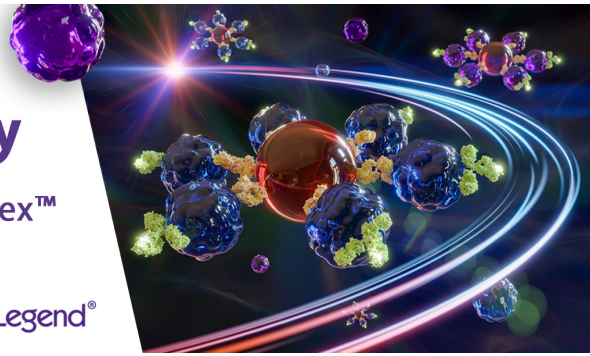


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Additive Inhibition of Complement Deposition by Pneumolysin and PspA Facilitates *Streptococcus pneumoniae* Septicemia¹

Jose Yuste,* Marina Botto,[†] James C. Paton,[‡] David W. Holden,[§] and Jeremy S. Brown^{2*}

Streptococcus pneumoniae is a common cause of septicemia in the immunocompetent host. To establish infection, *S. pneumoniae* has to overcome host innate immune responses, one component of which is the complement system. Using isogenic bacterial mutant strains and complement-deficient immune naive mice, we show that the *S. pneumoniae* virulence factor pneumolysin prevents complement deposition on *S. pneumoniae*, mainly through effects on the classical pathway. In addition, using a double *pspA*⁻/*ply*⁻ mutant strain we demonstrate that pneumolysin and the *S. pneumoniae* surface protein PspA act in concert to affect both classical and alternative complement pathway activity. As a result, the virulence of the *pspA*⁻/*ply*⁻ strain in models of both systemic and pulmonary infection is greatly attenuated in wild-type mice but not complement deficient mice. The sensitivity of the *pspA*⁻/*ply*⁻ strain to complement was exploited to demonstrate that although early innate immunity to *S. pneumoniae* during pulmonary infection is partially complement-dependent, the main effect of complement is to prevent spread of *S. pneumoniae* from the lungs to the blood. These data suggest that inhibition of complement deposition on *S. pneumoniae* by pneumolysin and PspA is essential for *S. pneumoniae* to successfully cause septicemia. Targeting mechanisms of complement inhibition could be an effective therapeutic strategy for patients with septicemia due to *S. pneumoniae* or other bacterial pathogens. *The Journal of Immunology*, 2005, 175: 1813–1819.

Streptococcus pneumoniae is one of a limited number of bacterial pathogens that commonly cause septicemia in a previously healthy host. Even if treated with appropriate antibiotics *S. pneumoniae* septicemia has a mortality of 20–25%, and identifying the mechanisms allowing *S. pneumoniae* survival and growth within the systemic circulation is a high priority. To establish invasive infection, bacterial pathogens have to evade important components of the innate immune response, including the complement system. Complement proteins form three enzyme cascades (termed the classical, mannose-binding lectin (MBL)³ and alternative pathways), activation of which results in opsonization of pathogens by C3b deposition on their surface, lysis of Gram-negative bacteria by the membrane attack complex, and the release of proinflammatory signals such as C3a and C5a (1). Both clinical and laboratory studies provide strong evidence that the complement system is important for innate immunity to *S. pneumoniae* (2–7). *S. pneumoniae* infections are common in patients with deficiencies of complement components (2, 5), and in mouse models of infection loss of complement activity results in a rapidly progressive septicemia and impaired cellular immune responses (7). Innate immunity to *S. pneumoniae* is critically dependent on both the classical and alter-

native pathways, whereas the MBL pathway seems to be only of minor importance (7, 8).

The central importance of complement for innate immunity suggests that inhibition of complement activity may be important for *S. pneumoniae* virulence. Several *S. pneumoniae* proteins interact with complement components in vitro, including the cell surface proteins PspA and CbpA, and the cytotoxin pneumolysin (9). Experiments with an isogenic *pspA*⁻ capsular serotype 3 *S. pneumoniae* strain have shown that PspA reduces complement deposition on *S. pneumoniae* by inhibiting both alternative and classical pathways by an unknown mechanism (8, 10–12). In addition the reduced virulence of this strain was fully restored in factor B-deficient mice, suggesting that inhibition of alternative pathway activity is one mechanism by which PspA aids *S. pneumoniae* virulence (10). Pneumolysin is an intracellular protein but is released into the extracellular environment during infection, and is essential for the full virulence of *S. pneumoniae* (13–16). In vitro pneumolysin activates the classical pathway by binding to C1q, either directly due to its similarity to C-reactive protein or indirectly by binding to the Fc region of host IgG (17–19), and this could potentially affect complement-dependent immunity against *S. pneumoniae* (15). However, pneumolysin has pleiotropic effects that may influence virulence, including impairment of leukocyte function, inhibition of mucociliary clearance, and promotion of inflammation (15, 20–22), and the importance of the interaction with C1q for disease pathogenesis is unclear. Experiments using *S. pneumoniae* strains expressing different pneumolysin derivatives have shown distinct effects on infection for the pneumolysin cytotoxic and complement activation domains (23, 24), but the relationship between pneumolysin structure and its in vivo functions is complex and varies in different models of infection (16, 25, 26). Whether pneumolysin prevents complement deposition on *S. pneumoniae* and therefore inhibits complement-mediated immunity is not known.

