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The *Streptococcus pneumoniae* Capsule Inhibits Complement Activity and Neutrophil Phagocytosis by Multiple Mechanisms[▽]

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The *Streptococcus pneumoniae* capsule is vital for virulence and may inhibit complement activity and phagocytosis. However, there are only limited data on the mechanisms by which the capsule affects complement and the consequences for *S. pneumoniae* interactions with phagocytes. Using unencapsulated serotype 2 and 4 *S. pneumoniae* mutants, we have confirmed that the capsule has several effects on complement activity. The capsule impaired bacterial opsonization with C3b/iC3b by both the alternative and classical complement pathways and also inhibited conversion of C3b bound to the bacterial surface to iC3b. There was increased binding of the classical pathway mediators immunoglobulin G (IgG) and C-reactive protein (CRP) to unencapsulated *S. pneumoniae*, indicating that the capsule could inhibit classical pathway complement activity by masking antibody recognition of subcapsular antigens, as well as by inhibiting CRP binding. Cleavage of serum IgG by the enzyme IdeS reduced C3b/iC3b deposition on all of the strains, but there were still marked increases in C3b/iC3b deposition on unencapsulated TIGR4 and D39 strains compared to encapsulated strains, suggesting that the capsule inhibits both IgG-mediated and IgG-independent complement activity against *S. pneumoniae*. Unencapsulated strains were more susceptible to neutrophil phagocytosis after incubation in normal serum, normal serum treated with IdeS, complement-deficient serum, and complement-deficient serum treated with IdeS or in buffer alone, suggesting that the capsule inhibits phagocytosis mediated by Fcγ receptors, complement receptors, and nonopsonic receptors. Overall, these data show that the *S. pneumoniae* capsule affects multiple aspects of complement- and neutrophil-mediated immunity, resulting in a profound inhibition of opsonophagocytosis.

The Gram-positive pathogen *Streptococcus pneumoniae* is one of the most common causes of pneumonia, septicemia, and meningitis in children and adults in both industrialized and developing parts of the world (10). This large burden of disease is compounded by the increased incidence of *S. pneumoniae* infections associated with HIV and by increasing antibiotic resistance among clinical isolates, and there is a strong need to understand the molecular pathogenesis of *S. pneumoniae* infections to assist the development of new therapeutic targets. Probably the most important virulence factor for *S. pneumoniae* is the extracellular capsule, a layer consisting of chains of monosaccharides that surrounds the bacteria. For *S. pneumoniae* strains, there are 91 antigenically distinct capsular serotypes, dictated by the order and type of the monosaccharide units within the polysaccharide chain and by different side branches (5, 27). The importance of the *S. pneumoniae* capsule for virulence is demonstrated by the facts that (i) all clinical isolates causing invasive disease are encapsulated; (ii) loss of the capsule by either genetic mutation or enzymatic degradation dramatically reduces *S. pneumoniae* virulence in animal models of infection (6, 28, 29, 43, 49); (iii) different capsular serotypes vary in the ability to cause invasive disease (9), and swapping capsular serotypes between strains affects virulence in animal models (21); and (iv) *S. pneumoniae* opaque-phase variants (which express a thicker capsule than transparent-phase variants) predominate during invasive infection (35). Furthermore, the capsule is the target for existing *S. pneumoniae* vaccines and widespread vaccination has led to the evolution of vaccine escape mutants expressing nonvaccine capsular serotypes, increasing the importance of a better understanding of how the capsule can affect virulence.

One component of the immune system that is likely to be affected by the *S. pneumoniae* capsule is the complement system. Clinical and experimental evidence has shown the vital role of complement for host immunity to *S. pneumoniae* and that neutrophil phagocytosis of *S. pneumoniae* is largely dependent on complement activity (8, 15, 19, 22, 39, 52, 53). The complement system is organized into three enzyme cascades termed the classical, alternative, and mannan binding lectin (MBL) pathways (42). The classical complement pathway is activated by specific immunoglobulin G (IgG) and was generally considered an effector of the adaptive immune response, but recent data have demonstrated an important role for the classical pathway as part of the innate immune response to *S. pneumoniae*. *S. pneumoniae* cell wall phosphorylcholine (PC) is recognized by the serum proteins C-reactive protein (CRP) and serum amyloid P (SAP) (collectively termed pentraxins due to their structurally similarity) (40) and also by natural IgM (4). In addition, the cell surface lectin SIGN-R1 binds to the *S. pneumoniae* capsule (20). Recognition of *S. pneumoniae* by the pentraxins, natural IgM, and SIGN-R1 results in binding

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of the first component of the classical pathway, C1q, to the bacterial surface and complement activation. The MBL pathway is activated by binding of MBL to certain sugar residues found on the surface of pathogens. However, MBL binds poorly to *S. pneumoniae* and seems to have little effect on complement deposition on *S. pneumoniae* (8, 31), although MBL or other ficolins may directly opsonize microorganisms independent of complement activity. The alternative pathway is spontaneously activated unless the target cell is coated in sialic acid or complement-inhibitory proteins such as factor H (FH) (42) and is therefore a component of the innate immune response to *S. pneumoniae*. The alternative pathway probably also amplifies the amount of C3b/iC3b deposited on the bacterial surface once complement activation has been initiated by the classical or MBL pathway (8, 42). Each pathway leads to the formation of a C3 convertase that cleaves the central complement component C3, resulting in deposition of C3b on the surface of the pathogen that is further processed to iC3b. C3b and iC3b are opsonins mediating phagocytosis mainly through the complement receptor CR1 and CR3 receptor, respectively. As well as opsonizing bacteria, complement activation aids the inflammatory response through release of anaphylaxins such as C5a (42) and improves the adaptive immune response to *S. pneumoniae* through direct stimulation of B cells by the C3 breakdown product C3d (13).

The external position of the capsule means it is ideally situated to modulate interactions between *S. pneumoniae* and host proteins and cells. Unencapsulated mutants have been shown to be more susceptible to phagocytosis, and there are limited data showing increased levels of complement deposition on their surface (1, 32, 47), but despite the importance of the capsule for *S. pneumoniae* virulence, there are few data on the mechanisms involved (32). Data obtained for other pathogens have shown a variety of mechanisms by which polysaccharide capsules can inhibit complement activity. The group B *Streptococcus* (GBS) and *Neisseria meningitidis* capsules contain sialic acid, which is thought to prevent alternative complement activity by creating a nonactivating surface and by binding to FH (23, 25, 46). Alternatively, the capsule may inhibit recognition of surface antigens by specific IgG, thereby preventing classical pathway activation or directly prevent binding of complement components to subcapsular targets of complement activity (37). In contrast, the capsule of *Cryptococcus neoformans* is a potent activator of alternative pathway activity and this is thought to aid immunity by depleting complement (50). For *S. pneumoniae*, whether the capsule prevents complement deposition indirectly through impairing recognition of the bacteria by IgG or has direct effects on bacterial interactions with non-IgG complement activators or other aspects of complement activity is not known. Given the importance of complement for neutrophil phagocytosis of *S. pneumoniae* (53), inhibition of opsonization with C3b/iC3b by the capsule could account for all of the effects of the capsule on phagocytosis. However, IgG bound to the bacterial surface and nonopsonic phagocytic molecules such as the mannose and scavenger receptors can mediate phagocytosis independently of complement, and these mechanisms of phagocytosis potentially could also be affected by the *S. pneumoniae* capsule. Indeed, recent data showing increased phagocytosis of an unopsonized, unencapsulated serotype 6B strain suggest that there can be a capsular effect on nonopsonic phagocytosis (44). Given the importance of the capsule for *S. pneumoniae* virulence and as a vaccine candidate, a more detailed understanding of the interactions of the capsule with complement and neutrophils would be beneficial.

