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The 13-valent pneumococcal conjugate vaccine (PCV13) elicits cross-functional opsonophagocytic killing responses in humans to *Streptococcus pneumoniae* serotypes 6C and 7A

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Abstract

The introduction of a 7-valent pneumococcal conjugate vaccine (PCV7) in 2000 dramatically reduced the incidence of invasive pneumococcal disease (IPD) caused by the seven serotypes covered by the vaccine. Following the introduction of PCV7, which contains a serotype 6B conjugate, some decrease in IPD due to serotype 6A was noted suggesting that the serotype 6B conjugate provided some partial cross-protection against serotype 6A. However, no effect on serotype 6C was observed. In 2010, a pneumococcal conjugate vaccine with expanded serotype coverage (PCV13) was introduced that expanded the serotype coverage to 13 serotypes including serotype 6A. To assess whether the 6A conjugate in PCV13 could potentially induce functional anti-6C antibody responses, an opsonophagocytic assay (OPA) for serotype 6C was developed. Randomly chosen subsets of immune sera collected from infants receiving three doses of PCV7 or PCV13 were tested in OPA assays for serotype 6A, 6B and 6C. PCV7 immune sera demonstrated strong OPA responses, defined as percentage of subjects having an OPA titer $\geq 1:8$, to serotype 6B (100% responders), partial responses to serotype 6A (70% responders) but only minimal responses to serotype 6C (22% responders). In contrast, PCV13 immune sera showed strong OPA responses to serotypes 6A (100% responders), 6B (100% responders) and 6C (96% responders). Furthermore, during pre-clinical work it was observed that serotype 7F (included in PCV13) and serotype 7A (not included in PCV13) shared serogroup-specific epitopes. To determine whether such epitopes also may be eliciting cross-functional antibody, PCV13 immune sera were also tested in serotype 7A and 7F OPA assays. All PCV13 immune sera demonstrated OPA responses to both of these serotypes. Taken together these results suggest that immunization with PCV13 has the potential to induce cross-protective responses to related serotypes not directly covered by the vaccine.

Keywords

Streptococcus pneumoniae; opsonophagocytic killing assay; OPA; PCV13

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1.0 Introduction

Streptococcus pneumoniae can persistently colonize the nasopharynx of human infants without causing clinical symptoms or can cause disease ranging from *otitis media* to more serious and invasive pneumococcal diseases (IPD) including sepsis, meningitis and pneumonia [1]. More than 90 serotypes of *S. pneumoniae* have been identified, but the vast majority of human disease is caused by fewer than 30 serotypes [2].

In 2000, a 7-valent pneumococcal conjugate vaccine (PCV7, Prevnar®/Prevenar®) was licensed in the United States that contains capsular polysaccharides corresponding to serotypes 4, 6B, 9V, 14, 18C, 19F and 23F conjugated to the carrier protein CRM₁₉₇, a nontoxic variant of diphtheria toxin, thereby providing coverage for the seven most prevalent serotypes causing IPD in infants and children in the United States at the time [3, 4]. In 2010, a 13-valent pneumococcal conjugate vaccine (PCV13, Prevnar 13™/Prevenar 13™) was licensed in the United States that expanded the serotype coverage to additionally include serotypes 1, 3, 5, 6A, 7F and 19A. PCV13 is expected to provide protection for the 13 most prevalent serotypes causing pneumococcal disease in infants and children globally [5–8].

The capsular polysaccharides of *S. pneumoniae* are important determinants of pathogenicity, and protective immune responses to pneumococci in humans are typically directed against the capsular polysaccharides. Pneumococcal serotypes are defined by the chemical structure of the capsular polysaccharides, and pneumococcal serogroups consist of structurally related serotypes that in some cases share some immunological cross-reactivity. However, cross-functional responses, as defined by the opsonophagocytic activity (OPA) assay, and cross-protection provided by immune responses to related serotypes may vary between individual serotypes within serogroups. While strong immunological cross-reactivity by ELISA has been demonstrated in some cases, *e.g.*, serotypes 19A and 19F, little cross-functional activity in OPA assays and no evidence of cross-protection has been observed in human populations for these two related serotypes [9–12].

