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# *Streptococcus pneumoniae* Capsular Serotype Invasiveness Correlates with the Degree of Factor H Binding and Opsonization with C3b/iC3b

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**Different capsular serotypes of *Streptococcus pneumoniae* vary markedly in their ability to cause invasive infection, but the reasons why are not known. As immunity to *S. pneumoniae* infection is highly complement dependent, variations in sensitivity to complement between *S. pneumoniae* capsular serotypes could affect invasiveness. We have used 20 capsule-switched variants of strain TIGR4 to investigate whether differences in the binding of the alternative pathway inhibitor factor H (FH) could be one mechanism causing variations in complement resistance and invasive potential between capsular serotypes. Flow cytometry assays were used to assess complement factor binding and complement-dependent neutrophil association for the TIGR4 capsule-switched strains. FH binding varied with the serotype and inversely correlated with the results of factor B binding, C3b/iC3b deposition, and neutrophil association. Differences between strains in FH binding were lost when assays were repeated with *pspC* mutant strains, and loss of PspC also reduced differences in C3b/iC3b deposition between strains. Median FH binding was high in capsule-switched mutant strains expressing more invasive serotypes, and a principal component analysis demonstrated a strong correlation between serotype invasiveness, high FH binding, and resistance to complement and neutrophil association. Further data obtained with 33 clinical strains also demonstrated that FH binding negatively correlated with C3b/iC3b deposition and that median FH binding was high in strains expressing more invasive serotypes. These data suggest that variations in complement resistance between *S. pneumoniae* strains and the association of a serotype with invasiveness could be related to capsular serotype effects on FH binding.**

The nasopharyngeal commensal *Streptococcus pneumoniae* is also a common bacterial pathogen, responsible for a high proportion of cases of pneumonia, meningitis, and septicemia. The capsule is the most important *S. pneumoniae* virulence factor and is classified into over 90 antigenically distinct capsular serotypes (1). The biology of *S. pneumoniae* varies markedly between serotypes (2–8), and understanding the mechanisms causing these differences will help identify characteristics required for virulence. For example, for each episode of carriage in the nasopharynx, some serotypes of *S. pneumoniae* are much more likely than others to cause invasive disease (defined as isolation of *S. pneumoniae* from a sterile site, mainly the blood) (5, 6, 8). Epidemiological studies have used the ratio of cases to carriers to provide estimates for relative serotype invasiveness, which can be represented as the number of cases of invasive disease per 100,000 colonization events (the attack rate) (5) or as case-to-carrier ratios (6, 8). With some exceptions, the different data sets identify similar sets of serotypes as being more invasive, but the reasons underpinning the link between a serotype and invasiveness are poorly understood. Relative serotype invasiveness is of increasing importance due to the changes in *S. pneumoniae* serotype ecology following universal vaccination with conjugated polysaccharide vaccine. This vaccine has dramatically reduced the prevalence of strains expressing serotypes included in the vaccine but is associated with an increase in nonvaccine serotypes in carriage and invasive disease (9, 10), which is called serotype replacement. To date, serotype replacement has been essentially complete in nasopharyngeal carriage, with the overall *S. pneumoniae* carriage prevalence in

vaccinated populations about the same as that before vaccination. However, in children, invasive disease has declined overall, suggesting that the invasiveness of the replacing serotypes is less than that for the types in the vaccine (9, 11).

The *S. pneumoniae* capsule can inhibit several aspects of host immunity, including neutrophil extracellular traps and both complement-dependent and complement-independent neutrophil phagocytosis (12, 13). Complement is vital for protection against septicemia (14–16), suggesting that the effect of the capsule on complement resistance is one major reason why the capsule is vital for the development of invasive disease. Complement resistance varies markedly between *S. pneumoniae* strains, and this is related to both the capsular serotype (2, 3, 17, 18) and other genetic variation between strains (18, 19). Infection with serotypes that were resistant to complement resulted in greater bacterial CFU in the blood in mouse models of sepsis (2, 4), supporting a hypothesis that resistance to complement-mediated immunity could partially explain differences in invasiveness between serotypes. How-

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ever, serotype-dependent effects on complement-mediated immunity have not been correlated to invasive potential for a large number of serotypes, and the mechanism by which serotype affects complement activity is not known.

*S. pneumoniae* has evolved a number of mechanisms to evade complement-mediated immunity, including the binding of the host inhibitor of the alternative pathway inhibitor factor H (FH) to the cell wall proteins PspC and PhtD (20, 21). FH binding prevents formation of the factor B (Bf)-dependent C3b convertase on the bacterial surface, thereby potentially reducing *S. pneumoniae* opsonization with C3b (20–22). FH also assists degradation of C3b to iC3b. PspC and PhtD are subcapsular proteins, and FH binding to *S. pneumoniae* is inhibited by the capsule (12). Furthermore, both the accessibility of PspC and the degree of FH binding varied between a small number of *S. pneumoniae* mutants expressing different capsular serotypes on the TIGR4 background (4, 22). These data demonstrate that the capsular serotype affects FH binding, supporting the hypothesis that differences in FH binding could be one mechanism by which the serotype causes variation between *S. pneumoniae* strains in resistance to complement-mediated immunity. Furthermore, high levels of FH binding could potentially increase the invasive potential of particular *S. pneumoniae* serotypes by preventing complement-mediated immunity in the blood.

We have therefore investigated the relationship between FH binding and resistance to complement and opsonophagocytosis by neutrophils for a large number of capsule-switched mutants. In addition, the results of FH binding and resistance to complement for capsule-switched variants and representative clinical strains from common serotypes were correlated with data for serotype-associated invasiveness reported previously by epidemiological studies.

## MATERIALS AND METHODS

**Strains and culture conditions.** The isogenic capsular transparent-phase variants of the *S. pneumoniae* strain TIGR4 used in this study have been described previously (2, 4, 23, 24). The 33 clinical strains used represent common multilocus sequence type (MLST) clones for 8 serotypes, and all isolates were obtained from nasopharyngeal cultures of asymptomatic children or from invasive *S. pneumoniae* infections in adults and children and were described by Hyams et al. (19) (kind gifts from Brian Spratt, Imperial College, and Birgitta Henriques-Normak, Karolinska). PspC mutants of capsule-switched variants of TIGR4 were created by transformation using conventional methods with a *pspC* deletion construct made by overlap extension PCR as described previously (22). Bacteria were cultured at 37°C in 5% CO<sub>2</sub> on blood agar plates or in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY) to a mean optical density at 580 nm of 0.41 (standard deviation [SD], 0.02) for the capsule-switched strains (mean CFU,  $1.09 \times 10^8$  per ml; SD,  $0.09 \times 10^8$ ) and 0.40 (SD, 0.01) for the clinical strains (mean CFU,  $1.17 \times 10^8$  per ml; SD,  $0.02 \times 10^8$ ) and stored at –70°C in 10% glycerol as single-use aliquots.