Using unencapsulated mutants from serotype 2 and 4 *S. pneumoniae* strains that are otherwise isogenic to the encapsulated parental strain, we have investigated the effect of the capsule on IgG-dependent and -independent complement deposition on the bacterial cell surface and on the binding of various complement mediators. We have also assessed the effects of the capsule on complement-dependent and complement-independent neutrophil phagocytosis.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *S. pneumoniae* TIGR4 wild-type and TIGR4 unencapsulated (TIGR4*cps*, made using the Janus cassette as previously described [38]) strains were a kind gift from Jeffrey Weiser, University of Pennsylvania. The *S. pneumoniae* D39 wild-type strain and the unencapsulated strain derived from D39 containing a deletion of *cpsD* (D39-DΔ) (28) were a kind gift from James Paton, University of Adelaide. Bacteria were cultured at 37°C in 5% CO_2 on blood agar plates (supplemented when necessary with erythromycin at 0.2 μg ml^{-1}) or in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY) to an optical density at 580 nm (OD_{580}) of 0.4 (approximately 10^8 CFU/ml) and stored at −70°C in 10% glycerol as single-use aliquots. Growth of the unencapsulated strains in THY and in human serum was identical to that of the parental wild-type strain (data not presented).

EM. Mid-log-phase *S. pneumoniae* bacteria were incubated at 37°C for 20 min in serum or phosphate-buffered saline (PBS), fixed in 1% paraformaldehyde, and prepared for electron microscopy (EM) using a ruthenium red and London resin protocol as previously described (16). Bacteria were viewed using a JEOL 1010 transmission electron microscope (100 kV), and Image J software was used to determine capsule thickness. The cross-sectional area of the whole bacterium, including and excluding the capsule, was obtained and, by assuming circularity, used to calculate the bacterial radius with or without the capsule and hence the average width of the capsule layer. Data were obtained for 10 or more randomly chosen bacteria of each strain investigated.

Serum sources and complement binding assays. The majority of experiments were performed using pooled serum obtained from unvaccinated normal human volunteers (53). Total IgG binding to *S. pneumoniae* was assessed using flow cytometry and R-phycoerythrin goat anti-human IgG (Jackson ImmunoResearch) as described previously (53). Serum with single complement component deficiencies ($C9^-$, $C3^-$, $C1q^-$, and Bf^- sera) were supplied by Calbiochem (53). Sera were stored as single-use aliquots at −70°C. C3b/iC3b deposition and C1q, CRP, or SAP binding to *S. pneumoniae* after incubation in human serum were measured using previously described flow cytometry assays and a fluorescein isothiocyanate (FITC)-conjugated polyclonal anti-human C3 antibody (Ab; ICN), an unlabeled polyclonal anti-human iC3b mouse Ab (Technoclone), polyclonal goat C1q (Calbiochem), or rabbit anti-human CRP or SAP (Calbiochem) with appropriate FITC-labeled secondary Abs (8, 51, 53). Markers for identifying bacteria positive for each molecule were set using bacteria incubated in PBS and then incubated with the secondary Ab. In order to combine the percentage of bacteria positive for a given factor and the intensity of the binding, the results of complement factor and protein binding assays are presented in arbitrary units as a fluorescence index (FI, proportion of positive bacteria expressed as a percentage multiplied by the geometric mean fluorescence intensity), a method that has been used extensively to combine changes in both intensity of binding and the proportion of bacteria affected (51, 53). To ensure consistent results for each strain, flow cytometry assays were repeated using two or more different sources of stock for each strain (that is, stocks cultured on separate days before storage as single-use aliquots). Complement was deactivated in serum by heat treatment at 65°C for 20 min, which is known to denature complement but leave Ab activity unaffected (53). Human IgG activity against *S. pneumoniae* was abrogated in serum using purified IgG-degrading enzyme of *Streptococcus pyogenes* (IdeS, a kind gift from Mattias Collin and Lars Björck, Lund University), a cysteine proteinase which cleaves IgG with a unique degree of specificity for the hinge region (41, 45). One percent IdeS or bovine serum albumin (BSA) was incubated with human serum for 45 min at 37°C before use for complement and phagocytosis assays as described above. After IdeS treat-

ment, the mean FI of total IgG binding to the TIGR4 strain in serum was 30 ± 12, compared to 480 ± 40 in BSA-treated serum.

Whole-cell ELISAs. The protocols for the whole-cell enzyme-linked immunosorbent assay (ELISA) to confirm loss of IgG were adapted from Roche et al. (33). *S. pneumoniae* strain TIGR4 was grown in THY to mid-log phase (OD, 0.6), washed in PBS, and resuspended at an of OD of 1.0. ELISA plates (Nunc Maxisorp) were coated with the bacterial suspension at 50 μl/well and refrigerated overnight before washing and blocking for 1 h at 37°C with PBS–1% BSA (Merck). Human serum was preincubated at a 1:20 dilution in the presence or absence of IdeS (12.5 μg/ml) for 1 h at 37°C prior to addition to the ELISA plates. The plates were developed using mouse monoclonal anti-human IgG heavy-chain alkaline phosphatase conjugate (Sigma), and Ab levels were assessed by measuring ODs at different serum dilutions.

Immunoblot assays for C3 breakdown products. To assess C3 activation in human serum, different concentrations of bacteria were incubated in 1 ml of 10% pooled human serum for 20 min at 37°C, followed by centrifugation at 13,000 rpm for 15 min. The supernatants were removed, diluted to a final serum running concentration of 1% in sodium dodecyl sulfate (SDS) sample buffer with 2-mercaptoethanol, boiled at 95°C for 10 min, separated by 10% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes using standard methods, and probed using an anti-C3 Ab (ICN) conjugated with horseradish peroxidase (20).

Neutrophil phagocytosis. Phagocytosis was investigated using an established flow cytometry assay, neutrophils extracted from fresh human blood (34), and fluorescent *S. pneumoniae* labeled with 6-carboxyfluorescein succinimidyl ester (FAMSE; Molecular Probes) incubated in human serum for 20 min at 37°C (53). Each reaction used 10^5 neutrophils and a multiplicity of infection (MOI) of 10 to 1, and a minimum of 10,000 cells were analyzed by flow cytometry to identify the mean (standard deviation [SD]) percentage of neutrophils associated with bacteria (22, 53) using neutrophils that had not been incubated with bacteria to identify the negative population. To prevent phagocytosis, neutrophils were incubated with 5 μM cytochalasin D (Sigma) for 30 min at room temperature to inhibit actin polymerization. Trypan blue (Sigma) at a final concentration of 0.5% was used to quench FAMSE fluorescence from extracellular bacteria adhering to neutrophils. For the killing assays, *S. pneumoniae* strains previously incubated in different concentrations of serum at room temperature for 30 min were added to fresh human neutrophils in Hanks balanced salt solution (HBSS) with divalent cations at an MOI of 1:800. After 45 min at 37°C, the number of surviving bacteria was calculated by plating serial dilutions and expressed as a percentage of the number of bacterial CFU used as the inoculum for each reaction (11).

Statistics. Results, expressed as means and SDs, were compared between strains by using Student unpaired *t* tests (two-way comparisons) and one-way analyses of variance (ANOVAs) with post-hoc tests (three [or more]-way comparisons). Results presented as medians (interquartile ranges) were compared using the Kruskal-Wallis test with Dunn's multiple-comparison test (multiple groups) or the Mann-Whitney U test (for two groups). Data are representative of results obtained with repeated assays with at least three replicates per experimental condition.

RESULTS

Confirmation of loss of the capsule layer in the TIGR4*cps* and D39-DΔ strains. Unencapsulated mutants from parental *S. pneumoniae* strains D39 (serotype 2) and TIGR4 (serotype 4) have previously been characterized and shown to have the correct mutation and be otherwise isogenic to the encapsulated parental (wild-type) strain (28, 30, 38). To further confirm that the capsule locus mutations resulted in loss of capsule expression, a colorimetric assay for the determination of mucopolysaccharides was used (14). Both the TIGR4*cps* and D39-DΔ strains had very low levels of staining for polysaccharides compared to those of their encapsulated parental strains (OD_{640} of TIGR4, 2.13 ± 0.18 versus 0.51 ± 0.12 for TIGR4*cps*; OD_{640} of D39, 1.96 ± 0.07 versus 0.51 ± 0.12 for D39-DΔ [Student unpaired *t* test, $P < 0.001$]). In addition, when measured using EM and a lysine acetate and ruthenium red protocol (16) to preserve the capsule, the TIGR4*cps* and D39-DΔ strains had only a minimal visible extracellular layer outside the cell wall with average thicknesses of 12 ± 4 and 24 ± 9 nm, respectively (Fig. 1). In contrast, the TIGR4 and D39 parental strains had visible extracellular layers outside the cell wall corresponding to the capsule 185 ± 19 and 104 ± 11 nm thick, respectively. Although thicker than the D39 capsule, the TIGR4 capsule ($P < 0.001$) was not as thick as that of serotype 3 strain 0100993 (265 ± 16 nm, $P < 0.001$), a serotype that is known to have a relatively thick polysaccharide capsule (16).