Pneumococcal serogroup 6 currently includes four structurally-related serotypes: 6A, 6B, 6C and 6D [13, 14]. Due to the similarity in polysaccharide structure between serotypes 6A and 6B, it was expected, also based on immunological cross-reactivity as measured by IgG ELISA, that the serotype 6B conjugate in PCV7 would provide at least partial cross-protection against serotype 6A in human populations. Post marketing IPD effectiveness studies of IPD did indeed report a decline in invasive disease due to serotype 6A, following the introduction of PCV7, but the decline in disease was less extensive than that seen for serotype 6B disease [15, 16]. The interpretation of the effectiveness data for serotype 6A however, was complicated by the increasing prevalence of serotype 6C, which was only recently identified in 2007 [14]. Serotype 6C shares many epitopes with serotype 6A, and had been indistinguishable from serotype 6A using conventional serotyping reagents. Retrospective studies carried out using specific methodologies to distinguish between serotypes 6A and 6C have demonstrated that the prevalence of serotype 6C in IPD and carriage isolates from the US has been steadily increasing [17–21]. Since serotype 6A has greater structural similarity and immunological cross-reactivity with serotype 6C than does serotype 6B [14], PCV13, which contains a serotype 6A conjugate, may elicit cross-protective responses to serotype 6C. To assess cross-functional responses for serotypes 6A, 6B, and 6C elicited by PCV7 or PCV13 immunization, sera from infants immunized with a 3-dose series of PCV7 or PCV13 were tested in OPA assays with serotype 6A, 6B, or 6C target pneumococcal strains. In addition to the potential for cross-protection for the pneumococcal serogroup 6, pre-clinical studies have also identified monoclonal antibodies (mAbs) that identified cross-reactive epitopes between the related serotypes 7F (present in

PCV13) and 7A (not present in PCV13). To address whether these cross-reactive epitopes may induce cross-functional antibodies, sera from PCV13 immunized children were also assessed in OPA assays using serotype 7A and 7F pneumococcal target strains.

2.0 Material and Methods

Sera

Serum specimens were obtained from children approximately one month after the third dose of a 2, 3, and 4 month (infant series) administration schedule of either PCV7 (Prevna[®]/Prevena[®], Wyeth) or PCV13 (Prevna 13[™]/Prevena 13[™], Pfizer) in a parallel-group, randomized, active-controlled, double-blind, multicenter trial [7]. The trial was conducted in Germany in accordance with the ethical principles that have their origin in the Declaration of Helsinki. The protocol was reviewed and approved by the responsible ethics committee. Written informed consent was obtained from all parents/guardians prior to the subject being enrolled into the study. Randomly chosen subsets were used in the analyses described here.

Opsonophagocytic Activity (OPA) Assay

PCV13 and PCV7 human immune serum samples were evaluated in opsonophagocytic activity (OPA) assays for serotypes 6A, 6B, 6C, 7A and 7F. Pneumococcal target strains were as follows: 6A (ATCC-6306), 6B (CDC-DS2212-94), 6C (CHPA388), 7A (SSI-2040/37), and 7F (ATCC-10351). OPA assay procedures were based on previously described methods [22] with the following modifications. Briefly, heat-inactivated sera were serially diluted 2.5-fold in buffer. Target bacteria were added to assay plates and were incubated for 30 min at 25°C on a shaker. Baby rabbit complement (3–4 week old, Pel-Freez, 12.5% final concentration) and differentiated HL-60 cells, were then added to each well at an approximate effector to target ratio of 200:1. Assay plates were incubated for 45 min at 37°C on a shaker. To terminate the reaction, 80 µL of 0.9% NaCl was added to all wells, mixed, and a 10-µL aliquot were transferred to the wells of Millipore, MultiScreenHTS HV filter plates containing 200 µL of water. Liquid was filtered through the plates under vacuum, and 150 µL of HySoy medium was added to each well and filtered through. The filter plates were then incubated at 37°C, 5% CO₂ overnight and were then fixed with Destain Solution (Bio-Rad). The plates were then stained with Coomassie Blue and destained once. Colonies were imaged and enumerated on a Cellular Technology Limited (CTL) ImmunoSpot Analyzer[®]. The OPA antibody titer was interpolated from the reciprocal of the two serum dilutions encompassing the point of 50% reduction in the number of bacterial colonies when compared to the control wells that did not contain immune serum.

Polysaccharide Competition OPA Assay

OPA assays were performed as described above except that free polysaccharide (125 µg/mL final concentration, from Pfizer, Pearl River, NY) was added to the initial reaction mixture of target bacterial cells and heat-inactivated human test serum to compete for polysaccharide-specific antibody. The phagocytosis step and enumeration of colonies were then completed as described above.

Statistical Analysis

Pearson two-tailed correlations and Fisher's exact test were calculated using GraphPad Prism software.

