

Passive protection of mice against *Streptococcus pneumoniae* challenge by naturally occurring and vaccine-induced human anti-PhtD antibodies

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Abbreviations: cfu, colony forming units; CI, confidence interval; ED50, dose providing 50% survival; ELISA, enzyme-linked immunosorbent assay; EU, ELISA units; PhtD, pneumococcal histidine triad protein D

Currently marketed *Streptococcus pneumoniae* vaccines are based on polysaccharide capsular antigens from the most common strains. Pneumococcal histidine triad protein D (PhtD) is a conserved surface protein that is being evaluated as a candidate for a vaccine with improved serotype coverage. Here, we measured the functional activity of human anti-PhtD antibodies in a passive protection model wherein mice were challenged with a lethal dose of *S. pneumoniae* by intravenous injection. This functional activity was compared with anti-PhtD antibody concentrations measured by enzyme-linked immunosorbent assay (ELISA) to estimate the 50% protective dose (ED50). Anti-PhtD antibodies affinity purified from pooled normal human sera passively protected mice with an ED50 of 1679 ELISA units/ml (95% confidence interval, 1420–1946). Sera from subjects injected with aluminum-adsorbed PhtD in a phase I trial had similar activity per unit of antibody (ED50 = 1331 ELISA units/ml [95% confidence interval, 762–2038]). Vaccine-induced activity in the passive protection model was blocked by pre-incubation with recombinant PhtD but not by a control *S. pneumoniae* antigen (LytB). These results show that human anti-PhtD antibodies, whether naturally acquired or induced by the PhtD candidate vaccine, are functional. This supports the development of the PhtD candidate as part of a broadly protective pneumococcal vaccine.

Each year, *Streptococcus pneumoniae* causes more than 800,000 deaths worldwide in children under 5 years of age.¹ Currently marketed *S. pneumoniae* vaccines, which are based on polysaccharide capsular antigens from the most common strains, have substantially reduced pneumococcal disease rates.² However, because serotypes can vary between countries or regions, coverage may be incomplete in some cases.³ Moreover, serotype replacement might eventually render these vaccines less effective.^{4,5} To provide broader, more diverse, and possibly infection stage-specific protection, vaccines based on conserved proteins are being investigated.^{2,6,7}

Pneumococcal histidine triad protein D (PhtD) is a conserved surface protein that mediates attachment to respiratory epithelial cells^{6,7} and can elicit a protective immune response.^{8–11} In mice, intranasal immunization with PhtD generates robust serum antibody and CD4 Th1-biased immune memory responses and confers protection against pneumococcal colonization.¹² A second study in mice showed that vaccination with PhtD protects against

nasopharyngeal and lung colonization.¹³ In a primate study, vaccination with PhtD and chemically detoxified pneumolysin induced high levels of antibodies and protected against a challenge with *S. pneumoniae* serotype 19F.¹⁴ A phase I trial in adults 18–50 years of age showed that an aluminum phosphate-adsorbed PhtD vaccine candidate was well tolerated, immunogenic, and could be boosted by a second vaccine dose.¹⁵ During development of an enzyme-linked immunosorbent assay (ELISA) to measure antibody responses in the phase I trial, we found that individual and pooled serum from unimmunized healthy adults contained substantial PhtD-binding antibody (data not shown). To further investigate the immune response elicited by a PhtD-based pneumococcal vaccine, we developed a murine passive protection sepsis model for assessing the functional activity of human anti-PhtD antibodies.

Naturally occurring human PhtD-binding antibody was purified from a commercial pooled serum (obtained from

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approximately 200 healthy individuals; Sigma, St. Louis, MO). The concentration of anti-PhtD antibody was determined by ELISA, and its purity and specificity were confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Western blotting, and competition with recombinant PhtD (data not shown). The purified PhtD-binding antibodies were passively transferred by intraperitoneal injection (200 μ l) to 6- to 8-week-old female CBA/CaHN-Btk xid /J (CBA/N) mice (Jackson Laboratories, Bar Harbor, ME). After 1 h, the mice were challenged by intravenous injection with a lethal dose (50 colony forming units [cfu] in 200 μ l) of *S. pneumoniae* strain A66.1 (serotype 3) (obtained from D. Briles, University of Alabama-Birmingham). The proportion of mice surviving at 14 days post-challenge increased with the concentration of anti-PhtD antibody (Fig. 1A). The dose providing 50% survival (ED50) was estimated to be 1679 ELISA units (EU)/ml (95% confidence interval [CI], 1420–1946) by logistic regression with probit link.

