Pneumolysin, PspA, and PspC Contribute to Pneumococcal Evasion of Early Innate Immune Responses during Bacteremia in Mice

Lisa R. Quin, Quincy C. Moore III, and Larry S. McDaniel

Departments of Microbiology, Surgery, and Medicine, The University of Mississippi Medical Center, Jackson, Mississippi 39216

Received 27 October 2006/Returned for modification 15 December 2006/Accepted 4 January 2007

The pneumococcal virulence factors include capsule, PspA, PspC, and Ply. Cytometric analysis demonstrated that the greatest levels of C3 deposition were on a Ply PspA− PspC− mutant. Also, Ply, PspA, and PspC expression resulted in C3 degradation in vitro and in vivo. Finally, blood clearance assays demonstrated that there was enhanced clearance of Δply PspA− PspC− pneumococci compared to the clearance of nonencapsulated pneumococci.

Streptococcus pneumoniae possesses virulence factors that function in evasion of complement (3, 27–29). The capsular polysaccharide (CPS) is considered a key factor in complement resistance (13, 21), and the interaction of pneumococci with complement varies according to the CPS type (1, 15, 19). Ply can activate the classical pathway, diverting complement activation (26, 29). Pneumococcal surface protein A (PspA) can also interfere with complement deposition, blocking recruitment of alternative pathway (AP) proteins (4, 24). Pneumococcal surface protein C (PspC; also called CbpA and SpsA) also interfere with complement deposition, blocking recruitment (26, 29). Pneumococcal surface protein A (PspA) can activate the classical pathway, diverting complement activation (26, 29). Pneumococcal surface protein C (PspC; also called CbpA and SpsA) interacts with factor H (7, 22). Factor H regulates the AP by serving as a cofactor during factor I-mediated cleavage of C3b to iC3b (10, 11, 16, 25). Studies have demonstrated that more C3b is deposited on nonencapsulated pneumococci (30) and to iC3b (10, 11, 16, 25). Pneumococcal surface protein A (PspA) can activate the classical pathway, diverting complement activation (26, 29).

Pneumolysin, PspA, and PspC Contribute to Pneumococcal Evasion of

Early Innate Immune Responses during Bacteremia in Mice

Lisa R. Quin, Quincy C. Moore III, and Larry S. McDaniel

Departments of Microbiology, Surgery, and Medicine, The University of Mississippi Medical Center, Jackson, Mississippi 39216

Received 27 October 2006/Returned for modification 15 December 2006/Accepted 4 January 2007

The pneumococcal virulence factors include capsule, PspA, PspC, and Ply. Cytometric analysis demonstrated that the greatest levels of C3 deposition were on a Ply PspA− PspC− mutant. Also, Ply, PspA, and PspC expression resulted in C3 degradation in vitro and in vivo. Finally, blood clearance assays demonstrated that there was enhanced clearance of Δply PspA− PspC− pneumococci compared to the clearance of nonencapsulated pneumococci.

Streptococcus pneumoniae possesses virulence factors that function in evasion of complement (3, 27–29). The capsular polysaccharide (CPS) is considered a key factor in complement resistance (13, 21), and the interaction of pneumococci with complement varies according to the CPS type (1, 15, 19). Ply can activate the classical pathway, diverting complement activation (26, 29). Pneumococcal surface protein A (PspA) can also interfere with complement deposition, blocking recruitment of alternative pathway (AP) proteins (4, 24). Pneumococcal surface protein C (PspC; also called CbpA and SpsA) interacts with factor H (7, 22). Factor H regulates the AP by serving as a cofactor during factor I-mediated cleavage of C3b to iC3b (10, 11, 16, 25). Studies have demonstrated that more C3b is deposited on nonencapsulated pneumococci (30) and to iC3b (10, 11, 16, 25). Pneumococcal surface protein A (PspA) can activate the classical pathway, diverting complement activation (26, 29).

