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Pneumococcal Surface Protein A Inhibits Complement Deposition on the Pneumococcal Surface by Competing with the Binding of C-Reactive Protein to Cell-Surface Phosphocholine

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In the presence of normal serum, complement component C3 is deposited on pneumococci primarily via the classical pathway. Pneumococcal surface protein A (PspA), a major virulence factor of pneumococci, effectively inhibits C3 deposition. PspA's C terminus has a choline-binding domain that anchors PspA to the phosphocholine (PC) moieties on the pneumococcal surface. C-reactive protein (CRP), another important host defense molecule, also binds to PC, and CRP binding to pneumococci enhances complement C3 deposition through the classical pathway. Using flow cytometry of PspA$^+$ and PspA$^-$ strains, we observed that the absence of PspA led to exposure of PC, enhanced the surface binding of CRP, and increased the deposition of C3. Moreover, when the PspA$^-$ mutant was incubated with a pneumococcal eluate containing native PspA, there was decreased deposition of CRP and C3 on the pneumococcal surface compared with incubation with an eluate from a PspA$^-$ strain. This inhibition was not observed when a recombinant PspA fragment, which lacks the choline-binding region of PspA, was added to the PspA$^-$ mutant. Also, there was much greater C3 deposition onto the PspA$^-$ pneumococcus when exposed to normal mouse serum from wild-type mice as compared with that from CRP knockout mice. Furthermore, when CRP knockout mouse serum was replenished with CRP, there was a dose-dependent increase in C3 deposition. The combined data reveal a novel mechanism of complement inhibition by a bacterial protein: inhibition of CRP surface binding and, thus, diminution of CRP-mediated complement deposition. *The Journal of Immunology*, **2012, 189: 5327–5335.**

Streptococcus pneumoniae (pneumococcus) is a Gram-positive bacterium responsible for much of the pneumonia, bacteremia, meningitis, otitis media, and septicemia in children <2 y of age, elderly adults, and immunocompromised hosts (1). Pneumococci asymptomatically colonize the nasopharynx in ~30% of healthy children and a lower percentage of adults. It is only when the bacterium successfully migrates to organs such as the lungs, nasal sinuses, brain, and middle ear that symptomatic disease can occur (2). The World Health Organization estimates that ~2 million children <5 y of age die of pneumonia each year, and the pneumococcus is the largest cause of these deaths (3).

The complement system consists of >30 circulating and membrane-bound proteins that play a major effector role in the immune response to pathogens. Complement must be activated to mediate antimicrobial activity. Serum components, such as C-reactive protein (CRP) and Ab, can initiate the complement cascade. There are at least three overlapping pathways of complement activation: the classical pathway, the alternative pathway, and the lectin pathway, of which the best known is the mannose-binding pathway (4). These pathways converge on the activation of C3, which leads to downstream events responsible for most of complement's effector functions. Complement eliminates bacteria through two major mechanisms: opsonization and membrane attack complex–mediated lysis. Pneumococci and other Gram-positive bacteria are normally protected from complement-mediated lysis by their rigid cell wall (5, 6). Opsonization of microbes, via covalent attachment of C3 and its fragments, fosters the recognition of the bacteria by complement receptors on professional phagocytes and promotes bacterial ingestion. Complement-dependent opsonophagocytosis is critical for the clearance of pneumococci from the bloodstream of the host (5, 7). Complement activation also results in the release of soluble bioactive fragments (e.g., C3a, C4a, C5a) that stimulate mast cells, macrophages, and neutrophils during the inflammatory response.

Pneumococci produce several molecules that help them evade complement deposition (8). Among these is pneumococcal surface

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Abbreviations used in this article: CBA/N, CBA/CaHN-Btk^{xid}/J; CRP, C-reactive protein; CRPKO, CRP knockout; FL-PspA, full-length native pneumococcal surface protein A; GVB, gelatin veronal buffer; MFI, mean fluorescence intensity; NHS, normal human serum; NMS, mouse serum from nonimmunized mice; PC, phosphocholine; PFA, paraformaldehyde; PspA, pneumococcal surface protein A; PspC, pneumococcal surface protein C; rCRP, recombinant CRP; SAP, serum amyloid P; THY, Todd–Hewitt broth supplemented with 5% yeast extract; WT, wild-type.

protein A (PspA), which is present on virtually all strains of pneumococci and is highly immunogenic (9–12). There are two major families of PspA, family 1 and family 2, which are further divided into clades (13, 14). Any given strain expresses only a single PspA allele, and thus expresses PspA of only one family. Strains of each major capsular type includes strains that express PspA family 1 and strains that express PspA family 2 (10, 14). Both families of PspA are able to inhibit C3 deposition on the pneumococcal surface and confer virulence on pneumococci (12). Proteins within PspA families are immunologically cross-reactive (15, 16), and immunity to a member of either family is generally protective against members of the same family and frequently cross-protective against strains of the other PspA family (9, 17, 18).

Full-length native PspA (FL-PspA) consists of five distinct domains. From the N to C terminus, these are a signal peptide, a highly charged antiparallel coiled coil α-helical domain, a proline-rich region, a choline-binding domain, and a C-terminal 17-aa tail (19–21). PspA is a member of a family of choline-binding proteins on the pneumococcus, which are anchored to the pneumococcal surface via their choline-binding domains (22). Choline-binding domains recognize the phosphocholine (PC) on the cell wall and cell-membrane-associated teichoic acids (23). Mutant PspA proteins lacking the choline-binding repeats are largely absent from the bacterial surface (12, 23, 24), and mutant strains lacking surface PspA show reduced virulence and greater complement deposition in normal serum (11, 12, 25). An isogenic strain lacking 6 of its 10 choline-binding repeats showed intermediate surface expression of PspA, reduced virulence, and intermediate deposition of C3 (12). It has been shown using normal serum lacking detectable anti-pneumococcal Ab that complement deposition on pneumococci occurs via the classical pathway (26, 27). In the absence of PspA, the classical pathway–dependent, C1q-dependent deposition of complement on pneumococci in normal serum is greatly increased (12, 27). Accordingly, $PspA^-$ strains show greater clearance from blood compared with $PspA^+$ strains (11, 28). However, the mechanism accounting for PspA's effect on complement has not been described.

CRP is found in low amounts (generally 1–3 μg/ml) in the serum of healthy humans but can increase ≥500-fold in response to inflammation or infection (29, 30). CRP is evolutionarily conserved, and healthy mice have blood concentrations of CRP comparable with healthy humans (31). Importantly, however, mouse CRP is a minor acute-phase reactant and can only increase in concentration ~2-fold during inflammation (31). Like PspA, CRP can bind PC on the pneumococcal cell surface (32) where it activates the classical pathway in a C1q-dependent manner (33, 34) and can protect against invasive infection (35, 36). Because PspA is the most abundant pneumococcal choline-binding protein (22, 37), its absence should significantly increase: 1) PC exposure on pneumococcal surface, 2) binding by CRP, 3) complement activation, and 4) greater opsonization of pneumococci. In this study, we tested the hypothesis that surface-bound PspA minimizes the binding of CRP, thus decreasing CRP-mediated activation of the complement pathway. $PspA^+$ and $PspA^-$ strains were examined for their exposure of PC and their ability to bind both CRP and natural Ab to PC. We also examined the ability of these strains to be targets of complement deposition when exposed to complement from wild-type (WT) versus CRP knockout (CRPKO) mice.

Materials and Methods

Bacterial strains and cultures

The strains used in this study included capsular serotype 2 pneumococci D39 ($PspA^+$) (38) and its isogenic mutants (JY182) and (TRE108) lacking PspA or pneumococcal surface protein C (PspC), respectively (24, 27, 39). The PspA of D39 is family 1, clade 2 (40). We confirmed that the respective PspA and PspC proteins were not expressed in these mutants by immunoblot assay. For the PspA mutants, we also examined PspA expression by flow cytometry where JY182 showed only background fluorescence with anti-PspA and D39 showed 7-fold higher mean fluorescence intensity (MFI). PspA expression was not changed in the *pspC* mutant. Both JY182 and TRE108 showed somewhat more capsule than strain D39; however, this difference was not statistically significant (data not shown). Capsule quantitation was done using a multiplex immunoassay developed in Dr. Moon Nahm's laboratory at the University of Alabama at Birmingham (41). Strains D39, JY182, and TRE108 were grown in triplicate in 3-ml volumes of Todd–Hewitt broth supplemented with 5% yeast extract (THY), with or without erythromycin (0.3 μg/ml), to OD_{600} 0.5. Next, 500 μl of each culture was lysed in 14× lysis buffer (41). Ten-fold dilutions ranging from 1/10 to $1/10^7$ lysates were used to inhibit the ability of type 2 anti-capsule Abs to bind polysaccharide-coated latex beads. Purified type 2 polysaccharide ATCC 16-X (American Type Culture Collection, Manassas, VA) was used as the standard.

