.001$; **, $P < 0.01$; and ns (not significant), $P > 0.05$.

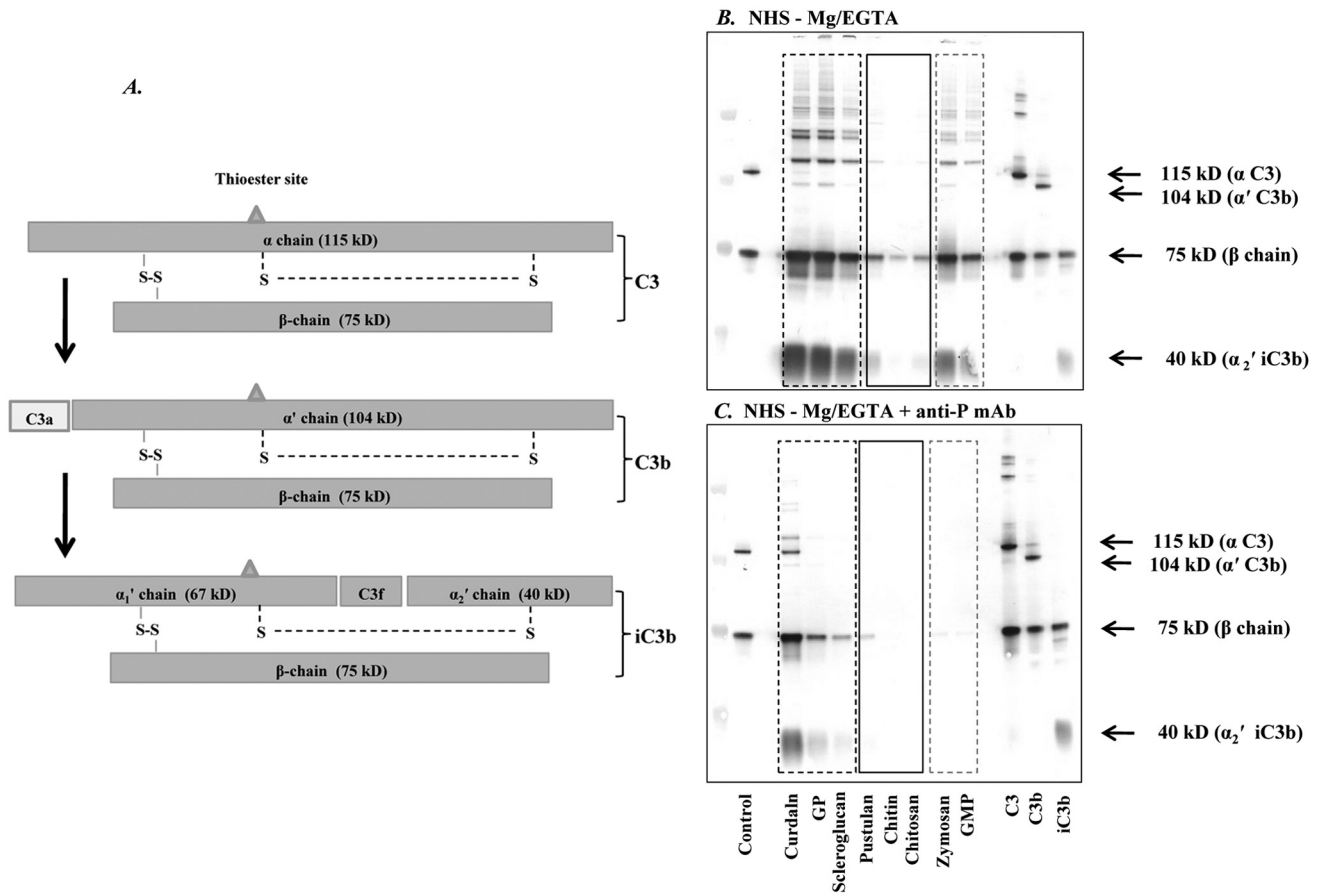


FIG 5 C3 fragment deposition on the β -glycan particles. (A) Schematic representation of C3 degradation products. (B) Particles were incubated with 40% NHS-Mg-EGTA, washed, and digested with 4 \times LDS sample buffer containing 10% β -mercaptoethanol. Samples were electrophoresed on a 4 to 12% Bis-Tris gel, and Western blotting was performed. C3 fragments were identified using affinity-purified sheep anti-human C3 antibody. Controls included 2 μ l of the 200 \times diluted NHS-Mg-EGTA (labeled Control), purified C3, C3b, and iC3b (6.25 ng of each protein per lane). (C) C3 fragments bound to the glycan particles in the absence of functional properdin. The particles were incubated in 40% NHS-Mg-EGTA that contained antiproperdin MAb 233 (anti-P mAb) and processed as described above for panel B, and Western blotting was performed as described above. Controls are described above for panel B, except that all specimens contained antiproperdin MAb 233. The identities of the C3 fragments are indicated in parentheses on the right (α C3). The results of one representative experiment of three separately performed and reproducibly repeated experiments are shown. kD, kilodaltons.

only particles that contained β 1 \rightarrow 3 linkages (curdalan, GP, and scleroglucan) generated C3a and C5a above baseline levels when properdin function was blocked. Pustulan (β 1 \rightarrow 6 linkages only) did not activate AP in the absence of properdin function (Fig. 4B). Although the total amounts of C3a and C5a generated in the absence of functional properdin were, on average, 10-fold less than when properdin was active, it is worth noting that the amounts of C3a and C5a spontaneously generated in the control reactions (serum plus buffer alone) when properdin was nonfunctional were \sim 30-fold and \sim 5-fold less for C3a and C5a generation, respectively (Fig. 4A and B and Fig. 4C and D, respectively). As expected, chitin, chitosan, and the soluble glycans did not generate C3a and C5a when properdin was nonfunctional. Interestingly, zymosan and GMP (both consisting of β -glucans, including β 1 \rightarrow 3 and β 1 \rightarrow 6 glucans and mannans) generated C3a and C5a in the presence of Mg-EGTA-treated NHS (properdin active) but did not significantly do so in the absence of properdin. Similar C3a and C5a generation were observed using Mg-EGTA-treated properdin-depleted serum (data not shown).