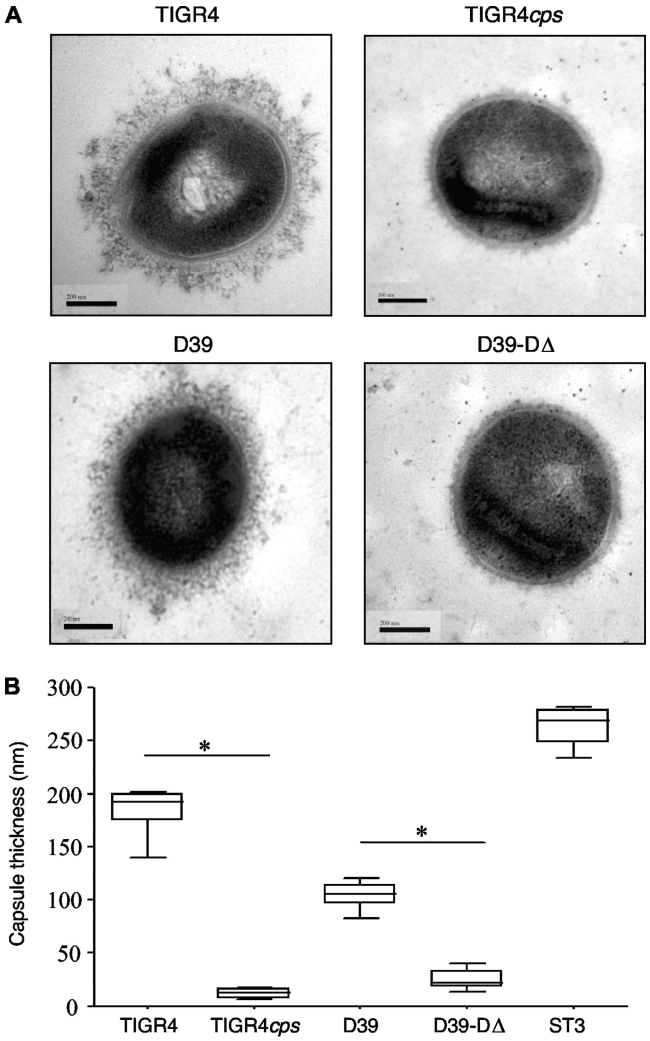

FIG. 1. Measurement of capsule layer diameter by EM. (A) Examples of EM of encapsulated and unencapsulated strains. Bars, 200 nm. (B) Capsule layer widths (nm) for the TIGR4 and D39 strains, their unencapsulated counterparts TIGR4*cps* and D39-DΔ, and a capsular serotype 3 strain, presented as medians and interquartile ranges. For comparisons between unencapsulated and encapsulated strains, an asterisk indicates a *P* value of <0.001 (Mann-Whitney U test).

The capsule inhibits C3b/iC3b deposition on both TIGR4 and D39 *S. pneumoniae* strains. Using an established flow cytometry assay and increasing concentrations of human serum (8, 51, 53), we confirmed that the deposition of C3b/iC3b was markedly increased on unencapsulated TIGR4 and

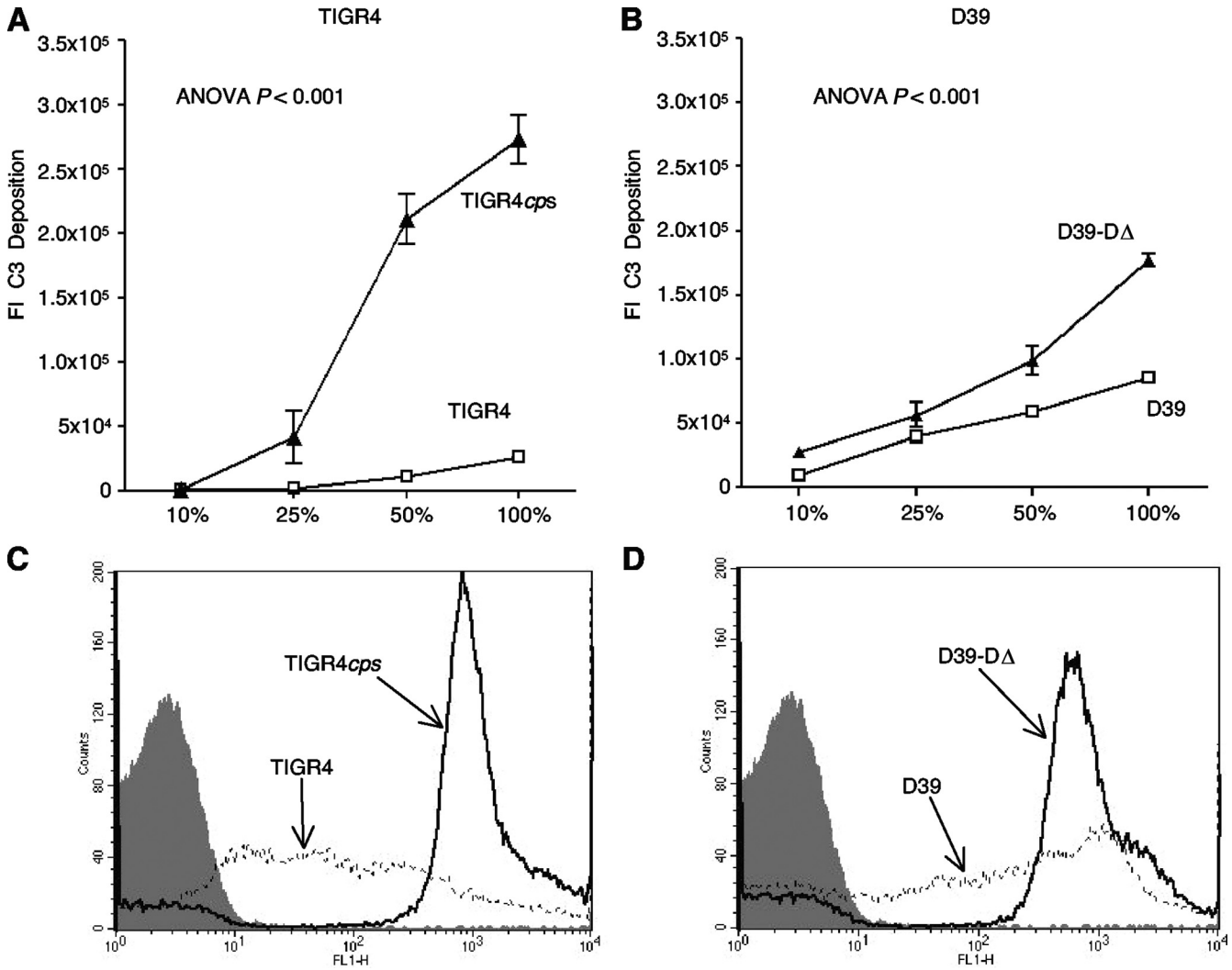

FIG. 2. Effect of the *S. pneumoniae* capsule on C3b/iC3b deposition. (A and B) FI of C3b/iC3b deposition measured using a flow cytometry assay on the TIGR4 (A) and D39 (B) strains for encapsulated (□) and unencapsulated strains (TIGR4*cps* and D39-DΔ) (▲) in increasing concentrations of human serum. (C) and (D) Examples of flow cytometry histograms for C3b/iC3b deposition on TIGR4 and D39 wild-type and unencapsulated strains in 100% human serum. Gray shading indicates the results for bacteria incubated in PBS alone. In panels A and B, error bars represent SDs, and for the differences between encapsulated and unencapsulated organisms, the P value is <0.001 (ANOVA).