We have used isogenic bacterial mutant strains and mouse models of infection in wild-type and complement-deficient mice to

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³ Abbreviations used in this paper: MBL, mannose-binding lectin; CI, competitive index; BAL, bronchoalveolar lavage.

investigate the ability of pneumolysin to prevent complement deposition on *S. pneumoniae* and whether this property affects disease pathogenesis. As pneumolysin and PspA may both affect complement activity, we have also investigated the effects of both proteins in combination on complement-mediated innate immunity to *S. pneumoniae*. The results demonstrate that the *pspA*⁻/*ply*⁻ strain is highly sensitive to complement, and we have exploited this property to identify when and at what anatomical sites complement assists innate immunity to *S. pneumoniae*.

Materials and Methods

Bacteria

The capsular serotype 2 strain, D39, and its previously described isogenic derivatives containing a deletion affecting the pneumolysin gene (originally called ΔPly and deficient in both the cytolytic and complement activities of pneumolysin; termed *ply*⁻ in this article) and/or a disruption affecting the PspA gene (*pspA*⁻) (13, 16, 27) were used for this study. Additional experiments were performed using a capsular serotype 23 strain, Io11697 (kind gift from B. Spratt, Imperial College, London, U.K.) and its isogenic *ply*⁻ and *pspA*⁻ derivatives (constructed by transformation with genomic DNA from the corresponding D39 mutant strains using standard protocols) (28). To limit the effects of phase variation on infection experiments, *S. pneumoniae* capsular serotype 2 strains were passaged through mice by i.p. inoculation and recovery of bacteria from the spleen. Bacteria were cultured at 37°C in 95% air/5% CO₂ on Columbia agar containing 5% horse blood or in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY), supplemented when necessary with erythromycin 0.2 μg/ml. Strains were stored at -80°C as single-use aliquots of THY broth culture (OD₅₈₀ = 0.3 - 0.4) in 10% glycerol.

C3 deposition assays

C3 deposition assays were performed as previously described using equal numbers of bacteria for each *S. pneumoniae* strain compared (7). A purified r43-kDa N-terminal truncated component of PspA, and full-length pneumolysin (modified by Trp⁴³³ → Phe substitution to have minimal cytotoxic activity but retaining complement-binding activity) and PsaA (a negative control protein) were obtained by overexpression of previously described plasmids in *Escherichia coli* (29). Protein concentrations were calculated using the BCA Protein Assay kit (Pierce). For experiments assessing protein inhibition of C3 deposition on *S. pneumoniae*, serum was incubated with protein for 30 min before addition of the bacteria. The data are presented as a percentage of the value for the wild-type strain and represent means and SD for three to four reactions using different serum (pooled from three to five mice or human volunteers) and bacterial stocks for each strain. Human serum was obtained from healthy subjects unvaccinated against *S. pneumoniae*. Experiments with the serotype 2 strains used neat mouse serum and a 1 in 5 dilution of human serum, and with the serotype 23 strains a 1 in 5 dilution of mouse serum. Data were analyzed using absolute values and two-tailed *t* tests.

Infection models

Infection experiments conformed to institutional and government guidelines for working with animals. Wild-type C57BL/6 mice were obtained from a regulated breeding establishment and complement-deficient mice (*C3*^{-/-}, *C1qa*^{-/-}, and *Bf*^{-/-}) supplied by the investigators (M. Botto) (30-32). Mixed infection experiments used mice aged from 8 to 16 wk with no prior exposure to *S. pneumoniae* (7). Mixtures of the two bacterial strains being compared were inoculated in a 1:1 ratio, target organs recovered at fixed time points and homogenized (spleens and lungs) then, along with the inoculum, diluted and plated onto plain and antibiotic medium to determine the ratios of the two strains using antibiotic sensitivities. The relative virulence of the strains is presented as a competitive index (CI), defined as the ratio of the test strain (single or double mutant strain) to the reference strain (wild-type or a single mutant strain) recovered from the mice divided by the ratio of the test strain to the reference strain in the inoculum (28). A CI of <1.0 indicates that the test strain is reduced in virulence compared with the reference strain. The lower the CI the greater the reduction in virulence. Mice were inoculated by the i.p. route (a model of septicemia) with a total of 5 × 10⁴ CFU and the CI calculated for bacteria recovered from the spleen after 24 h, or the intranasal route (under halothane anesthesia, a model of pneumonia) with a total of 5 × 10⁶ CFU and the CIs calculated for bacteria recovered from bronchoalveolar fluid (BAL), lung, blood, and spleen after 4 or 24 h (7, 28). Mixed infection

experiments were in general repeated twice using three to six mice in each group, and CIs compared using two-tailed *t* tests.

Results

Complement deposition on *S. pneumoniae ply*⁻ strains in human serum

Whether pneumolysin prevents complement deposition on *S. pneumoniae* was analyzed by measuring C3 deposition in human serum on capsular serotype 2 wild-type and *ply*⁻ *S. pneumoniae* strains using a flow cytometry assay. The effect of PspA alone and in combination with pneumolysin was assessed using *pspA*⁻ and double-mutant *pspA*⁻/*ply*⁻ strains. The proportion of bacteria positive for C3 was increased on the *ply*⁻ strain compared with the wild-type strain, but the intensity of C3 deposition was not affected (Fig. 1, A-C). In contrast, on the *pspA*⁻ strain the intensity of C3 deposition was increased but the proportion of bacteria positive for C3 was less affected compared with the wild-type strain. The pattern of C3 deposition on the double-mutant *pspA*⁻/*ply*⁻ strain was a combination of the different patterns observed with the individual *pspA*⁻ and *ply*⁻ strains, with both an increased intensity of C3 deposition and an increased proportion of bacteria positive for C3 compared with the wild-type strain. These results demonstrate that pneumolysin does prevent C3 deposition on *S. pneumoniae*, and that the combination of both pneumolysin and PspA are additive and highly effective in inhibiting complement activity.

