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A bivalent pneumococcal histidine triad protein D-choline-binding protein A vaccine elicits functional antibodies that passively protect mice from Streptococcus pneumoniae challenge

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ABSTRACT

Vaccines based on conserved pneumococcal proteins are being investigated because serotype coverage by pneumococcal polysaccharide and polysaccharide conjugate vaccines is incomplete and may eventually decrease due to serotype replacement. Here, we examined the functionality of human antibodies induced by a candidate bivalent choline-binding protein A- pneumococcal histidine triad protein D (PcpA-PhtD) vaccine. Pre- and post-immune sera from subjects who had been vaccinated with the PcpA-PhtD candidate vaccine were tested in an established passive protection model in which mice were challenged by intravenous injection with *Streptococcus pneumoniae* serotype 3 strain A66.1. Serum antibody concentrations were determined by enzyme-linked immunosorbent assay (ELISA). Bacterial surface binding by serum antibodies was determined by a flow cytometry-based assay. Sera from 20 subjects were selected based on low activity of pre-immune samples in the passive protection model. Bacterial surface binding correlated more strongly with anti-PcpA (0.87; $p < 0.0001$) than with anti-PhtD (0.71; $p < 0.0001$). The odds ratio for predicting survival in the passive protection assay was higher for the anti-PcpA concentration (470 [95% confidence interval (CI), 46.8 to >999.9]) than for the anti-PhtD concentration (3.4 [95% CI, 1.9 to 5.6]) or bacterial surface binding (9.4 [95% CI, 3.6 to 24.3]). Pooled post-immune serum also protected mice against a challenge with *S. pneumoniae* serotype 3 strain WU2. Both anti-PcpA and anti-PhtD antibodies induced by the bivalent candidate vaccine mediate protection against *S. pneumoniae*. The results also showed that the ELISA titer might be useful as a surrogate for estimating the functional activity of antibodies induced by pneumococcal protein vaccines.

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Introduction

Although vaccines have substantially reduced the rates of pneumococcal disease, each year, *S. pneumoniae* still causes more than 800,000 deaths worldwide in children under 5 y of age.[1] Currently marketed vaccines are based on polysaccharide capsular antigens from the most common strains.[2] Coverage, however, is incomplete because serotype circulation can vary between countries or regions,[3] and protection may eventually decrease due to serotype replacement.[4,5] Vaccines based on conserved pneumococcal proteins are therefore being investigated.[2,6,7]

Pneumococcal histidine triad protein D (PhtD) and pneumococcal choline-binding protein A (PcpA) are *S. pneumoniae* surface proteins that are being studied as candidate antigens for a pneumococcal protein vaccine. PhtD is a highly conserved virulence factor that induces an effective immune response in infected individuals.[8-13] The function of PcpA is less clear, although it may play a role in pneumococcal adherence.[14] Immunization with PhtD elicits protection against pneumococcal nasopharyngeal and lung colonization in mice[15-17] and reduces pneumococcal burden in primates.[18] Also, naturally acquired anti-PhtD antibodies protect mice against pneumococcal colonization.[14] A monovalent PhtD vaccine was shown to be well tolerated and immunogenic in a phase I trial,[19] and we have confirmed that the antibodies induced by the vaccine are functional in a mouse passive protection sepsis model.[20] Likewise, immunization with PcpA, another highly conserved surface protein,[21,22] has been shown to be protective in active immunization murine models of both sepsis and pneumonia.[22] Furthermore, expression of PcpA is increased in environments low in Mn^{2+}, including serum and other internal sites.[22,23]

The safety and immunogenicity of a candidate bivalent PcpA-PhtD protein vaccine have been evaluated in a phase I trial in which 60 subjects were vaccinated twice 30 d apart with 10, 25, or 50 μg of each antigen.[24] Here, we tested sera from these subjects for the presence of functional antibodies using the same passive protection mouse model that we previously used to examine antibodies induced by a monovalent PhtD candidate vaccine.[20] We also investigated the relationship between activity

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in this passive protection model, serum anti-PhtD and anti-PcpA antibody concentrations, and activity in a bacterial surface binding assay.