D39 *S. pneumoniae* strains (Fig. 2, ANOVA, P<0.001). Interestingly, the extent to which the capsule prevented complement deposition varied between the D39 and TIGR4 strains, with a larger difference between the results of C3b/iC3b deposition on the TIGR4*cps* strain and the TIGR4 strain than between the D39-DΔ and D39 strains. The level of C3b/iC3b deposition on the complemented TIGR4*cps* strain expressing serotype 4 capsule was similar to that found on the TIGR4 strain (FI of 10,890 [SD, 1,270] in 50% serum), confirming that loss of the capsule was responsible for the increased C3b/iC3b deposition on the TIGR4*cps* strain. To assess whether the capsule affected the relative proportions of the opsonins C3b and iC3b on the bacterial surface, a flow cytometry assay specific for iC3b was performed for the TIGR4*cps*, TIGR4, D39-DΔ, and D39 strains and the results were expressed as a proportion of the total C3b/iC3b deposition. For both strains, loss of the capsule resulted in an increase in the FI for iC3b deposition proportional to the total C3b/iC3b deposition (Table 1).

Increased breakdown of C3 in human serum incubated with unencapsulated *S. pneumoniae*. Although the polysaccharide

TABLE 1. Relative proportion of the FI for iC3b deposition expressed as a percentage of the FI for total C3b/iC3b deposition on encapsulated and unencapsulated *S. pneumoniae* bacteria after incubation in 50% human serum

Strain	Mean FI for iC3b deposition as % of FI for total C3b/iC3b deposition ± SD		*P* value[a]
	Encapsulated	Unencapsulated	
TIGR4	25 ± 0.6	43 ± 10.4	<0.01
D39	24 ± 1.1	32 ± 2.6	<0.01

[a] *P* values represent comparisons between the results for unencapsulated and encapsulated strains using Student unpaired *t* tests.

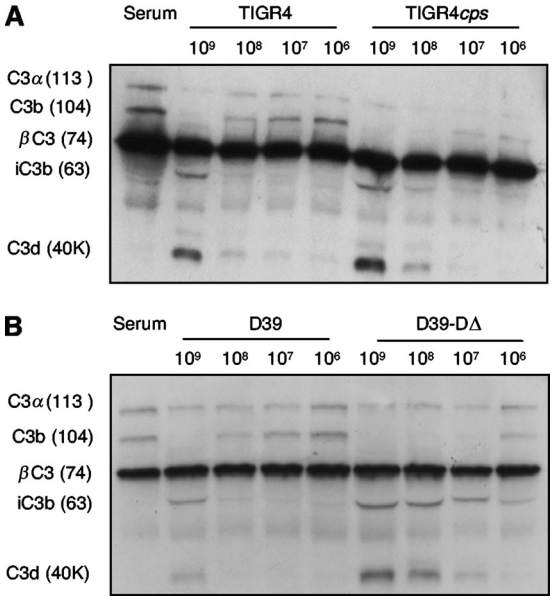

FIG. 3. Representative immunoblot assays of serum incubated with differing numbers of CFU of encapsulated and unencapsulated TIGR4 (A) and D39 (B) bacteria and then probed with an Ab to C3 and its breakdown products. The positions of breakdown products of C3 (with their approximate sizes in kDa in parentheses) are shown to the left of each panel. Similar results were obtained with repeated immunoblot assays.

capsule does not prevent Ab binding to cell wall-associated structures (7, 22, 24), the capsule might relatively restrict Ab access to cell surface-bound C3b/iC3b, which could affect the results of the C3b/iC3b deposition flow cytometry assays. Hence, an immunoblot assay against C3 was used to support the results of the flow cytometry C3b/iC3b assays by assessing complement activation in serum incubated with bacteria. A range of doses of each bacterial strain were incubated in 10% serum at 37°C for 20 min, and the relative quantities of the C3 breakdown products iC3b and C3d were assessed by probing with an Ab that recognizes C3 and all of its breakdown products. Bands representing iC3b and C3d were more obvious in serum incubated with the TIGR4*cps* and D39-DΔ strains than in serum incubated with the TIGR4 and D39 strains, respectively, and conversely, bands representing C3b were reduced in intensity (Fig. 3). These data suggest that there was increased activation of the complement system by unencapsulated bacteria.

The *S. pneumoniae* capsule prevents both classical and alternative complement pathway activity. To investigate the contribution of the alternative or classical complement pathways to C3b/iC3b deposition on unencapsulated strains, the flow cytometry assays were repeated using commercially obtained serum depleted of either C9 (a terminal complement component not involved in C3b/iC3b deposition used as a positive control), C1q (an essential mediator of the classical pathway), or factor B (Bf; an essential mediator of the alternative pathway). Loss of either classical or alternative pathway activity resulted in a marked reduction in total C3b/iC3b deposition on the bacterial surface for both the TIGR4*cps* and D39-DΔ strains (Fig. 4A to D), indicating that the capsule prevents complement deposition mediated by both pathways. Large increases in C3b/iC3b deposition on the unencapsulated strains compared to the encapsulated strains persisted in serum depleted of either C1q or Bf, confirming that both the classical and alternative pathways are inhibited by the capsule (Fig. 4E and F).

The capsule affects binding of mediators of complement activity. To investigate further how the capsule affects C3b/iC3b deposition on *S. pneumoniae*, the binding of the classical pathway mediators IgG, IgM, and the pentraxins CRP and SAP (8, 36, 51) was investigated by using flow cytometry assays. In addition, the binding of the alternative pathway inhibitor FH was assessed. For both the TIGR4*cps* and D39-DΔ strains, there was an increased proportion of bacteria positive for CRP but decreased binding of SAP compared to the TIGR4 and D39 strains, respectively (Fig. 5A and B). These data suggest that SAP, as well as binding to PC (51), may bind to the *S. pneumoniae* capsule but give no clear indication of whether the capsule inhibits pentraxin-mediated classical pathway activation. FH binding was actually increased on the TIGR4*cps* and D39-DΔ strains, which would be predicted to reduce rather than increase alternative pathway activity (Fig. 5C). IgG binding was markedly increased against the unencapsulated bacteria for both strains (Fig. 6A), suggesting that the effect of the capsule on complement activity is at least partially mediated through masking of subcapsular antigens from Ab recognition. Although IgM binding to *S. pneumoniae* was also increased in the absence of the capsule, the effect was weak and may not be particularly biologically significant (Fig. 6B). To assess the functional consequences of these effects on classical pathway mediators, we also used flow cytometry to assess the binding of the classical pathway component C1q to *S. pneumoniae*. Compatible with the increased binding of Ab and CRP to the unencapsulated bacteria, there was also increased binding of C1q to the TIGR4*cps* and D39-DΔ strains (Fig. 6C).

Role of IgG in capsule-dependent effects on complement activation. In order to characterize the relative importance of increased IgG binding for the increased complement activity against unencapsulated strains compared to any potential direct inhibition of complement activity by the capsule, the C3b/iC3b deposition assays were repeated with serum treated with IdeS to cleave IgG. IdeS treatment resulted in complete abrogation of IgG binding to *S. pneumoniae* when tested using flow cytometry (FI of IgG binding to TIGR4 of 31 [SD, 13] in IdeS-treated serum, compared to 676 [SD, 70] in BSA-treated serum) (Fig. 6A) and no detectable IgG binding to the TIGR4 or D39 strain when tested using a whole-cell ELISA (data not shown). For all of the strains, C3b/iC3b deposition was reduced in IdeS-treated or control serum (incubated with BSA rather than IdeS) compared to untreated serum, probably due to the breakdown of complement during the protein treatment process prior to the C3b/iC3b assays (Table 2). Similar to the results for untreated serum (Fig. 2), there was increased C3b/iC3b deposition on the unencapsulated strains in control serum treated with BSA. IdeS treatment reduced C3b/iC3b deposition on the TIGR4*cps* and D39-DΔ strains, demonstrating that complement deposition on unencapsulated strains is partially dependent on IgG. However, there was a persisting increase in C3b/iC3b deposition on unencapsulated strains compared to that on encapsulated strains in IdeS-treated serum