Pneumolysin prevents classical pathway-dependent complement deposition on *S. pneumoniae*

Whether pneumolysin and PspA affects either the alternative or classical pathways was assessed using serum from wild-type, *C1qa*^{-/-}, and *Bf*^{-/-} mice for C3 deposition assays on wild-type, *pspA*⁻, *ply*⁻, and *pspA*⁻/*ply*⁻ *S. pneumoniae* strains. Although C3 deposition on the mutant *S. pneumoniae* strains was increased in serum from wild-type mice, the patterns of C3 deposition differed compared with those in human serum (Fig. 1, D-F). Both the proportion of bacteria positive for C3 and the intensity of C3 deposition were increased for the *ply*⁻ strain, but the increase in C3 deposition on the *pspA*⁻ strain was not statistically significant when compared with the wild-type strain. Furthermore, in mouse serum the increase in C3 deposition on the *pspA*⁻/*ply*⁻ strain compared with the *pspA*⁻ and *ply*⁻ strains was more marked than in human serum. Similar experiments were also performed with a capsular serotype 23 strain. There was increased C3 deposition on the capsular serotype 23 *ply*⁻ and *pspA*⁻ strain compared with the wild-type strain, and C3 deposition was strikingly increased on the capsular serotype 23 *pspA*⁻/*ply*⁻ strain compared with the *pspA*⁻ and *ply*⁻ strains (Fig. 1, G-I). Hence, the combination of PspA and pneumolysin strongly inhibit C3 deposition on both capsular serotype 2 and 23 strains, although the effect was markedly stronger for the serotype 23 strain suggesting there is variation in the efficacy of these proteins between *S. pneumoniae* strains.

In serum from *C1qa*^{-/-} mice (in which only alternative pathway-dependent C3 deposition on *S. pneumoniae* can occur) there was a significant increase in both the proportion of bacteria positive for C3 and intensity of C3 deposition on the serotype 2 *pspA*⁻ strain compared with the wild-type strain, whereas there was only a small increase in the intensity of C3 deposition on the *ply*⁻ strain with no significant increase in the proportion of bacteria positive for C3 (Fig. 2, A and B). In contrast, in serum from *Bf*^{-/-} mice (in which only classical pathway-dependent C3 deposition on *S. pneumoniae* can occur) there was a significant increase in the proportion of bacteria positive for C3 on the *ply*⁻ strain compared with the wild-type strain (Fig. 2C). There was also an increase in the proportion of bacteria positive for C3 on the *pspA*⁻ strain, but this

