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Preclinical Evaluation of the Pht Proteins as Potential Cross-Protective Pneumococcal Vaccine Antigens[▽]

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Current pneumococcal vaccines are composed of capsular polysaccharides (PS) of various serotypes, either as free PS or as protein-PS conjugates. The use of pneumococcus protein antigens that are able to afford protection across the majority of serotypes is envisaged as a relevant alternative and/or complement to the polysaccharides. In this context, based on several studies, the Pht protein family emerged as relevant vaccine candidates. The purpose of the present study was to evaluate the Pht protein family in several preclinical mouse models. Immunization with these antigens was compared with immunization with other pneumococcal antigens, such as CbpA, PspA, and PsaA. In a nasopharyngeal colonization model and in a lung colonization model, the Phts were found to be superior to the other candidates in terms of efficacy of protection and serotype coverage. Likewise, vaccination with PhtD allowed higher animal survival rates after lethal intranasal challenge. Finally, a passive transfer model in which natural anti-PhtD human antibodies were transferred into mice demonstrated significant protection against lethal intranasal challenge. This indicates that natural anti-PhtD human antibodies are able to protect against pneumococcal infection. Our findings, together with the serotype-independent occurrence of the Phts, designate this protein family as valid candidate antigens to be incorporated in protein-based pneumococcal vaccines.

Streptococcus pneumoniae is responsible for a large spectrum of infections, such as otitis media, meningitis, and pneumonia (23, 34). Despite the availability of vaccination, this pathogen is still a leading cause of morbidity and mortality worldwide, especially in high-risk populations, such as infants, the elderly, and immunocompromised individuals.

Current vaccines are composed of epidemiologically dominant serotype-specific pneumococcal capsular polysaccharides, conjugated or not to a carrier protein (12, 16, 33, 48). These vaccines have greatly helped to reduce the burden of pneumococcal diseases. However, the exclusive serotype-specific protection can be an issue in some regions of the world where serotypes other than those included in the vaccine are dominant (11). In addition, serotype-based vaccination is suspected of enabling the carriage of, and possibly the occurrence of disease associated with, nonvaccine serotypes (29, 39, 47). For these reasons, efforts are being made to investigate pneumococcal protein candidates, ideally virulence factors common to all serotypes, that may be used in pneumococcal vaccines either alone or in combination with capsular polysaccharide conjugates. In that respect, the use of several proteins has been envisaged. Examples include the cholesterol-binding cytotoxin pneumolysin (Ply) or its nontoxic genetically derived pneumolysin toxoid mutants (2, 17, 44); the choline-binding proteins PspA, CbpA (PspC), and PcPA (7, 10, 13, 20); the metal-binding lipoprotein PsaA (4, 15); the iron uptake ABC transporters PiuA and PiaA (8, 49); the putative lipoate protein ligase (Lpl) and the ClpP protease (37); and the cell wall separation protein of group B streptococcus (PcsB) and serine/threonine protein kinase (StkP) (19). In the studies cited above, the antigens were shown to afford a significant level of protection against systemic challenge with one or several pneumococcal strains, but the protection remained lower than that obtained with polysaccharide-based vaccines. For broader protection, combinations of these antigens were tested, with different outcomes depending on the model and the antigens used (5, 6, 8, 40). The combination of PdB, PspA, and CbpA was claimed to offer the best protection (41). However, immunological cross-reactivity of these antigens across pneumococcal strains might not always be optimal (30, 36, 38, 46). More recently, another combination, including Ply, Lpl, and ClpB, was shown to be effective in animal models against different selected pneumococcal strains (51). Nevertheless, the search for common pneumococcus antigens able to afford protection against the majority of serotypes is still a concern.

In this context, the Pht protein family is worth considering. These proteins, which are restricted to the genus *Streptococcus*, are characterized mainly by a histidine triad motif that is repeated five to six times in their amino acid sequences. Four members of this family, with high sequence conservation between them, have been described: PhtA, PhtB, PhtD, and PhtE (1). The family possesses several assets to qualify them as valid vaccine candidates. Phts are well conserved across the pneumococcal species (21, 52), and members of the family have been shown to be antibody targets in infected individuals (3, 26). Results from antibody-labeling and flow cytometry experiments demonstrated that the Pht proteins are exposed on the surface of the encapsulated bacterium (21). In mouse immunization studies, all members of the Pht family have been shown to afford a high level of protection in pneumococcal animal challenges with a number of different strains/serotypes (1, 3, 21, 41, 50, 52). In addition, by signature-tagged mutagenesis, it has been suggested that PhtA, PhtB, and PhtD are

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involved in lung-specific virulence (24). Although the function of this protein family remains largely unknown, it has been recently suggested that these proteins are required to inhibit complement deposition on the bacterial surface through the recruitment of complement factor H (42). Other studies pointed out the affinity of the Phts for divalent cations, particularly zinc (1, 43, 45), which led us to the conclusion that these proteins are major actors in zinc homeostasis (45a).