Influence of properdin on the deposition of C3 fragments onto glycan particles. Deposition of C3b on the surface of a glycan particle reflects the number of proximate -OH groups that can serve as electron donors to form covalent ester bonds with activated C3b. We characterized C3 deposition on different particles by Western blotting. The 75-kDa β chain present in both C3b and iC3b, freed by reducing conditions prior to SDS-PAGE and Western blotting, is a measure of total C3 deposited onto particles (Fig. 5A). The amount of C3 deposited onto a particle may be paralleled by the amount of C3a and C5a generated, although properdin binding also enhances C3a and C5a production by prolonging the survival of the AP C3 and C5 convertases that result in increased cleavage of C3 and C5. This is exemplified above for zymosan particles (Fig. 4A and C). In addition, β linkage specificity of the glucans also accounted for differences in C3 deposition.

Figure 5 shows that among the β 1 \rightarrow 3 and/or β 1 \rightarrow 6 linkage-containing glucans, C3b was deposited when the AP was fully active in the presence of functional properdin (Fig. 5B), but little

or no C3 was deposited when properdin was blocked on pustulan ($\beta 1 \rightarrow 6$ glucan) and on zymosan and GMP (Fig. 5B and C). In the absence of functional properdin, only the $\beta 1 \rightarrow 3$ linkage-containing glucans (curdlan, GP, and scleroglucan) bound C3, consistent with their ability to generate C3a and C5a under these conditions (Fig. 4B and D); minimal C3 was deposited on pustulan, zymosan, and GMP in the absence of functional properdin.

Collectively, our data suggest that when properdin is functional, $\beta 1 \rightarrow 3$ and $\beta 1 \rightarrow 6$ linkage-containing glucans are potent activators of the alternative pathway. However, in the absence of functional properdin, only $\beta 1 \rightarrow 3$ linkage-containing glucans can activate the alternative pathway, albeit to a much lower level than when properdin is functional. In the case of zymosan and GMP, $\beta 1 \rightarrow 3$ glycans that are present may be less exposed at the surface because the outer mannan “layer” shields the glycans from interacting with C3 molecules.