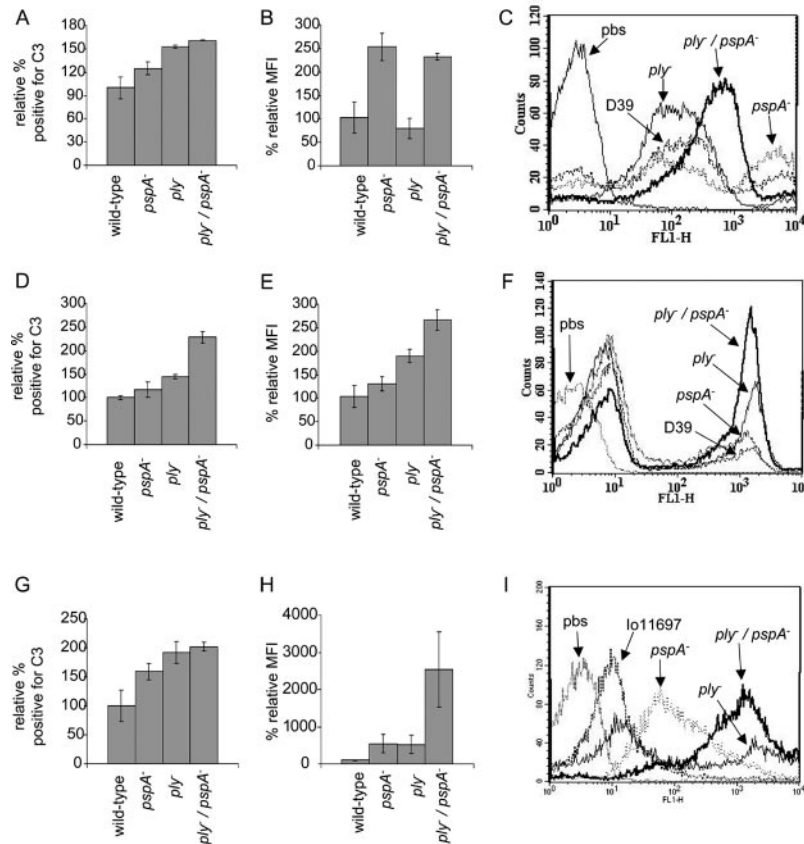


FIGURE 1. C3 deposition on the *pspA*⁻, *ply*⁻, and *pspA*⁻/*ply*⁻ *S. pneumoniae* strains expressed as a percentage of the results for the corresponding wild-type strain. Error bars represent SDs. **A–C**, Results using human serum and capsular serotype 2 strains, SDs. **A**, Proportion of bacteria positive for C3. Values of *p*: *ply*⁻ vs wild type = 0.0006; *pspA*⁻ vs wild type = 0.01; and *pspA*⁻/*ply*⁻ vs wild type = 0.0005. **B**, Geometric mean fluorescence intensity (MFI) of C3 deposition. Values of *p*: *ply*⁻ vs wild type = 0.17; *pspA*⁻ vs wild type = 0.006; and *pspA*⁻/*ply*⁻ vs wild type = 0.0002. **C**, Examples of flow cytometry histograms of C3 deposition on the D39 wild-type, *ply*⁻, *pspA*⁻, and *pspA*⁻/*ply*⁻ strains in human serum. **D–F**, Results using wild-type mouse serum and capsular serotype 2 strains. **D**, Proportion of bacteria positive for C3. Values of *p*: *ply*⁻ or *pspA*⁻/*ply*⁻ vs wild type <0.0001; *pspA*⁻ vs wild type = 0.10; and *pspA*⁻/*ply*⁻ vs *ply*⁻ or *pspA*⁻ <0.001. **E**, Geometric MFI of C3 deposition. Values of *p*: *ply*⁻ or *pspA*⁻/*ply*⁻ vs wild type <0.006; *pspA*⁻ vs wild type = 0.10; and *pspA*⁻/*ply*⁻ vs *ply*⁻ or *pspA*⁻ <0.001. **F**, Examples of flow cytometry histograms of C3 deposition in serum from wild-type mice on the D39 wild-type, *pspA*⁻, *ply*⁻, and *pspA*⁻/*ply*⁻ strains. **G–I**, Results using wild-type mouse serum and capsular serotype 23 strains. **G**, Proportion of bacteria positive for C3. Values of *p*: *ply*⁻ vs wild type = 0.015; *pspA*⁻ vs wild type = 0.002; and *pspA*⁻/*ply*⁻ vs wild type = 0.003. **H**, Geometric MFI of C3 deposition. Values of *p*: *ply*⁻ vs wild type = 0.13; *pspA*⁻ vs wild type = 0.022; and *pspA*⁻/*ply*⁻ vs wild type = 0.05. **I**, Examples of flow cytometry histograms of C3 deposition in serum from wild-type mice on the Io11697 wild-type, *pspA*⁻, *ply*⁻, and *pspA*⁻/*ply*⁻ strains. For **C**, **F**, and **I**, The line marked pbs represents the negative control (wild-type bacteria incubated in pbs).

did not reach statistical significance. Due to loss of alternative pathway-mediated amplification of C3 deposition, the intensity of C3 deposition on bacteria in serum from *Bf*^{-/-} mice was too low for differences between mutant strains to become apparent (Fig. 2D). These results indicate that pneumolysin prevents complement deposition on *S. pneumoniae* mainly through effects on the classical pathway, and that PspA mainly inhibits alternative pathway activity although some effect on the classical pathway may also occur.

In contrast to the results with serum from wild-type mice, the increase in complement deposition on the *pspA*⁻/*ply*⁻ strain in serum from *C1qa*^{-/-} mice was not statistically significantly different to the *pspA*⁻ strain, and in serum from *Bf*^{-/-} mice was no greater than the additive effects of the single *pspA*⁻ and *ply*⁻ mutations (Fig. 2, A–C). Hence the marked increase in C3 deposition on the *pspA*⁻/*ply*⁻ strain in wild-type serum requires intact classical and alternative pathways.

Complement deposition on *S. pneumoniae* in the presence of exogenous PspA and pneumolysin

To investigate further whether PspA and pneumolysin in combination are more effective at preventing C3 deposition on *S. pneumoniae*

than each protein alone, C3 deposition assays were performed with the serotype 2 *pspA*⁻/*ply*⁻ strain in the presence of a truncated purified rPspA and a noncytotoxic derivative of pneumolysin (29). Addition of PsaA, a *S. pneumoniae* lipoprotein with no known interaction with complement, was used as a negative control. Small doses of protein (10 μg/ml, data not shown) and addition of PspA or pneumolysin in combination with PsaA had no consistent statistically significant effect on complement deposition on *S. pneumoniae*. However, in both mouse and human serum addition of 100 μg/ml PspA in combination with 100 μg/ml of pneumolysin reduced the intensity of C3 deposition on the serotype 2 *pspA*⁻/*ply*⁻ strain (Fig. 3), providing further evidence that the proteins in combination effect complement activity to a stronger degree than individual proteins.

Mixed infections with *pspA*⁻ and *ply*⁻ strains in complement deficient mice

To investigate whether the effects of pneumolysin on complement deposition impair innate immune responses to *S. pneumoniae*, infection experiments with the serotype 2 *ply*⁻ strains were performed in wild-type and complement-deficient mice. If pneumolysin prevents