In the present work, the potential of Pht proteins to be incorporated in pneumococcal vaccines was further evaluated by using various animal challenge models.

MATERIALS AND METHODS

Animals. The OF1 and CBA/J female mice used in this study were purchased from Charles River Laboratories (Lyon, France). BALB/c mice were from Harlan (Horst, Netherlands). All experiments and assays were performed at GlaxoSmithKline Biologicals (GSK, Rixensart, Belgium) in accordance with the Belgian national guidelines for animal experimentation.

Bacteria. Strain 2/D39 was kindly provided by J. C. Paton (University of Adelaide, Australia). Strains 4/CDC and 6B/CDC were obtained from the Centers for Disease Control and Prevention (CDC), and the 19F/2737 strain was obtained from the American Type Culture Collection (ATCC). Strain 3/43 was provided by E. Yourassowski (Brugmann Hospital, University of Brussels, Brussels, Belgium).

Antigens. CbpA (or PspC) is a truncated recombinant protein, as described in reference 7, and was kindly provided by J. C. Paton. The protein was constructed from the sequence of the D39 strain and thus belongs to clade A. PspA (clade 2) and PsaA are recombinant proteins originating from the 2/D39 strain (40); both were provided by J. C. Paton.

The Pht proteins were cloned from the N4 strain (serotype 4) and produced in *Escherichia coli*. All Pht proteins except PhtD were His tagged. The proteins were purified from bacterial lysate through multiple chromatography steps.

Determination of humoral responses. Sera were taken from the immunized mice on the day of the challenge. The levels of specific antibodies were determined by enzyme-linked immunosorbent assay (ELISA). Briefly, an ELISA microtiter plate was coated with PhtD, PspA, or CbpA (1 μg/ml in phosphate-buffered saline [PBS]) for 2 h at 37°C for the detection of anti-PhtD, anti-PspA, or anti-CbpA antibodies, respectively. After washing, serial dilutions of the respective sera in PBS–0.05% Tween 20 (PBS-T) were added to the microtiter plate and incubated overnight at 4°C. The reference wells were coated with polyclonal goat anti-mouse antibodies (Jackson Immunoresearch; 1 μg/ml in PBS), and serial dilutions of mouse IgG whole molecule (Jackson Immunoresearch; 1 μg/ml in PBS-T) were added. After washing, peroxidase-conjugated goat anti-mouse IgG (Jackson Immunoresearch; 1/5,000 in PBS-T) was added to the reference and sample wells for 20 min of incubation at room temperature. Afterwards, the plate was revealed by addition of the peroxidase substrate *o*-phenylenediamine in 0.1 M citrate buffer, pH 4.5, in the presence of hydrogen peroxide. The colorimetric reaction was stopped by addition of 1 N HCl, and the plate was read in an ELISA reader at 490 and 620 nm.

Mouse pneumococcal lethal intranasal challenge. To evaluate the protection afforded by the members of the Pht family in a mouse pneumococcal intranasal challenge, OF1 female mice (4 weeks old; $n = 20$/group) were immunized intramuscularly (i.m.) at days 0 and 14 with 1 μg of PhtD, PhtA, PhtB, or PhtE formulated with the AS02 adjuvant system, which consists of an oil-in-water emulsion supplemented with 3-*O*-desacyl-4′-monophosphoryl lipid A (MPL) and QS21 (18). Control animals were injected with AS02 only. At day 28, the mice were challenged intranasally with the type 3/43 pneumococcal strain (10^5 CFU in 50 μl). Mortality was recorded for 10 days after the challenge.

In other experiments, vaccination with 1 μg of PhtD was compared with vaccination with 10 μg of PspA or 10 μg of CbpA. All antigens were formulated with the AS04 adjuvant system, consisting of aluminum salts with MPL (18). The i.m. immunizations occurred at days 0, 14, and 28. Control animals were vaccinated with adjuvant only. At day 42, the mice were challenged intranasally with *S. pneumoniae* type 4/CDC (5×10^6 CFU), type 2/D39 (2×10^5 CFU), or type 3/43 (10^5 CFU) in 50 μl. The mortality was recorded for 10 days after the challenge.

Mouse nasopharyngeal colonization model. BALB/c mice (4 weeks old; $n = 10$/group) were immunized at days 0, 14, and 28 by the intranasal route with 5 μg of PhtD, PhtA, PhtB, or PhtE supplemented with 0.2 μg of *E. coli* labile toxin (LT) as an adjuvant (except in the last immunization). Another experiment with the same protocol (schedule and dosages) consisted of comparing PhtD with CbpA, PsaA, and PspA. Control mice were injected with LT alone. At day 42, mice were challenged intranasally with 7×10^4 CFU of the type 6B/CDC, type 4/CDC, or type 2/D39 strain. The challenges were performed using a small bacterial inoculum volume (10 μl). Bacterial colonies in nasal washings collected 2 and 6 days after the challenge were counted. Nasal washings were obtained by flushing 500 μl of PBS inside the nasal cavities of anesthetized mice. Next, to count the bacterial colonies, 100 μl of nasal washing was diluted 10-fold in Todd-Hewitt broth. From this, 10 μl was plated onto Difco blood agar base supplemented with defibrated, sterile sheep blood and gentamicin (3 μg/ml). The petri dish was tilted to spread the sample, and the colonies were enumerated after incubation overnight at 37°C.