DISCUSSION

We have defined how monosaccharide composition and linkage specificity of β -glucans ($\beta 1 \rightarrow 3$ and $\beta 1 \rightarrow 6$ linked) together influence activation of the alternative pathway (AP). Importantly, the role played by properdin in linkage-specific β -glucan-mediated AP activation has also been defined. β -Glucans are found naturally in fungi, algae, plants, and some bacteria; both $\beta 1 \rightarrow 3$ and $\beta 1 \rightarrow 6$ glucans are major constituents of the cell walls of fungi. A small percentage of the fungal cell wall is composed of chitin ($\beta 1 \rightarrow 4$ D-N-acetylglucosamine). As shown previously (25), we also demonstrated that all glycan particles (GP) with either $\beta 1 \rightarrow 3$ and/or $\beta 1 \rightarrow 6$ glucan linkage activated C3 when the AP was intact. In contrast, chitin or chitosan ($\beta 1 \rightarrow 4$ linkages) activated the AP (measured by C3a and C5a generation) only minimally. In the absence of functional properdin, only glycan particles that express surface-exposed $\beta 1 \rightarrow 3$ linkages (GP, scleroglucan, and curdlan) generated C3a and C5a, albeit at concentrations about 10-fold lower than when properdin was functional. Pustulan (contains only $\beta 1 \rightarrow 6$ glucan linkages) was a potent AP activator in the presence of properdin but generated minimal C3a and C5a when properdin function was blocked. These data suggest that glycan particles that express surface-exposed $\beta 1 \rightarrow 3$ glucan linkages can activate C3 in the absence of properdin, but particles with only $\beta 1 \rightarrow 6$ glucan linkages require properdin to activate C3.

Following C3 activation, the metastable C3b molecule must quickly bind to an electron-donating $-OH$ group through the thioester bond; failure to do so results in hydrolysis of the thioester, and the C3b molecule remains in solution. Free C3b can form alternative pathway C3 and C5 convertases (C3b,Bb and C3bC3b,Bb) in solution that can further activate C3 and C5 and generate more C3a and C5a, respectively. However, the amount of C3b deposited on the glycan particles depends on the availability of hydroxyl ($-OH$) groups on ringed monosaccharides, principally hexoses, and serves to explain the apparent discordance between C3a and C5a generation (Fig. 4A and C) and C3 fragment deposition (Fig. 5B). The particulate $\beta 1 \rightarrow 3$ glucans used in this study exist primarily in a triple helix conformation (37). Based on the results of glycan fiber diffraction studies (37), the hydroxyl groups at positions 2 and 4 are buried within the polysaccharide chain participating in intra- and interchain hydrogen bonds. The hydroxyls at position 6 are exposed to solvent and are the likely electron donors for reaction with C3. Removal of primary $-OH$ groups from position 6 of monosaccharides decreases the effi-

ciency of C3 binding (38). Consistent with our observations (Fig. 5B), the $\beta 1 \rightarrow 3$ particulate glucans with exposed free $-OH$ groups at position 6 (curdlan, GP, and scleroglucan) bound C3 more effectively than $\beta 1 \rightarrow 6$ glucans, whose position 6 is occupied by the linkage to the adjacent monosaccharide (pustulan). Also consistent with our observations, the $\beta 1 \rightarrow 3$ particulate glucans with free $-OH$ groups at position 6 masked by mannans (zymosan and glucan-mannan particles [GMP]) bound C3 less effectively. Higher efficiency of $\beta 1 \rightarrow 3$ glucan binding to nascent C3 likely facilitates C3b binding to $-OH$ at position 6, even when the AP lacks properdin (Fig. 5C).