Pneumolysin, PspA, and PspC Contribute to Pneumococcal Evasion of

Early Innate Immune Responses during Bacteremia in Mice

Lisa R. Quin, Quincy C. Moore III, and Larry S. McDaniel

Departments of Microbiology, Surgery, and Medicine, The University of Mississippi Medical Center, Jackson, Mississippi 39216

Received 27 October 2006/Returned for modification 15 December 2006/Accepted 4 January 2007

The pneumococcal virulence factors include capsule, PspA, PspC, and Ply. Cytometric analysis demonstrated that the greatest levels of C3 deposition were on a Ply PspA− PspC− mutant. Also, Ply, PspA, and PspC expression resulted in C3 degradation in vitro and in vivo. Finally, blood clearance assays demonstrated that there was enhanced clearance of Δply PspA− PspC− pneumococci compared to the clearance of nonencapsulated pneumococci.

Streptococcus pneumoniae possesses virulence factors that function in evasion of complement (3, 27–29). The capsular polysaccharide (CPS) is considered a key factor in complement resistance (13, 21), and the interaction of pneumococci with complement varies according to the CPS type (1, 15, 19). Ply can activate the classical pathway, diverting complement activation (26, 29). Pneumococcal surface protein A (PspA) can also interfere with complement deposition, blocking recruitment of alternative pathway (AP) proteins (4, 24). Pneumococcal surface protein C (PspC; also called CbpA and SpsA) interacts with factor H (7, 22). Factor H regulates the AP by serving as a cofactor during factor I-mediated cleavage of C3b to iC3b (10, 11, 16, 25). Studies have demonstrated that more C3b is deposited on nonencapsulated pneumococci (30) and on PspA− or Ply− strains (24, 31). PspC mutants are less able to inhibit AP activation (17) and have reduced virulence (9). We investigated C3 deposition, complement inactivation, and blood clearance of pneumococci in the absence of Ply, PspA, and PspC.

Pneumococcal strains, growth conditions, and CPS determination. The pneumococci used are listed in Table 1 and include R36A, D39, and isogenic mutants of D39. LM91, TRE108, and TRE121 are insertion-duplication mutants, and ΔPLY2 and ΔPAC (generated for this study by deleting ply of TRE121) were generated by allelic replacement (29). Bacteria were grown to mid-log phase as described previously (22). When necessary, erythromycin (0.5 μg/ml), tetracycline (15 μg/ml), and trimethoprim (50 μg/ml) were added to media.

To investigate the combined role of Ply, PspA, and PspC in C3 deposition, we generated PAC. Experiments with PAC were performed as described previously (24), except that pneumococci (10⁶ CFU/ml) were incubated with normal human serum (NHS) (CompTech) as a complement source. C3 was then detected using goat anti-human C3-biotinylated (1:100 dilution) (CompTech) and strepavidin-conjugated Alexa Fluor 488 (Molecular Probes). Fluorescence-activated cell sorting was used to calculate the percentage of C3-positive bacteria and mean fluorescence intensities (MFI) (14) (Fig. 1).

C3 deposition on the pneumococcal surface. C3 deposition assays were performed as described previously (24), except that pneumococci (10⁷ CFU/ml) were incubated with normal human serum (NHS) (CompTech) as a complement source. C3 was then detected using goat anti-human C3-biotinylated (1:100 dilution) (CompTech) and strepavidin-conjugated Alexa Fluor 488 (Molecular Probes). Fluorescence-activated cell sorting was used to calculate the percentage of C3-positive bacteria and mean fluorescence intensities (MFI) (14). The results demonstrated that the percentages of ΔPAC, TRE121, and single mutants that were C3 positive were consistently greater than the percentage of D39 that was C3 positive (Fig. 2A). The MFI of D39, LM91, TRE108, ΔPLY2, TRE121, R36A, and ΔPAC were 7.8 ± 4, 57.7 ± 7, 71.3 ± 8, 75.7 ± 5, 102 ± 16.1, respectively.

TABLE 1. Pneumococcal strains used in complement deposition and virulence studies

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phenotype</th>
<th>Antibiotic resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>D39</td>
<td>Wild type</td>
<td>None</td>
</tr>
<tr>
<td>R36A</td>
<td>Nonecapsulated</td>
<td>None</td>
</tr>
<tr>
<td>LM91</td>
<td>PspA−</td>
<td>Ery'</td>
</tr>
<tr>
<td>TRE108</td>
<td>PspC−</td>
<td>Ery'</td>
</tr>
<tr>
<td>ΔPLY2</td>
<td>Ply−</td>
<td>Tmp'</td>
</tr>
<tr>
<td>TREG121</td>
<td>PspC− PspA−</td>
<td>Ery' Tetr'</td>
</tr>
<tr>
<td>ΔPAC</td>
<td>PspC− PspA− Ply−</td>
<td>Ery' Tetr' Tmp'</td>
</tr>
</tbody>
</table>

* Corresponding author. Mailing address: Department of Microbiology, University of Mississippi Medical Center, 2500 North State Street, Jackson, MS 39216. Phone: (601) 984-6880. Fax: (601) 984-1708. E-mail: LMcDaniel@microbio.unmsmed.edu.