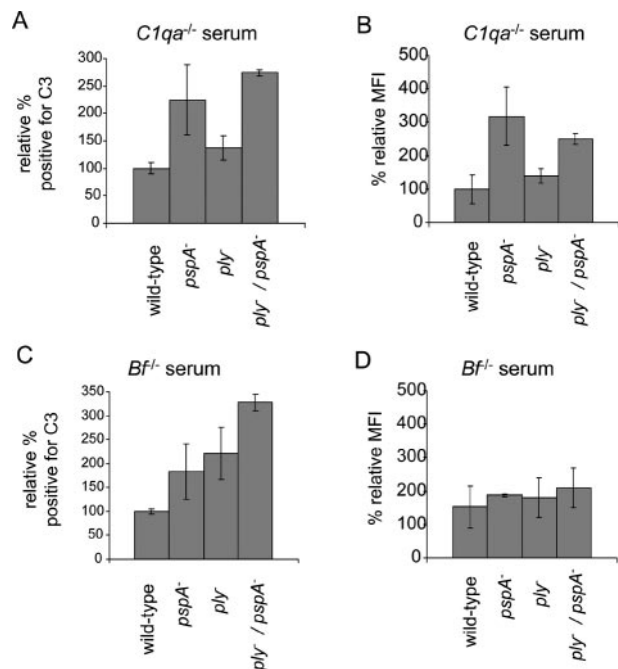


FIGURE 2. C3 deposition in mouse serum on the capsular serotype 2 *pspA*⁻, *ply*⁻, and *pspA*⁻/*ply*⁻ *S. pneumoniae* strains expressed as a percentage of the results for the D39 wild-type strain. **A**, Proportion of bacteria positive for C3 in serum from *C1qa*^{-/-} mice. Values of *p*: *pspA*⁻ vs wild type = 0.014; *pspA*⁻ vs *pspA*⁻/*ply*⁻ and *ply*⁻ vs wild type >0.12; and *pspA*⁻/*ply*⁻ vs wild type = 0.001. **B**, Geometric MFI of C3 deposition in serum from *C1qa*^{-/-} mice. Values of *p*: *ply*⁻ vs wild type = 0.025; *pspA*⁻ vs wild type = 0.014; *pspA*⁻/*ply*⁻ vs wild type = 0.008; and *pspA*⁻/*ply*⁻ vs *ply*⁻ = 0.02. **C**, Proportion of bacteria positive for C3 in serum from *Bf*^{-/-} mice. Values of *p*: *ply*⁻ vs wild type = 0.043; *pspA*⁻ vs wild type = 0.11; *pspA*⁻/*ply*⁻ vs wild type <0.001; *pspA*⁻/*ply*⁻ vs *pspA*⁻ = 0.037; and *ply*⁻ vs *pspA*⁻/*ply*⁻ = 0.058. **D**, Geometric MFI of C3 deposition in serum from *Bf*^{-/-} mice (no statistics performed).

complement-dependent immunity, then the *ply*⁻ strain will have reduced virulence in wild-type but not complement-deficient mice. However, using bacterial CFU in target organs or the rate of disease development to compare the virulence of different bacterial strains given as single strain inocula to wild-type and complement-deficient mice will be confounded by the rapid replication of all *S. pneumoniae* strains in complement-deficient mice (7, 8, 10). Hence infection with a mutant bacterial strain attenuated in virulence will progress faster in complement-deficient mice than in wild-type mice independent of whether the bacterial mutation affects a protein that interacts with complement. To avoid this problem and to provide a specific assessment of the interaction of pneumolysin with complement during infection, the relative virulence of wild-type and *ply*⁻ bacterial strains were compared using mixed infections within individual wild-type and complement-deficient mice and calculating a CI. As PspA influences virulence in mice through inhibition of the alternative pathway (8, 10), the *pspA*⁻ strain was used to validate the ability of mixed infection experiments and CIs to assess the specific interaction of bacterial proteins and the host immune system.

After i.p. inoculation the *pspA*⁻ strain was greatly reduced in virulence in wild-type mice, with a CI of 0.019. However, in keeping with previous reports (8, 10, 11) and demonstrating the utility of the mixed infection approach, the CI of the *pspA*⁻ strain was increased in *C3*^{-/-}, *Bf*^{-/-}, and, to a lesser extent, in *C1qa*^{-/-} mice (Table I), indicating that during infection PspA affects alternative and classical pathway activity. The *ply*⁻ strain had a less marked reduction in virulence in wild-type mice compared with

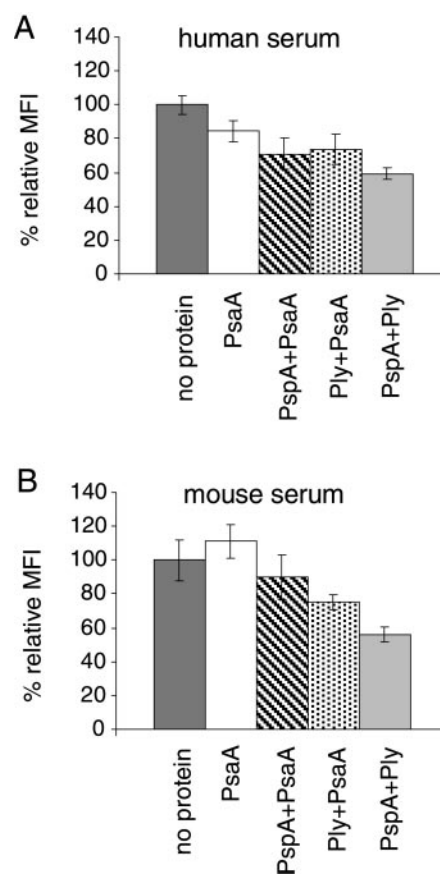


FIGURE 3. Relative geometric MFI of C3 deposition in pooled human (A) or wild-type mouse (B) serum on the capsular serotype 2 *pspA*⁻/*ply*⁻ strain in the presence of 200 µg/ml purified protein. The results are expressed as a percentage of the results in the absence of additional protein. Error bars represent SDs. Which proteins are present is written beneath each column. Values of *p* for: **A**, PspA + Ply vs PsaA + Ply = 0.025; PspA + Ply vs PsaA + PspA = 0.017; **B**, PspA + Ply vs PsaA + Ply = 0.031; PspA + Ply vs PsaA + PspA = 0.007.