Mouse lung colonization model. The mouse lung colonization model was adapted from that described by Briles et al. (6). CBA/J female mice (4 weeks old; 30/group) were immunized i.m. at days 0, 14, and 28 with 3 μg of PhtD adjuvanted with AS02. At day 42, the mice were challenged intranasally with 2×10^7 CFU/50 μl of *S. pneumoniae* 19F/2737. Control mice were injected with adjuvant only. The bacterial load was measured by colony counting in lungs collected at 3, 4, and 5 days postchallenge.

Passive transfer of natural human PhtD antibodies. Human anti-PhtD antibodies from naturally exposed human sera were purified by means of an affinity column. This column was prepared by coupling PhtD to CNBr-activated Sepharose 4B (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions. Bound antibodies were eluted from the column with 0.1 M glycine, pH 2.5, and immediately dialyzed against phosphate-buffered saline. To ascertain purity, the purified anti-PhtD preparation was used in ELISA to screen different pneumococcal antigens. There was no reaction to Ply, CbpA, PS3, CPS, PspA, PsaA, or SP91. However, cross-reactions with PhtA and PhtB were observed.

Sixty (medium dose) or 20 (low dose) μg of human anti-PhtD antibodies was transferred intraperitoneally into OF1 mice (8 weeks old; $n = 20$/group) at 1 h before intranasal challenge with 10^5 CFU/50 μl of the *S. pneumoniae* type 3/43 strain. Alternatively, as a reference, 100 μg (high dose) of rabbit immunization-induced anti-PhtD was transferred in another group of mice. Control animals were injected with PBS. The mortality induced by infection was monitored for 10 days.

Statistical analyses. Survival data were analyzed with the Mantel-Haenszel log rank test. All colony counting data, after normalization, were compared by analysis of variance (ANOVA), followed by the Dunnett posttest when results were found to be significant by ANOVA.

RESULTS

Immunization with Pht proteins confers protection in a mouse pneumococcal lethal intranasal challenge model. Mice were vaccinated with the different Pht proteins individually. After challenge with the *S. pneumoniae* 3/43 strain, mouse survival was recorded for 10 days (Fig. 1). The results indicate that vaccination with either of the Pht proteins allowed the survival of approximately 60% of mice, while only 20% of the animals in the control group survived.

In subsequent experiments, other groups of animals were vaccinated with three different pneumococcal antigens, i.e., PspA, CbpA, and one protein of the Pht family, PhtD. The extent of the humoral response was evaluated, and the animals were challenged with three different pneumococcal strains at 2 weeks after the last immunization. Mouse survival was recorded for all antigen/strain combinations.

The resulting levels of antibodies were antigen dependent (Fig. 2A). Vaccination with 1 μg of PhtD elicited higher antibody titers than vaccination with 10 μg of CbpA or PspA. Nevertheless, the levels of protection against intranasal lethal challenge with the 2/D39 strain, from which CbpA and PspA originate, were similar for the three antigens, with around 70% survival (Fig. 3A). Differences between the antigens were evidenced when other strains were used. Indeed, vaccination with PhtD allowed 60% and 80% of mice to survive the challenge with the 3/43 and the 4/CDC strains, respectively. In contrast,

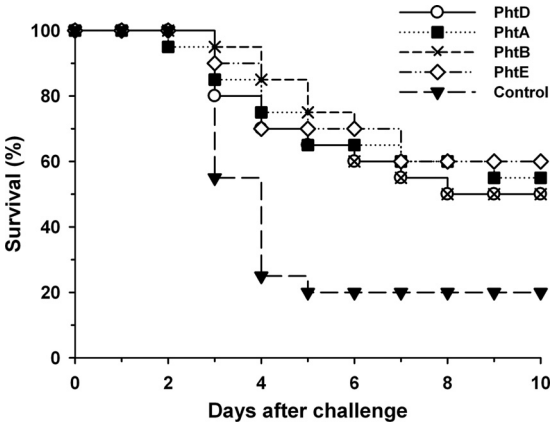

FIG. 1. Mouse survival upon lethal *S. pneumoniae* intranasal challenge. Mice (n = 20/group) were immunized with AS02-adjuvanted PhtD, PhtA, PhtB, or PhtE or with AS02 alone (control) before they were challenged with the type 3/43 pneumococcal strain. Statistical analyses were carried out with the log rank test, compared with the control: PhtD, $P = 0.0126$; PhtA, $P = 0.0103$; PhtB, $P = 0.0038$; PhtE, $P = 0.0033$.