† Published ahead of print on 12 January 2007.
138 ± 39.5, and 468.4 ± 50, respectively. Histograms demonstrated that more C3 was present on ΔPAC (Fig. 2B). These data support the findings of other studies that demonstrated the synergistic roles of Ply and PspA in complement inhibition (31) and suggest that in the absence of virulence proteins, pneumococci are more vulnerable to complement than a non-encapsulated strain is.

In vitro and in vivo C3 processing by pneumococci expressing Ply, PspA, and PspC. We first investigated the role of Ply, PspA, and PspC in complement inactivation using in vitro C3 degradation assays. Western analysis was performed as described previously to detect iC3b (6, 23), except that pneumococci (10⁹ CFU/ml) were incubated with 20% NHS for 30 min at 37°C. C3 fragments in supernatants were analyzed by Western blotting using an anti-human iC3b monoclonal antibody (Quidel). This antibody detects generation of iC3b, which serves as an indicator of AP activity. The remaining incubation procedures were performed as described previously (6, 23).

Degradation of C3b to iC3b resulted in the appearance of 75- and 40-kDa fragments. With D39 and R36A we detected degradation of human C3b (109 kDa) to iC3b, as indicated by the appearance of 75- and 40-kDa bands. TRE121 exhibited some ability to degrade C3b, whereas ΔPAC was unable to cleave C3b as efficiently as the other strains (Fig. 3A). These results

FIG. 1. Detection of type 2 CPS: electron micrographs of D39 (A), ΔPAC (B), and R36A (C) stained with Alcian Blue to visualize pneumococcal CPS (indicated by arrows).

FIG. 2. Detection of C3 deposition on the surface of pneumococci using flow cytometry analysis. (A) Percentages of the populations that were positive for C3 deposition. Student’s t test was used to compare C3 deposition data, and a P value of <0.05 was considered significant. The P values for comparisons with D39 were as follows: R36A, P = 0.0001; ΔPAC, P = 0.0002; TRE121, P = 0.006; LM91, P = 0.013; TRE108, P = 0.02; and ΔPLY2, P = 0.01. (B) Representative histograms for C3 deposition on D39 (MFI, 7.8 ± 4.0 [mean ± standard error for three or more independent experiments]), R36A (138 ± 39.5), TRE121 (102 ± 16.1), and ΔPAC (468.4 ± 50). The fluorescence intensity of C3 detected on encapsulated ΔPAC was significantly greater (P = 0.007) than that detected on non-encapsulated R36A.