Results

Selection of sera

Sera were available from 60 subjects who had received the PhtD-PcpA candidate vaccine. Twenty pairs of pre- and post-immune samples were selected based on low activity of the pre-immune sample in the mouse passive protection model (≤ 1 of 5 mice surviving at day 4; Supplemental Fig. 1). In all cases, the post-immune serum protected more mice from death at day 4 than the matched pre-immune serum, and for 16 of 20, the difference was significant (Table 1). Mean time to death was also longer in all cases with the post-immune serum than with the matched pre-immune serum and significantly longer in 18 of 20 cases. Furthermore, more mice overall were protected by post-immune sera than by pre-immune sera (84/100 vs. 4/100; $p < 0.0001$).

Relationship between serum antibody concentrations determined by passive protection, ELISA, and surface binding

We next compared activity in the passive protection model with the anti-PcpA and anti-PhtD antibody concentrations determined by ELISA (Table 2). Survival at day 4 tended to correspond with higher antibody concentrations (Fig. 1A). The ED50 for anti-PcpA IgG was estimated to be 9310 EU/ml (95% CI, 7318–11843) by logistic regression analysis (Fig. 1B). An ED50 could not be estimated for the anti-PhtD antibody because of a lack of fit to the logistic regression model (Fig. 1C).

We also compared activity in the passive protection model with the extent of bacterial surface binding by serum antibodies. For 19 of 20 subjects, bacterial surface binding was greater for the post-immune serum than for the pre-immune serum (range, 1.3–8.9) (Table 2). Correlations between surface binding and antibody concentration were statistically significant for both antibodies, but the correlation was stronger for anti-PcpA (0.87; $p < 0.0001$) than for anti-PhtD (0.71; $p < 0.0001$).

Survival at day 4 could be predicted by both ELISAs as well as by bacterial surface binding. However, the odds ratio for predicting survival was higher with the anti-PcpA IgG ELISA (470 [95% CI, 46.8 to > 999.9]) than with the anti-PhtD IgG ELISA (3.4 [95% CI, 1.9 to 5.6]) or the surface binding assay (9.4 [95% CI, 3.6 to 24.3]).

Functional activity using S. pneumoniae strain WU2 in place of strain A66.1

To explore the role of anti-PhtD antibody in more detail, we examined passive protection against *S. pneumoniae* strain WU2, which produces more PhtD than strain A66.1 when grown in the same low-Mn^{2+} medium (data not shown). For these experiments, samples were pooled from 4 subjects whose

