Antibodies to PcpA and PhtD protect mice against \textit{Streptococcus pneumoniae} by a macrophage- and complement-dependent mechanism

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Currently marketed \textit{Streptococcus pneumoniae} (Spn) vaccines, which contain polysaccharide capsular antigens from the most common Spn serotypes, have substantially reduced pneumococcal disease rates but have limited coverage. A trivalent pneumococcal protein vaccine containing pneumococcal choline-binding protein A (PcpA), pneumococcal histidine triad protein D (PhtD), and detoxified pneumolysin is being developed to provide broader, cross-serotype protection. Antibodies against detoxified pneumolysin protect against bacterial pneumonia by neutralizing Spn-produced pneumolysin, but how anti-PhtD and anti-PcpA antibodies protect against Spn has not been established. Here, we used a murine passive protection sepsis model to investigate the mechanism of protection by anti-PhtD and anti-PcpA antibodies. Depleting complement using cobra venom factor eliminated protection by anti-PhtD and anti-PcpA monoclonal antibodies (mAbs). Consistent with a requirement for complement, complement C3 deposition on Spn in vitro was enhanced by anti-PhtD and anti-PcpA mAbs and by sera from PhtD- and PcpA-immunized rabbits and humans. Moreover, in the presence of complement, anti-PhtD and anti-PcpA mAbs increased uptake of Spn by human granulocytes. Depleting neutrophils using anti-Ly6G mAbs, splenectomy, or a combination of both did not affect passive protection against Spn, whereas depleting macrophages using clodronate liposomes eliminated protection. These results suggest anti-PhtD and anti-PcpA antibodies induced by pneumococcal protein vaccines protect against Spn by a complement- and macrophage-dependent opsonophagocytosis.

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with cobra venom factor alone survived for the entire surveillance period (10 days) (Supplementary Table 1).

In agreement with this requirement for complement in the passive protection model, complement deposition on Spn was promoted by anti-PcpA or anti-PhtD mAbs, hyperimmune sera from rabbits immunized with PcpA- or PhtD-monovalent vaccines, and post-immune sera from human subjects vaccinated with a PcpA- and PhtD-bivalent PPrV14 (Fig. 2). Therefore, and because Spn is resistant to the complement membrane attack complex, anti-PcpA and anti-PhtD antibodies likely promote clearance by enhancing complement-mediated phagocytosis.

Because neutrophils and macrophages are the major cell types mediating phagocytosis of Spn, we next examined which of these cell types are required for passive protection by anti-PcpA and anti-PhtD mAbs. Depleting neutrophils using anti-Ly6G mAb (clone 1A8; BioXCell, #BE0075) to deplete neutrophils as described previously. Control mice received PBS alone. Depletion of blood neutrophils by at least 90% was achieved by administering 0.1 mg/kg buprenorphine.

As described in Fig. 1, CBA/N mice received an intraperitoneal injection of anti-PcpA, anti-PhtD, or irrelevant mAbs. Mice were challenged 1 h later with single intraperitoneal injections of a lethal dose of Spn A66.1 or WU2 strains. Survival was followed for 10 days.