**Serum source, flow cytometry, and ELISAs.** Pooled human serum was obtained from unvaccinated normal human volunteers and stored in single use aliquots at –70°C. Capsule-specific antibody titers for clinically relevant serotypes were measured using standardized enzyme-linked immunosorbent assays (ELISAs) with preabsorption with C-polysaccharide and serotype 22F capsular polysaccharide as described (<http://www.vaccine.uab.edu/ELISA%20Protocol.pdf>). Total IgG binding to *S. pneumoniae* (representing both anticapsular and antiprotein antigen activity), C3b/iC3b deposition, iC3b, FH, and Bf binding to *S. pneumoniae* after incubation in 20% serum for 20 min at 37°C were measured using flow cytometry assays and R-phycoerythrin goat anti-human IgG (Jackson

ImmunoResearch) or fluorescein isothiocyanate (FITC)-conjugated anti-human C3 or iC3b, anti-FH, or anti-Bf (ICN) as described previously (2, 12, 15, 16, 19). As complement factor binding to *S. pneumoniae* is often biphasic with strongly positive and weakly positive populations of bacteria, results are presented as a fluorescence index (FI; percentage of positive bacteria multiplied by the geometric mean fluorescence index (MFI) in arbitrary units) (2, 12, 15); this ensures both intensity (geometric mean MFI) of complement factor binding and the proportion of positive bacteria are included in the data analysis. Each strain gave highly consistent results using stocks from independent THY cultures (correlations for data from two stocks of the same capsule-switched strain gave an R<sup>2</sup> of 0.94 for C3b/iC3b deposition and 1.0 for FH binding). Neutrophil association (a measure of phagocytosis) (12) was investigated using flow cytometry to identify the proportion of fresh human neutrophils ( $10^5$  per reaction) associated with 6-carboxyfluorescein succinimidyl ester (FAMSE; Molecular Probes) labeled *S. pneumoniae* ( $10^6$  per reaction) after preincubation in 20% serum for 20 min at 37°C as described previously (2, 12). Neutrophil killing assays were performed using freshly isolated human neutrophils and bacteria (in a ratio of 800 cells per bacterium) preincubated in phosphate-buffered saline (PBS) with heat-treated (65°C for 20 min to neutralize complement activity) or untreated serum as described previously (12).

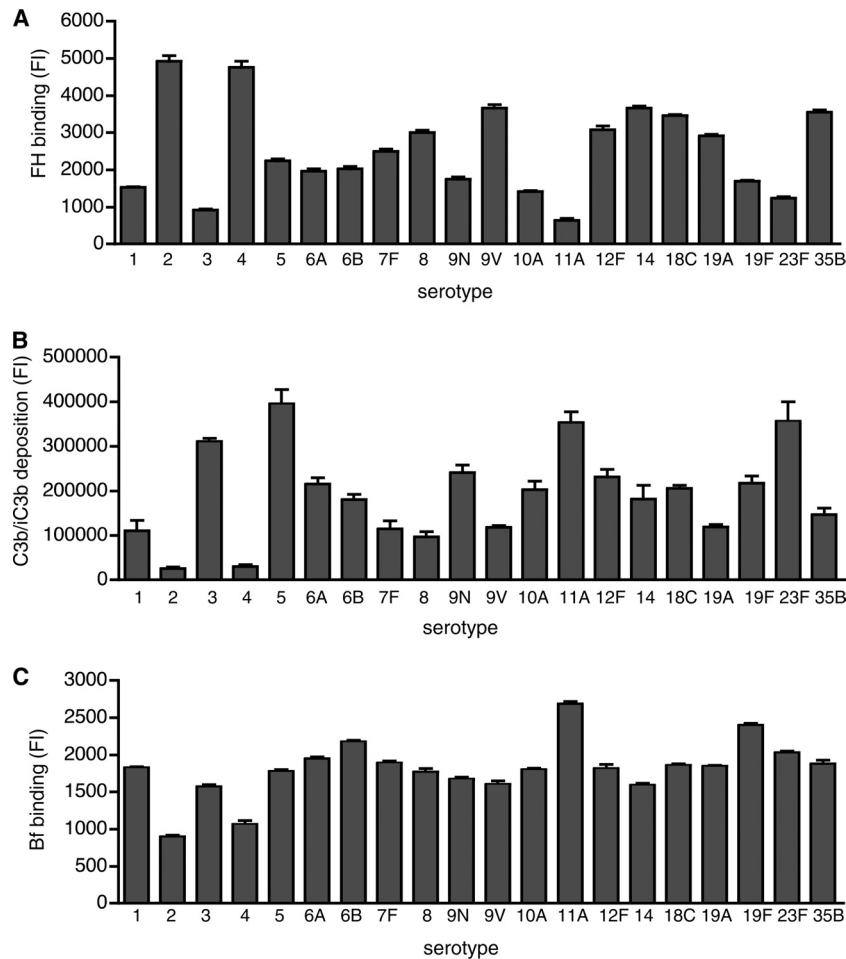
**Immunogold electron microscopy.** Electron microscopy (EM) was performed using mid-log-phase capsule-switched *S. pneumoniae* strains (2) incubated at 37°C for 20 min in serum, a Jeol 1010 transmission electron microscope (100 kV), and a ruthenium red and London Resin protocol. C3b/iC3b deposition was detected by an immunogold detection method using 1/100 FITC goat polyclonal anti-C3 (ICN Biomedicals) and 1/100 immunogold conjugated rabbit anti-goat antibodies as described previously (12). The numbers of gold particles associated with bacteria were counted for 10 or more randomly chosen bacteria per strain and expressed as medians (interquartile ranges [IQRs]).

**Statistics.** Flow cytometry data between individual strains were analyzed using one-way analyses of variance (ANOVAs) with Dunn's *post hoc* test and presented as standard errors of the means (SEM). Complement factor binding and phagocytosis data for individual strains were correlated using Spearman's correlation test for nonparametric data. Immunogold EM data were compared using a Kruskal-Wallis test with Dunn's *post hoc* test and presented as IQRs. Pooled data for serotypes with high or low attack rates were compared using Mann-Whitney U tests. Log-transformed data from each of the measurements above were used for a principal component (PC) analysis. For the PC analysis, variance in complement component binding and phagocytosis assays between different serotypes was assessed in R v2.11.1 ([www.r-project.org](http://www.r-project.org)) to obtain scores for each serotype and loading values for each measurement parameter in all PCs.

