Pertussis Toxin Improves Immune Responses to a Combined Pneumococcal Antigen and Leads to Enhanced Protection against Streptococcus pneumoniae

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Pneumococcal surface protein A (PspA) is a candidate antigen for the composition of protein-based vaccines against Streptococcus pneumoniae. While searching for efficient adjuvants for PspA-based vaccines, our group has described the potential of combining PspA with the whole-cell pertussis vaccine (wP). When given to mice through the nasal route, a formulation composed of PspA from clade 5 (PspA5) and wP (PspA5-wP) induced high levels of antibodies and protection against challenges with different pneumococcal strains. PspA5-wP also induced the secretion of interleukin 17 (IL-17) by splenocytes and the infiltration of leukocytes in the lungs after challenge. Here, we show that protection against a pneumococcal invasive challenge was completely abrogated in μMT mice, which are deficient in the maturation of B cells, illustrating the importance of antibodies in the survival elicted by the PspA5-wP vaccine. Moreover, passive immunization showed that IgG purified from the sera of mice immunized with PspA5-wP conferred significant protection to naive mice, whereas the respective F(ab’)2 did not. Additionally, in vivo depletion of complement abolished protection against the pneumococcal challenge. The combination of PspA5 with wild-type or mutant Bordetella pertussis strains or with purified components showed that the pertussis toxin (PT)-containing formulations induced the highest levels of antibodies and protection. This suggests that the adjuvant activity of wP in the PspA5 model is mediated at least in part by PT. The sera from mice immunized with such formulations displayed high IgG binding and induction of complement deposition on the pneumococcal surface in vitro, which is consistent with the in vivo results.

Streptococcus pneumoniae is an important cause of noninvasive infections, such as pneumonia and otitis media, as well as of invasive diseases, such as bacteremia and meningitis. The most affected population is children <5 years old, and estimates for the year 2000 accounted for around 1 million deaths in this age group around the world (1). Pneumococcal conjugated vaccines have greatly contributed to the decrease in this disease incidence in several countries (2, 3). However, epidemiologic studies in vaccinated populations have shown changes in the prevalences of serotypes, which may account for the decrease in vaccine efficacy after a period of use (4, 5).

Pneumococcal surface protein A (PspA) is a virulence factor that mediates evasion of the immune system by inhibiting the deposition of complement on the pneumococcal surface as well as the bactericidal activity of apolactoferrin present on mucosal surfaces (6, 7). Several proposals of protein-based vaccines as alternatives to conjugated vaccines include PspA. PspA-based vaccines were shown to be very effective against pneumococcal infections in animal models (8). The N-terminal part of PspA is exposed at the bacterial surface and contains protective epitopes (9, 10). However, this region also shows sequence variability between strains, and a portion at the end of the N-terminal region (the clade-defining region) is the basis for classifying PspAs in six clades that can be grouped into three families (11). More than 99% of the pneumococcal isolates around the world express PspAs from families 1 and 2 (12–14). Cross-reactivity between clades from the same family is observed (15, 16), suggesting that using one member from each family may be sufficient for designing a broad-coverage vaccine. In addition, some molecules, such as the PspA from clade 5 (PspA5) used in this work, were shown to induce antibodies with even broader cross-reactivity, as they can recognize molecules from different families (17, 18). We have shown that nasal immunization of mice with a formulation composed of PspA5 and a whole-cell pertussis vaccine (wP), used as an adjuvant, protects animals against challenges with different pneumococcal strains (19). Combining PspA5 with wP offers the benefit of the adjuvant properties of a vaccine administered to children at 2, 4, and 6 months in many countries in the world, with boosters at 15 months and 4 years of age (20).

The adjuvant properties of wP, alone or in diphtheria-tetanus-wP (DTwP) formulations, were already reported for different combined antigens (both in animal models and in humans). These include influenza, hepatitis B, conjugated Haemophilus influenzae B, and conjugated pneumococcal vaccines (21–26). wP is known to modulate immune responses toward Th1- and Th17-
type responses (27, 28), and several Bordetella pertussis components, such as lipopolysaccharides (LPS), pertussis toxin (PT), or adenylate cyclase toxin (ACT), contribute to this property (29-31). When nasally delivered to mice, the combination of PspA5 with wP (PspA5-wP) induces high levels of mucosal and systemic anti-PspA5 antibodies, with balanced IgG1-to-IgG2a ratios, antigen-specific interleukin 17 (IL-17) secretion by spleen cells, and controlled inflammatory responses in the respiratory tract after an invasive challenge with the S. pneumoniae ATCC 6303 strain (32). The depletion of CD4+ T, CD8+ T, or B lymphocytes in immunized mice during the pneumococcal invasive challenge did not impair survival (32). On the other hand, passive immunization of the total sera from mice immunized with PspA5-wP conferred protection to naive mice challenged with the ATCC 6303 pneumococcal strain (19). To further characterize the mechanisms of protection elicited by PspA5-wP, we address here the role of IgG and complement in this model. In addition, we evaluated the components of B. pertussis that are involved in the adjuvant activity to PspA5 in the wP context, and we analyzed the adjuvant activity of purified pertussis components in combination with PspA5.