**Surviving, n (%)**

| Spn strain | mAb | Splenectomya | Neutrophils depletedb | Macrophages depletedc | No. Mice | Day 1 | Day 2 | Day 10 |
|------------|-----|--------------|----------------------|-----------------------|----------|------|------|-------|
| A66.1      | Anti-PcpA | -             | -                    | -                     | 8       | 100.0 | 100.0 | 100.0 |
|            | Anti-PhtD  | -             | -                    | -                     | 8       | 100.0 | 100.0 | 100.0 |
|            | Irrelevant | -             | -                    | -                     | 8       | 100.0 | 100.0 | 100.0 |
|            | Anti-PcpA  | -             | -                    | +                     | 16      | 93.7  | 93.7  | 13.1  |
|            | Anti-PhtD  | -             | -                    | +                     | 16      | 100.0 | 100.0 | 100.0 |
|            | Irrelevant | -             | -                    | +                     | 15      | 100.0 | 85.3  | 1.3   |
| WU2        | Anti-PhtD  | -             | -                    | +                     | 8       | 100.0 | 80.0  | 100.0 |
|            | Anti-PhtD  | -             | +                    | -                     | 8       | 100.0 | 80.0  | 100.0 |
|            | Irrelevant | -             | +                    | -                     | 8       | 100.0 | 25.0  | 0.0   |
| WU2        | Anti-PhtD  | -             | +                    | -                     | 16      | 100.0 | 100.0 | 100.0 |
|            | Anti-PhtD  | -             | +                    | -                     | 16      | 100.0 | 0.0   | 0.0   |
|            | Irrelevant | -             | +                    | -                     | 16      | 100.0 | 0.0   | 0.0   |

As described in Fig. 1, CBA/N mice received an intraperitoneal injection of anti-PcpA, anti-PhtD, or irrelevant mAbs. Mice were challenged 1 h later with single intraperitoneal injections of a lethal dose of Spn A66.1 or WU2 strains. Survival was followed for 10 days.

aSplenectomy was performed on anesthetized mice 2 weeks before passive immunization and lethal challenge with the indicated Spn strain (D0). Control mice were sham-operated. Before and 1 day after surgery, mice were subcutaneously administered 0.1 mg/kg buprenorphine.

b1 day before and 3 and 7 days after bacterial challenge, mice were treated by intraperitoneal injection with PBS containing 600 μg of anti-Ly-6G mAb (clone 1A8; BioXCell, #BE0075) to deplete neutrophils as described previously.控制 mice received PBS alone. Depletion of blood neutrophils by at least 90% was confirmed by flow cytometry (data not shown).

c3 days before and 1 day after bacterial challenge, mice were injected intravenously with 1 mg clodronate liposomes (from Dr N. Van Rooijen, clodronateliposome.org, #283539) in PBS to deplete macrophages as previously described. Control mice received PBS alone.
Figure 2. PcpA- and PhtD-specific mAbs and sera promote complement C3 deposition on Spn. Spn strains WU2 or A66.1 (1.3 × 10⁶ colony-forming units) in 20 μl assay buffer (phosphate-buffered saline + 1% bovine serum albumin) were incubated for 30 min at 37°C with an equal volume of pooled anti-PcpA or anti-PhtD mAbs (see Figure 1 legend; 50 μg/ml final concentration of each mAb) (A), hyperimmune sera from rabbits vaccinated with monovalent PcpA or PhtD vaccines formulated with a proprietary squalene-based TLR4 adjuvant (1:40 final concentration; Sanofi, Montpellier) (B), or pooled pre- or post-immune sera from human subjects vaccinated with a bivalent PcpA-PhtD PPrV in a clinical trial (1:320 final concentration) (C). To deplete complement, all sera were heated before mixing with Spn. Opsonized bacteria were then washed twice in assay buffer and incubated with 13% (A and B) or 9% (C) baby rabbit complement (in-house preparation) for 90 min at 37°C. Next, bacteria were incubated for 30 min at 37°C with 1:100 fluorescein isothiocyanate-conjugated goat anti-rabbit C3 antibody (MP Biomedical, #0855654), and the percentage of antibody-bound bacteria was determined using an Accuri C6 flow cytometer (Becton Dickinson) and analyzed using CSampler software (Becton Dickinson). Bars indicate means and error bars indicate standard deviations. In A, results depict the means of five determinations for anti-PcpA and anti-PhtD mAbs and two determinations for irrelevant mAbs; in B, of three determinations; and in C, of two determinations. All flow cytometry evaluations were based on ≥ 20,000 gated events.
Complement is similarly required for human anti-pneumococcal IgG to protect against infection and bacteremia-associated complications and is further required for maximal induction of phagocytosis by antibodies against pneumococcal surface protein A (PspA).  