CbpA and PspA afforded no or very weak protection against the type 3 and 4 challenges. PhtD was thus the only antigen able to afford protection against the three strains.

Immunization with Pht proteins protects mice against *S. pneumoniae* nasopharyngeal colonization. To assess the protective activity of vaccination against nasopharyngeal carriage, BALB/c mice were immunized intranasally with the different Pht proteins before they were challenged via the same route with the 2/D39 strain. As can be seen in Fig. 4, although only vaccination with PhtD or PhtE afforded significant protection against the challenge with the type 2 strain, all members of the Pht family were able to reduce the bacterial loads in the nasopharynges of the vaccinated animals. Due to the better performance of PhtD in this model, this member of the Pht family was chosen for further experiments, which consisted of comparing PhtD with other pneumococcal proteins. Therefore, mice were immunized with different pneumococcal antigens, including PhtD, and were subsequently challenged with a type 2, a type 4, or a type 6B strain.

As was observed after systemic immunization, the humoral responses elicited after intranasal immunization were antigen dependent (Fig. 2B). Particularly, CbpA elicited lower antibody titers than PspA and PhtD. However, the level of protection afforded by CbpA against the clade-homologous 2/D39 strain was similar to that afforded by PspA and PhtD (Fig. 5A).

When the type4/CDC strain was used for the challenge, only immunization with PhtD could protect the animals against nasopharyngeal colonization, whereas immunization with CbpA, PsaA, or PspA was not statistically distinguishable from treatment with the LT control (Fig. 5B). Finally, there was no difference in protection at day 2 postchallenge with type 6B/CDC whether the animals were immunized with CbpA, PspA, or PhtD (Fig. 5C). Only PsaA seemed to be less efficient in that respect. At day 6 postchallenge, there was no statistical difference between all groups. However, a careful examination of the results for PhtD revealed that the majority of animals were protected against nasopharyngeal colonization and that the unfavorable statistical conclusion was probably due only to the presence of two outliers. In conclusion, PhtD was the only antigen able to afford some protection against the three strains in this model of nasopharyngeal colonization.

Immunization with PhtD protects mice against *S. pneumoniae* lung colonization. CBA/J mice, a strain susceptible to pneumococcal infections, were vaccinated with PhtD before they were challenged with a moderately virulent 19F bacterial strain. Such a protocol allows for the induction of a focal pneumonia without generalized sepsis. The number of living bacteria in the lungs was evaluated at days 3, 4, and 5 after the challenge.

It was shown that vaccination with PhtD reduced the bacterial load in the lungs to a great extent (more than 95%) compared with placebo treatment (Fig. 6). The efficacy of PhtD vaccination was particularly evident when analyzing the number of noncolonized mice, since up to 80% of vaccinated mice remained free of bacteria at day 5, compared with 10% in the control group (Fig. 6).

Natural response of human adults to PhtD. The biological functionality of naturally acquired human anti-PhtD antibodies was evaluated by passive transfer of immunoaffinity-purified human antibodies into naïve recipient mice. The passive

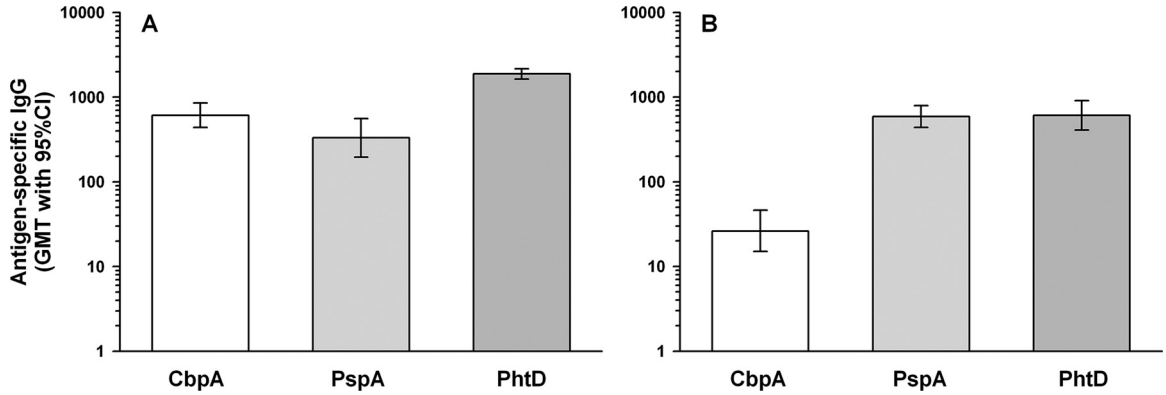

FIG. 2. Antibody levels after immunization. (A) Mice were immunized systemically with AS02-adjuvanted CbpA, PspA, or PhtD. (B) Mice were immunized intranasally with LT-adjuvanted CbpA, PspA, or PhtD. In both cases, blood was taken on day 42, and the levels of specific antibodies were measured by ELISA. Error bars indicate 95% confidence intervals (95% CI).