Strain Rx1 ($PspA^+$) (11, 42) is a nonencapsulated derivative of D39. WG44.1 is a *pspA*-null mutant of Rx1. $PspA^+$ WT strains TIGR4, P.765, P.1547, and P.1121 are capsular types 4, 6B, 6A, and 23F, respectively (43–45). A *pspA* mutant in the 6A strain was made by double crossover insertion–deletion as described previously (44). The *pspA* mutation was transferred to the type 4, 6B, and 23F strains by sequential back transformation and selecting with 200 μg/ml spectinomycin (Sigma-Aldrich, St. Louis, MO). A 6B revertant was prepared by transforming the 6B *pspA* mutant with a PCR product of the 6B *pspA* gene including ~800 bp flanking regions and screening for PspA protein expression by colony immunoblot with clade-specific anti-PspA sera (46). The revertant was confirmed by loss of spectinomycin resistance and by DNA sequence analysis. Strains P.765, P.1547, and P.1121 are PspA family 1, and are PspA clades 1, 2, and 1, respectively. Capsular type 4 strain TIGR4 is family 2, clade 3 PspA.

Bacteria that were used in the CRP binding and C3 deposition assays were grown directly from frozen stocks on the day of assay and washed in PBS before use. The cells were grown in THY (with erythromycin added to the mutant strains) to an OD_{600} 0.4, diluted back to an OD_{600} 0.1, and then regrown in THY to an OD_{600} 0.4 for use in the assays. For enumeration of pneumococci, cultures were serially diluted, plated on blood agar with or without erythromycin, and grown in a candle jar overnight.

Sera and buffers

Fresh-frozen "normal" mouse serum from nonimmunized mice (NMS) was obtained from CBA/CaHN-Btk^{xid}/J (CBA/N) mice, which have no natural Ab to PC (47); BALB/cJ mice, which have natural Ab to PC (48); and CRPKO mice ($Crp^{-/-}$ [C57BL/6J background]), which do not express any CRP (49). NMS for use as a complement source was pooled from groups of 8–10 mice of each genotype. The sera from all sources were aliquoted and stored at −80°C. In assays to measure CRP or C3 deposition, all buffers had a pH of ~7.3; all dilutions were in gelatin veronal buffer (GVB) containing 0.1% gelatin, 5 mM veronal, 0.15 mM calcium, and 0.5 mM magnesium (GVB^{2+}; CompTech, Tyler, TX); and all the washing steps were performed using PBS without Mg^{2+} or Ca^{2+} (Mediatech, Manassas, VA). These studies were conducted in accordance with the principles set forth in the *Guide for Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Research Council, revised 1996), and approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

Isolation of eluates containing FL-PspA

Rx1 and WG44.1 pneumococci were grown to an OD_{600} 0.4 in 200 ml of chemically defined medium containing 0.03% choline chloride. FL-PspA was isolated and dialyzed as previously described (37).

One-fourth serial dilutions of cell eluates were immunoblotted on nitrocellulose membranes, blocked with 1% BSA in PBS, incubated with PspA-specific mAb, XiR278 (50), and developed with biotinylated goat anti-mouse Ig, alkaline phosphatase-conjugated streptavidin (Southern Biotechnology Associates, Birmingham, AL) and nitroblue tetrazolium substrate with 5-bromo-4-chloro-3-indoyl phosphate *p*-toluidine salt (Fisher Scientific, Norcross, GA), to qualitatively detect PspA. The presence of eluted PspA was further confirmed by a Western immunoblot assay (37, 50). The amount of total protein in these bacterial eluate samples was determined by Bio-Rad protein assay (Bio-Rad, Hercules, CA), and the amount of BSA added in the FL-PspA isolation step was subtracted to yield net (total) bacterial eluate protein.

The amount of PspA in the cell eluate from Rx1 was determined using an ELISA. Microtiter 96-well plates (NUNC, Wiesbaden, Germany) were

coated overnight at 4°C with 100 μl of a mixture of mAb XiR278 (50) and RX1003 (Genschmer and Briles, manuscript in preparation) at 2 μg/ml PBS total concentration (1 μg/ml each). Plates were washed, blocked with 150 μl 1% BSA/0.2% casein (BSA/casein) for 1 h, and washed; then Rx1 ($PspA^+$) and WG44.1 ($PspA^-$) cell eluates were titrated out on the plate in one-third dilutions in BSA/casein starting at 100, 10, and 1 μg/ml total protein. Finally, the plates were incubated at room temperature for 1 h with rabbit anti-PspA (1:1000) (14), washed, incubated for 1 h with 1:10,000 biotin-conjugated donkey anti-rabbit Ig antiserum IgG (H+L; Southern Biotechnology Associates), washed, incubated for 1 h with 1:4000 streptavidin conjugated to alkaline phosphatase (Southern Biotechnology Associates), and developed with p-nitrophenyl phosphate (Sigma-Aldrich), and the absorbance read at 405 nm. rUAB055 (51) was used as a standard at 60 ng/ml titered 1:3 in BSA/casein. The total PspA concentration in the cell eluate was calculated and expressed as ng/ml PspA.

Detection of exposed PspA and PC on the pneumococcal surface

Pneumococci (D39, JY182, TRE108, Rx1, and WG44.1) were grown as above and resuspended to 1×10^9 CFU/ml in GVB. For the PspA expression assay, D39, JY182, and TRE108 were incubated in 100 μl of 1:500 PspA-specific mAb (XiR278) at 37°C for 30 min with shaking. Samples were then washed and incubated with 50 μl FITC-labeled goat anti-mouse Ab (Southern Biotechnology Associates) diluted 1:100 in GVB at 37°C for 30 min with shaking. They were then washed and fixed with 500 μl 2% paraformaldehyde (PFA; Electron Microscopy Sciences, Hatfield, PA) for analysis by flow cytometry. The PC exposure assay was carried out the same way as the PspA expression assay except that the IgA hybridoma TEPC15 (Sigma-Aldrich) was used to detect PC on strains and mutants for 20,000 gated events.

Ab binding to pneumococcal surface

Types 6B, 4, 6A, and 23F, together with their *pspA* mutants (and revertant, in the case of 6B), were grown to midlog phase, washed, and resuspended in 1% BSA/PBS containing primary Ab. Ab for primary incubation was as follows: human IgG to PC (1:10) purified from pooled γ-globulin as previously described (52), normal human serum (NHS; 1:100) obtained from healthy adults, and NMS (1:10) pooled from BALB/C mice. Bacteria were incubated with primary Ab at 37°C for 30 min, washed, and bound Ab was detected using the appropriate species-specific anti-IgG or anti-IgM conjugated to FITC (1:200) (Sigma-Aldrich) at room temperature for 30 min. Finally, bacteria were washed and resuspended in 2% PFA for analysis by flow cytometry for 20,000 gated events.

CRP binding assay

D39, JY182, and TRE108 strains were grown fresh from frozen stocks. Cultures were centrifuged at 3000 rpm, washed twice, and pneumococci were resuspended in GVB to a concentration of 1×10^9 CFU/ml. Two hundred microliters of bacterial suspension was incubated with the indicated concentrations of human recombinant CRP (rCRP; US Biologicals, Swampscott, MA) at 37°C for 30 min. For the assays where cell eluates from Rx1 and WG44.1 were used, bacteria were first incubated with appropriate concentration of eluate for 1 h at 37°C followed by the incubation step with CRP. These were washed twice and then incubated with 50 μl FITC-conjugated mouse anti-human CRP (1:50; HyTest, Turku, Finland) at 37°C for 30 min. Finally, bacteria were then washed and fixed with 500 μl 2% PFA. The controls included bacteria incubated with only GVB and bacteria incubated with only FITC-conjugated secondary Ab. Flow cytometric analysis was carried out using an FACSCalibur instrument (Becton Dickinson, Mountain View, CA) for 40,000 gated events. To make sure that the rCRP was not agglutinating pneumococci, we examined bacteria by slide agglutination. Even at concentrations as high as 250 μg/ml, rCRP was not able to aggregate strains D39, JY182, TRE108, and a nonencapsulated mutant of D39, AM1000 (53) (data not shown). We also failed to see evidence of CRP-induced aggregation in our flow cytometry studies with D39, JY182, and TRE108. These findings are consistent with the fact that all PC binding sites of CRP are on the same face of the CRP pentamer (54, 55).

Complement deposition assay

Pneumococci at 1×10^9 CFU/ml in GVB were prepared as described earlier. Fresh-frozen NMS (complement) from CBA/N mice or CRPKO mice was quick thawed and added to the bacterial culture at a final concentration of 10%, incubated for 30 min at 37°C, and washed twice. Fifty microliters of FITC-conjugated goat IgG Ab to C3 (1:100) was added to the washed cells and incubated at 37°C for 30 min. The bacteria were subsequently washed and fixed with 500 μl 2% PFA for flow cytometric analysis using 40,000 gated events. Most of the assays had three controls: 1) bacteria incubated with GVB, 2) bacteria incubated with FITC conjugated to secondary Ab, and 3) bacteria incubated with complement alone. In all cases, control values were at or near background levels and <5% of maximum MFI. For assays using pneumococcal eluates or rPspA to inhibit C3 deposition, comparable amounts of Rx1 or WG44.1 eluate were added to 200 μl bacteria, incubated for 1 h at 37°C, and the bacteria were washed and fixed in 2% PFA before the addition of the complement source. Flow cytometric analysis was carried out for 40,000 gated events.