MATERIALS AND METHODS

Bacterial strains and growth conditions. S. pneumoniae ATCC 6303 (serotype 5, PspA5a clade 5) was grown in Todd-Hewitt broth (Difco, Detroit, MI, USA) supplemented with 0.5% yeast extract (THY) at 37°C, without shaking. The bacteria were plated in blood agar and grown overnight at 37°C before inoculation in THY. The stocks were maintained at −80°C in THY containing 20% glycerol. The B. pertussis strains used in this work were BPSM (a streptomycin-resistant derivative of Tohama I) (33), BPLow (a BPSM derivative in which the entire bvgA gene and the 5′ portion of the bvgS gene, both from the Bordetella virulence control locus bvg/S, were deleted) (34), and BPRA (a BPSM derivative in which the ptx gene, which encodes the pertussis toxin, was deleted) (35). These strains were grown in Bordet-Gengou medium (Difco) supplemented with 1% glycerol, 20% sheep blood, and 100 μg/ml streptomycin, at 35°C. wP was derived from the B. pertussis NIH 137 strain and was produced at the Instituto Butantan, São Paulo, Brazil (36).

Recombinant proteins and vaccine formulations. The N-terminal fragment of PspA from clade 5 (from strain S. pneumoniae 122/02, Instituto Adolpho Lutz, São Paulo, Brazil) was expressed in Esherichia coli strain BL21-S1 (Invitrogen, Carlsbad, CA, USA) and purified by chromatography, as previously described (17). Inactivation of the different B. pertussis strains was performed according to a protocol based on the production of wP at the Instituto Butantan (São Paulo, Brazil). Briefly, the bacteria were grown as described above until the exponential phase. Formaldehyde (0.2% [vol/vol]) was added, and the samples were incubated for 24 h at 35°C. The bacteria were centrifuged, washed in saline, suspended in 1/10 of the original volume, and maintained at 4°C until use. Purified ACT and PT oligomer B (OligB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pertussis toxin (PT) and filamentous hemagglutinin (FHA) were purchased from Sigma-Aldrich and The Native Plant Proteins Co (St. Louis, MO, USA). Pertussis toxin (PT) and filamentous hemagglutinin (FHA) were purchased from Sigma-Aldrich and The Native Plant Proteins Co (St. Louis, MO, USA). Pertussis toxin (PT) and filamentous hemagglutinin (FHA) were purchased from Sigma-Aldrich and The Native Plant Proteins Co (St. Louis, MO, USA). Pertussis toxin (PT) and filamentous hemagglutinin (FHA) were purchased from Sigma-Aldrich and The Native Plant Proteins Co (St. Louis, MO, USA). Pertussis toxin (PT) and filamentous hemagglutinin (FHA) were purchased from Sigma-Aldrich and The Native Plant Proteins Co (St. Louis, MO, USA). Pertussis toxin (PT) and filamentous hemagglutinin (FHA) were purchased from Sigma-Aldrich and The Native Plant Proteins Co (St. Louis, MO, USA). Pertussis toxin (PT) and filamentous hemagglutinin (FHA) were purchased from Sigma-Aldrich and The Native Plant Proteins Co (St. Louis, MO, USA). Pertussis toxin (PT) and filamentous hemagglutinin (FHA) were purchased from Sigma-Aldrich and The Native Plant Proteins Co (St. Louis, MO, USA). Pertussis toxin (PT) and filamentous hemagglutinin (FHA) were purchased from Sigma-Aldrich and The Native Plant Proteins Co (St. Louis, MO, USA). Pertussis toxin (PT) and filamentous hemagglutinin (FHA) were purchased from Sigma-Aldrich and The Native Plant Proteins Co (St. Louis, MO, USA). Pertussis toxin (PT) and filamentous hemagglutinin (FHA) were purchased from Sigma-Aldrich and The Native Plant Proteins Co (St. Louis, MO, USA). Pertussis toxin (PT) and filamentous hemagglutinin (FHA) were purchased from Sigma-Aldrich and The Native Plant Proteins Co (St. Louis, MO, USA). Pertussis toxin (PT) and filamentous hemagglutinin (FHA) were purchased from Sigma-Aldrich and The Native Plant Proteins Co (St. Louis, MO, USA). Pertussis toxin (PT) and filamentous hemagglutinin (FHA) were purchased from Sigma-Aldrich and The Native Plant Proteins Co (St. Louis, MO, USA). Pertussis toxin (PT) and filamentous hemagglutinin (FHA) were purchased from Sigma-Aldrich and The Native Plant Proteins Co (St. Louis, MO, USA). Pertussis toxin (PT) and filamentous hemagglutinin (FHA) were purchased from Sigma-Aldrich and The Native Plant Proteins Co (St. Louis, MO, USA). Pertussis toxin (PT) and filamentous hemagglutinin (FHA) were purchased from Sigma-Aldrich and The Native Plant Proteins Co (St. Louis, MO, USA). Pertussis toxin (PT) and filamentous hemagglutinin (FHA) were purchased from Sigma-Aldrich and The Native Plant Proteins Co (St. Louis, MO, USA). Pertussis toxin (PT) and filamentous hemagglutinin (FHA) were purchased from Sigma-Aldrich and The Native Plant Proteins Co (St. Louis, MO, USA). Pertussis toxin (PT) and filamentous hemagglutinin (FHA) were purchased from Sigma-Aldrich and The Native Plant Proteins Co (St. Louis, MO, USA). Pertussis tox
harvested by centrifugation at 3,200 × g for 10 min. The bacteria were washed, suspended in phosphate-buffered saline (PBS), and incubated with 5% of the different sera for 30 min at 37°C. The samples were washed once with PBS before incubation for 30 min on ice with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (MP Biomedicals, Santa Ana, CA, USA) diluted 1:100 in PBS. For the complement deposition assays, the sera were preheated at 56°C for 30 min and incubated with bacteria at a concentration of 5% at 37°C for 30 min. The samples were washed once with PBS and incubated with 10% normal mouse serum as a source of complement in gelatin veronal buffer (Sigma-Aldrich), at 37°C for 30 min. After washing, the samples were incubated with FITC-conjugated anti-mouse C3 IgG (MP Biomedicals) in PBS for 30 min on ice. The samples were fixed with 200 µl of Cytofix (BD Biosciences) after two washing steps and stored at 4°C. Flow cytometry analysis was conducted using FACSCanto II (BD Biosciences, San Jose, CA, USA), and 10,000 gated events were recorded. Fluorescence was analyzed in histograms using the FlowJo 7.6.1 software, and the median values of the curves were used to compare the groups.