## RESULTS

### Effects of capsular serotype on binding to complement factors.

To investigate the effect of the capsular serotype on FH binding to *S. pneumoniae* independent of other genetic variation between strains, FH binding to a panel of 20 capsule-switched mutants of the TIGR4 strain (23, 24) was measured using flow cytometry. After incubation in human serum, there was wide variation in FH binding between these strains, with over a 7.5-fold difference between the serotype with the highest binding to FH (serotype 2) and the serotype with the lowest binding (serotype 11A) (Fig. 1A). To assess whether these differences in FH binding affected strain sensitivity to opsonization with complement, we measured the deposition of the activated complement component C3b/iC3b and the binding of Bf (as a measure of alternative pathway activity) for each strain. As previously shown by our group and others (2, 18), C3b/iC3b deposition showed major variations between capsule-switched strains, with a 15-fold difference between the sero-



**FIG 1** Flow cytometry data for the binding of complement factors to the TIGR4 capsule-switched strains. Data are presented as the mean (SEM) FI of FH binding (A), C3b/iC3b deposition (B), and factor B binding (C).

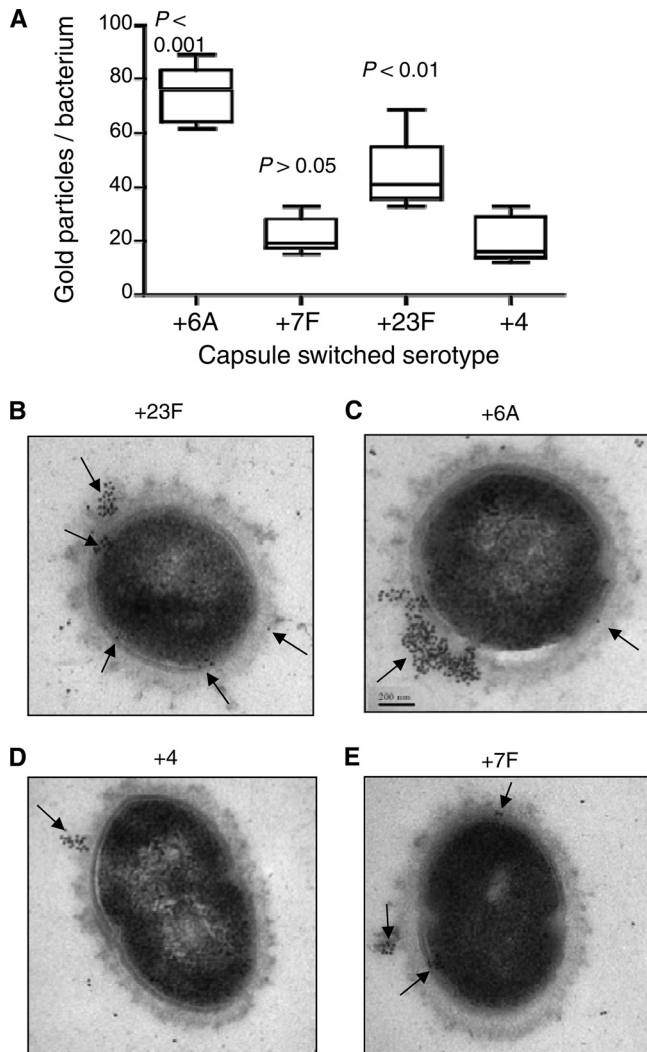
type with the highest level of C3b/iC3b deposition (serotype 5) and the serotype with the lowest level (serotype 2) (Fig. 1B). In addition, Bf binding also varied significantly between capsule-switched strains (Fig. 1C). To confirm differences in C3b/iC3b deposition between capsule-switched strains identified by flow cytometry and to locate sites of C3b/iC3b binding, immunogold EM was performed on selected capsule-switched strains using an antibody to C3b/iC3b. The total number of C3b/iC3b particles detected were higher for the serotype 23F and 6A capsule-switched strains than serotype 4 and 7F capsule-switched strains (Fig. 2A), conforming to the pattern observed using a flow cytometry assay. Although some C3b/iC3b was detected in the subcapsule region, the majority of the differences in C3 binding between strains were due to focal clusters of C3b/iC3b forming on the capsule (Fig. 2B to E).

The level of FH binding to each capsular mutant showed a strong negative correlation to both Bf binding and C3b/iC3b deposition (Fig. 3A and B). FH binding did not correlate with capsule thickness, the numbers of carbon atoms or presence of hydroxyl groups in the polysaccharide repeating unit for each serotype (data not shown) (24). These results suggest that the relative degree of FH binding between the capsule-switched strains was functionally important, with high levels of FH binding

resulting in reduced alternative pathway activity and increased resistance to opsonization with complement. As FH binding may alter processing of C3b bound to the bacterial surface to iC3b, flow cytometry was used to assay specific iC3b deposition on selected capsule-switched strains, two with high levels of FH binding and two with low levels of FH binding (Fig. 3C). However, one of each pair of strains had relatively high levels of iC3b deposition, with the other having low levels, showing there was no consistent pattern between FH and iC3b levels.

**Effects of PspC on differences in FH binding and C3b/iC3b deposition between selected capsule-switched strains.** To further assess the role of FH binding for differences in C3b/iC3b deposition between capsule-switched strains, *pspC* was deleted from the serotype 4 strain and two strains with relatively low levels of FH binding, serotypes 6A and 23F. FH binding of all three *pspC* mutant strains was markedly reduced, and in contrast to the data for *pspC*<sup>+</sup> parental strains, there were no differences in FH binding between the three *pspC* mutant strains (Fig. 4A and B). The increase in C3b/iC3b deposition on the *pspC*<sup>+</sup> serotype 6A and 23F strains compared to the serotype 4 strain were reduced by 39% and 71%, respectively, when the assay was repeated with the corresponding *pspC* mutants (Fig. 4C). These results demonstrate