the *pspA*⁻ strain with a CI of 0.29 (Table I), perhaps because pneumolysin is released into the extracellular environment and in mixed infections the *ply*⁻ strain could be partially complemented by the *ply*⁺ wild-type strain. Nevertheless, the CI of the *ply*⁻ strain was increased over 2-fold in *C3*^{-/-} and *C1qa*^{-/-} mice (CIs of 0.77 and 0.58, respectively), but not *Bf*^{-/-} mice (CI of 0.30) (Table I). These results mirror the results of the C3 deposition assays and demonstrate that a major role for pneumolysin during systemic infection is prevention of immunity mediated by the classical but not alternative pathway.

The pspA⁻/*ply*⁻ strain is highly sensitive to complement-dependent immunity

Whether the high levels of complement deposition on the *pspA*⁻/*ply*⁻ strain results in a synergistic decrease in virulence was also investigated using mixed infections. Mixed infection of the *pspA*⁻/*ply*⁻ strain vs the wild-type D39 strain in wild-type mice gave a very low CI of 0.004, whereas the CI in *C3*^{-/-} mice was 27-fold higher at 0.11 (Table I), indicating that loss of both PspA and pneumolysin results in a strain which is greatly attenuated in virulence in the presence of an intact complement system. To confirm that loss of both PspA and pneumolysin results in a synergistic reduction in virulence, mixed infections of the *pspA*⁻/*ply*⁻ strain vs the *ply*⁻ strain were performed. As the *ply*⁻ mutation is present in both strains, if the effects on virulence of mutations in *pspA* and

Table I. CI \pm SD for serotype 2 *pspA*⁻, *ply*⁻, and *pspA*⁻/*ply*⁻ mutant strains recovered from spleens of wild-type and complement-deficient mice 24 h after i.p. inoculation^a

Mixed Infection Comparison Strains	Mouse Strain			
	Wild type	<i>C3</i> ^{-/-}	<i>Bf</i> ^{-/-}	<i>C1qa</i> ^{-/-}
<i>pspA</i> ⁻ vs D39	0.019 \pm 0.01 (n = 10)	0.16 \pm 0.05 ^b (n = 10)	0.16 \pm 0.08 ^b (n = 10)	0.11 \pm 0.06 ^b (n = 7)
<i>ply</i> ⁻ vs D39	0.29 \pm 0.13 (n = 8)	0.77 \pm 0.22 ^c (n = 8)	0.30 \pm 0.09 ^d (n = 6)	0.58 \pm 0.12 ^c (n = 5)
<i>pspA</i> ⁻ / <i>ply</i> ⁻ vs D39	0.004 \pm 0.003 (n = 5)	0.11 \pm 0.06 ^b (n = 5)	nd	nd
<i>pspA</i> ⁻ / <i>ply</i> ⁻ vs <i>ply</i> ⁻	0.005 \pm 0.007 (n = 8)	1.11 \pm 0.24 ^b (n = 7)	0.13 \pm 0.05 ^e (n = 4)	0.33 \pm 0.19 ^e (n = 5)

^a nd, not done. Versus the CI for the same mixed infection in wild-type mice: ^b *p* < 0.011; ^c *p* < 0.002; ^d *p* = 0.12; ^e *p* < 0.02.

ply⁻ were independent of each other the CI for the *pspA*⁻/*ply*⁻ vs *ply*⁻ strains should be similar to the CI for the *pspA*⁻ vs wild-type strain (28, 33). However, after i.p. inoculation in wild-type mice the CI for the *pspA*⁻/*ply*⁻ strain vs the *ply*⁻ strain was nearly four times lower than the CI for the *pspA*⁻ strain vs the wild-type D39 strain (0.005 vs 0.019, *p* = 0.006) (Table I), demonstrating that loss of both pneumolysin and PspA has synergistic effects on virulence.

Mixed infections of the *pspA*⁻/*ply*⁻ strain vs the *ply*⁻ strain were also performed in *C3*^{-/-}, *C1qa*^{-/-}, and *Bf*^{-/-} mice (Table I). Strikingly, in *C3*^{-/-} mice the CI was 1.1, showing that the reduced virulence of the *pspA*⁻/*ply*⁻ strain was not apparent in mice with no effective complement activity. The CIs of the *pspA*⁻/*ply*⁻ strain vs the *ply*⁻ strain in *C1qa*^{-/-} and *Bf*^{-/-} mice were also increased but only to 0.33 and 0.13, respectively, demonstrating that both classical and alternative pathway activity are required for the full loss of virulence of the *pspA*⁻/*ply*⁻ strain to be apparent. These results are compatible with the C3 deposition data, and confirm that inhibition of complement deposition on *S. pneumoniae* by the combination of PspA and pneumolysin is essential for full virulence during systemic infection.