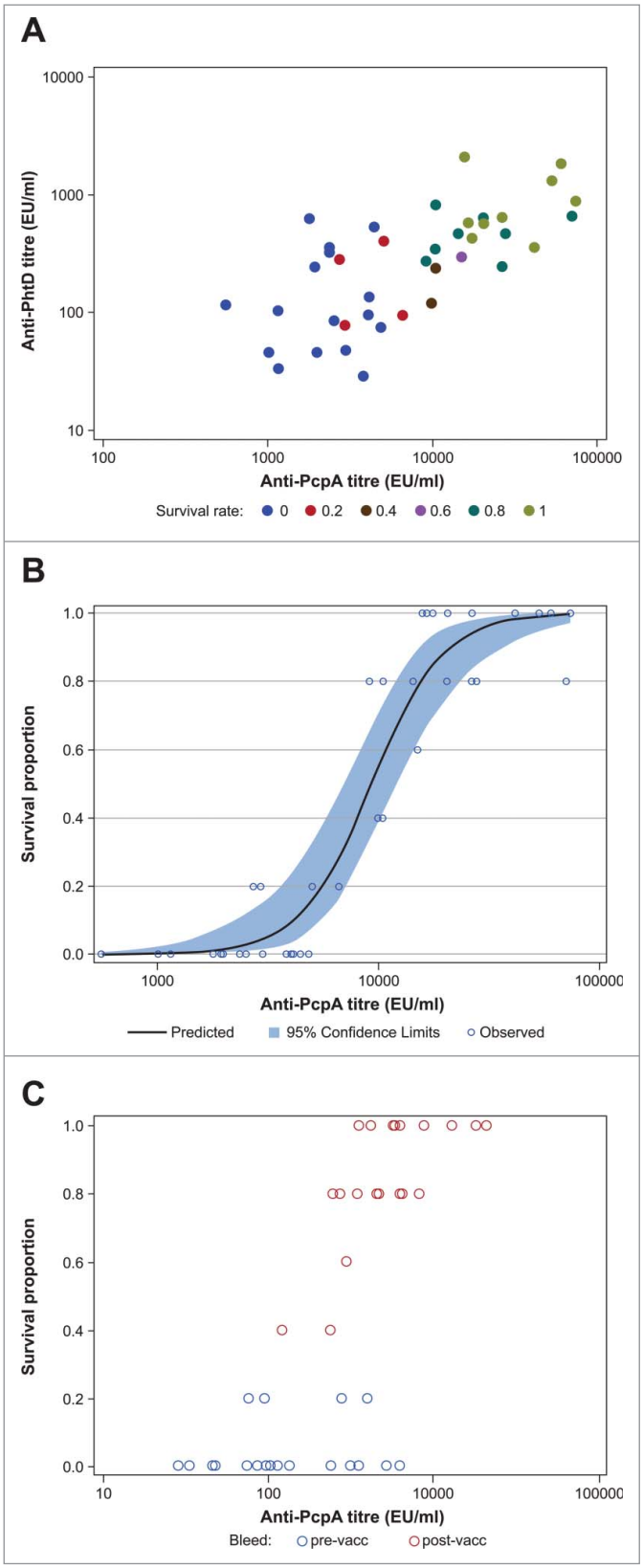

Figure 1. Relationship between post-vaccination anti-PcpA and anti-PhtD antibody concentrations and passive protection of mice. Shown are the relationships between survival at day 4 and post-vaccination anti-PcpA and anti-PhtD antibody concentrations (A), pre- and post-vaccination anti-PcpA concentrations (B), and pre- and post-vaccination anti-PhtD concentrations (C).

Table 1. Activity of pre- and post-immune sera in the mouse passive protection model.

Clinical trial subject no.	Vaccine dose	Serum dilution tested	No. of mice surviving at day 4			Time to death (h), mean ± standard deviation		
			Pre-immune serum	Post-immune serum	P-value	Pre-immune serum	Post-immune serum	P-value
10	10 μg	1:20	0	5	0.004	46 ± 11	336 ± 0	0.002
23	10 μg	1:20	0	5	0.004	49 ± 10	336 ± 0	0.002
61	25 μg	1:40	0	4	0.024	87 ± 46	296 ± 89	0.008
70	25 μg	1:40	0	4	0.024	71 ± 24	291 ± 100	0.005
80	25 μg	1:40	0	4	0.024	154 ± 50	282 ± 120	0.034
84	25 μg	1:40	1	4	0.103	134 ± 116	291 ± 100	0.044
94	25 μg	1:20	0	5	0.004	46 ± 11	336 ± 0	0.002
101	25 μg	1:20	0	3	0.083	47 ± 10	233 ± 144	0.016
105	25 μg	1:40	0	5	0.004	46 ± 11	336 ± 0	0.002
108	50 μg	1:20	0	5	0.004	100 ± 132	336 ± 0	0.013
110	50 μg	1:40	0	4	0.024	51 ± 21	282 ± 121	0.007
112	50 μg	1:40	0	2	0.222	51 ± 13	193 ± 142	0.053
115	50 μg	1:40	1	2	0.50	104 ± 130	161 ± 160	0.787
117	50 μg	1:20	0	5	0.004	66 ± 29	336 ± 0	0.002
121	50 μg	1:20	1	5	0.024	115 ± 127	336 ± 0	0.013
122	50 μg	1:20	0	4	0.024	52 ± 15	282 ± 121	0.009
123	50 μg	1:40	0	4	0.024	41 ± 0	278 ± 130	0.003
124	50 μg	1:20	0	5	0.004	84 ± 36	336 ± 0	0.002
127	50 μg	1:20	0	4	0.024	41 ± 0	277 ± 132	0.014
129	50 μg	1:20	1	5	0.024	121 ± 124	336 ± 0	0.013