Statistical analysis. The differences in the antibody concentrations were analyzed by the Mann-Whitney U test. Overall survival was analyzed by Fisher’s exact test, and the survival curves were analyzed by the log rank test. Statistical analyses were performed using the Prism 5.03 software, and a P value of ≤0.05 was considered significant.

RESULTS
Protection elicited by PspA5-wP requires the induction of antibodies. Nasal immunization of mice with PspA5-wP was previously shown to induce high levels of antibodies and protect mice against respiratory challenges with different pneumococcal strains (19). The passive transfer of sera from immunized mice to naive mice was shown to confer survival to 75% of the animals after challenge with the ATCC 6303 pneumococcal strain, suggesting an important role for antibodies (19). On the other hand, the depletion of CD4+ T, CD8+ T, or B lymphocytes during the challenge did not impair protection in this model (32). In order to evaluate the role of antibodies in protection, C57BL/6 and C57BL/6 µMT−/− mice were immunized with 6 nasal doses of PspA5-wP. The control groups received wP only. This protocol was chosen since it induces high levels of anti-PspA5 antibodies and antigen-specific IL-17 secretion by splenocytes (19, 32). High levels of anti-PspA5 IgG were observed in the wild-type mice immunized with PspA5-wP but not in mice immunized with wP. As expected, no anti-PspA5 IgG was found in C57BL/6 µMT−/− mice immunized with PspA5-wP (see Fig. S1 in the supplemental material). The mice were then challenged through the intranasal route with a lethal dose of the ATCC 6303 pneumococcal strain. Whereas 100% of the vaccinated C57BL/6 mice survived the challenge, the vaccine did not protect C57Bl/6 µMT−/− mice (Table 1). Next, naive BALB/c mice were inoculated i.p. with sera from BALB/c mice immunized with six doses of PspA5-wP (PspA5-wP serum containing 50 µg of IgG per animal), 50 µg of IgG purified from these sera (PspA5-wP IgG), or from hyperimmune sera of mice immunized with a nonrelated antigen (NR IgG). The mice were challenged 2 h later with the ATCC 6303 pneumococcal strain. Both the PspA5-wP sera and the respective purified IgG conferred partial but significant protection to mice (50% and 41.6% survival, respectively) (Fig. 1A). No survival was observed in the group of mice immunized with NR IgG (P < 0.05 by Fisher’s exact test, or P < 0.01 by the log rank analyses of the survival curves, in a comparison of mice that received PspA5-wP IgG with mice that received NR IgG). To test the capacity of the F(ab′)2 derived from the PspA5-wP IgG to confer protection, 20 µg of IgG or the same molar mass of the respective F(ab′)2 (PspA5-wP F(ab′)2 or NR F(ab′)2) was inoculated i.p. into naive mice 2 h before challenge with the ATCC 6303 pneumococcal strain (Fig. 1B). In this experiment, reduced amounts of IgG were inoculated, since we had difficulties producing large amounts of purified F(ab′)2. Although only 33.3% of the mice inoculated with the PspA5-wP sera or PspA5-wP IgG survived the challenge under these conditions, the survival curves showed a significant increase in the survival time of mice inoculated with PspA5-wP IgG compared to that of mice inoculated with PspA5-wP F(ab′)2 (P < 0.01, log rank survival analyses). Importantly, both purified PspA5-wP