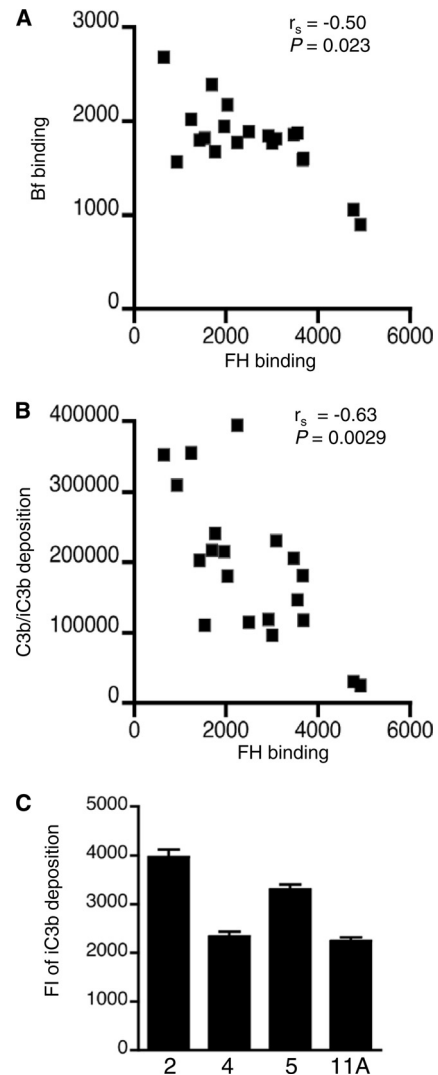




**FIG 2** Immunogold EM of Cb/iC3b deposition on selected capsule-switched strains. (A) Median (IQR) number of gold particles associated with transparent phase variants of serotype 4, 6A, 7F, and 23F capsule-switched strains on ultrathin EM sections. *P* values for comparisons to serotype 4 data using the Kruskal-Wallis test with Dunnett's multiple comparisons are stated above the corresponding column. (B to E) Representative EM pictures for the serotype 23F (B), 6A (C), 4 (D), and 7F (E) strains. Arrows indicate gold particles that represent the location of C3b/iC3b deposition.

that differences in FH binding between capsule-switched strains were PspC dependent and made a significant contribution to variation in C3b/iC3b deposition results between serotypes.

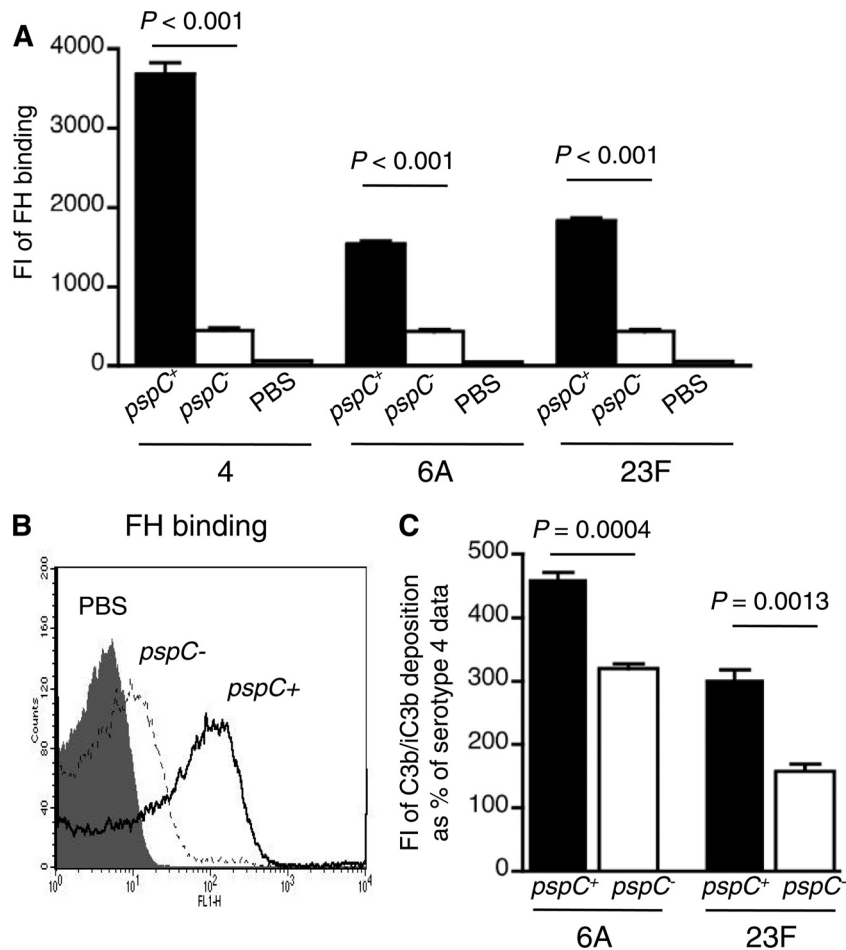
**Relationship of complement factor binding to capsule-switched mutants and neutrophil association.** Neutrophil phagocytosis of *S. pneumoniae* is strongly dependent on complement activity (12, 25) and therefore should be affected by the variation in opsonization with complement between capsule-switched strains. After incubation in human serum, the proportion of bacteria associated with human neutrophils (which is strongly related to phagocytosis) (12, 25) varied significantly between capsule-switched strains from 19% (serotype 2) to 71% (serotype 11A) (Fig. 5A). The degree of neutrophil association had a strong negative correlation with FH binding (Fig. 5B) and a strong positive correlation with C3b/iC3b deposition (Fig. 5C),



**FIG 3** Spearman's correlation of the FI for the FH binding results for the capsule-switched variants to FI for Bf binding (A) and C3b/iC3b deposition (B). (C) Flow cytometry data for specific iC3b deposition on selected capsule-switched strains (serotypes 2, 4, 5, and 11A) presented as mean FIs with error bars representing SEM.

demonstrating that differences in complement factor binding between these strains were functionally important. To support the flow cytometry data, neutrophil killing assays were performed for selected capsule-switched strains, two with high levels (5 and 11A) and two with low (2 and 4) levels of neutrophil association. Neutrophil killing of the serotype 2 and 4 strains was very similar under all conditions. In contrast, after opsonization with serum, 16% and 21% more of the serotype 5 and 11A strains were killed, respectively, than the serotype 4 strain, falling to 8% for both serotypes after opsonization in heat-treated serum or 6% and 7% after incubation in PBS (Fig. 5D). These data confirmed that strains with high levels of neutrophil association were more readily killed by neutrophils and that this effect was largely dependent on complement.