Statistics

All experiments were conducted two or more times under the same conditions, and in some cases, representative experiments are shown. In most experiments, data points were obtained from duplicates or triplicates, and error bars show SE or the range of the duplicates. Because duplicates invariably had very similar results, additional data points were not examined and no further attempt was made to statistically analyze those data. In the study where the PspA-containing eluate was used to inhibit C3 binding, three studies were done at singlicate at two different levels of inhibitor with similar results at each dose of inhibitor in each study. In that study, the results within each inhibitor in each experiment were ranked. Because both doses of inhibitor gave similar results, all six ranked data points for each inhibitor were analyzed together. The ranked data from the three experiments were analyzed in a single two-tailed Mann–Whitney two-sample rank test as is indicated in the text describing this experiment. Unpaired *t* tests were used for some data, as indicated in the relevant figure legends.

Results

There is more PC exposed on the surface of $PspA^-$ than on WT pneumococci

Our hypothesis was that PspA bound to PC blocks CRP binding to PC. If this was the case, then there should be more PC exposed on $PspA^-$ pneumococci than on $PspA^+$ pneumococci. To test this, we evaluated PC expression on WT strain D39 and its *pspA* and *pspC* mutants. The *pspA* mutant of D39 had ~10-fold more exposure of PC as compared with WT. The absence of PspC also increased the exposure of PC, but it was only a 2-fold change (Fig. 1A, D39). Similar results were seen when we compared PC exposure on the *pspA* mutant WG44.1 (11) with that of its parent strain Rx1 (42), a nonencapsulated variant of D39 (data not shown). We also looked at three additional strains of encapsulated pneumococci with isogenic mutations in *pspA* (Fig. 1A, 6B, 6A, and 23F). In the case of the capsular type 6B and 6A strains, an absence of PspA resulted in 7.5- and 10.5-fold increased exposure of PC, respectively, compared with their respective WT strains. When the PspA deficiency on the 6B mutant strain was restored by replacing the original *pspA* gene, the PC exposure was reduced back to WT levels (Fig. 1A, 6B strain, Fig. 1B). This observation confirmed that the increase in PC exposure on the *pspA* mutant of the type 6B strain was due to the lack of PspA expression and not the result of an unintended genetic event. In the case of the 23F strain, the PspA mutation caused only a modest 1.36-fold increase in PC exposure in the *pspA* mutant (Fig. 1A, 23F). The reason for this much smaller effect of the PspA mutant on the type 23F strain is unknown but may indicate that PspA covered up by a much smaller fraction of the PC on that particular strain than it had been on the three other serotypes we tested.

Because NHS and NMS contain Abs to PC (47, 52, 56), it was also expected that the *pspA* mutants incubated with NHS or NMS would bind more human and mouse Ig than would WT strains. This expectation was confirmed for both NHS and BALB/c NMS for three *pspA* mutant strains (Supplemental Fig. 1). Human IgG to PC purified from pooled NHS (52) was observed to bind 2-fold better in the absence of PspA to 6B pneumococci. This increase in binding was lost in the revertant (Fig. 1C). These combined data with natural Ab to PC support the hypothesis that the absence of PspA exposes PC. The smaller effect seen with NHS versus mAb

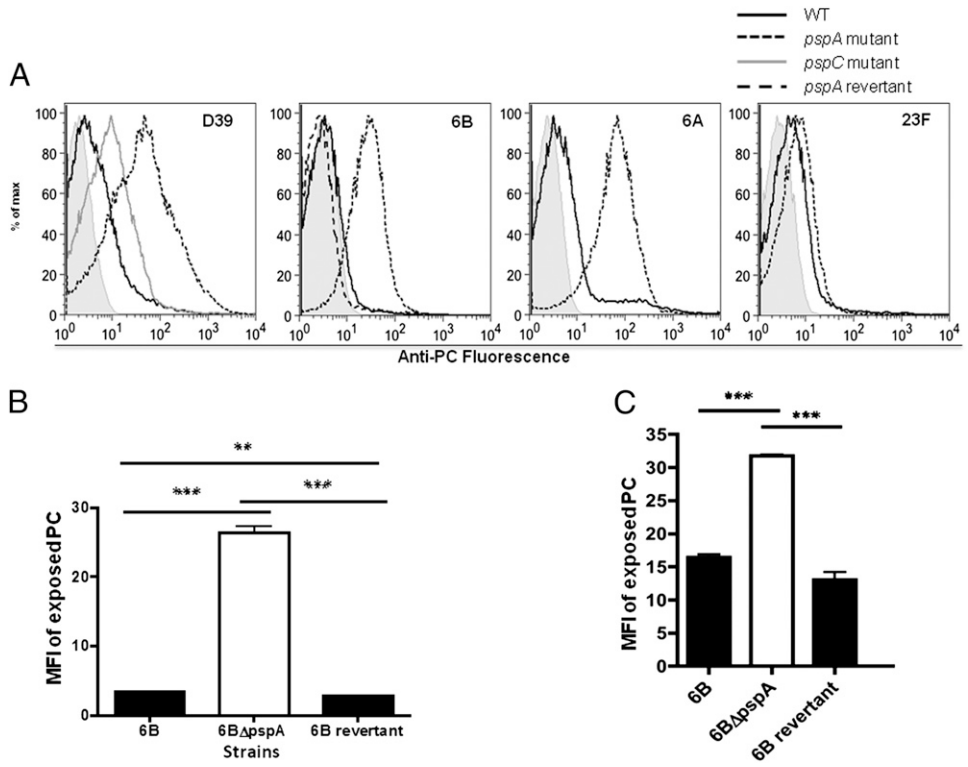

FIGURE 1. Effect of PspA on PC surface exposure. (**A**) Representative flow histograms of TEPC 15 IgA mAb binding to PC on four WT strains of pneumococci of capsular types 2 (D39), 6B, 6A, and 23F, together with their PspA mutants. Also shown is the effect on PC exposure of a *pspC* mutant of strain D39 and a *pspA* revertant of the type 6B strain. Data in the vertical scale show the events (bacteria detected) for each channel of each sample normalized (% of maximum events) based on the highest number of events in any channel examined for that sample. Fluorescence intensity is shown on the horizontal scale. Data shown as the gray peaks represent the background fluorescence of samples incubated without the primary Ab to PC, but with the secondary fluorescein-conjugated Ab. (**B**) MFI of binding of TEPC 15 mAb to PC to a 6B strain, its *pspA* mutant (6BΔ*pspA*), and a revertant re-expressing PspA. (**C**) The MFI for the 6B strain, its Δ*pspA* mutant, and revertant incubated with human IgG Ab to PC that had been purified from pooled γ-globulin. $^{**}p < 0.01$, $^{***}p < 0.001$, unpaired *t* test.

to PC probably reflects the fact that NHS invariably contains Ab to PspA (57), which would bind more strongly to WT than mutant pneumococci.

There is greater binding of CRP to the surface of the pspA mutant strain than to its WT parent

CRP is known to efficiently deposit C3 on encapsulated pneumococci (32, 34), so it was likely that CRP might contribute to increased complement deposition on PspA$^-$-deficient pneumococci. The WT D39 strain was examined along with its *pspA* mutant (JY182) and *pspC* mutant (TRE108) to determine the relative effects of PspA and PspC on the binding of CRP. As predicted, the *pspA* mutant showed an increase of 2.28-fold in CRP binding as compared with the WT strain (Fig. 2). A measurable increase in CRP binding to the *pspC* mutant was not observed, consistent with PspC's smaller effect on PC exposure (Fig. 2).

The CRP present in normal serum is sufficient for C3 deposition on WT pneumococci and pspA mutant pneumococci

CRP is known to efficiently deposit C3 on encapsulated pneumococci (32, 34), so it was likely that increased CRP binding because of the absence of PspA might contribute to increased complement deposition on PspA-deficient pneumococci. Despite low levels of CRP in NMS, we suspected that there would be enough CRP to support the classical pathway-dependent C3 deposition that has been observed on WT and PspA-deficient pneumococci in NMS (26, 27). In the presence of WT NMS, we observed much stronger C3 deposition than in the presence of NMS from CRPKO mice (Fig. 3A). Moreover, in the presence of normal serum, there was more complement deposition on PspA$^-$ versus PspA$^+$ D39 pneumococci. However, when CRPKO serum was the complement source, the amount of C3 deposited was essentially the same as when we incubated the bacteria without complement (Fig. 3A). These data confirmed that CRP was necessary for the deposition of C3 on pneumococci.