Selected paired pre- and post-immune sera were passively transferred by intraperitoneal injection to 6- to 8-week-old female CBA/CaHN-Btk xid /J (CBA/N) mice (5 mice/group). After 1 h, the mice were challenged by intravenous injection with a lethal dose of *S. pneumoniae* strain A66.1 (serotype 3). Survival was followed for 14 d.
[a]P-value was determined by a one-sided Fisher exact test.
[b]Time to death was determined by Kaplan-Meier analysis.
[c]P-value determined by logistic regression with logit link and a random subject effect.

post-immune serum had high activity in the standard (strain A66.1) passive protection model. Protection from death was dose-dependent (Fig. 2A). At a dilution of 1:40, the pooled pre-immune serum protected no mice (0/5), whereas the pooled post-immune serum protected 100% (5/5).

To examine the specificity of this protection, the pooled sera were pre-incubated with recombinant PcpA or PhtD. Pre-incubation with PcpA or PhtD reduced protection equally, and the combination of the 2 recombinant proteins eliminated protection (Fig. 2B).

Discussion

This study showed that human antibodies induced by a bivalent PcpA-PhtD candidate vaccine are functional as indicated by their activity in an established mouse passive protection model.

Table 2. Antibody concentrations and surface binding titres.

Vaccine Dose	Subject no.	Anti-PcpA IgG(EU/ml)			Anti-PhtD IgG(EU/ml)			Surface binding titer (1/dilution)		
		Pre-immune serum	Post-immune serum	Fold difference	Pre-immune serum	Post-immune serum	Fold difference	Pre-immune serum	Post-immune serum	Fold difference
10 μg	10	1988	20530	10.3	46	568	12.3	7613	17160	2.3
	23	1780	15747	8.8	631	2106	3.3	8542	15430	1.8
25 μg	61	1114	21063	18.9	228	1662	7.3	7868	18922	2.4
	70	2017	18178	9.0	92	550	6.0	9044	16543	1.8
	80	2307	53015	23.0	67	497	7.4	12234	41659	3.4
	84	5438	20835	3.8	561	693	1.2	15127	20417	1.3
	94	2353	26516	11.3	352	640	1.8	6734	18298	2.7
	101	4829	14985	3.1	76	298	3.9	9725	16374	1.7
	105	8111	35068	4.3	193	852	4.4	10936	27825	2.5
50 μg	108	1939	73777	38.0	242	891	3.7	10936	43139	3.9
	110	4740	28536	6.0	640	932	1.5	9186	18115	2.0
	112	7626	21001	2.8	57	480	8.4	11208	10951	1.0
	115	5846	19855	3.4	154	243	1.6	8300	11649	1.4
	117	4407	60249	13.7	531	1835	3.5	7564	30156	4.0
	121	6614	41405	6.3	95	357	3.8	7417	23496	3.2
	122	2991	27715	9.3	48	473	9.9	4466	15697	3.5
	123	5048	40599	8.0	171	1264	7.4	10539	24156	2.3
	124	4137	53033	12.8	136	1308	9.6	12544	37357	3.0
	127	1151	70214	61.0	105	661	6.3	3484	30948	8.9
	129	5029	16603	3.3	403	582	1.4	10112	16342	1.6

Anti-PcpA and anti-PhtD IgG concentrations were determined by ELISA. For surface binding, diluted sera were mixed with *S. pneumoniae* strain A66.1 for 1 h and then incubated with fluorescein isothiocyanate-conjugated rabbit $F(ab')_2$-anti-human IgG H^+L, after which fluorescence was detected by flow cytometry.