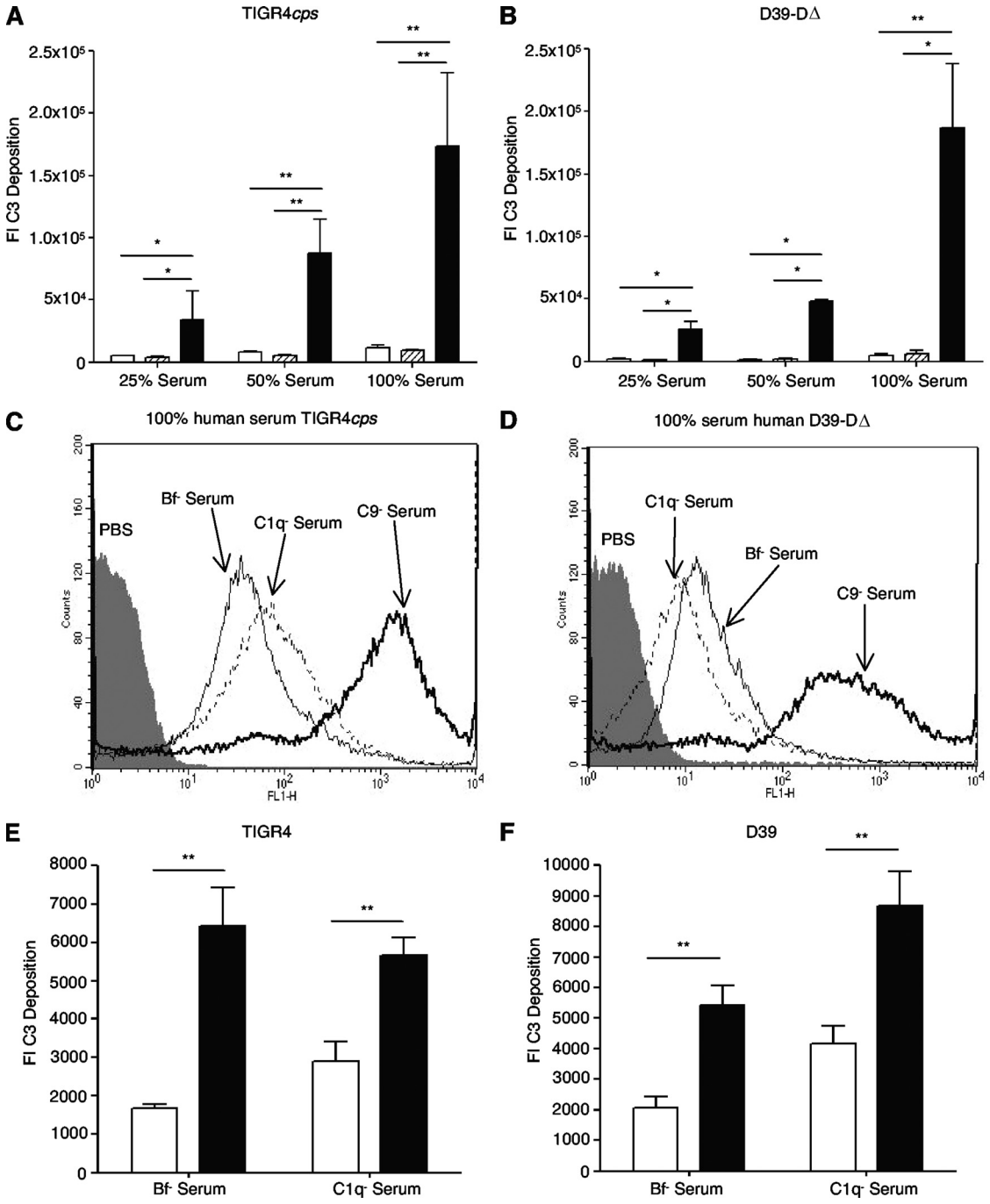

FIG. 4. Effects of classical and alternative pathways on C3b/iC3b deposition on unencapsulated strains. (A and B) FI for flow cytometry results of C3b/iC3b deposition on the TIGR4*cps* (A) and D39-DΔ (B) strains in different concentrations of human serum depleted of C9 (black bars), C1q (open bars), or Bf (diagonally shaded bars). (C and D) Examples of flow cytometry histograms for C3b/iC3b deposition on strain TIGR4*cps* (C) and D39-DΔ (D) bacteria incubated in 100% $C9^-$ (thick black line), $C1q^-$ (thin black line), or Bf^- (dashed line) human serum. (E) Comparison of the FIs for C3b/iC3b deposition in C1q ($C1q^-$)- or Bf (Bf^-)-depleted serum on the TIGR4 (white bars) and TIGR4*cps* (black bars) strains. (F) Comparison of the FIs for C3b/iC3b deposition in C1q ($C1q^-$)- or Bf (Bf^-)-depleted serum on the D39 (white bars) and D39-DΔ (black bars) strains. In panels A, B, E, and F, error bars represent SDs and single and double asterisks indicate a P value of < 0.01 and < 0.001, respectively (ANOVAs with post-hoc analysis).

(Table 2), confirming that the capsule has a significant IgG-independent effect on complement activation. In addition, in IdeS-treated serum, although C1q binding to *S. pneumoniae* was (as expected) reduced, there were still significant increases in C1q binding to unencapsulated compared to encapsulated strains (Table 2). Overall, the data for the C3b/iC3b deposition and complement factor binding assays suggest that the capsule inhibits both IgG-dependent and -independent (C1q-medi-