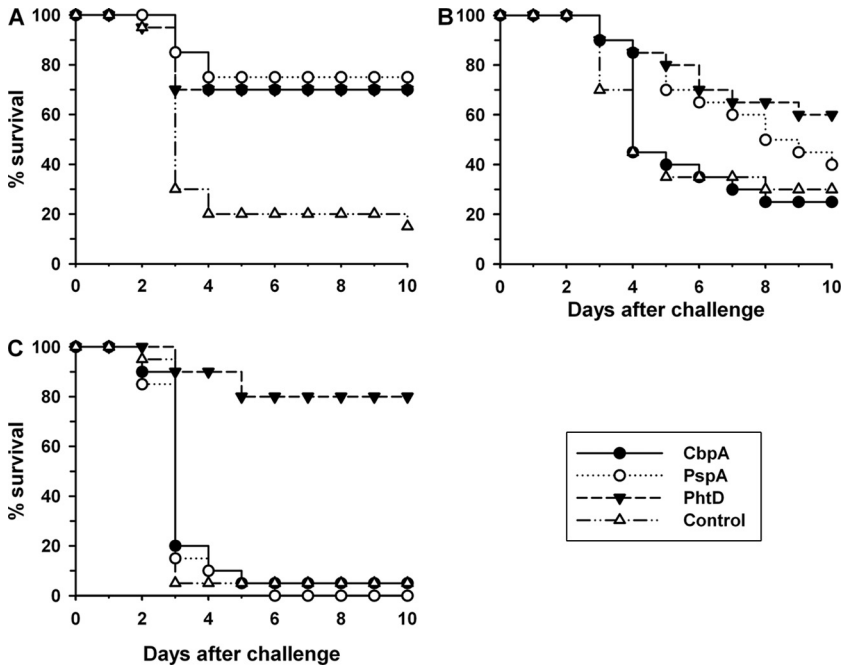

FIG. 3. Mouse survival upon lethal *S. pneumoniae* intranasal challenge. Mice were immunized with AS02-adjuvanted CbpA, PspA, or PhtD or with AS02 alone (control) before they were challenged with the type 2/D39 (A), type 3/43 (B), or type 4/CDC (C) pneumococcal strain. Statistical analyses were carried out with the log rank test, compared with control. (A) CbpA, $P = 0.0002$; PspA, $P = 0.0001$; PhtD, $P = 0.0009$. (B) CbpA, $P = 0.885$; PspA, $P = 0.184$; PhtD, $P = 0.027$. (C) CbpA, $P = 0.825$; PspA, $P = 0.538$; PhtD, $P < 0.0001$.

immunization was performed 1 h before lethal intranasal challenge with a type 3 strain. The mortality induced by infection was monitored for 10 days. Immunization-induced anti-PhtD rabbit antibodies were used as a positive comparator. The results show that naturally induced human anti-PhtD antibodies were functional and efficient in preventing lethal infection (Fig. 7).

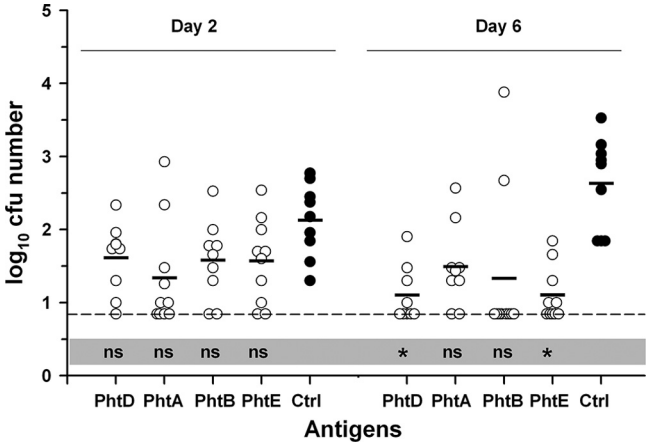

FIG. 4. Vaccine efficacy in an *S. pneumoniae* nasopharyngeal colonization model. BALB/c mice were immunized with PhtD, PhtA, PhtB, PhtE, or LT alone (control [Ctrl]) before they were intranasally challenged with the 2/D39 pneumococcal strain. Bacterial colonies in nasal washings were counted at day 2 and at day 6 postchallenge and expressed as $\log_{10}$ mean CFU. Each dot represents a mouse. Black horizontal bars are geometric means. The dashed line indicates the limit of detection (at 0.84). Statistical analyses were carried out per day with ANOVA. All significant differences, compared with the control, are shown. *, $P < 0.05$; ns, not significant.