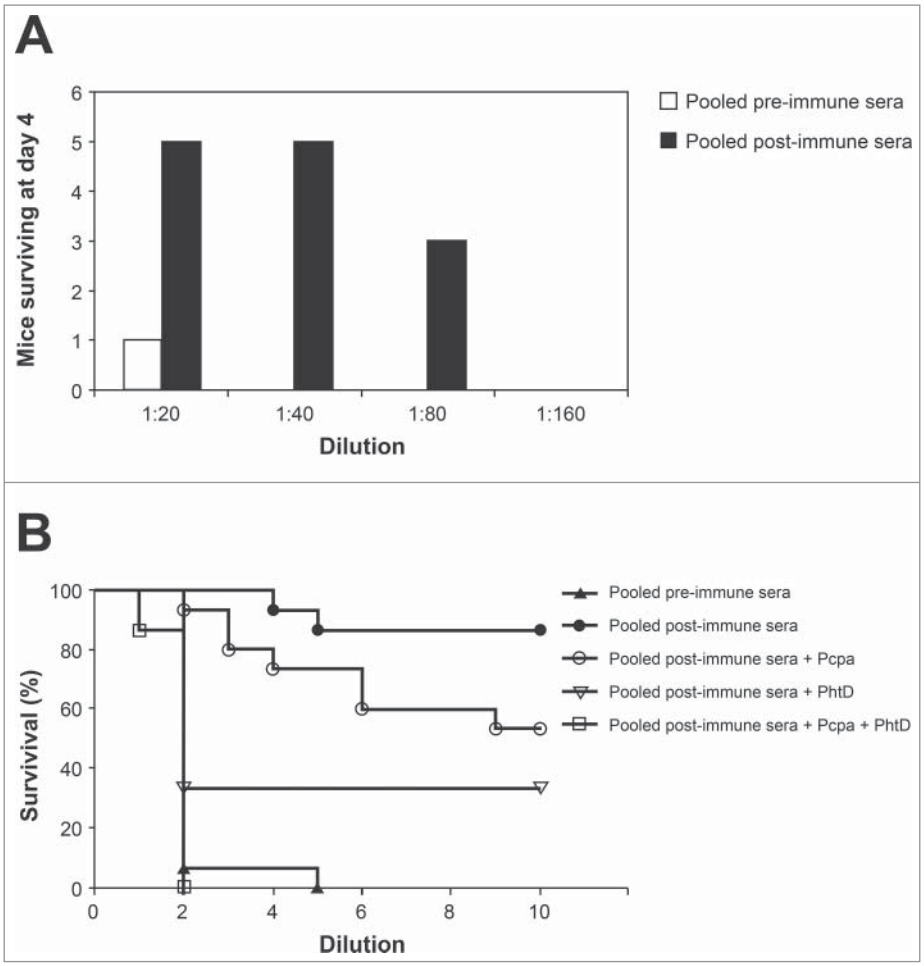

Figure 2. Protection of mice in the passive protection model using strain WU2. Sera from subjects 10, 121, 122, and 124 were pooled and tested in the murine passive protection model using serotype 3 strain WU2 in place of strain A66.1. (A) Pooled pre- and post-immune sera were first tested at 1:20 to 1:160 (5 mice/group) to identify a dilution where the pre-immune pooled serum did not provide protection. Shown is the number of mice surviving at day 10. (B) Pooled pre- and post-immune serum were tested at a dilution of 1:40 (15 mice/group). To test specificity, pooled post-immune serum was pre-incubated for 1 h with no addition, 400 μg/ml PcpA, 400 μg/ml PhtD, or both before testing in the passive protection model. Shown is the proportion surviving at each day up to day 10.