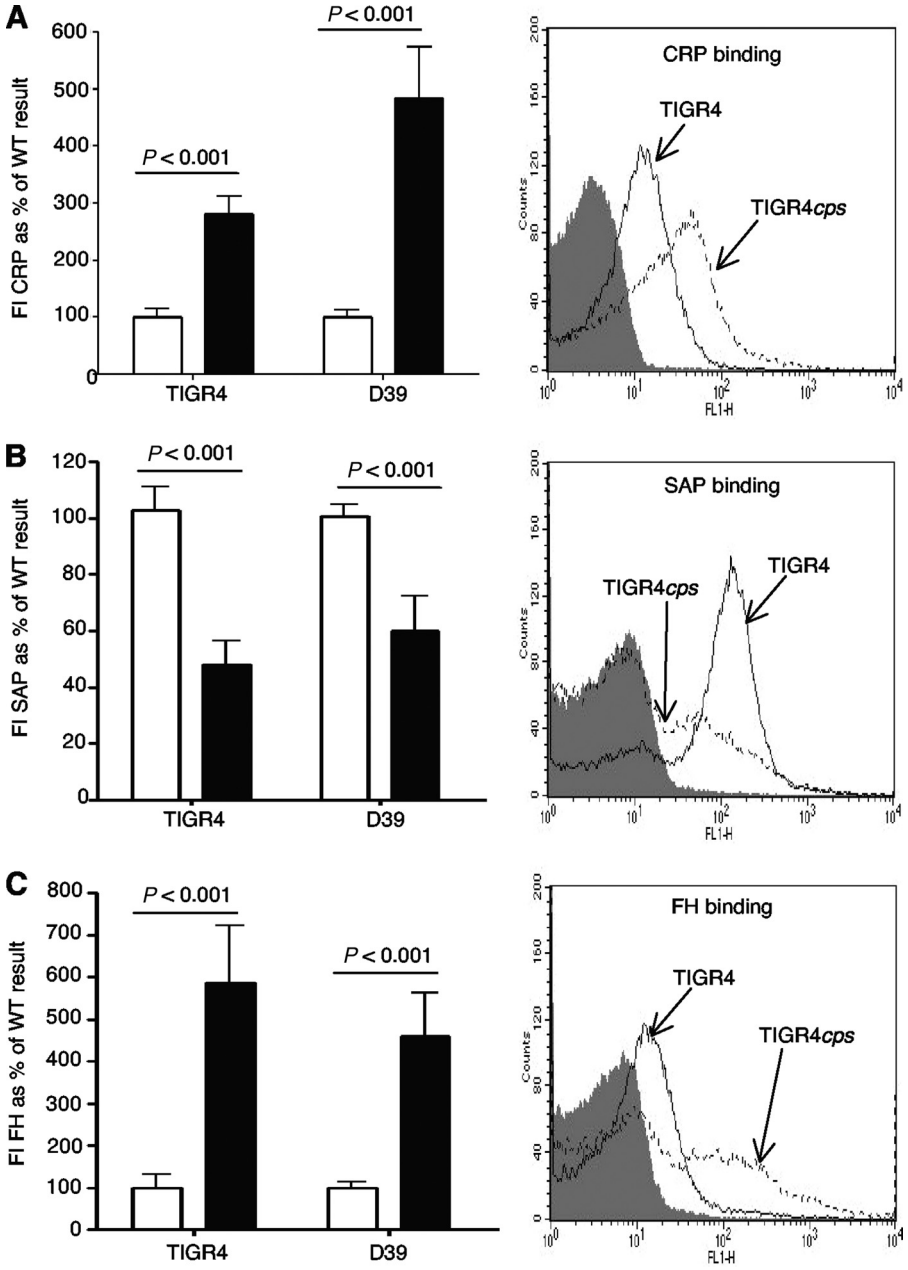

FIG. 5. Flow cytometry assays of binding of the pentraxins CRP and SAP and the alternative pathway inhibitor FH to unencapsulated and encapsulated *S. pneumoniae* in 50% human serum. (A) FIs of CRP binding to the TIGR4 and D39 strains (white bars), compared to the TIGR4*cps* and D39-DΔ strains (black bars), and an example of the flow cytometry histogram for the TIGR4 strains. (B) FIs of SAP binding to the TIGR4 and D39 strains (white bars), compared to the TIGR4*cps* and D39-DΔ strains (black bars), and an example of the flow cytometry histogram for the TIGR4 strains. (C) FIs of FH binding to the TIGR4 and D39 strains (white bars), compared to the TIGR4*cps* and D39-DΔ strains (black bars), and an example of the flow cytometry histogram for the TIGR4 strains. In all panels, error bars represent SDs and *P* values were obtained using unpaired Student *t* tests. For the representative flow cytometry histograms, gray shading indicates the results for TIGR4*cps* incubated in PBS alone.

ated) classical pathway complement activity against *S. pneumoniae*.

The *S. pneumoniae* capsule inhibits complement-dependent neutrophil phagocytosis. An established flow cytometry assay of neutrophil phagocytosis and freshly isolated human neutrophils were used to examine the functional consequences of the increased C3b/iC3b deposition on unencapsulated *S. pneumoniae* strains (8, 51, 53). Compared to the encapsulated strains, there were markedly higher levels of association with human neutrophils of both fluorescent TIGR4*cps* and D39-DΔ strains (Fig. 7A and B) in 20% human serum, showing that the capsule inhibits serum-dependent bacterial association with neutrophils. In order to determine the effect of the capsule on the proportion of *S. pneumoniae* bacteria that were cell surface associated or phagocytosed, the neutrophil phagocytosis assays were repeated with 20% serum using cytochalasin D to block

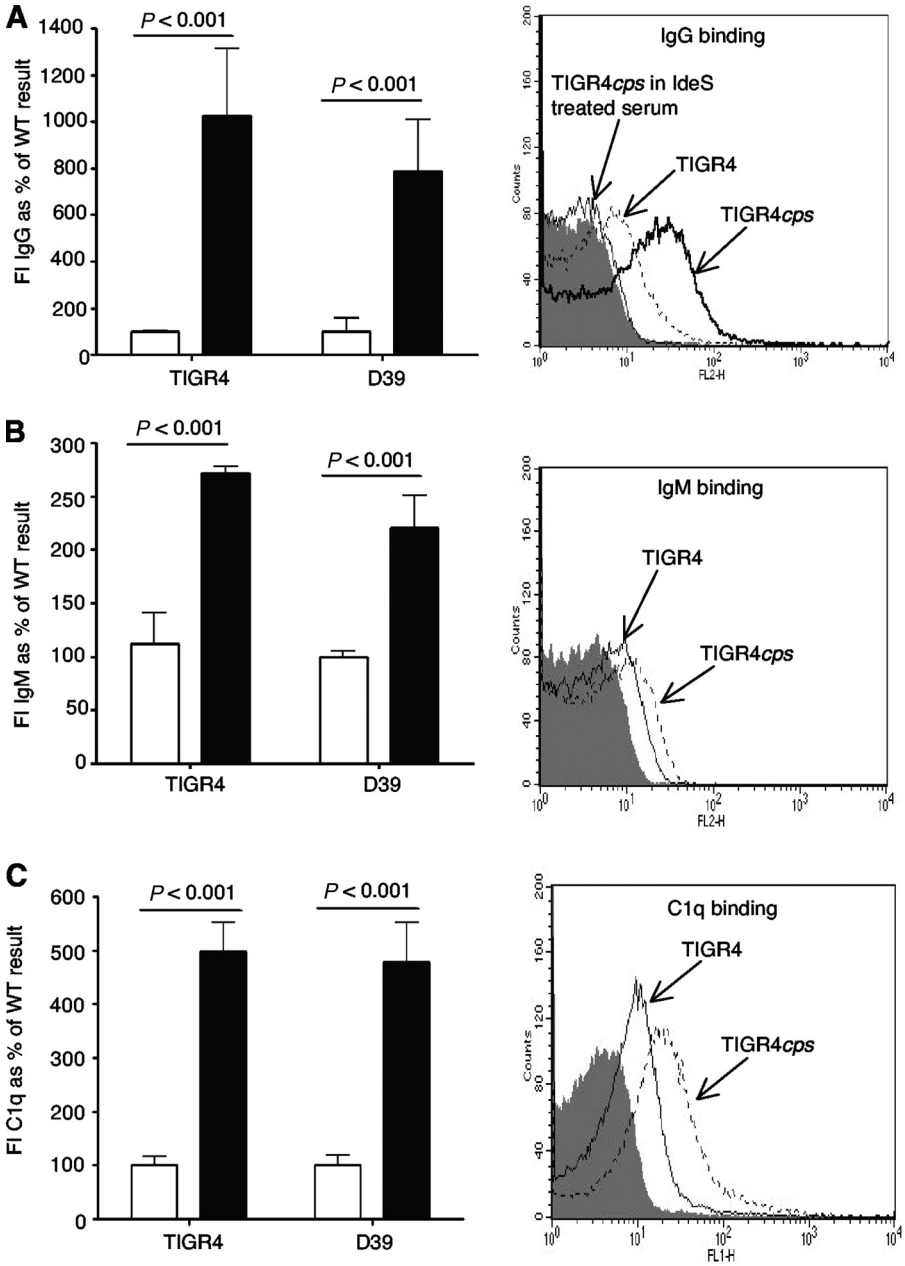

FIG. 6. Binding of IgG, IgM, and C1q to unencapsulated *S. pneumoniae* in 50% human serum. (A) FIs of IgG binding to the TIGR4 and D39 strains (white bars), compared to the TIGR4*cps* and D39-DΔ strains (black bars) and an example of the flow cytometry histogram for the TIGR4 strains, including an example of the results obtained with serum treated with IdeS. (B) FIs of IgM binding to the TIGR4 and D39 strains (white bars), compared to the TIGR4*cps* and D39-DΔ strains (black bars), and an example of the flow cytometry histogram for the TIGR4 strains. (C) FIs of C1q binding to the TIGR4 and D39 strains (white bars), compared to the TIGR4*cps* and D39-DΔ strains (black bars), and an example of the flow cytometry histogram for the TIGR4 strains. In all panels, error bars represent SDs and *P* values were obtained using Student unpaired *t* tests. WT, wild type.

phagocytosis. Treatment with cytochalasin D caused large decreases in the association of unencapsulated bacteria with neutrophils (Fig. 7A and B), suggesting that the main effect of the *S. pneumoniae* capsule is to inhibit phagocytosis rather than simply reducing association of the bacteria with the neutrophil surface.