Release of anaphylatoxins C3a and C5a during activation of complement has also been shown to promote neutrophil influx to sites of infection. Human neutrophils release properdin upon stimulation (12), which likely serves to augment AP activation (39). $\beta 1 \rightarrow 6$ glucans, in particular, stimulate human neutrophils and mediate engulfment, production of reactive oxygen species, and expression of heat shock proteins more efficiently than $\beta 1 \rightarrow 3$ glucans (40). In one study, human neutrophils rapidly ingested beads coated with $\beta 1 \rightarrow 6$ glucan, but not beads coated with $\beta 1 \rightarrow 3$ glucan (40), suggesting that stimulation of neutrophils may be directed by the specific location of C3b binding on glycan surfaces. The effects may not be limited to neutrophils, as recently, it was shown that C3a and C5a generation can regulate interleukin 17A (IL-17A) responses (41), a critical component of host defense against many fungal infections. Selective reduction of properdin function may be of therapeutic benefit. For example, in a model of cardiopulmonary bypass, treatment with antiproperdin antibodies resulted in significantly reduced neutrophil and platelet activation (14). However, our data suggest that neutralizing properdin could increase the risk of fungal infections by decreasing AP activation.

In addition to affecting innate immune responses, generation of C3a and C5a can affect adaptive immune responses (41, 42). For example, recently, Lajoie et al. demonstrated the abilities of C3a and C5a to reciprocally regulate T_H1 and T_H17 responses in an airway hyperresponsiveness mouse model (41). As T_H1 and T_H17 responses are critical components of host defense against many fungal infections (43, 44), the contribution of complement activation by fungal cell walls to T_H skewing deserves further study.

We have shown that purified properdin in its native forms (dimers, trimers, or tetramers) binds directly to zymosan (27, 29), but data from the present study suggest that properdin does not bind directly to either GP or zymosan in the context of serum but instead binds to deposited C3 (Fig. 3A and B). Thus, it seems unlikely that properdin itself initiates AP activation in the presence of serum, confirming that properdin serves to stabilize AP C3 convertases (26–28). Serum amyloid P component has been reported to interfere with properdin’s ability to bind to complement activator surfaces (30). Although zymosan and GMP contain both $\beta 1 \rightarrow 3$ and $\beta 1 \rightarrow 6$ glucan linkages and activated the AP in the presence of properdin, C3 was minimally activated by these particles in the absence of properdin. We speculate that mannans present on the surfaces of zymosan and GMP may limit covalent linkage formation between nascent C3b and the underlying $\beta 1 \rightarrow 3$ glucans. The finding that properdin is required for AP activation by mannan-coated particles may be clinically relevant, considering the fact that most pathogenic fungi have an outer mannan layer.

Because hollow and porous β -glucan particles allow for high antigen loading (20, 21, 35), they can serve as an effective antigen-presenting cell receptor-targeted vaccine delivery system. Recently, orally administered β -glucan particles have also been shown to function as effective adjuvants for tumor immunotherapy (45). Although the relative contributions of complement receptors and β -glucan receptors remain to be defined, efficient coating of β 1 \rightarrow 3 linkage-containing particles such as GP with C3 fragments likely contributes to their excellent ability to act as an adjuvant. Goodridge et al. (46) recently demonstrated that Dectin-1, a pattern recognition receptor, was activated only by particulate β -glucans and not by soluble β -glucans (46). Particulate β -glucans clustered around the receptor to form synapse-like structures activating phagocytosis (46). Binding of C3 fragments to antigens facilitates their uptake by subcapsular sinus macrophages in lymph nodes (47). Transfer of the antigen-C3 fragment complexes to mature B cells involves binding of the C3d-coated antigen to the B cell coreceptor (CD21/CD19/CD81), which lowers the threshold for B cell activation (48) and results in B cell expansion and migration to the T cell-B cell boundary in lymph nodes. Following interaction with follicular T helper cells, B cells undergo somatic cell hypermutation and class switch recombination and develop into plasma cells or memory B cells (reviewed in reference 47). Our observations regarding how properdin facilitates AP activation by glucans in fungal cell walls contributes to our understanding of the complexity of complement activation by fungi and has implications for the use of glucan particles in biomedical applications.