**Role of antibody binding.** Recognition by specific antibody has a strong influence on complement activity against *S. pneumoniae* (26–28), and the serotype could potentially affect antibody

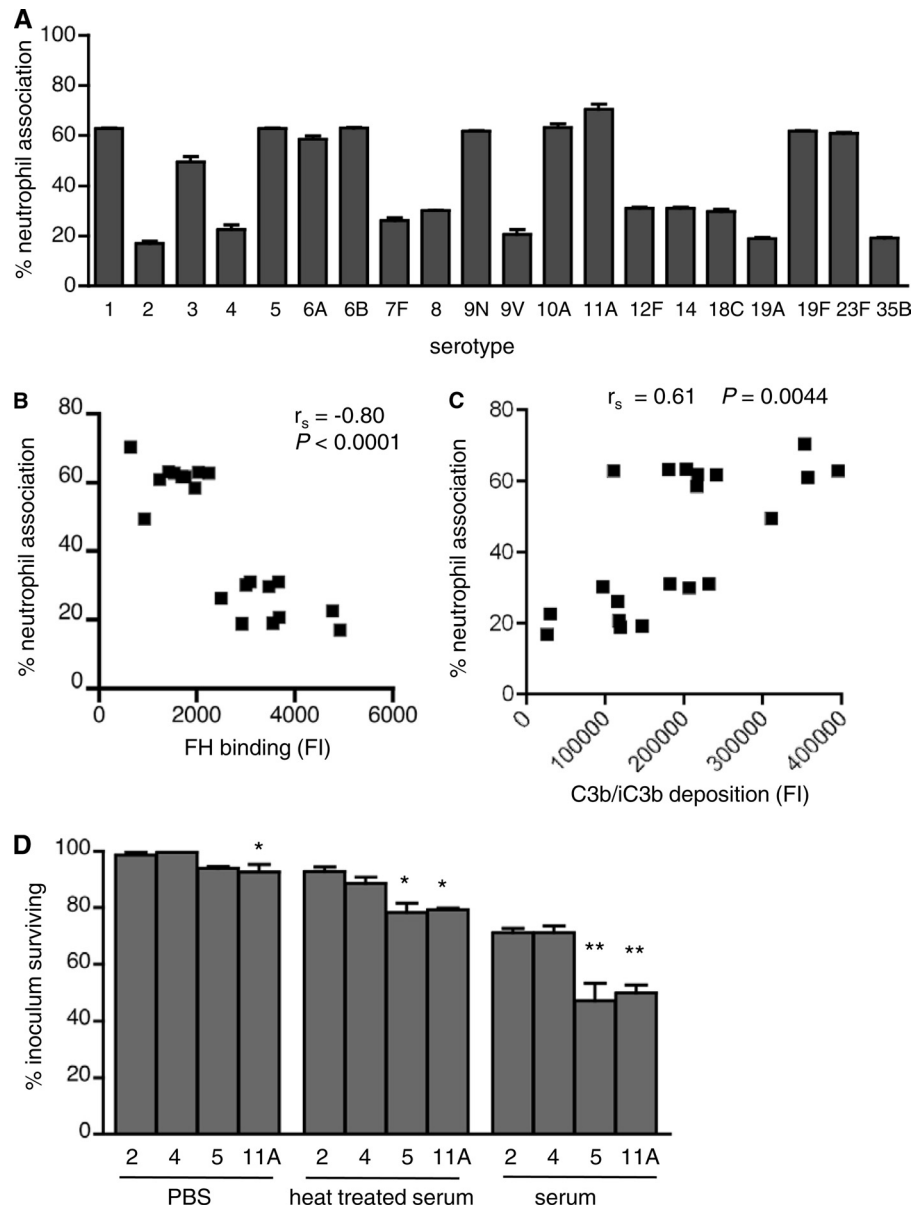


**FIG 4** Role of PspC for FH binding and differences in C3b/iC3b deposition between capsule-switched strains. (A) Flow cytometry data for FH binding to selected capsule-switched strains and their *pspC* mutant derivatives. Data are presented as mean FI for FH binding, black columns represent *pspC*<sup>+</sup> strains, white columns represent *pspC* mutant strains, and gray columns represent *pspC*<sup>+</sup> strains incubated in PBS (negative control). (B) Example of a flow cytometry histogram for FH binding to serotype 4 *pspC*<sup>+</sup> and *pspC* mutant strains. (C) Mean FI of C3b/iC3b deposition on the *pspC*<sup>+</sup> and *pspC* mutant serotype 6A and 23F capsule-switched strains expressed as a mean percentage of the results for the corresponding serotype 4 strain. Error bars represent SEM, and *P* values comparing *pspC*<sup>+</sup> and *pspC* mutant data in panels A and C were obtained by unpaired *t* tests.

binding to *S. pneumoniae* through modifying access to subcapsular antigens or due to differences in levels of anticapsular antibody between serotypes. Hence, total IgG binding (both to capsular and subcapsular antigens) to each capsule-switched strain was measured using flow cytometry in the serum used for the complement and phagocytosis experiments (Fig. 6A). Although there were significant differences in total IgG binding between capsule-switched strains, these were relatively small (with the exception of the serotype 4 strain) and did not positively correlate with C3b/iC3b deposition (Fig. 6B). Furthermore, serum anticapsular antibody levels (data available for 11 serotypes) also did not correlate with C3b/iC3b deposition results (Fig. 6C). Hence, differences between capsule-switched strains in their sensitivity to complement were not related to differences in IgG binding.

**Relationship of FH binding to serotype invasiveness.** We hypothesized that serotypes resistant to complement due to high levels of FH binding would be more likely to cause invasive disease. To investigate this, FH binding results for the capsule-switched strains were correlated with invasiveness for the corresponding serotypes represented by the attack rate calculated by