3.0 Results and Discussion

Serotype 6C OPA Responses

Functional antibodies generated to pneumococcal capsular polysaccharide (CPS) following vaccination with polysaccharide conjugate vaccines may specifically recognize the structures of the saccharide repeat units of the CP, linkages between the repeat units, side chains, or other modifications (*e.g.*, O-acetyl groups) of the CPS. Within the pneumococcal serogroup 6, there are four serotypes with related CPS (Figure 1). The CPS structures of serotypes 6A vs 6B and serotypes 6C vs 6D differ only in the specific linkages between the rhamnose and the ribitol sugar residues: $\alpha 1 \rightarrow 3$ for serotypes 6A and 6C and $\alpha 1 \rightarrow 4$ for serotypes 6B and 6D. A galactose residue in serotype 6A and 6B CPS is also replaced by a glucose residue in serotype 6C and 6D CPS, resulting in 4 distinct but related CP structures. Therefore, the CPS structure of serotype 6C is more similar to the CPS structure of serotype 6A (one change present) than it is to the structure of serotype 6B CPS (two changes present). Based on the similarity in CPS structure between serotypes 6A and 6C, we predicted that immune sera elicited by a serotype 6A conjugate containing vaccine would be more likely to kill serotype 6C pneumococci than immune sera elicited by a serotype 6B conjugate containing vaccine. To investigate the cross-functional response against serotype 6C, immune sera from PCV7 (containing a serotype 6B conjugate) and PCV13 (containing serotype 6A and 6B conjugates) immunized infants were evaluated in serotype 6A, 6B and 6C OPA assays.

A subset of serum samples (PCV7 N = 54; PCV13 N = 52) was selected from a phase 3, randomized, active-controlled, double-blinded clinical trial comparing PCV7 and PCV13 vaccines in healthy infants in Germany [7]. Samples were tested in previously validated OPA assays for serotypes 6A and 6B and in a newly developed OPA assay for serotype 6C (Figure 2A). A serum was called positive in the OPA assays if it demonstrated a titer $\geq 1:8$. Of the 54 PCV7 sera, 100% had positive serotype 6B OPA titers as expected as serotype 6B conjugate is present in the vaccine. Of these same samples, 70% also had a positive serotype 6A OPA titer. However, only 22% of the samples had a positive serotype 6C OPA titer, demonstrating that serotype 6B shows greater cross-functional reactivity with serotype 6A than with serotype 6C. In contrast, 100% of the 52 PCV13 sera had positive serotype 6A and serotype 6B OPA titers as expected as both serotype 6A and 6B conjugates are present in the vaccine. Additionally, 96% of the PCV13 samples had a positive serotype 6C OPA titer. The difference in the responder rate to serotype 6C in PCV7 sera (22%) versus PCV13 sera (96%) was highly significant (Fisher's exact test, $p < 0.0001$). Taken together these data suggest that the majority of the cross-functional antibody response to serotype 6C in PCV13 sera is induced by the serotype 6A conjugate, since the serotype 6B conjugate in PCV7 demonstrated only marginal OPA responses to serotype 6C. Furthermore, there was better a correlation between the serotype 6A and 6C OPA titers (Pearson correlation of $r = 0.78$) than there was between the serotype 6B and 6C OPA titers (Pearson correlation of $r = 0.21$) (Figure 2B). These results demonstrate that there is greater functional cross-reactivity between serotypes 6A and 6C than between serotypes 6B and 6C, as was suggested by the polysaccharide structures.

To confirm the specificity of the cross-functional OPA responses in the serotype 6C OPA assay, competition OPAs were conducted using serotype 6A, 6B and 6C free capsular polysaccharide (CPS) to inhibit the OPA reactions of 12 serotype 6C OPA positive serum samples. Serotype 6C CPS inhibited the OPA activity of 100% of the serum samples to below the limit of detection of the assay (titer of 1:8) (Table 1). Serotype 6A CPS inhibited the OPA activity of 92% of the serum samples below the limit of detection of the assay, while only 33% of the samples were inhibited by serotype 6B CPS. Overall, the results

support the conclusion that the cross-functional antibody responses to serotype 6C in the PCV13 immune sera were primarily elicited by the serotype 6A conjugate.

Despite the limited sample size available for these studies, these results suggest that the serotype 6A conjugate in PCV13 will routinely elicit functional responses to serotype 6C and that immunization with PCV13 will protect against serotype 6C disease in a high proportion of humans. PCV13 post-approval effectiveness studies will assess the full impact of the introduction of PCV13 on serotype 6C disease.

Serotype 7F OPA Responses

Serogroup 7 consists of four serotypes: 7F, 7A, 7B and 7C. Serotype 7F is one of the six additional serotypes covered by PCV13. The polysaccharide structure of serotypes 7F and 7A are identical except for an additional galactose residue present in serotype 7F [23, 24] (Figure 1), suggesting the potential for the existence of cross-reactive epitopes. Furthermore, during pre-clinical work, monoclonal antibodies were identified that could bind to both serotype 7F and serotype 7A CPS. To determine if these cross-reactive epitopes could induce cross-functional antibody responses, PCV13 immune sera were tested side by side in serotype 7F and 7A OPAs.