DISCUSSION

Polysaccharide-based pneumococcal vaccines have helped to significantly reduce the incidence of pneumococcal diseases. To address the remaining disease due to nonvaccine types, common pneumococcal proteins are evaluated as vaccine candidates. In this context, the members of the Pht protein family were evaluated in various studies (1, 21, 41, 52). These proteins are well conserved across the various pneumococcal serotypes and have been described as virulence factors (24), making them promising vaccine candidates. Among their putative roles, neutralization of the complement factor C3b through factor H binding has been suggested (27, 42), which is further sustained by the fact that SHT, the group B *Streptococcus* Pht homolog, seems to bind factor H as well (32). However, other authors, through the use of Pht-deficient strains, concluded that these proteins do not bind factor H (35). In addition, due to the high number of histidine residues in the histidine triads, it has been suggested that the Pht proteins may be involved in DNA and/or metal binding (1). A study by our group (45a) suggested that Phts are regulators of metal homeostasis. As such, they may play a role in ion storage, particularly zinc storage, in order to make the ion available when the bacterium faces ion-restricted environments, as is the case during early stages of infection (9, 22). By targeting the Pht protein family, the immune system thus may impede the ability of the bacteria to store and use ions, which might be crucial for the invasion process. In the present work, we evaluated with several animal

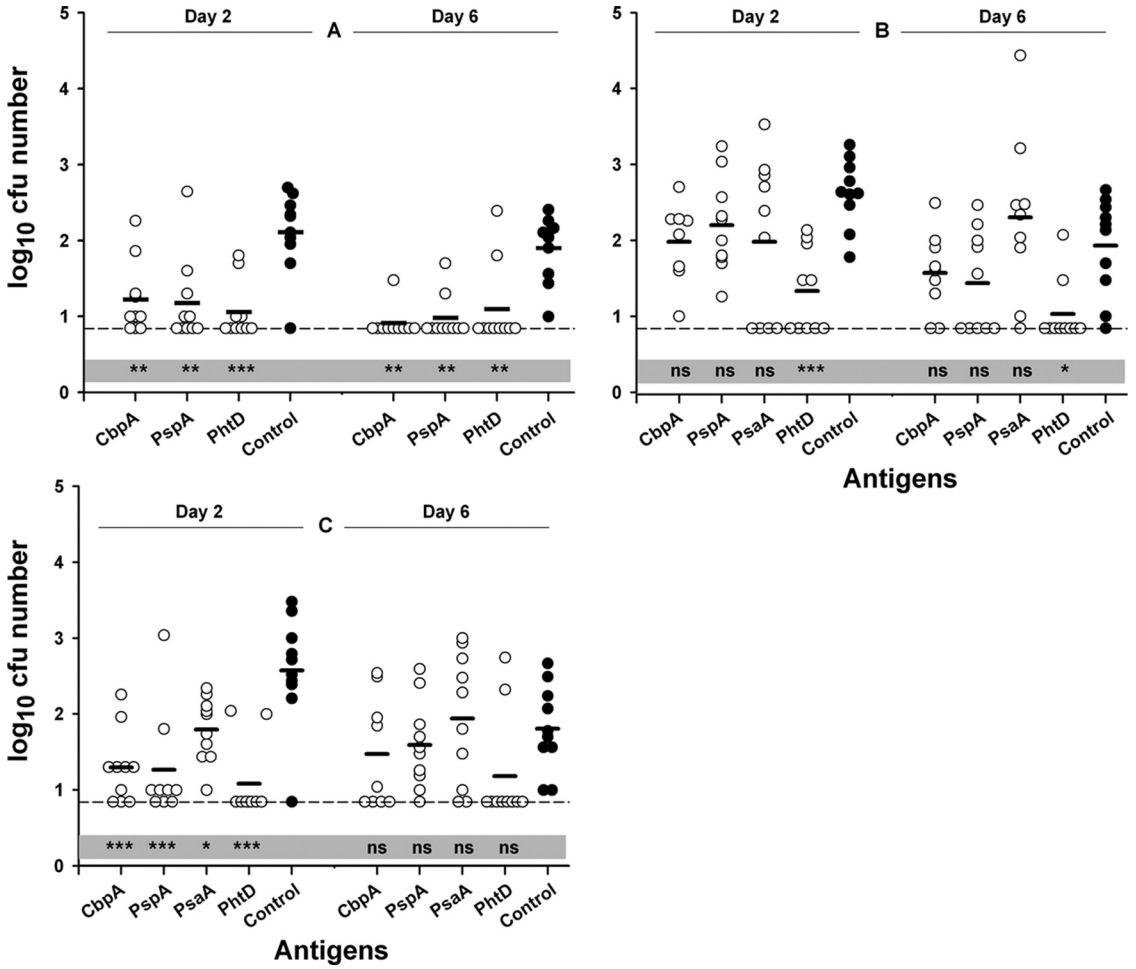

FIG. 5. Vaccine efficacy in an *S. pneumoniae* nasopharyngeal colonization model. BALB/c mice were immunized with either CbpA, PspA, PhtD, PsaA, or LT alone (control) before they were intranasally challenged with either the 2/D39 (A), the 4/CDC (B), or the 6B/CDC (C) pneumococcal strain. Bacterial colonies in nasal washings were counted at day 2 and at day 6 postchallenge and expressed as $\log_{10}$ mean CFU. Each dot represents a mouse. Dashed lines indicate the limit of detection (at 0.84). Black horizontal bars are geometric means. Statistical analyses were carried out per day with ANOVA. All significant differences, compared with the control, are shown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, ns, not significant.