We previously used the same passive protection model to show that human antibodies induced by a monovalent PhtD candidate vaccine are functional.[20] A trivalent vaccine containing PhtD, PcpA, and detoxified pneumolysin has also been shown to protect infant mice against nasal challenge with a lethal dose of *S. pneumoniae* serotype 6A and 3.[25]

Passive protection is used to measure the potential functional activity of antibodies induced by pneumococcal protein vaccines, but passive protection assays are too cumbersome and time-consuming for screening large numbers of samples. We therefore examined whether the ELISA titer or surface binding activity could be used as a surrogate for functional activity, with the aim of simplifying testing and allowing for high-throughput screening, while reducing the need for animal testing. Both anti-PcpA and anti-PhtD antibody concentrations and surface binding activity correlated with passive protection in the mouse model, although the anti-PcpA concentration was the most predictive. The fact that the anti-PcpA concentration correlated better with passive protection than anti-PhtD suggests that the anti-PcpA antibodies played a more important role in protection, but this may have been due to the specific challenge strain used (A66.1). In fact, when we replaced strain A66.1 with WU2, which expresses higher levels of PhtD, we found that anti-PcpA and anti-PhtD antibodies contributed equally to the passive protection activity. Also, the results showed that the surface binding assay could be useful, at least as a secondary assay, for testing clinical trial samples for the presence of functional antibodies. Surface binding of antibodies is the initial step to subsequent opsonophagocytosis, but we did not measure opsonophagocytosis in the current investigation.

This study confirms our previous findings that vaccine-induced anti-PhtD antibodies are functional.[20] Similarly, Verhoeven et al. reported that immunization with monovalent PhtD and PcpA candidate vaccines protected infant and adult mice and improved bacterial clearance and survival following an intranasal challenge with *S. pneumoniae* serotype 6A.[17] Conversely, Kaur et al. found that vaccine-induced anti-PhtD but not anti-PcpA antibodies protected infant mice against colonization following an intranasal inoculation of *S. pneumoniae* serotype 4.[14] Our findings suggest that these differences may be related to the specific bacterial strains used, although other factors may also be involved, including differences in the route and site of infection, the route of serum administration, or the age and strain of the mice.

We used serotype 3 strain A66.1 for our initial experiments because it was previously used in mouse passive protection studies to test human clinical trial sera.[26] We added serotype 3 strain WU2, which has been used in passive protection studies to test monoclonal antibodies, because of its higher expression of PhtD.[27] Using these 2 serotype 3 strains, we showed that both the anti-PcpA and the anti-PhtD antibodies induced by the bivalent candidate vaccine protect mice against *S. pneumoniae*, information that can help in the development of a pneumococcal protein vaccine. These results highlight the benefits of using more than one functional model or at least more than one pneumococcal strain in evaluating the functional activity of vaccine-induced antibodies. Finally, we showed that anti-PcpA ELISA titres might be a useful surrogate for estimating the functional activity of antibodies induced by pneumococcal protein vaccines.

Materials and methods

Sera

Sera tested in this study were from a phase I trial in which adults 18–50 y of age were vaccinated twice, 30 d apart, with an aluminium-adjuvanted PcpA-PhtD vaccine containing 10, 25, or 50 μg/antigen (ClinicalTrials.gov no. NCT01444339).[24] Sera were stored at −20°C and were heated to 56°C for 30 min to inactivate complement components.

Bacteria

S. pneumoniae serotype 3 strains A66.1 and WU2 were from D. Briles, University of Alabama-Birmingham, USA. Bacteria were grown in Todd Hewitt Broth with 0.5% yeast extract adjusted to pH 7 with MOPS. Because expression of PcpA is repressed by Mn^{2+},[23] the medium was depleted of metals using a Chelex-100 column (Bio-Rad, Hercules, CA, USA) and then adjusted to 1 mM $FeSO_4$, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $ZnCl_2$, and 0.1 μM $MnSO_4$.