MATERIALS AND METHODS

Particles used in this study. Glucan particles (GP) and glucan-mannan particles (GMP) were prepared from *Saccharomyces cerevisiae* using a succession of alkaline and acidic extraction steps as previously described (21, 31, 35, 36). GP are comprised of >85% β 1 \rightarrow 6 branched β 1 \rightarrow 3 D-glucan polymers, 2% chitin, and <1% lipids and protein, with the remainder being mostly ash and moisture, and GMP are comprised of ~50% β 1 \rightarrow 6 branched β 1 \rightarrow 3 D-glucan polymers, ~20% mannans, ~15% protein, 1% chitin, and <1% lipids (31, 49). Zymosan (β -glucans, mannans, and chitin), also prepared from *S. cerevisiae*, was obtained from Sigma (St. Louis, MO). All particulate and soluble glycans used in this study and their source and linkage composition are listed in Table 1.

Sera and complement reagents. Hemolytically active sera obtained from 10 healthy adults (normal human serum [NHS]) were pooled and stored at -80°C until used. To permit selective activation of the alternative pathway (AP), NHS was treated with 10 mM EGTA and 10 mM Mg^{2+} (Mg-EGTA-NHS). The role of properdin in AP was determined by using Mg-EGTA-treated properdin-depleted serum (P-depleted serum; catalog no. A512; Quidel), or Mg-EGTA-NHS containing antiproperdin monoclonal antibody (MAb) (catalog no. A233 [see below]; Quidel) used at a final concentration of 50 $\mu\text{g}/\text{ml}$ as described previously (27). Serum depleted of C3 by immunoaffinity chromatography was purchased from Complement Technology, Inc., Tyler, TX (catalog no. A314). C3 was purified from human plasma by polyethylene glycol (PEG) precipitation and DEAE Sephacel chromatography as described previously (50). The final concentration of serum in all reaction mixtures was 40%. In some experiments, all complement pathways were inactivated by heating serum for 30 min at 56°C .

Antibodies. Two antiproperdin MAbs were purchased from Quidel (catalog no. A233 and A235). MAb A233 blocks properdin function, while MAb A235 binds to properdin but does not block the function of properdin. C3 (C3b plus iC3b) deposition on GP and zymosan was detected by anti-human C3c conjugated to fluorescein isothiocyanate (FITC) from BioDesign (now Meridian Life Science, Inc., Saco, ME) for both flow

cytometry and confocal microscopy. For Western blot analysis, C3 deposition was detected using affinity-purified sheep anti-human C3 antibody (Immunology Consultants Laboratory, Newburg, OR) followed by anti-sheep IgG alkaline phosphatase (Sigma).

C3 deposition and properdin binding to GP and zymosan by flow cytometry. C3 deposition on GP and zymosan was determined by flow cytometry. Briefly, 10 μg of GP or zymosan particles were suspended in Hanks' balanced salt solution (HBSS). Because GP and zymosan particles are similarly sized and shaped, an approximately equal amount of surface area should have been available for both types for C3 deposition. The particles were washed once with HBSS containing 10 mM EGTA and 10 mM Mg^{2+} (HBSS-Mg-EGTA) and incubated for 30 min at 37°C with either NHS (all complement pathways active), Mg-EGTA-NHS (only AP active), or heat-inactivated NHS (complement activity inactivated). A reaction mixture that lacked serum was also included as a control. Total C3 (C3b plus iC3b) deposited on GP or zymosan was detected by flow cytometry using FITC-conjugated anti-C3c antibody as described previously (51, 52). To demonstrate the dependence of C3 deposition on GP or zymosan for properdin binding, particles were incubated with C3-depleted serum (Complement Technologies, Inc.) or C3-depleted serum reconstituted with C3 to a concentration of 1 mg/ml, and binding of properdin was determined using anti-human properdin MAb (catalog no. A235; Quidel) at a dilution of 1:100, followed by anti-mouse IgG conjugated to Alexa Fluor 647 (1:400) as described previously (27). Positive and negative controls included particles incubated with Mg-EGTA-NHS or Mg-EGTA-treated, properdin-depleted serum (Quidel), respectively. Data were collected on an LSR II flow cytometer (Becton Dickinson [Franklin Lakes, NJ]) and analyzed using the FlowJo analysis software program (version 7.2.4; TreeStar Inc. [Ashland, OR]).