A random subset of samples from the PCV13 cohort of the clinical trial described above [7] (N=28) was selected and was tested side-by-side in the validated OPA assay for serotype 7F and a newly developed OPA for serotype 7A. All of the PCV13 immune serum samples tested had positive OPA titers ($\geq 1:8$) for both serotype 7F and 7A, suggesting that immunization with serotype 7F conjugate elicits a strong functional antibody response not just to serotype 7F but also to serotype 7A (Figure 3A). Furthermore, there was good correlation between the serotype 7F and 7A OPA titers: Pearson correlation of $r = 0.61$ (Figure 3B).

Serotype 7A OPA specificity could not be directly confirmed as serotype 7A free CPS was not available. However, due to the similarity in CPS structure between serotype 7F and 7A, we expected that serotype 7F CPS, which was available, should also inhibit fully serotype 7A OPA. Therefore specificity of the cross-functional OPA responses to the serotype 7A strain was demonstrated with a competition OPA assay using serotype 7F CPS to inhibit OPA reactions. The serotype 7A OPA activity of ten PCV13 serum samples tested was completely inhibited (titers reduced to below the limit of detection) when 7F CPS was used to compete for antibody (Table 2). These results indicate that the cross-functional response to serotype 7A is most likely elicited by the serotype 7F conjugate in the PCV13 vaccine. Furthermore, these results suggest that immunization with PCV13 will likely elicit protection against serotype 7A disease.

We have demonstrated two instances of cross-functional OPA responses against pneumococcal serotypes not directly covered by PCV13. Our data indicate that PCV13 will likely provide some degree of protection against disease from serotype 6C and serotype 7A due to cross-functional antibodies elicited by the serogroup-related serotype 6A and 7F conjugates, respectively. This is in sharp contrast to the pneumococcal serogroup 19, for which it is clear that little cross-functional activity to serotype 19A is elicited by serotype 19F conjugates in PCV7 [9] and PCV10 [25]; and no protection against serotype 19A IPD in human populations has been observed [9]. However, there may be other cases of functional cross-reactivity between PCV13 serotypes and related serotypes that may result in cross-protection against disease caused by related serotypes. The potential for cross-protection should be considered when designing future pneumococcal conjugate vaccines in regards to the selection of additional serotype conjugates.

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Abbreviations

PCV13	13-valent pneumococcal conjugate vaccine
PCV7	7-valent pneumococcal conjugate vaccine
OPA	opsonophagocytic activity
GMT	geometric mean titer
IPD	invasive pneumococcal disease
mAb	monoclonal antibody
CPS	capsular polysaccharide

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Figure 1.
Comparison of polysaccharide structures: **A.** Serotypes 6A, 6B, 6C and 6D. **B.** Serotypes 7F and 7A.

Figure 2.

OPA responses to pneumococcal serotypes 6A, 6B and 6C elicited by PCV7 or PCV13 immunization. A subset of post-immunization infant sera from PCV7 and PCV13 cohorts were assessed for functional antibody in serotypes 6A, 6B and 6C OPA assays. A. The percent of samples determined to be OPA positive (titer $\geq 1:8$) are indicated above each group; bars indicate the geometric mean titer. B. Correlation plots of PCV13 OPA titers between Serotype 6A and 6C; 6B and 6C with Pearson correlation indicated.

3A

3B

Figure 3.

Cross-functional OPA responses within serogroup 7. A subset of post-immunization infant sera from the PCV13 cohort positive for serotype 7F OPA responses, were assessed for OPA responses against the serotype 7A target strain. The percent of samples determined to be OPA positive are indicated above each group; bars indicate the geometric mean titer. B. Correlation plot of PCV13 OPA titers between Serotype 7F and 7A with Pearson correlation indicated.

Table 1

Specificity of the serotype 6C OPA response.

Sample	Serotype 6C OPA Titer ^a			
	No Inhibitor	6A CPS	6B CPS	6C CPS
1	1,862	4	4	4
2	229	4	4	4
3	475	4	4	4
4	931	4	498	4
5	5,421	4	5,241	4
6	465	4	4	4
7	1,235	1,012	875	4
8	3,119	4	1,489	4
9	7,457	4	1,393	4
10	3,088	4	465	4
11	2,877	4	875	4
12	2,393	4	2,124	4

^aThe OPA titer of 12 serotype 6C OPA positive serum samples was determined in the presence of no inhibitor or in the presence of the indicated free CPS at a final concentration of 125 µg/mL.

Table 2

Specificity of the serotype 7A OPA response.

Sample	Serotype 7A OPA Titer ^a	
	No Inhibitor	7F CPS
1	39,622	4
2	15,451	4
3	2,923	4
4	14,365	4
5	49,005	4
6	3,587	4
7	7,835	4
8	2,824	4
9	18,414	4
10	6,669	4

^aThe OPA titer of 10 serotype 7A OPA positive serum samples was determined in the presence of no inhibitor or in the presence of the serotype 7F CPS at a final concentration of 125 µg/mL.