We next examined the activity of vaccine-induced antibodies in the passive protection model. In the phase I clinical trial, adults were vaccinated on weeks 0 and 4 with 6, 25, or 100 μ g of the candidate aluminum phosphate-adsorbed PhtD vaccine.¹⁵ As described above, we found substantial pre-existing anti-PhtD antibodies in pooled sera from vaccine-naïve healthy adults. We therefore selected subjects who had the lowest pre-immune passive protection activity and the highest post-immune PhtD antibody titers for testing in the passive protection assay. For testing in subsequent experiments, we selected a dilution for each subject where the pre-immune serum was not protective in the passive protection assay. All samples were initially tested at a dilution of 1:20, and survival was compared to control mice injected with PBS. Human pre-immune sera that were protective at a 1:20 dilution and for which corresponding post-immune sera (week 8) had an increase in titer of at least 1000 EU/ml were further tested at a 1:40 dilution. Pre-immune sera shown to be protective at a 1:40 dilution and for which corresponding post-immune sera had an increase in titer of at least 2000 EU/ml over baseline were further tested at a 1:60 dilution.

Of the 54 serum pairs tested, we identified appropriate dilutions for 18 (Table 1). Vaccination increased the anti-PhtD antibody titer in all subjects at all vaccine doses (6, 25, and 100 μ g). Post-immune sera from 10 of these subjects (nos. 21, 36, 37, 41, 43, 45, 51, 57, 58, and 61) significantly delayed death in the murine challenge model compared to the same dilution of the corresponding pre-immune serum. In addition, 2 of these post-immune sera (from subject nos. 37 and 43) significantly improved survival compared to the corresponding pre-immune serum. As with the naturally acquired antibody, survival increased with the concentration of anti-PhtD antibody as determined by ELISA (Fig. 1B). The ED50 in this case was estimated to be 1331 EU/ml (95% CI, 762–2038).

Finally, we performed ligand competition experiments to investigate the specificity of the vaccine-induced activity. For these experiments, we selected 5 serum pairs (subject nos. 4, 21, 37, 43, and 61) that spanned the different vaccine doses and functional activities. Sera from subject nos. 4 and 61 were selected because the post-immune sera significantly increased