Effects of the capsule on neutrophil-mediated killing of *S. pneumoniae*. Whether increased phagocytosis resulted in increased bacterial killing was assessed using a neutrophil *S. pneumoniae* killing assay. In a lower concentration of normal serum (12.5%), there was increased killing of the D39-DΔ and TIGR4*cps* strains compared to that of the D39 and TIGR4 strains, respectively (Fig. 7C and D). However, the D39 strain was more sensitive to neutrophil killing that the TIGR4 strains at higher concentrations of serum, with the majority of both the D39-DΔ and D39 strains killed when incubated in 25%

TABLE 2. Effects of IgG depletion using IdeS on C3b/iC3b deposition and C1q binding to unencapsulated and encapsulated D39 and TIGR4 strains in 50% human serum

Assay and strain	IdeS treatment	Mean FI ± SD of C3b/iC3b deposition or C1q binding to:		*P* value[a]
		Encapsulated strain	Unencapsulated strain	
C3b/iC3b deposition				
D39	No	12,118 ± 2,200	28,776 ± 3,900	<0.001
	Yes	9,466 ± 440	19,856 ± 4,400	<0.001
TIGR4	No	20,385 ± 2,600	38,056 ± 3,400	<0.001
	Yes	12,133 ± 1,900	20,978 ± 1,900	<0.001
C1q binding				
D39	No	1,073 ± 110	3,524 ± 190	<0.001
	Yes	719 ± 150	1,357 ± 100	<0.001
TIGR4	No	1,041 ± 210	4,520 ± 200	<0.0001
	Yes	933 ± 110	1,648 ± 210	<0.001

[a] *P* values represent comparisons between the results for unencapsulated and encapsulated strains using Student unpaired *t* tests.

serum, therefore obscuring any effects of the capsule (Fig. 7D). In contrast, there was only a low level of killing of the encapsulated TIGR4 strain in 25% serum, and large differences in the results of the killing assays for the TIGR4*cps* and TIGR4 strains persisted (Fig. 7C).

The *S. pneumoniae* capsule inhibits complement-independent and IgG-independent neutrophil phagocytosis. As well as the differences in neutrophil phagocytosis between unencapsulated and encapsulated strains incubated in serum, there was an increased association of the TIGR4*cps* and D39-DΔ strains with neutrophils after incubation in PBS alone (Fig. 7A and B) or in heat-killed serum (data not shown). These data suggest that there is also a complement-independent effect of the capsule on neutrophil phagocytosis, and this possibility was investigated further by repeating the assays using human serum deficient in C3 and serum treated with IdeS to cleave IgG. After incubation in $C3^-$ serum, bacterial association with neutrophils was significantly reduced for all of the strains, demonstrating the importance of complement for the phagocytosis of *S. pneumoniae*. There was increased phagocytosis of both the TIGR4*cps* and D39-DΔ strains than of the TIGR4 and D39 strains in $C3^-$ serum (Table 3), confirming a complement-

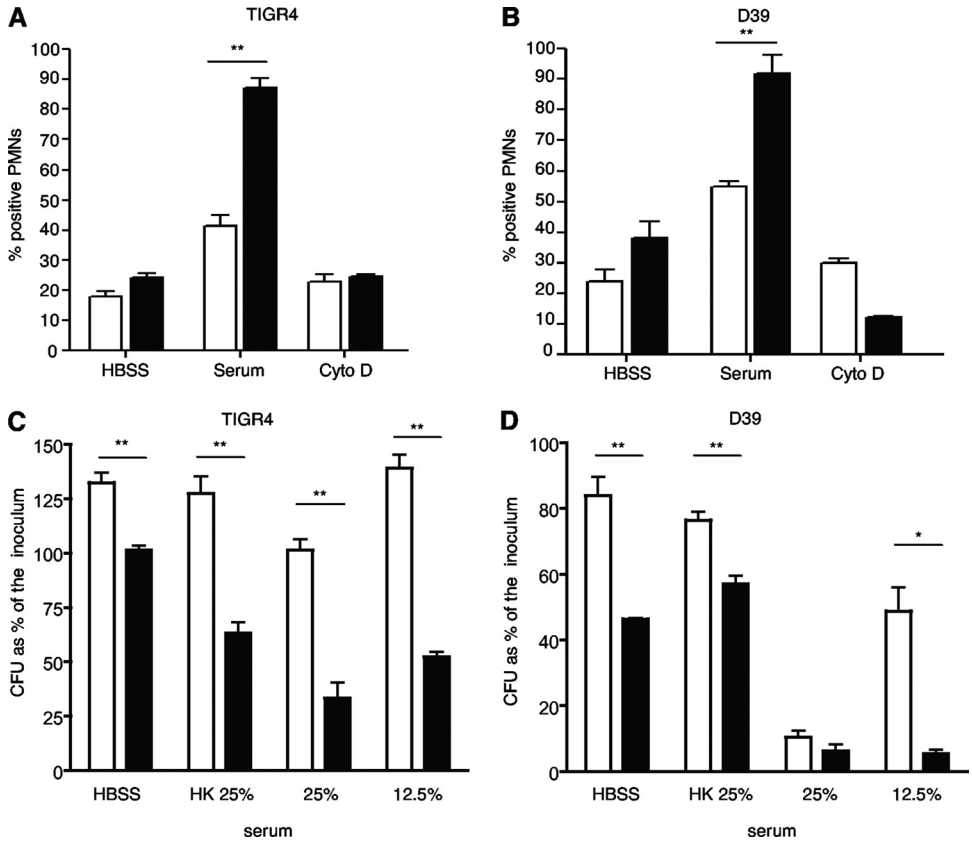

FIG. 7. Effect of the capsule on the interactions of *S. pneumoniae* with human neutrophils. (A and B) Percent association of fresh human neutrophils with strain TIGR4 (A) or D39 (B) (open bars) and strain TIGR4*cps* or D39-DΔ (black bars) when opsonized in 20% serum with or without inhibition of phagocytosis using cytochalasin D. Error bars represent SDs, and single and double asterisks represent P values of < 0.01 and < 0.001, respectively (ANOVA with post-hoc tests). (C and D) Proportion of *S. pneumoniae* inoculum surviving after incubation with fresh human neutrophils for 30 min when opsonized with 12.5 or 25% human serum, 25% heat-treated serum (HK), or HBSS. (C) Results for strains TIGR4 (white bars) and TIGR4*cps* (black bars). (D) Results for strains D39 (white bars) and D39-DΔ (black bars). Error bars represent SDs, and single and double asterisks represent P values of < 0.05 and < 0.01, respectively, for comparisons of unencapsulated to encapsulated bacteria under each opsonization condition (Student *t* test). PMN, polymorphonuclear neutrophils; WT, wild type.

TABLE 3. Effects of IgG depletion using IdeS on association of unencapsulated and encapsulated D39 and TIGR4 strains with neutrophils after incubation in normal human serum or C3-deficient serum

Serum and strain	IdeS treatment	Mean % association with neutrophils ± SD		*P* value[a]
		Encapsulated strain	Unencapsulated strain	
NHS[b]				
D39	No	39.2 ± 2.5	79.0 ± 5.6	<0.0001
	Yes	28.5 ± 0.5	61.0 ± 2.8	<0.0001
TIGR4	No	32.0 ± 3.9	85.9 ± 3.9	<0.0001
	Yes	28.9 ± 1.5	70.2 ± 1.2	<0.0001
$C3^{-}$[c]				
D39	No	23.5 ± 0.8	39.9 ± 1.3	<0.0001
	Yes	16.8 ± 1.1	25.8 ± 1.2	<0.0001
TIGR4	No	21.2 ± 3.2	40.2 ± 2.7	<0.0001
	Yes	12.8 ± 0.8	28.5 ± 1.1	<0.0001

[a] *P* values represent comparisons between the results for unencapsulated and encapsulated strains using Student unpaired *t* tests.
[b] NHS, 20% normal human serum.
[c] $C3^{-}$, 20% C3-deficient serum.

independent effect of the capsule on neutrophil phagocytosis. Treatment of serum with IdeS reduced the neutrophil phagocytosis of all of the strains in both normal and $C3^{-}$ serum (Table 3). However, there was a persisting increase in the phagocytosis of unencapsulated compared to encapsulated strains after incubation in IdeS-treated normal and $C3^{-}$ serum, suggesting that the capsule has effects on phagocytosis even in the absence of IgG alone and in the absence of both complement and IgG, respectively (Table 3). These data were supported by the results of the neutrophil killing assays, which showed that the TIGR4*cps* and D39-DΔ strains were more susceptible to killing by neutrophils when opsonized with buffer alone or heat-treated serum than were the TIGR4 and D39 strains, respectively (Fig. 7C and D).