challenge models the potential of the Pht protein family as vaccine candidates.

To assess the level of protection induced by Pht immunization, we used a mouse pneumococcal lethal intranasal challenge model, making use of a pneumococcal type 3 serotype for the challenge. In this model, all Phts induced similar levels of protection. The proteins were further used in a mouse nasopharyngeal colonization model. It is relevant to evaluate the Pht proteins in such model, since pneumococcal colonization of the nasopharynx precedes the development of invasive disease and is the main gateway of transmission of the pathogen between individuals. In a mouse nasopharyngeal colonization model, immunization with the Pht proteins, particularly PhtD and PhtE, was shown to protect against nasopharyngeal colonization. After demonstration of the efficacy of Pht immunization, it seemed relevant to compare it with that of other candidate antigens. To that end, we selected PhtD among the different members of the Pht family. Although immunization with PhtE was also significantly protective, it was estimated to be less representative of the family, since its amino acid sequence is most divergent from those of the other Phts.

PhtD was compared with other pneumococcal candidate antigens in pneumococcal lethal intranasal challenge models with serotype 2, 3, or 4 strains. The results showed that PspA and CbpA immunizations were efficient in protecting animals against type 2 pneumococcal challenge, but vaccination with these antigens afforded little or no protection against a challenge with the selected serotype 3 and 4 strains. This finding is in accordance with previous studies showing that different clades of PspA exist (25) and are not necessarily immunologically cross-reactive (14, 36, 38, 46). The PspA antigen used for immunization in our study originates from the type 2/D39 strain and belongs to clade 2. The *pspA* genes of the type 3/43 and type 4/CDC challenge strains were sequenced (data not shown), and it was determined that the respective encoded PspA antigens belong to clade 5 and clade 3, respectively, thus being different from the immunization clade. The fact that PspA vaccination protects only against homologous challenge

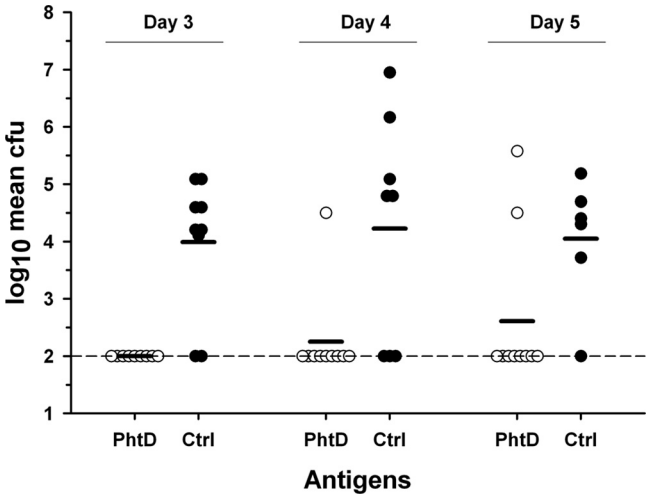

FIG. 6. Vaccine efficacy in an *S. pneumoniae* lung colonization model. CBA/J mice were immunized with AS02-adjuvanted PhtD or with AS02 only (Ctrl), before they were challenged with the moderately virulent 19F/2737 pneumococcal strain. Lungs were taken at day 3, 4, or 5 postchallenge, and the bacterial load was evaluated by colony counting (CFU). Each dot represents a mouse. The dashed line indicates the limit of detection (at 2). Black horizontal bars are geometric means. The groups were compared by two-way ANOVA over the 3 days, followed by the Tukey HSD test ($P < 0.0001$).