The deposition of C3 onto the surface of PspA$^-$ pneumococci is CRP dose dependent

The interpretation that CRPKO NMS failed to support complement deposition because of its lack of CRP (Fig. 3A) depends, in part, on the assumption that the CRPKO NMS contained sufficient C3 to cause detectable deposition onto pneumococci if CRP had been present. To test this, we incubated JY182 (PspA$^-$) with NMS from CRPKO mice at 37°C, with and without the addition of human rCRP. C3 binding to the surface of JY182 was measured by flow cytometry. As a negative control, pneumococci received neither CRP nor CRPKO NMS. Compared with pneumococci incubated with CRPKO sera, there was a 1.5-, 2.2-, and 2.8-fold increase in C3 binding after addition of 50, 100, and 150 μg/ml CRP, respectively (Fig. 3B). Thus, these data make it clear that the very low C3 deposition in the presence of CRPKO NMS is due to a lack of CRP and not due to inadequate complement levels.

A pneumococcal eluate containing FL-PspA inhibited CRP binding to the surface of JY182

The earlier data are consistent with the premise that PspA on pneumococci reduces PC exposure leading to impaired binding by CRP and the deposition of C3. Even though independent *pspA*

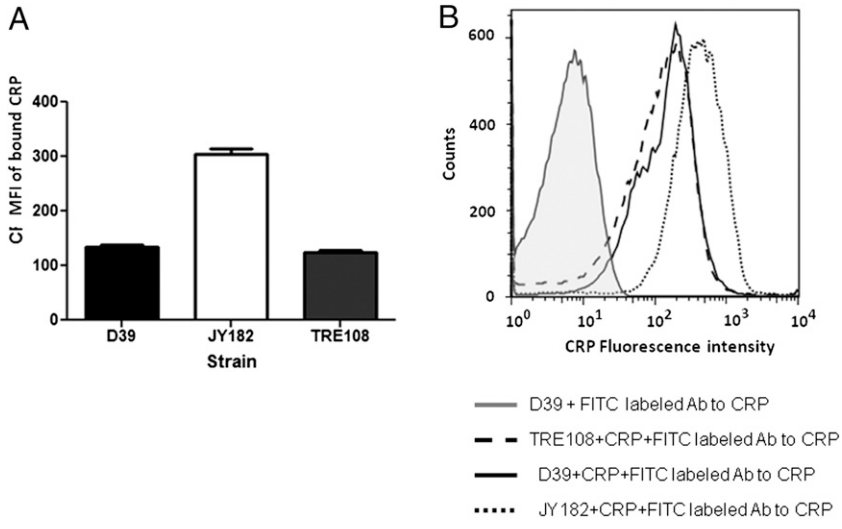

FIGURE 2. Relative effects of *pspA* and *pspC* mutations on CRP binding. WT (D39), PspA$^-$ mutant (JY182), and PspC$^-$ mutant (TRE108) strains were examined for their ability to bind CRP in the presence of 100 μg/ml human rCRP using flow cytometry as described. (**A**) The average MFI of anti-CRP is shown in the bar plots. Error bars represent the range of duplicate samples. Bacteria that were not exposed to added CRP showed <10 on the MFI scale (not shown). (**B**) Flow cytometry histogram of the number of events at different fluorescent intensities is shown for D39, JY182, and TRE108. This experiment was conducted twice with duplicates. A representative experiment is shown. Gray peak represents D39 in the absence of CRP, but with FITC-labeled Ab to CRP.

mutants were used in some of the experiments and all mutants are well characterized, the possibility exists that differences between the WT and mutant strains may have been caused by unknown changes in non-*pspA* genes. To independently determine whether PspA can inhibit CRP deposition and C3 deposition, we added FL-PspA to JY182 before the addition of CRP or NMS.

FL-PspAs, in contrast with rPspA fragments that lack the choline-binding domain, are quite unstable and cannot be isolated and concentrated without extensive denaturation and loss. However, FL-PspA can be recovered in 2% choline-chloride eluates of WT pneumococci (37). A cell eluate containing FL-PspA was isolated in this manner from the nonencapsulated strain Rx1 that had been grown in a chemically defined medium. A control lysate was prepared from WG44.1, a *pspA* null mutant of strain Rx1. Rx1 and WG44.1 cell eluates were incubated separately at 5 and 10 μg/ml total eluate protein with JY182 (PspA$^-$), washed to remove unbound FL-PspA, incubated with CRP, and developed to detect CRP binding to the bacteria. The eluates of Rx1 (PspA$^+$) at 5 and 10 μg/ml contained 9.9 and 19.8 ng/ml PspA, respectively, as determined by a PspA-capture assay. This assay detected no PspA (<0.1 ng/ml) in the 5 and 10 μg/ml concentrations of the eluate of WG44.1 (PspA$^-$). The eluate from Rx1, but not WG44.1, inhibited CRP deposition onto JY182 (Fig. 4A). Based on MFI in the presence of 5 and 10 μg/ml total protein from the two eluates, there was 1.13- and 1.37-fold more CRP deposition, respectively, in the presence of the WG44.1 versus the Rx1 extract. Because the only difference between the two eluates was the presence of PspA, the results indicate that FL-PspA can inhibit CRP deposition on JY182.

A FL-PspA–containing pneumococcal eluate inhibited C3 deposition onto the surface of JY182

The FL-PspA–containing eluate of Rx1 and the control eluate from WG44.1 lacking FL-PspA were added to JY182 before the addition of NMS to measure its blocking effect on C3 deposition (Fig. 4B). The PspA-containing Rx1 cell eluate reduced C3 deposition compared with the WG44.1 eluate. At 20 and 40 μg/ml protein added, there was a 1.32- and 1.28-fold greater deposition of C3 on bacteria treated with WG44.1 cell eluate compared with those treated with Rx1 (Fig. 4B). Although these differences were small, they were significant at $p = 0.002$ (see legend for Fig. 4B). The fact that the effect was not larger with the higher dose may mean that the lower dose had already saturated the binding sites for PspA. These results show that treatment of PspA$^-$ pneumococci with an extract containing FL-PspA was able to inhibit subsequent C3 deposition. Importantly, when the same experiment was repeated using CRPKO NMS as the complement source, there was ~1/15 as much C3 deposited on the surface of JY182. Moreover, in the presence of CRPKO NMS, no significant difference was found

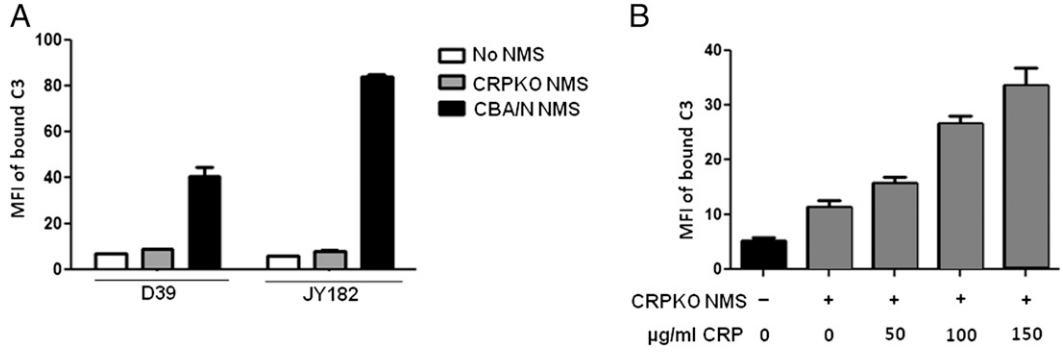

FIGURE 3. Effect of CRP on complement C3 deposition on pneumococci. (**A**) D39 (PspA$^+$) and JY182 (PspA$^-$) were incubated with 10% NMS from either CBA/N or CRPKO mice as the complement source for 30 min at 37°C with shaking. The cells were then incubated with 1:100 FITC-labeled Ab to C3 and fixed for flow cytometric analysis to detect bound C3. (**B**) Effect of exogenously added CRP on C3 deposition on the surface of JY182 (PspA$^-$) using CRPKO NMS as complement source. Ten percent CRPKO NMS was added to all the samples except for the negative control. Human rCRP was added at concentrations of 0, 50, 100, or 150 μg/ml. The negative control had no CRP added. The mixtures were incubated at 37°C for 1 h. C3 was detected by the addition of 1:100 anti-mouse C3 and 1:100 FITC-labeled goat Ab in separate 30-min incubations. Cells were fixed and analyzed by flow cytometry. Both experiments (A, B) were conducted with duplicates only once. Error bars represent the range of duplicate samples.