FIG. 3. Western blot analysis of pneumococci following complement inactivation assays. (A) Following incubation with NHS, activated human C3b (represented by the band at 109 kDa) and its cleaved fragments, iC3b (represented by bands at 75 and 40 kDa), were detected using an anti-iC3b monoclonal antibody. D39 (lane 1), R36A (lane 2), and TRE121 (lane 3) exhibited enhanced cleavage of C3b to iC3b compared to the cleavage in ΔPAC (lane 4). (B) Following challenge of mice with 10⁸ CFU of a strain, blood was collected at 5 min (lanes 1), 10 min (lanes 2), 20 min (lanes 3), and 30 min (lanes 4). C3 processing was detected using a mouse C3 antiserum. C3 (represented by bands at approximately 120 to 170 kDa) was detected in naïve mouse blood and in blood by 5 min after infection with ΔPAC, R36A, and D39. Degradation fragments of iC3b (corresponding to bands at approximately 70 and 43 kDa) were detected in D39- and R36A-infected mice by 10 min (lanes 2 to 4).
To demonstrate C3 processing in vivo, we used previously described methods (12), except that naïve CBA/N mice (Jackson Laboratories) were challenged intravenously with 10^6 CFU of D39, R36A, or ΔPAC suspended in 0.2 ml of lactated Ringer’s solution. Blood samples were collected from mice by retroorbital bleeding at 5, 10, 20, and 30 min postinfection. Serum was collected and diluted 1:25 with 2× sodium dodecyl sulfate loading buffer, and 20 μl of each sample was used in a Western analysis as described previously (12) with polyclonal goat anti-mouse C3 (Immunology Consultants Laboratory). Figure 3B shows the C3, C3b, and inactivated fragments in mouse serum at 5, 10, 20, and 30 min after infection with different strains. C3 (bands at approximately 120 to 170 kDa) was detected in naive mouse serum and in serum 5 min after infection with ΔPAC, R36A, and D39. iC3b was more evident in serum collected after challenge with D39 or R36A, as indicated by the presence of bands at approximately 70 and 43 kDa at 10 min. This pattern of C3 inactivation was not as evident after infection with ΔPAC. These observations supported the results of the in vitro C3 assays and further suggest that expression of Ply, PspA, and PspC, independent of the CPS, accelerates C3 inactivation that could enhance pneumococcal survival.

**Pneumococcal clearance during infection.** Clearance assays were performed as described previously (2, 18, 23), and groups of five CBA/N mice per challenge strain were infected intravenously with 2 × 10^6 CFU of pneumococci (Table 1). Blood was collected at zero time, 10 and 20 min, and 24 h. Pneumococci in blood were enumerated by plating serial dilutions on blood agar. Three independent experiments were performed, and all animal experiments were conducted by following the University of Mississippi Medical Center IACUC guidelines. We also monitored the mortality of mice for up to 21 days. Data from clearance assays demonstrated that mice challenged with D39 had the highest number of pneumococci in their blood at 24 h (Fig. 4). A significant reduction in the number of nonencapsulated pneumococci in the bloodstream occurred after 20 min, whereas the numbers of the combination mutants were significantly reduced by 10 min and at 20 min (Fig. 4). This indicates that in the absence of Ply, PspA, and PspC the type 2 CPS cannot effectively evade early innate responses and suggests that the rapid clearance of TRE121 and ΔPAC could be due to their inability to successfully evade complement deposition.

Together, our results obtained using ΔPAC extend previous observations demonstrating that PspC has an additive role in complement deposition and emphasize the importance of Ply, PspA, and PspC in the establishment of pneumococcal disease. Since other workers have used C3-deficient mice to demonstrate that virulence can be restored in PspA-Ply, and PspC mutants (13, 31), it would be of value to use complement-deficient mice to investigate complement-independent virulence mechanisms employed by ΔPAC. The results of such studies using Ply-, PspA-, and PspC-deficient mutants belonging to different serotypes may identify common complement-dependent and -independent pneumococcal virulence mechanisms.

We are grateful to Moon H. Nahm (supported by grant AI30021) for providing the anticapsular antibody and to Glenn Hoskins for preparing the electron micrographs. We also thank Edwin Swiatlo for his critical reading of the manuscript. Finally, we appreciate the technical assistance of Justin Thornton and Chihwendi Owubiko. This study was supported by National Institutes of Health grant AI43653 to L.S.M.

**REFERENCES**


---

**FIG. 4.** Pneumococcal blood clearance assays. The numbers of CFU detected in the bloodstream following infection with TRE121 and following infection with ΔPAC were significantly reduced by 10 min (*P* = 0.003 [one asterisk]) and *P* = 0.004 [two asterisks], respectively, for comparisons with R36A). The numbers of nonencapsulated R36A in the blood were significantly reduced by 20 min (*P* = 0.01 for a comparison with D39). Three independent experiments using groups of five mice per challenge strain were performed, and the mortality of mice was monitored for 21 days after infection. Mice challenged with D39 succumbed to infection by 36 h, and mice challenged with pneumococci lacking only one of the virulence proteins succumbed to infection between 48 and 72 h after infection. The values are the numbers of pneumococci in blood (log CFU/ml; mean ± the standard error of the mean), and Student’s *t* test was used to compare pneumococcal clearance data. A *P* value of <0.05 was considered significant.

Editor: J. N. Weiser