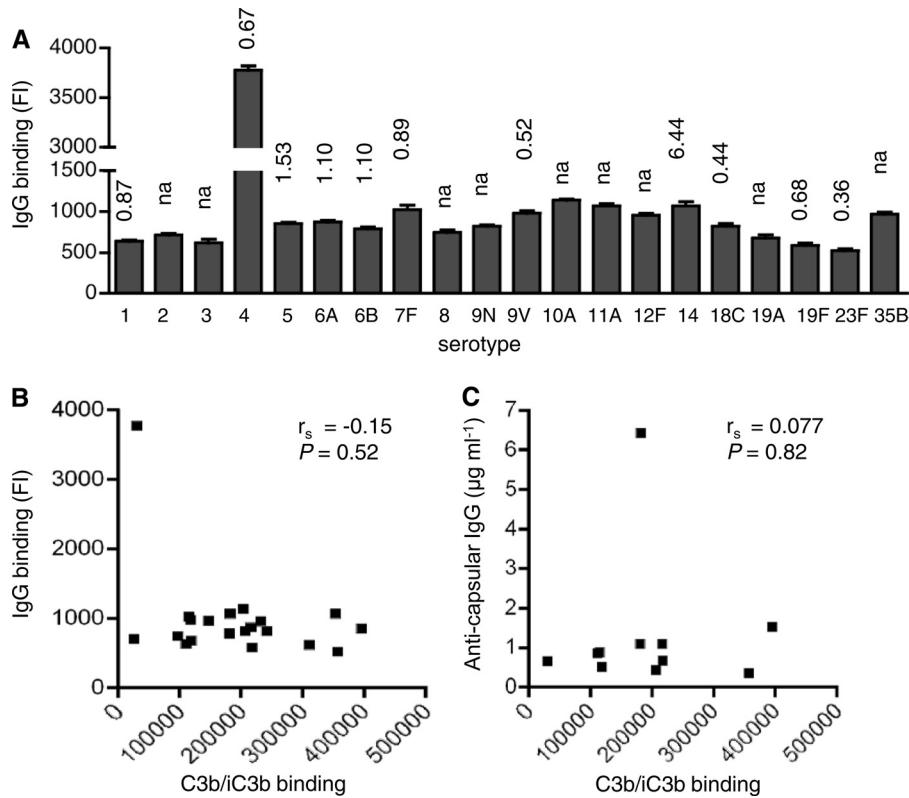
Sleeman et al. (Fig. 7A) (5). Serotypes 2 and 35B were excluded, as there were no estimates of their invasiveness. Estimates of invasiveness are also not available for serotypes 1 and 5, as they are found in cases of invasive disease but rarely as commensals, and were assumed to have the highest attack rate identified among other serotypes (assumed attack rate of 75) (5). FH binding positively correlated with attack rates ( $r_s = 0.62$ ,  $P = 0.0062$ ), supporting the hypothesis that differences in FH binding could be influencing serotype-dependent invasiveness. In addition, median FH binding for 10 capsule-switched strains expressing highly invasive serotypes (attack rates  $>20$ ) was higher than that for the 8 strains expressing weakly invasive serotypes (attack rates  $<10$ ) (Fig. 5B). Serotype 1 and 5 strains were clear outliers for these analyses with low FH binding despite an assumed high attack rate (Fig. 7A); their exclusion strengthened the correlation between FH binding and the calculated serotype attack rates ( $r_s = 0.81$ ,  $P = 0.0001$ ) and increased differences in median FH between highly invasive and weakly invasive serotypes (Fig. 7B). To strengthen the association of FH binding with *S. pneumoniae* invasiveness, FH results were correlated with the relative serotype invasiveness data described



**FIG 5** Complement-dependent neutrophil phagocytosis of capsule-switched variants. (A) Mean (SD) proportion of fresh human neutrophils associated with each TIGR4 capsule-switched variants after opsonization with 20% serum measured using flow cytometry. (B and C) Spearman's correlation of the neutrophil association results presented in panel A to the results for FH binding (B) or C3b/iC3b deposition (C) (both represented as FIs). (D) Neutrophil killing assay data for selected capsule-switched strains (serotypes 2, 4, 5, and 11A). Data are presented as mean (SEM) proportion of the inoculum surviving after incubation with human neutrophils for 1 h and given for bacteria preincubated in PBS and heat-treated and untreated 20% serum. Asterisks indicate strains with significant differences to the serotype 4 strain (ANOVA with Dunnett's *post hoc* comparisons; \*,  $P < 0.05$ , \*\*,  $P < 0.01$ ).

by Trotter (6) and Yildirim (8) independently of Sleeman (5). With the Trotter data, median FH binding to highly invasive serotype strains (pediatric disease case-to-carrier ratios of  $>100$ ; serotypes 7F, 9V, 14, and 18C) was higher than that for weakly invasive serotype strains (case-to-carrier ratios of  $<100$ ; serotypes 3, 6A, 6B, 9N, 11A, 19A, 19F, 23F, and 35B) (Fig. 5C) and FH binding showed a positive Spearman's correlation with case-to-carrier ratios (not shown) ( $r_s = 0.62$ ,  $P = 0.023$ ) (6). The Yildirim data for serotype invasiveness also showed a positive Spearman's correlation with FH binding, but only if the outlying serotype 35B (which had high FH binding but was never found during invasive disease) (8) was excluded (not shown) ( $r_s = 0.58$ ,  $P = 0.049$ ).

**PC analysis.** To further assess the relationship between the complement factor, neutrophil phagocytosis, and the attack rate (5), a PC analysis was used to explore the relationships between these measurements in a multivariate analysis. PC1, which included a large positive loading for FH binding and a large negative loading for C3b/iC3b deposition, varied markedly between strains (Fig. 8A). PC1 also showed less negative loadings for the neutrophil association and Bf binding results (Fig. 8B). This supports the hypothesis that these factors were cocorrelated and that the serotype has a common effect on FH binding, C3b/iC3b deposition, and neutrophil phagocytosis. Importantly, PC1 results for each capsule-switched strain had a strong correlation with the serotype

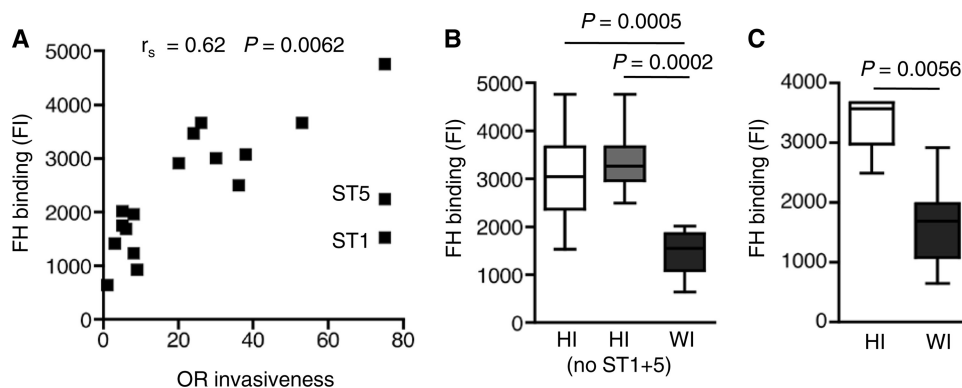


**FIG 6** Antibody binding to capsule-switched strains. (A) Mean (SD) FI of total IgG binding to TIGR4 capsule-switched variants measured by flow cytometry. Levels ( $\mu\text{g ml}^{-1}$ ) of anticapsular serotype-specific IgG measured by ELISA are shown above each column (na, not available). (B and C) Spearman's correlation of total IgG (B) or anticapsular serotype-specific antibody levels (C) to FI of C3b/iC3b deposition on capsule-switched strains.

attack rate ( $r_s = 0.55$ ,  $P = 0.019$  or  $r_s = 0.76$ ,  $P = 0.0007$ , excluding serotype 1 and 5 data). In this analysis, strains with high levels of FH binding but low levels of C3b/iC3b deposition and neutrophil association expressed serotypes that epidemiological data suggest are more likely to cause invasive infection (Fig. 8C).