Site and timing of complement-dependent immunity to *S. pneumoniae*

As the CI for the *pspA*⁻/*ply*⁻ strain vs *ply*⁻ strain mixed infection was largely restored in *C3*^{-/-} mice, mixed infections comparing

the CI for this combination of strains in different target organs and at different time points between wild-type and *C3*^{-/-} mice can be used as a biological indicator for the effects of complement-mediated immunity. After intranasal inoculation in wild-type and *C3*^{-/-} mice, CIs for the *pspA*⁻/*ply*⁻ strain vs *ply*⁻ strain mixed infection were obtained in BAL and lung at 4 and 24 h and in blood and spleen at 24 h (the first time point at which systemic infection consistently develops in wild-type mice in this model). At 4 h the CIs in BAL and lung in wild-type mice had fallen to 0.36 and 0.26, respectively (Fig. 4A). However, these CIs were close to 1 in *C3*^{-/-} mice, demonstrating that the reduced virulence of the *pspA*⁻/*ply*⁻ strain compared with the *ply*⁻ strain was complement-dependent and that complement has a role in early innate immunity in the lung. By 24 h this effect was not noticeable, with similar CIs in BAL and lung between *C3*^{-/-} and wild-type mice (Fig. 4B). The CIs in blood and spleen for *C3*^{-/-} mice at 24 h were also similar to those for BAL and lung, with the *pspA*⁻/*ply*⁻ strain representing over one in five of the colonies recovered from all four sites (Fig. 4B), demonstrating that in the absence of complement the *pspA*⁻/*ply*⁻ can readily spread from the lungs to the blood. In marked contrast, in wild-type mice no *pspA*⁻/*ply*⁻ colonies were recovered from culture of the blood and spleen at 24 h despite the recovery of large numbers of the *ply*⁻ strain, giving a CI of <0.0004 (Fig. 4B). To ensure that the differences in CI between wild-type and *C3*^{-/-} mice within the blood and spleen represent failure of the *pspA*⁻/*ply*⁻ strain to survive in the systemic circulation rather than an inability to cross from the lung or peritoneum into the blood, mixed infections comparing the *pspA*⁻/*ply*⁻ strain vs the *ply*⁻ strain 6 h after i.v. inoculation were performed. Later time points were not possible as the numbers of bacteria recovered from wild-type mice were too low. The CIs for bacteria recovered from the spleen were reduced in wild-type mice (0.37 \pm 0.18) but remained close to 1 in *C3*^{-/-} mice (0.81 \pm 0.04, *p* = 0.0016 compared with the CI for wild-type mice), confirming that the *pspA*⁻/*ply*⁻ strain has a particular problem with surviving within the blood. These results demonstrate that complement activity is essential for preventing spread of *S. pneumoniae* from the pulmonary compartment to the systemic circulation, and that the combination of pneumolysin and PspA is required for *S. pneumoniae* to overcome this aspect of innate immunity.

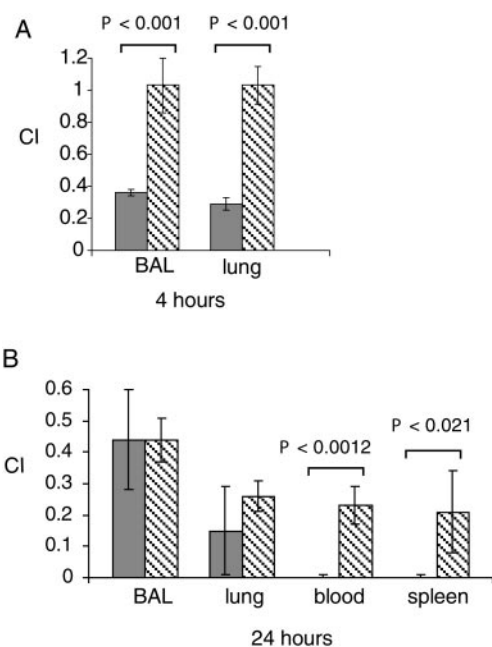


FIGURE 4. CIs for the *pspA*⁻/*ply*⁻ strain compared with the *ply*⁻ strain in BAL, lungs, blood, or spleen at 4 (A) and 24 (B) h after intranasal inoculation of wild-type (gray shading) or *C3*^{-/-} (diagonal shading) mice.

Discussion

Both pneumolysin and PspA are well-recognized virulence factors for the important human pathogen *S. pneumoniae*. However, the multiple biological properties of pneumolysin and PspA have made evaluation of how these proteins assist *S. pneumoniae* disease pathogenesis difficult to assess. In particular, it has not been clear whether the in vitro observation that pneumolysin interacts with C1q protects *S. pneumoniae* against complement-dependent immunity during infection. Experiments with *S. pneumoniae* strains expressing pneumolysin derivatives with intact cytotoxic but impaired C1q-binding properties have given conflicting results

about the importance of the complement-binding domain, with some studies suggesting that this domain aids the progress of infection in mouse models and others suggesting that it has little influence (14, 23–25). In this paper we have used bacterial strains deficient in pneumolysin and/or PspA and genetically engineered complement-deficient mice to characterize the consequences of the interactions of these proteins with the complement system during infection.