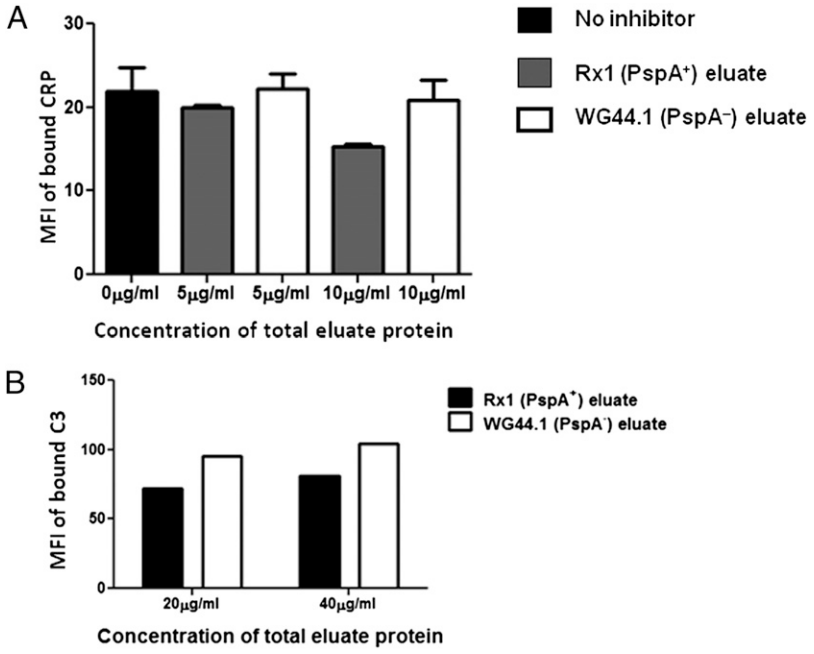

FIGURE 4. Inhibition of CRP and C3 binding with a cell eluate containing FL-PspA. Cell eluate from strains Rx1 (PspA$^+$) and WG44.1 (PspA$^-$) were incubated with JY182 (PspA$^-$) for 1 h at 37°C with shaking before the addition of CRP or C3. The amounts of FL-PspA in 5, 10, 20, and 40 μg/ml Rx1 eluate were 9.9, 19.8, 39.6 and 79.2 ng/ml, respectively. (**A**) To measure CRP binding, we incubated the cells with 100 μg/ml rCRP and examined them by flow cytometry for CRP deposition. MFI is shown. Experiment was repeated three times with duplicates showing similar results; a representative experiment is shown. Error bars represent the range of duplicate samples. (**B**) To measure C3 binding, we incubated the cells with 10% CBA/N NMS and examined them by flow cytometry for C3 deposition. The assay was preformed three times singly with similar results; a representative experiment is shown. The data for the inhibited and noninhibited samples in each study were ranked, and the combined ranks from the 20 and 40 μg/ml experiments were pooled and evaluated by a Mann–Whitney two-sample rank test. The result indicated that values were lower ($p = 0.0025$) for the samples treated with full-length PspA–containing eluate than non-PspA–containing eluate.

between the C3 deposited in the presence of the PspA$^+$ and PspA$^-$ eluates (data not shown). These results confirm a role for CRP in complement activation and deposition on the surface of PspA$^-$ pneumococci. Furthermore, CRP-dependent activity could be inhibited by the addition of PspA expressing an intact choline-binding domain.

To test the hypothesis that the choline-binding domain was essential to inhibit the deposition of CRP and C3, we repeated the inhibition studies using rPspA, UAB055 (51), that contains the N-terminal α-helical region of PspA and 14 aa of the proline-rich domain, but completely lacks PspA's choline-binding domain. There was no blocking effect with either 5 or 10 μg/ml rPspA on the binding of CRP to pneumococci or the deposition of C3 on pneumococci (Fig. 5). This lack of inhibition is especially striking because the concentrations of rPspA used were up to 500 times the concentration of FL-PspA used in the prior experiments. This observation is consistent with our hypothesis that because native PspA binds to PC, it is able to inhibit CRP binding and thus indirectly blocks CRP-dependent activation of C3 and deposition of C3b onto the pneumococcal surface.

Discussion

Both the classical and alternative complement pathways are known to be important for host defense against pneumococci (12, 26, 27, 58). Total complement deposition is greatly affected by defects in the alternative pathway because it acts to amplify any complement deposited on the pneumococcus via the classical or lectin pathways. However, the classical pathway appears to be the primary trigger for almost all complement deposition on highly virulent strains of pneumococci. This is true even in the presence of nonimmune sera where complement deposition on pneumococci is dependent on the initial complement activation by the classical pathway (26, 27, 59). PspA is a choline-binding protein of pneumococci that binds to the PC epitopes on teichoic acids (23), and its virulence role of PspA in bacteremia and sepsis has been attributed largely to its ability to interfere with Ab-independent activation of the complement system (12, 27, 60).

CRP, when bound to the PC of teichoic acids, is able to bind C1q and activate complement via the classical pathway (34). CRP can activate complement when bound to *S. pneumoniae* and *Haemophilus influenzae* (61, 62). CRP is present at a few micrograms per

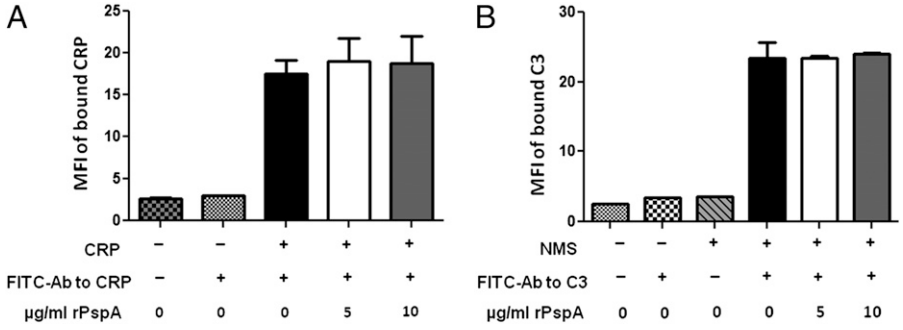

FIGURE 5. Effect of rPspA, lacking the choline-binding domain, on CRP and C3 deposition on the surface of JY182. These studies were carried out and depicted similarly to those in Fig. 4 except that rPspA UAB055, which lacks the choline-binding domain, was used as the inhibitor in place of cell eluates containing native Rx1 PspA. Five and 10 μg/ml rPspA were incubated with JY182 (PspA$^-$) for 1 h at 37°C with shaking before the addition of CRP or C3. (**A**) To measure CRP binding, we incubated the cells with 100 μg/ml rCRP and examined them by flow cytometry for CRP deposition. MFI is shown. Experiment was repeated three times with duplicates showing similar results; a representative experiment is shown. Error bars represent the range of duplicate samples. (**B**) To measure C3 binding, we incubated the cells with 10% CBA/N NMS and examined them by flow cytometry for C3 deposition. Each experiment was performed three times with duplicates with similar results. (A) Average and SE for the six values for each determination. (B) Average and range of duplicates from a representative experiment.

milliliter in all human and mouse sera (29, 31). It can protect mice from pneumococcal infection (35, 36), and this protection requires complement (61). PspA can significantly reduce complement deposition on pneumococci in normal serum, and this additional complement deposition is all C1q dependent (27). In this study, we hypothesized that the ability of PspA to reduce C1q-dependent complement activation (27) by pneumococci in normal serum was by inhibiting the binding of CRP to pneumococci, thus reducing CRP-mediated, C1q-dependent activation of the classical complement pathway (26, 34).

Our prior studies have shown that, in addition to exhibiting greater complement deposition in normal serum, $PspA^-$ pneumococci exhibit greater in vitro phagocytosis and faster clearance in nonimmune mice as compared with $PspA^+$ WT strains (11, 12, 25, 63). In this study, we showed that mutant pneumococci lacking PspA had significantly higher exposure of PC, greater deposition of rCRP, and greater deposition of C3 from NMS than was observed with WT pneumococci that expressed PspA. Moreover, enhanced C3 deposition on the strain lacking PspA was not observed in the presence of nonimmune mouse serum from CRPKO mice. However, when human rCRP was added to CRPKO serum, the deposition of C3 was restored. The role of PspA's choline-binding activity in inhibiting C3 deposition was strengthened by the observation that FL-PspA, but not rPspA lacking a choline binding domain, could inhibit CRP binding and C3 deposition. These results support our hypothesized mechanism of classical pathway inhibition by PspA. The results also indicate that in normal serum, complement activation via the classical pathway can be largely attributed to CRP. The importance of this inhibitory effect by PspA on complement deposition is probably magnified during a pneumococcal infection in human where CRP is an acute-phase protein, which can increase in concentration >500-fold during an infection (30).

Virtually all mice, except for Xid mice, produce Ab to PC in their NMS (47, 64), and these Abs can have a protective effect against in vivo infection (47, 63). However, the fact that so little C3 deposition was observed with the CRPKO sera indicates that it is the CRP, and not the Ab to PC, that accounts for the bulk of the C3 deposition on WT pneumococci in NMS. In fact, in Fig. 3A, we observed much higher C3 deposition using CBA/N NMS, which lack Ab to PC but have CRP, than we did with CRPKO NMS, which lacks CRP but should contain IgM Ab to PC.