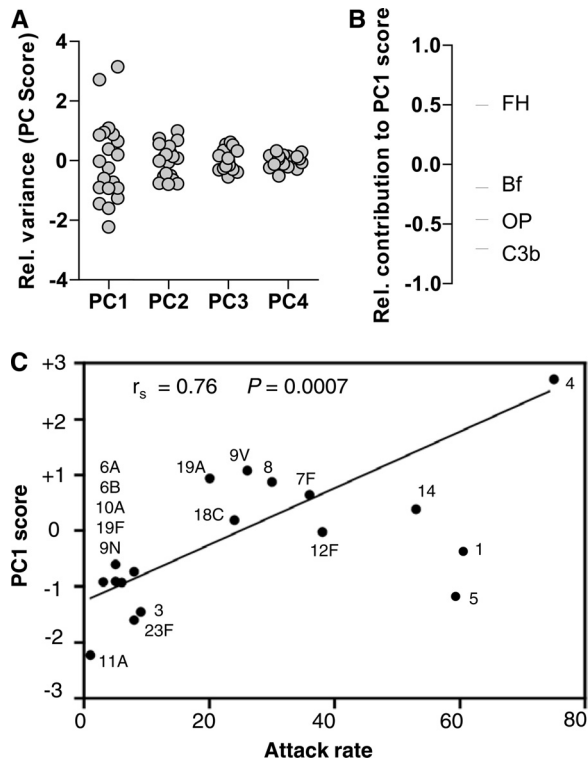
**FH binding by clinical *S. pneumoniae* isolates.** To further support the hypothesis that differences in FH binding might influence strain invasiveness, FH binding was analyzed using a set of

33 clinical isolates from 8 common serotypes with different genetic backgrounds (19). FH binding varied significantly between strains from different serotypes (e.g., serotype 1 strain versus serotype 6A strain) and between strains expressing the same capsular serotype (e.g., serotype 4 and serotype 14) (Fig. 9A). To assess if these differences could affect complement sensitivity binding, the previously published C3b/iC3b deposition data for these strains (19) were correlated with the FH binding results. FH bind-



**FIG 7** Relationship between FH binding to TIGR4 capsule-switched strains and *S. pneumoniae* serotype-related invasiveness. (A) Spearman's correlation of FH binding results for capsule-switched strains to the estimated attack rate for 18 serotypes (5). The outlier serotypes (serotype 1 and serotype 5) are marked, excluding serotype 1 and 5 data ( $r_s = 0.81$ ,  $P = 0.0001$ ). (B and C) Median (IQR) FH binding to capsule-switched mutants divided according to Sleeman et al. (B) (5) or Trotter et al. (C) (6) into highly invasive (HI; attack rate  $>20$  or case-to-carrier ratios  $>100$ ) or weakly invasive (WI; attack rate  $<10$  or case-to-carrier ratios  $<100$ ) serotypes.  $P$  values are shown for Mann-Whitney U tests.





**FIG 8** PC analysis of FH, Bf, C3b/iC3b, and neutrophil opsonophagocytosis data for the TIGR4 capsule-switched strains. (A) Relative variance of each PC. (B) Contribution of each variable to the variance between strains in PC1. (C) The correlation between variance of PC1 with serotype invasiveness represented by the attack rate (5). Data points represent individual serotypes and the linear regression is shown for the relationship between the attack rate and PC1. Spearman's  $r_s$  and  $P$  values are given for data, excluding the serotype 1 and serotype 5 strains.

ing was negatively correlated with C3b/iC3b deposition on the clinical isolates ( $r_s = -0.76$ ,  $P < 0.0001$ ) (Fig. 9B), supporting the hypothesis that variation in FH binding has an important effect on complement resistance for *S. pneumoniae*. Furthermore, although there was considerable overlap between the groups, median FH binding was higher on clinical strains from highly invasive capsular serotypes (1, 4, 9V, and 14) than strains from weakly invasive capsular serotypes (6A, 6B, 19F, and 23F) (Fig. 9C).

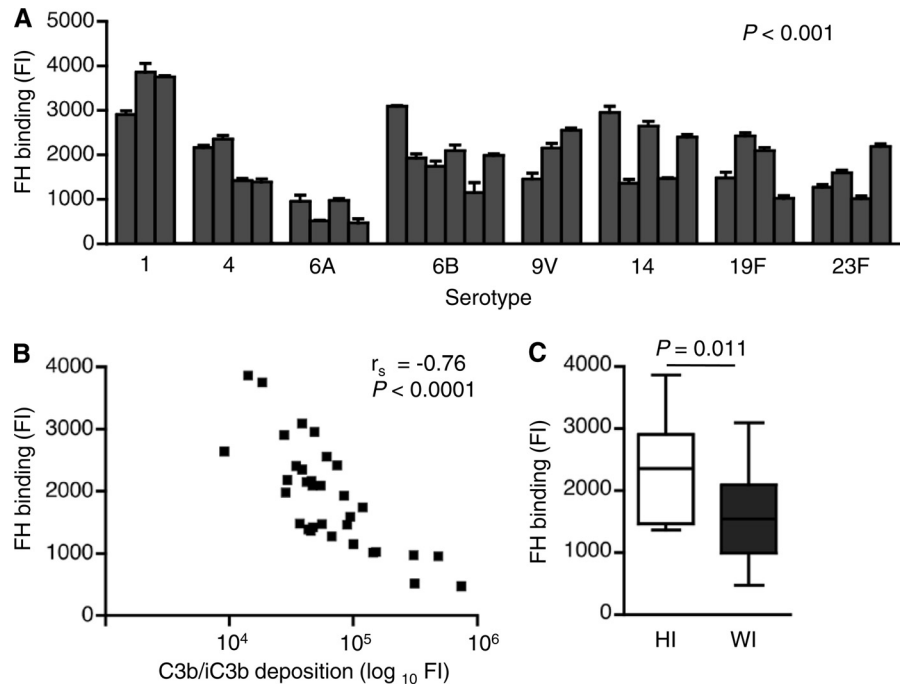
## DISCUSSION

There are surprisingly large variations in sensitivity to complement between *S. pneumoniae* strains (2, 3, 17–19). These differences partly depend on the capsular serotype (2, 3, 18), but the mechanisms involved have not been identified. Using a large number of capsule-switched strains in the TIGR4 background, we have now shown that serotype-dependent differences in FH binding negatively correlated with differences in Bf binding, C3b/iC3b deposition, and sensitivity to complement-dependent neutrophil association. The flow cytometry data were supported by similar results obtained using immunogold EM for selected strains. The site of C3b/iC3b localization may also affect complement efficacy at promoting phagocytosis, and the immunogold EM data suggested that most of the increased C3b/iC3b deposition on some serotypes was located on the capsule rather than at the cell wall level where it may be a more effective opsonin (27). For the se-

lected capsule-switched strains investigated, FH binding was largely dependent on the cell wall protein PspC, and loss of PspC greatly reduced differences in C3b/iC3b deposition between strains. These results support the hypothesis that the effects of the serotype on FH binding to the subcapsular protein PspC could be one mechanism causing differences in complement sensitivity binding between different *S. pneumoniae* capsular serotypes. The thickness of the capsule layer varies between the TIGR4 capsule-switched strains used for these studies (24), but neither this nor the numbers of carbon atoms or the presence of hydroxyl groups correlated with the FH binding results, and how the serotype affects FH binding will need further investigation. Of note, minor changes may have large effects; for example, serotypes 19A and 19F differ in structure by a single bond in the trisaccharide repeating unit (1) yet have significant differences in FH binding and complement sensitivity.