We have shown that in serum obtained from unvaccinated humans and from mice with no prior exposure to *S. pneumoniae* C3 deposition is increased on *ply*⁻ strains. Similar results were obtained for a serotype 23 strain in mouse serum, demonstrating that the increased deposition of C3 on *ply*⁻ strains is not just a property of serotype 2 strains. Although the human serum used for these experiments may contain low levels of specific Ab due to prior *S. pneumoniae* infection, the mouse serum does not (7), and, broadly, our results indicate that pneumolysin is important for overcoming complement-dependent innate immunity. We have previously shown that during innate immunity the classical pathway strongly affects the proportion of bacteria positive for C3 whereas the alternative pathway mainly affects the intensity of C3 deposition on *S. pneumoniae* (7). In human serum there is an increased proportion of the *ply*⁻ strain positive for C3 but little effect on the intensity of C3 deposition, a pattern that suggests pneumolysin prevents classical pathway-dependent C3 deposition on *S. pneumoniae*. This was confirmed by experiments using serum from mice with genetic deficiencies in the classical or alternative pathways, and would be predicted from the in vitro observation that pneumolysin binds C1q (17, 18). Furthermore, mixed infection experiments in wild-type and complement-deficient mice demonstrated that the effects of pneumolysin on complement assist *S. pneumoniae* infection only in mice with an intact classical pathway. These data are the first direct evidence that pneumolysin affects classical pathway-mediated complement immunity. The results of the C3 deposition assays and mixed infections for the *pspA*⁻ strain are in keeping with previous results (10, 12), and demonstrate that PspA assists virulence by inhibiting both alternative and, to a lesser extent, classical pathway activity. The CIs for both the *ply*⁻ and *pspA*⁻ strains were not restored completely in *C3*^{-/-} deficient mice, suggesting that the respective cytotoxic and lactoferrin-binding properties of these proteins also contribute to virulence in these models of infection (15, 20–22, 34, 35). The relatively preserved virulence of the *ply*⁻ strain when assessed using a mixed infection model is probably due to extracellular complementation of the *ply*⁻ strain by pneumolysin released from the wild-type strain (23).

PspA is highly expressed on the surface of *S. pneumoniae* during infection (36) and therefore ideally situated to prevent complement binding to *S. pneumoniae*, although the exact mechanism by which it prevents alternative pathway activity is not known. Although pneumolysin is located in the cytoplasm of *S. pneumoniae*, it is steadily released into the culture medium during growth in vitro, either through a basal level of autolysis (37) or via a yet to be characterized export mechanism (38). Free pneumolysin has also been detected in vivo during experimental infections (39). By binding C1q, pneumolysin, rather than inhibit the classical pathway, may actually increase complement activity, and how it is able to prevent complement deposition on *S. pneumoniae* is unclear at present. Complement deposition is highly localized to molecules within the immediate vicinity of the activating stimulus (1), and release of pneumolysin into extracellular fluid could divert complement activity away from the bacterial surface, as well as consume the

available C3 (15). Uncontrolled complement activation distant to *S. pneumoniae* may prevent effective phagocytosis of the bacteria and aid bacterial growth by increasing vascular permeability, thereby allowing an influx of nutrient-containing serum to the site of infection. In addition, the increase in complement activity may damage host cells and so promote virulence. Further research is necessary to characterize in detail the mechanisms by which the interaction of pneumolysin with the classical pathway results in reduced C3 deposition on *S. pneumoniae* and increased virulence.

Research into bacterial pathogenesis classically involves assessing the phenotypes of mutant strains containing deletions of a single virulence gene. However this approach will not identify important biological effects caused by the interactions between different virulence factors. As both pneumolysin and PspA affect complement activity, we have investigated whether pneumolysin and PspA in combination results in additional inhibition of complement activity compared with the individual proteins. In human or mouse serum C3 deposition was markedly increased on the *pspA*⁻/*ply*⁻ strains compared with the single-mutant and wild-type strains. Exogenous pneumolysin and PspA in combination reversed this effect, although a truncated form of PspA was used for these experiments and may have only been partially effective. As previously demonstrated (27) the double *pspA*⁻/*ply*⁻ mutant strain was markedly attenuated in virulence compared with the single mutant and wild-type bacterial strains in wild-type mice, and we have now shown that this attenuation in virulence is not seen in complement-deficient mice. Therefore, the synergistic effect on virulence of PspA and pneumolysin is mainly due to their combined inhibition of complement-dependent immunity. The interaction of PspA, pneumolysin, and other *S. pneumoniae* proteins that affect complement such as CbpA (40) requires further investigation.

Complement components are found in high concentration within serum, but are also found in bronchial secretions and inflammatory exudates induced by infection. Hence, there are several points during *S. pneumoniae* infection when complement-mediated immunity may be important. Using the sensitivity of the *pspA*⁻/*ply*⁻ strain to complement as an indicator for when complement-mediated immunity is active, we have demonstrated that early in infection there is significant complement activity in the lungs against *S. pneumoniae*, and presumably other bacteria. After 24 h there was no detectable effect of complement in the pulmonary compartment, perhaps suggesting that within the lungs complement aids the early rapid clearance of bacteria but other immune mechanisms are dominant at later time points. However, the most striking effect of complement was preventing the spread of *S. pneumoniae* from the lungs to the blood, with *pspA*⁻/*ply*⁻ bacteria only able to successfully invade the systemic circulation in *C3*^{-/-} mice. These data combined with the results of the C3 deposition assays suggest that prevention of both classical and alternative pathway-mediated C3 deposition on *S. pneumoniae* by pneumolysin and PspA greatly facilitates the development of *S. pneumoniae* septicemia. Other pathogens which commonly cause septicemia such as *Staphylococcus aureus* and *Streptococcus pyogenes* also have multiple mechanisms affecting complement activity (9), and inhibition of complement may be an essential step for many bacterial pathogens to be able to cause septicemia in a healthy host. Preventing the bacterial mechanisms that inhibit complement activity could be a potential novel therapy for patients with septicemia.

Disclosures

The authors have no financial conflict of interest.

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