The absence of another choline-binding protein, PspC, resulted in only a small exposure of PC, did not increase CRP binding (this study), and does not increase classical pathway complement deposition (27). It is likely that the reason PspA, but not PspC, significantly inhibited CRP binding was because PspA is present in much higher concentrations on the pneumococcal surface than PspC, and is thus able to cover much more PC. Although strict comparisons have not been made between the numbers of molecules of PspA and PspC expressed, it is noted that the PspC (CbpA) protein, and other choline-binding proteins, could not be originally detected in pneumococcal surface extracts without using pneumococci with a *pspA* mutation to remove the coisolated PspA from the extracts (22). It was also noted in early studies where PspA was isolated by elution with choline chloride from the pneumococcal surface that the major product was PspA (23, 37).

The observation that PspA's effect on complement deposition resided in its choline-binding domain is in contrast with the observation that all known protective mAb to PspA are directed against the α-helical or proline-rich domains of PspA (50, 65–67). Thus, the protective effects of Ab to PspA are not because they interfere with PspA's ability to inhibit complement. Rather, the ability of Ab to PspA to enhance complement deposition on pneumococci and be protective (59, 68) probably depends on its ability to bind C1q and activate the classical pathway in the same manner as Abs to other Ags.

The α-helical and proline-rich regions of PspA are highly exposed to Ab at the pneumococcal surface (66, 69) where they can be targeted by protective Ab (50, 65–67). To evolutionarily justify the surface exposure of PspA, there must be a function mediated by the exposed portions of the molecule. The α-helical end of PspA blocks the bactericidal activity of cationic bactericidal peptides released by the degradation of apolactoferrin by binding strongly to the bactericidal peptides (21, 70, 71). PspA may block the bactericidal function of cationic peptides in general because it has been observed to bind and inhibit killing by other bactericidal peptides (S. Mirza, B. Hatcher, and D. Briles, unpublished observations).

Like CRP, serum amyloid P (SAP) is another PC-binding pentraxin that has been shown to opsonize pneumococci with C3b via the classical pathway and reduce infection in vivo in mice (33). SAP, however, has a different fine specificity from CRP in that it binds better to phosphorylethanolamine (72, 73), which is not normally expressed on pneumococci (74). Few direct in vivo comparisons between the two pentraxins have been conducted, but in early studies performed in mice, SAP was less protective against pneumococcal infection than CRP (36). Even so, it is anticipated that SAP and CRP each contribute to complement deposition on pneumococci and subsequent bacterial clearance.

Finally, many mucosal pathogens, including *S. pneumoniae*, *H. influenzae*, *Pseudomonas aeruginosa*, *Neisseria meningitidis* (62, 75), *Salmonella enterica* Typhimurium (76), *Proteus morganii* (77), and the nonmucosal parasitic worms *Brugia malayi* (78) express PC on their surface. In most cases, the PC can be detected with the same T15 idiotype Abs to PC that are optimally protective against pneumococci (79). The expression of PC may aid these pathogens in the mucosal environment by facilitating adherence and colonization (62, 80). The frequent expression of PC by such pathogens may explain, in part, why mammalian hosts have evolved choline-binding triggers of innate immunity such as CRP and SAP. Mice and men also make strong (in the case of mice, germline) (81) anti-PC Ab responses. Both mouse (79) and human (52, 63) Abs to PC can protect against pneumococcal infection. Not only have pneumococci developed a means to mask their PC with PspA (this study), they (43) and some Gram-negative mucosal pathogens (62, 75) modify PC exposure by phase variation. In pneumococci, the invariant PC epitope has been masked by PspA, which, unlike PC, is not detected by the innate immune system and is antigenically variable.

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References

1. Gray, B. M., and D. M. Musher. 2008. The history of pneumococcal disease. In *Pneumococcal Vaccines.* G. R. Seiber, K. P. Klugman, and P. H. Makela, eds. ASM Press, Washington, DC, p. 3–18.