Neutrophil phagocytosis is thought to be a major mechanism of bacterial killing of *S. pneumoniae* and is highly dependent on intact complement activity (12, 22, 29). The negative correlation between FH binding and neutrophil phagocytosis of serum opsonized *S. pneumoniae* (supported by the neutrophil killing assay for selected strains) confirms that the differences in FH binding and C3b/iC3b deposition between capsule-switched strains is functionally relevant. In addition, variations in FH binding between clinical isolates negatively correlated with C3b/iC3b deposition, demonstrating that FH binding could also be important for variations in complement sensitivity between clinical isolates as well as the capsule-switched strains. FH binding varied between strains expressing the same capsular serotypes, indicating that capsule-independent mechanisms such as differences in PspC expression or allelic variation in PspC structure (30) may also affect FH interactions with *S. pneumoniae*.

The essential role for complement to protect against systemic infection caused by *S. pneumoniae* (14–16) suggests that variations in complement sensitivity between strains should affect invasiveness. In support of this hypothesis, we found a positive relationship between FH binding to the capsule-switched strains and different sets of epidemiological data on invasiveness of each serotype. Furthermore, the PC analysis demonstrated that capsule-switched strains expressing weakly invasive serotypes had low levels of FH binding, relatively high levels of factor B and C3b/iC3b binding, and high levels of neutrophil association. Overall, our data suggest that the degree of binding to FH could be a critical factor in explaining some of the variation in invasiveness between *S. pneumoniae* strains. By binding high levels of FH, invasive *S. pneumoniae* serotypes are relatively resistant to complement-dependent neutrophil phagocytosis, so they can evade complement-mediated systemic immunity, and this may make them more likely to cause septicemia. Differences in FH binding between serotype were dependent on PspC, and the capsular serotype could potentially also affect other functions of PspC. These include bacterial adhesion to and invasion across epithelial layers (31, 32), which could provide another explanation of why high levels of FH binding positively correlate with serotype invasiveness independent of the effects on complement activity. Interestingly, the neutrophil association of serum-opsonized capsule-switched strains gave a different serotype rank order for resistance to phagocytosis compared to complement-independent neutrophil phagocytosis obtained using the same set of capsule-switched



**FIG 9** FH binding to representative clinical isolates for common serotypes. (A) Mean (SD) FI of FH binding to 33 clinical strains from 8 serotypes measured using flow cytometry. The  $P$  value was obtained using ANOVA. (B) Spearman's correlation of C3b/iC3b deposition ( $\log_{10}$  FI; previously published data) (19) to the results of FH binding to the clinical isolates. (C) Median (IQR) FH binding to the clinical strains divided into highly invasive (HI; attack rate  $>20$ ; serotypes 1, 4, 9V, and 14) and weakly invasive (WI; attack rate  $<10$ ; serotypes 6A, 6B, 19F, and 23F) serotypes (5). The  $P$  value was calculated using the Mann-Whitney U test.

strains (24). Hence, the effects of each serotype on interactions with the host may depend on opsonizing conditions.

With the correlations between complement factor binding and serotype invasiveness, the capsule-switched serotype 1 and 5 strains were significant outliers with relatively low FH binding but an assumed high invasiveness. As a consequence, although FH binding positively correlated with invasiveness when these strains were included in the analyses, the strength of the association improved markedly when they were excluded. *S. pneumoniae* serotypes have markedly different biological and genetic characteristics, and it is perhaps not surprising that some serotypes did not conform to the general pattern. Serotype 1 is the only capsular serotype that is zwitterionic, and both serotypes 1 and 5 have unusual epidemiological characteristics. They have a relatively flat age-incidence pattern and are rarely found in the nasopharyngeal carriage (5, 7); as a consequence of the latter, invasiveness has not been calculated for these serotypes and, for our analysis, an assumed attack rate of 75 had to be used, which may not be accurate. The biological reasons for the differences in the epidemiology of serotype 1 and serotype 5 strains may also account for why they are highly invasive independent of their complement sensitivity phenotype.

Although we have shown strong correlations between FH binding, complement resistance, and serotype invasiveness, it is difficult to prove that differences in FH binding cause variations in invasiveness. Demonstrating that differences between capsule-switched strains in complement sensitivity and invasiveness are abrogated in the absence of FH would provide strong support for this hypothesis. However, FH knockout mice are functionally C3 deficient due to uncontrolled complement activity. Furthermore,

comparing virulence between capsule-switched strains using mouse models may be difficult to interpret; the *S. pneumoniae* strain background has unpredictable effects in mouse infection models, and TIGR4 capsule-switched strains expressing serotype 6A, 23F, and 7F did not cause significant disease in some models (4). Alternatively, experiments could be repeated using *pspC* mutant strains. However, the deletion of *pspC* increases the quantity of cell-associated PspA (J. S. Brown, unpublished data), another choline-binding protein that affects complement activity (16, 33). This will confound the interpretation of results obtained using *pspC* mutants and perhaps explains why there has been a variable effect of the deletion of *pspC* on complement sensitivity between strains (22). Instead, we have sought additional support for the hypothesis by comparing median FH binding to clinical strains when divided by serotype into highly or weakly invasive groups. Invasive potential varies between strains with the same capsular serotype, which will partially confound these comparisons (34). Despite this, there was still a significantly higher level of FH binding to clinical isolates expressing more invasive serotypes.

In conclusion, we have shown that the capsular serotype has significant effects on FH binding by *S. pneumoniae* and that serotypes with high levels of FH binding were resistant to complement-mediated immunity and are associated with increased invasiveness. The data suggest that the capsule does not simply aid virulence by blocking bacterial interactions with host proteins. Instead, more invasive capsular serotypes tend to have increased binding to a specific host protein (FH), and this is one plausible mechanism that might explain their increased invasiveness compared to other strains. How capsular serotype can cause variations

in FH binding and how this could affect invasiveness will need further investigation.

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