Recombinant proteins

Pneumococcal proteins PhtD and PcpA were cloned from strain 14453 (serotype 6B) and expressed in *Escherichia coli* as soluble proteins lacking their secretion signals. The choline-binding domain was also deleted from PcpA in the construct. The proteins were purified to ≥ 90% purity by ion-exchange chromatography as described previously.[24,28]

Passive protection assay

Mouse passive protection assays were performed as described previously.[20] Briefly, sera were diluted in phosphate-buffered saline and passively transferred by intraperitoneal injection to 6- to 8-week-old female CBA/CaHN-Btk xid /J (CBA/N) mice, a model of human X-linked immunodeficiency (Sanofi, Montpellier, France or Jackson Laboratories, Bar Harbor, ME, USA). Except where indicated, 5 mice were tested per condition. One hour after passive transfer, the mice were challenged by intravenous injection with a lethal dose of *S. pneumoniae* strain serotype 3 A66.1 (50 colony forming units) or strain WU2 (600 colony forming units). Survival was monitored for 14 d. Technicians performing the passive protection assays were blinded to the serum antibody concentration (determined by ELISA) and the dose of PhtD-PcpA vaccine that had been administered in the clinical trial. For specificity experiments, pooled sera were diluted in phosphate-buffered saline containing no addition, 400 μg/ml PcpA, 400 μg/ml PhtD, or 400 μg/ml PcpA + 400 μg/ml PhtD and then incubated for 1 h at 37°C before passive transfer.

Enzyme-linked immunosorbent assay (ELISA)

Antigen-specific IgG concentrations were analyzed as described previously.[24] Briefly, ELISA plates coated with recombinant PhtD or PcpA were incubated with duplicate serial dilutions of test sera or human AB serum standard (Sigma, St. Louis, MO, USA). Bound antibody was detected using horseradish peroxidase-conjugated goat-anti-human IgG (Jackson ImmunoResearch, West Grove, PA, USA) followed by tetramethylbenzidine substrate (Sigma-Aldrich, St. Louis, MO, USA). Antibody concentrations were determined by comparison with the human AB serum standard curve. The lower limit of quantitation was 0.123 ELISA units (EU)/ml for PhtD and 1.11 EU/ml for PcpA.

Antibody surface binding assay

Serum samples were diluted to 1:50 in IF buffer (phosphate-buffered saline + 1% bovine serum albumin) and then serially diluted (5-fold dilutions) in the same buffer. Serum dilutions (20 μl/well) or buffer alone were added in duplicate to 96-well deep round-bottom plates (Ritter, Schwabmünchen, Germany) with 20 μl/well *S. pneumoniae* strain A66.1 suspended in IF buffer at 2.25×10^7 colony forming units/ml. After incubation with shaking for 1 h at 37°C, plates were washed twice with IF buffer and incubated with shaking for 1 h with a 1:100 fluorescein isothiocyanate-conjugated rabbit $F(ab')_2$-anti-Human IgG H^+L (Southern Biotech, Birmingham, AL, USA). After further washing with IF buffer, antibody surface binding was measured using a FC500 cytometer (Beckman Coulter, Villepente, France). The bacterial population was gated for forward and side scatter. The antibody titer was calculated using Softmax Pro (Molecular Devices, Sunnyvale, CA, USA) as the inverse of the dilution giving a X-mean 50.0 of 1.0.

Statistical analysis

Statistical analysis was performed using SAS version 9.1 (SAS Institute, Cary, NC). Differences in survival rates with pre- and post-immune sera were compared using logistic regression with logic link and a random subject effect. Time to death was assessed by Kaplan-Meier analysis and compared between groups by a one-sided Fisher exact test. The antibody concentration (in EU/ml) producing 50% survival (ED50) was estimated by logistic regression analysis with probit link and a random subject effect. Pearson correlation coefficients were calculated between activities for pairs of different in vitro assays

(anti-PhtD ELISA, anti-PcpA ELISA, and surface binding). $P < 0.05$ was considered to be statistically significant.

Abbreviations

CI	confidence interval
ELISA	enzyme-linked immunosorbent assay
EU	ELISA units
IF buffer	phosphate-buffered saline + 1% bovine serum albumin
PcpA	pneumococcal choline-binding protein A
PhtD	pneumococcal histidine triad protein D

Disclosure of potential conflicts of interest

All authors were employees of Sanofi Pasteur.

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