2. Fedson, D. S., and D. M. Musher. 2004. Pneumococcal polysaccharide vaccine. In *Vaccines.* S. A. Plotkin and W. A. Orenstein, eds. W.B. Saunders, Philadelphia, p. 529–588.
3. Bryce, J., C. Boschi-Pinto, K. Shibuya, and R. E. Black; WHO Child Health Epidemiology Reference Group. 2005. WHO estimates of the causes of death in children. *Lancet* 365: 1147–1152.
4. Frank, M. M., and J. P. Atkinson. 2001. Complement system. In *Samter's Immunologic Diseasse.* K. F. Austin, M. M. Frank, J. P. Atkinson, and H. Cantor, eds. Lippincott Williams & Wilkins, New York, p. 282–298.
5. Joiner, K., E. Brown, C. Hammer, K. Warren, and M. Frank. 1983. Studies on the mechanism of bacterial resistance to complement-mediated killing. III. C5b-9 deposits stably on rough and type 7 *S. pneumoniae* without causing bacterial killing. *J. Immunol.* 130: 845–849.
6. Hyams, C., E. Camberlein, J. M. Cohen, K. Bax, and J. S. Brown. 2010. The *Streptococcus pneumoniae* capsule inhibits complement activity and neutrophil phagocytosis by multiple mechanisms. *Infect. Immun.* 78: 704–715.
7. Yuste, J., S. Ali, S. Sriskandan, C. Hyams, M. Botto, and J. S. Brown. 2006. Roles of the alternative complement pathway and C1q during innate immunity to *Streptococcus pyogenes. J. Immunol.* 176: 6112–6120.
8. Jarva, H., T. S. Jokiranta, R. Würzner, and S. Meri. 2003. Complement resistance mechanisms of streptococci. *Mol. Immunol.* 40: 95–107.
9. Briles, D. E., S. K. Hollingshead, J. King, A. Swift, P. A. Braun, M. K. Park, L. M. Ferguson, M. H. Nahm, and G. S. Nabors. 2000. Immunization of humans with rPspA elicits antibodies, which passively protect mice from fatal infection with *Streptococcus pneumoniae* bearing heterologous PspA. *J. Infect. Dis.* 182: 1694–1701.
10. Croney, C. M., M. T. Coats, M. H. Nahm, D. E. Briles, and M. J. Crain. 2012. PspA family distribution, unlike capsular serotype, remains unaltered following introduction of the heptavalent pneumococcal conjugate vaccine. *Clin. Vaccine Immunol.* 19: 891–896.
11. McDaniel, L. S., J. Yother, M. Vijayakumar, L. McGarry, W. R. Guild, and D. E. Briles. 1987. Use of insertional inactivation to facilitate studies of biological properties of pneumococcal surface protein A (PspA). *J. Exp. Med.* 165: 381–394.
12. Ren, B., A. J. Szalai, O. Thomas, S. K. Hollingshead, and D. E. Briles. 2003. Both family 1 and family 2 PspA proteins can inhibit complement deposition and confer virulence to a capsular serotype 3 strain of Streptococcus pneumoniae. *Infect. Immun.* 71: 75–85.
13. Hollingshead, S. K., L. Baril, S. Ferro, J. King, P. Coan, and D. E. Briles; Pneumococcal Proteins Epi Study Group. 2006. Pneumococcal surface protein A (PspA) family distribution among clinical isolates from adults over 50 years of age collected in seven countries. *J. Med. Microbiol.* 55: 215–221.
14. Melin, M. M., S. K. Hollingshead, D. E. Briles, W. P. Hanage, M. Lahdenkari, T. Kaijalainen, T. M. Kilpi, and H. M. Käyhty. 2008. Distribution of pneumococcal surface protein A families 1 and 2 among *Streptococcus pneumoniae* isolates from children in Finland who had acute otitis media or were nasopharyngeal carriers. *Clin. Vaccine Immunol.* 15: 1555–1563.
15. Vela Coral, M. C., N. Fonseca, E. Castañeda, J. L. Di Fabio, S. K. Hollingshead, and D. E. Briles. 2001. Pneumococcal surface protein A of invasive Streptococcus pneumoniae isolates from Colombian children. *Emerg. Infect. Dis.* 7: 832–836.
16. Nabors, G. S., P. A. Braun, D. J. Herrmann, M. L. Heise, D. J. Pyle, S. Gravenstein, M. Schilling, L. M. Ferguson, S. K. Hollingshead, D. E. Briles, and R. S. Becker. 2000. Immunization of healthy adults with a single recombinant pneumococcal surface protein A (PspA) variant stimulates broadly cross-reactive antibodies to heterologous PspA molecules. *Vaccine* 18: 1743–1754.
17. Briles, D. E., S. K. Hollingshead, G. S. Nabors, J. C. Paton, and A. Brooks-Walter. 2000. The potential for using protein vaccines to protect against otitis media caused by *Streptococcus pneumoniae. Vaccine* 19(Suppl. 1): S87–S95.
18. Roche, H., B. Ren, L. S. McDaniel, A. Håkansson, and D. E. Briles. 2003. Relative roles of genetic background and variation in PspA in the ability of antibodies to PspA to protect against capsular type 3 and 4 strains of *Streptococcus pneumoniae. Infect. Immun.* 71: 4498–4505.
19. Yother, J., and D. E. Briles. 1992. Structural properties and evolutionary relationships of PspA, a surface protein of *Streptococcus pneumoniae*, as revealed by sequence analysis. *J. Bacteriol.* 174: 601–609.
20. McDaniel, L. S., D. O. McDaniel, S. K. Hollingshead, and D. E. Briles. 1998. Comparison of the PspA sequence from *Streptococcus pneumoniae* EF5668 to the previously identified PspA sequence from strain Rx1 and ability of PspA from EF5668 to elicit protection against pneumococci of different capsular types. *Infect. Immun.* 66: 4748–4754.
21. Senkovich, O., W. J. Cook, S. Mirza, S. K. Hollingshead, I. I. Protasevich, D. E. Briles, and D. Chattopadhyay. 2007. Structure of a complex of human lactoferrin N-lobe with pneumococcal surface protein a provides insight into microbial defense mechanism. *J. Mol. Biol.* 370: 701–713.
22. Rosenow, C., P. Ryan, J. N. Weiser, S. Johnson, P. Fontan, A. Ortqvist, and H. R. Masure. 1997. Contribution of novel choline-binding proteins to adherence, colonization and immunogenicity of *Streptococcus pneumoniae. Mol. Microbiol.* 25: 819–829.
23. Yother, J., and J. M. White. 1994. Novel surface attachment mechanism of the Streptococcus pneumoniae protein PspA. *J. Bacteriol.* 176: 2976–2985.
24. Yother, J., G. L. Handsome, and D. E. Briles. 1992. Truncated forms of PspA that are secreted from *Streptococcus pneumoniae* and their use in functional studies and cloning of the *pspA* gene. *J. Bacteriol.* 174: 610–618.
25. Ren, B., J. Li, K. Genschmer, S. K. Hollingshead, and D. E. Briles. 2012. The absence of PspA or presence of antibody to PspA facilitates the complement-dependent phagocytosis of pneumococci in vitro. *Clin. Vaccine Immunol.* 19: 1574–1582.
26. Brown, J. S., T. Hussell, S. M. Gilliland, D. W. Holden, J. C. Paton, M. R. Ehrenstein, M. J. Walport, and M. Botto. 2002. The classical pathway is the dominant complement pathway required for innate immunity to *Streptococcus pneumoniae* infection in mice. *Proc. Natl. Acad. Sci. USA* 99: 16969–16974.
27. Li, J., D. T. Glover, A. J. Szalai, S. K. Hollingshead, and D. E. Briles. 2007. PspA and PspC minimize immune adherence and transfer of pneumococci from erythrocytes to macrophages through their effects on complement activation. *Infect. Immun.* 75: 5877–5885.
28. Briles, D. E., R. C. Tart, E. Swiatlo, J. P. Dillard, P. Smith, K. A. Benton, B. A. Ralph, A. Brooks-Walter, M. J. Crain, S. K. Hollingshead, and L. S. McDaniel. 1998. Pneumococcal diversity: considerations for new vaccine strategies with emphasis on pneumococcal surface protein A (PspA). *Clin. Microbiol. Rev.* 11: 645–657.
29. Claus, D. R., A. P. Osmand, and H. Gewurz. 1976. Radioimmunoassay of human C-reactive protein and levels in normal sera. *J. Lab. Clin. Med.* 87: 120–128.
30. Szalai, A. J., A. Agrawal, T. J. Greenhough, and J. E. Volanakis. 1999. C-reactive protein: structural biology and host defense function. *Clin. Chem. Lab. Med.* 37: 265–270.
31. Whitehead, A. S., K. Zahedi, M. Rits, R. F. Mortensen, and J. M. Lelias. 1990. Mouse C-reactive protein. Generation of cDNA clones, structural analysis, and induction of mRNA during inflammation. *Biochem. J.* 266: 283–290.
32. Volanakis, J. E., and M. H. Kaplan. 1971. Specificity of C-reactive protein for choline phosphate residues of pneumococcal C-polysaccharide. *Proc. Soc. Exp. Biol. Med.* 136: 612–614.
33. Yuste, J., M. Botto, S. E. Bottoms, and J. S. Brown. 2007. Serum amyloid P aids complement-mediated immunity to *Streptococcus pneumoniae. PLoS Pathog.* 3: 1208–1219.
34. Volanakis, J. E., and M. H. Kaplan. 1974. Interaction of C-reactive protein complexes with the complement system. II. Consumption of guinea pig complement by CRP complexes: requirement for human C1q. *J. Immunol.* 113: 9–17.
35. Mold, C., S. Nakayama, T. J. Holzer, H. Gewurz, and T. W. Du Clos. 1981. C-reactive protein is protective against *Streptococcus pneumoniae* infection in mice. *J. Exp. Med.* 154: 1703–1708.
36. Yother, J., J. E. Volanakis, and D. E. Briles. 1982. Human C-reactive protein is protective against fatal *Streptococcus pneumoniae* infection in mice. *J. Immunol.* 128: 2374–2376.
37. Briles, D. E., J. D. King, M. A. Gray, L. S. McDaniel, E. Swiatlo, and K. A. Benton. 1996. PspA, a protection-eliciting pneumococcal protein: immunogenicity of isolated native PspA in mice. *Vaccine* 14: 858–867.
38. Avery, O. T., C. M. Macleod, and M. McCarty. 1944. Studies on the chemical nature of the substance inducing transformation of pneumococcal types. Induction of transformation by a desoxyribonucleic acid fraction isolated from pneumococcus type III. *J. Exp. Med.* 79: 137–158.
39. Balachandran, P., A. Brooks-Walter, A. Virolainen-Julkunen, S. K. Hollingshead, and D. E. Briles. 2002. Role of pneumococcal surface protein C in nasopharyngeal carriage and pneumonia and its ability to elicit protection against carriage of Streptococcus pneumoniae. *Infect. Immun.* 70: 2526–2534.
40. Hollingshead, S. K., R. S. Becker, and D. E. Briles. 2000. Diversity of PspA: mosaic genes and evidence for past recombination in *Streptococcus pneumoniae. Infect. Immun.* 68: 5889–5900.
41. Yu, J., J. Lin, K. H. Kim, W. H. Benjamin, Jr., and M. H. Nahm. 2011. Development of an automated and multiplexed serotyping assay for *Streptococcus pneumoniae. Clin. Vaccine Immunol.* 18: 1900–1907.
42. Shoemaker, N. B., and W. R. Guild. 1974. Destruction of low efficiency markers is a slow process occurring at a heteroduplex stage of transformation. *Mol. Gen. Genet.* 128: 283–290.
43. Kim, J. O., and J. N. Weiser. 1998. Association of intrastrain phase variation in quantity of capsular polysaccharide and teichoic acid with the virulence of *Streptococcus pneumoniae. J. Infect. Dis.* 177: 368–377.
44. Roche, A. M., S. J. King, and J. N. Weiser. 2007. Live attenuated *Streptococcus pneumoniae* strains induce serotype-independent mucosal and systemic protection in mice. *Infect. Immun.* 75: 2469–2475.
45. McCool, T. L., and J. N. Weiser. 2004. Limited role of antibody in clearance of *Streptococcus pneumoniae* in a murine model of colonization. *Infect. Immun.* 72: 5807–5813.
46. Darrieux, M., A. T. Moreno, D. M. Ferreira, F. C. Pimenta, A. L. de Andrade, A. P. Lopes, L. C. Leite, and E. N. Miyaji. 2008. Recognition of pneumococcal isolates by antisera raised against PspA fragments from different clades. *J. Med. Microbiol.* 57: 273–278.
47. Briles, D. E., M. Nahm, K. Schroer, J. Davie, P. Baker, J. Kearney, and R. Barletta. 1981. Antiphosphocholine antibodies found in normal mouse serum are protective against intravenous infection with type 3 *streptococcus pneumoniae. J. Exp. Med.* 153: 694–705.
48. Claflin, J. L., and M. Cubberley. 1980. Clonal nature of the immune response to phosphocholine. VII. Evidence throughout inbred mice for molecular similarities among antibodies bearing the T15 idiotype. *J. Immunol.* 125: 551–558.
49. Jones, N. R., M. A. Pegues, M. A. McCrory, S. W. Kerr, H. Jiang, R. Sellati, V. Berger, J. Villalona, R. Parikh, M. McFarland, et al. 2011. Collagen-induced arthritis is exacerbated in C-reactive protein-deficient mice. *Arthritis Rheum.* 63: 2641–2650.
50. McDaniel, L. S., B. A. Ralph, D. O. McDaniel, and D. E. Briles. 1994. Localization of protection-eliciting epitopes on PspA of *Streptococcus pneumoniae* between amino acid residues 192 and 260. *Microb. Pathog.* 17: 323–337.