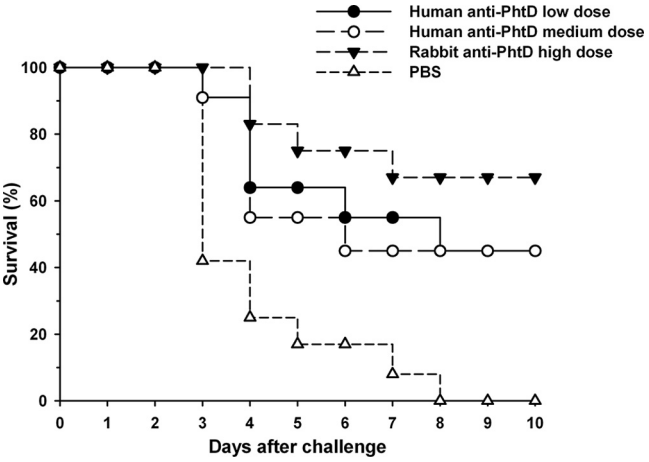

FIG. 7. Mouse survival upon lethal *S. pneumoniae* intranasal challenge. Naturally occurring human anti-PhtD antibodies (20 μg, low dose; 60 μg, high dose) were transferred into mice ($n = 20$/group) before the animals were challenged with the 3/43 strain. Anti-PhtD antibodies (100 μg) from PhtD-immunized rabbits were used as positive controls. Statistical analyses were carried out with the log rank test. Compared with PBS: human anti-PhtD low dose, $P < 0.0054$; human anti-PhtD high dose, $P < 0.0029$; rabbit anti PhtD, $P < 0.0001$.

strongly indicates that immunization with this antigen does not elicit cross-reactive immunity. The same applied to CbpA. Two different clades of CbpA have been referenced, namely, clade A and clade B (7). The recombinant CbpA used in our study is a clade A molecule, from the serotype 2/D39 strain. Immunization with this molecule protected against homologous challenge with type 2/D39 but not against heterologous challenge with type 3/43 or type 4/CDC. Absence of protection against the type 3/43 is understandable since this strain has a *hic* gene instead of *cbpA* (28), but the absence of protection against the type 4/CDC strain, expressing CbpA of clade A, is more difficult to interpret unless one considers the existence of intraclade immunogenic differences. Together, these results indicate that CbpA and PspA as single antigens may not be appropriate for incorporation into a pneumococcal vaccine that necessitates broad coverage. In contrast, we showed in this study that PhtD immunization seems to afford interstrain cross-protection, which might be related to the fact that PhtD is well conserved across pneumococcal serotypes (45a).

The same kind of observation was made when PhtD was compared with PsaA, PspA, and CbpA in the nasopharyngeal colonization model using three different pneumococcal strains. The data showed that only PhtD vaccination was able to protect mice against the different bacterial strains to the same extent, which indicates that vaccination with PhtD has the possibility of limiting carriage of various serotypes, while this promise is less with PsaA, PspA, and CbpA. Here again, the existence of the different clades of PspA and CbpA may be responsible for the absence of immunologic cross-reactivity. Indeed, the 6B/CDC strain that was used for the nasopharyngeal colonization model expresses PspA clade 3 (as determined by sequencing of the *pspA* gene [data not shown]), which is different from the vaccine clade. Concerning CbpA, 6B/CDC expresses a clade A molecule, which is similar to the vaccine clade, suggesting again that intraclade immunogenic differences exist.

In addition to prevention of nasopharyngeal colonization, we also showed in this study a reduction of the bacterial load in the lungs of mice nonlethally challenged with serotype 19F, which suggests that PhtD immunity protects mice against infection by interfering also with lung bacterial colonization.

It must be emphasized that protection against *S. pneumoniae* is suspected to be a two-pronged system. Although antibodies were shown in multiple experiments to mediate the protection against invasive disease, the cellular compartment of immunity, and more particularly the $CD4^+$ Th17 cells, has been proposed in the last few years to be the main effector of the protection against colonization (31). This has not been investigated in our experiments. However, the fact that different levels of specific antibodies were associated with a similar level of protection against nasopharyngeal colonization (and this was particularly evident for CbpA) indicates that other immune actors besides the antibodies that have been elicited may be important. Also, the fact that intranasal immunization was required to induce protection against nasopharyngeal colonization in our experiments may be explained by the need to induce responses other than systemic antibodies. Further investigation in our murine model is required to conclude whether the protection against colonization is mediated by T cells and/or by antibodies.

Finally, questions may arise as to whether PhtD-induced protection is a mouse-restricted phenomenon, inherent to an artificial immunization model. To address this question, we purified anti-PhtD antibodies from naturally exposed humans and could demonstrate, after transfer in mice, that these antibodies were able to afford protection against lethal challenge. This shows that anti-PhtD human antibodies are functional,

which is a good indicator of the relevance of a PhtD-based vaccine.

Several pneumococcal antigens have been proposed to be incorporated in pneumococcal vaccines. However, none of them combines high immunogenicity and protection with good conservation across the various pneumococcal strains. Therefore, there is still a need for more common pneumococcal antigens to afford broad cross-protection. In the present study, PhtD immunization was shown to prevent nasopharyngeal colonization by several different serotypes, to reduce the bacterial load in lungs of nonlethally challenged animals, and to prevent death from several serotypes in otherwise lethally challenged mice. These findings, together with the ubiquitous serotype-independent nature of the Pht proteins, designate this family as valid vaccine candidates.

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