TABLE 1 Survival of mice against challenge with the ATCC 6303 pneumococcal strain: evaluation of the role of antibodies on protection induced by PspA5-wP vaccine

| Mouse group | Vaccination | No. of mice alive/total no. of mice | % survival | P value |
|-------------|-------------|---------------------------------|------------|---------|
| µMT−/−      | wP          | 0/4                             | 0          |         |
| PspA5-wP    | 0/4         | 0                               | 1          |         |
| C57BL/6     | wP          | 0/6                             | 0          |         |
| PspA5-wP    | 6/6         | 100                             | 0.005      |         |

*For the challenge, 1 × 105 CFU of the ATCC 6303 pneumococcal strain was inoculated through the nasal route, and survival was monitored for 10 days.

**By Fisher’s exact test. Comparisons were made with the respective group immunized with wP.

FIG 1 Effect of passive immunization on mouse survival against the respiratory challenge with the pneumococcal ATCC 6303 strain. Naive mice (6 per group) were inoculated i.p. with serum, purified IgG, or purified F(ab′)2 from mice immunized with PspA5-wP or with nonrelated antigen (NR) 2 h before the respiratory challenge with the ATCC 6303 pneumococcal strain. Survival was monitored for 10 days. The survival curves were analyzed by the log rank test. (A) Mice received serum containing 50 µg of IgG or the same amount of purified IgG; **, P = 0.001 in a comparison of mice that received PspA5-wP IgG with NR IgG. The results were collected from 2 independent experiments. (B) Mice received serum containing 20 µg of IgG, the same amount of purified IgG, or the same molar mass of purified F(ab′)2; **, P < 0.01 in a comparison of mice that received PspA5-wP serum or the respective purified IgG with mice that received purified F(ab′)2 from the same sample.
IgG and PspA5-wP F(\text{ab}')\text{2} were able to bind to the surface of the ATCC 6303 pneumococcal strain in vitro. However, the deposition of complement C3 on the surface of the bacteria was induced only by PspA5-wP IgG (see Fig. S2 in the supplemental material).

**Protection elicited by PspA5-wP depends on complement activity.** Since the Fc portion of IgG may contribute to the protection elicited by PspA5-wP, we next tested the role of complement in an *in vivo* model. BALB/c mice were vaccinated through the nasal route with 6 doses of PspA5-wP in order to induce high levels of anti-PspA5 IgG. The control mice received PspA5 or wP alone. As expected, high concentrations of antibodies were observed in all animals vaccinated with PspA5-wP (see Fig. S3 in the supplemental material). Twenty-four hours before challenge with the ATCC 6303 pneumococcal strain, a group of mice inoculated with PspA5-wP was treated with CVF (a snake toxin known to deplete complement components in serum) (42) via i.p. injection. This group received two additional CVF injections on days one and three after challenge. Survival monitoring showed that 100% of the mice vaccinated with PspA5-wP and treated with CVF succumbed to the infection (Table 2). In contrast, 100% of the mice vaccinated with PspA5-wP but not treated with CVF survived the challenge, and this was the only group that showed a statistically significant difference from the controls inoculated with wP (Table 2; \( P = 0.01 \), Fisher’s exact test). Two additional mice were vaccinated with PspA5-wP and treated with CVF but were not challenged. No signs of disease due to the three injections of CVF were observed (data not shown).

**PT-deficient *B. pertussis* strains are less effective as adjuvants for PspA5.** We have shown that the adjuvant activity of wP in PspA5-wP does not depend on the LPS present in the vaccine (19), suggesting that other components of *B. pertussis* exert adjuvant activity when combined with PspA5. In order to identify these components, we first determined the minimal dose of PspA5-wP that confers significant protection. A single nasal dose of PspA5-wP (containing 5 \( \mu \)g of the protein and 1/8 of the wP human dose) was enough to protect 80% to 100% of the BALB/c mice against challenge with the ATCC 6303 pneumococcal strain. Therefore, all the following experiments were performed under these conditions. We first tested preparations of the *B. pertussis* BPLOW strain, a mutant deficient for BvgA and thereby unable to produce different virulence factors, including PT and FHA (34). Nasal immunization of BALB/c mice with a single dose of PspA5-BPLOW induced high levels of anti-PspA5 IgG that were significantly higher than those observed in the group immunized with BPLOW or PspA5 alone (Fig. 2A). However, the levels of IgG induced by PspA5-BPLOW were slightly but significantly lower than those induced by PspA5-wP (Fig. 2A). Evaluation of the IgG subtypes showed no differences in the anti-PspA5 IgG1-to-IgG2a ratios induced by immunizing mice with PspA5-BPLOW or PspA5-wP, with slightly larger amounts of IgG1 than IgG2a in both cases (data not shown). After challenge with the ATCC 6303 pneumococcal strain, only 50% of the mice immunized with a single dose of PspA5-BPLOW were protected, and this result was at the limit of the significance compared with the nonimmunized group (\( P = 0.05 \)) (Table 3), but it did not reach significance in a comparison with mice immunized with BLOW or PspA5 (\( P > 0.05 \), Fisher’s exact test). In contrast, 83% of the mice immunized with PspA5-wP survived the challenge (Table 3), with a significant difference from the nonimmunized group or the groups immu-

| Vaccination given | No. of mice alive/total no. of mice | % survival | \( P^b \) |
|-------------------|-------------------------------------|------------|----------|
| wP                | 1/6                                 | 16.6       |          |
| PspA5             | 4/6                                 | 66.6       | 0.24     |
| PspA5-wP          | 6/6                                 | 100        | 0.01     |
| PspA5-wP (CVF)\(^c\) | 0/6                               | 0          | 1        |