DISCUSSION

There are considerable data which support the vital role of complement for immunity against *S. pneumoniae* (8, 15, 19, 22, 39, 52, 53). Complement is essential for efficient phagocytosis of *S. pneumoniae* by neutrophils and seems to be especially important at preventing systemic infection with *S. pneumoniae* (52, 53), and capsule inhibition of complement activity is therefore likely to be an important mechanism by which the capsule aids systemic virulence. Data showing that the capsular serotype can affect the site and quantity of complement deposition on *S. pneumoniae* (17, 48, 53) suggest an important role for the capsule in modulating complement activity, but there are few data directly comparing the interactions with complement and neutrophils of encapsulated and unencapsulated *S. pneumoniae*. Several mechanisms for capsule inhibition of complement activity have been described for other microbial pathogens, but whether these are relevant for *S. pneumoniae* requires clarification. Furthermore, the capsule could affect phagocytosis mainly through its effects on complement but may also affect IgG-mediated and nonopsonic phagocytic receptor-mediated phagocytosis directly (44). A better understanding of how the capsule affects interactions with the immune system and thereby improves virulence may help identify why some capsular serotypes are more able to cause invasive disease in humans (9) and perhaps assist our understanding of the implications of changes in capsular serotype ecology in response to vaccination (18).

We have investigated the consequences of loss of the capsule on interactions with complement and neutrophils for two *S. pneumoniae* strains, TIGR4 and D39, both of which have been used extensively for pathogenesis studies and have had their genomes sequenced. The isogenic unencapsulated derivatives of these strains were constructed by different methods, the TIGR4*cps* strain by complete replacement of the capsular locus with the Janus cassette (30) and the D39-DΔ strain by an in-frame deletion of *cpsD*, a gene that encodes an enzyme required for regulation of capsule synthesis (28). Both strains have been previously well characterized, and we have further shown the absence of the capsule using a biochemical assay and EM. Using flow cytometry, we have confirmed the finding of other investigators that the capsule inhibits opsonization of the D39 *S. pneumoniae* strain with C3b/iC3b (32) and have shown that this is also true for the TIGR4 strain. For both strains, the capsule also inhibited breakdown of C3b to iC3b, with a decreased ratio of iC3b to total C3b/iC3b on encapsulated bacterial surfaces. Our data obtained with serum depleted of C1q or Bf demonstrating that both pathways are required for the increase in C3b/iC3b deposition on unencapsulated *S. pneumoniae* suggest that the capsule may inhibit the activity of both pathways.

In serum, the classical pathway initiates C3b/iC3b deposition on *S. pneumoniae* by recognition of *S. pneumoniae* by Ab (acquired IgG and IgM, as well as natural IgM) and by the pentraxins CRP and SAP (8, 36, 51). Our data show that the capsule inhibits the binding of IgG, IgM, and CRP to *S. pneumoniae* but not that of SAP, and this was associated with an increase in C1q binding. Although anticapsular Ab is important for immunity to *S. pneumoniae* in vaccinated individuals, our data showing that IgG binding to *S. pneumoniae* is inhibited by the capsule suggest that a significant component of naturally occurring IgG in unvaccinated individuals recognizes subcapsular antigens, presumably cell wall PC or cell surface protein antigens. Previous data suggest that the capsule masks specific subcapsular antigens from host IgG (12), and the decrease in C3b/iC3b deposition on *S. pneumoniae* in serum treated with IdeS to cleave IgG suggests that this is one mechanism by which the capsule inhibits classical pathway activation. However, there were persisting increases in C3b/iC3b deposition and C1q binding to unencapsulated compared to encapsulated strains in IgG-depleted serum, demonstrating that the capsule also inhibits non-IgG-mediated complement activity. IgM binding to subcapsular antigens may be one IgG-independent mechanism of complement activation prevented by the capsule, but we only found low levels of IgM binding to *S. pneumoniae* in our serum. In addition, we found that the capsule inhibited CRP binding to *S. pneumoniae*. CRP is thought to initiate classical pathway activity through binding of C1q, and increased CRP binding could therefore cause the persisting increase in C1q binding to the D39-DΔ and TIGR4*cps* strains in IdeS-treated serum and thereby aid classical pathway activity.

Although the GBS and *N. meningitidis* capsules inhibit complement activity by binding FH (26), we found that the *S. pneumoniae* capsule actually decreases FH binding. Capsule inhibition of FH binding (presumably by masking of the FH binding protein PspC) would be predicted to increase alternative pathway activity against encapsulated strains, but the data obtained with C1q-deficient serum (representing C3b/iC3b deposition mediated mainly through the alternative pathway) demonstrated that, in fact, the capsule inhibits alternative-pathway-mediated C3b/iC3b deposition on *S. pneumoniae*. Hence, loss of the capsule has effects on alternative pathway activity that counterbalance the increase in FH binding. As C1q-deficient serum has no classical pathway activity, the increase in alternative pathway-mediated C3b/iC3b deposition on unencapsulated strains cannot be caused by amplification of complement activity initiated through increased classical pathway activity. Instead, the capsule must prevent alternative pathway activity directly, perhaps by inhibiting the access of alternative pathway proteins to the bacterial cell wall and impaired formation of the alternative pathway C3 convertase on the bacterial surface. Alternatively, although the MBL pathway is not a major activator of complement activity against encapsulated *S. pneumoniae* (8), it is possible that it may contribute to the increased C3b/iC3b deposition on unencapsulated strains in C1q-deficient serum. The non-IgG-dependent mechanisms by which the capsule inhibits complement activity need further investigation.

Neutrophil phagocytosis is considered one of the major elements of immunity to *S. pneumoniae* and is markedly dependent on opsonization of *S. pneumoniae* with complement (53). Hence, the increased phagocytosis of unencapsulated strains when opsonized with serum is an expected consequence of the increase in C3b/iC3b deposition and possibly the higher ratio of iC3b to C3b on these strains compared to the corresponding encapsulated strains. Inhibition of actin polymerization with cytochalasin D demonstrated that the differences between unencapsulated and encapsulated bacteria were largely due to increased internalization of unencapsulated bacteria, and this would also explain the increased neutrophil killing of the unencapsulated *S. pneumoniae* strains. However, as well as complement-dependent effects of the capsule on phagocytosis, there were also significant impairments of the association or killing of encapsulated TIGR4 and D39 with neutrophils when the bacteria were opsonized with complement-deficient serum, IgG-deficient serum, or combined complement and IgG-deficient serum or HBSS. These results show that, as well as inhibiting neutrophil phagocytosis by reducing opsonization with C3b/iC3b, the capsule also prevents IgG and non-IgG complement-independent mechanisms of phagocytosis. Hence, the capsule can prevent phagocytosis mediated by complement receptors (through reduced opsonization with C3b/iC3b) or Fcγ receptors (by decreasing IgG binding to *S. pneumoniae*) and by inhibiting bacterial interactions with nonopsonic phagocytic receptors such as mannose or scavenger receptors (2, 3).

In summary, we have shown that the *S. pneumoniae* capsule can affect several aspects of complement activity against *S. pneumoniae*. These include preventing binding of both IgG and CRP to *S. pneumoniae* and thereby inhibiting classical pathway activity, reducing alternative pathway activity through unexplained mechanisms, and decreasing the degradation of C3b bound to the bacterial surface to iC3b. The effects on C3b/iC3b deposition prevent phagocytosis of encapsulated bacteria, but the data also suggest that the capsule inhibits phagocytosis mediated directly by IgG and by nonopsonic phagocytic receptors. The results clarify some of the mechanism by which the *S. pneumoniae* capsule could mediate immune evasion. Further research is required to investigate whether differences between capsular serotypes in their interactions with the host immune response may partially explain why *S. pneumoniae* strains vary in the ability to cause invasive disease.

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