51. Brooks-Walter, A., D. E. Briles, and S. K. Hollingshead. 1999. The *pspC* gene of *Streptococcus pneumoniae* encodes a polymorphic protein, PspC, which elicits cross-reactive antibodies to PspA and provides immunity to pneumococcal bacteremia. *Infect. Immun.* 67: 6533–6542.
52. Goldenberg, H. B., T. L. McCool, and J. N. Weiser. 2004. Cross-reactivity of human immunoglobulin G2 recognizing phosphorylcholine and evidence for protection against major bacterial pathogens of the human respiratory tract. *J. Infect. Dis.* 190: 1254–1263.
53. Magee, A. D., and J. Yother. 2001. Requirement for capsule in colonization by *Streptococcus pneumoniae*. *Infect. Immun.* 69: 3755–3761.
54. Roux, K. H., J. M. Kilpatrick, J. E. Volanakis, and J. F. Kearney. 1983. Localization of the phosphocholine-binding sites on C-reactive protein by immunoelectron microscopy. *J. Immunol.* 131: 2411–2415.
55. Du Clos, T. W., and C. Mold. Pentraxins (CRP, SAP) in the process of complement activation and clearance of apoptotic bodies through Fcγ receptors. *Curr. Opin. Organ Transplant.* In press.
56. Briles, D. E., G. Scott, B. Gray, M. J. Crain, M. Blaese, M. Nahm, V. Scott, and P. Haber. 1987. Naturally occurring antibodies to phosphocholine as a potential index of antibody responsiveness to polysaccharides. *J. Infect. Dis.* 155: 1307–1314.
57. Virolainen, A., W. Russell, M. J. Crain, S. Rapola, H. Käyhty, and D. E. Briles. 2000. Human antibodies to pneumococcal surface protein A in health and disease. *Pediatr. Infect. Dis. J.* 19: 134–138.
58. Ren, B., M. A. McCrory, C. Pass, D. C. Bullard, C. M. Ballantyne, Y. Xu, D. E. Briles, and A. J. Szalai. 2004. The virulence function of *Streptococcus pneumoniae* surface protein A involves inhibition of complement activation and impairment of complement receptor-mediated protection. *J. Immunol.* 173: 7506–7512.
59. Ren, B., A. J. Szalai, S. K. Hollingshead, and D. E. Briles. 2004. Effects of PspA and antibodies to PspA on activation and deposition of complement on the pneumococcal surface. *Infect. Immun.* 72: 114–122.
60. Tu, T., R. L. Fulgham, M. A. McCrory, D. E. Briles, and A. J. Szalai. 1999. Pneumococcal surface protein A inhibits complement activation by Streptococcus pneumoniae. *Infect. Immun.* 67: 4720–4724.
61. Szalai, A. J., D. E. Briles, and J. E. Volanakis. 1996. Role of complement in C-reactive-protein-mediated protection of mice from Streptococcus pneumoniae. *Infect. Immun.* 64: 4850–4853.
62. Weiser, J. N., N. Pan, K. L. McGowan, D. Musher, A. Martin, and J. Richards. 1998. Phosphorylcholine on the lipopolysaccharide of *Haemophilus influenzae* contributes to persistence in the respiratory tract and sensitivity to serum killing mediated by C-reactive protein. *J. Exp. Med.* 187: 631–640.
63. Briles, D. E., C. Forman, J. C. Horowitz, J. E. Volanakis, W. H. Benjamin, Jr., L. S. McDaniel, J. Eldridge, and J. Brooks. 1989. Antipneumococcal effects of C-reactive protein and monoclonal antibodies to pneumococcal cell wall and capsular antigens. *Infect. Immun.* 57: 1457–1464.
64. Lieberman, R. M., M. Potter, E. B. Mushinski, W. Humphrey, Jr., and S. Rudikoff. 1974. Genetics of a new IgVH (T15 idiotype) marker in the mouse regulating natural antibody to phosphorylcholine. *J. Exp. Med.* 139: 983–1001.
65. Roche, H., A. Håkansson, S. K. Hollingshead, and D. E. Briles. 2003. Regions of PspA/EF3296 best able to elicit protection against *Streptococcus pneumoniae* in a murine infection model. *Infect. Immun.* 71: 1033–1041.
66. Daniels, C. C., P. Coan, J. King, J. Hale, K. A. Benton, D. E. Briles, and S. K. Hollingshead. 2010. The proline-rich region of pneumococcal surface proteins A and C contains surface-accessible epitopes common to all pneumococci and elicits antibody-mediated protection against sepsis. *Infect. Immun.* 78: 2163–2172.
67. Darrieux, M., E. N. Miyaji, D. M. Ferreira, L. M. Lopes, A. P. Lopes, B. Ren, D. E. Briles, S. K. Hollingshead, and L. C. Leite. 2007. Fusion proteins containing family 1 and family 2 PspA fragments elicit protection against Streptococcus pneumoniae that correlates with antibody-mediated enhancement of complement deposition. *Infect. Immun.* 75: 5930–5938.
68. Ochs, M. M., W. Bartlett, D. E. Briles, B. Hicks, A. Jurkuvenas, P. Lau, B. Ren, and A. Millar. 2008. Vaccine-induced human antibodies to PspA augment complement C3 deposition on *Streptococcus pneumoniae*. *Microb. Pathog.* 44: 204–214.
69. Daniels, C. C., T. C. Briles, S. Mirza, A. P. Håkansson, and D. E. Briles. 2006. Capsule does not block antibody binding to PspA, a surface virulence protein of *Streptococcus pneumoniae*. *Microb. Pathog.* 40: 228–233.
70. Mirza, S., L. Wilson, W. H. Benjamin, Jr., J. Novak, S. Barnes, S. K. Hollingshead, and D. E. Briles. 2011. Serine protease PrtA from *Streptococcus pneumoniae* plays a role in the killing of *S. pneumoniae* by apolactoferrin. *Infect. Immun.* 79: 2440–2450.
71. Shaper, M., S. K. Hollingshead, W. H. Benjamin, Jr., and D. E. Briles. 2004. PspA protects Streptococcus pneumoniae from killing by apolactoferrin, and antibody to PspA enhances killing of pneumococci by apolactoferrin [corrected]. *Infect. Immun.* 72: 5031–5040.
72. Omtvedt, L. A., T. N. Wien, T. Myran, K. Sletten, and G. Husby. 2004. Serum amyloid P component in mink, a non-glycosylated protein with affinity for phosphorylethanolamine and phosphorylcholine. *Amyloid* 11: 101–108.
73. Mikolajek, H., S. E. Kolstoe, V. E. Pye, P. Mangione, M. B. Pepys, and S. P. Wood. 2011. Structural basis of ligand specificity in the human pentraxins, C-reactive protein and serum amyloid P component. *J. Mol. Recognit.* 24: 371–377.
74. Tomasz, A. 1968. Biological consequences of the replacement of choline by ethanolamine in the cell wall of Pneumococcus: chanin formation, loss of transformability, and loss of autolysis. *Proc. Natl. Acad. Sci. USA* 59: 86–93.
75. Weiser, J. N., J. B. Goldberg, N. Pan, L. Wilson, and M. Virji. 1998. The phosphorylcholine epitope undergoes phase variation on a 43-kilodalton protein in *Pseudomonas aeruginosa* and on pili of *Neisseria meningitidis* and *Neisseria* gonorrhoeae. *Infect. Immun.* 66: 4263–4267.
76. Allaoui-Attarki, K., S. Pecquet, E. Fattal, S. Trollé, E. Chachaty, P. Couvreur, and A. Andremont. 1997. Protective immunity against *Salmonella typhimurium* elicited in mice by oral vaccination with phosphorylcholine encapsulated in poly (DL-lactide-co-glycolide) microspheres. *Infect. Immun.* 65: 853–857.
77. Claflin, J. L., J. George, C. Dell, and J. Berry. 1989. Patterns of mutations and selection in antibodies to the phosphocholine-specific determinant in *Proteus morganii*. *J. Immunol.* 143: 3054–3063.
78. Lal, R. B., R. S. Paranjape, D. E. Briles, T. B. Nutman, and E. A. Ottesen. 1987. Circulating parasite antigen(s) in lymphatic filariasis: use of monoclonal antibodies to phosphocholine for immunodiagnosis. *J. Immunol.* 138: 3454–3460.
79. Briles, D. E., C. Forman, S. Hudak, and J. L. Claflin. 1982. Anti-phosphorylcholine antibodies of the T15 idiotype are optimally protective against Streptococcus pneumoniae. *J. Exp. Med.* 156: 1177–1185.
80. Cundell, D. R., N. P. Gerard, C. Gerard, I. Idanpaan-Heikkila, and E. I. Tuomanen. 1995. Streptococcus pneumoniae anchor to activated human cells by the receptor for platelet-activating factor. *Nature* 377: 435–438.
81. Perlmutter, R. M., S. T. Crews, R. Douglas, G. Sorensen, N. Johnson, N. Nivera, P. J. Gearhart, and L. Hood. 1984. The generation of diversity in phosphorylcholine-binding antibodies. *Adv. Immunol.* 35: 1–37.