\(^a\) For the challenge, \( 3 \times 10^5 \) CFU of the ATCC 6303 pneumococcal strain was inoculated through the nasal route, and survival was monitored for 10 days.

\(^b\) By Fisher’s exact test. Comparisons were made with the group immunized with wP.

\(^c\) Cobra venom factor (CVF) was administered 24 h before the challenge, and two additional injections were given at a 48-h interval.

**TABLE 2** Survival of mice against challenge with the ATCC 6303 pneumococcal strain: evaluation of the role of complement on protection induced by the PspA5-wP vaccine

**FIG 2** Induction of anti-PspA5 IgG by immunizing mice with PspA5 combined with different *B. pertussis* preparations. Serum samples were collected 21 days after the immunization. Anti-PspA5 IgG was measured by ELISA, using IgG standard curves as references. The data for each mouse are presented, with the median values of the groups (horizontal line). (A) Group that received PspA5 was significantly different from the nonimmunized group (Non); ***, \( P = 0.001 \). Significant differences (****, \( P < 0.0001 \)) were also observed in a comparison of the groups that received PspA5-BPLOW or PspA5-wP with the respective adjuvant controls or with the group immunized with PspA5. PspA5-wP also showed significant differences from the group immunized with PspA5-BPLOW (**, \( P < 0.01 \)). The results were collected from two independent experiments. (B) Significant differences (*, \( P < 0.05 \)) were observed in a comparison of the group immunized with PspA5-BPRA with the group immunized with BPR or with PspA5. Immunization with PspA5-BPSM or PspA5-wP induced significantly higher levels of anti-PspA5 IgG in a comparison with all other groups, including PspA5-BPRA (***, \( P < 0.001 \)). Statistical analyses were performed using the Mann-Whitney U test. The results are representative of two independent experiments.
TABLE 3 Survival of mice against challenge with the ATCC 6303 pneumococcal strain: evaluation of the adjuvant activity of a *B. pertussis*-attenuated strain

| Vaccination given | No. of mice alive/total no. of mice | % survival | P* |
|-------------------|------------------------------------|------------|----|
| Non              | 0/6                                | 0          |    |
| BPLOW            | 1/12                               | 8.3        | 1  |
| wP               | 2/12                               | 16.6       | 0.53 |
| PspA5            | 4/12                               | 33.3       | 0.24 |
| PspA5-BPLOW      | 6/12                               | 50.0       | 0.05 |
| PspA5-wP         | 10/12                              | 83.3       | 0.002 |

*For the challenge, 3 × 10^5 CFU of the ATCC 6303 pneumococcal strain was inoculated through the nasal route, and survival was monitored for 10 days. The results were collected from two independent experiments.

*Non, nonimmunized. BPLOW is a BPRA derivative in which the entire *bvgA* gene and the 5' portion of the *bvgS* gene were deleted.

**By Fisher's exact test. Comparisons were made with the nonimmunized group.

TABLE 4 Survival of mice against challenge with the ATCC 6303 pneumococcal strain: evaluation of the adjuvant activity of a *B. pertussis* mutant strain lacking PT

| Vaccination given | No. of mice alive/total no. of mice | % survival | P* |
|-------------------|------------------------------------|------------|----|
| Non              | 0/6                                | 0          |    |
| BPRA             | 0/6                                | 0          | 1  |
| BPSM             | 1/6                                | 16.7       | 1  |
| wP               | 0/6                                | 0          | 1  |
| PspA5            | 1/6                                | 16.7       | 1  |
| PspA5-BPRA       | 1/6                                | 16.7       | 1  |
| PspA5-BPSM       | 5/6                                | 83.3       | 0.02 |
| PspA5-wP         | 4/6                                | 66.7       | 0.06 |

*For the challenge, 3 × 10^5 CFU of the ATCC 6303 pneumococcal strain was inoculated through the nasal route, and survival was monitored for 10 days.

*Non, nonimmunized. BPSM is a streptomycin-resistant derivative of Tohama I. BPRA is a BPSM derivative in which the *ptx* gene was deleted.