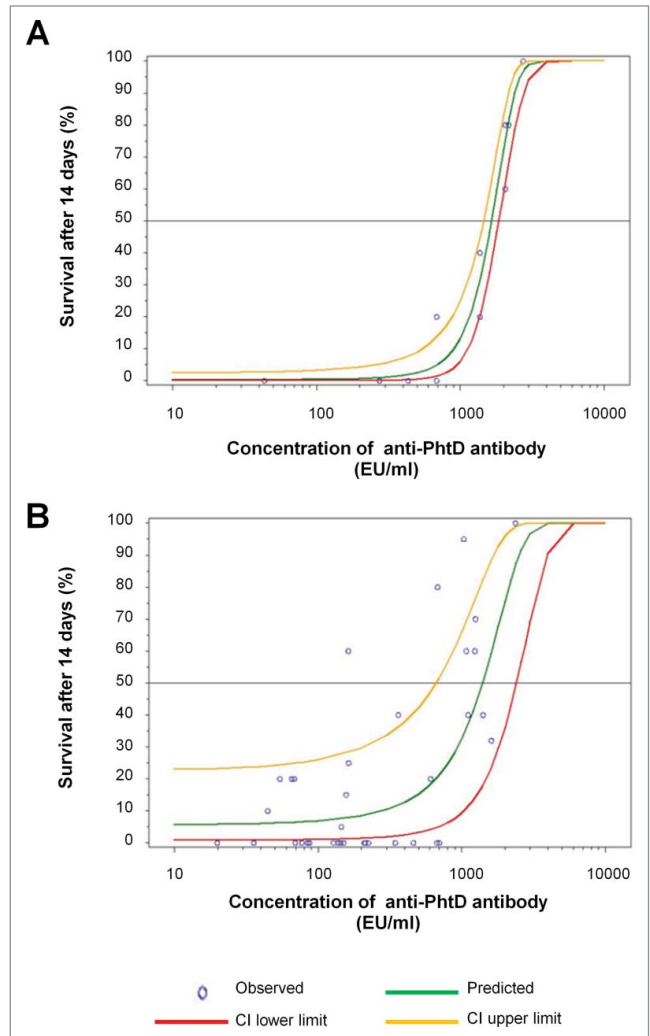


Figure 1. Dose-response of antibody activity in the passive protection model. 6- to 8-week-old female naïve CBA/N mice ($n = 5$ /group) received an intraperitoneal injection of 200 μ l test sample or PBS. Control mice received PBS. After 1 h, mice were challenged intravenously with a lethal dose of *S. pneumoniae* strain A66.1 (serotype 3), and survival was monitored for 14 days. Data were analyzed using logistic regression with probit link under PROC GLIMMIX in SAS version 8.2 to determine the ED50. In each plot, circles indicate survival data for individual samples, and the best fit regression is shown as a green line, with the upper and lower limits of the 95% confidence interval shown as yellow and red lines, respectively. The horizontal line indicates a median response of 50% survival. In (A), mice were injected with 2.8–27.5 EU (1–10 μ g) of purified anti-PhtD antibody or PBS. Survival data were from 4 passive protection experiments, which included 13 survival points. In five of the 13 cases (206, 275, 687, 1375, 2063, and 2750 EU/ml), the passive protection experiment was performed twice, and for 2 of these (275 and 2750 EU/ml), the points overlap and appear as a single data point. Protection experiments for 3 cases (43, 435, and 2178 EU/ml) were not repeated and were included to provide a more appropriate range of antibody concentrations. In (B), mice were injected with 1:20 to 1:60 post-immune sera. To account for the baseline levels of protection seen in the pre-immune sera, the analysis was corrected for over-dispersion using PROC GENMOD with the SCALE = option in SAS version 9.13 and based on survival data from mice that received pre- and post-immune sera.

Table 1. Anti-PhtD titers and survival mediated by the 18 selected paired pre-/post-immune sera

Subject	Vaccine dose	Anti-PhtD titer (EU/ml)			Mice surviving at day 14 (n/N)			Significant difference (P<0.05)	
		Pre-immune serum (week 0)	Post-immune serum (week 8)	Difference	Serum dilution tested	Pre-immune serum (week 0)	Post-immune serum (week 8)	Delay to death	Protection
4	6 µg	163	1252	1088	1:20	1/5	3/5		
6	6 µg	85	345	261	1:20	0/5	0/5		
8	6 µg	138	225	86	1:20	0/5	0/5		
15	6 µg	87	214	127	1:20	0/5	0/5		
20	6 µg	66	363	297	1:20	1/5	2/5		
21	6 µg	77	157	80	1:20	0/5	2/5	X	
23	6 µg	210	610	400	1:20	0/5	1/5		
25	25 µg	137	1349	1212	1:40	1/5	0/5		
36	25 µg	109	1364	1256	1:40	1/5	4/5	X	
37	25 µg	145	1039	893	1:20	0/5	5/5	X	X
41	25 µg	20	463	443	1:20	0/5	0/5	X	
43	25 µg	89	2478	2389	1:40	1/5	5/5	X	X
45	100 µg	69	1412	1343	1:20	0/5	2/5	X	
51	100 µg	256	1404	1148	1:40	0/5	0/5	X	
56	100 µg	302	2223	1921	1:40	0/5	2/5		
57	100 µg	107	3239	3132	1:60	0/5	3/5	X	
58	100 µg	326	4743	4417	1:40	6/10	10/10	X	
61	100 µg	288	3231	2944	1:40	0/10	4/10	X	

In the phase I clinical trial, healthy adults were vaccinated twice (week 0, 4) with the candidate aluminum phosphate-adsorbed PhtD vaccine.¹⁵ Dilutions (1:20) of pre-immune sera (200 µl) were passively transferred by intraperitoneal injection to 6- to 8-week-old female naïve CBA/N mice (n = 5/group). After 1 h, the mice were challenged by intravenous injection of a lethal dose (50 cfu in 200 µl) of *S. pneumoniae* strain A66.1 (serotype 3). Survival was followed for 14 days. Pre-immune sera that were protective at a 1:20 dilution and for which corresponding post-immune sera (week 8) had an increase in titer of at least 1000 EU/ml was further tested at a 1:40 dilution. Pre-immune sera shown to be protective at a 1:40 dilution and for which corresponding post-immune sera had an increase in titer of at least 2000 EU/ml over baseline were further tested at a 1:60 dilution. Using this method, appropriate dilutions for testing were identified for the 18 subjects shown. Paired pre-immune and post-immune sera from these subjects were tested in the passive protection assay, and survival after 14 days is shown. Delay to death was assessed by survival distribution functions using the product-limit approach and compared between groups of mice by log-rank test using PROC LIFETEST in SAS version 9.13. Differences in protection were compared by one-sided Fisher's exact test using PROC FREQ with the exact option in SAS version 9.13.