Confocal microscopy. C3 fragment deposition on and properdin binding to GP and zymosan were also examined by confocal microscopy. Briefly, 10 μg of GP or zymosan was washed once with HBSS-Mg-EGTA and incubated with either Mg-EGTA-NHS or Mg-EGTA-treated, properdin-depleted serum for time points between 10 and 30 min. The particles were washed three times, and C3 and properdin associated with GP or zymosan were detected using FITC-conjugated anti-C3c and anti-human properdin MAb (catalog no. A235; Quidel) at a dilution of 1:100, followed by anti-mouse IgG conjugated to Alexa Fluor 647 (1:400). The stained particles were resuspended in HBSS containing 1% paraformaldehyde and spotted onto a 35-mm petri dish with a 10-mm microwell (MatTek Corporation, Ashland, MA) and imaged using a $63\times$ plan apochromat objective lens (Zeiss) with an inverted TCS SP2 AOBs laser scanning confocal microscope (Leica, Wetzlar, Germany). To demonstrate the dependence of C3 deposition on GP or zymosan for properdin binding by confocal microscopy, the particles were incubated with C3-depleted serum that had been treated with Mg-EGTA (C3-depleted serum-Mg-EGTA) or C3-depleted serum-Mg-EGTA reconstituted with purified C3 (1 mg/ml) at 37°C for 30 min. Positive and negative controls included incubation of particles with Mg-EGTA-NHS and Mg-EGTA-treated, properdin-depleted serum, respectively. The particles were washed three times, and properdin associated with GP or zymosan was detected using anti-human properdin MAb (catalog no. A235; Quidel) at a dilution of 1:100, followed by anti-mouse IgG conjugated to Alexa Fluor 647 (1:400). The stained particles were resuspended in HBSS containing 1% paraformaldehyde and imaged as mentioned above.

C3a and C5a assays. Activation of C3 is accompanied by release into solution of the C3a fragment from the N terminus of the α chain of C3. Furthermore, AP activation results in formation of C5 convertase (C3bC3b,Bb) that cleaves C5 and releases the C5a fragment. Briefly, 10 μg of each glycan particle or soluble glycan (listed in Table 1) was incubated at 37°C for 30 min with 40% NHS-Mg-EGTA alone or with 40% NHS-Mg-EGTA containing MAb 233, which blocks properdin function, or 40% NHS-Mg-EGTA depleted of properdin. Reactions were stopped by adding EDTA to a final concentration of 20 mM, and particles were separated from the supernatants by centrifugation at $10,000\times g$ for 5 min.

The amounts of C3a and C5a present in the supernatants were measured using the MicroVue C3a enzyme immunoassay (EIA) kit (catalog no. A015; Quidel) or the MicroVue C5a EIA kit (catalog no. A021; Quidel), respectively.

AP-mediated C3 deposition on glycan particles. C3 fragment deposition on glycan particles was determined by Western blotting as described previously (53). Briefly, 10 μg of each of the particles listed in Table 1 was incubated with either Mg-EGTA-NHS- or Mg-EGTA-treated serum containing antiproperdin MAb 233 (final serum concentration of 40% [vol/vol]) in a final reaction volume of 100 μl for 30 min at 37°C. The particles were washed four times in HBSS and lysed in 4 \times lithium dodecyl sulfate (LDS) sample buffer (Invitrogen) containing 10% 2-mercaptoethanol (2-ME). The proteins were separated on NuPAGE Novex 4 to 12% Bis-Tris gradient gels using NuPAGE 3-morpholinopropanesulfonic acid running buffer (Invitrogen). The proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA), and C3 fragments were detected using affinity-purified sheep anti-human C3 antibody followed by goat anti-sheep IgG conjugated to alkaline phosphatase.

Statistical analysis. Differences between C3a and C5a generation were calculated between the C3a and C5a generated by NHS-Mg-EGTA in the presence or absence of functional properdin alone compared to glycans incubated with the corresponding serum using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer test (GraphPad InStat, San Diego, CA).

ACKNOWLEDGMENTS

This work was supported by grants from the National Institute of Health, grants AI32725, AI084048 (P.A.R.), AI025780 (S.M.L.), and AI054544 (S.R.).

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