**By Fisher's exact test. Comparisons were made with the nonimmunized group.

FIG 3 Induction of anti-PspA5 IgG by immunization of mice with PspA5 combined with different purified *B. pertussis* components. Serum samples were collected 21 days after the immunization. Anti-PspA5 IgG was measured by ELISA, using IgG standard curves as references. The data for each mouse are presented, with the median of the group (horizontal lines). (A) Significant differences (**, P < 0.01) were observed in a comparison of the groups that received PspA5-ACT, PspA5-FHA, or PspA5-PT with the nonimmunized group or with the respective adjuvant controls. Immunization with PspA5 induced significantly higher levels of antibodies (**, P < 0.05) than those in the nonimmunized group. *P < 0.05 and **P < 0.01. Statistical analyses were performed using the Mann–Whitney U test. The results are representative of two independent experiments.

Combination of PspA5 with purified PT increases the induction of antibodies and protection against the pneumococcal challenge. Since PT in the wP may be one of the molecules that expresses adjuvant activity in this model, we directly tested this and other purified *B. pertussis* components with known immunomodulating properties. As shown in Fig. 3A, the highest anti-PspA5 IgG levels were observed in BALB/c mice vaccinated with PspA5 combined with PT (PspA5-PT). Although the levels of anti-PspA5 IgG induced by the PspA5-FHA vaccine were also significantly higher than those of the control groups (P < 0.01) and of the groups immunized with PspA5 or PspA5-ACT (P ≤ 0.05), they were lower than those induced by PspA5-PT (P < 0.05). ACT did not appear to display adjuvant functions, since the levels of antibodies observed in the PspA5-ACT group were not signifi-

Protection (Table 4). Only 1 out of 6 mice from this group survived the challenge. In contrast, 5 out of 6 mice immunized with PspA5-BPSM and 4 out of 6 mice immunized with PspA5-wP survived the challenge.
TABLE 5 Survival of mice against challenge with the ATCC 6303 pneumococcal strain: evaluation of the adjuvant activity of purified *B. pertussis* componentsa

| Vaccination given | No. of mice alive/total no. of mice | % survival | P* |
|-------------------|-------------------------------------|------------|----|
| Non               | 0/6                                 | 0          |    |
| ACT               | 0/6                                 | 0          | 1  |
| FHA               | 0/6                                 | 0          | 1  |
| PT                | 0/6                                 | 0          | 1  |
| PspA5             | 0/6                                 | 0          |    |
| PspA5-ACT         | 1/6                                 | 16.6       | 1  |
| PspA5-FHA         | 3/6                                 | 50         | 0.18 |
| PspA5-PT          | 6/6                                 | 100        | 0.002 |

a For the challenge, 3 × 10^6 CFU of the ATCC 6303 pneumococcal strain was inoculated through the nasal route, and survival was monitored for 10 days.

b Non, nonimmunized; ACT, adenylate cyclase toxin; FHA, filamentous hemagglutinin; PT, pertussis toxin.

c By Fisher’s exact test. Comparisons were made with the nonimmunized group.

PT Enhances Immune Responses to PspA

Protein vaccines are being proposed as alternatives to the available polysaccharide conjugate vaccines, with the objective of inducing serotype-independent immunity against *S. pneumoniae*. A great body of data supports the use of PspA for these purposes. Although sequence variability may counteract the development of PspA-based vaccines, some studies have shown that the choice of PspA molecules that induce broad-reactive antibodies, or the combination of two PspA molecules, may overcome this problem. The enhancement of the immune responses against the antigen by the addition of adjuvants may also improve cross-reactivity. Sera from mice immunized with PspA5 combined with wP as an adjuvant were shown to better recognize PspAs from different clades than sera from the mice immunized with PspA5 alone. When given to mice through the nasal route, PspA5-wP induced protection against respiratory invasive challenges with pneumococcal strains expressing PspAs from clades 5 and 2 (*S. pneumoniae* ATCC 6303 and A66.1 strains, respectively) as well as against nasal colonization with the *S. pneumoniae* 0603 strain, which expresses PspA from clade 1. Repeated nasal immunizations with PspA5-wP induce high levels of systemic and mucosal anti-PspA antibodies, IL-17 responses in the spleen, and a peak of proinflammatory responses in the lungs after pneumococcal challenge. Here, we show that the induction of IgG by the PspA5-wP vaccine is essential for protection against invasive challenge with the ATCC 6303 pneumococcal strain. PspA5-wP failed to confer protection against pneumococcal challenge in µMT−/− mice, which showed undetectable amounts of anti-PspA5 IgG after immunization. Moreover, passive immunization with IgG purified from the sera of mice immunized with PspA5-wP conferred significant protection to naive BALB/c mice.

PspA inhibits the deposition of complement on the pneumococcal surface, thereby enhancing bacterial survival during systemic infection. The deletion of the *pspA* gene or the presence of anti-PspA antibodies abolishes this inhibitory effect. The inhibition of complement deposition by PspA occurs through the choline-binding domain present at the C-terminal end of the molecule. The attachment of PspA occurs via the interaction of this molecule with the C-reactive protein, an important activator of the complement cascade, to phosphocholine, resulting in diminished complement deposition at the bacterial surface. This interaction impairs the binding of the C-reactive protein, an important activator of the complement cascade, to phosphocholine, resulting in diminished complement deposition at the bacterial surface.