Vaccination with a trivalent PPrV has been suggested to enhance early clearance of Spn from the lungs of mice by increasing phagocytosis by neutrophils. Similarly, transfer of pneumococcus-immunized serum increases Spn uptake by mature splenic neutrophils, and this uptake is complement-dependent. Consistent with these findings, we showed that human granulocytes phagocytosed Spn in the presence of complement and anti-PcpA or anti-PhtD antibodies. However, neutrophils were not needed for anti-PcpA and anti-PhtD antibodies to passively protect CBA/N mice against an intravenous lethal challenge with Spn. This discrepancy might be explained by the partially impaired neutrophil maturation and function in this mouse strain.  

Indeed, a recent study in C57BL/6 mice showed mature splenic neutrophils are integral for Spn clearance. In our mouse model, macrophages and complement were indispensable for protection by anti-PcpA and anti-PhtD antibodies. Interestingly, macrophages, but not neutrophils, are similarly required for Spn clearance by mAbs against Spn polysaccharide capsule antigens. Further study in other Spn infection models will be needed to make a more definitive conclusion about the role of neutrophils in passive protection by anti-PcpA and anti-PhtD mAbs.

Splenectomized patients are highly susceptible to infection by new Spn strains for which they do not have pre-existing immunity. This is not only because they have impaired IgM antibody responses to polysaccharide antigen, but also because splenic macrophages and neutrophils are likely to control the early stages of Spn infection before sufficient antibody levels are raised. In our study, splenectomy did not affect passive protection by anti-PcpA and anti-PhtD mAbs, as previously observed for passive protection by anti-PspA antibodies. This suggests macrophages outside of the spleen eliminated opsonized Spn, likely those in the liver. Liver-resident Kupffer cells, for example, clear C3-opsonized bacteria in the circulation via their CR1g receptors.

In addition to clarifying the mechanism of protection by the trivalent PPrV, our results indicate some options for developing assays to rapidly assess functional antibody responses to PPrVs in clinical trials. For example, antibody responses could be measured by complement deposition assays or by a modified opsonophagocytosis assay similar to that proposed to study anti-PspA antibodies.

In conclusion, our study suggests that anti-PhtD and anti-PcpA antibodies induced by PPrVs protect against Spn by a complement- and macrophage-dependent opsonophagocytosis.

Note: The findings presented in this manuscript were derived from repeat experiments and are supported by clear-cut differences between compared experimental conditions (such as 0% versus 100% survival), which indicates the results’ practical significance; hence, no analysis to show statistical significance was performed.

Disclosure of potential conflicts of interest

All authors were employees of Sanofi Pasteur when this study was conducted.

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Figure 3. PcpA- and PhtD-specific rabbit sera promote Spn phagocytosis by human granulocytes. Spn WU2 fluorescently labeled with 6-carboxyfluorescein succinimidyl ester (CFSE; Thermo Fisher, #C1311) (1 × 10⁵ colony-forming units in 50 μl) were mixed with 50 μl of a 1:1:1 mixture of assay buffer (RPMI1640 + 5% fetal calf serum), 2% baby rabbit sera (in-house preparation) as the source of complement, and dilutions of heat-inactivated hyperimmune sera from rabbits immunized with monovalent PcpA or PhtD vaccines (A) or 1:200 hyperimmune sera (Becton Dickinson) and CSampler software (Becton Dickinson). Mean percentages of CFSE-positive phagocytic cells were determined using an Accuri C6 flow cytometer (Becton Dickinson) and CSampler software (Becton Dickinson). Mean percentages of CFSE-positive cells are shown with error bars indicating the standard deviation. All flow cytometry evaluations were based on ≥ 10,000 gated events. Results depict the means of (A) three determinations, and (B) one determination (without complement) or four determinations (with complement).
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