survival at day 14 compared to the pre-immune sera, and sera from subject nos. 37 and 43 were selected because the post-immune sera increased survival at day 14 and delayed death compared to the pre-immune serum. We also selected sera from subject no. 21 because the post-immune serum had weak or no functional activity, as indicated by a non-significant delay to death (P = 0.06) and small increase (79.9 EU/ml) in anti-PhtD titer compared to the pre-immune serum. Prior to testing in the passive protection model, the selected serum pairs were incubated for 1 h with recombinant PhtD to block PhtD-binding antibodies. In parallel reactions, sera were incubated with LytB, another *S. pneumoniae* surface protein, as a control. Compared to the pre-immune sera, post-immune sera from all subjects except no. 21 significantly increased mouse survival when incubated with LytB, whereas survival was not significantly increased by any of the post-immune sera incubated with PhtD (Table 2). Therefore, most or all of the protective activity in the post-immune sera was due to anti-PhtD antibodies.

In summary, our results show that the candidate PhtD vaccine induces anti-PhtD antibodies that can protect against *S. pneumoniae* infection. Another PhtD candidate vaccine, adjuvanted with AS02_V, was recently reported to be immunogenic and well tolerated in adults and to induce antibodies that passively protect mice against a lethal dose of *S.*

pneumoniae.^{16,17} Vaccination with the AS02_V-adjuvanted candidate vaccine increased the protective activity in sera from older but not younger adults. The authors concluded that the difference between the 2 age groups was due to higher baseline activity in the younger adults. In the current study, we tried to avoid interference from such baseline functional activity by testing serum dilutions at which the corresponding pre-immune sera was not protective. In addition, we performed competition experiments to confirm that the functional activity in the sera was specifically due to anti-PhtD antibodies and not to antibodies to other pneumococcal antigens, which could have arisen from a *S. pneumoniae* infection during the trial.

According to logistic analysis, naturally acquired and vaccine-induced antibodies had overlapping activities in the passive protection model (ED₅₀ = 1679 EU/ml [95% CI, 1420–1946] for naturally acquired antibodies vs. 1331 EU/ml [95% CI, 762–2038] for vaccine-induced antibodies). Another study also found that naturally acquired anti-PhtD antibody from human serum can protect mice against a lethal *S. pneumoniae* intranasal challenge; however, the study did not examine the relationship between functional activity and vaccination, antibody quantity, or antibody quality.¹³ Accordingly, our current results indicate that the candidate PhtD

Table 2. Specificity of vaccine-induced antibody

Subject	Dilution tested	Survival, n (%)					
		+ LytB			+ PhtD		
		Pre-immune	Post-immune	P-value	Pre-immune	Post-immune	P-value
4	1:20	4 (26.7)	11 (73.3)	0.013	1 (6.7)	3 (20.0)	NS
21	1:20	0 (0)	1 (6.7)	NS	1 (6.7)	0 (0.0)	NS
37	1:20	1 (6.7)	14 (93.3)	<0.001	0 (0.0)	0 (0.0)	NS
43	1:40	1 (6.7)	7 (46.7)	0.018	0 (0.0)	2 (13.3)	NS
61	1:40	0 (0.0)	4 (26.7)	0.0498	0 (0.0)	0 (0.0)	NS

Paired pre- and post-immune sera from subject nos. 4, 21, 37, 43, and 61 were incubated at the indicated dilutions with 20 µg/ml PhtD or LytB proteins for 1 h. The sera (200 µl) were then administered by intraperitoneal injection to 6- to 8-week-old female naïve CBA/N mice (n = 15/group). After 1 h, the mice were challenged intravenously with a lethal dose (50 cfu in 200 µl) of *S. pneumoniae* strain A66.1 (serotype 3), and survival was monitored for 14 days. P-values were calculated using a one-sided Fisher's exact test comparing survival in mice injected with matched post-immune and pre-immune sera in SAS version 8.2. NS, not significant (P ≥ 0.05).

vaccine increases functional activity, at least in part, by increasing the antibody concentration. Whether vaccination also improves antibody quality is less clear. Our preliminary epitope mapping studies and experiments measuring serum binding to bacterial surfaces suggest that vaccination can also expand the epitope repertoire of anti-PhtD antibodies (data not shown).

Collectively, these results showed that human anti-PhtD antibodies, whether naturally acquired or induced by the PhtD candidate vaccine, are functional. This supports the development of the PhtD candidate as part of a broadly protective pneumococcal vaccine.

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Disclosure of Potential Conflicts of Interest

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