DISCUSSION

cently different from those observed in mice immunized with PspA5 alone. The immunization of mice with PspA5-ACT, PspA5-FHA, or PspA5-PT induced immune responses with a drift toward Th2, showing higher levels of IgG1 than IgG2a. However, no differences in the IgG1-to-IgG2a ratios among these groups were observed (data not shown).

Only PspA5-PT conferred 100% survival in the mice after challenge with the ATCC 6303 pneumococcal strain, although PspA5-FHA conferred partial protection, with 50% survival (Table 5).

The immunization of BALB/c mice with a combination of PspA5 and the B oligomer of PT (PspA5-OligB) induced levels of anti-PspA5 IgG similar to those induced by PspA5-PT (Fig. 3B), indicating that the enzymatic activity of PT is not essential for the expression of its adjuvant activity. Both formulations conferred significant protection against challenge with the ATCC 6303 pneumococcal strain, with around 80% of the mice surviving in both cases (Table 6).

Sera from mice immunized with PT-containing vaccines showed higher capacity to bind to and to induce complement deposition on pneumococcal surface in vitro. Pools of sera collected from the groups of mice were evaluated for IgG binding and complement deposition on the surface of the ATCC 6303 pneumococcal strain. The sera from mice immunized with formulations containing wild-type *B. pertussis* bacterial preparations (PspA5-wP and PspA5-BPSM) showed high IgG binding (median fluorescence intensities of 1,098 and 519, respectively) and induced complement deposition (median fluorescence intensities of 4,736 and 1,406, respectively) on the pneumococcal surface (Fig. 4A and B). In contrast, the sera from mice immunized with PspA5-BPRA showed median fluorescence intensity values that were only slightly above the median values observed for the control sera (115 for IgG binding and 349 for complement deposition). Regarding the formulations composed of PspA5 and purified *B. pertussis* components (Fig. 4C and D), only sera from mice immunized with PspA5-PT showed significant IgG binding and complement deposition (median fluorescence intensity values of 1,339 for binding and 806 for complement deposition). IgG binding slightly above the controls was observed with the sera from mice immunized with PspA5-ACT or PspA5-FHA (median fluorescence intensity values of 90 and 113, respectively). No effects of these sera on complement deposition were observed.

**TABLE 6 Survival of mice against challenge with the ATCC 6303 pneumococcal strain: evaluation of the adjuvant activity of PTa**

| Vaccination given | No. of mice alive/total no. of mice | % survival | P* |
|-------------------|-------------------------------------|------------|----|
| Non               | 0/6                                 | 0          |    |
| OligB             | 1/6                                 | 16.7       | 1  |
| PT                | 0/6                                 | 0          | 1  |
| PspA5             | 1/6                                 | 16.7       | 1  |
| PspA5-OligB       | 5/6                                 | 83.3       | 0.02 |
| PspA5-PT          | 4/5                                 | 80.0       | 0.02 |

a For the challenge, 3 × 10^6 CFU of the ATCC 6303 pneumococcal strain was inoculated through the nasal route, and survival was monitored for 10 days.

b Non, nonimmunized; OligB, pertussis toxin oligomer B; PT, pertussis toxin.

c By Fisher’s exact test. Comparisons were made with the nonimmunized group.
suggesting that the protective effects of anti-PspA antibodies are probably related to the direct binding of their Fc portion to the complement C1q, activating the classical complement pathway (44). The PspA5 antigen used here comprises the N-terminal region up to the proline-rich region and lacks the choline-binding region. Passive immunization with IgG purified from PspA5-wP sera conferred partial but increased survival against the pneumococcal invasive challenge, whereas the respective F(ab’)2 did not, supporting this hypothesis.

Repeated nasal immunizations with PspA5-wP induce high levels of anti-PspA IgG and IgA in the serum and in the respiratory mucosa of mice (19), but the depletion of complement in mice immunized with up to 6 doses of PspA5-wP completely abrogated protection against respiratory invasive challenge with the ATCC 6303 strain. This result supports the importance of the complement system in the protection elicited by PspA5-wP. Similarly, a nasal vaccine composed of a hybrid protein that targets PspA to the human Fcγ receptor type I (anti-hFcγRI-PspA) induced high levels of anti-PspA antibodies and complement-dependent protection against a challenge with a serotype 3 pneumococcal strain in transgenic mice expressing hFcγRI (40).

We previously showed that the LPS present in wP is not essential to its adjuvant activity. A modified wP vaccine containing low levels of LPS, produced at the Instituto Butantan (São Paulo, Brazil), showed adjuvant properties similar to those of wP. In addition, PspA5-wP induced high levels of antibodies and protection against the pneumococcal invasive challenge in C3H/HeJ mice, which lack signaling through Toll-like receptor 4 (TLR4) (19). Therefore, we decided to evaluate the role of other B. pertussis components in the adjuvant activity in this model. Here, we found that even a single nasal dose of PspA5-wP consistently induced high levels of anti-PspA IgG and conferred survival to around 80% of the BALB/c mice after invasive challenge with the ATCC 6303 pneumococcal strain. The inactivated virulent B. pertussis BPSM strain, derived from Tohama I, displayed adjuvant activity equivalent to that of our previous wP preparations. In contrast,
BPLOW, the attenuated mutant BPSM derivative, showed a decrease in the induction of anti-PspA IgG compared with that of wP. Moreover, immunizing mice with a single dose of PspA5-BPLOW induced only partial protection (50% survival) in mice after challenge with the ATCC 6303 pneumococcal strain. In BPLOW, the two-component regulatory system BvgA/S, which regulates the expression of most B. pertussis virulence genes, including those encoding PT, FHA, and ACT, was disrupted (34, 45). Since these proteins are known to possess immunomodulating properties (46–48), we investigated their adjuvant properties in the PspA5 model. Decreased adjuvant activity was observed for the BPRA strain, in which the ptx gene was deleted. The levels of anti-PspA IgG were significantly lower in the mice immunized with PspA5-BPRA than in the mice immunized with PspA5-BPSM or PspA5-wP, and no protection was observed in this group, suggesting that the PT contributes to the adjuvant activity. The combination of PspA5 with purified B. pertussis components confirmed the adjuvant activity of PT in this model. Although a single nasal immunization of PspA5 combined with FHA induced higher levels of anti-PspA IgG than the immunization with PspA5 alone, the highest levels of antibodies were observed when PT was used as the adjuvant. The levels of protection correlated with the levels of antibodies, with the highest survival percentages observed in the mice immunized with PspA5-PT. Immunization with PspA5-FHA protected 50% of the mice compared to no significant protection after immunization with PspA5 alone. However, vaccination with PspA5-PT showed significantly enhanced protection compared to vaccination with PspA5-FHA. ACT was previously shown to express adjuvant activity in mice when administered in combination with ovalbumin or pertactin through the nasal route, inducing antibodies and T-cell responses against the antigens (49). However, in our experiments, we did not observe such adjuvant activity. The levels of anti-PspA IgG induced by PspA5-ACT were not different from those induced by PspA5. The possible reasons for these differences are the administration of a single dose using 1 μg of ACT in our experiments, whereas in previous work, three doses with 10 μg of ACT were used.

In agreement with the in vivo results that indicate the importance of IgG and complement in the protection mediated by PspA5-wP, the highest levels of complement deposition on the pneumococcal surface in vitro were induced by the sera from mice immunized with PspA5 combined with preparations from virulent B. pertussis strains (wP and BPSM) or with PT. No differences in the IgG1-to-IgG2a ratios were observed when PspA5 was combined with the different B. pertussis preparations. All the combinations induced balanced Th1/Th2 responses. On the other hand, immunization with PspA5 combined with the purified B. pertussis components induced responses with a Th2 character with higher levels of IgG1 than IgG2a. PspA-based vaccines able to induce Th1 responses were shown to be more efficient at eliciting protection against pneumococcal infections in mice due to the induction of relatively high levels of IgG2a, a subtype with a higher capacity to mediate complement deposition on the pneumococcal surface (50, 51). However, in our experiments, this may have been compensated by the induction of high levels of total IgG (including IgG1 and IgG2a) observed in mice immunized with PspA5-wP, PspA5-BPSM, or PspA5-PT.

PT is composed of two moieties called the A protomer and the B oligomer. While the A subunit (also known as S1) expresses enzymatic activity, catalyzing the ADP-ribosylation of signal-transducing G proteins in the host cells, the B oligomer (composed of five subunits named S2 to S5) promotes binding to the PT receptors at the host cell surface. Enzymatically inactive PT with mutations in the S1 subunit but that is able to bind to host cells was shown to enhance Th1 and Th2 responses to coinfected antigens. This activity depends on the receptor-binding activity of the B moiety (46). In agreement with these observations, OligB displayed adjuvant activity similar to that of PT in the PspA5 model. High levels of anti-PspA5 IgG and protection against pneumococcal challenge were observed in mice immunized with a single dose of PspA5-OligB.

Several studies have shown the effect of formaldehyde treatment on PT structure and activity (52, 53). Increasing concentrations of formaldehyde can differently affect the enzymatic and carbohydrate-binding activities, as well as the presence of native epitopes (52). In addition, reversion of the formaldehyde effects, mainly with respect to the carbohydrate-binding activity, has been observed (53). These studies highlighted the importance of achieving a balance between low toxicity and immunogenicity when developing PT-containing pertussis vaccines. We did not assess the properties of PT in the formaldehyde-inactivated whole-cell pertussis preparations, but the results presented here show that the PT-deficient strain displays reduced adjuvant activity compared with those of the PT-producing strains, suggesting that formaldehyde treatment did not affect the adjuvant activity of PT in a major manner. In summary, we show here that the protective effect of PspA5-wP against pneumococcal invasive challenge is mainly conferred by the induction of anti-PspA5 IgG and depends on complement. PT-containing formulations can improve the immune responses against PspA5, leading to enhanced protection.

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